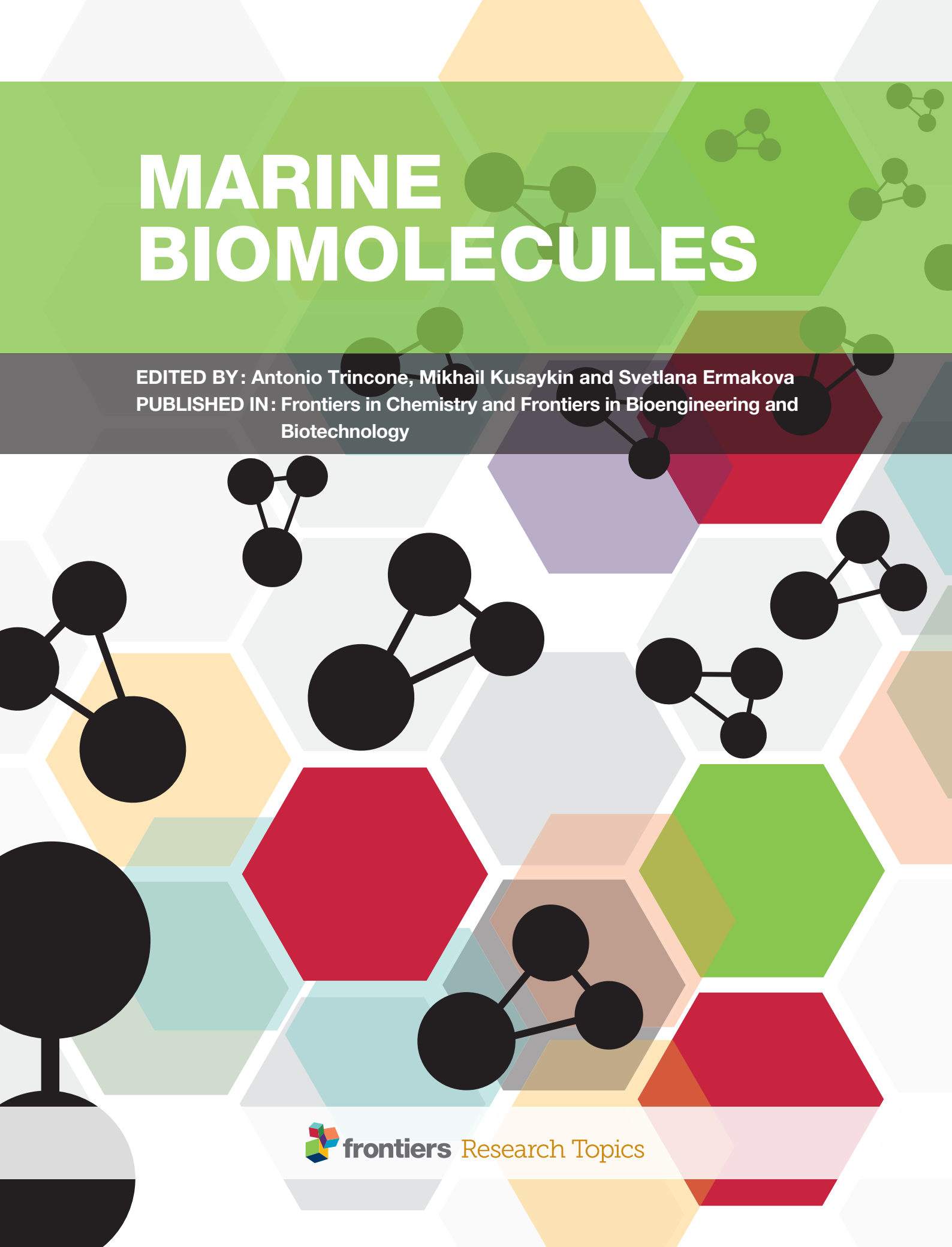
MARINE BIOMOLECULES

EDITED BY: Antonio Trincone, Mikhail Kusaykin and Svetlana Ermakova
PUBLISHED IN: Frontiers in Chemistry and Frontiers in Bioengineering and Biotechnology



frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88919-661-6
DOI 10.3389/978-2-88919-661-6

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view.
By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: **researchtopics@frontiersin.org**

MARINE BIOMOLECULES

Topic Editors:
Antonio Trincone, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Italy
Mikhail Kusaykin, G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Russia
Svetlana Ermakova, G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far Eastern Branch, Russian Academy of Sciences, Russia

Oceans include the greatest extremes of pressure, temperature and light, and habitats can range from tropical waters to ocean trenches, several kilometers below sea level at high pressure. With its 70% of the surface of our planet marine ecosystem still remains largely unexplored, understudied and underexploited in comparison with terrestrial ecosystems, organisms and bioprocesses. The biological adaptation of marine organisms to a wide range of environmental conditions in the specific environment (temperature, salinity, tides, pressure, radiation, light, etc.) has made them an enormous reservoir of interesting biological material for both basic research and biotechnological improvements. As a consequence marine ecosystem is valued as a source of enzymes and other biomolecules exhibiting new functions and activities to fulfill human needs. Indeed, in recent years it has been recognised as an untapped source of novel enzymes and metabolites even though, with regard to the assignment of precise biological functions to genes, proteins and enzymes, it is still considered as the least developed.

Using metagenomics to recover genetic material directly from environmental samples, this biogenetic diversification can be accessed but despite the contributions from metagenomic technologies the new field requires major improvements.

A few words on the complexity of marine environments should be added here. This complexity ranges from symbiotic relationships to biology and chemistry of defence mechanisms and from chemoecology of marine invasions up to the strategies found in prokaryotes to adapt to extreme environments. The interdisciplinary study of this complexity will enable researchers to find an arsenal of enzymes and pathways greatly demanded in biotechnological applications.

As far as marine enzymes are concerned they may carry novel chemical and stereochemical properties, thus biocatalytically oriented studies (testing of suitable substrates, appropriate checking of reaction conditions, study of stereochemical asset of catalysis) should be performed to appropriately reveal this "chemical biodiversity" which increases interest for these enzymes.

Among other biomolecules, polysaccharides are the most abundant renewable biomaterial found on land and in oceans. Their molecular diversity is very interesting; except polysaccharides used traditionally in food and non-food industries, the structure and the functionality of most of them are unknown and unexplored. Brown seaweeds synthesize unique bioactive polysaccharides: laminarans, alginic acids and fucoidans. A wide range of biological activities (anticoagulant, antitumor, antiviral, anti-inflammation, etc.) have been attributed to fucoidans and their role with respect to structure-activity relationship is still under debate.

In this Research Topic, we wish to centralize and review contributions, idea and comments related to the issues above. In particular results of enzymatic bioprospecting in gross marine environment will be acknowledged along with research for structural characterization and biological function of biomolecules such as marine polysaccharides and all kind of research related to the complexity of bioprocesses in marine environments. Inter- and multi-disciplinary approach to this field is favoured in this Research Topic and could greatly be facilitated by the web and open access nature as well.

Citation: Trincone, A., Kusaykin, M., Ermakova, S., eds. (2015). Marine Biomolecules. Lausanne: Frontiers Media. doi: 10.3389/978-2-88919-661-6

Table of Contents

frontiers in Chemistry

EDITORIAL
published: 19 August 2015
doi: 10.3389/fchem.2015.00052

Editorial: Marine biomolecules

Antonio Trincone[1]*, *Mikhail Kusaykin*[2] *and Svetlana Ermakova*[2]

[1] *Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Pozzuoli, Italy,* [2] *G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russia*

Keywords: marine biomoleculers, marine oligosaccharides, marine compounds, marine enzymes, marine biocatalysts

Even before the development of the idea to exploit marine biomolecules for human needs (pharmaceuticals, cosmetics, etc.), the interest for such compounds is implicit when one ponders the astonishingly basic definition of marine biotechnology as "a natural extension of cultural practices of garnering food from the ocean" by the anthropologist Helmreich who beautifully summarizes the origins of marine biotechnology and its deep significance to human society (Helmreich, 2003). Free from any sectarian, discipline-directed bias for a particular type of molecules and, as a consequence of this inclusive unfiltered consideration of all types of molecule, is the great flexibility of the biological adaptation of marine organisms to the wide range of environmental conditions found (temperature, salinity, tides, pressure, radiation, light, etc.). Indeed this adaptation has empowered marine livings with an enormous reservoir of any type of precious biological material for both basic research and biotechnological improvements. Relative to the terrestrial well-known ecosystem, the prevalent and unknown marine environment is valued as a source of enzymes exhibiting new functions and activities to fulfill human needs (Trincone, 2013), as well as other biomolecules such as important polysaccharides which are the most abundant renewable biomaterial found in oceans. This list cannot be completed without the inclusion of small molecular weight compounds characterized by various molecular skeletons isolated from marine organisms (sponges, corals, and other marine invertebrates), which possess interesting activities. Moreover, it is still widely accepted that the assignment of precise biological functions to genes, proteins, and enzymes in marine environment is the least developed aspect.

The above consideration was the motivation for the proposal of this Research topic that aims to centralize review contributions, idea and comments simply related to all marine biomolecules. In particular, results of enzymatic bioprospecting in a gross marine environment were expected, along with research for structural characterization and biological function of marine polysaccharides and all kind of research related to the complexity of bioprocesses in marine environments, with an inter- and multi-disciplinary approach. Probably the short lifetime of the research topic hampered a more crowded collection of different contributions spanning all class of biomolecules. Nevertheless, 78 authors from the most prestigious research institutions from different parts of world have submitted articles reporting original results along with reviews, opinions, and perspective contributions all composing this e-book. Two new research topics dedicated to marine oligosaccharides and to marine compounds in food domain, are directly derived from this success and currently active in Frontiers in Marine Science under the Specialty of Marine Biotechnology.

In order to understand the mechanism of action of the α-galactosidase of the marine bacterium *Pseudoalteromonas* sp. KMM 701, the stereochemistry of its hydrolytic reaction has been analyzed by ^{1}H NMR spectroscopy for the identification of the first anomer of the sugar formed, before mutarotation equilibrium. The data showed that the enzymatic hydrolysis of substrates proceeds with retention of configuration due to a double displacement mechanism of reaction. Hydrolysis of terminal α-linked galactose residues from glycoconjugates has found

OPEN ACCESS

Edited and reviewed by:
John D. Wade,
Florey Institute of Neuroscience and Mental Health, Australia

***Correspondence:**
Antonio Trincone,
antonio.trincone@icb.cnr.it

Specialty section:
This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry

Received: *21 July 2015*
Accepted: *05 August 2015*
Published: *19 August 2015*

Citation:
Trincone A, Kusaykin M and Ermakova S (2015) Editorial: Marine biomolecules. Front. Chem. 3:52. doi: 10.3389/fchem.2015.00052

a number of useful potential biotechnological and medical applications with direct research involvement by the same group of authors (Balabanova et al., 2010). Worldwide efforts focused on the subject are also recently reviewed (Bakunina et al., 2014).

From the same prestigious Russian institution, G.B. Elyakov Pacific Institute of Bioorganic Chemistry, two other groups have contributed to this Research topic with a review and an opinion article. The review presented a survey in collaboration with the Department of Biochemistry, Dong-A University, South Korea, on marine triterpene glycosides having very low toxicity and considered potential suitable agents for the prevention and treatment of cancer. The conclusion affirms that fine details of the structural characteristics including carbohydrate moiety and sulfation are involved in the biological activities of marine triterpene glycosides and the study is essential for developing marine drugs.

The opinion article evaluates the status of different research on marine polysaccharides. The molecular diversity of these molecules is very interesting with new structures and, except those used traditionally in food and non-food industries, with functionalities that are unknown and unexplored so that biomolecular tools for study in this field are very important. The authors specially focused on fucoidan and fucoidanases on which the Russian group is intensively working (Kusaykin et al., 2008; Vishchuk et al., 2011, 2013; Silchenko et al., 2013; Shevchenko et al., 2015). Despite the obvious prospects for exploitation in medicine, fucoidan has not yet been declared a drug and the question of the title makes sense in view of the biological effects of a food supplement called Fucolam® recently registered in Russia that in addition to the immunomodulatory, antibacterial, antiviral, and antitumoral activities, has probiotic, hepatoprotective, glucose, and cholesterol lowering effects.

The importance of the carbohydrate class of biomolecules in marine environment is highlighted by the presence of other contributions. An original article from the Station Biologique de Roscoff, France, reports on an iota-carrageenan sulfatase detected in the cell-free lysate of the marine bacterium *Pseudoalteromonas carrageenovora*. Not only do the rheological properties of these carrageenans depend on their sulfate content but the enzymatic manipulation of sulfates is an enabling technology of paramount importance for modulation of activity of sulfate containing biomolecules.

Furthermore, from the Laboratory of Marine Biotechnology and Microbiology Division of Applied Marine Life Science of Hokkaido University, Japan, a study is presented on an excellent source of carbohydrate active marine enzymes, the *Aplysia* genus. In this case the characterization of a cellulase, from the common sea hare *Aplysia kurodai* is the topic related to the importance of marine carbohydrate biomasses as a source material for ethanol fermentation.

A review directly linked to this topic is provided by the National Institute of Renewable Energy, Punjab, India, detailing recent findings and advanced developments of algal biomass for biofuel production, highlighting the great impact of fossil fuels on carbon cycle (carbon balance) related to combustion. According to this green chemistry approach that aims to bypass competition with agricultural food and feed production, researchers, and entrepreneurs have focused interest on algal biomass and other marine bioresources as alternative feed-stock for improved production of biofuels.

Turning back to small molecular agents, the very interesting perspective article from the Institute of Biomolecular Chemistry, Italy, is worth mentioning last. Some of the volatile terpenoids found in marine sponges and nudibranchs have also been found in terrestrial plants and insects. Being odorant molecules that are hydrophobic in nature, they cannot be effective in any form of remote sensing based on diffusion in water. Olfaction is generally regarded as a distance sense, while gustation is a contact sense, and exactly the same volatile molecules, that are almost insoluble in water, would be considered at the same time as being odorant on land, and tasted by contact at sea. After an in-depth analysis from ecological, chemical, and biological perspective of the actual view about selection of odorant receptors for hydrophobic interactions, the authors report a new perspective aiming at a radical solution preserving the usual taste-smell dichotomy.

References

Bakunina, I. Y., Balabanova, L. A., Pennacchio, A., and Trincone, A. (2014). Hooked on α-D-galactosidases: from biomedicine to enzymatic synthesis. *Crit. Rev. Biotechnol.* doi: 10.3109/07388551.2014.949618. [Epub ahead of print].

Balabanova, L. A., Bakunina, I. Y., Nedashkovskaya, O. I., Makarenkova, I. D., Zaporozhets, T. S., Besednova, N. N., et al. (2010). Molecular characterization and therapeutic potential of a marine bacterium *Pseudoalteromonas* sp. KMM 701 α-galactosidase. *Mar. Biotechnol.* 12, 111–120. doi: 10.1007/s10126-009-9205-2

Helmreich, S. (2003). A tale of three seas: from fishing through aquaculture to marine biotechnology in the life history narrative of a marine biologist. *Marit. Stud.* 2, 73–94.

Kusaykin, M., Bakunina, I., Sova, V., Ermakova, S., Kuznetsova, T., Besednova, N., et al. (2008). Structure, biological activity, and enzymatic transformation of fucoidans from the brown seaweeds. *Biotechnol. J.* 3, 904–915. doi: 10.1002/biot.200700054

Shevchenko, N. M., Anastyuk, S. D., Menshova, R. V., Vishchuk, O. S., Isakov, V. I., Zadorozhny, P. A., et al. (2015). Further studies on structure of fucoidan from brown alga *Saccharina gurjanovae*. *Carbohydr. Polym.* 121, 207–216. doi: 10.1016/j.carbpol.2014.12.042

Silchenko, A. S., Kusaykin, M. I., Kurilenko, V. V., Zakharenko, A. M., Isakov, V. V., Zaporozhets, T. S., et al. (2013). Hydrolysis of fucoidan by fucoidanase isolated from the marine bacterium, *Formosa algae*. *Mar. Drugs* 11, 2413–2430. doi: 10.3390/md11072413

Trincone, A. (ed.). (2013). "Marine enzymes for biocatalysis: sources, biocatalytic characteristics and bioprocesses of marine enzymes," in *Biomedicine*, (Cambridge: Woodhead Publishing), Series No.38. Available online at: http://www.woodheadpublishing.com/en/book.aspx?bookID=2822

Vishchuk, O. S., Ermakova, S. P., and Zvyagintseva, T. N. (2011). Sulfated polysaccharides from brown seaweeds *Saccharina japonica* and *Undaria pinnatifida*: isolation, structural characteristics, and antitumor activity. *Carbohydr. Res.* 346, 2769–2776. doi: 10.1016/j.carres.2011.09.034

Vishchuk, O. S., Ermakova, S. P., and Zvyagintseva, T. N. (2013). The effect of sulfated (1→3)-α-L-fucan from the brown alga *Saccharina cichorioides* Miyabe on resveratrol-induced apoptosis in colon carcinoma cells. *Mar. Drugs* 11, 194–212. doi: 10.3390/md1101 0194

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in CHEMISTRY

ORIGINAL RESEARCH ARTICLE
published: 06 August 2014
doi: 10.3389/fchem.2014.00060

Characterization of a GHF45 cellulase, AkEG21, from the common sea hare *Aplysia kurodai*

Mohammad M. Rahman[1,2], Akira Inoue[1] and Takao Ojima[1]*

[1] *Laboratory of Marine Biotechnology and Microbiology, Division of Applied Marine Life Science, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Japan*
[2] *Department of Fisheries Biology and Genetics, Bangladesh Agricultural University, Mymensingh, Bangladesh*

Edited by:
Antonio Trincone, Istituto di Chimica Biomolecolare, Italy

Reviewed by:
Joana Costa, Center for Neuroscience and Cellular Biology, Portugal
Irina Bakunina, G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Russia

****Correspondence:***
Takao Ojima, Laboratory of Marine Biotechnology and Microbiology, Division of Applied Marine Life Science, Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan
e-mail: ojima@fish.hokudai.ac.jp

The common sea hare *Aplysia kurodai* is known to be a good source for the enzymes degrading seaweed polysaccharides. Recently four cellulases, i.e., 95, 66, 45, and 21 kDa enzymes, were isolated from *A. kurodai* (Tsuji et al., 2013). The former three cellulases were regarded as glycosyl-hydrolase-family 9 (GHF9) enzymes, while the 21 kDa cellulase was suggested to be a GHF45 enzyme. The 21 kDa cellulase was significantly heat stable, and appeared to be advantageous in performing heterogeneous expression and protein-engineering study. In the present study, we determined some enzymatic properties of the 21 kDa cellulase and cloned its cDNA to provide the basis for the protein engineering study of this cellulase. The purified 21 kDa enzyme, termed AkEG21 in the present study, hydrolyzed carboxymethyl cellulose with an optimal pH and temperature at 4.5 and 40°C, respectively. AkEG21 was considerably heat-stable, i.e., it was not inactivated by the incubation at 55°C for 30 min. AkEG21 degraded phosphoric-acid-swollen cellulose producing cellotriose and cellobiose as major end products but hardly degraded oligosaccharides smaller than tetrasaccharide. This indicated that AkEG21 is an endolytic β-1,4-glucanase (EC 3.2.1.4). A cDNA of 1013 bp encoding AkEG21 was amplified by PCR and the amino-acid sequence of 197 residues was deduced. The sequence comprised the initiation Met, the putative signal peptide of 16 residues for secretion and the catalytic domain of 180 residues, which lined from the N-terminus in this order. The sequence of the catalytic domain showed 47–62% amino-acid identities to those of GHF45 cellulases reported in other mollusks. Both the catalytic residues and the N-glycosylation residues known in other GHF45 cellulases were conserved in AkEG21. Phylogenetic analysis for the amino-acid sequences suggested the close relation between AkEG21 and fungal GHF45 cellulases.

Keywords: *Aplysia kurodai*, AkEG21, endo-β-1,4-glucanase, cellulase, GHF45, cDNA cloning, primary structure, phylogenic analysis

INTRODUCTION

Cellulose, a structural polysaccharide comprising 1,4-linked β-D-glucopyranose residues, exists mainly in plant cell wall as crystalline microfibrils (Jagtap and Rao, 2005). Since plant cellulose accounts for almost a half of total carbohydrate biomass on the Earth, intensive uses of the cellulose are expected to solve various problems that we are facing in ecological, environmental and energy fields (Agbor et al., 2011; Yang et al., 2011). In this respect, degradation of cellulosic materials by cellulose-degrading enzymes will be a fundamentally important technique because the cellulose-degrading enzyme can convert insoluble cellulose to soluble oligosaccharides and glucose without consuming high energy and producing hazardous byproducts (Michel and Czjzek, 2013; Ojima, 2013; Tsuji et al., 2013). The resulted sugars are applicable for foods, feeds, pharmaceutics, fermentation substrates, etc.

Complete enzymatic degradation of cellulose is usually achieved by the collaborative actions of three enzymes, namely, (1) endo-β-1,4-glucanase (EC 3.2.1.4) which randomly cleaves internal β-1,4-linkages of amorphous regions in cellulose fibers, (2) cellobiohydrolase (EC 3.2.1.91) which releases cellobiosyl unit from non-reducing end of cellulose chain, and (3) β-D-glucosidase (EC 3.2.1.21) which releases glucose unit from cello-oligosaccharides (Lynd et al., 2002; Perez et al., 2002; Bayer et al., 2004). Although individual enzyme alone cannot completely depolymerize crystalline cellulose, the synergistic action of three enzymes efficiently promotes the depolymerization of cellulose. Among the three enzymes, endo-β-1,4-glucanase is the primarily important for the depolymerization of cellulose since it first acts on cellulose and provides new substrate sites for cellobiohydrolase and β-D-glucosidase. Accordingly, endo-β-1,4-glucanase is generally called "cellulase." Fungal and microbial cellulases have already been used in various purposes, e.g., detergent, textile, food, paper, pulp, brewing and winery (Sheehan and Himmel, 1999; Bhat, 2000; Zaldivar et al., 2001; Kuhad et al., 2011; Mojsov, 2012). Cellulases are also expected as a biocatalyst

in the production of biofuels from cellulose. If fermentable sugars can be produced from unused cellulosic materials at low cost, food-fuel conflicts in the bioethanol production using edible crops will be circumvented.

Cellulase distributes over various organisms, e.g., archaea (Gueguen et al., 1997; Li et al., 2003), bacteria (Tomme et al., 1995; Hong et al., 2002; Masuda et al., 2006; Fibriansah et al., 2007), fungi (de la Cruz et al., 1995; Tomme et al., 1995), plants (Pesis et al., 1978; Castresana et al., 1990), and herbivorous invertebrates such as termite, cockroach, crayfish and mollusks (Watanabe et al., 1998; Yan et al., 1998; Byrne et al., 1999; Tokuda et al., 1999; Watanabe and Tokuda, 2001; Xu et al., 2001; Sugimura et al., 2003; Suzuki et al., 2003; Davison and Blaxter, 2005; Nishida et al., 2007; Sakamoto et al., 2007; Sakamoto and Toyohara, 2009; Tsuji et al., 2013). Previously, cellulase activities detected in the invertebrate animals were considered to be originated from symbiotic microbes in their digestive tracts or contamination by foods (Cleveland, 1924; Martin and Martin, 1978). However, recent biochemical and genomic studies have revealed that cellulases found in insects, crustaceans, annelids, mollusks, echinoderms and nematodes are their own gene products.

To date, a large number of primary structures of cellulases have been enrolled in CAZy data base (Cantarel et al., 2009). These cellulases have been classified under GHF (glycosyl hydrolase family) 5, 6, 7, 8, 9, 10, 11, 12, 26, 44, 45, 48, 51, and 74 on the basis of hydrophobic cluster analysis for amino-acid sequences (Henrissat et al., 1989; Henrissat, 1991; Henrissat and Bairoch, 1993). Invertebrate cellulases are enrolled in five families, i.e., GHF5 (nematodes: *Globodera rostochiensis* and *Heterodera glycines*; Smant et al., 1998), GHF6 (sea squirt: *Ciona savignyi*; Matthysse et al., 2004), GHF9 (termite: *Reticulitermes speratus*, Watanabe et al., 1998; abalone: *Haliotis discus hannai*, Suzuki et al., 2003; sea urchin: *Strongylocentrotus nudus*, Nishida et al., 2007), GHF10 (freshwater snails: *Ampullaria crossean*, Wang et al., 2003; *Pomacea canaliculata*, Imjongjirak et al., 2008), and GHF45 (bivalve: *Mytilus edulis*, Xu et al., 2001; freshwater snail: *A. crossean*, Guo et al., 2008; freshwater bivalve: *Corbicula japonica*, Sakamoto and Toyohara, 2009). Among these cellulases, GHF9-type cellulases appear to be most widespread in nature and well characterized (Davison and Blaxter, 2005). In molluscan cellulases, GHF9 enzyme was identified in *H. discus hannai* (Suzuki et al., 2003) and *A. kurodai* (Tsuji et al., 2013), while both GHF10 and GHF45 enzymes were identified in *P. canaliculata* (Imjongjirak et al., 2008) and *A. crossean* (Ding et al., 2008), and GHF45 enzymes were identified in *M. edulis* (Xu et al., 2001), *A. crossean* (Guo et al., 2008), *C. japonica* (Sakamoto and Toyohara, 2009) and *A. kurodai* (Tsuji et al., 2013). Some mollusks possess plural cellulases, e.g., GHF9 and GHF45 cellulases (Sakamoto et al., 2007; Guo et al., 2008; Li et al., 2009; Sakamoto and Toyohara, 2009; Tsuji et al., 2013). The synergistic action of multiple enzymes appeared to improve the production of glucose from seaweed cellulose (Tsuji et al., 2013). Among the molluscan cellulases, GHF45 enzyme has been characterized by its smaller molecular size compared with other cellulases. Namely, the molecular size of GHF45 enzymes is ∼25 kDa, while those of GHF9 and GHF10 enzymes are 45–63 kDa. The small size of GHF45 cellulases appeared to be advantageous in performing protein-engineering and crystallography studies, since low molecular mass proteins are usually heat stable and easily produced by heterogeneous expression systems. Actually, the GHF45-type cellulase CjCel45 from freshwater clam was successfully produced by the *Escherichia coli* expression system (Sakamoto and Toyohara, 2009) and the three-dimensional structure of Cel45A from *M. edulis* could be analyzed by X-ray crystallography (PDB ID, 1WC21006).

To date, GHF45 cellulase genes have been identified only in a few mollusks (Xu et al., 2001; Guo et al., 2008; Sakamoto and Toyohara, 2009) and enzyme proteins have been isolated only from *M. edulis* (Xu et al., 2000), *A. crossean* (Li et al., 2005), and *A. kurodai* (Tsuji et al., 2013). Molluscan GHF45 cellulases were suggested to be acquired by horizontal gene transfer from fungi by phylogenetic analyses (Scholl et al., 2003; Kikuchi et al., 2004; Sakamoto and Toyohara, 2009); however, accumulation of primary structure data seems to be still insufficient for detailed discussion about the origin and molecular evolution of molluscan GHF45 cellulases.

The common sea hare *A. kurodai* is a good source for polysaccharide-degrading enzymes since it harbor much digestive fluid in gastric lumen (Kumagai and Ojima, 2009; Rahman et al., 2010; Zahura et al., 2010; Tsuji et al., 2013; Kumagai et al., 2014). Recently, four cellulase isozymes, i.e., 21, 45, 66, and 95 K cellulases, were isolated from the digestive fluid of *A. kurodai* (Tsuji et al., 2013). Among these enzymes, the 21K enzyme was suggested to be GHF45 cellulase. We also had noticed that the digestive fluid of *A. kurodai* contained plural cellulases and the smallest enzyme was of ∼21 kDa. This enzyme was considered to correspond to the 21K cellulase reported by Tsuji et al. (2013). Although partial amino-acid sequences of the 21K cellulase were reported, no entire primary structure has been determined yet.

In the present study, we isolated the ∼21 kDa enzyme from the digestive fluid of *A. kurodai* and investigated its general properties. Further, we cloned the cDNA encoding this enzyme and confirmed that this enzyme is a member of GHF45. This cDNA will provide the basis for protein-engineering studies on *Aplysia* GHF45 cellulase.

MATERIALS AND METHODS

MATERIALS

Sea hares identified as *A. kurodai* (average body length and weight; ∼15 cm and ∼400 g, respectively) were collected in the shore of Hakodate, Hokkaido Prefecture of Japan in July 2012. Approximately 112 mL of digestive fluid was obtained from the gastric lumen of 14 animals after dissection. The digestive fluid was dialyzed against 2 mM sodium phosphate buffer (pH 7.0) for 2 h and centrifuged at 10,000 × g for 10 min to remove insoluble materials. The supernatant (crude enzyme) was used for purification of cellulase. Carboxymethyl cellulose (CMC, medium viscosity) was purchased from ICN Bio medicals, Inc. (OH, USA). TOYOPEARL CM-650M was purchased from Toyo Soda Mfg, Co. (Tokyo, Japan) and Superdex 200 10/300 GL from GE Healthcare UK Ltd. (Little Chalfont, Buckingham shire, England). Cellooligosaccharides (disaccharide – hexasaccharide, G2 – G6) were prepared by limited acid hydrolysis. Briefly, 1 g of cellulose powder (Wako Pure Chemical Industries Co. Ltd.

Osaka, Japan) was hydrolyzed with 100 mL of 0.2 N HCl at 100°C for 1 h, and the supernatant containing cellulose fragments was neutralized with 1 N NaOH. Approximately 50 mg of cellulose fragments were subjected to gel-filtration through a column of BioGel-P2 (2 × 100 cm) and cellooligosaccharides were separately eluted with 10 mM sodium phosphate buffer (pH 7.0) and stored at −20°C until use. RNAiso Plus and Oligotex dT30 were purchased from TaKaRa (Tokyo, Japan). cDNA synthesis kit and 5′- and 3′-Full RACE kits were purchased from TaKaRa and TA-PCR cloning kit comprising pTAC-1 and *E. coli* DH5α was from Biodynamics (Tokyo, Japan). Restriction endonucleases, T4 DNA ligase, agarose, *E. coli* strain DH5α were purchased from TaKaRa. AmpliTaq Gold PCR Master Mix and BigDye-Terminator Cycle Sequencing kit were from Applied Biosystems (Foster city, CA, USA). Bacto-tryptone, Bacto-yeast extract and other reagents were from Wako Pure Chemicals Industries Ltd. (Osaka, Japan).

PURIFICATION OF *A. KURODAI* CELLULASE

Crude enzyme from *A. kurodai* (∼100 mL) was first subjected to ammonium sulfate fractionation. Cellulase activity was detected in the fraction precipitated between 40 and 60% saturation of ammonium sulfate. This fraction was collected by centrifugation at 10,000 × g for 20 min, dissolved in and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) for 24 h. The dialysis bag was changed every 2 h to avoid puncturing by cellulase action. The dialysate was then applied to a TOYOPEARL CM-650M column (1.5 × 20 cm) pre-equilibrated with the same buffer. Proteins adsorbed to the column were developed by linear gradient of NaCl from 0 to 0.3 M. Fractions showing cellulase activity were pooled and dialyzed against 10 mM sodium phosphate buffer (pH 7.0) and lyophilized. The dried material was dissolved in 0.05 M NaCl—10 mM sodium phosphate buffer (pH 6.0) and subjected to AKTA-FPLC (GE Healthcare) equipped by Superdex 200 10/300 GL column. Cellulase was eluted with 0.05 M NaCl—10 mM sodium phosphate buffer (pH 6.0) at a flow rate of 1 mL/min.

ASSAY FOR CELLULASE ACTIVITY

Standard assay for cellulase activity was carried out with a reaction mixture containing 0.5% CMC, 10 mM sodium phosphate (pH 6.0), and 0.01–0.1 mg/mL of enzyme at 30°C. Reducing sugar released by the reaction was determined by the method of Park and Johnson (1949). One unit of cellulase activity was defined as the amount of enzyme that produces reducing sugar equivalent to 1 μmol of glucose per 1 min. Temperature dependence of the cellulase was determined at 10–70°C and pH 6.0. pH dependence was determined at 30°C in reaction mixtures adjusted to pH 4.0–10.0 with 50 mM sodium phosphate. Thermal stability was assessed by measuring the residual activity in the standard assay condition after heating at 10–70°C for 30 min. The average values of triplicate measurements were shown with standard deviations.

THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography (TLC) for degradation products of cellulose and cellooligosaccharides was carried out with Silica gel-60 TLC plates (Merck KGaA, Darmstadt, Germany). Two μL of the degradation products (∼5 mg/mL) were applied to the TLC plate and developed with 1-butanol/acetic acid/water (2:1:1, v/v/v). The sugars separated on the plate were detected by heating at 120°C for 15 min after spraying 10% (v/v) sulfuric acid in ethanol.

SDS-PAGE

SDS-PAGE was carried out by the method of Porzio and Pearson (1977) using 10% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS. After the electrophoresis, the gel was stained with 0.1% (w/v) Coomassie Brilliant Blue R-250–50% (v/v) methanol–10% (v/v) acetic acid, and the background of the gel was destained with 5% (v/v) methanol–7% (v/v) acetic acid. Molecular masses of proteins were estimated with a Protein Marker, Broad Range (New England Biolabs, Inc. MA, USA).

PROTEIN CONCENTRATION

Protein concentration was determined by either the biuret method (Gornall et al., 1949) or the method of Lowry et al. (1951) using bovine serum albumin fraction V as a standard protein.

DETERMINATION OF PARTIAL AMINO-ACID SEQUENCES

The N-terminal amino-acid sequence of cellulase was determined with specimens electro-blotted to polyvinylidene difluoride membrane and ABI 492 protein sequencer (Applied Biosystems). The internal amino-acid sequences of cellulase were determined by mass spectrometry with tryptic and lysylendopeptidyl fragments prepared by the digestion with 1/200 (w/w) enzymes at 37°C for 12 h. The fragments were subjected to matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) using Proteomics Analyzer 4700 (Applied Biosystems) and the amino-acid sequences of the fragments were analyzed by MS/MS mode with DeNovo Explorer software (Applied Biosystems). Homology searches for the amino-acid sequences to the public databases were performed with the BLAST program (http://blast.ddbj.nig.ac.jp/top-j.html) provided by DNA Data Bank of Japan.

cDNA CLONING

Total RNA was extracted from ∼0.1 g of hepatopancreas of *A. kurodai* using RNAiso Plus and mRNA was selected from the total RNA with Oligotex-dt(30) (TaKaRa). Hepatopancreas cDNA was synthesized from the mRNA with a cDNA synthesis kit (TaKaRa) using random hexanucleotide primers. Cellulase cDNAs were amplified by the PCR using the hepatopancreas cDNA and degenerated primers synthesized on the basis of partial amino-acid sequences. PCR was performed in 20 μL of reaction mixture containing 50 mM KCl, 15 mM Tris–HCl (pH 8.0), 0.2 mM each of dATP, dTTP, dGTP, and dCTP, 2.5 mM $MgCl_2$, 5 pmol/μL primers, 1 ng/μL template DNA, and 0.5 units AmpliTaq Gold DNA polymerase (Applied Biosystems). A successive reaction consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s was repeated 40 cycles with Thermal Cycler Dice mini (TaKaRa). Sizes of amplified cDNAs were estimated by 1.2% agarose-gel electrophoresis and the target cDNAs were excised from the gel and cloned with TA-PCR cloning kit (Invitrogen).

The transformed *E. coli* was grown in 2 × YT medium supplemented by 50 μg/mL ampicillin at 37°C for 14 h with shaking at 150 rpm/min. The plasmids extracted from the transformants were subjected to sequence analysis with BigDye-Terminator Cycle Sequencing kit and ABI 3130*xl* Genetic Analyzer (Applied Biosystems). The 3′-RACE and 5′-RACE PCRs were carried out with specific primers synthesized on the basis of nucleotide sequences of above amplified cDNAs with a successive reaction at 94°C for 30 s, 57°C for 30 s, and 72°C for 1.0 min, which was repeated 30 cycles. The amplified DNAs were cloned and sequenced as described above.

PHYLOGENETIC ANALYSIS FOR GHF45 CELLULASES

Phylogenetic analysis was carried out with amino-acid sequence data of *A. kurodai* cellulase and other GHF45 cellulases which are enrolled in GenBank (http://www.ncbi.nlm.nih.gov/) and CAZy database (http://www.cazy.org/fam/acc_GH.html). The amino-acid sequences of GHF45 cellulases were first aligned with ClustalW2 program (Larkin et al., 2007). The alignment was then corrected by trimming the sequences with Gblocks (Castresana, 2000; Talavera and Castresana, 2007). The maximum-likelihood algorithm implemented in MEGA6 software (Tamura et al., 2013) was used to generate phylogenetic tree. The bootstrap values were calculated from 1,000 replicates.

RESULTS

ISOLATION AND CHARACTERIZATION OF *APLYSIA* 21 kDa CELLULASE

Cellulase activity was detected in four peak fractions (*P-1–P-4*) in TOYOPEARL CM-650M chromatography performed for the proteins obtained by the ammonium sulfate fractionation (**Figure 1**). The N-terminal amino-acid sequences of major proteins in *P-1–P-4* fractions were analyzed with the samples blotted to PVDF membranes after SDS-PAGE. According to BLAST search analyses, the 40 kDa protein in *P-1* fraction (N-terminal sequence: RLHIQNGHFVLNGQRVFLSG) was identified as *A. kurodai* β-mannanase AkMan (Zahura et al., 2010). The 21 kDa protein in *P-2* fraction (N-terminal sequence: EQKCQPNSHGVRVYQGKKCA) was considered to be a GHF45 cellulase corresponding to 21K cellulase previously reported by Tsuji et al. (2013). The 45 kDa protein in *P-4* fraction (N-terminal sequence: AKNYGQALGLSIKFYEAQ) was regarded as a GHF9 cellulase similar to *H. discus hannai* HdEG66 (Suzuki et al., 2003) and 45K cellulase reported by Tsuji et al. (2013). While 38 kDa protein (N-terminal sequence: RLTVSGKTFRLNNQQVFLSG) was regarded as the β-mannanase-like protein that had been annotated in *A. california* genome (GenBank accession number, XP_005100017). The 21 kDa cellulase in *P-2* fraction was recovered in high yield, while the GHF9-type cellulase in *P-4* fraction poorly recovered. The GHF9-type cellulase exhibited similar properties as abalone cellulase HdEG66 (Suzuki et al., 2003) and Aplysia 66K cellulase (Tsuji et al., 2013). Therefore, in the present study, we focused on the 21 kDa cellulase in *P-2* fraction to characterize it as a GHF45 cellulase.

The 21 kDa cellulase in *P-2* fraction was purified by gel-filtration through Superdex 200 (**Figure 2**). In this chromatography, the 21 kDa cellulase eluted as a single peak showing a single band on SDS-PAGE. Thus, we named this enzyme AkEG21 (*Aplysia kurodai* endo-β-1,4-glucanase with 21 kDa). By the above purification procedure, AkEG21 was purified at a yield of 3.3% with the specific activity 67.3 U/mg (**Table 1**). Optimal pH of AkEG21 was 4.5 and more than 80% of maximal activity was retained in a pH range from 4.3 to 5.6 (**Figure 3A**). AkEG21 showed an optimal temperature at around 40°C and it was resistant to the incubation at 55°C for 30 min. The temperature that caused a half inactivation of AkEG21 during 30 min incubation was ∼65°C (**Figures 3B,C**). These results indicated that AkEG21 was relatively heat-stable among the molluscan cellulases reported so far.

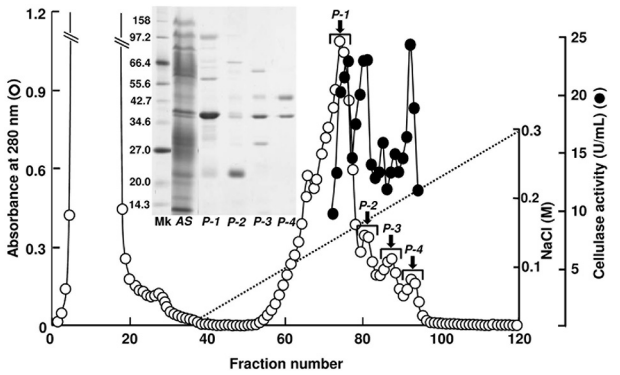


FIGURE 1 | TOYOPEARL CM-650M column chromatography for *A. kurodai* cellulase. Proteins precipitated between 40 and 60% saturation of ammonium sulfate from the crude enzyme was applied to a TOYOPEARL CM-650M column (1.5 × 20 cm) and eluted with a linear gradient of 0–0.3 M NaCl in 10 mM sodium phosphate buffer (pH 7.0) at a flow rate of 15 mL/h. Each fraction contains 7.0 mL. SDS-PAGE of the peak fractions are shown in the inset.

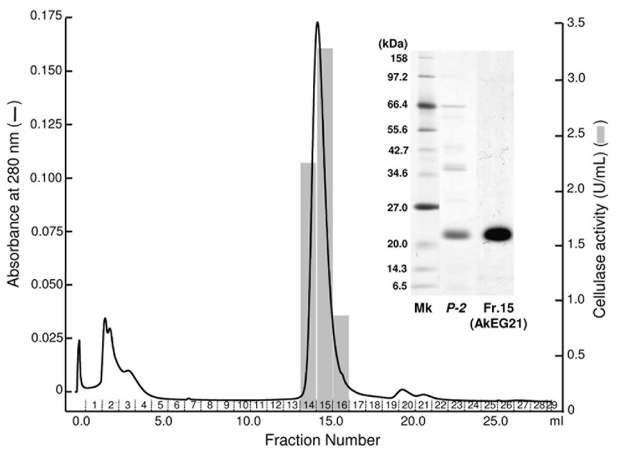


FIGURE 2 | Purification of AkEG21 by Superdex 200 gel filtration. The *P-2* fraction obtained in the TOYOPEARL CM-650M column chromatography was subjected to gel filtration through Superdex 200 10/300 GL. Fractions 14–16 were pooled as the purified AkEG21. SDS-PAGE for cellulase preparations from *A. kurodai* is shown in the inset. Mk, marker protein; *P-2*, the sample after TOYOPEARL CM-650M chromatography; *Fr. 15*, purified AkEG21.

Table 1 | Summary of purification of AkEG21.

Purification	Total protein (mg)	Total activity (U)	Specific activity (U[a]/mg)	Purification (fold)	Yield (%)
Crude enzyme[b]	1440	11,552	8.0	1	100
AS[c]	798	8912	11.2	1.4	77.2
CM[d]	8.9	529.8	59.8	7.5	4.6
Gel filtration[e]	5.6	376.1	67.3	8.4	3.3

[a] *One unit (U) of cellulase was defined as the amount of enzyme that produces reducing sugar equivalent to 1 μmol of glucose per minute from 0.5% CMC.*

[b] *Crude enzyme after the dialysis against 2 mM sodium phosphate buffer (pH 7.0).*

[c] *Fraction precipitated between 40 and 60% saturation of ammonium sulfate.*

[d] *Active fraction obtained by TOYOPEARL CM-650M chromatography.*

[e] *AkEG21 purified by the gel filtration through Superdex 200 10/300 GL.*

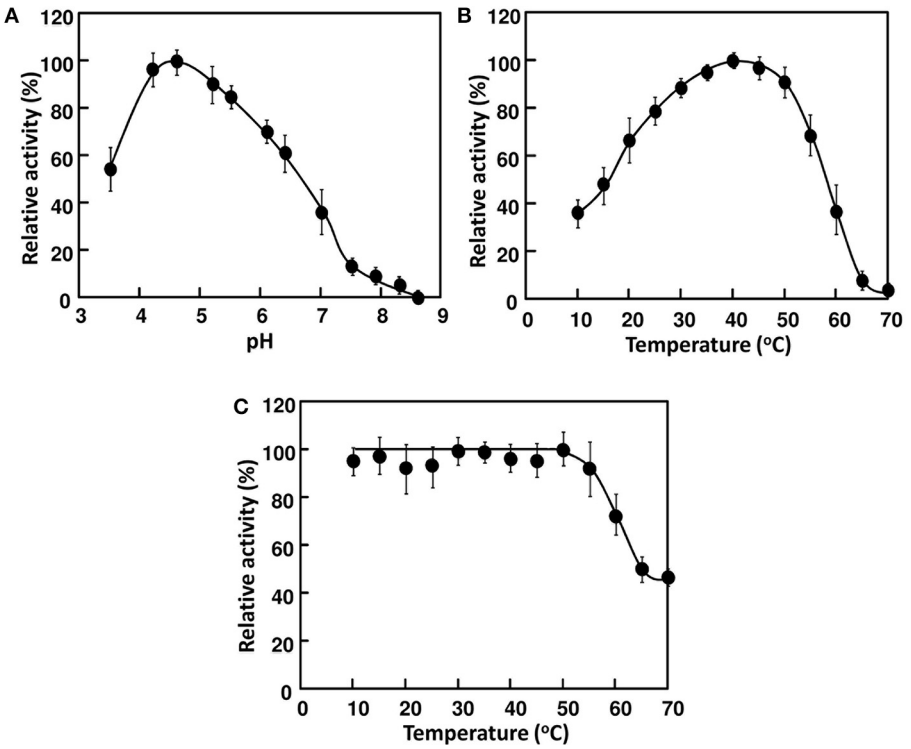


FIGURE 3 | Effects of pH and temperature on the activity of AkEG21. (A) pH dependence of AkEG21 was examined at 30°C using reaction mixtures adjusted to pH 4–10 with 50 mM sodium phosphate buffer. **(B)** Temperature dependent activity of AkEG21 was measured at 10–60°C in a reaction mixture containing 0.5% CMC and 10 mM sodium phosphate (pH 6.0). **(C)** Thermal stability of AkEG21 was assessed by measuring the activity remaining after the heat-treatment at 10–70°C for 30 min.

Degradation products of cellulose and cellooligosaccharides produced by AkEG21 were analyzed by TLC. As shown in **Figure 4A**, AkEG21 degraded amorphous cellulose producing cellobiose and cellotriose. Cellooligosaccharides larger than cellotriose were not detected in the reaction products even after 16 h incubation. On the other hand, AkEG21 showed high activity toward hexaose (G6) and pentaose (G5) and weak activity toward tetraose (G4), but no activity toward triose (G3) (**Figure 4B**). AkEG21 readily degraded G5 to G2 and G3 (plus trace amount of G4 and glucose), and degraded G6 to G2 and G4 along with small amount of G3. These degradation profiles were practically unchanged even in the longer reaction time and higher enzyme concentrations although the amounts of the products were increased (data not shown). These results indicate that AkEG21 is an endo-β-1,4-D-glucanase (EC 3.2.1.4). However, readily production of cellobiose and small amount of cellotriose from amorphous cellulose without larger intermediate oligosaccharides may indicate that this enzyme can act as cellobiohydrolase-like enzyme as suggested by Tsuji et al. (2013).

CLONING OF AkEG21 cDNA

The N-terminal amino-acid sequence of AkEG21 was determined as EQKCQPNSHGVRVYQGKKCA- by the protein sequencer (**Table 2**). This sequence shared ∼40, 40, 55, and 60% amino-acid

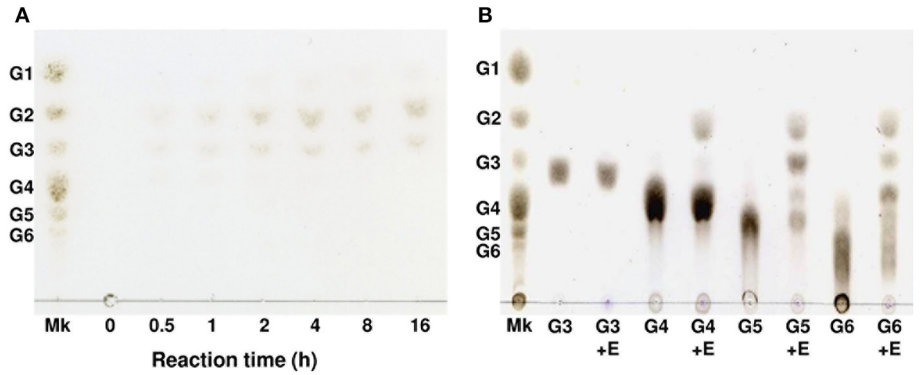


FIGURE 4 | Thin-layer chromatography of degradation products of phosphoric acid-swollen cellulose and cello-oligosaccharides produced by AkEG21. (A) The reaction mixture of 1.0 mL containing 2.5 mg of phosphoric acid-swollen cellulose, 10 mM sodium phosphate (pH 6.0), and 7 U of AkEG21 was incubated at 37°C for up to 16 h. The supernatant of the reaction mixture was subjected to TLC. **(B)** The reaction mixture of 0.05 mL containing 0.05 mg of G3–G6, 10 mM sodium phosphate (pH6.0), and 7 U of AkEG21 was incubated at 30°C for 24 h, then subjected to TLC. G1, glucose; G2, cellobiose; G3, cellotriose; G4, cellotetraose; G5, cellopentaose; G6, cellohexaose. Mk, marker sugars.

Table 2 | N-terminal and internal amino-acid sequences of AkEG21.

Peptides[a]	Amino-acid sequences	Similarity (%) to other molluscan GHF45 enzymes[b]
AkEG21	EQKCQPNSHGVRVYQGKKCA-	*A. crossean* (60.0%, 17–36) *H. discus discus* (55.0%, 18–36)
L-1	VNDHGYEAHFDLQNNK	*A. crossean* (68.8%, 143–158) *H. discus discus* (68.8%, 141–156)
L-2	LTPTGGFVPGNGK	*A. crossean* (84.6%, 95–107) *H. discus discus* (61.5%, 92–104)
T-1	CQPNSHGVR	*A. crossean* (77.8%, 20–28) *H. discus discus* (44.4%, 21–28)
T-2	VWCGQSGKPGTNK	*A. crossean* (61.5%, 130–142) *H. discus discus* (46.2%, 128–140)
T-3	YNDGHR	*A. crossean* (66.7%, 41–46) *H. discus discus* (83.3%, 41–46)

[a] L-1 and L-2, Lysylendopeptidyl fragments; T-1–T-3, tryptic fragments.

*[b] Residue numbers for corresponding regions in the sequences of A. crossean and H. discus discus (see **Figure 6**) are also shown in the parentheses.*

Table 3 | Primers used for amplification of AkEG21-cDNA.

Primer names	Sequences
PCR	
cDNA-1(Fw)	5′-AARACNCARCCNAAYWSNCAYGGNGTNMGNATG-3′[a] (KTQPNCHGVRM)[b]
cDNA-1(Rv)	5′-TCRAARTGNGCYTCRTANCCRTGRTC-3′ (DHGYEAHFD)
3′-RACE	
3Fw	5′-TGACCAATAGCTGCCCTATC-3′
3Adapt	5′-CTGATCTAGAGGTACCGGATCC-3′
5′-RACE	
5RACE(Fw2)	5′-TGGATTCGTTCCTGGCAACG-3′
5RACE(Rv2)	5′-GTTTGGACATGTTCCAGTCG-3′
CONFIRMATION	
5FullFw	5′-ATCTCAGATCTAGAGAACCC-3′
3FullRv	5′-CGCAAATCTCACGAAAATCGCG-3′

[a] R, adenine or guanine; Y, cytosine or thymine; W, adenine or thymine; S, cytosine or guanine; M, adenine or cytosine; N, adenine or guanine or cytosine or thymine.

[b] Amino-acid sequences used for designing the degenerated primers are in the parentheses.

identities to those of Eg from *M. edulis* (DDBJ accession number, CAC59694), CjCel45A from *C. japonica* (DDBJ accession number, AB468959), endo-β-1,4-glucanase 1 from *H. discus discus* (DDBJ accession number, EF103350) and EG27I from *A. crossean* (DDBJ accession number, EF471315), respectively. The sequences of tryptic and lysylendopeptidyl fragments of AkEG21 also showed 62–85% identities with the corresponding sequences of above molluscan cellulases (**Table 2**). Such high sequence similarities of partial amino-acid sequences between AkEG21 and other GHF45 cellulases suggested that AkEG21 also belongs to GHF45.

To determine the entire amino-acid sequence of AkEG21, we amplified AkEG21 cDNA by the PCR using degenerated forward and reverse primers, cDNA-1(Fw) and cDNA-1(Rv), respectively (**Table 3**). The amplified cDNA comprised 405 bp and encoded an amino-acid sequence of 135 residues. Then, 3RACE-cDNA of 519 bp covering the 3′-terminal region was amplified by 3′-RACE PCR with specific primers designed on the basis of the nucleotide sequence of the first amplified cDNA. Finally, 5RACE-cDNA of 363 bp covering 5′-terminal region was amplified by 5′-RACE PCR with a series of specific primers synthesized on the basis of the sequence of the amplified cDNA. By overlapping the nucleotide sequences of 5RACE-cDNA, first amplified cDNA and 3RACE-cDNA in this order, the nucleotide sequence of total 1013 bp including the complete translational region of AkEG21 was determined (**Figure 5**). The transcription-initiation codon (ATG) was found in the nucleotide positions from 162 to 164, while the termination codon (TGA) was in 753–755. Accordingly, the coding region of AkEG21 cDNA was found to locate in the nucleotide positions from 162 to 752 and encode 197 amino-acids. All the partial amino-acid sequences determined with

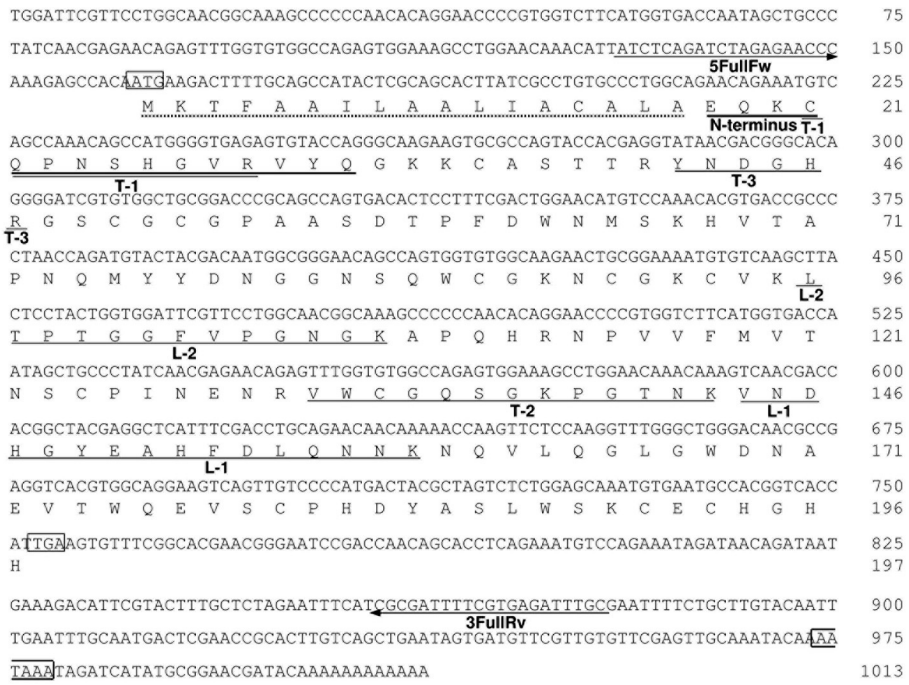


FIGURE 5 | The nucleotide and deduced amino-acid sequences of AkEG21. Residue numbers for nucleotides and amino-acids are indicated in the right of each row. The translational initiation codon ATG, termination codon TGA, and a putative polyadenylation signal AATAAA are boxed. A putative signal peptide is indicated by a dotted underline. The amino-acid sequences determined with intact AkEG21 (N-terminus) and peptide fragments (L-1, L2, T1-T-3) are indicated with solid lines under the amino-acid sequence. The positions of 5FullFw and 3FullRv primers are indicated with arrows under the nucleotide sequence. The sequence data are available from the DNA Data Bank of Japan with an accession number, AB920344.

peptide fragments, L-1, L-2, and T-1–T-3 (**Table 2**), were found in the deduced sequence (**Figure 5**). A putative polyadenylation-signal sequence (AATAAA) located at 22 nucleotides upstream from the poly $(A)^+$ tail. This suggested that the origin of AkEG21cDNA was not intestinal prokaryotes but eukaryote, i.e., *Aplysia* itself. The N-terminus of mature AkEG21 protein was identified as Glu18 in the deduced sequence indicating that the N-terminal region of 16 residues except for initiation Met was the signal peptide for secretion. Indeed, KTFAAILAALIACALA located in the N-terminus of the deduced sequence was predicted as the signal peptide by SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). Accordingly, the mature AkEG21 was concluded to comprise 180 amino-acid residues with the calculated molecular mass of 19,854.0 Da (**Figure 5**). The nucleotide and the deduced amino-acid sequences are available from the DNA Data Bank of Japan with the accession number AB920344.

The amino-acid sequence of AkEG21 was aligned with those of other molluscan GHF45 cellulases (**Figure 6**) and 47, 49, 54, and 62% identities were calculated between AkEG21 and Eg from *M. edulis* (GenBank accession number, CAC59695), CjCel45A from *C. japonica* (GenBank accession number, BAH23793), endo-β-1,4-glucanase 1 from *H. discus discus* (GenBank accession number, ABO26608), and EG27I from *A. crossean* (GenBank accession number, ABR92637), respectively. The consensus amino-acid sequence T-T-R-Y-X-D that has been shown to take part in the catalytic site of GHF45 enzymes (Girard and Jouanin, 1999; Guo et al., 2008) was conserved in AkEG21 as Thr39–Asp44. The N-glycosylation site (Asn-X-Thr/Ser) was also conserved as Asn64–Ser66 where Ans64 was the N-glycosylation residue according to the analyses with NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). Twelve Cys residues that form six disulfide bonds stabilizing the catalytic domain were also conserved in AkEG21. Two Asp residues that function as catalytic nucleophile and proton donor in GHF45 enzymes were conserved as Asp44 and Asp154, respectively, in AkEG21. These features in the amino-acid sequence of AkEG21 indicate that this enzyme belongs to GHF45.

PHYLOGENETIC ANALYSIS

To reveal the structural relationship between AkEG21 and other GHF45 cellulases, phylogenetic analysis was performed using amino-acid sequence data of GHF45 cellulases from mollusk, fungi, insects, nematode, protists and bacteria. The tree topology drawn by the maximum likelihood analysis revealed that molluscan GHF45 cellulases are assembled as a large clade (bootstrap values above 50%) with some fungal enzymes (**Figure 7**). Whereas, enzymes from insects, nematode, protists, bacteria and some other fungi formed another paraphyletic group. These clustering results suggest that molluscan GHF45 cellulases have been deviated from other animal cellulases but closely related to some fungal cellulases.

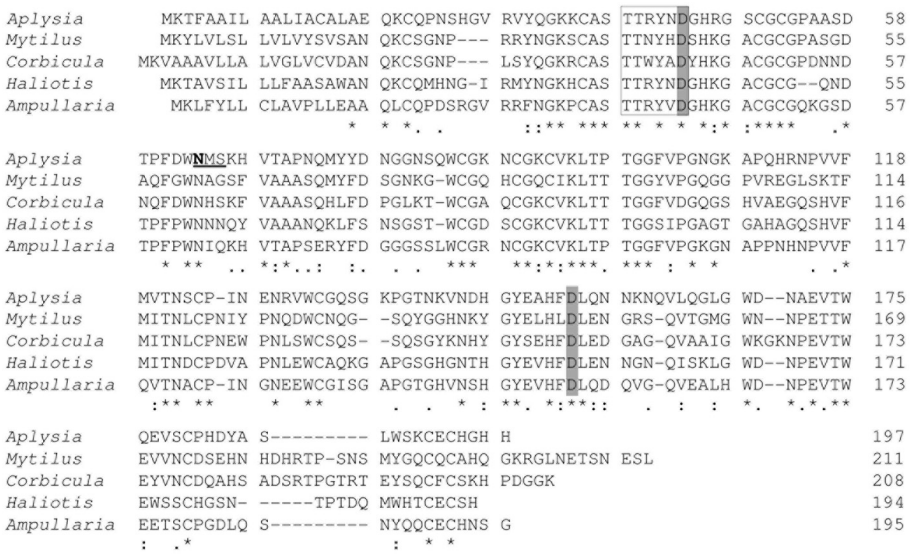


FIGURE 6 | Alignment of the amino-acid sequences of AkEG21 and other molluscan GHF45 cellulases. The amino-acid sequence of AkEG21 (DDBJ accession number, AB920344) was aligned with those of endoglucanase (Eg) from *M. edulis* (DDBJ accession number, CAC59694), CjCel45A from *C. japonica* (DDBJ accession number, AB468959), endo-β-1,4-glucanase 1 from *H. discus discus* (DDBJ accession number, EF103350), and EG27I from *A. crossean* (DDBJ accession number, EF471315). Identical, highly conservative, and conservative residues among sequences are indicated by asterisk (*), colon (:), dot (.), respectively. The consensus amino-acid sequence and residues in catalytic sites of GHF45 cellulases are boxed and shaded, respectively. The putative N-glycosylation site is underlined and N-glycosylated residue is indicated with bold letter.

DISCUSSION

The ocean that covers 70% of surface on the Earth is rich in biodiversity, e.g., organisms from 34 of 38 animal phyla are living in the ocean. Through the adaptation to diverse physical and chemical conditions of marine environments, marine organisms are believed to have deviated along with acquiring specific phenotypes. Thus, the marine organisms have become capable of producing variety of characteristic chemical compounds relating to lipids, proteins and carbohydrates, as a result of adaptation to marine environments. Such marine bio-products are promising materials for functional food additives, pharmaceutics, cosmetics, industrial materials, energy sources, etc. Among the marine bio-products, polysaccharides produced by marine algae, e.g., agar, carrageenan and alginate, which have already been used as gelling agents, viscosifiers and dietary fibers in food, are also important materials for producing functional oligosaccharides and fermentable sugars (Nishida et al., 2007; Kumagai and Ojima, 2009; Rahman et al., 2010; Zahura et al., 2010; Takeda et al., 2011; Wargacki et al., 2012; Tsuji et al., 2013; Yanagisawa et al., 2013; Enquist-Newman et al., 2014; Kumagai et al., 2014). Actually, enzymatically degraded seaweed polysaccharides were shown to exhibit beneficial activities to human (Deville et al., 2007; Wang et al., 2012; Thomas and Kim, 2013). Further, monosaccharides produced by the degradation of alginate were found to be available as a source material for ethanol fermentation (Takeda et al., 2011; Wargacki et al., 2012; Enquist-Newman et al., 2014). While, sea lettuce *Ulva pertusa* was also used as feedstock for acetone and ethanol (Yanagisawa et al., 2011; van der Wal et al., 2012). These trends have stimulated the exploration of new enzymes that convert seaweeds' polysaccharides to value-added materials.

Herbivorous mollusks produce various kinds of polysaccharide-degrading enzymes, e.g., alginate lyase (Wong et al., 2000; Shimizu et al., 2003; Suzuki et al., 2006; Rahman et al., 2010), mannanase (Zahura et al., 2010, 2011), laminarinase (Kozhemyako et al., 2004; Kovalchuk et al., 2006, 2009; Kumagai and Ojima, 2009, 2010; Pauchet et al., 2009), amylase (Kumagai et al., 2012) and cellulase (Suzuki et al., 2003; Guo et al., 2008; Sakamoto and Toyohara, 2009). Among these enzymes, cellulase appears to be most widely distributed in mollusks (Elyakova, 1972; Nishida et al., 2007; Sakamoto and Toyohara, 2009; Ravindran et al., 2010; Nagano et al., 2011). However, information about enzymatic properties and physiological significance of molluscan cellulases are still poorly understood compared with the enzymes from microbes (Tomme et al., 1995; Hong et al., 2002; Masuda et al., 2006; Fibriansah et al., 2007). In the present study, we isolated a GHF45-type cellulase AkEG21 from the common sea hare *A. kurodai* and determined its general properties.

AkEG21 showed an optimum pH at around 4.5 and more than 80% of maximal activity retained at pH range from 4.3 to 5.6. This pH range is consistent with the pH range of the digestive fluid of *A. kurodai*, i.e., pH 4–6 (Zahura et al., 2011; Tsuji et al., 2013). Although optimal pH of microbial cellulases is known to vary from acidic to alkaline range (Hurst et al., 1977; Ito et al., 1989; Park et al., 2002), those of animal GHF45 cellulases are usually in acidic pH range. For example, optimal pHs of EG27 from *A. crossean* (Li et al., 2005) and Cel45A from *M. edulis* (Xu et al., 2000) were shown at around 4.4–4.8. While, heat stability of AkEG21 was found to be considerably high, i.e., it retained more than 80% of maximal activity after the pre-incubation at

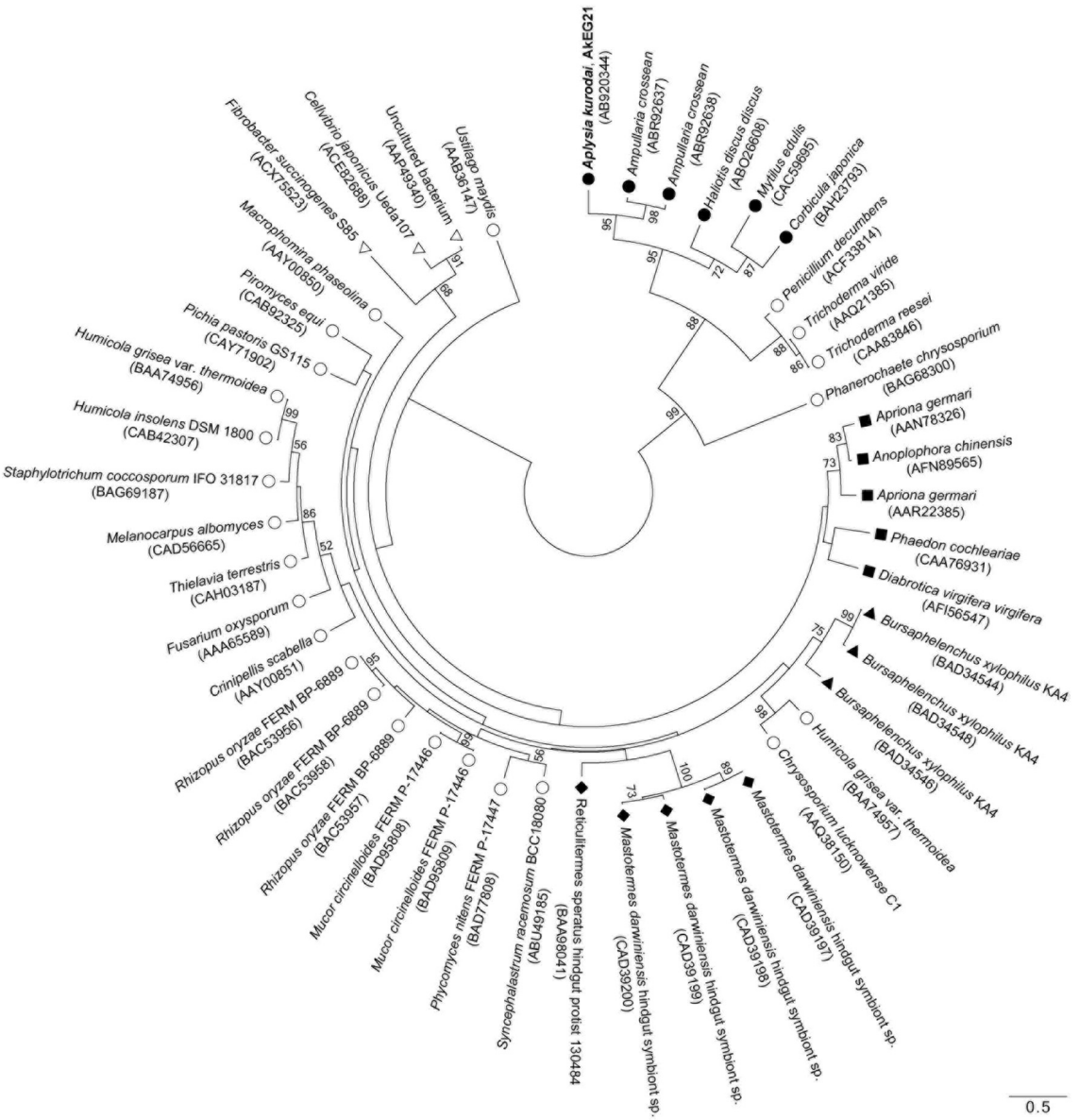


FIGURE 7 | Phylogenic relationship for GHF45 cellulases. An unrooted phylogenetic tree for catalytic domains of AkEG21 and GHF45 enzymes from CAZy database was generated using MEGA 6 software. Bootstrap values over 50 are indicated on the branches. The scale bar indicates 0.50 amino-acid substitutions. Symbols shown with scientific names of organisms indicate as follows: solid circles, mollusks; open circles, fungi; solid squares, insects; solid triangles, nematode; solid diamonds, protists; open triangles, bacteria. GenBank accession numbers for each sequence data are shown in the parentheses after the names of organisms.

55°C for 30 min and was not completely inactivated even at 70°C (**Figure 3**). Such high heat stability was also reported in EG27 from *A. crossean* (Li et al., 2005), Cel45A from *M. edulis* (Xu et al., 2000) and 21K cellulase from *A. kurodai* (Tsuji et al., 2013). EG27 retained ∼85% of maximal activity after the incubation at 60°C for 24 h (Li et al., 2005) and Cel45A retained more than 70% of the activity after the incubation in boiling water bath for 10 min (Xu et al., 2000). Such stabilities of GHF45 cellulases in acidic and high temperature conditions may be due to the formation of plural disulfide bonds in the catalytic domain. Such stability of GHF45 cellulase will be advantageous in performing both basic researches and biotechnological applications. On the other hand, *M. edulis* cellulase Cel45A was reported to show an unusual psychrophilic feature, i.e., it retains 55–60% of its maximum activity even at 0°C (Xu et al., 2000). AkEG21 also showed relatively high activity in low temperature conditions, e.g., it retained ∼40% of the maximal activity at 10°C (**Figure 3**). In this respect, molluscan GHF45 cellulases may be applicable for cellulose degradation in acidic and broad temperature conditions.

AkEG21 produced cellotriose and cellobiose as major products from amorphous cellulose and efficiently hydrolyzed cellohexaose and cellopentaose, and moderately cellotetraose, but not cellotriose and cellobiose. These indicated that AkEG21 recognized cellotetraose unit in cellulose chain and split the central glycosyl linkage of tetraose. Such substrate-recognition profiles of AkEG21 were essentially the same as those of from Cel45A from *M. edulis* (Xu et al., 2000) and 21K cellulase from *Aplysia* (Tsuji et al., 2013).

By the cDNA method, an entire amino-acid sequence of AkEG21 comprising 197 residues was predicted. The sequence of catalytic domain comprising 180 residues shared 47–62% amino-acid identities with the other molluscan GHF45 cellulases and conserved T-T-R-Y-X-D motif and two Asp residues which were identified as catalytic site and residues of GHF45 enzymes (Girard and Jouanin, 1999; Bourne and Henrissat, 2001; Guo et al., 2008) (**Figure 6**). AkEG21 possessed a typical N-glycosylation motif (Asn-X-Thr/Ser) at amino-acid positions of 64–66, and the Ans64 was predicted to be the N-glycosylation residue (**Figure 6**). The 21K cellulase from *Aplysia* was shown to be glycosylated (Tsuji et al., 2013). This indicated that AkEG21 was a glycosylated enzyme. Cel45A from *M. edulis* (Xu et al., 2000) and CjCel45A from *C. japonica* (Sakamoto and Toyohara, 2009) were also suggested to be glysocylated at the N-glycosylation sites, while no N-glycosylation site was found in GHF45 cellulase from *A. crossean* (Guo et al., 2008) and *H. discus discus* (GenBank accession number, ABO26608) (**Figure 6**). On the other hand, coleopteran GHF45 cellulases, e.g., Ag-EGase I (contain 2 N-glycosylation sites) and Ag-EGase II (contain 3 N-glycosylation sites) from *Apriona germari*, and Oa-EGase II (contain 2 N-glycosylation sites) from *Oncideresalbomarginata chamela* were found to be N-glycosylated and the N-glycosylations were important for secretion and enzyme activity (Wei et al., 2006; Calderon-Cortes et al., 2010). Previous report showed that 90% of proteins possessing the sequence Asn-X-Ser/Thr were glycosylated (Gavel and von Heijne, 1990). The roles of glycosylation are known to vary from protein to protein (Bisaria and Mishra, 1989; Wang and Gao, 2000). It is necessary to examine the physiological significance of the N-glycosylation in AkEG21 using recombinant enzymes expressed in prokaryote cells where no glycosylation takes place.

AkEG21 contained 12 Cys residues. This suggested that occurrence of six disulfide bonds in AkEG21, which may structurally stabilize the catalytic domain. All the molluscan GHF45 possess 12 Cys residues in common positions, suggesting that the stabilization by 6 disulfide bridges is a common feature among the GHF45 cellulases. Extremely high thermal stability of Cel45A from *M. edulis*, which withstands the heat-treatment at 100°C for 10 min (Xu et al., 2000), may be derived from such disulfide bonds. AkEG21 was also considerably heat stable probably due to the multiple disulfide formations.

In most organisms, cellulases are produced as modular enzymes made up of a catalytic domain and cellulose-binding domain(s) (CBD) that facilitates adsorption of the catalytic domain to insoluble cellulose (Gilkes et al., 1991; Henrissat and Davies, 2000). However, AkEG21 lacked cellulose-binding domain (CBD). Lack of CBD was also the cases of Cel45A from *M. edulis* (Xu et al., 2000), EG27 from *A. crossean* (Guo et al., 2008) and CjCel45 from *C. japonica* (Sakamoto and Toyohara, 2009). Physiological meaning of the lack of CBD in molluscan GHF45 cellulases is currently obscure; however, low affinity of enzyme to cellulose substrate may rather suitable for the turnover of enzyme in the digestive fluid to digest amorphous seaweed cellulose.

Kinds of animal digestive enzymes appeared to be closely related to the staple foods of animals (Baldwin, 1949). However, distribution of cellulase in animal kingdom was found to be more closely related to their phylogenetic relationships than their feeding habits (Yokoe and Yasumasu, 1964). GHF45 cellulases have been found in fungi, bacteria, protists, and some invertebrate animals (Henrissat and Bairoch, 1993; http://www.cazy.org/Glycoside-Hydrolases.html). Phylogenetic analysis revealed that molluscan GHF45 cellulases and some fungal enzymes were clustered as a distinct group (**Figure 7**). Such clustering of molluscan GHF45 cellulases suggested that they have evolved from the same origin. Relatively close relation between molluscan cellulases and fungal cellulases suggests that molluscan enzymes were acquired by horizontal gene transfer from fungi as suggested by Sakamoto and Toyohara (2009). On the other hand, presence of potential N-glycosylation sites in all molluscan GHF45 cellulases is in common with some coleopteran cellulases may suggest that the molluscan cellulases share the common ancestor with insect GHF45 cellulases and have diverged from them during the evolutionary process (Davison and Blaxter, 2005; Watanabe and Tokuda, 2010). Rigorous investigation is necessary before concluding that the animal cellulases are acquired by horizontal gene transfer from fungi (Ochman et al., 2000; Genereux and Logsdon, 2003).

Besides AkEG21, a GHF9 cellulase of 45 kDa was also found in the digestive fluid of *A. kurodai* (see **Figure 1**). Occurrence of multiple cellulase genes belonging to different GHFs in mollusks has already been reported (Zhang et al., 1999; Wang et al., 2003; Li et al., 2005; Sakamoto et al., 2007; Guo et al., 2008; Sakamoto and Toyohara, 2009) and synergistic action of multiple cellulases was recently reported (Tsuji et al., 2013). It may be reasonable to consider that herbivorous mollusks rely on plural cellulases to degrade cellulose to obtain carbohydrate nutrient from seaweeds. Indeed, the GHF9 cellulase of *A. kurodai* exhibited relatively higher specific activity compared with the GHF45 cellulase AkEG21 upon degradation of amorphous cellulose (Tsuji et al., 2013). Such differences in enzymatic properties were attributed to the differences in enzymatic parameters (Tsuji et al., 2013). The protein-engineering study on AkEG21 for application of this enzyme as biocatalyst for degradation of cellulosic materials from seaweeds is now under the investigation.

ACKNOWLEDGMENTS

This study was supported in part by the grants for project research (Construction of Marine Science Bases in Tohoku) of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

Agbor, V. B., Cicek, N., Sparling, R., Berlin, A., and Levin, D. B. (2011). Biomass pretreatment: fundamentals toward application. *Biotechnol. Adv.* 29, 675–685. doi: 10.1016/j.biotechadv.2011.05.005

Baldwin, E. (1949). *Dynamic Aspects of Biochemistry*. London: Cambridge University Press.

Bayer, E. A., Belaich, J. P., Shoham, Y., and Lamed, R. (2004). The cellulosomes: multi-enzyme machines for degradation of plant cell wall polysaccharides. *Ann. Rev. Microbiol.* 58, 521–554. doi: 10.1146/annurev.micro.57.030502.091021

Bhat, M. K. (2000). Cellulases and related enzymes in biotechnology. *Biotechnol. Adv.* 18, 355–383. doi: 10.1016/S0734-9750(00)00041-0

Bisaria, V. S., and Mishra, S. (1989). Regulatory aspects of cellulase biosynthesis and secretion. *Crit. Rev. Biotechnol.* 9, 61–103. doi: 10.3109/07388558909040616

Bourne, Y., and Henrissat, B. (2001). Glycoside hydrolases and glycosyltransferases: families and functional modules. *Curr. Opin. Struct. Biol.* 11, 593–600. doi: 10.1016/S0959-440X(00)00253-0

Byrne, K. A., Lehnert, S. A., Johnson, S. E., and Moore, S. S. (1999). Isolation of a cDNA encoding a putative cellulase in the red claw crayfish *Cherax quadricarinatus*. *Gene* 239, 317–324. doi: 10.1016/s0378-1119(99)00396-0

Calderon-Cortes, N., Watanabe, H., Cano-Camacho, H., Zavala-Paramo, G., and Quesada, M. (2010). cDNA cloning, homology modelling and evolutionary insights into novel endogenous cellulases of the borer beetle *Oncideresalbomarginata chamela* (Cerambycidae). *Insect Mol. Biol.* 19, 323–336. doi: 10.1111/j.1365-2583.2010.00991.x

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009). The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. *Nucleic Acids Res.* 37, D233–D238. doi: 10.1093/nar/gkn663

Castresana, C., de Carvalho, F., Gheysen, G., Habets, M., Inze, D., and Montagu, M. V. (1990). Tissue-specific and pathogen-induced regulation of a *Nicotiana plumbaginifolia* β-1,3-glucanase gene. *Plant Cell* 2, 1131–1143. doi: 10.1105/tpc.2.12.1131

Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* 17, 540–552. doi: 10.1093/oxfordjournals.molbev.a026334

Cleveland, L. R. (1924). The physiological and symbiotic relationships between the intestinal protozoa of termites and their host, with special reference to *Reticulitermes flavipes* Kollar. *Biol. Bull.* 46, 177–215.

Davison, A., and Blaxter, M. (2005). Ancient origin of glycosyl hydrolase family 9 cellulase genes. *Mol. Biol. Evol.* 21, 1273–1284. doi: 10.1093/molbev/msi107

de la Cruz, J., Pintor-Toro, J. A., Benitez, T., Llobell, A., and Romero, L. C. (1995). A novel endo-beta-1,3-glucanase, BGN13.1, involved in the mycoparasitism of *Trichoderma harzianum*. *J. Bacteriol.* 177, 6937–6945.

Deville, C., Gharbi, M., Dandrifosse, G., and Peulen, O. (2007). Study on the effects of laminarin, a polysaccharide from seaweed, on gut characteristics. *J. Sci. Food Agric.* 87, 1717–1725. doi: 10.1002/jsfa.2901

Ding, M., Teng, Y., Yin, Q., Zhao, J., and Zhao, F. (2008). The N-terminal cellulose-binding domain of EGXA increases thermal stability of xylanase and changes its specific activities on different substrates. *Acta Biochim. Biophys. Sin.* 40, 949–954. doi: 10.1111/j.1745-7270.2008.00481.x

Elyakova, L. A. (1972). Distribution of cellulases and chitinases in marine invertebrates. *Comp. Biochem. Physiol. B* 43, 67–70. doi: 10.1016/0305-0491(72)90202-7

Enquist-Newman, M., Faust, A. M., Bravo, D. D., Santos, C. N., Raisner, R. M., Hanel, A., et al., (2014). Efficient ethanol production from brown macroalgae sugars by a synthetic yeast platform. *Nature* 505, 239–243. doi: 10.1038/nature12771

Fibriansah, G., Masuda, S., Koizumi, N., Nakamura, S., and Kumasaka, T. (2007). The 1.3 Å crystal structure of a novel endo-beta-1,3-glucanase of glycoside hydrolase family 16 from alkaliphilic *Nocardiopsis* sp. strain F96. *Proteins* 69, 683–690. doi: 10.1002/prot.21589

Gavel, Y., and von Heijne, G. (1990). Sequence differences between glycosylated and non-glycosylated Asn-X-Thr/Ser acceptor sites: implications for protein engineering. *Protein Eng.* 3, 433–442. doi: 10.1093/protein/3.5.433

Genereux, D. P., and Logsdon, J. M. (2003). Much ado about bacteria-to-vertebrate lateral gene transfer. *Trends Genet.* 19, 191–195. doi: 10.1016/S0168-9525(03)00055-6

Gilkes, N. R., Henrissat, B., Kilburn, D. G., Miller, R. C. Jr., and Warren, R. A. (1991). Domains in microbial beta-1,4-glycanases: sequence conservation, function, and enzyme families. *Microbiol. Rev.* 55, 303–315.

Girard, C., and Jouanin, L. (1999). Molecular cloning of cDNAs encoding a range of digestive enzymes from a phytophagous beetle, *Phaedon cochleariae*. *Insect Biochem. Mol. Biol.* 29, 1129–1142. doi: 10.1016/S0965-1748(99)00104-6

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* 177, 751–766.

Gueguen, Y., Chemardin, P., Labrot, P., Arnaud, A., and Galzy, P. (1997). Purification and characterization of an intracellular β-glucosidase from a new strain of *Leuconostoc mesenteroides* isolated from cassava. *J. Appl. Microbiol.* 82, 469–476. doi: 10.1046/j.1365-2672.1997.00136.x

Guo, R., Ding, M., Zhang, S. L., Xu, G. J., and Zhao, F. K. (2008). Molecular cloning and characterization of two novel cellulase genes from the mollusc *Ampullaria crossean*. *J. Comp. Physiol. B* 178, 209–215. doi: 10.1007/s00360-007-0214-z

Henrissat, B., and Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 293, 781–788.

Henrissat, B., Claeyssens, M., Tomme, P., Lemesle, L., and Mornon, J. P. (1989). Cellulase families revealed by hydrophobic cluster analysis. *Gene* 81, 83–95.

Henrissat, B., and Davies, G. J. (2000). Glycoside hydrolases and glycosyltransferases. Families, modules, and implications for genomics. *Plant Physiol.* 124, 1515–1519. doi: 10.1104/pp.124.4.1515

Henrissat, B. A. (1991). A Classification of glycosyl hydrolases based on amino acid sequence similarities. *Biochem. J.* 280, 309–316.

Hong, T. Y., Cheng, C. W., Huang, J. W., and Meng, M. (2002). Isolation and biochemical characterization of an endo-1,3-beta-glucanase from *Streptomyces sioyaensis* containing a C-terminal family 6 carbohydrate-binding module that binds to 1,3-beta-glucan. *Microbiology* 148, 1151–1159.

Hurst, P. L., Nielsen, J., Sullivan, P. A., and Shepherd, M. G. (1977). Purification and properties of a cellulase from *Aspergillus niger*. *Biochem. J.* 165, 33–41.

Imjongjirak, C., Amparyup, P., and Sittipraneed, S. (2008). Cloning, genomic organization and expression of two glycosyl hydrolase family 10 (GHF10) genes from golden apple snail (*Pomacea canaliculata*). *DNA Seq.* 19, 214–236. doi: 10.1080/10425170701517911

Ito, S., Shikata, S., Ozaki, K., Kawai, S., Okamoto, K., Inoue, S., et al. (1989). Alkaline cellulase for laundry detergent: production by *Bacillus* sp. KSM-635 and enzymatic properties. *Agric. Biol. Chem.* 53, 1275–1281.

Jagtap, S., and Rao, M. (2005). Purification and properties of a low molecular weight β-1,4-glucan glucohydrolase having one active site for carboxymethyl cellulose and xylan from an alkalothermophilic *Thermomonospora* sp. *Biochem. Biophys. Res. Commun.* 329, 111–116. doi: 10.1016/j.bbrc.2005.01.102

Kikuchi, T., Jones, J., Aikawa, T., Kosaka, H., and Ogura, N. (2004). A family of glycosyl hydrolase family 45 cellulases from the pine wood nematode *Bursaphelenchus xylophilus*. *FEBS Lett.* 572, 201–205. doi: 10.1016/j.febslet.2004.07.039

Kovalchuk, S. N., Bakunina, I. Y., Burtseva, Y. V., Emelyanenko, V. I., Kim, N. Y., Guzev, K. V., et al. (2009). An *endo*-(1→3)-β-$_D$-glucanase from the scallop *Chlamys albidus*: catalytic properties, cDNA cloning and secondary-structure characterization. *Carbohydr. Res.* 344, 191–197. doi: 10.1016/j.carres.2008.10.028

Kovalchuk, S. N., Sundukova, E. V., Kusaykin, M. I., Guzev, K. V., Anastiuk, S. D., Likhatskaya, G. N., et al. (2006). Purification, cDNA cloning and homology modelling of endo-1,3-β-$_D$-glucanase from scallop *Mizuhopecten yessoensis*. *Comp. Biochem. Physiol. B* 143, 473–485. doi: 10.1016/j.cbpb.2005.12.024

Kozhemyako, V. B., Rebrikov, D. V., Lukyanov, S. A., Bogdanova, E. A., Marin, A., Mazur, A. K., et al. (2004). Molecular cloning and characterization of an endo-1,3-β-D-glucanase from the mollusk *Spisula sachalinensis*. *Comp. Biochem. Physiol. B* 137, 169–178. doi: 10.1016/j.cbpc.2003.10.018

Kuhad, R. C., Gupta, R., and Singh, A. (2011). Microbial cellulases and their industrial applications. *Enzym. Res.* 2011, 1–10. doi: 10.4061/2011/280696

Kumagai, Y., and Ojima, T. (2009). Enzymatic properties and the primary structure of a β-1,3-glucanases from the digestive fluid of the Pacific abalone *Haliotis discus hannai*. *Comp. Biochem. Physiol. B* 154, 113–120. doi: 10.1016/j.cbpb.2009.05.005

Kumagai, Y., and Ojima, T. (2010). Isolation and characterization of two types of beta-1,3-glucanases from the common sea hare Aplysia kurodai. *Comp. Biochem. Physiol. B* 155, 138–144. doi: 10.1016/j.cbpb.2009.10.013

Kumagai, Y., Satoh, T., Inoue, A., and Ojima, T. (2012). Enzymatic properties and primary structures of two α-amylase isozymes from the Pacific abalone Haliotis discus hannai. *Comp. Biochem. Physiol. B* 164, 80–88. doi: 10.1016/j.cbpb.2012.11.001

Kumagai, Y., Satoh, T., Inoue, A., and Ojima, T. (2014). A laminaribiose-hydrolyzing enzyme, AkLab, from the common sea hare *Aplysia kurodai* and its transglycosylation activity. *Comp. Biochem. Physiol. B* 167, 1–7. doi: 10.1016/j.cbpb.2013.07.008

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948. doi: 10.1093/bioinformatics/btm404

Li, L., Fröhlich, J., Pfeiffer, P., and König, H. (2003). Termite gut symbiotic archaezoa are becoming living metabolic fossils. *Eukaryot. Cell* 2, 1091–1098. doi: 10.1128/EC.2.5.1091-1098.2003

Li, Y., Yin, Q., Ding, M., and Zhao, F. (2009). Purification, characterization and molecular cloning of a novel endo-β-1,4-glucanases AC-EG65 from the mollusc *Ampullaria crossean*. *Comp. Biochem. Physiol. B* 153, 149–156. doi: 10.1016/j.cbpb.2009.02.011

Li, Y. H., Guo, R., Yin, Q. Y., Ding, M., Zhang, S. L., Xu, G. J., et al. (2005). Purification and characterization of two endo-β-1,4-glucanases from mollusca, *Ampullaria crossean*. *Acta Biochem. Biophys. Sin.* 37, 702–708. doi: 10.1111/j.1745-7270.2005.00099.x

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem*. 193, 265–275.

Lynd, L. R., Weimer, P. J., vanZyl, W. H., and Pretorious, I. S. (2002). Microbial cellulose utilization: fundamentals and biotechnology. *Microbiol. Mol. Biol. Rev*. 66, 506–577. doi: 10.1128/MMBR.66.3.506-577.2002

Martin, M. M., and Martin, J. S. (1978). Cellulose digestion in the midgut of the fungus-growing termite *Macrotermes natalensis*: the role of acquired digestive enzymes. *Science* 199, 1453–1455. doi: 10.1126/science.199.4336.1453

Masuda, S., Endo, K., Koizumi, N., Hayami, T., Fukazawa, T., Yatsunami, R., et al. (2006). Molecular identification of a novel β-1,3-glucanase from alkaliphilic *Nocardiopsis* sp. strain F96. *Extremophiles* 10, 251–255. doi: 10.1007/s00792-006-0514-3

Matthysse, A. G., Deschet, K., Williams, M., Marry, M., White, A. R., and Smith, W. C. (2004). A functional cellulose synthase from ascidian epidermis. *Proc. Natl. Acad. Sci. U.S.A.* 101, 986–991. doi: 10.1073/pnas.0303623101

Michel, G., and Czjzek, M. (2013). "Polysaccharide-degrading enzymes from marine bacteria," in *Marine Enzymes for Biocatalysis: Sources, Biocatalytic Characteristics and Bioprocesses of Marine Enzymes*, ed A. Trincone (Philadelphia, PA: Woodhead Publishing Series in Biomedicine, Number 38), 429–464.

Mojsov, K. (2012). Microbial cellulases and their applications in textile processing. *Int. J. Mark. Technol*. 2, 1219.

Nagano, N., Matsui, S., Kuramura, T., Taoka, Y., Honda, D., and Hayashi, M. (2011). The distribution of extracellular cellulase activity in marine *Eukaryotes*, *thraustochytrids*. *Mar. Biotechnol*. 13, 133–136. doi: 10.1007/s10126-010-9297-8

Nishida, Y., Suzuki, K., Kumagai, Y., Tanaka, H., Inoue, A., and Ojima, T. (2007). Isolation and primary structure of a cellulase from the Japanese sea urchin *Strongylocentrotus nudus*. *Biochimie* 89, 1002–1011. doi: 10.1016/j.biochi.2007.03.015

Ochman, H., Lawrence, J. G., and Groisman,. E. A. (2000). Lateral gene transfer and the nature of bacterial innovation. *Nature* 405, 299–304. doi: 10.1038/35012500

Ojima, T. (2013). "Polysaccharide-degrading enzymes from herbivorous marine invertebrates," in *Marine Enzymes for Biocatalysis: Sources, Biocatalytic Characteristics and Bioprocesses of Marine Enzymes*, ed A. Trincone (Philadelphia, PA: Woodhead Publishing Series in Biomedicine, Number 38), 333–371.

Park, J. T., and Johnson, M. J. (1949). A submicrodetermination of glucose. *J. Biol. Chem*. 181, 149–151.

Park, S. R., Cho, S. J., Kim, M. K., Ryu, S. K., Lim, W. J., An, C. L., et al. (2002). Activity enhancement of Cel5Z from *Pectobacterium chrysanthemi* PY35 by removing C-terminal region. *Biochem. Biophys. Res. Commun*. 291, 425–430. doi: 10.1006/bbrc.2002.6437

Pauchet, Y., Freitak, D., Heidel-Fischer, H. M., Heckel, D. G., and Vogel, H. (2009). Immunity or digestion: glucanase activity in a glucan-binding protein family from lepidoptera. *J. Biol. Chem*. 284, 2114–2124. doi: 10.1074/jbc.M806204200

Perez, J., Munoz-Dorado, J., de la Rubia, T., and Martinez, J. (2002). Biodegradation and biological treatments of cellulose, hemicellulose, and lignin: an overview. *Int. Microbiol*. 5, 53–63. doi: 10.1007/s10123-002-0062-3

Pesis, E., Fuchs, Y., and Zauberman, G. (1978). Cellulase activity and fruit softening in avocado. *Plant Physiol*. 61, 416–419. doi: 10.1104/pp.61.3.416

Porzio, M. A., and Pearson, A. M. (1977). Improved resolution of myofibrillar proteins with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochim. Biophys. Acta* 490, 27–34. doi: 10.1016/0005-2795(77)90102-7

Rahman, M. M., Inoue, A., Tanaka, H., and Ojima, T. (2010). Isolation and characterization of two alginate lyase isozymes, AkAly28 and AkAly33, from the common sea hare *Aplysia kurodai*. *Comp. Biochem. Physiol. B* 157, 317–325. doi: 10.1016/j.cbpb.2010.07.006

Ravindran, C., Naveenan, T., and Varatharajan, G. R. (2010). Optimization of alkaline cellulase production by the marine-derived fungus *Chaetomium* sp. Using agricultural and industrial wastes as substrates. *Bot. Mar*. 53, 275–282. doi: 10.1515/BOT.2010.026

Sakamoto, K., Touhata, K., Yamashita, M., Kasai, A., and Toyohara, H. (2007). Cellulose digestion by common Japanese freshwater clam *Corbicula japonica*. *Fish. Sci*. 73, 675–683. doi: 10.1111/j.1444-2906.2007.01381.x

Sakamoto, K., and Toyohara, K. (2009). Molecular cloning of glycoside hydrolase family 45 cellulase genes from brackish water clam *Corbicula japonica*. *Comp. Biochem. Physiol. B* 152, 390–396. doi: 10.1016/j.cbpb.2009.01.010

Scholl, E. H., Thorne, J. L., McCarter, J. P., and Bird, D. M. (2003). Horizontally transferred genes in plant-parasitic nematodes: a high-throughput genomic approach. *Genome Biol*. 4, R39.1–R39.12. doi: 10.1186/gb-2003-4-6-r39

Sheehan, J., and Himmel, M. (1999). Enzymes, energy, and the environment: a strategic perspective on the U.S. Department of Energy's research and development activities for bioethanol. *Biotechnol. Prog*. 15, 817–827. doi: 10.1021/bp990110d

Shimizu, E., Ojima, T., and Nishita, K. (2003). cDNA cloning of an alginate lyase from abalone, *Haliotis discus hannai. Carbohydr. Res.* 338, 2841–2852. doi: 10.1016/j.carres.2003.08.009

Smant, G., Stokkermans, J. P., Yan, Y., de Boer, J. M., Baum, T. J., Wang, X., et al. (1998). Endogenous cellulases in animals: isolation of β-1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes. *Proc. Natl. Acad. Sci. U.S.A*. 95, 4906–4911. doi: 10.1073/pnas.95.9.4906

Sugimura, M., Watanabe, H., Lo, N., and Saito, H. (2003). Purification, characterization, cDNA cloning and nucleotide sequencing of a cellulase from the yellow spotted longicorns beetle, *Psacothea hilaris*. *Eur. J. Biochem*. 270, 3455–3460. doi: 10.1046/j.1432-1033.2003.03735.x

Suzuki, H., Suzuki, K., Inoue, A., and Ojima, T. (2006). A novel oligoalginate lyase from abalone, Haliotis discus hannai, that releases disaccharide from alginate polymer in an exolytic manner. *Carbohydr. Res*. 14, 1809–1819. doi: 10.1016/j.carres.2006.04.032

Suzuki, K., Ojima, T., and Nishita, K. (2003). Purification and cDNA cloning of a cellulase from abalone *Haliotis discus hannai*. *Eur. J. Biochem*. 270, 771–778. doi: 10.1046/j.1432-1033.2003.03443.x

Takeda, H., Yoneyama, F., Kawai, S., Hashimoto, W., and Murata, K. (2011). Bioethanol production from marine biomass alginate by metabolically engineered bacteria. *Energy Environ. Sci*. 4, 2575–2581. doi: 10.1039/C1EE01236C, Paper

Talavera, G., and Castresana, J. (2007). Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Syst. Biol*. 56, 564–577. doi: 10.1080/10635150701472164

Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol*. 30, 2725–2729. doi: 10.1093/molbev/mst197

Thomas, N. V., and Kim, S. K. (2013). Beneficial effects of marine algal compounds in cosmeceuticals. *Mar. Drugs* 11, 146–164. doi: 10.3390/md11010146

Tokuda, G., Lo, N., Watanabe, H., Slaytor, M., Matsumoto, T., and Noda, H. (1999). Metazoan cellulase genes from termites: intron/exon structures and sites of expression. *Biochim. Biophys. Acta* 1447, 146–159. doi: 10.1016/S0167-4781(99)00169-4

Tomme, P., Warren, R. A., and Gilkes, N. R. (1995). Cellulose hydrolysis by bacteria and fungi. *Adv. Microb. Physiol*. 37, 1–81. doi: 10.1016/S0065-2911(08)60143-5

Tsuji, A., Tominaga, K., Nishiyama, N., and Yuasa, K. (2013). Comprehensive enzymatic analysis of the cellulolytic system in digestive fluid of the Sea Hare *Aplysia kurodai*. Efficient glucose release from sea lettuce by synergistic action of 45 kDa endoglucanase and 210 kDa ß-glucosidase. *PLoS ONE* 8:e65418. doi: 10.1371/journal.pone.0065418

van der Wal, H., Sperber, B. L., Houweling-Tan, B., Bakker, R. R., Brandenburg, W., and López-Contreras, A. M. (2012). Production of acetone, butanol, and ethanol from biomass of the green seaweed *Ulva lactuca*. *Bioresour. Technol*. 128, 431–437. doi: 10.1016/j.biortech.2012.10.094

Wang, J., Ding, M., Li, Y. H., Chen, Q. X., Xu, G. J., and Zhao, F. K. (2003). Isolation of a multifunctional endogenous cellulase gene from mollusc, *Ampullaria crossean*. *Acta Biochim. Biophys. Sin*. 35, 941–946.

Wang, J., and Gao, P. (2000). *In vitro* expression of *Penicillium janthinellum* cellobiohydrolase I gene in a coupled transcription–translation system. *J. Biotechnol*. 81, 205–209. doi: 10.1016/S0168-1656(00)00285-6

Wang, W., Wang, S. X., and Guan, H. S. (2012). The antiviral activities and mechanisms of marine polysaccharides: an overview. *Mar. Drugs.* 10, 2795–2816. doi: 10.3390/md10121795

Wargacki, A. J., Leonard, E., Win, M. N., Regitsky, D. D., Santos, C. N., Kim, P. B., et al. (2012). An engineered microbial platform for direct biofuel production from brown macroalgae. *Science* 335, 308–313 doi: 10.1126/science.1214547

Watanabe, H., Noda, H., Tokuda, G., and Lo, N. (1998). A cellulase gene of termite origin. *Nature* 394, 330–331. doi: 10.1038/28527

Watanabe, H., and Tokuda, G. (2001). Animal cellulases. *Cell. Mol. Life Sci*. 58, 1167–1178. doi: 10.1007/PL00000931

Watanabe, H., and Tokuda, G. (2010). Cellulolytic systems in insects. *Ann. Rev. Entomol*. 55, 609–632. doi: 10.1146/annurev-ento-112408-085319

Wei, Y. D., Lee, K. S., Gui, Z. Z., Yoon, H. J., Kim, I., Je, Y. H., et al. (2006). N-linked glycosylation of a beetle (*Apriona germari*) cellulase Ag-EGase II is necessary for enzymatic activity. *Insect Biochem. Mol. Biol*. 36, 435–441. doi: 10.1016/j.ibmb.2006.03.007

Wong, T. Y., Preston, L. A., and Schiller, N. L. (2000). ALGINATE LYASE: review of major sources and enzyme characteristics, structure-function analysis, biological roles, and applications. *Annu. Rev. Microbiol*. 54, 289–340. doi: 10.1146/annurev.micro.54.1.289

Xu, B., Hellman, U., Ersson, B., and Janson, J. C. (2000). Purification, characterization and amino-acid sequence analysis of a thermostable, low molecular mass endo-β-1,4-glucanase from blue mussel, *Mytilus edulis*. *Eur. J. Biochem*. 267, 4970–4977. doi: 10.1046/j.1432-1327.2000.01533.x

Xu, B., Janson, J. C., and Sellos, D. (2001). Cloning and sequencing of a molluscan endo-beta-1,4glucanase gene from the blue mussel, *Mytilus edulis*. *Eur. J. Biochem*. 268, 3718–3727. doi: 10.1046/j.1432-1327.2001.02180.x

Yan, Y., Smant, G., Stokkermans, J., Qin, L., Helder, J., Baum, T., et al. (1998). Genomic organization of four β-1,4-endoglucanase genes in plant-parasitic cyst nematodes and its evolutionary implications. *Gene* 210, 61–70. doi: 10.1016/S0378-1119(98)00413-2

Yanagisawa, M., Kawai, S., and Murata, K. (2013). Strategies for the production of high concentrations of bioethanol from seaweeds: production of high concentrations of bioethanol from seaweeds. *Bioengineered* 4, 214–235. doi: 10.4161/bioe.23396

Yanagisawa, M., Ojima, T., and Nakasaki, K. (2011). Bioethanol from sea lettuce with use of crude enzymes derived from waste. *J. Mat. Cycl. Waste Man*. 14, 321–326. doi: 10.1007/s10163-011-0026-9

Yang, B., Dai, Z., Ding, S. Y., and Wyman, C. E. (2011). Enzymatic hydrolysis of cellulosic biomass. *Biofuels* 2, 421–450. doi: 10.4155/bfs.11.116

Yokoe, Y., and Yasumasu, I. (1964). The distribution of cellulase in invertebrates. *Comp. Biochem. Physiol.* 13, 323–338. doi: 10.1016/0010-406X(64)90027-1

Zahura, U. A., Rahman, M. M., Inoue, A., Tanaka, H., and Ojima, T. (2010). An endo-β-1,4- mannanase, AkMan, from the common sea hare *Aplysia kurodai*. *Comp. Biochem. Physiol. B* 157, 137–143. doi: 10.1016/j.cbpb.2010.05.012

Zahura, U. A., Rahman, M. M., Inoue, A., Tanaka, H., and Ojima, T. (2011). cDNA cloning and bacterial expression of an endo-β-1,4-mannanase, AkMan, from *Aplysia kurodai*. *Comp. Biochem. Physiol. B* 159, 217–235. doi: 10.1016/j.cbpb.2011.05.001

Zaldivar, J., Nielsen, J., and Olsson, L. (2001). Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. *Appl. Microbiol. Biotechnol*. 56, 17–34. doi: 10.1007/s002530100624

Zhang, Z., Zhao, H., Zhou, X. W., Chen, L. F., Chen, T. S., and Chen, Q. X. (1999). Preliminary studies on isolation, purification and some properties of β-glucosidase from *Ampullarium crossean*. *J. Xiamen. Univ*. 38, 287–291.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 18 June 2014; paper pending published: 28 June 2014; accepted: 15 July 2014; published online: 06 August 2014.

Citation: Rahman MM, Inoue A and Ojima T (2014) Characterization of a GHF45 cellulase, AkEG21, from the common sea hare Aplysia kurodai. Front. Chem. ***2****:60. doi: 10.3389/fchem.2014.00060*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

ORIGINAL RESEARCH ARTICLE
published: 26 August 2014
doi: 10.3389/fchem.2014.00067

Discovery of a novel iota carrageenan sulfatase isolated from the marine bacterium *Pseudoalteromonas carrageenovora*

Sabine M. Genicot[1,2]*, Agnès Groisillier[1,2], Hélène Rogniaux[3], Laurence Meslet-Cladière[1,2,4], Tristan Barbeyron[1,2] and William Helbert[1,2,5]

[1] UMR 8227, Integrative Biology of Marine Models, Station Biologique de Roscoff, Sorbonne Universités, UPMC Université Paris 06, Roscoff, France
[2] UMR 8227, Centre National de la Recherche Scientifique, Integrative Biology of Marine Models, Station Biologique de Roscoff, Roscoff, France
[3] INRA, UR1268 Biopolymers Interactions Assemblies, Nantes, France
[4] EA3882, LUBEM, Technopôle Brest-Iroise, Université de Bretagne Occidentale, Plouzané, France
[5] Centre de Recherches sur les Macromolécules Végétales (CERMAV, UPR-CNRS 5301), Université Joseph Fourier, and Member of the Institut de Chimie Moléculaire de Grenoble (ICMG, FR-CNRS 2607), Grenoble, France

Edited by:
Antonio Trincone, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Italy

Reviewed by:
David J. Vocadlo, Simon Fraser University, Canada
Zvyagintseva Tatiana, Far-Eastern Branch, The Russian Academy of Sciences, Russia

***Correspondence:**
Sabine M. Genicot, UMR 8227, Centre National de la Recherche Scientifique, Integrative Biology of Marine Models, Station Biologique de Roscoff, Place Georges Teissier, 29680 Roscoff, France
e-mail: genicot@sb-roscoff.fr

Carrageenans are sulfated polysaccharides extracted from the cell wall of some marine red algae. These polysaccharides are widely used as gelling, stabilizing, and viscosifying agents in the food and pharmaceutical industries. Since the rheological properties of these polysaccharides depend on their sulfate content, we screened several isolated marine bacteria for carrageenan specific sulfatase activity, in the aim of developing enzymatic bioconversion of carrageenans. As a result of the screening, an iota-carrageenan sulfatase was detected in the cell-free lysate of the marine bacterium *Pseudoalteromonas carrageenovora* strain Psc^T. It was purified through Phenyl Sepharose and Diethylaminoethyl Sepharose chromatography. The pure enzyme, *Psc* ι-CgsA, was characterized. It had a molecular weight of 115.9 kDaltons and exhibited an optimal activity/stability at pH ∼8.3 and at 40 ± 5°C. It was inactivated by phenylmethylsulfonyl fluoride but not by ethylene diamine tetraacetic acid. *Psc* ι-CgsA specifically catalyzes the hydrolysis of the 4-S sulfate of iota-carrageenan. The purified enzyme could transform iota-carrageenan into hybrid iota-/alpha- or pure alpha-carrageenan under controlled conditions. The gene encoding *Psc* ι-CgsA, a protein of 1038 amino acids, was cloned into *Escherichia coli*, and the sequence analysis revealed that *Psc* ι-CgsA has more than 90% sequence identity with a putative uncharacterized protein Q3IKL4 from the marine strain *Pseudoalteromonas haloplanktis* TAC 125, but besides this did not share any homology to characterized sulfatases. Phylogenetic studies show that *P. carrageenovora* sulfatase thus represents the first characterized member of a new sulfatase family, with a C-terminal domain having strong similarity with the superfamily of amidohydrolases, highlighting the still unexplored diversity of marine polysaccharide modifying enzymes.

Keywords: sulfatase, novel family, iota carrageenan, marine bacteria, sulfated polysaccharides, bioconversion

INTRODUCTION

Although sulfated biomolecules are present throughout the tree of life in terrestrial and marine environments, most of the sulfated carbohydrates are found in the marine environment, as illustrated by the structural diversity of sulfated polysaccharides encountered in the cell wall of macroalgae (Lahaye and Robic, 2007; Pomin and Mourao, 2008; Popper et al., 2011; Usov, 2011). As such polysaccharides are absent in the cell wall of fresh water and land plants; their loss has been postulated to be a consequence of the land colonization (Michel et al., 2010). These polysaccharides are thought to be involved in the phenomena of ionic and osmotic regulations. They are supposed to confer to the algae resistance to water currents and, due to their highly soluble nature, they allow water retention thereby slowing the drying of the algae at low tide (Kloareg and Quatrano, 1988).

The cell wall sulfated galactans of the red algae, referred to as agars or carrageenans, consist of a linear backbone of galactose residues linked by alternating β-1,4 and α-1,3 glycosidic bonds. While all the β-linked residues are in the D configuration (G monomer), the α-linked galactose units are in the L configuration in agars (L monomer) and in the D configuration in carrageenans (D monomer). In carrageenans, the repeating disaccharide units are classified according to the number and the position of ester sulfate (S) and by the presence of a 3,6-anhydro-bridge (DA) in the 4-linked residue (Knutsen et al., 1994). DA units are found in gelling carrageenans such as the kappa (κ; G4S-DA) and the

iota (ι; G4S-DA2S)-carrageenan (**Figure 1**). Other substitutions, such as methyl or pyruvate groups, have also been observed, increasing the diversity of carrageenans which depends also on the algal source, the growth conditions and the extraction procedures (Pereira and Mesquita, 2004; Pereira et al., 2009). The physico-chemical properties of carrageenans, which are extensively used as thickeners and stabilizers in the food and cosmetic industries (de Ruiter and Rudolph, 1997), depend on their molecular weight, the occurrence of anhydrogalactose and their sulfate content and it is well established that higher levels of ester sulfate induce a decrease of the gel strength (Necas and Bartosikova, 2013). The use of specific enzymes to modify the sulfate pattern of carrageenans would therefore offer a biotechnological approach to control their sulfate content and thereby their rheological properties.

The heterogeneous structure of carrageenans, their gelling properties, and their interactions with the other components of red algal cell walls challenge the microorganisms using these polymers as carbon and energy sources. To breakdown the complex polysaccharides, marine bacteria secrete specific glycoside hydrolases (GHs), referred to as agarases and carrageenases, which catalyze the hydrolysis of the β-1,4 glycosidic bond between two galactopyranose units of their respective substrate (Michel et al., 2006). However, these enzymes are not sufficient alone to lead to the complete substrate assimilation. As revealed by the increasing number of sequenced marine microbial genomes, marine bacteria possess a large number of sulfatases. Although their precise function has not been elucidated yet, it is likely that they play an important role in the degradation of algal sulfated polysaccharides (Glöckner et al., 2003).

While genomic and metagenomic approaches offer promising strategies for marine biodiscovery (Ekborg et al., 2006; Shin et al., 2010), there are still some limitations, as screening of such libraries is indeed either sequence based or function based (Kennedy et al., 2008). These limitations sometimes do not allow assigning new functions to proteins annotated as hypothetical, and in such cases, it is still necessary to go through the isolation and purification of a defined activity in order to ascribe a function to a gene.

As both exo- and endo-acting sulfatases have been demonstrated in the case of glycoaminoglycans, we postulate their existence in the case of carrageenans. We therefore screened marine bacteria for endo-sulfatases to specifically modify the sulfation pattern of carrageenans in a polymeric state. In a previous work, a carrageenan sulfatase converting ι- in α-carrageenan was isolated from *P. atlantica* T6C and recombinantly overexpressed (Préchoux et al., 2013). Analysis of its mode of action confirmed the endo-character of this sulfatase removing the sulfate ester groups, most likely in a random pattern along the polysaccharide chain. Aiming at monitoring the physico-chemical properties of carrageenan, we were looking for further sulfatases potentially having different properties as this would allow to fine tune the rheological properties of these hydrocolloids or to adapt to different industrial conditions. In this context, we isolated and purified to homogeneity an endo-sulfatase from the marine bacterium *P. carrageenovora* Psc^T. Despite the fact that this enzyme has the same specificity as the ι-carrageenan sulfatase from *P. atlantica* (Préchoux et al., 2013), sequence analysis revealed that the *P. carrageenovora* sulfatase did not share any homology to already known sulfatases. It has, however, more than

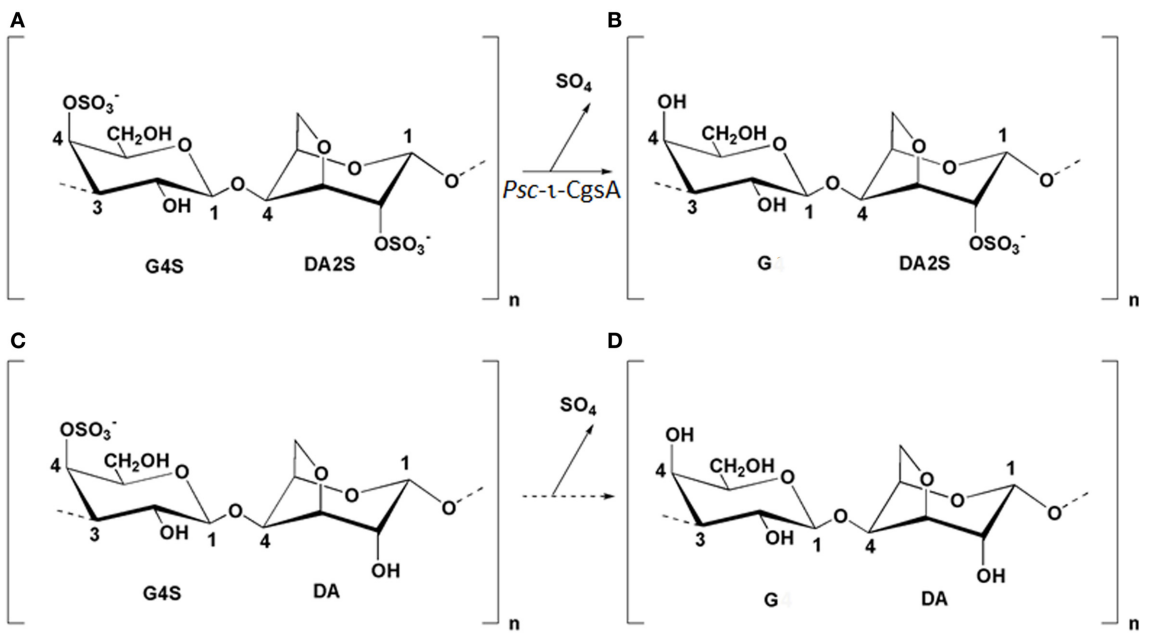


FIGURE 1 | Structure of the idealized repeating units of ι-(G4S-DA2S) (A), α-(G-DA2S) (B), κ-(G4S-DA) (C), and β-(G-DA) (D) carrageenans. The arrow between G4S-DA2S and G-DA2S illustrates the reaction catalyzed by *Psc* ι-CgsA. Enzymatic production of β-carrageenan **(D)** from κ-carrageenan **(C)** has not been yet demonstrated and is represented by a dashed arrow.

90% sequence identity with a putative uncharacterized protein, Q3IKL4, from the marine strain *Pseudoalteromonas haloplanktis* TAC 125. Phylogenetic studies show that *P. carrageenovora* sulfatase thus represents the first characterized member of a new sulfatase family, with a C-terminal domain having strong similarity with the superfamily of amidohydrolases, highlighting the still unexplored diversity of marine polysaccharide modifying enzymes.

MATERIALS AND METHODS

MATERIALS

λ-carrageenan (GENU X-7055) was extracted from tetrasporophytic plants of *Gigartina skottsbergii*, κ-carrageenan (GENU X-6913) was extracted from *Eucheuma cottonii* and ι-carrageenan (GENU X-6908) was extracted from *Eucheuma spinosum*. All of these samples were kindly provided by CP Kelco (Copenhagen). The marine bacteria *P. carrageenovora* strain Psc^T (ATCC 43555^T) (Bellion et al., 1982) and *Pseudoalteromonas haloplanktis* 545^T (DSMZ 6060^T) were obtained from the American Type Culture Collection and the Deutsche Sammlung von Mikroorganismen und Zellkulturen collection respectively.

PRODUCTION AND PURIFICATION OF THE ι-CARRAGEENAN SULFATASE FROM *P. carrageenovora* (PSC ι-CgsA)

Unless otherwise stated, all purification steps were performed at 4°C. Hydrophobic interaction chromatography and anion exchange chromatography were carried out at 18°C. *P. carrageenovora* strain Psc^T was grown in 5 L of sulfate free ZoBell medium (ZoBell, 1941) containing 1g L^{-1} of λ-carrageenan. After 36 h of incubation at 15°C, the culture medium was centrifuged for 60 min at 1400–1800 *g*. The supernatant was discarded and the cells were slowly suspended for about 1 h in 30 mL of buffer A (50 mM Tris-HCl, pH 8.3) before lysis with a French Press. The lysate was centrifuged for 1 h at 29000 *g*. The cell-free supernatant was brought to 30% $(NH_4)_2SO_4$ saturation (16.4 g $(NH_4)_2SO_4$ per 100 mL of extract) by slow addition of $(NH_4)_2SO_4$ salt. The sample was then centrifuged for 1 h at 25000 *g* and the pH of the supernatant was adjusted to 8.3 with 1 M Tris-HCl buffer before being loaded on a Phenyl Sepharose 6 fast flow high sub column (2.0 × 19.5 cm; GE Healthcare) equilibrated in buffer B (30% saturation $(NH_4)_2SO_4$ in 50 mM Tris-HCl, pH 8.3). The stationary phase was washed with buffer B at a flow rate of 1.5 mL min^{-1} until the absorbance at 280 nm of the effluent was negligible. Elution of the bound proteins was then achieved at the same flow rate by applying a linear decreasing gradient from buffer B to buffer A during 20 column volumes (CVs). Fractions of 6.5 mL were collected.

The active fractions were pooled and dialyzed for 96 h against buffer A. The desalted sample (about 140 mL) was loaded at a flow rate of 2 mL min^{-1} on a Diethylaminoethyl (DEAE) Sepharose fast flow column (1.6 × 13 cm; GE Healthcare) equilibrated in buffer A. The column was washed at the same flow rate with buffer A until the absorbance measured at 280 nm was negligible. Elution of bound proteins was achieved at a flow rate of 2 mL min^{-1} with a linear increasing gradient from 0 to 1 M NaCl in buffer A. The final concentration of NaCl was reached after 20 CVs and 5.5 mL fractions were collected. The fractions containing pure *Psc* ι-CgsA were pooled and stored at 4°C in buffer A.

At the different steps of purification, the fractions were tested for sulfatase activity using standard ι-carrageenan as substrate. The standard reaction mixture contained 100 µL of protein fraction and 100 µL of 1.2% (*w/v*) ι-carrageenan both in 50 mM Tris-HCl buffer, pH 8.3. Incubation was performed for 12 h at 35°C. The reaction was stopped by diluting the incubation medium 2-fold and by centrifuging the samples in a Microcon-10 (Millipore) to remove the carrageenan. For each sample, the corresponding blank was performed in the same conditions but using the sample previously boiled for 10 min. The amount of free sulfate present in the filtrate was assayed by high pressure anion exchange chromatography (HPAEC) using a Dionex 500 chromatography system as described previously (Genicot-Joncour et al., 2009). Briefly, the anions present in the reaction medium were separated on an AS11 anion-exchange column (4 × 200 mm, Dionex) equipped with an AG11 guard column (4 × 50 mm, Dionex). Elution was performed with an isocratic gradient of 12 mM NaOH at a flow rate of 1 mL min^{-1} using a GP40 gradient pump (Dionex). Detection of the anions was carried out with an ED40 electrochemical detector in the conductivity mode. The peak of sulfate eluted separately from the other ions at 3 min and the concentration of sulfate was deduced from the signal intensity and calculated from a standard sulfate calibration curve. The active fractions were analyzed by SDS-PAGE (Laemmli and Favre, 1973) using 12% Criterion precast Bis-Tris gels (Bio-Rad). Gels were stained routinely with Coomassie blue R-250 and colloidal Coomassie blue staining when subjected to mass spectrometry analyses (Candiano et al., 2004). Protein quantification was performed according to Bradford (1976) using the Bio-Rad protein assay. Bovine serum albumin was used as a standard. Protein concentration of pure sulfatase was also estimated at 280 nm using a Nanodrop 2000 Spectrophotometer (Thermofisher). A molar extinction coefficient of 172.120 M^{-1} cm^{-1} and a molecular weight of 115.916 kDaltons (kDa), both deduced from the protein sequence (see below), were used to calculate the concentration of the enzyme.

PROTEIN SEQUENCE DETERMINATION OF THE Psc ι-CgsA PROTEIN

The band corresponding to the *Psc* ι-CgsA sulfatase was excised from Coomassie blue stained SDS-PAGE and was subjected to in-gel tryptic digestion as described in Larré et al. (2010). Briefly, the gel slice was washed with 100 µL of 25 mM NH_4HCO_3, followed by dehydration with 100 µL of 50% (*v/v*) acetonitrile in 25 mM NH_4HCO_3. Proteins were reduced and alkylated by incubation for 1 h at 57°C in the presence of 10 mM Dithiothreitol (DTT), followed by 45 min of incubation at room temperature with 55 mM iodoacetamide. The gel slice was further washed with NH_4HCO_3 and dehydrated as described before. The band was then incubated overnight at 37°C with 10 µL of trypsin (sequencing grade, Promega) solubilized at 12.5 ng μL^{-1} in 25 mM NH_4HCO_3.The supernatant was collected and the tryptic fragments were analyzed by Matrix Assisted Laser Desorption Ionization coupled to a Time-of-Flight analyzer (MALDI-TOF) and nanoscale capillary liquid chromatography-tandem mass spectrometry (LC-MS/MS). MALDI-TOF mass spectrometry was

performed on a M@LDI LR instrument (Waters). One microliter of the sample was mixed with 1 µL of the matrix preparation (2.5 g L^{-1} α-cyano-4-hydroxycinnamic, 2.5 g L^{-1} 2,5-dihydroxy benzoic acid, 70% (*v/v*) acetonitrile, and 0.1% (*w/v*) trifluoroacetic acid) and deposited onto the MALDI sample probe. Mass spectra were acquired on the mass-to-charge ratio range from 800 to 3000. LC-MS/MS analysis was performed using a nanoflow high pressure liquid chromatography (HPLC) system (Switchos-Ultimate II, Dionex) coupled to a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Global, Waters). Chromatographic separations were conducted on a reverse-phase capillary column (75 µm i.d., Pepmap C18, Dionex) at a flow rate of 200 nL min^{-1} using a gradient from 2 to 50% of 0.08% (*w/v*) formic acid in acetonitrile. Mass data were recorded in "data dependent" mode: one MS spectrum was recorded on the mass-to-charge ratio range 400 to 1500 within 1 s, after which the three most intense ions were selected and fragmented in the collision cell. Raw data obtained by MALDI-TOF or LC-MS/MS were processed by means of the Protein Lynx Global Server v. 2.1. Software (Waters).

Protein identification was carried out by comparing the collected LC-MS/MS data against Uniprot databank. Databank searches were performed through the use of the Mascot server v. 2.2. program (Matrix Science). The mass tolerance was set to 120 parts per million (ppm) for parent ions (MS mode) and 0.3 Da for fragment ions (MS/MS mode), and one missed cut per peptide was allowed. Some MS/MS spectra were *de-novo* sequenced using the Protein Lynx Global Server v. 2.1. Software. This procedure was facilitated by the use of the OVNIp program (Tessier et al., 2010).

CLONING, HETEROLOGOUS EXPRESSION AND PURIFICATION OF THE Psc ι-CgsA

Genomic DNA from *P. carrageenovora* Psc^T was prepared as previously described (Barbeyron et al., 1984). The primers forward (5′-CCCCCCGAATTCTTATTTGTTGTTTTCAAAATAAAGTG GTTTAC-3′; *Eco*RI restriction site is underlined) and reverse (5′-GGGGGGGGATCCCAACAAGACGATGAGCCAAAATGG-3′; *Bam*HI restriction site is underlined), deduced from the gene PSHAa1171 of *P. haloplanktis* TAC 125 (Uniprot accession number Q3IKL4), were used to amplify the *Psc ι-cgsA* gene. The SignalP 3.0 program (Bendtsen et al., 2004) predicted the presence of a signal peptide with a cleavage site between residues Ala^{22} and Gln^{23} in the Q3IKL4 protein. Here, the gene without the signal peptide was cloned in between the *Bam*HI/*Eco*RI sites of the expression vector pFO4 (Groisillier et al., 2010) which encompass an N-terminal fused six-histidine-tag (6 His-tag). The sequence of the gene was checked using a genetic analyzer ABI 3130xl (Applied Biosystems) equipped with 50 cm capillaries and POP7™ polymer. The amplified and verified gene sequence of *Psc ι-cgsA* was deposited at GenBank with accession number JN228253. The *Psc ι-cgsA* gene was also optimized for *Escherichia coli* codon use by GENEART (Life Technologies), amplified using the forward primer (5′-CCGGGGATCCCAGCAGGAT GATGAACCG-3′; *Bam*HI restriction site is underlined) and the reverse primer (5′-GGCCGAATTCTTATTTGTTATTTTCAAA ATACAG-3′; *Eco*RI restriction site is underlined) and cloned into the same expression vector.

For protein expression, transformed *E. coli* strains BL21(DE3) (Novagen®) were either grown at 20°C for 72 h in ZYP 5052 medium containing 200 µg. mL^{-1} ampicillin (Studier, 2005) or in Luria Bertani (LB) medium. In the latter case, the recombinant *E. coli* BL21 (DE3) cells were grown at 37°C in LB medium containing 100 µg.mL^{-1} ampicillin and 0.025% (*w/v*) glucose until the optical density at 600 nm reached ∼1.2–1.5. The culture medium was then diluted twice with an equal volume of cold LB medium and buffered with HEPES buffer pH 7 to a 20 mM final concentration. Induction was performed by addition of lactose (0.6%) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (2 mM) (Korf et al., 2005). The cultivation was further continued for ∼18 h at 20°C until the optical density at 600 nm reached ∼5–8. *E. coli* BL21 (DE3) bearing pFO4 without insert was used as the negative control. Culture was stopped by centrifugation at 1400–1800 *g* for 60 min. The pellet was then suspended in 50 mM Tris-HCl (pH 8.3) buffer containing 200 mM NaCl and 15 mM imidazole (buffer C) before lysis with a French Press. The lysate was centrifuged for 1 h at 29,000 *g*. The cell-free supernatant was then 0.2 µM filtered before being loaded onto a HisPrep FF 16/10 column (1.6 × 10 cm, GE Healthcare) equilibrated in buffer C. Elution of the protein was performed in buffer C using a linear gradient, increasing from 15 mM to 500 mM imidazole. The final concentration of imidazole was reached after 10 CVs and 2 mL fractions were collected. Fractions containing the recombinant tagged enzyme were estimated by SDS-PAGE analysis and by Western blot. Transfer from SDS gel onto ready to use 0.2 µm nitrocellulose membrane (BioRad) was performed using a Trans Blot Turbo system in the conditions specified by the manufacturer (BioRad). Monoclonal anti-polyhistidine peroxidase conjugate (Sigma) was used at a final concentration of 1/10,000 to specifically recognize the his-tagged fusion proteins. Immuno-detection was performed by chemiluminescence using the Clarity Western ECL Substrate kit (BioRad) and visualization was achieved using the Chemi-Capt 50001 software. Recombinant enzyme activity was tested using 4- methylumbeliferyl sulfate (potassium salt, Sigma), further on called MUFS, as substrate. Unless explicitly indicated in the text, the standard conditions included 8–20 µg sulfatase, 50 mM Tris-HCl pH 8.3, 200 mM NaCl, 850 µM MUFS in a 150 µl reaction volume. The reaction was carried out for up to 120 min at 35°C and the increase of absorbance was measured as a function of time at 360 nm using a Saphire2 microplate reader (Tecan, Männedorf, Switzerland). For each reaction, a blank was made using the negative control at the same protein concentration than that of the sample.

BIOCHEMICAL CHARACTERIZATION OF THE NATIVE Psc ι-CgsA

Sulfatase activity of the native enzyme was characterized using ι-carrageenan as substrate and the amount of sulfate released was determined by HPAEC as described above. pH optimum determination was performed at 35°C in a pH range of 5.8–9.5. Assays were carried out by incubating 50 µl of ι-carrageenan (1.8% *w/v* in water) with 80 µl of 500 mM buffer and 40 µl of sulfatase solution (600 µg mL^{-1} in 50 mM Tris-HCl pH8). The solutions used to buffer the reactions were as follows: 500 mM

2-(*N*-morpholino) ethanesulfonic acid (MES) (pH 5.5 to pH 6.9), 500 mM Bis-Tris Propane (pH 6.5 to pH 9.5) and 500 mM Tris-HCl (pH 7 to pH 9). The optimal temperature was determined by measuring the amount of sulfate released upon incubation of 35 µl of sulfatase solution (600 µg mL^{-1}) with 115 µl of ι-carrageenan (0.6%, *w/v*) both in 50 mM Tris-HCl pH 8.0 in a range of 5–55°C in steps of 5°C. The effect of ionic strength on enzyme activity was assessed using increasing NaCl concentration from 0 to 500 mM. The influence of phenylmethylsulfonyl fluoride (PMSF), magnesium chloride ($MgCl_2$), and DTT on the sulfatase activity was tested at final concentrations of 1 and 2 mM, whilst chelators agents were tested at final concentrations ranging from 2 to 10 mM. All these assays were performed at 30°C by incubating 50 µl of ι-carrageenan (1.5% *w/v in* 50 mM Tris-HCl pH8) with 20 µl of sulfatase solution (670 µg mL^{-1}) in 120 µl total volume The relative activity was defined as the percentage of the activity observed without addition of these reagents.

BIOCHEMICAL CHARACTERIZATION OF THE RECOMBINANT SULFATASE

Sulfatase activity of the recombinant enzyme was characterized using MUFS in the standard conditions described above. The influence of pH on the activity was determined using the same buffers as for the native enzyme. The effect of different additives on the recombinant enzyme activity was studied in the same concentration conditions as those used for the characterization of the native enzyme. However, prior to the determination of the effect of ionic strength on the enzyme activity, the sample of recombinant enzyme was dialyzed overnight against 50 mM Tris-HCl pH8.3. In the same way as for the native enzyme, the relative activity was defined as the percentage of the activity observed without addition of these reagents.

KINETIC PARAMETERS OF NATIVE AND RECOMBINANT Psc ι-CgsA

As the ι-carrageenan is viscous even at low concentration, it was not possible to prepare a sufficient range of ι-carrageenan concentrations to allow for the accurate determination of kinetic parameters of the enzymes. For this reason, the Michaelis parameters of both enzymes, purified from *P. carrageenovora* culture medium and recombinantly expressed in *E. coli*, were determined at optimum conditions of the native enzyme, using MUFS as substrate. Kinetic measurements were conducted in 150 µl final volume and 80 µl of sulfatase were incubated with 2 µl of MUFS at final concentrations ranging from 5.2 to 206.5 µM. The formation of methylumbelliferone was measured as a function of time at 360 nm (molar extinction coefficient of 5143 M^{-1} cm^{-1}) (Viladot et al., 1998), using a Saphire2 microplate reader (Tecan, Männedorf, Switzerland). The experiments were carried out in triplicates. Kinetic experiments were performed for up to 120 min at 35°C. The initial velocities were measured on the linear section of the kinetics plots and the apparent K_m and V_{max} were calculated from an hyperbolic regression analysis using the software Hyper32 version 1.0.0. (http://homepage.ntlworld.com/john.easterby).

NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

The ι-carrageenan incubated with pure native *Psc* ι-CgsA was freeze dried, exchanged twice with 99.97 atom % ^2H_2O and then dissolved in ^2H_2O at a final concentration of 10 mg mL^{-1}. The product was then transferred into a 5 mm NMR tube and ^{1}H-NMR spectra were recorded at 70°C, using a BRUKER Advance DRX 500 spectrometer equipped with an indirect 5 mm gradient probehead TXI $^1H/^{13}C/^{31}P$. Chemical shifts are expressed in ppm in reference to trimethylsilylpropionic acid (TSP), which was used as an external standard. No suppression of the HOD signal was performed.

SEQUENCE AND PHYLOGENETIC ANALYSIS

Searches for protein sequence similarities were performed in Uniprot database using the BlastP program (Altschul et al., 1997). Protein structure prediction was performed with the Phyre2 server (Kelley and Sternberg, 2009). A multiple alignment was generated with 421 sequences using the MAFFT program and L-INS-i algorithm (Katoh et al., 2005) and manually refined. From the multiple alignments, 171 positions were used to build a phylogenetic tree by the Maximum Likelihood method using the Whelan and Goldman evolution model (Whelan and Goldman, 2001). Reconstruction of the tree and bootstrap analysis (resampling of 100) were conducted with the MEGA v. 5.05. Software (Tamura et al., 2011).

RESULTS

SCREENING AND PURIFICATION OF THE Psc ι-CgsA

The marine bacterium *P. carrageenovora* was screened for potential sulfatase activity capable to catalyze the hydrolysis of sulfate from carrageenans. As a consequence the use of carrageenans as substrate was mandatory during the screening and the sulfate assay was performed by HPAEC as described previously (Genicot-Joncour et al., 2009). To avoid interference of ions with the detection of sulfate groups during chromatography analysis, *P. carrageenovora* strain Psc^T was grown in ZoBell medium.

Carrageenan-sulfatase activities were detected only when the culture medium was supplemented with κ-, ι- or λ-carrageenan. As reported in **Table 1**, carrageenan-sulfatase activities were measured both in the bacterial pellet and in the culture supernatant. ι- and λ-carrageenan proved to be good inducers of carrageenan-sulfatases that were active on κ-, ι- and λ-carrageenan. In this study, we focus our attention on the ι-carrageenan sulfatase activity detected in the pellet of *P. carrageenovora* cultures induced by the λ-carrageenan.

The newly detected enzyme, further on named *Psc* ι-CgsA, was purified by a combination of ammonium sulfate fractionation, hydrophobic interaction chromatography and ion exchange chromatography. The first chromatographic step using hydrophobic interaction chromatography on phenyl-Sepharose was efficient in purifying the sulfatase as this one elutes between 91 and 78 mS cm^{-1}, just before the majority of the proteins (**Figure 2A**). All the active fractions were pooled and dialyzed before being loaded on top of the anion exchange chromatography column (DEAE Sepharose). At this stage, the sulfatase eluted between 300 and 370 mM NaCl i.e., 29.5 and 32.3 mS cm^{-1} respectively (**Figure 2B**). The purified enzyme gave a single band with an apparent molecular mass of 110 kDa in SDS-PAGE under reducing conditions (**Figure 2C**).

Table 1 | Sulfatase activity in cultures of *P. carrageenovora* Psc^T and *P. haloplanktis* 545^T.

	Inducer	Total Proteins	Substrate		
			κ-carrageenan	ι-carrageenan	λ-carrageenan
Supernatant	κ-carrageenan	1.6 (1.1)	n.d. (1.5)	n.d. (13.4)	0.6 (n.d.)
	ι-carrageenan	1.6 (0.5)	6.3 (n.d.)	11.9 (n.d.)	n.d. (n.d.)
	λ-carrageenan	2.35 (0.8)	n.d. (n.d.)	n.d. (73.2)	4.5 (7.7)
Pellet	κ-carrageenan	6.5 (4.1)	0.9 (n.d.)	n.d. (n.d.)	n.d. (n.d.)
	ι-carrageenan	10.5 (3.8)	4.0 (n.d.)	4.7 (n.d.)	n.d. (n.d.)
	λ-carrageenan	30 (5.9)	2.1 (n.d.)	10.4 (n.d.)	0.4 (n.d.)

Cultures of P. carrageenovora Psc^T and P. haloplanktis 545^T were induced with 0.1% (w/v) κ-, ι-, or λ-carrageenan. Incubations were performed for 12 h as described in material and methods using κ-, ι- or λ-carrageenan as substrate. Activities are expressed in nmole of sulfate released per minute and per mg of total proteins (nmole min^{-1} mg^{-1}). The amount of total proteins are expressed in mg. Data in brackets are relative to Pseudoalteromonas haloplanktis 545^T. n.d. means that no activity was detected.

IDENTIFICATION OF THE Psc ι-CgsA PROTEIN, A NOVEL SULFATASE

Following digestion of the pure *Psc* ι-CgsA protein by trypsin, the peptide fragments were analyzed by LC-MS/MS and compared to the Uniprot database. Eight peptides (YVEPTFSPDGK, VIENGVIITDGK, DLGEPMFSPDGR, SLGAGEVWLYHK, YVYFSHDATPGK, FTQNLDTDEFDVK, LLNSPAWSPDGDYLVAR, VSPDGQYLAFAER) were identical to peptides found in a putative uncharacterized protein Q3IKL4 (117.873 kDa), encoded in the genome of the marine strain *P. haloplanktis* TAC 125 (Medigue et al., 2005). In depth sequencing by MALDI-MS and by *de novo* allowed to cover 14 additional sequence stretches (LYESEHATEFR, QQVIEAGR, TDVWNHPR, AGENLS, LVYTTW, VLDG-PL, WSLNPG-YSV, LTSDLA—QPR, QPQFG—DR, YELF-QYSR, ELF-QYSR, VTPFVE——LNSP, LF-A-TM–VGKK, ELETVL). Altogether, peptide sequencing of the *Psc* ι-CgsA provided coverage of 23% of the 1060 amino acids of the *P. haloplanktis* Q3IKL4 protein (**Figure 3**). The sequence of the here described novel *Psc* ι-CgsA was further confirmed as described in the following section.

SEQUENCE DETERMINATION, CLONING AND OVEREXPRESSION OF THE ι-CgsA SULFATASE

The nucleotide sequence encoding the Q3IKL4 protein without the peptide signal was used to design specific oligonucleotide primers that were used to clone and sequence the gene encoding the *Psc* ι-CgsA (*ι-cgsA*) from *P. carrageenovora*. After successful expression and sequencing, the translation of the *ι-cgsA* gene yielded a protein of 1037 amino acids (GenBank accession number JN228253) with a theoretical isoelectric point (pI) value of 6.78 and a molecular weight of 115.9 kDa. This mass corresponds to the apparent molecular mass of the purified native and expressed *Psc* ι-CgsA (∼110 kDa), deduced from SDS-PAGE. As expected, all the peptide sequences identified by mass spectrometry analysis were present in the protein sequence. The *Psc* ι-CgsA shares 92.6% sequence identity with the putative uncharacterized protein Q3IKL4 of *P. haloplanktis* TAC 125 (**Figure 3**).

Psc ι-CgsA was expressed as a soluble protein at about 6.5 mg L^{-1} in *E. coli* BL21 (DE3) when induced with 0.2% lactose (ZYP 5052 medium). The yield was more than doubled (16.9 mg L^{-1}) when induction was carried out with both lactose (0.6%) and IPTG (2 mM). After affinity chromatography on a HisPrep sepharose column, the enzyme was pure (**Figure 2D**). As shown on **Figure 2D**, the use of anti-histidine antibody allows recognizing the his-tagged protein which migrates as a single band at the expected size.

BIOCHEMICAL CHARACTERIZATION OF THE NATIVE Psc ι-CgsA PROTEIN

The purified sulfatase was kinetically evaluated using carrageenan as substrates. The amount of sulfate released after incubation of the pure sulfatase using ι-, κ- or λ-carrageenan was monitored by HPAEC. In the presence of κ- and λ-carrageenan, no desulfation was observed, even after prolonged incubation. In contrast, strong desulfation occurred when ι-carrageenan or hybrid ι-carrageenan containing about 20% of ν-carrabiose was used as substrate, highlighting the specificity of this sulfatase toward the ι-carrabiose moiety.

As shown in **Figure 4**, the desulfation reaction of ι-carrageenan by the Psc-ι sulfatase was slow, in the range of hours. The rate of sulfate release increased with the concentration of ι-carrageenan up to 0.6% (*w/v*). At higher concentrations the medium became very viscous interfering with the enzyme diffusion. When 0.5% (*w/v*) ι-carrageenan was used, the rate of desulfation was linear for the first 15 h. Under these experimental conditions the temperature optimum was determined to be 40 ± 5°C and the pH optimum was measured at 8.3 (**Table 2**). The *Psc* ι-CgsA could be kept for several months at 4°C at this pH. Addition of sodium chloride at high concentrations affects the enzyme activity. Indeed, at 500 mM NaCl, the activity drops to 17.5% of the optimal activity, observed at 200 mM NaCl (**Table 2**).

With the aim to determine the specificity of the ι-carrageenan sulfatase more precisely, the structural modification of ι-carrageenan was monitored, as a function of time, by ^{1}H NMR (**Figure 4**). The signals of ^{1}H NMR spectrum of the standard ι-carrageenan (**Figure 4** top spectrum: 0 h) were attributed on the basis of previously reported analyses (van de Velde et al., 2002). The signal observed at 5.32 ppm corresponds

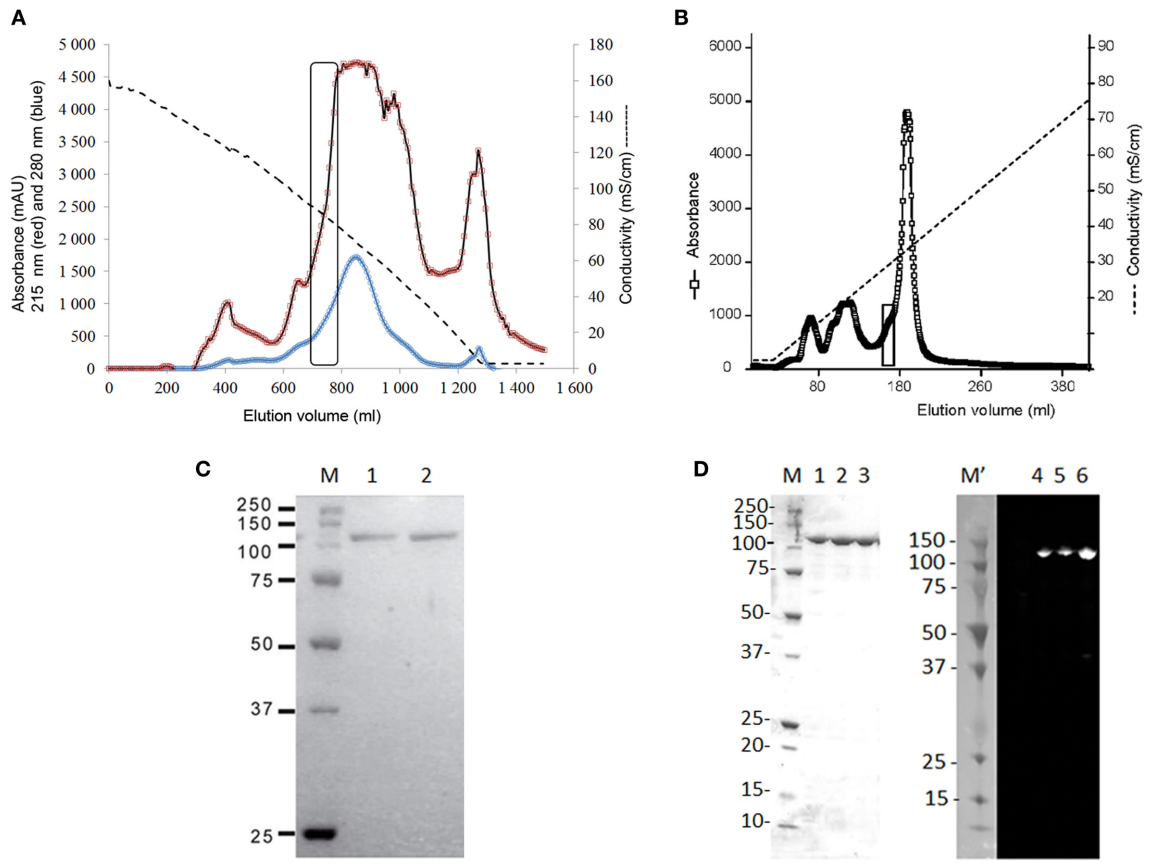


FIGURE 2 | Purification of the ι-carrageenan sulfatase. (A) Purification of the native ι-carrageenan sulfatase on a Phenyl Sepharose 6 fast flow high sub column (2.0 × 19.5 cm). Elution of proteins was performed using a decreasing gradient from 30% saturation $(NH_4)_2SO_4$ in buffer A to 0% (dashed lines). The absorbance of the fractions (6.5 ml) was measured both at 280 nm (blue curve) and at 215 nm (red curve). Active fractions containing sulfatase activity are surrounded with a rectangle. They were pooled and dialyzed against buffer A prior being loaded on top of the anion exchange chromatography, as described in material and methods. **(B)** Purification of the native ι-carrageenan sulfatase on a DEAE-Sepharose fast flow column (1.6 × 13 cm). Elution of proteins from the anion exchange chromatography was performed using an increasing gradient from 0 to 1 M NaCl (dashed line). The absorbance of the elution fractions was measured at 215 nm (open squares). Fractions containing pure *Psc* ι-CgsA are surrounded with a rectangle. **(C)** SDS- PAGE analysis of the native ι-carrageenan sulfatase isolated from *P. carrageenovora*. Elution fractions from the DEAE Sepharose chromatography were separated on 12% SDS-PAGE and stained with Colloïdal Coomassie Blue (Lanes 1–2). Precision protein standards from Bio-Rad were used as markers (Lane M, from top to bottom: 250 kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa, 37 kDa, and 25 kDa). **(D)** SDS- PAGE and Western Blot analysis of the recombinant his-tagged ι-carrageenan sulfatase after affinity chromatography. Lane M corresponds to the molecular weight markers (as above and in addition 20 kDa, 15 kDa, and 10 kDa). Lanes 1 to 3: elution fractions from the HisPrep chromatography. Precision Plus protein dual color standards from BioRad were used as makers for the Western blot (Lane M', sizes same as lane M). Lanes 4, 5, and 6 correspond to the Western blot analysis of the his-tagged sulfatase. Revelation was performed by chemiluminescence as described in material and methods.

to the anomeric proton of the 4-linked anhydrogalactose unit (DA2S-H1). The G4S-H4 and DA2S-H3 protons, which signals are at 4.91 ppm and 4.85 ppm respectively, are also indicated on the spectrum. These signals decreased after 15 h of incubation of 41.25 mg of ι-carrageenan with 1.12 mg of ι-CgsA whilst two other signals, at 5.26 ppm and 4.80 ppm, appeared (**Figure 4**, middle spectrum: 15 h). To overcome heat inactivation of the enzyme, 300 µg of *Psc* ι-CgsA were added after 60 h. This addition, combined with prolonged enzymatic incubation (up to 120 h), allowed the complete modification of the ι-carrageenan (**Figure 4**, bottom spectrum: 120 h). Based on the analyses reported by Falshaw et al. (1996), this spectrum is characteristic of alpha (α)-carrageenan. Signals assignable to κ- (5.1 ppm) or beta (β)-carrabiose units (5.09 ppm) or to the production of reducing and non-reducing ends were not observed. This suggests that *Psc* ι-CgsA specifically catalyzes the conversion of ι-carrabiose into α-carrabiose units (**Figure 1**) within a polysaccharide chain and is devoid of glycoside hydrolase activity.

BIOCHEMICAL CHARACTERIZATION OF THE RECOMBINANT ι-CgsA PROTEIN

The purified recombinant ι-CgsA was able to specifically remove sulfate from ι-carrageenan. After 24 h of incubation at 35°C and pH 8.3, the amount of sulfate released represents roughly 3% of the total sulfate of the polymer. Although this is not sufficient to detect the conversion of ι-carrageenan into α-carrageenan by ^{1}H NMR, this low but significant activity confirmed that the

FIGURE 3 | Sequence alignment of *Psc* ι-CgsA and Q3IKL4_PSEHT from *P. haloplanktis* TAC 125 using the program MEGA 5. The ESPript 3 program (Robert and Gouet, 2014) was used to enhance the conserved amino acids which are shown in white letters on a red background.

product of the cloned gene was a ι-carrageenan sulfatase. As a consequence of the low activity, all further characterization of the recombinant enzyme was performed using an artificial substrate which is MUFS. As shown on **Table 3A**, both native and recombinant enzymes are most active at 35°C and pH 8.3. Although the recombinant sulfatase shows an activity in MES comparable to that observed in the Tris buffer, its activity drops drastically in the 3-morpholinopropane-1-sulfonic acid (MOPS) buffer and in the phosphate/citrate buffer deviating from the trend of pH profile (data not shown). This therefore suggests that, in the case of the later buffer, inorganic phosphate may be an inhibitor of the ι-sulfatase.

The activity of the recombinant ι-CgsA was investigated in presence of different additives (**Table 3A**), to be compared to the native enzyme. Among them, Mg^{2+} and DTT inhibit slightly the enzyme whilst the addition of chelating agents such as EDTA an EGTA at 2 mM does not affect the activity. EDTA has to be used at a concentration of 10 mM to induce a slight decrease of the activity. The recombinant enzyme is completely inhibited when the concentration of NaCl reaches 500 mM. At 200 mM NaCl, the recombinant enzyme has 89.6% of its optimal activity observed at 100 mM NaCl.

The Michaelis parameters of both enzymes using an artificial substrate have been determined in optimal conditions using MUFS as substrate (**Figure 5**). These preliminary experiments show that while the K_m of both enzymes are in the same order of magnitude, the *kcat* of the native enzyme is about 3 times that of the recombinant enzyme (**Table 3B**).

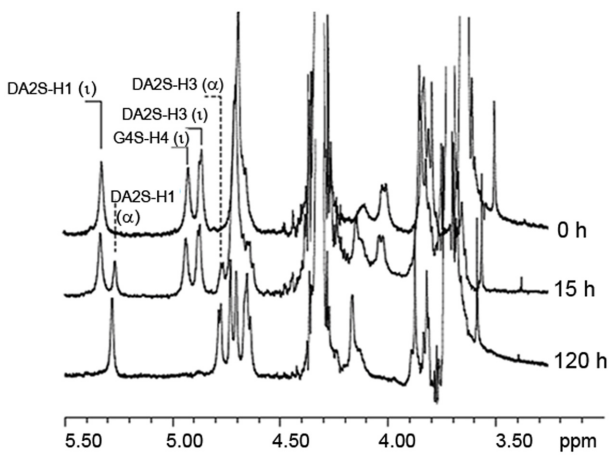


FIGURE 4 | ι-carrageenan desulfation with pure native ι-carrageenan sulfatase. ^{1}HNMR spectra monitoring the bioconversion of standard ι-carrageenan (Top spectrum, 0 h) into α-carrageenan (Bottom spectrum). This conversion was performed after 120 h of incubation of ι-carrageenan with pure *Psc* ι-CgsA (Bottom spectrum, 120 h). Intermediate structures observed after 15 h of incubation (Middle spectrum, 15 h), are hybrid ι-/α-carrageenans whose composition was deduced by integrating the α-anomeric signal of DA2S-H1 (ι) or (α) indicated on the spectra (Falshaw et al., 1996; van de Velde et al., 2002).

DISCUSSION

DISCOVERY OF A ι-CARRAGEENAN SULFATASE, ι-CgsA, SPECIFICALLY RELEASING SULFATE FROM THE D-GALACTOSE-4-SULFATE UNITS IN ι-CARRAGEENAN

Many genes encoding sulfatases have been cloned from all kingdoms of life (Sasaki et al., 1988; de Hostos et al., 1989; Paietta, 1989; Yang et al., 1989; Hallmann and Sumper, 1994; Ferrante et al., 2002; Medigue et al., 2005; Sardiello et al., 2005; Frese et al., 2008), but the number of characterized sulfatases remains limited and does not reflect the huge chemical diversity of sulfated biomolecules. The recent discovery that a vast number of sulfatases are present in marine bacteria (Glöckner et al., 2003; Barbeyron, personal communication) highlights the untapped resource of this type of activity in the marine environment. Indeed, most of the characterized sulfatases are specific of metabolizing glycosaminoglycans (Buono and Cosma, 2010), due to their importance in pathogenicity or human health. Nevertheless, it has been shown that the sulfatase from *Sphingomonas* sp. AS6330 (Kim et al., 2004) and the arylsufatase AtsA from *P. carrageenovora* Psc^T (Lim et al., 2004; Kim et al., 2005) catalyze the desulfation of agar. Recently, a formylglycine-dependent endo-4S-ι-carrageenan sulfatase from the marine bacterium *P. atlantica* T6c has been purified and characterized (Préchoux et al., 2013). In the present study we have identified, sequenced

Table 2 | Biochemical characterization of the native *Psc* ι-CgsA compared to two other polysaccharide sulfatases described in the literature.

Characteristics	Native *Psc* ι-CgsA	*P. atlantica* sulfatase	*P. carrageenovora* arylsulfatase
Sulfatase family	New sulfatase family	Formylglycine family	β- lactamase superfamily New sulfatase family
Polysaccharide substrate	ι-carrageenan	ι-carrageenan	Agar
Molecular mass	115.9 kDa	55.7 kDa	35.8 kDa
Theoretical pI	6.8	7.1	5.4
Optimal temperature	35–45°C	35°C	45°C
Optimal pH	8.3	7.5	7.0–8.5
Optimal (NaCl]	200 mM	~25 mM	~500 mM
EDTA 1 mM	0% inhibition	Data not available	53.2% inhibition
PMSF 1 mM	91% inhibition	Data not available	26% inhibition
Phosphate buffer	82% inhibition	Data not available	Inhibition

Both the native Psc ι-CgsA and the ι-carrageenan sulfatase from P. atlantica (Préchoux et al., 2013) were characterized using ι-carrageenan as substrate. The data are compared to those of the arylsulfatase isolated from P. carrageenovora (Barbeyron et al., 1995; Lim et al., 2004; Kim et al., 2005).

and characterized a novel ι-carrageenan sulfatase, *Psc* ι-CgsA, first member of a new family of sulfatases. NMR studies of the reaction products unambiguously demonstrate that *Psc* ι-CgsA catalyzes the removal of the sulfate ester group localized on the position C4 of the G4S moieties from the ι-carrabiose units, leading to the formation of α-carrabiose units. Despite the complete absence of sequence homology, this activity appears to be the same as that already described for the formylglycine-dependent sulfatase from the marine bacterium *P. atlantica* T6c, indicating that this catabolic activity plays an important role in carrageenan metabolization.The desulfation is ι-carrageenan specific, since neither the *P. atlantica* 4S-ι-carrageenan sulfatase nor the here described *Psc* ι-CgsA sulfatase are able to catalyze the removal of the 4S sulfate group from κ-carrabiose units. In κ-carrageenan, the G4S residues are located between two neutral anhydrogalactose residues whilst in ι-carrageenan the G4S moieties are positioned between two sulfated anhydrogalactose residues (**Figure 1**). This finding therefore allows the assumption that the substrate specificity in these endo-sulfatases includes the recognition of at least one additional sulfated anhydrogalactose in their substrate binding site to display the observed specificity.

Table 3 | Characterization of the native and the recombinant *Psc* ι-CgsA.

(A) BIOCHEMICAL CHARACTERIZATION		
Characteristics	**Native *Psc* ι-CgsA**	***Recombinant Psc* ι-CgsA**
Optimal temperature (°C)	35–45	35
Optimal pH	8.3	8.4 ± 0.3
Optimal [NaCl] (mM)	200	100
EDTA 10 mM	132.8% ± 12.9	93.6% ± 3.3
EGTA 10 mM	125.5% ± 9.2	100.9% ± 8.5
$MgCl_2$ up to 2 mM	37.8% ± 2.8	81.7% ± 2.5
DTT 1 mM	46.8% ± 4.2	74.8% ± 6.9
PMSF 2 mM	0%	34.1% ± 13.4

Characterization of the native enzyme has been performed using ι- carrageenan whereas MUFS was used for the recombinant enzyme. As stated in material and methods, incubation with ι- carrageenan was performed during 12 h whilst kinetics with MUFS were carried out for 2 h. Unless otherwise stated, the activity is expressed as the percentage of the initial activity observed without addition of reagents.

(B) KINETIC PARAMETERS			
Enzyme	**Apparent Km (μM)**	***kcat* (min^{-1})**	***Kcat*/Km (μM^{-1} min^{-1})**
Native *Psc* ι-CgsA	21.9 ± 8.2	6.32 ± 0.38	0.280 ± 0.05
Recombinant ι-CgsA	13.4 ± 4.9	1.64 ± 0.23	0.12 ± 0.04
Arylsulfatase from *P. carrageenovora*	68	ND	ND

Preliminary kinetic parameters of the native and recombinant Psc ι-CgsA were measured using the optimal reaction conditions of the enzymes as determined above and the artificial substrate MUFS at concentrations ranging from 5.2 to 206.5 μM. Data are compared to that of the P. carrageenovora arylsulfatase (Barbeyron et al., 1995) using the same substrate. N.D. means not determined.

THE ι-CARRAGEENAN SULFATASE ι-CgsA IS A MODULAR ENZYME BELONGING TO A NOVEL CLASS OF SULFATASES

Despite their identical substrate specificity, the ι-carrageenan sulfatases of *P. carrageenovora* Psc^T and *P. atlantica* T6c have very different biochemical characteristics (**Table 2**) and can also be distinguished by their primary sequence that do not share enough similarity to allow an accurate sequence alignment. The ι-carrageennan sulfatase from *P. atlantica* T6c is indeed a formylglycine sulfatase and in this respect it has the characteristic signatures of family 1 formylglycine-dependent sulfatases, namely [SAPG]-[LIVMST]-[CS]-[STACG]-P-[STA]-R-x(2)-[LIVMFW](2)-[TAR]-G and G-[YV]-x-[ST]-x(2)-[IVAS]-G-K-x(0,1)-[FYWMK]-[HL] (Prosite signatures PS00523 and PS00149 respectively), which are well conserved in this family of enzymes. These signatures are not present in the *Psc* ι-CgsA sulfatase, thus indicating that this latter enzyme does not belong to the well-defined and represented family of formylglycine-dependent sulfatases. Sequence alignment of the Psc-ι sulfatase with the protein Q3IKL4 from *P. haloplanktis* and 9 other proteins chosen amongst the ones representing the new sulfatase family revealed that no cystein amino acids are conserved (Supplementary Figure 1). However, several conserved serine could potentially be subjected to post-translational modification. Therefore, and based on sequence data alone, it cannot be completely excluded (although improbable) that this sulfatase potentially reveals the existence of a new formylglycine-dependent family. It would however involve a serine modification. Interestingly, a ι-carrageenan sulfatase was revealed to be very active in *P. haloplanktis* 545^T extracts. This enzyme was induced in similar conditions to those observed for induction of the *Psc* ι-CgsA (**Table 1**, data in brackets). Since the genomes of both sequenced strains of *P. haloplanktis* TAC 125 (Medigue et al., 2005) and *P. haloplanktis* ANT/505 (GenBank ADOP00000000.1) do not contain any formylglycine-dependent sulfatase genes, it is tempting to assume that the sulfatase activity detected in *P. haloplanktis* 545^T is due to a ι-CgsA-type enzyme, such as in *P. carrageenovora*. Indeed, as described above, proteins orthologous to *Psc* ι-CgsA are present both in *P. haloplanktis* TAC 125 and ANT/505.

A BlastP sequence similarity search with the *Psc* ι-CgsA used as query sequence against the Uniprot database revealed that *Psc* ι-CgsA showed more than 90% identity with putative uncharacterized proteins from several marine species of *Pseudoalteromonas*. Moreover, many proteins from different marine strains such as *Colwellia psychrerythraea* 34H (Methe et al., 2005) and *Shewanella sediminis* HAW-EB3, belonging to the amidohydrolase superfamily, exhibited more than 60% of identity with the *Psc* ι-CgsA sulfatase (Supplementary Figure 1). Three-dimensional structure modeling of *Psc* ι-CgsA using the Phyre2 (Kelley and Sternberg, 2009) tool reveals that *Psc* ι-CgsA most likely has a multi-modular arrangement that covers 94% of the residues and are modeled with more than 90% of confidence. From this model it is hypothesized that *Psc* ι-CgsA consists of an N-terminal module (from residues 24 to 618 approximately) featuring a first β-propeller fold composed of six blades (from residues 24 to 300 approximately), and a second β-propeller fold composed of seven blades (from residues 310 to 618 approximately). This type of organization has already been observed among carbohydrate processing

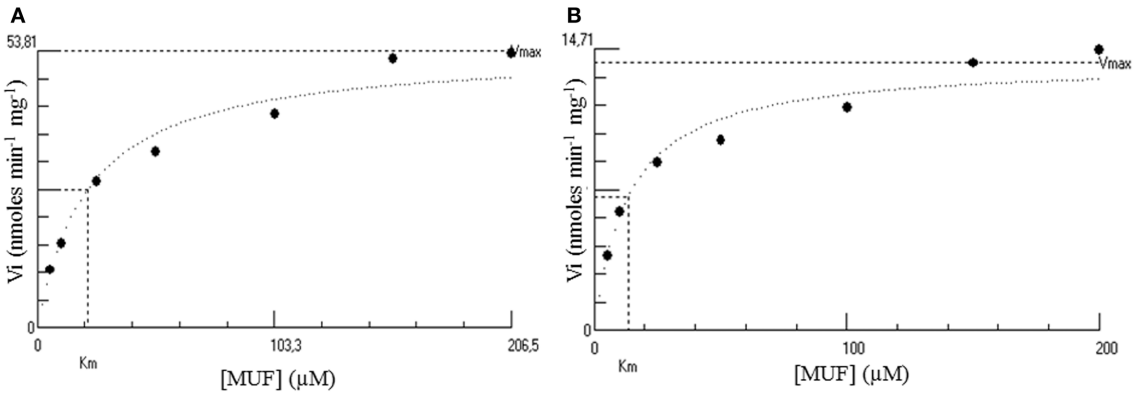


FIGURE 5 | Hyperbolic regression determination of the kinetic parameters of both the native (A) and the His-tagged (B) *Psc* ι-CgsA. Kinetics were performed at 35°C using MUFS at different concentrations from 5.2 to 206.5 µM. These plots have been performed using the software Hyper32 version 1.0.0. (http://homepage.ntlworld.com/john.easterby). They are representative of experiments performed in triplicates.

enzymes, such as glycoside hydrolases from the clans GHE, GHF, GHJ, and for λ-carrageenases (Guibet et al., 2007), but also in the PL11 family of polysaccharide lyases involved in the degradation of anionic polysaccharides. The C-terminal module (comprising amino acids 663–1060 in *Psc* ι-CgsA) displays a typical TIM-barrel fold found in the amidohydrolase superfamily. This superfamily includes an outstanding set of enzymes that catalyze the hydrolysis of a wide range of substrate having amide or ester groups. For example, urease, amidohydrolase, guanine deaminases and phosphoesterase share the same three-dimensional fold describing this superfamily (Seibert and Raushel, 2005).

A phylogenetic analysis of the C-terminal module found in *Psc* ι-CgsA and in 420 sequences of enzymes classified as belonging to the amidohydrolases superfamily showed that *Psc* ι-CgsA was not related to any known activity in this superfamily (**Figure 6**). In contrast, it appears that *Psc* ι-CgsA belongs to a clade composed of uncharacterized proteins only. In this cluster, a sub-clade supported by a node with a relatively good bootstrap value (73%) is composed of 122 sequences displaying the same modular organization as *Psc* ι-CgsA (i.e., the presence of β-propeller modules at the N-terminal extremity) (Supplementary Table 1). The topology of the phylogenetic tree suggests that *Psc* ι-CgsA and these 122 sequences represent a novel family of sulfatases, which might have different substrate specificities, as supported by the low percentage of sequence identity between some proteins within this clade.

PRODUCTION AND ANALYSIS OF THE RECOMBINANT ι-CARRAGEENAN SULFATASE

In addition to the identification of the native *Psc* ι-CgsA enzyme, the gene coding for *Psc* ι-CgsA was successfully cloned and heterologously expressed in *E. coli*, although the purified recombinant enzyme displayed lower activity on the natural substrate than the native form. The biochemical characteristics of the native and the recombinant enzymes, measured on the artificial substrate MUFS, are very similar (**Table 3A**), but the *kcat* of the native enzyme is roughly about 3 times that of the recombinant enzyme (**Table 3B**). The slightly different biochemical behavior of native vs. recombinant enzyme might be due to intrinsic elements in the native protein that *E. coli* does not supply, such as potential post-translational modifications and/or the requirement of a cofactor or a chaperone during protein folding, preventing the production of a fully active recombinant enzyme. Future work and a more detailed biochemical depiction of different recombinant homologs of this novel sulfatase family are necessary, and will shed more light on the precise catalytic mechanism and mode of action of this newly discovered enzyme family.

DECIPHERING A NEW PATHWAY FOR CARRAGEENAN BIODEGRADATION

The degradation pathway of κ-carrageenan by *P. carrageenovora* Psc^T has been partially determined and involves a κ-carrageenase (Weigl and Yaphe, 1966a; McLean and Williamson, 1979a; Barbeyron et al., 1994; Michel et al., 2001) to produce oligosaccharides which are readily degraded into neocarrabiose through the concerted action of a glycosulfatase (Weigl and Yaphe, 1966b; McLean and Williamson, 1979b) and a neocarratetraose monosulfate hydrolase (McLean and Williamson, 1981). For the catabolism of ι-carrageenan a similar pathway might be assumed as a low ι-carrageenase activity has been detected in the crude extract of *P. carrageenovora* Psc^T (Henares et al., 2010). However, based on our results with *Psc* ι-CgsA, we propose an alternative mechanism in which ι-carrageenan is first desulfated and converted into α-carrageenan. The latter likely constitutes a metabolic intermediate probably subject to further degradation by an α-carrageenase and/or desulfation by other sulfatases, leading to the end-product of galactose residues. Except for the conversion of ι- into α-carrageenan, these steps are speculative and need to be corroborated.

BIOCONVERSION OF RED ALGAL POLYSACCHARIDES

α-Carrageenan has been observed in the cell wall of several red algae such as *Catenella nipae* Zanardini (Zablackis and Santos, 1986), *Sarconema filiforme* (Sonder) Kylin (Chiovitti et al., 1998; Kumar et al., 2011) and in some *Solieria* spp. (Chiovitti et al., 1997; Bondu et al., 2010). Although structural studies revealed

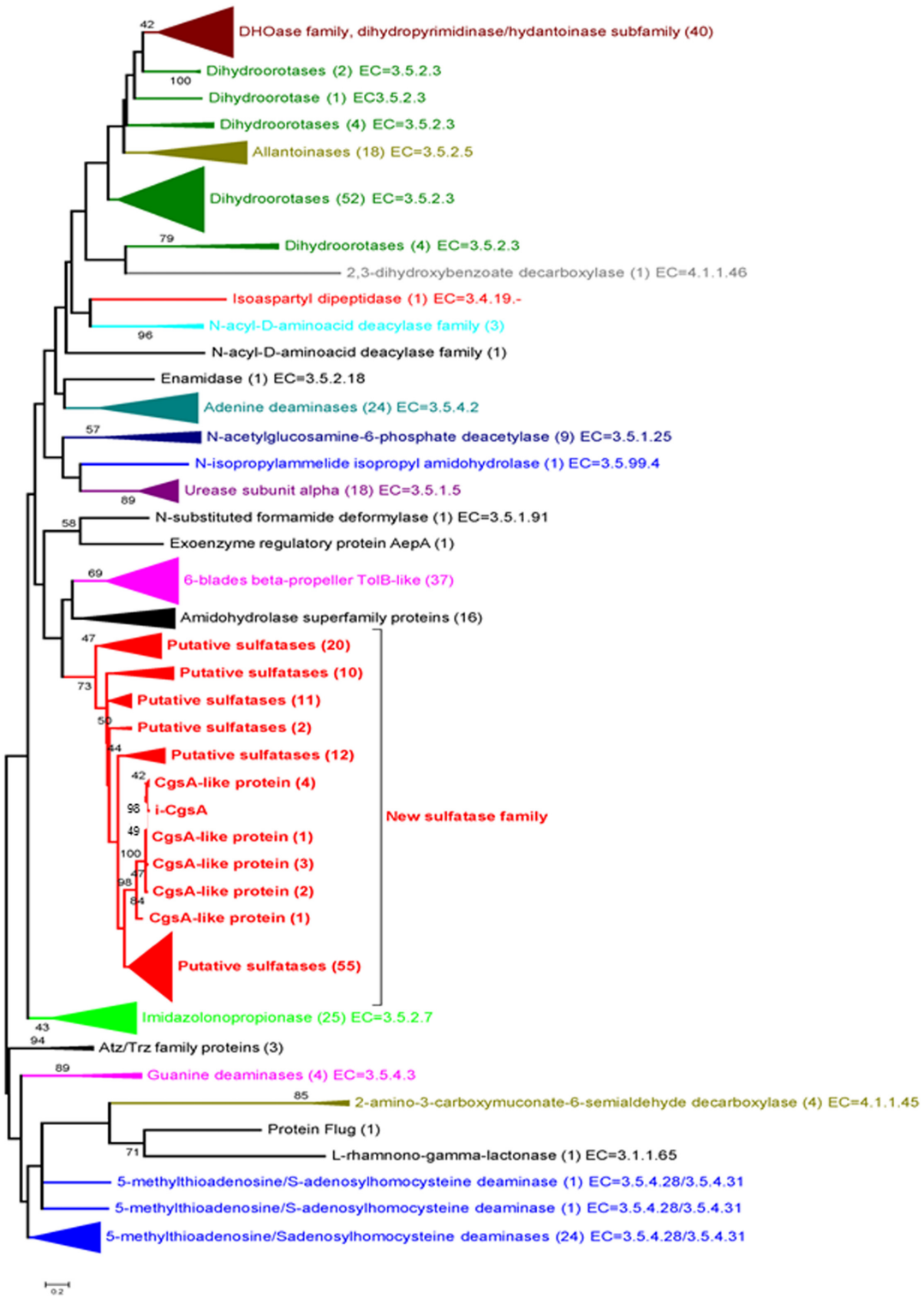


FIGURE 6 | Phylogenetic tree of the amidohydrolase module present in *Psc* L-CgsA and in 420 sequences of enzymes classified as amidohydrolases. The maximum likelihood tree was built with the MEGA v. 5.05. Software, using the substitution model WAG. Evolutionary rate differences among sites (5 categories) were modeled using a discrete Gamma distribution, from an alignment produced by the MAFFT program with the L-INSi algorithm (Katoh et al., 2005). Phylogenetic analysis was performed using 171 positions, defined from the multiple sequence alignment. All ambiguous positions were removed for each sequence pair. Only the bootstrap values higher than 40 are shown. The potential new sulfatase family (in red) was delimited on the basis of sequence similarities to the here identified sulfatase and the bootstrap value (73) of the best node (the deepest, the first red node) in the tree. Numbers in brackets indicate the number of sequences involved in each group.

that this carrageenan referred to as hybrids of α-/ι-carrageenan in which the α-carrabiose content does not exceed 30–40% (mol/mol) (Falshaw et al., 1996), it has been shown that the sodium salt of such hybrids exhibit twice the capacity to suspend cacao particles in milk compared to κ-carrageenan which is commonly used for that purpose (Zablackis and Santos, 1986). As suggested by **Figure 4**, the Psc-ι-CgsA sulfatase could be used to produce carrageenan with controlled ratio of ι-/α-carrabiose and even pure α-carrageenan. It is therefore more than likely that new hybrids of α-/ι-carrageenans or pure α-carrageenan will harbor new and interesting functional properties.

Recent marine genomic projects have shown that marine bacteria are a potential source of large sulfatase diversity, with the presence of huge multigenic sulfatase families, as exemplified by the formylglycine-dependent sulfatase family (up to 300 genes in the *Lentisphaera araneosa* HTCC2155^T genome) (Thrash et al., 2010). Since the origin of these bacteria is marine, it is tempting to assume that a large portion of these enzymes are involved in the degradation of sulfated polysaccharides from marine algae (Glöckner et al., 2003). In addition to these sulfatases accessible through genome mining, our work demonstrates that the sulfatase diversity extends even beyond those that have already characterized members. By screening a marine bacterium for sulfatase activity on carrageenan substrate, we have successfully identified and characterized a new sulfatase family, members of which were annotated "hypothetical proteins with unknown function" before hand. Altogether, these data open up a large field for future work, necessitating substantial experimental effort to provide all the biochemical details to assign precise substrate specificities to these newly identified sulfatases. The immediate gain of such efforts will be a large panel of new marine polysaccharide sulfatases that represent interesting enzymatic tools to fine-tune the physico-chemical properties of algal polysaccharides.

ACKNOWLEDGMENTS

We thank Audrey Geairon for excellent technical assistance in mass spectrometry analyses. Special thanks also to Nelly Kervarec and Morgan Perennou for NMR analysis and DNA sequencing respectively. We also thank Dr. Fanny Gaillard for her help with the mass spectrometry analyses, Dr. Thierry Tonon, Dr. Simon Dittami and Dr. Mirjam Czjzek for critical reading of the manuscript. This work has received funding from the European Community's Seventh Framework Program (FP7/2007-2013) under grant agreement No. 222628 (Large collaborative project PolyModE, http://www.polymode.eu/).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fchem.2014.00067/abstract

REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., et al. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389–3402. doi: 10.1093/nar/25.17.3389

Barbeyron, T., Henrissat, B., and Kloareg, B. (1994). The gene encoding the kappa-carrageenase of *Alteromonas carrageenovora* is related to beta-1,3-1,4-glucanases. *Gene* 139, 105–109. doi: 10.1016/0378-1119(94)90531-2

Barbeyron, T., Kean, K., and Forterre, P. (1984). DNA adenine methylation of GATC sequences appeared recently in the *Escherichia coli* lineage. *J. Bacteriol.* 160, 586–590.

Barbeyron, T., Potin, P., Richard, C., Collin, O., and Kloareg, B. (1995). Arylsulphatase from *Alteromonas carrageenovora*. *Microbiology* 141, 2897–2904. doi: 10.1099/13500872-141-11-2897

Bellion, C., Hamer, G. K., and Yaphe, W. (1982). The degradation of *Eucheuma spinosum* and *Eucheuma cottonii* carrageenans by ι-carrageenases and κ-carrageenases from marine bacteria. *Can. J. Microbiol.* 28, 874–880. doi: 10.1139/m82-129

Bendtsen, J. D., Nielsen, H., von Heijne, G., and Brunak, S. (2004). Improved prediction of signal peptides: signalp 3.0. *J. Mol. Biol.* 340, 783–795. doi: 10.1016/j.jmb.2004.05.028

Bondu, S., Deslandes, E., Fabre, M., Berthou, C., and Guangli, Y. (2010). Carrageenan from *Solieria chordalis* (Gigartinales): structural analysis and immunological activities of the low molecular weight fractions. *Carbohydr. Polym.* 81, 448–460. doi: 10.1016/j.carbpol.2010.02.046

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Buono, M., and Cosma, M. P. (2010). Sulfatase activities towards the regulation of cell metabolism and signaling in mammals. *Cell. Mol. Life Sci.* 67, 769–780. doi: 10.1007/s00018-009-0203-3

Candiano, G., Bruschi, M., Musante, L., Santucci, L., Ghiggeri, G. M., Carnemolla, B., et al. (2004). Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* 25, 1327–1333. doi: 10.1002/elps.200305844

Chiovitti, A., Bacic, A., Craik, D. J., Munro, S. L., Kraft, G. T., and Liao, M. (1997). Cell-wall polysaccharides from Australian red algae of the family *Solieriaceae* (Gigartinales, Rhodophyta): novel, highly pyruvated carrageenans from the genus *Callophycus*. *Carbohydr. Res.* 299, 229–243. doi: 10.1016/S0008-6215(97)00017-7

Chiovitti, A., Bacic, A., Craik, D., Kraft, G. T., Liao, M., Falshaw, R., et al. (1998). A pyruvated carrageenan from Australian specimens of the red alga *Sarconema filiforme*. *Carbohydr. Res.* 310, 77–83. doi: 10.1016/S0008-6215(98)00170-0

de Hostos, E. L., Schilling, J., and Grossman, A. R. (1989). Structure and expression of the gene encoding the periplasmic arylsulfatase of *Chlamydomonas reinhardtii*. *Mol. Gen. Genet.* 218, 229–239. doi: 10.1007/BF00331273

de Ruiter, G. A., and Rudolph, B. (1997). Carrageenan biotechnology. *Trends Food Sci. Technol.* 8, 389–395. doi: 10.1016/S0924-2244(97)01091-1

Ekborg, N. A., Taylor, L. E., Longmire, A. G., Henrissat, B., Weiner, R. M., and Hutcheson, S. W. (2006). Genomic and proteomic analyses of the agarolytic system expressed by *Saccharophagus degradans* 2-40. *Appl. Environ. Microbiol.* 72, 3396–3405. doi: 10.1128/AEM.72.5.3396-3405.2006

Falshaw, R., Furneaux, R. H., Wong, H., Liao, M., Bacic, A., and Chandrkrachang, S. (1996). Structural analysis of carrageenans from Burmese and Thai samples of *Catenella nipae* Zanardini. *Carbohydr. Res.* 285, 81–98. doi: 10.1016/S0008-6215(96)90174-3

Ferrante, P., Messali, S., Meroni, G., and Ballabio, A. (2002). Molecular and biochemical characterisation of a novel sulphatase gene: arylsulfatase G (ARSG). *Eur. J. Hum. Genet.* 10, 813–818. doi: 10.1038/sj.ejhg.5200887

Frese, M. A., Schulz, S., and Dierks, T. (2008). Arylsulfatase G, a novel lysosomal sulfatase. *J. Biol. Chem.* 283, 11388–11395. doi: 10.1074/jbc.M709917200

Genicot-Joncour, S., Poinas, A., Richard, O., Potin, P., Rudolph, B., Kloareg, B., et al. (2009). The cyclization of the 3,6-anhydro-galactose ring of iota-carrageenan is catalyzed by two D-galactose-2,6-sulfurylases in the red alga *Chondrus crispus*. *Plant Physiol.* 151, 1609–1616. doi: 10.1104/pp.109.144329

Glöckner, F. O., Kube, M., Bauer, M., Teeling, H., Lombardot, T., Ludwig, W., et al. (2003). Complete genome sequence of the marine planctomycete *Pirellula* sp. strain 1. *PNAS* 100, 8298–8303. doi: 10.1073/pnas.1431443100

Groisillier, A., Hervé, C., Jeudy, A., Rebuffet, E., Pluchon, P. F., Chevolot, Y., et al. (2010). MARINE-EXPRESS: taking advantage of high throughput cloning and expression strategies for the post-genomic analysis of marine organisms. *Microb. Cell. Fact.* 9, 45–55. doi: 10.1186/1475-2859-9-45

Guibet, M., Colin, S., Barbeyron, T., Genicot, S., Kloareg, B., Michel, G., et al. (2007). Degradation of lambda-carrageenan by *Pseudoalteromonas carrageenovora* lambda-carrageenase: a new family of glycoside hydrolases

unrelated to kappa- and iota-carrageenases. *Biochem. J.* 404, 105–114. doi: 10.1042/BJ20061359

Hallmann, A., and Sumper, M. (1994). An inducible arylsulfatase of *Volvox carteri* with properties suitable for a reporter-gene system. Purification, characterization and molecular cloning. *Eur. J. Biochem.* 221, 143–150. doi: 10.1111/j.1432-1033.1994.tb18723.x

Henares, B. M., Enriquez, E. P., Dayrit, F. M., and Rojas, N. R. (2010). Iota-carrageenan hydrolysis by *Pseudoalteromonas carrageenovora* IFO 12985. *Phil. J. Sci.* 139, 131–138. Available online at: http://philjournalsci.dost.gov.ph/vol139no2/pdfs/Iota%20carrageenan%20hydrolysis.pdf

Katoh, K., Kuma, K., Toh, H., and Miyata, T. (2005). MAFFT version 5: improvement in accuracy of multiple sequence alignment. *Nucleic Acids Res.* 33, 511–518. doi: 10.1093/nar/gki198

Kelley, L. A., and Sternberg, M. J. (2009). Protein structure prediction on the Web: a case study using the Phyre server. *Nat. Protoc.* 4, 363–371. doi: 10.1038/nprot.2009.2

Kennedy, J., Marchesi, J. R., and Dobson, A. D. W. (2008). Marine metagenomics strategies for discovery of novel enzymes with biotechnological applications from marine ecosystems. *Microb. Cell Fact.* 7:27. doi: 10.1186/1475-2859-7-27

Kim, D. E., Kim, K. H., Bae, Y. J., Lee, J. H., Jang, Y. H., and Nam, S. W. (2005). Purification and characterization of the recombinant arylsulfatase cloned from *Pseudoalteromonas carrageenovora*. *Prot. Expr. Purif.* 39, 107–115. doi: 10.1016/j.pep.2004.09.007

Kim, J. H., Byun, D. S., Godber, J. S., Choi, J. S., Choi, W. C., and Kim, H. R. (2004). Purification and characterization of arylsulfatase from *Sphingomonas* sp. AS6330. *Appl. Microbiol. Biotechnol.* 63, 553–559. doi: 10.1007/s00253-003-1463-8

Kloareg, B., and Quatrano, R. S. (1988). Structure of the cell-walls of marine-algae and ecophysiological functions of the matrix polysaccharides. *Oceanogr. Mar. Biol.* 26, 259–315.

Knutsen, S., Myslabodski, D., Larsen, B., and Usov, A. (1994). A modified system of nomenclature for red algal galactans. *Bot. Mar.* 37, 163–169. doi: 10.1515/botm.1994.37.2.163

Korf, U., Kohl, T., van der Zandt, H., Zahn, R., Schleeger, S., Ueberle, B., et al. (2005). Large-scale protein expression for proteome research. *Proteomics* 5, 3571–3580. doi: 10.1002/pmic.200401195

Kumar, S., Mehta, G. K., Prasad, K., Meena, R., and Siddhanta, A. K. (2011). Chemical investigation of carrageenan from the red alga *Sarconema filiforme* (Gigartinales, Rhodophyta) of Indian waters. *Nat. Prod. Commun.* 6, 1327–1332.

Laemmli, U. K., and Favre, H. (1973). Maturation of the head of bacteriophage T4. I DNA packaging events. *J. Biol. Chem.* 80, 575–599.

Lahaye, M., and Robic, A. (2007). Structure and functional properties of ulvan, a polysaccharide from green seaweeds. *Biomacromolecules* 8, 1765–1774. doi: 10.1021/bm061185q

Larré, C., Penninck, S., Bouchet, B., Lollier, V., Tranquet, O., Denery-Papini, S., et al. (2010). *Brachypodium distachyon* grain: identification and subcellular localization of storage proteins. *J. Exp. Bot.* 61, 1771–1783. doi: 10.1093/jxb/erq050

Lim, J. M., Jang, Y. H., Kim, H. R., Kim, Y. T., Choi, T. J., Kim, J. K., et al. (2004). Overexpression of arylsulfatase in *E. coli* and its application to desulfatation of agar. *J. Microbiol. Biotechn.* 14, 777–782. Available online at: http://www.jmb.or.kr/

McLean, M. W., and Williamson, F. B. (1979a). Glycosulphatase from *Pseudomonas carrageenovora*. Purification and some properties. *Eur. J. Biochem.* 101, 497–505. doi: 10.1111/j.1432-1033.1979.tb19744.x

McLean, M. W., and Williamson, F. B. (1979b). Kappa-Carrageenase from *Pseudomonas carrageenovora*. *Eur. J. Biochem.* 93, 553–558. doi: 10.1111/j.1432-1033.1979.tb12854.x

McLean, M. W., and Williamson, F. B. (1981). Neocarratetraose 4-O-monosulphate beta-hydrolase from *Pseudomonas carrageenovora*. *Eur. J. Biochem.* 113, 447–456. doi: 10.1111/j.1432-1033.1981.tb05084.x

Medigue, C., Krin, E., Pascal, G., Barbe, V., Bernsel, A., Bertin, P. N., et al. (2005). Coping with cold: the genome of the versatile marine Antarctica bacterium *Pseudoalteromonas haloplanktis* TAC125. *Genome Res.* 15, 1325–1335. doi: 10.1101/gr.4126905

Methe, B. A., Nelson, K. E., Deming, J. W., Momen, B., Melamud, E., Zhang, X., et al. (2005). The psychrophilic lifestyle as revealed by the genome sequence of *Colwellia psychrerythraea* 34H through genomic and proteomic analyses. *Proc. Natl. Acad. Sci. U.S.A.* 102, 10913–10918. doi: 10.1073/pnas.0504766102

Michel, G., Chantalat, L., Duee, E., Barbeyron, T., Henrissat, B., Kloareg, B., et al. (2001). The kappa-carrageenase of *P. carrageenovora* features a tunnel-shaped active site: a novel insight in the evolution of Clan-B glycoside hydrolases. *Structure* 9, 513–525. doi: 10.1016/S0969-2126(01)00612-8

Michel, G., Nyval-Collen, P., Barbeyron, T., Czjzek, M., and Helbert, W. (2006). Bioconversion of red seaweed galactans: a focus on bacterial agarases and carrageenases. *Appl. Microbiol. Biotechnol.* 71, 23–33. doi: 10.1007/s00253-006-0377-7

Michel, G., Tonon, T., Scornet, D., Cock, J. M., and and Kloareg, B. (2010). The cell wall polysaccharide metabolism of the brown alga *Ectocarpus siliculosus*. Insights into the evolution of extracellular matrix polysaccharides in Eukaryotes. *New Phytol.* 188, 82–97. doi: 10.1111/j.1469-8137.2010.03374.x

Necas, J., and Bartosikova, L. (2013). Carrageenan: a review. *Vet. Med.* 58, 187–205. Available online at: http://www.vri.cz/docs/vetmed/58-4-187.pdf

Paietta, J. V. (1989). Molecular cloning and regulatory analysis of the arylsulfatase structural gene of *Neurospora crassa*. *Mol. Cell. Biol.* 9, 3630–3637.

Pereira, L., Crichley, A. T., Amado, A. M., and Ribeiro-Claro, P. J. A. (2009). A comparative analysis of phycocolloids produced by underutilized versus industrially utilized carrageenophytes (Gigartinales, Rhodophyta). *J. Appl. Phycol.* 21, 599–605. doi: 10.1007/s10811-009-9447-4

Pereira, L., and Mesquita, J. (2004). Population studies and carrageenan properties of *Chondracanthus teedei* var. *lusitanicus* (Gigartinaceae, Rhodophyta). *J. Appl. Phycol.* 16, 369–383. doi: 10.1023/B:JAPH.0000047948.11892.67

Pomin, V. H., and Mourao, P. A. (2008). Structure, biology, evolution, and medical importance of sulfated fucans and galactans. *Glycobiology* 18, 1016–1027. doi: 10.1093/glycob/cwn085

Popper, Z. A., Michel, G., Hervé, C., Domozych, D. S., Willats, W. G. T., Tuohy, M. G., et al. (2011). Evolution and diversity of plant cell walls: from algae to flowering plants. *Annu. Rev. Plant Biol.* 62, 567–590. doi: 10.1146/annurev-arplant-042110-103809

Préchoux, A., Genicot, S., Rogniaux, H., and Helbert, W. (2013). Controlling carrageenan structure using a novel formylglycine-dependent sulfatase, an endo-4S-iota-carrageenan sulfatase. *Mar. Biotech.* 15, 265–274. doi: 10.1007/s10126-012-9483-y

Robert, X., and Gouet, P. (2014). Deciphering key features in protein structures with the new ENDscript server. *Nucl. Acids Res.* 42, W320–W324. doi: 10.1093/nar/gku316

Sardiello, M., Annunziata, I., Roma, G., and Ballabio, A. (2005). Sulfatases and sulfatase modifying factors: an exclusive and promiscuous relationship. *Hum. Mol. Genet.* 14, 3203–3217. doi: 10.1093/hmg/ddi351

Sasaki, H., Yamada, K., Akasaka, K., Kawasaki, H., Suzuki, K., Saito, A., et al. (1988). cDNA cloning, nucleotide sequence and expression of the gene for arylsulfatase in the sea urchin (*Hemicentrotus pulcherrimus*) embryo. *Eur. J. Biochem.* 177, 9–13.

Seibert, C., and Raushel, F. (2005). Structural and catalytic diversity within the amidohydrolase superfamily. *Biochemistry* 44, 6383–6390. doi: 10.1021/bi047326v

Shin, M. H., Lee, D. Y., Wohlgemuth, G., Choi, L. G., Fiehn, O., and Kilm, K. H. (2010). Global metabolite profiling of agarose degradation by *Saccharophagus degradans* 2-40. *New Biotechnol.* 27, 156–168. doi: 10.1016/j.nbt.2010.02.023

Studier, F. W. (2005). Protein production by auto-induction in high-density shaking cultures. *Prot. Exp. Pur.* 41, 207–234. doi: 10.1016/j.pep.2005.01.016

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* 28, 2731–2739. doi: 10.1093/molbev/msr121

Tessier, D., Yclon, P., Jacquemin, I., Larré, C., and Rogniaux, H. (2010). OVNIp: an open source application facilitating the interpretation, the validation and the edition of proteomics data generated by MS analyses and *de novo* sequencing. *Proteomics* 10, 1794–1801. doi: 10.1002/pmic.200800783

Thrash, J. C., Cho, J. C., Vergin, K. L., Morris, R. M., and Giovannoni, S. J. (2010). Genome sequence of *Lentisphaerae araneosa* HTCC2155T, the type species of the order *Lentisphaerales* in the phylum lentisphaerae. *J. Bacteriol.* 192, 2938. doi: 10.1128/JB.00208-10

Usov, A. I. (2011). Polysaccharides of the red algae. *Adv. Carbohydr. Chem. Biochem.* 65, 115–217. doi: 10.1016/B978-0-12-385520-6.00004-2

van de Velde, F., Knutsen, S. H., Usov, A. I., Rollema, H. S., and Cerezo, A. S. (2002). ^{1}H and ^{13}C high resolution NMR spectroscopy of carrageenans: application in

research and industry. *Trends Food Sci. Technol.* 13, 73–92. doi: 10.1016/S0924-2244(02)00066-3

Viladot, J. L., de Ramon, E., Durany, O., and Planas, A. (1998). Probing the mechanism of *Bacillus* 1,3-1,4-beta-D-glucan 4-glucanohydrolases by chemical rescue of inactive mutants at catalytically essential residues. *Biochemistry* 37, 11332–11342.

Weigl, J., and Yaphe, W. (1966a). The enzymic hydrolysis of carrageenan by *Pseudomonas carrageenovora*: purification of a kappa-carrageenase. *Can. J. Microbiol.* 12, 939–947.

Weigl, J., and Yaphe, W. (1966b). Glycosulfatase of *Pseudomonas carrageenovora*: desulfation of disaccharide from κ- carrageenan. *Can. J. Microbiol.* 12, 874–876.

Whelan, S., and Goldman, N. (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol.* 18, 691–699. doi: 10.1093/oxfordjournals.molbev.a003851

Yang, Q., Angerer, L. M., and Angerer, R. C. (1989). Structure and tissue-specific developmental expression of a sea urchin arylsulfatase gene. *Dev. Biol.* 135, 53–65.

Zablackis, E., and Santos, G. A. (1986). The carrageenan of *Catenella-nipae* Zanardini, a marine red alga. *Bot. Mar.* 29, 319–322.

ZoBell, C. E. (1941). Studies on marine bacteria. The cultural requirements of heterotrophic aerobes. *J. Mar. Res.* 4, 42–75.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 24 June 2014; accepted: 03 August 2014; published online: 26 August 2014.
Citation: Genicot SM, Groisillier A, Rogniaux H, Meslet-Cladière L, Barbeyron T and Helbert W (2014) Discovery of a novel iota carrageenan sulfatase isolated from the marine bacterium Pseudoalteromonas carrageenovora. Front. Chem. ***2****:67. doi: 10.3389/fchem.2014.00067*
This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

REVIEW ARTICLE
published: 09 September 2014
doi: 10.3389/fchem.2014.00077

Relationships between chemical structures and functions of triterpene glycosides isolated from sea cucumbers

Joo-In Park[1]*, Hae-Rahn Bae[2]*, Chang Gun Kim[1,3], Valentin A. Stonik[4] and Jong-Young Kwak[1,3]*

[1] *Department of Biochemistry, Dong-A University, Busan, South Korea*
[2] *Department of Physiology, School of Medicine, Dong-A University, Busan, South Korea*
[3] *Immune-Network Pioneer Research Center, Dong-A University, Busan, South Korea*
[4] *The Laboratory of Chemistry of Marine Natural Products, G. B. Elyakov Pacific Institute of Bioorganic Chemistry, Far-Eastern Branch of the Russian Academy of Science, Vladivostok, Russia*

Edited by:
Antonio Trincone, Consiglio Nazionale delle Ricerche, Italy

Reviewed by:
Robert Vianello, Rudjer Boskovic Institute, Croatia
Daniel Anthony Dias, The University of Melbourne, Australia

****Correspondence:***
Joo-In Park, Department of Biochemistry, Dong-A University College of Medicine, 32, Daesin-gongwon-ro, Seo-Gu, Busan 602-714, South Korea
e-mail: jipark@dau.ac.kr;
Hae-Rahn Bae, Department of Physiology, Dong-A University School of Medicine, 32, Daesin-gongwon-ro, Seo-Gu, Busan 602-714, South Korea
e-mail: hrbae@dau.ac.kr;
Jong-Young Kwak, Department of Biochemistry, Immune-network Pioneer Research Center, Dong-A University School of Medicine, Dong-A University, 32, Daesin-gongwon-ro, Seo-Gu, Busan 602-714, South Korea
e-mail: jykwak@dau.ac.kr

Many marine triterpene glycosides have *in vitro* and *in vivo* activities with very low toxicity, suggesting that they are suitable agents for the prevention and treatment of different diseases, particularly cancer. However, the molecular mechanisms of action of natural marine compounds in cancer, immune, and other various cells are not fully known. This review focuses on the structural characteristics of marine triterpene glycosides and how these affect their biological activities and molecular mechanisms. In particular, the membranotropic and membranolytic activities of frondoside A and cucumariosides from sea cucumbers and their ability to induce cytotoxicity and apoptosis have been discussed, with a focus on structure-activity relationships. In addition, the structural characteristics and antitumor effects of stichoposide C and stichoposide D have been reviewed along with underlying their molecular mechanisms.

Keywords: triterpene glycosides, frondoside A, cucumarioside, stichoposides, anticancer activity, membrane transporters

INTRODUCTION

Many marine natural products have biological activities and low toxicity suitable for administration and exhibit wide diversity in their mechanisms of action. Glycosides, substances consisting of a sugar moiety attached to a triterpene or steroid aglycone, are widely distributed in plants. Triterpene glycosides are also found in marine invertebrates and are characteristic secondary metabolites of echinoderms, octocorals, and sponges (Stonik et al., 1999; Kalinin et al., 2008; Bordbar et al., 2011).

Stichoposide C (STC) (compound 1) and stichoposide D (STD) (compound 2) are hexaosides isolated from the holothurian *Stichopus chloronotus* (**Figure 1**) (Kitagawa et al., 1981; Stonik et al., 1982a). These compounds are also found in other representatives of the family Stichopodidae such as *Thelenota ananas* (Stonik et al., 1982b). The structural differences between STC and STD are a sugar residue; STC has quinovose, while STD has glucose as the second monosaccharide unit (indicated as the asterisk in compound 1 and 2). Frondoside A (compound 3) and cucumariosides are derived from the edible sea cucumbers *Cucumaria frondosa* and *C. japonica*, respectively (Girard et al., 1990; Stonik et al., 1999) (**Figure 2**). *C. japonica* is a source of several different cucumariosides such as cucumarioside A_2-2 (compound 4), A_4-2 (compound 5), and A_7-1 (compound 6) (**Figure 2**) (Avilov et al., 1990; Drozdova et al., 1993, 1997; Stonik et al., 1999). Frondoside A and cucumariosides are pentaosides, with the main structural difference between the two compounds being the functional group at C-16 of the aglycone (acetoxy or keto group) and the third carbohydrate unit in the carbohydrate chain (indicated by an asterisk in compound 3 and 4). Interestingly, despite such similar structures, the biological activity and mechanism of frondoside A and cucumariosides appear to differ.

This review highlights the structural characteristics and mechanisms of action of marine triterpene glycosides, such as stichoposides, frondoside A, and cucumariosides. The biological activities and molecular mechanisms of several additional marine triterpene glycosides that have been studied are summarized.

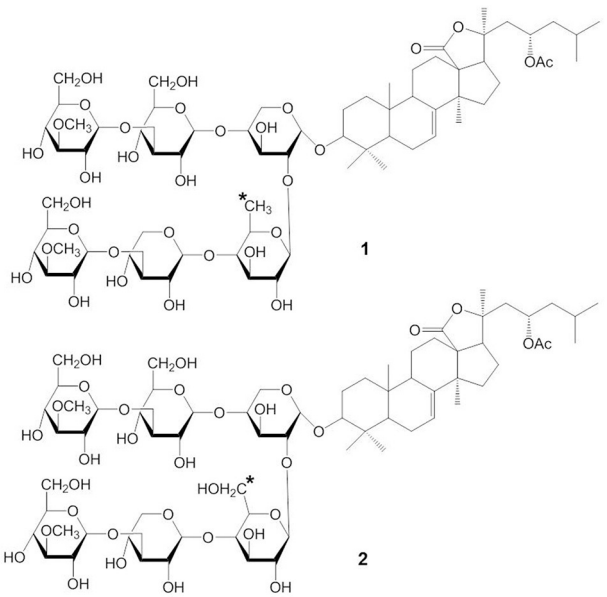


FIGURE 1 | Structures of STC (1) and STD(2).

THE STRUCTURAL CHARACTERISTICS OF MARINE TRITERPENE GLYCOSIDES

Triterpene glycosides are the most abundant secondary metabolites in terrestrial plants and sea cucumbers. Marine triterpene glycosides are the predominant secondary metabolites of holothurians and are suggested to be responsible for their general cytotoxicity (Zhang et al., 2006a,b,c,d; Kim and Himaya, 2012; Colorado-Ríos et al., 2013). Last 20 years more than 100 new triterpene glycosides were isolated. Really, only by Russian group from Pacific Institute of Bioorganic Chemistry (PIBOC) at Vladivostok, about 30 new glycosides were isolated from *Eupentacta (Cucumaria) fraudatrix* (Silchenko et al., 2011, 2012a,b,c,d, 2013b,c), 14 new glycosides from *Cucumaria frondosa* (Girard et al., 1990; Silchenko et al., 2005a,b, 2007b), 7 from *Stuarocucumis liouvillei* (Antonov et al., 2008, 2011), 6 from *Cladolabes schmeltzi* (Silchenko et al., 2013d), 6 from *Cucumaria okhotensis* (Silchenko et al., 2007a, 2008), 5 from *Synallactes nozawai* (Silchenko et al., 2002), 5 from *Actinocucumis typica* (Silchenko et al., 2013a), 4 from *Cucumaria conicospermium* (Avilov et al., 2003), 3 from Mediterranean species (Silchenko et al., 2005c), 3 from *Pentamera calcigera* (Avilov et al., 2000a,b), 3 from *Australostichopus (Stichopus) mollis* (Moraes et al., 2005), 3 from *Achlionice violaecuspidata* (Antonov et al., 2009), 2 from *Synapta maculata* (Avilov et al., 2008), 1 from *Cucumaria koriaiensis* (Avilov et al., 1997), 1 from *Psolus eximus* (Kalinin et al., 1997), and so on. Several series of structures were also reviewed (Kalinin et al., 2012; Kim and Himaya, 2012). Reviews, completely described all the last glycosides were not yet published. Several early reviews are mentioned in our papers (Stonik et al., 1999; Kalinin et al., 2005). These are grouped into four main structural categories, based on their aglycone structures: 3β-hydroxyholost-9(11)-ene aglycone skeleton (structure 7), 9βH-3β-hydroxyholost-7-ene skeleton (structure 8), other holostane type glycosides and nonholostane glycosides (**Figure 3**) (Kim and Himaya, 2012).

Triterpene glycosides of holothurians typically have carbohydrates and triterpenoid moieties (Kerr and Chen, 1995; Bordbar et al., 2011). The triterpenoid moieties consist of lanostane derivatives (Zhou et al., 2005) where the majority belongs to the holostane type (Dang et al., 2007). Holostane type triterpene glycosides include a 3β, 20S-dihydroxy-5α-lanostano-18(20)-lactone structural feature (structure 9). The glycone part of natural products isolated from the sea cucumbers consists of two to six sugar units and is linked to the C-3 position of the aglycone unit (Chiludil et al., 2003; Kalinin et al., 2005). Quinovose, glucose, 3-*O*-methylglucose, xylose, and 3-*O*-methylxylose are the main sugars present in the carbohydrate moieties of these glycosides (Iñiguez-Martinez et al., 2005). In the structure of the oligosaccharide chain, the first monosaccharide unit is always a xylose, whereas 3-*O*-methylglucose or 3-*O*-methylxylose is always at the terminus. In some glycosides, sulfate groups are attached to the oligosaccharide chain. Most of these are mono-sulfated glycosides with a few occurrences of di- and tri-sulfated glycosides (Chiludil et al., 2003).

MEMBRANOTROPIC AND MEMBRANOLYTIC EFFECTS OF TRITERPENE GLYCOSIDES

Membranolytic effects such as increased membrane permeability, loss of barrier function, and the rupture of cell membrane are considered to be the basic mechanisms underlying a variety of biological activities exerted by triterpene glycosides from both sea cucumbers and higher plants. However, the molecular mechanisms of action of these compounds in biomembranes are not fully understood. The triterpene glycosides attach to cell membranes, interact with membrane lipids, and form glycoside-sterol complexes in biomembranes, modulating the membrane microviscosity and the activities of embedded membrane proteins (Stonik et al., 1999; Pislyagin et al., 2012). The formation of multimeric channels in sterol-containing lipid bilayers by triterpene glycosides may also be a basic mechanisms involved in increasing the permeability of membranes to ions and peptides (Li et al., 2005).

Although there are many subtle structural and functional differences between marine and plant triterpene glycosides, knowledge from earlier research with plant triterpene glycosides suggests that marine triterpene glycosides may have similar effects on membranes. For example, extensive studies on the membranotropic effects of plant triterpene glycosides have been performed for decades, especially with natural compounds from *Panax ginseng C.A. Meyer* (Im and Nah, 2013). The structures of some pharmacologically important plant triterpene glycosides are shown in **Figure 4**. Ginsenosides or ginseng saponins are major pharmacologically active ingredients of ginseng, which are composed with an aglycone of a dammarane skeleton and one or more covalently linked sugar moieties (Nah, 2014). Ginsenoside Rb_1 has two glucopyranosyl sugar chains at C-3 and C-20 positions, respectively (compound 10) (**Figure 4**). Ginsenoside Re has one glucose-rhamnose disaccharide moiety at C-6 position and one glucopyranosyl moiety at C-20 position (compound 11). Ginsenoside Rg_3 has two glucopyranosyl

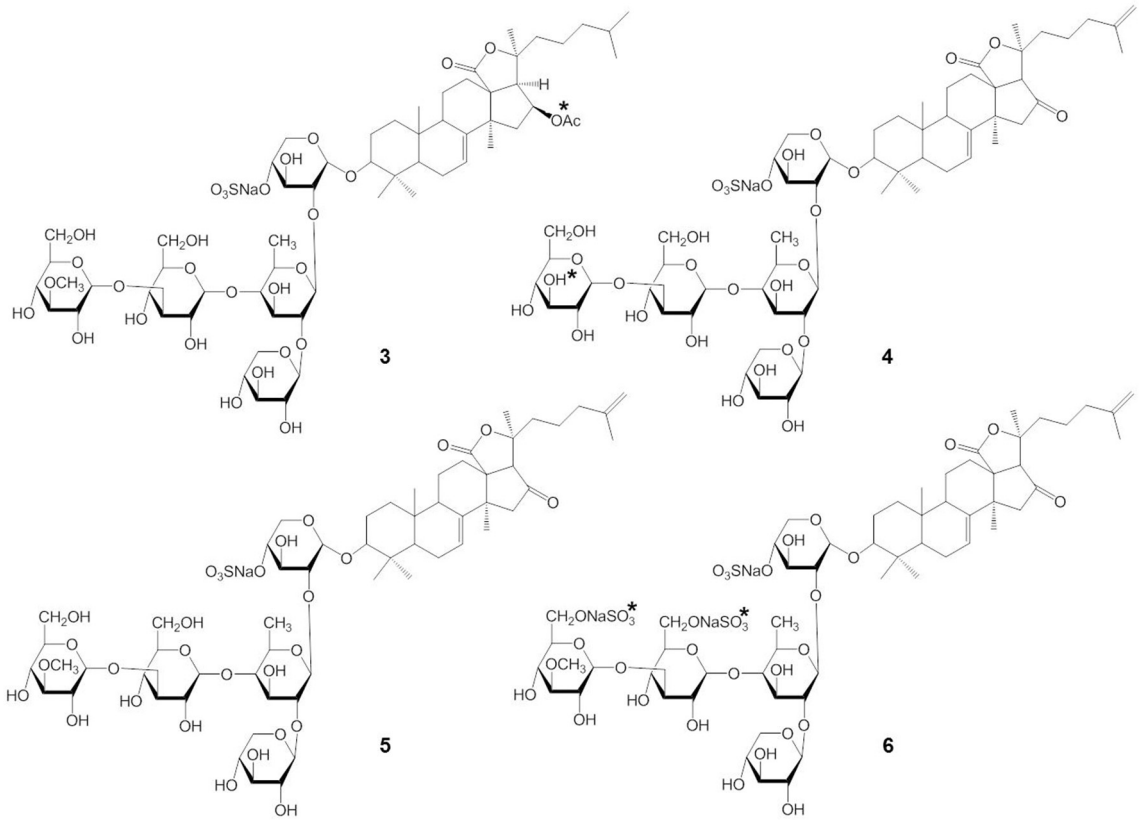


FIGURE 2 | Structures of frondoside A (3) and cucumariosides (4-6).

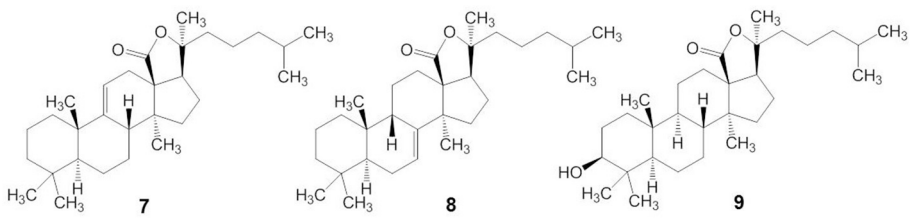


FIGURE 3 | Structures of aglycone skeleton systems with 9(11) double bond (7), 9β-H-7(8)-unsaturation (8) and 3β, 20S-Dihydroxy-5α-lanostano-18(20)-lactone (9).

sugar chains only at C-3 position (compound 12). Glycyrrhizin, the main sweet-tasting constituent of licorice root, consists of a disaccharide of two glucuronic acids linked at C-3 position of the pentacyclic triterpene aglycone, glycyrrhetinic acid (compound 13). Recently, a detailed mechanism for the membrane permeabilization induced by the triterpenoid monodesmosidic saponin, α- and δ-hederin (triterpene saponins) has been proposed (Lorent et al., 2013). This mechanism includes three steps of cholesterol-independent binding to the membrane, interaction with cholesterol and asymmetric lateral distribution of saponin, and pore formation and budding of the lipid bilayer due to the increased curvature stress (Lorent et al., 2013).

More recently, at lower concentrations (in the nanomolar or low micromolar ranges) than those causing hemolytic and cytotoxic effects, the triterpene glycosides from marine sponges and sea cucumbers were found to act on specific membrane transport proteins and change their activities. For example, frondoside A and cucumarioside A_2-2 inhibited the ATP-binding cassette (ABC) transporter, multidrug-resistance protein-1 (MDR1) (Wink et al., 2012; Menchinskaya et al., 2013). Membrane transporters which are modulated by triterpene glycosides and thus can be proposed as potential therapeutic targets are summarized in **Table 1**. Although the majority of researches on the membranotropic effects were done with plant triterpene glycosides, understanding the reported targets of membrane transport

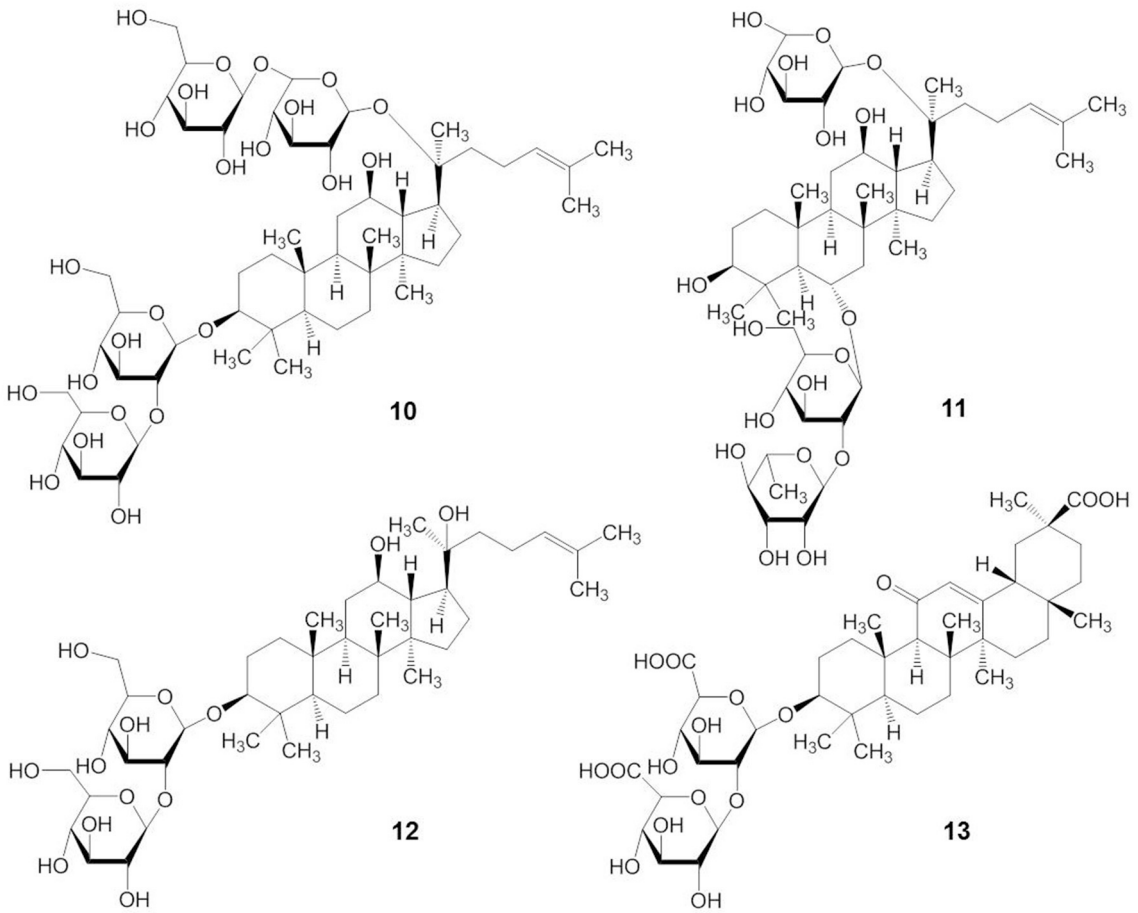


FIGURE 4 | Structures of plant triterpene glycosides.

proteins might provide a basis for the exploration of target molecules for marine triterpene glycosides and their development as drugs.

Selective inhibition of Na^+-K^+-ATPase and Ca^{2+}-ATPase in sarcoplasmic/endoplasmic reticulum (SERCA), in combination with increased Ca^{2+} influx through L-type voltage-gated calcium channels, transient receptor potential canonical (TRPC) channels, and the ryanodine receptor led to an increase of cytosolic Ca^{2+} levels. This might explain the positive inotropic effect of triterpene glycosides (Gorshkova et al., 1999; Wang et al., 2008; Lin et al., 2012; Hwang et al., 2013; Wong et al., 2013). Furthermore, triterpene glycosides inhibited voltage-gated Na^+ channels ($Na_V1.2$ and $Na_V1.4$) (Lee et al., 2008). In addition, triterpene glycosides could induce K^+ currents through voltage-gated K^+ channel ($K_V1.4$), calcium-activated K^+ channel (BK_{Ca}), and human Ether-*à*-go-go Related Gene (hERG) K^+ channels (Kv11.1), which might be responsible for their vasodilatory and antiarrhythmic effects (Lee et al., 2009; Choi et al., 2011a,b; Xu and Huang, 2012). The antiepileptic and neuroprotective effects of triterpene glycosides might be due to the inhibition of excitatory *N*-methyl-D-aspartate (NMDA) receptors and nicotinic acetylcholine receptors, as well as the activation of inhibitory γ-amino butyric acid (GABA) receptors (Lee et al., 2006, 2013a,b).

ANTICANCER ACTIVITIES OF MARINE TRITERPENE GLYCOSIDES

The antitumor action of the triterpene glycosides from sea cucumbers was discovered by Nigrelli (1952), and most of the marine triterpene glycosides that have been studied since that time are cytotoxic toward cancer cells. Nigrelli (1952) showed that injection of a "holothurin" solution inhibited the growth of Sarcoma-180 tumor cells and induced regression of the tumor. Injection of Krebs-2 ascitic tumor cells treated with holothurin into healthy mice failed to induce marked tumor growth up to 80 days (Sullivan et al., 1955). In addition, holothurin, which is a substance containing as a main constituent holothurin A, inhibited the growth of epidermal carcinoma (KB) tumor cells (Nigrelli et al., 1967).

Many triterpene glycosides from various species of sea cucumbers have diverse biological activities, including anticancer activity. For example, glycosides from 19 species of the families *Holothuriidae* and *Stichopodidae* (the glycosides in majority belong to holothurin A and B series) inhibited the growth

Table 1 | Membrane transporters as potential targets of triterpene glycosides from sea cucumbers and plants.

Type		Membrane transporter	Name of compound	Species	References
Pump		Na^+-K^+-ATPase	Glycyrrhizin	*Glycyrrhiza glabra*	Itoh et al., 1989
			Glycyrrhetinic acid		
			Psolusosides A and B	*Psolus fabricii*	Gorshkova et al., 1999
		Ca^{2+}-ATPase in sarcoplasmic reticulum	Cyclopiazonic acid	*Penicillium cyclopium*	Uyama et al., 1992
			Astragaloside IV	*Astragalus membranaceus*	Xu et al., 2008
		Multidrug-resistance protein-1	Saikosaponin-d	*Bupleurum falcatnum*	Wong et al., 2013
			Ginsenoside Rp_1	*Panax ginseng*	Yun et al., 2013
			Glycyrrhizin	*Glycyrrhiza glabra*	Fu et al., 2014
			Cucumarioside A_2-2	*Cucumaria japonica**	Menchinskaya et al., 2013
			Frondoside A	*Cucumaria okhotensis**	Menchinskaya et al., 2013
		Na^+-Ca^{2+} exchange	Echinoside-A and –B	*Pearsonothuria graeffei*	Yamasaki et al., 1987
Channel	Voltage-gated	Voltage-gated Na^+ channel	Ginsenoside Rg_3	*Panax ginseng*	Lee et al., 2008
			Ginsenoside Rb_1	*Panax ginseng*	Xu and Huang, 2012
			Ginsenoside Rg_3	*Panax ginseng*	Lee et al., 2009
		Calcium-activated K^+channel	Dehydrosoyasaponin I	*Desmodium adscendens*	McManus et al., 1993
			Ginsenoside Rg_3	*Panax ginseng*	Choi et al., 2011a
		Human ether-a-go-go related gene K^+ channel	Ginsenoside Rg_3	*Panax ginseng*	Choi et al., 2011b
		L-type voltage-gated calcium channel	Ginsenoside Rb_1	*Panax ginseng*	Lin et al., 2012
	Ligand-gated	Nicotinic acetylcholine receptor	Ginsenoside Rg_3	*Panax ginseng*	Lee et al., 2013a
		N-methyl-D-aspartate receptor	Ginsenoside Rh_2	*Panax ginseng*	Lee et al., 2006
			Ginsenoside Rg_3	*Panax ginseng*	Kim et al., 2002
		$GABA_A$ receptor	Ginsenoside Rg_3	*Panax ginseng*	Lee et al., 2013b
		Ryanodine receptor	Ginsenoside Re	*Panax ginseng*	Wang et al., 2008
	Mechanosensitive	Transient receptor potential canonical	20-O-β-d-Glucopyranosyl-20(S)-protopanaxadiol	*a metabolite of ginseng saponin*	Hwang et al., 2013
	Others	Auqaporin-1	Ginsenoside Rg_3	*Panax ginseng*	Pan et al., 2012
		Auqaporin-4	Astragaloside IV	*Astragalus membranaceus*	Li et al., 2013
Carrier		Glucose transporter (GLUT1, GLUT4)	Ginsenoside Rb_1	*Panax ginseng*	Shang et al., 2008

*Sea cucumbers.

of Sarcoma-37 cells at *in vitro* concentrations ranging from 6.2 to 100 μg/ml (Kuznetsova et al., 1982). Although the anticancer mechanisms of the triterpene glycosides have not been investigated in detail, the biologic actions, structure-activity relationships, and molecular mechanisms of stichoposide C, frondoside A, and cucumariosides have been most intensively studied (Aminin et al., 2001; Jin et al., 2009; Yun et al., 2012; Yun, 2014) and are discussed in the following sections. In addition, the potential molecular mechanisms of other triterpene glycosides have been described.

STRUCTURE-ACTIVITY RELATIONSHIPS OF MARINE TRITERPENE GLYCOSIDES

The molecular mechanisms of action of marine triterpene glycosides can be understood by uncovering the relationships between their structure and activities. However, the structure-activity relationships of marine triterpene glycosides have not been intensively studied. As shown in structure 14, the presence of an 18(20)-lactone in the aglycone, with at least one oxygen group nearby (indicated by an asterisk in structure 14), is significant for the biological activity of triterpene glycosides bearing a

9(11)-double bond (Kitagawa, 1988) (**Figure 5**). Glycosides that have a 7(8)-double bond in their aglycone without a 16-keto group are more active in hemolytic test than those with a 16-keto group (Kalinin et al., 1996). In general, the characteristics of the attached glycone structure may be related to the biological activities of the marine triterpene glycosides.

Many investigators have suggested that the bioactivity of the triterpene glycosides results from their strong membranolytic activity. It was reported that the membranolytic activity of triterpene glycosides was due to the formation of complexes between the glycosides and the 5(6)-unsaturated sterols within target cellular membranes (Kalinin, 2000). A linear tetrasaccharide chain of triterpene glycosides is necessary for the effects leading to modification of the cellular membrane (Kitagawa, 1988; Kalinin et al., 1992). Stichoposide A (STA) (compound 15), which had two monosaccharide units, and stichoposide E (STE) (compound 16), which has a xylose residue as the second monosaccharide unit (indicated by an asterisk in compound 16), had lesser membranotropic activity than other stichoposides (Kalinin et al., 2008) (**Figure 6**). Maltsev et al. (1985) reported that glycosides with quinovose as the second monosaccharide unit were more active hemolytics than other triterpene glycosides.

The presence or absence of a sulfate group in the sugar chain of triterpene glycosides influences their bioactivity (Kalinin, 2000; Kim and Himaya, 2012). A sulfate group at C-4 of the first xylose of non-branched glycosides with a linear tetrasaccharide unit (compound 17) does not significantly affect the activity of triterpene glycosides, but the absence of a sulfate group at C-4 of the xylose residue (compound 18) decreases their activity (indicated by an asterisk in compound 17 and 18) (**Figure 7**) (Kitagawa, 1988; Kalinin et al., 1992). In contrast, the presence of a sulfate at C-4 of the first xylose in branched pentaosides with 3-*O*-methyl group on the terminal monosaccharide increases their activities, while the same sulfate decreases the activity of branched pentaosides that have glucose as the terminal residue. Sulfate groups attached to the C-6 position of terminal glucose or 3-*O*-methylglucose residues in triterpene glycosides greatly reduce their activity (Kalinin, 2000; Kim and Himaya, 2012).

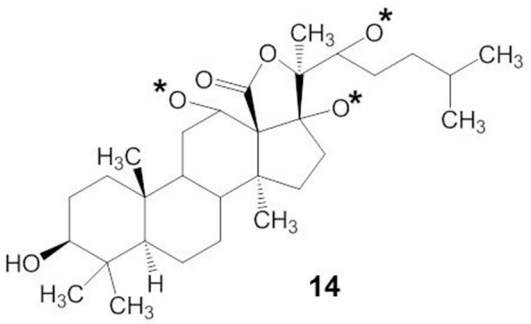


FIGURE 5 | Structures of 18(20)-lactone in the aglycone with oxygen group.

STICHOPOSIDES

STC

STC (also called stichloroside C1) is a quinovose-containing hexaoside, originally isolated from the holothurian *S. chloronotus* (Kitagawa et al., 1981; Stonik et al., 1982a) but is also found in other representatives of the family *Stichopodidae* such as *T. ananas* (Stonik et al., 1982b). STC has quinovose as the second monosaccharide unit. The antitumor activity of STC appears to be related to its membranotropic effects (Kalinin et al., 2008). We previously reported that STC induced apoptosis of human leukemia and colorectal cancer cells through the activation of both intrinsic and extrinsic pathways (Yun et al., 2012). Anticancer agents increase ceramide levels, to variable extents, in all types of cancer cells (Taha et al., 2006). Ceramide is generated either by *de novo* synthesis or by sphingomyelin hydrolysis through the action of several types of sphingomyelinase (SMase) such as acid, neutral, or alkaline SMase (Strum et al., 1997; Brown and London, 1998; Kolesnick et al., 2000; Hannun and Obeid, 2008). Both acid and neutral SMase are involved in ceramide generation in response to apoptotic stimuli (Levade and Jaffrezou, 1999; Goni and Alonso, 2002; Gulbins and Kolesnick, 2002). Moreover, under conditions where the classical apoptotic pathway fails, intracellular generation of ceramide may function as part of a backup system that enables caspase-independent programmed cell death (Taha et al., 2006). We demonstrated that STC induced apoptosis through the generation of ceramide by the activation of acid and neutral SMases (Yun et al., 2012). Therefore, the target of STC seems to be SMase leading to increases in ceramide and apoptosis.

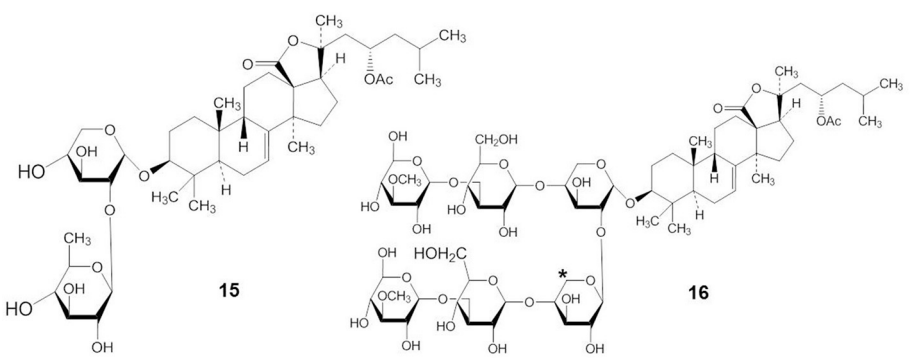


FIGURE 6 | Structures of STA (15) and STE (16).

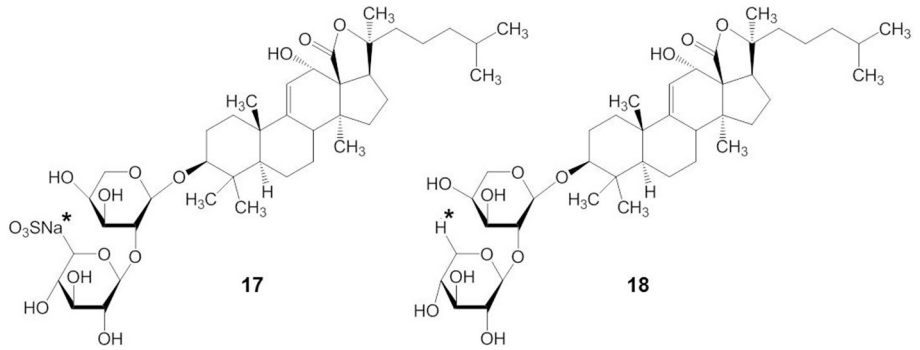


FIGURE 7 | Structure of compound with (17) or without (18) a sulfate group at C-4 of the xylose residue.

STD

STD is a hexaoside containing glucose at the second monosaccharide unit. We have shown that STD can induce apoptosis of human leukemia cells via the extrinsic and intrinsic pathways (Park et al., 2012a). We previously compared the potency of STC and STD in the induction of apoptosis using human leukemia K562 and HL-60 cells. STC was two to five times more potent than STD in inducing cell death [$IC_{50} = 0.5$ (K562 cells) and 0.3 (HL-60 cells) μM for STC; 1.0 (K562 cells), and 1.5 (HL-60 cells) μM for STD] (Park et al., 2012a). These results are consistent with the relative membranotropic activities of STC and STD, suggesting that their anticancer activities may be related to their membranotropic activities. More importantly, STC and STD did not have any toxicity in normal hematopoietic progenitor cells or in a mouse tumor model (Yun et al., 2012; Yun, 2014).

It was shown that STD induced apoptosis by activating ceramide synthase 6 (CerS6) and increasing cellular levels of ceramide (Yun, 2014). The activation of CerS6 appears to be subsequent to the activation of the death receptor Fas (CD95) by STD (Yun, 2014). These results suggest that the difference in only one sugar between STC and STD may influence both the potency and the molecular mechanisms for their activities. However, further studies on the relationship between the structure and the activity of these molecules are needed to improve the efficacy and safety of these compounds in treating cancer patients.

FRONDOSIDE A AND CUCUMARIOSIDES

BIOLOGICAL ACTIONS OF FRONDOSIDE A AND THE CUCUMARIOSIDES

Frondoside A has a sulfate, an acetoxy group at C-16 of the aglycone, penta-saccharide chain with xylose as the third monosaccharide residue, and 3-*O*-methylglucose as the terminal monosaccharide residue (Girard et al., 1990). Cucumarioside A_4-2 has a 16-keto group in the aglycone and a glucose residue as the third monosaccharide unit in the carbohydrate chain (Kalinin et al., 1992, 1996). Cucumarioside A_2-2 has 3-*O*-methylglucose instead of glucose as the terminal monosaccharide unit. Cucumarioside A_2-2 is probably biogenetically connected with A_4-2 (Kalinin et al., 1992). Therefore, the main structural differences between frondoside A and the cucumariosides, as shown in **Figure 2**, are in a functional group at C-16 of the aglycone and the third carbohydrate unit in the carbohydrate chain.

Frondoside A and cucumariosides show anticancer activities *in vitro* and suppress tumor growth *in vivo* (Tian et al., 2005; Tong et al., 2005; Li et al., 2008). The antitumor activity of frondoside A and cucumariosides is a result of their activity to induce apoptosis of cancer cells (Li et al., 2008; Jin et al., 2009; Roginsky et al., 2010), including HL-60, NB-4, and THP-1 leukemic cells (Jin et al., 2009).

The cancer inhibitory effect of frondoside A in tumor-bearing mice might partly result from other biological activities, including its antiangiogenic and antimetastatic effects (Li et al., 2008; Al Marzouqi et al., 2011; Ma et al., 2012; Attoub et al., 2013). In addition, frondoside A inhibited the invasion of breast cancer cells via its ability to decrease matrix metalloproteinase (MMP)-9 expression through inhibition of nuclear translocation and transactivation of NF-κB and AP-1 (Park et al., 2012b). Park et al. (2012b) also showed that frondoside A significantly inhibited PI3K/Akt, ERK-1/2, and p38 MAPK activation in 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA)-stimulated breast cancer cells, indicating that frondoside A inhibited TPA-induced NF-κB and AP-1 activation via inactivation of the PI3K/Akt, ERK1/2 and p38 MAPK pathways. Frondoside A also decreased AP-1-dependent transcriptional activities in JB6-LucAP-1 cells (Silchenko et al., 2008).

It is well established that prostaglandin E receptor, EP_4 that is expressed in a number of different malignancies, can promote the migration of tumor cells *in vitro* (Timoshenko et al., 2003; Wang and Dubois, 2010). EP_4 also promotes the invasive behavior of inflammatory breast cancers, one of the more aggressive forms of breast cancers (Robertson et al., 2010). Frondoside A inhibited metastasis of breast cancer cells by antagonizing EP_4 and EP_2 (Ma et al., 2012).

Cucumariosides increased the lysosomal activity and intracellular Ca^{++} concentrations of macrophages. These effects are related to the chemical structures of the molecules. For example, although there was no direct correlation, Silchenko et al. (2013c) suggested that the lysosomal activity and cytotoxicity of cucumarioside depended on features of both the aglycone and the carbohydrate chain. Holt et al. (2012) have investigated the effect of frondoside A on NK cells and demonstrated that prostaglandin E_2 (PGE_2) significantly suppressed the secretion of interferon-γ (IFNγ) from NK cells while frondoside A restored the capacity of NK cells to secrete IFNγ in the presence of PGE_2. Other

studies reported that *in vitro* treatment of peritoneal macrophages with cucumarioside A_2-2 stimulated cell adhesion as well as their spreading reaction and motility (Aminin et al., 2011), whereas frondoside A suppressed MMP-9 enzymatic activity, secretion, and expression in MBA-MB-231 human breast cancer cells, leading to inhibition of invasion and migration of these cells (Park et al., 2012b). Therefore, it is important to compare the effects of frondoside A and cucumariosides on the migration and spreading of various kinds of cells, including cancer and immune cells.

EFFECTS OF SULFATE GROUPS ON THE HEMOLYTIC ACTIVITY OF CUCUMARIOSIDES

The structures of the aglycone and carbohydrates in cucumariosides may confer membranolytic activity (Stonik et al., 1999). Kalinin et al. (1996) demonstrated that the membranolytic properties of cucumariosides correlated with their cytotoxicity to tumor cells. Cucumarioside A_2-2 had *in vitro* cytotoxic and hemolytic effects on sea urchin embryos with EC_{50s} of 0.45 and 5 μg/mL, respectively (Aminin et al., 2006, 2010). The LD_{50} of cucumarioside A_2-2 for mice was 10 mg/kg after intraperitoneal injection (Polikarpova et al., 1990). The membranolytic action of cucumariosides may be mediated through formation of molecular complexes with sterols in membranes and subsequent generation of solitary ion channels and large aqueous pores (Anisimov, 1987; Verbist, 1993; Kalinin et al., 2008). In addition, the glycosides effectively increased the microviscosity of the lipid bilayer of cell membranes (Pislyagin et al., 2012).

Marine triterpene glycosides contain different numbers of sulfate groups bound with sugars. Cucumarioside A_2-2 has a sulfate group at C-4 of the first xylose residue and cucumarioside A_6-2 has an additional sulfate group at C-6 of the terminal 3-*O*-methylglucose residue. The hemolytic activity of cucumarioside A_2-2 was significantly greater than its desulfated derivative and was higher than that of cucumarioside A_6-2 (Kalinin et al., 1996). Moreover, cucumarioside A_2-2 had more active hemolytic activity than cucumarioside A_3, which has an additional sulfate group at C-6 of the third monosaccharide unit (Kalinin et al., 1996). The increase in intracellular Ca^{2+} concentrations was also influenced by the number and positions of sulfate groups in the carbohydrate moiety of the molecules. Cucumarioside A_2-2 was more active in inducing a rapid increase in cytosolic Ca^{2+} content, when compared to the poly-sulfated derivative of A_2-2, cucumarioside A_7-1 (indicated by an asterisk in compound 5) (Agafonova et al., 2003). In addition, the mono-sulfated cucumariosides A_2-2 and A_4-2 stimulated peritoneal macrophage lysosomal activity, while desulfation of their carbohydrate moiety completely abolished this activity (Aminin et al., 2001). Therefore, the hemolytic and cytotoxic activities of triterpene glycosides may be dependent on the positions of sulfate groups attached to the carbohydrates.

CYTOTOXIC EFFECTS OF FRONDOSIDE A AND CUCUMARIOSIDES ON CANCER CELLS

Frondoside A showed potent cytotoxicity against various cancer cells, including HeLa, HL-60, and lung cancer cells such as LNM35, A549, and NCI-H460-Luc2 (Silchenko et al., 2008; Jin et al., 2009; Attoub et al., 2013). Moreover, frondoside A enhanced the inhibition of lung tumor growth induced by the anticancer agent, cisplatin (Attoub et al., 2013). The IC_{50} of frondoside A in HL-60 cells was approximately 5- to 10- fold lower than that of cucumarioside A_2-2 (Jin et al., 2009), although the *in vivo* toxicity of these two compounds for mice was similar (Polikarpova et al., 1990; Aminin et al., 2001). Overall, the structures of both the aglycone and the carbohydrate chain seem to be very important for the cytotoxic activity of frondoside A and the cucumariosides against cancer cells. However, some changes in the carbohydrate residues may not play a significant role in the cytotoxicity of triterpene glycosides because the cucumarioside A_2-2 and cucumarioside A_4-2 differ only in the structure of their terminal monosaccharide residue having glucose and methylglucose, respectively.

Silchenko et al. (2012d) suggested that amphiphilicity might affect the cytotoxic potency of cucumarioside by demonstrating that the presence of a 25-OH group in the aglycone moiety of triterpene glycosides (cucumarioside H_2) (indicated by an asterisk in compound 19) significantly decreased their cytotoxicity, but the cucumarioside having 25-ethoxy group (cucumarioside H_4) had potent cytotoxic activity against lymphocytes and very high hemolytic activity (**Figure 8**). Our study suggested that the acetyl group at C-16 of the aglycone moiety might play a significant role in the cytotoxicity of triterpene glycosides because frondoside A had more potent effects than cucumarioside A_2-2 (Jin et al., 2009). In contrast, the presence of acetyl groups in steroids increased their cytotoxic potency (Mimaki et al., 2001).

APOPTOTIC EFFECTS OF FRONDOSIDE A AND CUCUMARIOSIDES ON CANCER CELLS

Frondoside A caused a concentration-dependent reduction in the viability of lung cancer cells (LNM35, A549, and NCI-H460-Luc2), melanoma cells (MDA-MB-435), breast cancer cells (MCF-7), and hepatoma cells (HepG2) over 24 h, and increased the activities of caspases-3 and -7 in LNM35 lung cancer cells (Attoub et al., 2013). It was also shown that treatment of human pancreatic cancer cells with a low concentration of frondoside A induced apoptosis through increased activities of caspases-9, -3, and -7, increased bax, and decreased bcl-2 and

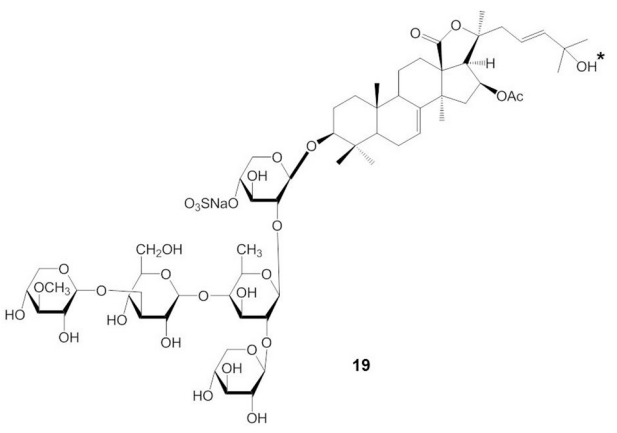


FIGURE 8 | Structure of cucumarioside H_2having 25-OH group.

mcl-1 (Li et al., 2008). Our results demonstrated that mitochondrial membrane permeability was not changed, and the accumulation of cytochrome c in the cytosolic fraction was not observed in HL-60 cells treated with frondoside A (Jin et al., 2009). Similarly, frondoside A had more potent effects than cucumarioside A_4-2 on apoptosis in leukemic cells but did not induce caspase activation before early apoptosis, whereas cucumariosides A_2-2 and A_4-2 showed similar effects on procaspase cleavage and mitochondrial permeability (Jin et al., 2009). Moreover, the annexin-V positivity induced by frondoside A was not inhibited by the pancaspase inhibitor, zVAD-fmk, whereas both the annexin-V positivity and cleavage of caspases induced by cucumarioside A_2-2 were efficiently blocked by zVAD-fmk. These results suggest that frondoside A initiates apoptosis in a caspase-independent manner in some cancer cells. Determination of the structural characteristics responsible for the differential effects of frondoside A and cucumariosides on inducing apoptosis in cancer cells will be essential to reveal their mechanisms of action.

OTHER MARINE TRITERPENE GLYCOSIDES

Previous studies demonstrated that marine triterpene glycosides from sea cucumbers had anticancer activities (Stonik, 1986; Stonik et al., 1999). However, the molecular mechanisms for their anticancer activities were only partly defined. Here, we briefly review potential molecular mechanisms for the anticancer activity of several marine triterpene glycosides. A summary of this information is shown in **Table 2**.

ECHINOSIDE A AND DS-ECHINOSIDE A

Echinoside A (EA) and DS-echinoside A (DSEA) belong to the holostane glycoside type with an 18(20)-lactone. Both have identical aglycone structures and there are only small structural

Table 2 | Potential molecular mechanisms for anticancer activity of marine triterpene glycosides.

Name	Species	Actions	Molecular mechanisms	IC_{50}	References
Frondoside A	*Cucumaria frondosa*	Inhibition of proliferation Induction of apoptosis	Increased expression of p21 Caspase-independent pathway, mitochondrial pathway, increased expression of p53 Decreased expression of Bcl-1 and Mcl-1, increased expression of Bax	4 μg/mL (AsPC-1 cells) 1 μM (HL-60 cells) 2. 5 μM (MDA-MB 231 cells) 4 μg/mL (AsPC-1 cells)	Li et al., 2008 Jin et al., 2009 Al Marzouqui et al., 2011
		Antimetastatic activity	Inhibition of MMP-9 activation Inhibition of prostaglandin receptors EP_4 and EP_2	1 μM (MDA-MB-231 cells) 0.5 μM (Line 66.1 cells)	Li et al., 2008 Park et al., 2012b Ma et al., 2012
Stichoposide C	*Thelenota anax*	Induction of apoptosis	Extrinsic and intrinsic pathway, activation of acid SMase and neutral SMase, ceramide generation	0.3 μM (HL-60 cells) 0.5 μM (K562 cells)	Yun et al., 2012
Stichoposide D	*Thelenota anax*	Induction of apoptosis	Extrinsic and intrinsic pathway, activation of ceramide synthase 6, ceramide generation	1.5 μM (HL-60 cells) 1.0 μM (K562 cells)	Park et al., 2012a Yun, 2014
Cucumaioside A_2-2, A_4-2	*Cucumaria japonica*	Induction of apoptosis	Caspase-dependent pathway	3 μM (HL-60 cells)	Jin et al., 2009
Echinoside A	*Holothuria nobilis* *Peasonothuria graeffei*	Induction of apoptosis Cell cycle arrest	Inhibition of the noncovalent binding of topoisomerase 2α to DNA Increased expression of *p16, p21, and c-myc,* decreased expression of cyclin D1	2.4 μM (human cancer cell lines) 2.7 μM (HepG2 cells)	Li et al., 2010 Zhao et al., 2012
Ds-echinoside A	*Peasonothuria graeffei*	Antimetastatic activity	Inhibition of NF-κB dependent MMP-9 and VEGF expression	2.7 μM (HepG2 cells)	Zhao et al., 2011
Philinopside A	*Pentacta quadrangularis*	Induction of apoptosis	Inhibition of receptor tyrosine kinase autophosphorylation	1.5−2.4 μM (Sarcoma 180, BEL-7402, MCF-7 cells)	Tong et al., 2005
Philinopside E	*Pentacta quadrangularis*	Antimetastatic activity	Inhibition of VEGFR2 signaling Inhibition of interaction between KDR and $\alpha_v\beta_3$ integrin	∼4 μM 2.5 μM	Tian et al., 2005 Tian et al., 2007

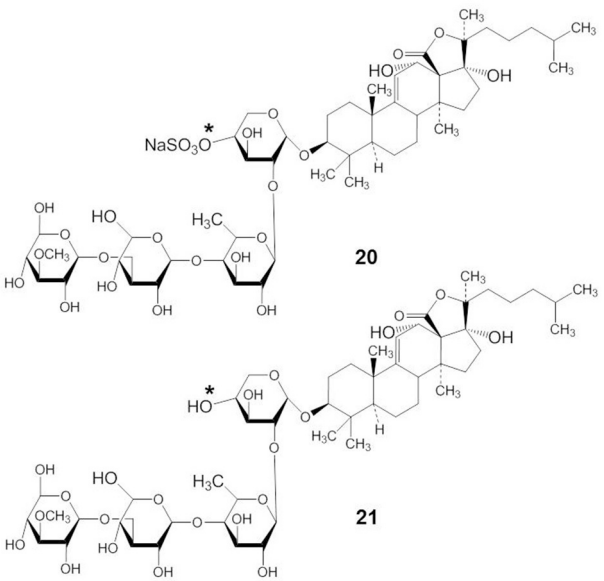


FIGURE 9 | Structures of EA (20) and DSEA (21).

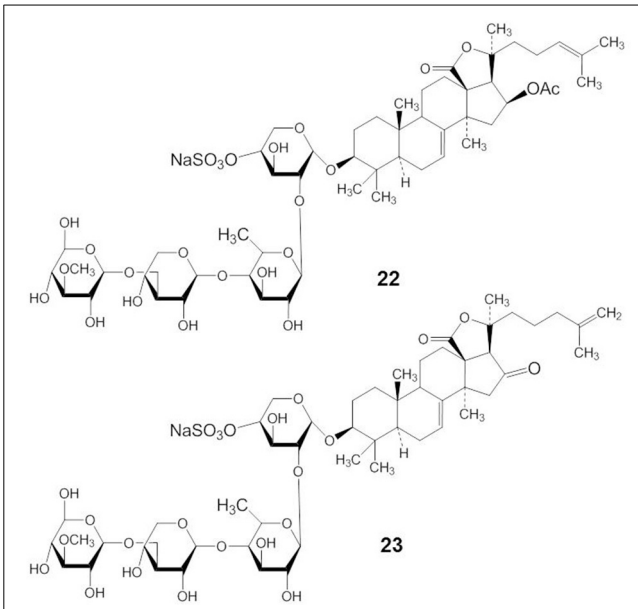


FIGURE 10 | Structures of PA (22) and PE (23).

differences in their carbohydrate chains. EA has a sulfate group at C-4 of the first xylose residue (compound 20) but DSEA is a non-sulfated triterpene glycoside (compound 21) (indicated by an asterisk in compound 20 and 21) (**Figure 9**). Li et al. (2010) have shown that EA, which was isolated from the sea cucumber *Holothuria nobilis*, displayed potent anticancer activities through inhibition of the noncovalent binding of topoisomerase 2α to DNA, resulting in double strand breaks and subsequent cell apoptosis. EA is the first marine-derived topoisomerase inhibitor identified with a saponin skeleton. Zhao et al. (2012) demonstrated that EA, isolated from *Pearsonothuria graeffei*, inhibited cell proliferation by arresting the cell cycle in the G_0/G_1 phase and inducing apoptosis, with DSEA exerting the strongest effect. DSEA exhibited more potent anticancer activity than EA. This suggests that the sulfate group at C-4 of the first xylose residue may reduce the anticancer activity of EA. Zhao et al. (2011) demonstrated that DSEA, isolated from the sea cucumber *Pearsonothuria graeffei*, inhibited the main steps involved in metastasis of HepG2 cells, including suppression of cell migration, adhesion, and invasion. DSEA suppressed MMP-9 and VEGF expression and enhanced the expression of TIMP by blocking the NF-κB signaling pathway in a dose-dependent manner. This indicated that a desulfation reaction at xylose C-2 might be related to NF-κB targeting in tumor metastasis.

PHILINOPSIDE A AND E

Philinopside A (PA) (compound 22) is a compound isolated from the sea cucumber *Pentacta quadrangularis* (**Figure 10**). PA exhibited antitumor effects both *in vitro* and *in vivo* through the inhibition of autophosphorylation of receptor tyrosine kinases, including growth factor receptor, platelet derived growth factor receptor-β, and fibroblast growth factor receptor (Tong et al., 2005).

Philinopside E (PE) (compound 23) is a new sulfated saponin from sea cucumbers. PE inhibits cell adhesion, migration, and invasion through the inhibition of vascular endothelial growth factor receptor 2 (VEGFR2) signaling leading to the suppression of Akt, ERK, focal adhesion kinase, and paxillin (Tian et al., 2005). In addition, Tian et al. (2007) have shown that PE specifically interacted with the extracellular domain of the kinase insert domain-containing receptor (KDR) to block its interaction with VEGF and inhibited downstream signaling. More specifically, PE markedly suppressed $\alpha_v\beta_3$ integrin-driven downstream signaling as a result of disturbing the physical interaction between KDR and $\alpha_v\beta_3$ integrin in human microvascular endothelial cells, followed by disruption of the actin cytoskeleton organization and decreased cell adhesion to vitronectin (Tian et al., 2007).

CONCLUSIONS

Sea cucumbers contain physiologically active triterpene glycosides. Biological effects, including anticancer activities of several marine triterpene glycosides are observed *in vitro* and *in vivo*. Research regarding the mechanisms of action of marine triterpene glycosides on membrane transporters is very limited despite extensive studies on similar compounds in plants. Taking into account the structural and functional differences between marine and plant triterpene glycosides, more intensive studies are required with natural marine triterpene glycosides to assess their potential as novel drugs for the treatment of diseases, including cancer. The STC has anticancer activity through the generation of ceramide by a different mechanism from STD because of the sugar moiety. The anticancer effects of frondoside A and cucumariosides might be through inhibition of tumorigenesis and metastasis and modulation of antitumor immune responses. However, both frondoside A and cucumariosides also possess membranolytic, cytotoxic, and apoptotic properties

with different potencies and mechanisms. Structural differences between frondoside A and cucumarioside seem to be responsible for their different biological activities. Thus, identification of the structural characteristics controlling the biological activities of marine triterpene glycosides is essential for developing marine drugs.

ACKNOWLEDGMENTS

This work was supported by the Pioneer Research Center Program through the National Research Foundation of Korea funded by the Ministry of Science, ICT & Future Planning (2012-0009583 and 2012-0009664).

REFERENCES

Agafonova, I. G., Aminin, D. L., Avilov, S. A., and Stonik, V. A. (2003). Influence of cucumariosides upon intracellular $[Ca_2^+]_i$ and lysosomal activity of macrophages. *J. Agric. Food Chem.* 51, 6982–6986. doi: 10.1021/jf034439x

Al Marzouqi, N., Iratni, R., Nemmar, A., Arafat, K., Ahmed Al Sultan, M., Yasin, J., et al. (2011). Frondoside A inhibits human breast cancer cell survival, migration, invasion and the growth of breast tumor xenografts. *Eur. J. Pharmacol.* 668, 25–34. doi: 10.1016/j.ejphar.2011.06.023

Aminin, D. L., Agafonova, I. G., Berdyshev, E. V., Isachenko, E. G., Avilov, S. A., and Stonik, V. A. (2001). Immunomodulatory properties of cucumariosides from the edible Far-Eastern holothurian *Cucumaria japonica*. *J. Med. Food* 4, 127–135. doi: 10.1089/109662001753165701

Aminin, D. L., Chaykina, E. L., Agafonova, I. G., Avilov, S. A., Kalinin, V. I., and Stonik, V. A. (2010). Antitumor activity of the immunomodulatory lead Cumaside. *Int. Immunopharmacol.* 10, 648–654. doi: 10.1016/j.intimp.2010.03.003

Aminin, D. L., Gorpenchenko, T. Y., Bulgakov, V. P., Andryjashchenko, P. V., Avilov, S. A., and Kalinin, V. I. (2011). Triterpene glycoside cucumarioside A_2-2 from sea cucumber stimulates mouse immune cell adhesion, spreading, and motility. *J. Med. Food* 14, 594–600. doi: 10.1089/jmf.2010.1274

Aminin, D. L., Pinegin, B. V., Pichugina, L. V., Zaporozhets, T. S., Agafonova, I. G., Boguslavski, V. M., et al. (2006). Immunomodulatory properties of Cumaside. *Int. Immunopharmacol.* 6, 1070–1082. doi: 10.1016/j.intimp.2006.01.017

Anisimov, M. M. (1987). Triterpene glycosides and structure-functional properties of biomembranes. *Nauchnye Doki. Vyss. Shkoly Biol. Nauki* 10, 49–63.

Antonov, A. S., Avilov, S. A., Kalinovsky, A. I., Anastyuk, S. D., Dmitrenok, P. S., Evtushenko, E. V., et al. (2008). Triterpene glycosides from Antartic sea cucumbers. 1. Structure of liouvillosides A_1, A_2, A_3, B_1, and B_2 from the sea cucumber *Staurocucumis liouvillei*: new procedure for separation of highly polar glycoside fractions and taxonomic revision. *J. Nat. Prod.* 71, 1677–1685. doi: 10.1021/np800173c

Antonov, A. S., Avilov, S. A., Kalinovsky, A. I., Anastyuk, S. D., Dmitrenok, P. S., Kalinin, V. I., et al. (2009). Triterpene glycosides from Antarctic sea cucumbers. 2. Structure of Achlioniceosides A_1, A_2, and A_3 from the sea cucumber Achlionice violaecuspidata (=Rhipidothuria racowitzai). *J. Nat. Prod.* 72, 33–38. doi: 10.1021/np800469v

Antonov, A. S., Avilov, S. A., Kalinovsky, A. I., Dmitrenok, P. S., Kalinin, V. I., Taboada, S., et al. (2011). Triterpene glycosides from Antartic sea cucumbers. III. Structures of liouvillosides A_4 and A_5, two minor disulphated tetraosides containing 3-O-methylquinovose as terminal monosaccharide units from the sea cucumber *Staurocucumis liouvillei* (Vaney). *Nat. Prod. Res.* 25, 1324–1333. doi: 10.1080/14786419.2010.531017

Attoub, S., Arafat, K., Gélaude, A., Al Sultan, M. A., Bracke, M., Collin, P., et al. (2013). Frondoside A suppressive effects on lung cancer survival, tumor growth, angiogenesis, invasion, and metastasis. *PLoS ONE* 8:e53087. doi: 10.1371/journal.pone.0053087

Avilov, S. A., Antonov, A. S., Drozdova, O. A., Kalinin, V. I., Kalinovsky, A. I., Riguera, R., et al. (2000a). Triterpene glycosides from the far eastern sea cucumber *Pentamera calcigera* II: disulfated glycosides. *J. Nat. Prod.* 63, 65–71. doi: 10.1021/np9903447

Avilov, S. A., Antonov, A. S., Drozdova, O. A., Kalinin, V. I., Kalinovsky, A. I., Stonik, V. A., et al. (2000b). Triterpene glycosides from the Far Eastern sea cucumber *Pentamera calcigera*. 1. Monosulfated glycosides and cytotoxicity of their unsulfated derivatives. *J. Nat. Prod.* 63, 65–71. doi: 10.1021/np9903447

Avilov, S. A., Antonov, A. S., Silchenko, A. S., Kalinin, V. I., Kalinovsky, A. I., Dmitrenok, P. S., et al. (2003). Triterpene glycosides from the Far Eastern sea cucumber *Cucumaria conicospermium*. *J. Nat. Prod.* 66, 910–916. doi: 10.1021/np030005k

Avilov, S. A., Kalinovsky, A. I., Kalinin, V. I., Stonik, V. A., Riguera, R., and Jimenez, C. (1997). Koreoside A, a new nonholostane triterpene glycoside from the sea cucumber *Cucumaria koraiensis*. *J. Nat. Prod.* 60, 808–810. doi: 10.1021/np970152g

Avilov, S. A., Silchenko, A. S., Antonov, A. S., Kalinin, V. I., Kalinovsky, A. I., Smirnov, A. V., et al. (2008). Synaptosides A and A_1, two triterpene glycosides from the sea cucumber *Synapta maculata* containing 3-*O*-methylglucuronic acid and their cytotoxic activity against tumor cells. *J. Nat. Prod.* 71, 525–531 doi: 10.1021/np070283+

Avilov, S. A., Stonik, V. A., and Kalinovsky, A. I. (1990). Structures of four new triterpene glycosides from the sea cucumber *Cucumaria japonica*. *Khim. Prirodn. Soedin.* 6, 787–792.

Bordbar, S., Anwar, F., and Saari, N. (2011). High-value components and bioactives from sea cucumbers for functional foods- a review. *Mar. Drugs* 9, 1761–1805. doi: 10.3390/md9101761

Brown, D. A., and London, E. (1998). Functions of lipid rafts in biological membranes. *Annu. Rev. Cell. Dev. Biol.* 14, 111–136. doi: 10.1146/annurev.cellbio.14.1.111

Chiludil, H. D., Murray, A. P., Seldes, A. M., and Maier, M. (2003). Biologically active triterpene glycosides from the sea cucumbers (*Holothuroidea, Echinodermata*). *Stud. Nat. Prod. Chem.* 28, 587–591. doi: 10.1016/S1572-5995(03)80150-3

Choi, S. H., Shin, T. J., Hwang, S. H., Lee, B. H., Kang, J., Kim, H. J., et al. (2011b). Ginsenoside Rg_3 decelerates hERG K^+ channel deactivation through Ser631 residue interaction. *Eur. J. Pharmacol.* 663, 59–67. doi: 10.1016/j.ejphar.2011.05.006

Choi, S. H., Shin, T. J., Lee, B. H., Hwang, S. H., Lee, S. M., Lee, B. C., et al. (2011a). Ginsenoside Rg_3 enhances large conductance Ca^{2+}-activated potassium channel currents: a role of Tyr360 residue. *Mol. Cells* 31, 133–140. doi: 10.1007/s10059-011-0017-7

Colorado-Ríos, J., Muñoz, D., Montoya, G., Márquez, D., Márquez, M. E., López, J., et al. (2013). HPLC-ESI-IT-MS/MS analysis and biological activity of triterpene glycosides from the Colombian marine sponge Ectyoplasia ferox. *Mar. Drugs* 11, 4815–4833. doi: 10.3390/md11124815

Dang, N. H., Thanh, N. V., Kiem, P. V., Huang, L. M., Minh, C. V., and Kim, Y. H. (2007). Two new triterpene glycosides from the Vietnamese sea cucumber *Holothuria scarbra*. *Arch. Pharm. Res.* 30, 1387–1391. doi: 10.1007/BF02977361

Drozdova, O. A., Avilov, S. A., Kalinin, V. I., Kalinovsky, A. I., Stonik, V. A., Rugiera, R., et al. (1997). Cytotoxic triterpene glycosides from Far-Eastern sea cucumber belonging to the genus *Cucumaria*. *Liebigs. Ann. Chem.* 11, 2351–2356. doi: 10.1002/jlac.199719971125

Drozdova, O. A., Avilov, S. A., Kalinovsky, A. I., Stonik, V. A., Milgrom, Y. M., and Rashkes, Y. W. (1993). Trisulfated glycosides from the sea cucumber *Cucumaria japonica*. *Khim. Prirodn. Soedin.* 3, 369–374.

Fu, Y., Zhou, E., Wei, Z., Liang, D., Wang, W., Wang, T., et al. (2014). Glycyrrhizin inhibits the inflammatory response in mouse mammary epithelial cells and a mouse mastitis model. *FEBS J.* 281, 2543–2557. doi: 10.1111/febs.12801

Girard, M., Belanger, J., ApSimon, J. W., Garneau, F. X., Harvey, C., and Brisson, J. R. (1990). Frondoside A, novel triterpene glycoside from the holothurian *Cucumaria frondosa*. *Can. J. Chem.* 68, 11–18. doi: 10.1139/v90-003

Goni, F. M., and Alonso, A. (2002). Sphingomyelinases: enzymology and membrane activity. *FEBS. Letter.* 531, 38–46. doi: 10.1016/S0014-5793(02)03482-8

Gorshkova, I. A., Kalinin, V. I., Gorshkov, B. A., and Stonik, V. A. (1999). Two different modes of inhibition of the rat brain Na^+, K^+-ATPase by triterpene glycosides, psolusosides A and B from the holothurian *Psolus fabricii*. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* 122, 101–108. doi: 10.1016/S0742-8413(98)10085-3

Gulbins, E., and Kolesnick, R. (2002). Acid sphingomyelinase-derived ceramide signaling in apoptosis. *Subcell. Biochem.* 36, 229–244. doi: 10.1007/0-306-47931-1_12

Hannun, Y. A., and Obeid, L. M. (2008). Principles of bioactive lipid signaling: lessons from sphingolipids. *Nat. Rev. Mol. Cell. Biol.* 9, 139–150. doi: 10.1038/nrm2329

Holt, D. M., Ma, X., Kundu, N., Collin, P. D., and Fulton, A. M. (2012). Modulation of host natural killer cell functions in breast cancer via prostaglandin E_2 receptors EP_2 and EP_4. *J. Immunother.* 35, 179–188. doi: 10.1097/CJI.0b013e318247a5e9

Hwang, J. A., Hwang, M. K., Jang, Y., Lee, E. J., Kim, J. E., Oh, M. H., et al. (2013). 20-O-β-d-glucopyranosyl-20(S)-protopanaxadiol, a metabolite of ginseng, inhibits colon cancer growth by targeting TRPC channel-mediated calcium influx. *J. Nutr. Biochem.* 24, 1096–1104. doi: 10.1016/j.jnutbio.2012.08.008

Im, D. S., and Nah, S. Y. (2013). Yin and Yang of ginseng pharmacology: ginsenosides vs gintonin. *Acta Pharmacol. Sin.* 34, 1367–1373. doi: 10.1038/aps.2013.100

Iñiguez-Martinez, A. M., Guerra-Rivas, G., Rios, T., and Quijano, L. (2005). Triterpenoid oligoglycosides from the sea cucumber *Stichopus parvimensis*. *J. Nat. Prod.* 68, 1669–1673. doi: 10.1021/np050196m

Itoh, K., Hara, T., Shiraishi, T., Taniguchi, K., Morimoto, S., and Onishi, T. (1989). Effects of glycyrrhizin and glycyrrhetinic acid on Na^+-K^+-ATPase of renal basolateral membranes *in vitro*. *Biochem. Int.* 18, 81–89.

Jin, J. O., Shastina, V. V., Shin, S. W., Xu, Q., Park, J. I., Rasskazov, V. A., et al. (2009). Differential effects of triterpene glycosides, frondoside A and cucumarioside A_2-2 isolated from sea cucumbers on caspase activation and apoptosis of human leukemia cells. *FEBS. Lett.* 583, 697–702. doi: 10.1016/j.febslet.2009.01.010

Kalinin, V. I. (2000). System-theoretical (holistic) approach to the modeling of structural functional relationships of biomolecules and their evolution: an example of triterpene glycosides from sea cucumbers (*Echinodermata, Holothurioidea*). *J. Theor. Biol.* 206, 151–168. doi: 10.1006/jtbi.2000.2110

Kalinin, V. I., Aminin, D. L., Avilov, S. A., Silchenko, A. S., and Stonik, V. A. (2008). "Triterpene glycosides from sea cucucmbers (Holothurioidae, Echinodermata), biological activities and functions," in *Studies in Natural Product Chemistry (Bioactive Natural Products)*, ed Atta-ur-Rahman (Amsterdam: Elsevier Science Publisher), 135–196.

Kalinin, V. I., Avilov, S. A., Kalinina, E. Y., Korolkova, O. G., Kalinovsky, A. I., Stonik, V. A., et al. (1997). Structure of eximisoside A, a novel triterpene glycoside from the Far-Eastern sea cucumber *Psolus eximius*. *J. Nat. Prod.* 60, 817–819. doi: 10.1021/np9701541

Kalinin, V. I., Ivanchina, N. V., Krasokhin, V. B., Makarieva, T. N., and Stonik, V. A. (2012). Glycosides from marine sponges (*Porifera, Demospongiae*): structures, taxonomical distribution, biological activities and biological roles. *Mar. Drugs* 10, 1671–1710. doi: 10.3390/md10081671

Kalinin, V. I., Prokofieva, N. G., Likhatskaya, G. N., Schentsova, E. B., Agafonova, I. G., Avilov, S. A., et al. (1996). Hemolytic activities of triterpene glycosides from holothurian order *Dendrochirotida*: some trends in the evolution of this group of toxins. *Toxicon* 34, 475–483. doi: 10.1016/0041-0101(95)00142-5

Kalinin, V. I., Silchenko, A. S., Avilov, S. A., Stonik, V. A., and Smirnov, A. V. (2005). Sea cucumbers triterpene glycosides, the recent progress in structural elucidation and chemotaxonomy. *Phytochem. Rev.* 4, 221–236. doi: 10.1007/s11101-005-1354-y

Kalinin, V. I., Volkova, O. V., Likhatskaya, G. N., Prokofieva, N. G., Agafonova, I. G., Anisimov, M. M., et al. (1992). Hemolytic activity of triterpene glycosides from *Cucumariidae* family holothurians and evolution of this group of toxins. *J. Nat. Toxins* 1, 17–30.

Kerr, R. G., and Chen, Z. (1995). *In vivo* and *in vitro* biosynthesis of saponins in sea cucumbers. *J. Nat. Prod.* 58, 172–176. doi: 10.1021/np50116a002

Kim, S., Ahn, K., Oh, T. H., Nah, S. Y., and Rhim, H. (2002). Inhibitory effect of ginsenosides on NMDA receptor-mediated signals in rat hippocampal neurons. *Biochem. Biophys. Res. Commun.* 296, 247–254. doi: 10.1016/S0006-291X(02)00870-7

Kim, S. K., and Himaya, S. W. (2012). Triterpene glycosides from sea cucumbers and their biological activities. *Adv. Food Nutr. Res.* 65, 297–319. doi: 10.1016/B978-0-12-416003-3.00020-2

Kitagawa, I. (1988). Research of biologically active marine natural products. *Yakugaku. Zasshi.* 108, 398–416.

Kitagawa, I., Kobayashi, M., Inamoto, T., Yosuzawa, T., and Kyogoku, Y. (1981). The structures of six antifungal oligoglycosides, Stichlorosides A_1, A_2, B_1, B_2, C_1 and C_2 from the sea cucumber *Stichopus chloronotus* (Brandt). *Chem. Phar. Bull.* 29, 2387–2391 doi: 10.1248/cpb.29.2387

Kolesnick, R. N., Goni, F. M., and Alonso, A. (2000). Compartmentalization of ceramide signaling: physical foundations and biological effects. *J. Cell. Physiol.* 184, 285–300. doi: 10.1002/1097-4652(200009)184:3<285::AID-JCP2>3.0.CO;2-3

Kuznetsova, T. A., Anisimov, M. M., Popov, A. M., Baranova, S. I., Afiyatullov, S. H., Kapustina, I. I., et al. (1982). A comparative study *in vitro* of physiological activity of triterpene glycosides of marine invertebrates of echinoderm type. *Comp. Biochem. Physiol. C.* 73, 41–43. doi: 10.1016/0306-4492(82)90165-4

Lee, B. H., Choi, S. H., Hwang, S. H., Kim, H. J., Lee, S. M., Kim, H. C., et al. (2013a). Effects of ginsenoside Rg_3 on $\alpha_9\alpha_{10}$ nicotinic acetylcholine receptor-mediated ion currents. *Biol. Pharm. Bull.* 36, 812–818. doi: 10.1248/bpb.b12-01009

Lee, B. H., Kim, H. J., Chung, L., and Nah, S. Y. (2013b). Ginsenoside Rg_3 regulates GABA receptor channel activity: involvement of interaction with the γ_2 subunit. *Eur. J. Pharmacol.* 705, 119–125. doi: 10.1016/j.ejphar.2013.02.040

Lee, E., Kim, S., Chung, K. C., Choo, M. K., Kim, D. H., Nam, G., et al. (2006). 20(S)-ginsenoside Rh_2, a newly identified active ingredient of ginseng, inhibits NMDA receptors in cultured rat hippocampal neurons. *Eur. J. Pharmacol.* 536, 69–77. doi: 10.1016/j.ejphar.2006.02.038

Lee, J. H., Choi, S. H., Lee, B. H., Shin, T. J., Pyo, M. K., Hwang, S. H., et al. (2009). The effects of ginsenoside Rg_3 on human Kv1.4 channel currents without the N-terminal rapid inactivation domain. *Biol. Pharm. Bull.* 32, 614–618. doi: 10.1248/bpb.32.614

Lee, J. H., Lee, B. H., Choi, S. H., Yoon, I. S., Shin, T. J., Pyo, M. K., et al. (2008). Involvement of batrachotoxin binding sites in ginsenoside-mediated voltage-gated Na^+ channel regulation. *Brain Res.* 1203, 61–67. doi: 10.1016/j.brainres.2008.01.078

Levade, T., and Jaffrezou, J. P. (1999). Signalling sphingomyelinases: which, where, how and why? *Biochim. Biophys. Acta* 1438, 1–17. doi: 10.1016/S1388-1981(99)00038-4

Li, M., Ma, R. N., Li, L. H., Qu, Y. Z., and Gao, G. D. (2013). Astragaloside IV reduces cerebral edema post-ischemia/reperfusion correlating the suppression of MMP-9 and AQP4. *Eur. J. Pharmacol.* 715, 189–195. doi: 10.1016/j.ejphar.2013.05.022

Li, M., Miao, Z. H., Chen, Z., Chen, Q., Gui, M., Lin, L. P., et al. (2010). Echinoside A, a new marine-derived anticancer saponin, targets topoisomerase 2α by unique interference with its DNA binding and catalytic cycle. *Ann. Oncol.* 21, 597–607. doi: 10.1093/annonc/mdp335

Li, X., Roginsky, A. B., Ding, X. Z., Woodward, C., Collin, P., and Newman, R. A. (2008). Review of the apoptosis pathways in pancreatic cancer and the anti-apoptotic effects of the novel sea cucumber compound, Frondoside A. *Ann. N.Y. Acad. Sci.* 1138, 181–198. doi: 10.1196/annals.1414.025

Li, X. X., Davis, B., Haridas, V., Gutterman, J. U., and Colombini, M. (2005). Proapoptotic triterpene electrophiles (avicins) form channels in membranes: cholesterol dependence. *Biophys. J.* 88, 2577–2584. doi: 10.1529/biophysj.104.049403

Lin, Z. Y., Chen, L. M., Zhang, J., Pan, X. D., Zhu, Y. G., Ye, Q. Y., et al. (2012). Ginsenoside Rb_1 selectively inhibits the activity of L-type voltage-gated calcium channels in cultured rat hippocampal neurons. *Acta Pharmacol. Sin.* 33, 438–444. doi: 10.1038/aps.2011.181

Lorent, J., Le Duff, C. S., Quetin-Leclercq, J., and Mingeot-Leclercq, M. P. (2013). Induction of highly curved structures in relation to membrane permeabilization and budding by the triterpenoid saponins, α- and δ-Hederin. *J. Biol. Chem.* 288, 14000–14017. doi: 10.1074/jbc.M112.407635

Ma, X., Kundu, N., Collin, P. D., Goloubeva, O., and Fulton, A. M. (2012). Frondoside A inhibits breast cancer metastasis and antagonizes prostaglandin E receptors EP_4 and EP_2. *Breast Cancer Res. Treat.* 132, 1001–1008. doi: 10.1007/s10549-011-1675-z

Maltsev, I. I., Stekhova, S. I., Schentsova, E. B., Anisimov, M. M., and Stonik, V. A. (1985). Antimicrobial activities of glycosides from the sea cucumbers of family *Stichopodidae*. *Khim. Pharm. Zhurn.* 19, 54–56.

McManus, O. B., Harris, G. H., Giangiacomo, K. M., Feigenbaum, P., Reuben, J. P., Addy, M. E., et al. (1993). An activator of calcium-dependent potassium channels isolated from a medicinal herb. *Biochemistry* 32, 6128–6133. doi: 10.1021/bi00075a002

Menchinskaya, E. S., Pislyagin, E. A., Kovalchuk, S. N., Davydova, V. N., Silchenko, A. S., Avilov, S. A., et al. (2013). Antitumor activity of cucumarioside A2-2. *Chemotherapy* 59, 181–191. doi: 10.1159/000354156

Mimaki, Y., Yokosuka, A., Kuroda, M., and Sashida, Y. (2001). Cytotoxic activities and structure–cytotoxic relationships of steroidal saponins. *Biol. Pharm. Bull.* 24, 1286–1289. doi: 10.1248/bpb.24.1286

Moraes, G., Northcote, P. T., Silchenko, A. S., Antonov, A. S., Kalinovsky, A. I., Dmitrenok, P. S., et al. (2005). Mollisosides A, B_1, and B_2: minor triterpene glycosides from the New Zealand and South Australian sea cucumber *Australostichopus mollis*. *J. Nat. Prod.* 68, 842–847. doi: 10.1021/np050049o

Nah, S. Y. (2014). Ginseng ginsenoside pharmacology in the nervous system: involvement in the regulation of ion channels and receptors. *Front. Physiol.* 5:98. doi: 10.3389/fphys.2014.00098

Nigrelli, R. F. (1952). The effect of holothurin on fish and mice with Sarcoma-180. *Zoologica* 37, 89–90.

Nigrelli, R. F., Stempien, M. F. Jr., Ruggieri, G. D., Liguori, V. R., and Cecil, J. T. (1967). Substances of potential biomedical importance from marine organisms. *Fed. Proc.* 26, 1197–1205.

Pan, X. Y., Guo, H., Han, J., Hao, F., An, Y., Xu, Y., et al. (2012). Ginsenoside Rg_3 attenuates cell migration via inhibition of aquaporin 1 expression in PC-3M prostate cancer cells. *Eur. J. Pharmacol.* 683, 27–34. doi: 10.1016/j.ejphar.2012.02.040

Park, E. S., Yun, S. H., Shin, S. W., Kwak, J. Y., and Park, J. I. (2012a). Induction of apoptosis and antitumor activity by stichoposide D through the generation of ceramide in human leukemia cells. *J. Life. Sci.* 22, 760–771. doi: 10.5352/JLS.2012.22.6.760

Park, S. Y., Kim, Y. H., Kim, Y., and Lee, S. J. (2012b). Frondoside A has an anti-invasive effect by inhibiting TPA-induced MMP-9 activation via NF-κB and AP-1 signaling in human breast cancer cells. *Int. J. Oncol.* 41, 933–940. doi: 10.3892/ijo.2012.1518

Pislyagin, E. A., Gladkikh, R. V., Kapustina, I. I., Kim, N. Y., Shevchenko, V. P., Nagaev, I. Y., et al. (2012). Interaction of holothurian triterpene glycoside with biomembranes of mouse immune cells. *Int. Immunopharmacol.* 14, 1–8. doi: 10.1016/j.intimp.2012.05.020

Polikarpova, S. I., Volkova, O. N., Sedov, A. M., Stonik, V. A., and Likhoded, V. G. (1990). Cytogenetic study of the mutagenicity of cucumarioside. *Genetika* 26, 1682–1685.

Robertson, F. M., Simeone, A. M., Lucci, A., McMurray, J. S., Ghosh, S., and Cristofanilli, M. (2010). Differential regulation of the aggressive phenotype of inflammatory breast cancer cells by prostanoid receptors EP_3 and EP_4. *Cancer* 116, 2806–2814. doi: 10.1002/cncr.25167

Roginsky, A. B., Ding, X.-Z., Woodward, C., Ujiki, M. B., Singh, B., Bell, R. H. Jr., et al. (2010). Anti-pancreatic cancer effects of a polar extract from the edible sea cucumber, *Cucumaria frondosa*. *Pancreas* 39, 646–652. doi: 10.1097/MPA.0b013e3181c72baf

Shang, W., Yang, Y., Zhou, L., Jiang, B., Jin, H., and Chen, M. (2008). Ginsenoside Rb_1 stimulates glucose uptake through insulin-like signaling pathway in 3T3-L1 adipocytes. *J. Endocrinol.* 198, 561–569. doi: 10.1677/JOE-08-0104

Silchenko, A. S., Avilov, S. A., Antonov, A. A., Kalinin, V. I., Kalinovsky, A. I., Smimov, A. V., et al. (2002). Triterpene glycosides from the deep-water North-Pacific sea cucumber *Synallactes nozawai Mitsukuri*. *J. Nat. Prod.* 65, 1802–1808. doi: 10.1021/np0202881

Silchenko, A. S., Avilov, S. A., Antonov, A. S., Kalinovsky, A. I., Dmitrenok, P. S., Kalinin, V. I., et al. (2005a). Glycosides from the sea cucumber *Cucumaria frondosa*. III. Structure of frondosides A_2-1, A_2-2, A_2-3, and A_2-6, four new minor monosulfated triterpene glycosides. *Can. J. Chem.* 83, 21–27. doi: 10.1139/v04-163

Silchenko, A. S., Avilov, S. A., Antonov, A. S., Kalinovsky, A. I., Dmitrenok, P. S., Kalinin, V. I., et al. (2005b). Glycosides from the sea cucumber *Cucumaria frondosa*. IV. Structure of frondosides A_2-4, A_2-7, and A_2-8, three new minor monosulfated triterpene glycosides. *Can. J. Chem.* 83, 2120–2126. doi: 10.1139/v05-243

Silchenko, A. S., Avilov, S. A., Kalinin, V. I., Kalinovsky, A. I., Dmitrenok, P. S., Fedorov, S. N., et al. (2008). Constituents of the sea cucumber *Cucumaria okhotensis*. Structures of okhotosides B_1-B_3 and cytotoxic activities of some glycosides from this species. *J. Nat. Prod.* 71, 351–356. doi: 10.1021/np0705413

Silchenko, A. S., Avilov, S. A., Kalinin, V. I., Stonik, V. A., Kalinovsky, A. I., Dmitrenok, P. S., et al. (2007a). Monosulfated triterpene glycosides from *Cucumaria okhotensis* Levin et Stepanov, a new species of sea cucumbers from sea of Okhotsk. *Russ. J. Bioorg. Chem.* 33, 73–82, doi: 10.1134/S1068162007010098

Silchenko, A. S., Avilov, S. A., Kalinovsky, A. I., Dmitrenok, P. S., Kalinin, V. I., Morre, J., et al. (2007b). Glycosides from the North Atlantic sea cucumber *Cucumaria frondosa* V- Structures of five new minor trisulfated triterpene oligoglycosides, frondosides A_7-1, A_7-2, A_7-3, A_7-4, and isofrondoside C. *Can. J. Chem.* 85, 626–636. doi: 10.1139/v07-087

Silchenko, A. S., Kalinovsky, A. I., Avilov, S. A., Andryjaschenko, P. V., Dmitrenok, P. S., Martyyas, E. A., et al. (2012a). Triterpene glycosides from the sea cucumber *Eupentacta fraudatrix*. Structure and biological action of cucumariosides A_1, A_3, A_4, A_5, A_6, A_{12} and A_{15}, seven new minor non-sulfated tetraosides and unprecedented 25-keto, 27-norholostane aglycone. *Nat. Prod. Commun.* 7, 517–525.

Silchenko, A. S., Kalinovsky, A. I., Avilov, S. A., Andryjaschenko, P. V., Dmitrenok, P. S., Martyyas, E. A., et al. (2012b). Triterpene glycosides from the sea cucumber *Eupentacta fraudatrix*. Structure and cytotoxic action of cucumariosides A_2, A_7, A_9, A_{10}, A_{11}, A_{13} and A_{14}, seven new minor non-sulfated tetraosides and an aglycone with an uncommon 18-hydroxy group. *Nat. Prod. Commun.* 7, 845–852.

Silchenko, A. S., Kalinovsky, A. I., Avilov, S. A., Andryjaschenko, P. V., Dmitrenok, P. S., Martyyas, E. A., et al. (2012c). Triterpene glycosides from the sea cucumber *Eupentacta fraudatrix*. Structure and biological activity of cucumariosides B_1 and B_2, two new minor non-sulfated unprecedented triosides. *Nat. Prod. Commun.* 7, 1157–1162.

Silchenko, A. S., Kalinovsky, A. I., Avilov, S. A., Andryjaschenko, P. V., Dmitrenok, P. S., Martyyas, E. A., et al. (2013a). Structures and biological activities of typicosides A_1, A_2, B_1, C_1 and C_2, triterpene glycosides from the sea cucumber *Actinocucumis typica*. *Nat. Prod. Commun.* 8, 301–310.

Silchenko, A. S., Kalinovsky, A. I., Avilov, S. A., Andryjaschenko, P. V., Dmitrenok, P. S., Martyyas, E. A., et al. (2013b). Triterpene glycosides from the sea cucumber *Eupentacta fraudatrix*. Structure and biological action of cucumariosides I_1, I_3, I_4, three new minor disulfated pentaosides. *Nat. Prod. Commun.* 8, 1053–1058.

Silchenko, A. S., Kalinovsky, A. I., Avilov, S. A., Andryjaschenko, P. V., Dmitrenok, P. S., Menchinskaya, E. S., et al. (2013c). Structure of cucumarioside I_2 from the sea cucumber *Eupentacta fraudatrix* (Djakonov *et* Baranova) and cytotoxic and immunostimulatory activities of this saponin and relative compounds. *Nat. Prod. Res.* 27, 1776–1783. doi: 10.1080/14786419.2013.778851

Silchenko, A. S., Kalinovsky, A. I., Avilov, S. A., Andryjaschenko, P. V., Dmitrenok, P. S., Yurchenko, E. A., et al. (2011). Structure of cucumariosides H_5, H_6, H_7 and H_8, triterpene glycosides from the sea cucumber *Eupentacta fraudatrix* and unprecedented aglycone with 16,22-epoxy-group. *Nat. Prod. Commun.* 6, 1075–1082.

Silchenko, A. S., Kalinovsky, A. I., Avilov, S. A., Andryjaschenko, P. V., Dmitrenok, P. S., Yurchenko, E. A., et al. (2012d). Structures and cytotoxic properties of cucumariosides H_2, H_3 and H_4 from the sea cucumber *Eupentacta fraudatrix*. *Nat. Prod. Res.* 26, 1765–1774. doi: 10.1080/14786419.2011.602637

Silchenko, A. S., Kalinovsky, A. I., Avilov, S. A., Andryjaschenko, P. V., Dmitrenok, P. S., Yurchenko, E. A., et al. (2013d). Structure and biological action of cladolosides B_1, B_2, C_1, C_2 and D, six new triterpene glycosides from the sea cucumber *Cladolabes schmeltzii*. *Nat. Prod. Commun.* 8, 1527–1534.

Silchenko, A. S., Stonik, V. A., Avilov, S. A., Kalinin, V. I., Kalinovsky, A. I., Zaharenko, A. M., et al. (2005c). Holothurins B_2, B_3, and B_4, new triterpene glycosides from Mediterranean sea cucumbers of the genus *holothuria*. *J. Nat. Prod.* 68, 564–567. doi: 10.1021/np049631n

Stonik, V. A. (1986). Some terpenoid and steroid derivatives from echinoderms and sponges. *Pure Appl. Chem.* 58, 423–436.

Stonik, V. A., Kalinin, V. I., and Avilov, S. A. (1999). Toxins from sea cucumbers (holothuroids): chemical structures, properties, taxonomic distribution, biosynthesis and evolution. *J. Nat. Toxins* 8, 235–248.

Stonik, V. A., Maltsev, I. I., and Elyakov, G. B. (1982b). Structures of thelenotoside-A and thelenoside-B from the sea cucumber *Thelenota ananas*. *Chem. Nat. Prod.* 624–627.

Stonik, V. A., Maltsev, I. I., Kalinovsky, A. I., Conde, K., and Elyakov, G. B. (1982a). Glycosides of marine- invertebrates. XI. The two novel triterpene glycosides from holothurian of *Stichopodidae* family. *Chem. Nat. Prod.* 194–199.

Strum, J. C., Ghosh, S., and Bell, R. M. (1997). Lipid second messengers. A role in cell growth regulation and cell cycle regulation. *Adv. Exp. Mol. Biol.* 407, 421–431. doi: 10.1007/978-1-4899-1813-0_63

Sullivan, T. D., Ladue, K. T., and Nigrelli, R. F. (1955). The effects of holothurin, a steroid saponin of animal origin, on Krebs-2 ascites tumors in Swiss mice. *Zoologica* 40, 49–52.

Taha, T. A., Mullen, T. D., and Obeid, L. M. (2006). A house divided: ceramide, sphingosine, and sphingosine-1-phosphate in programmed cell death. *Biochim. Biophys. Acta* 1758, 2027–2036. doi: 10.1016/j.bbamem.2006.10.018

Tian, F., Zhang, X., Tong, Y., Yi, Y., Zhang, S., Li, L., et al. (2005). PE, a new sulfated saponin from sea cucumber, exhibits anti-angiogenetic and antitumor activities *in vitro* and *in vivo*. *Cancer Biol. Ther.* 4, 874–882. doi: 10.4161/cbt.4.8.1917

Tian, F., Zhu, C. H., Zhang, X. W., Xie, X., Xin, X. L., Yi, Y. H., et al. (2007). Phillinopside E, a new sulfated saponin from sea cucumber, blocks the interaction between kinase insert domain-containing receptor (KDR) and $\alpha_v\beta_3$ integrin via binding to the extracellular domain of KDR. *Mol. Pharmacol.* 72, 545–552. doi: 10.1124/mol.107.036350

Timoshenko, A. V., Xu, G., Chakrabarti, S., Lala, P. K., and Chakraborty, C. (2003). Role of prostaglandin E_2 receptors in migration of murine and human breast cancer cells. *Exp. Cell Res.* 289, 265–274. doi: 10.1016/S0014-4827(03)00269-6

Tong, Y., Zhang, X., Tian, F., Yi, Y., Xu, Q., Li, L., et al. (2005). Philinopside A, a novel marine derived compound possessing dual anti-angiogenetic and antitumor effects. *Int. J. Cancer* 114, 843–853. doi: 10.1002/ijc.20804

Uyama, Y., Imaizumi, Y., and Watanabe, M. (1992). Effects of cyclopiazonic acid, a novel Ca^{2+}-ATPase inhibitor, on contractile responses in skinned ileal smooth muscle. *Br. J. Pharmacol.* 106, 208–214. doi: 10.1111/j.1476-5381.1992.tb14316.x

Verbist, J. E. (1993). "Pharmacological effects of compounds from Echinoderms," in *Echinoderm Studies*, Vol. 4, eds M. Jangoux and J. M. Lawrence (Rotterdam; Brookfild: A. A. Balkema), 111–186.

Wang, D., and Dubois, R. N. (2010). Eicosanoids and cancer. *Nat. Rev. Cancer* 10, 182–192. doi: 10.1038/nrc2809

Wang, Y. G., Zima, A. V., Ji, X., Pabbidi, R., Blatter, L. A., and Lipsius, S. L. (2008). Ginsenoside Re suppresses electromechanical alternans in cat and human cardiomyocytes. *Am. J. Physiol. Heart Circ. Physiol.* 295, H851–H859. doi: 10.1152/ajpheart.01242.2007

Wink, M., Ashour, M. L., and El-Readi, M. Z. (2012). Secondary metabolites from plants inhibiting ABC transporters and reversing resistance of cancer cells and microbes to cytotoxic and antimicrobial agents. *Front. Microbiol.* 3:130. doi: 10.3389/fmicb.2012.00130

Wong, V. K., Li, T., Law, B. Y., Ma, E. D., Yip, N. C., Michelangeli, F., et al. (2013). Saikosaponin-d, a novel SERCA inhibitor, induces autophagic cell death in apoptosis-defective cells. *Cell Death Dis.* 11, e720. doi: 10.1038/cddis.2013.217

Xu, L., and Huang, S. P. (2012). Effect of the ginsenoside Rb_1 on the spontaneous contraction of intestinal smooth muscle in mice. *World J. Gastroenterol.* 18, 5462–5469. doi: 10.3748/wjg.v18.i38.5462

Xu, X. L., Chen, X. J., Ji, H., Li, P., Bian, Y. Y., Yang, D., et al. (2008). Astragaloside IV improved intracellular calcium handling in hypoxia-reoxygenated cardiomyocytes via the sarcoplasmic reticulum Ca-ATPase. *Pharmacology* 81, 325–332. doi: 10.1159/000121335

Yamasaki, Y., Ito, K., Enomoto, Y., and Sutko, J. L. (1987). Alterations by saponins of passive Ca^{2+} permeability and Na^{+}-Ca^{2+} exchange activity of canine cardiac sarcolemmal vesicles. *Biochim. Biophys. Acta* 897, 481–487. doi: 10.1016/0005-2736(87)90445-7

Yun, S. H. (2014). *Study on the Molecular Mechanisms for Antitumor Activity of Stichoposide D, an Extract from Sea Cucumbers*. Ph.D. Thesis, Dong-A University, Busan, Korea.

Yun, S. H., Park, E. S., Shin, S. W., Na, W. Y., Han, J. Y., Jeong, J. S., et al. (2012). Stichoposide C induces apoptosis through the generation of ceramide in leukemia and colorectal cancer cells and shows *in vivo* antitumor activity. *Clin. Cancer Res.* 18, 5934–5948. doi: 10.1158/1078-0432.CCR-12-0655

Yun, U. J., Lee, J. H., Koo, K. H., Ye, S. K., Kim, S. Y., Lee, C. H., et al. (2013). Lipid raft modulation by Rp_1 reverses multidrug resistance via inactivating MDR-1 and Src inhibition. *Biochem. Pharmacol.* 85, 1441–1453. doi: 10.1016/j.bcp.2013.02.025

Zhang, S. H., Li, L., Yi, Y. H., and Sun, P. (2006a). Philipnosides E and F, two new sulfated triterpene glycosides from the sea cucumber *Pentacta quadrangularis*. *Nat. Prod. Res.* 20, 399–407. doi: 10.1080/14786410500185584

Zhang, S. Y., Yi, Y. H., and Tang, H. F. (2006b). Bioactive triterpene glycosides from the sea cucumber *Holothuria fuscocinerea*. *J. Nat. Prod.* 69, 1492–1495. doi: 10.1021/np060106t

Zhang, S. Y., Yi, Y. H., and Tang, H. F. (2006c). Cytotoxic sulfated triterpene glycosides from the sea cucumber *Pseudocolochirus violaceus*. *Chem. Biodivers.* 3, 807–817. doi: 10.1002/cbdv.200690083

Zhang, S. Y., Yi, Y., Tang, H. F., Li, L., Sun, P., and Wu, J. (2006d). Two new bioactive triterpene glycosides from the sea cucumber *Pseudocolochirus violaceus*. *J. Asian Nat. Prod. Res.* 8, 1–8. doi: 10.1080/10286020500034972

Zhao, Q., Liu, Z., Xue, Y., Wang, J., Li, H., Tang, Q., et al. (2011). Ds-echinoside A, a new triterpene glycoside derived from sea cucumber, exhibits antimetastatic activity via the inhibition of NF-κB-dependent MMP-9 and VEGF expressions. *J. Zhejang Univ. Sci. B* 12, 534–544. doi: 10.1631/jzus.B1000217

Zhao, Q., Xue, Y., Wang, J., Li, H., Long, T., Li, Z., et al. (2012). *In vitro* and *in vivo* anti-tumour activities of echinoside A and ds-echinoside A from *Pearnonothuria graeffei*. *J. Sci. Food. Agric.* 92, 965–974. doi: 10.1002/jsfa.4678

Zhou, Z., Yi, Y., Wu, H., Yao, X., Du, L., Jiuhong, W., et al. (2005). Intercedensides D-I, cytotoxic triterpene glycosides from the sea cucumber *Mensamaria intercedens Lampert*. *J. Nat. Prod.* 68, 540–546. doi: 10.1021/np040205b

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 28 June 2014; accepted: 21 August 2014; published online: 09 September 2014.

Citation: Park J-I, Bae H-R, Kim CG, Stonik VA and Kwak J-Y (2014) Relationships between chemical structures and functions of triterpene glycosides isolated from sea cucumbers. Front. Chem. ***2****:77. doi: 10.3389/fchem.2014.00077*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in
CHEMISTRY

REVIEW ARTICLE
published: 08 October 2014
doi: 10.3389/fchem.2014.00085

Exopolysaccharides produced by marine bacteria and their applications as glycosaminoglycan-like molecules

Christine Delbarre-Ladrat*, Corinne Sinquin, Lou Lebellenger, Agata Zykwinska and Sylvia Colliec-Jouault

EM³B Laboratory, Institut Français de Recherche pour l'Exploitation de la Mer, Nantes, France

Edited by:
Antonio Trincone, Consiglio Nazionale delle Ricerche, Italy

Reviewed by:
Analia Graciela Abraham, Centro de Investigacion y Desarrollo en Criotecnologia de Alimentos, Argentina
Jochen Schmid, Technische Universität München, Germany

****Correspondence:***
Christine Delbarre-Ladrat, Institut Français de Recherche pour l'Exploitation de la Mer, Centre Atlantique, EM³B Laboratory, Rue de l'Ile d'Yeu, BP21105, 44311 Nantes Cedex 3, France
e-mail: cladrat@ifremer.fr

Although polysaccharides are ubiquitous and the most abundant renewable bio-components, their studies, covered by the glycochemistry and glycobiology fields, remain a challenge due to their high molecular diversity and complexity. Polysaccharides are industrially used in food products; human therapeutics fall into a more recent research field and pharmaceutical industry is looking for more and more molecules with enhanced activities. Glycosaminoglycans (GAGs) found in animal tissues play a critical role in cellular physiological and pathological processes as they bind many cellular components. Therefore, they present a great potential for the design and preparation of therapeutic drugs. On the other hand, microorganisms producing exopolysaccharides (EPS) are renewable resources meeting well the actual industrial demand. In particular, the diversity of marine microorganisms is still largely unexplored offering great opportunities to discover high value products such as new molecules and biocatalysts. EPS-producing bacteria from the marine environment will be reviewed with a focus on marine-derived EPS from bacteria isolated from deep-sea hydrothermal vents. Information on chemical and structural features, putative pathways of biosynthesis, novel strategies for chemical and enzymatic modifications and potentialities in the biomedical field will be provided. An integrated approach should be used to increase the basic knowledge on these compounds and their applications; new clean environmentally friendly processes for the production of carbohydrate bioactive compounds should also be proposed for a sustainable industry.

Keywords: marine-derived polysaccharide, biodiversity, biological activity, structure, modification, GAG-mimetic, biosynthesis

GLYCOPOLYMERS AND THEIR IMPORTANCE IN GLYCOBIOLOGY

Polysaccharides are natural macromolecules composed of osidic monomers and present in all organisms: microorganisms, plants and animals. Polysaccharides, such as starch, glycogen, and cellulose, are common. However, some other specific classes of polysaccharides exist based on their origin, chemical structure and function. Although polysaccharides are ubiquitous, their chemical structure varies greatly from one to the other. They have various roles within the cells, from structural involvement to numerous biological activities including interactions. Therefore, studying the polysaccharide chemical structure and structure-related activities remains a challenge for glycochemists and glycobiologists. Polysaccharides can be distinguished by their osidic composition: homopolysaccharides, which contain a single type of monosaccharide, and heteropolysaccharides composed of different osidic residues and usually displaying a regular backbone structure with a repeating unit. This repeating unit may be linear or branched and may contain up to 10 monomers as well as organic or inorganic substituents such as phosphate, sulfate, and lactic, succinic, acetic and pyruvic acids. The chemical structure including monosaccharide composition and repeating unit sequence as well as non-carbohydrate substituents is species-specific (Decho, 1990) and may vary, most of the time, with production, culture conditions and the physiological state of the organism. The linkages most commonly found between monomers are β-1,4 or β-1,3 giving a more rigid backbone vs. α-1,2 and α-1,6 for more flexible zones. The overall physical properties of polysaccharides are also influenced by the monosaccharide composition, the osidic sequence and the network formed by the single polymer chains (Poli et al., 2010). These polymers are high-molecular weight macromolecules usually above 10^6 g mol^{-1}.

Most of the polysaccharides used in the industry are extracted from plants, algae, and animals. A few of them come from bacteria and have industrial applications in the paper, food, biotechnological, environmental or health industry (Finore et al., 2014). The most famous examples of microbial used macromolecules have been listed in **Table 1** (Kumar et al., 2007; Freitas et al., 2011). Owing mainly to their rheological properties, fungal exopolysaccharides (EPS) have also found several applications in the food, cosmetic and pharmaceutical industries as well as in oil recovery (Mahapatra and Banerjee, 2013). Scleroglucan produced by *Sclerotium rolfsii*, schizophyllan, a structurally similar molecule produced by *Schizophyllum commune* and pullulan from *Aureobasidium pullulans* are the most

Table 1 | Examples of microbial used macromolecules (adapted from Kumar et al., 2007; Freitas et al., 2011).

Exopolysaccharide	Source	Main applications
Xanthan	*Xanthomonas campestris*	food industry as texturizing agent, petroleum industry, health care
Alginate	*Pseudomonas aeruginosa, Azotobacter vinelandii*	food hydrocolloid, wound care, drug encapsulating agent
Dextran	*Leuconostoc mesenteroides*	food industry, biomedical as plasma volume expander biotechnological supports for separation
Cellulose	*Acetobacter xylinum*	food industry, biomedical as artificial temporary skin, biotechnological separations as hollow fiber and membranes
Hyaluronic acid	*Streptococcus equi, Streptococcus zooepidemicus*	human health cosmetics
Gellan	*Sphingomonas paucimobilis*	food industry, biotechnology (culture medium gelification)
Curdlan	*Sinorhizobium meliloti, Agrobacterium radiobacter, Alcaligenes faecalis*	food and pharmaceutical industries, bioremediation
Succinoglycan	*Sinorhizobium meliloti, Alcaligenes faecalis*	food and pharmaceutical industries, oil recovery
Levans	Various	food industry (prebiotic)

common fungal polysaccharides of high-added value (Survase et al., 2007; Mahapatra and Banerjee, 2013). Some of these polysaccharides are homolog counterparts of plant or animal macromolecules. Compared to these sources, microorganisms allow a better controlled production in bioreactors, devoid of no variation due to physiological state or season encountered for the highest organisms (Bertagnolli et al., 2014) and an easier extraction without any drastic or environmentally toxic compounds. However, downstream processing of bacterial polysaccharides still represents an important cost intensive step (Kreyenschulte et al., 2014). Moreover, microorganisms cultivation in fermenters allows the optimization of the growth and the production yield either by the study of physiology or by genetic engineering. For the high-added value pharmaceutical industry, bacterial polysaccharides can be produced at a viable economic cost. The production in controlled conditions is in agreement with the Good Manufacturing Practices (GMP) such as well-defined medium, controlled environment without the risk for viral or pathogen agents. The advantages of a bacterial source over plant, algal or animal source have made it attractive to obtain macromolecules for various industrial purposes and strengthened their study.

Complex carbohydrates and glycoconjugates have a basic importance in biological systems and cellular processes, either physiological or pathological. They play a major role as structural agents in connective tissues, and they are ubiquitously present on cell surfaces, mediating the interaction of the cells with other cells, with the extracellular matrix, with biotic or abiotic surfaces and with other molecules. But their study that falls into the field of the glycobiology was greatly hindered by technical issues; no sequencing tool such as that existing in proteomics or genomics is available to date while glycopolymers potential chemical diversity is far greater than proteins and nucleic acids (Turnbull and Field, 2007; Merritt et al., 2013). While genes and proteins syntheses are based on a template, polysaccharide biosynthesis is regulated by a number of physiological and metabolic parameters including the availability of sugar precursors and the expression level of enzymes. The number of osidic residues combined with the anomeric configuration of the linkages and the possibility of branching result in unique complexity and diversity of carbohydrates molecules. Nonetheless, some advances in analytical techniques have revealed the vast and diverse chemical structures of carbohydrates existing in nature (Grice and Wilson, 2010); insights into the structure-function relationship allow determining their role in diverse cellular processes. Glycobiology becomes therefore a major research field allowing the understanding of human diseases and the discovery of novel therapeutic compounds.

Glycosaminoglycans (GAGs) are glycopolymers found in animal tissues and composed of uronic acid and neutral or hexosamine residues. They are covalently bound to a core protein and are the major constituent of proteoglycans. Their carbohydrate backbone is unique for each cell type. GAGs are essential for life of animals since they are involved in their development and organogenesis (De Angelis et al., 2013). Two GAG macromolecules are frequently used in various industry fields, namely hyaluronic acid and heparin.

Hyaluronic acid is a polysaccharide ranging from 500 to 1000 10^3 g mol^{-1}, with a disaccharidic linear and non-sulfated repeating unit (**Table 2**). It is widely used in osteoarthritis treatment as synovial fluid because of its effect on the cartilage, in ophthalmology treatments and surgery because of its visco-elastic properties, and in wound healing as well as in the cosmetic industry (moisturizing agent, wrinkle filler).

Heparin is a polyanionic molecule of about 80 10^3 g mol^{-1} with 20 to 40% of sulfates exhibiting several biological activities such as anticoagulant, anti-thrombotic, anti-inflammatory, antiviral and anticancer (Hirsh et al., 2001). It has been discovered in the early twentieth century and used medically for over 70 years. The commercially available drug is extracted from a mucous lining of pig intestines. In parallel, low-molecular weight (LMW) heparin (6–10 10^3 g mol^{-1}) has been developed for use as an anticoagulant to increase benefit-risk ratios (Hirsh et al., 2001). In 1983, the pentasaccharide unit responsible for the anti-coagulant and anti-thrombin activity within the heparin molecule was identified, chemically synthesized (Choay et al., 1983) and it is now commercialized (Petitou et al., 2002) (**Table 2**). Although this molecule presents, as the unfractionated and LMW heparin, some undesirable bleeding side effects

Table 2 | Repeating unit chemical structures of main glycosaminoglycans and of some marine exopolysaccharides.

Heparin antithrombin-binding site pentasaccharide (Choay et al., 1983)

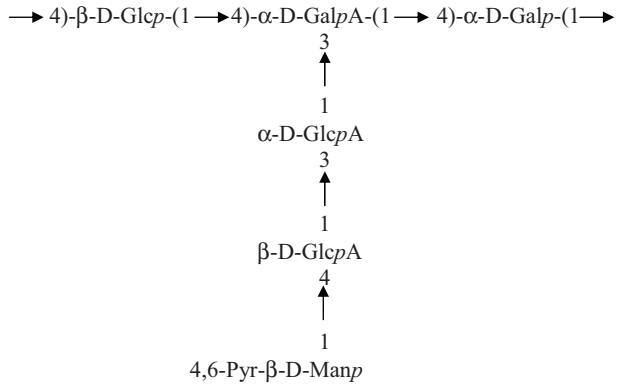
→4)-α-D-GlcN*p*2S6S-(1→4)-β-D-GlcA*p*-(1→4)-α-D-GlcN*p*2S3S6S-(1→4)-α-L-IdoA*p*2S-(1→4)-α-D-GlcN*p*2S6S-(1→

Hyaluronic acid

→3)-β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*A-(1→

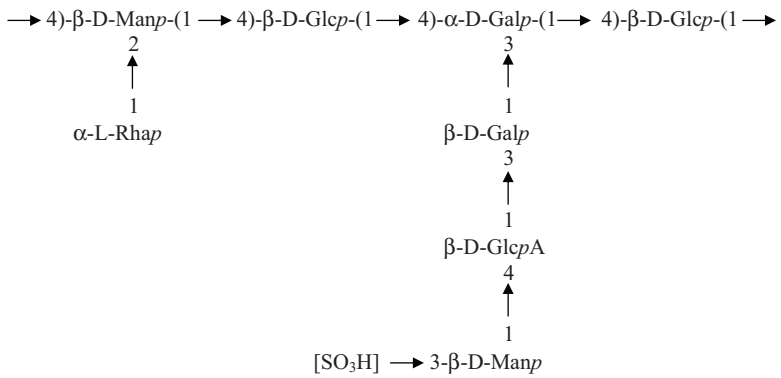
EPS ST716

from *Alteromonas macleodii* subsp. fijiensis (Raguenes et al., 1996; Rougeaux et al., 1998)



EPS HYD721

from *Pseudoalteromonas* sp. (Rougeaux et al., 1999a)



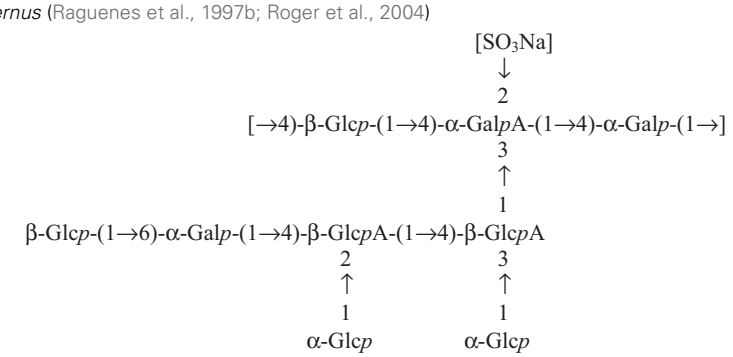
EPS HE800

from *Vibrio diabolicus* (Raguenes et al., 1997a; Rougeaux et al., 1999b)

→3)-β-D-GlcpNAc-(1→4)-β-D-GlcpA-(1 →4)-β-D-GlcpA -(1 →4)-α-D-GalpNAc-(1 →

EPS GY785

from *Alteromonas infernus* (Raguenes et al., 1997b; Roger et al., 2004)



Galp, galactose; GalpA, galacturonic acid; GalpNAc, N-Acetyl-galactosamine; Glcp, glucose; GlcAp or GlcpA, glucuronic acid; GlcpNAc, N-Acetyl-glucosamine; GlcNp2S6S, N-sulfate-6-O-sulfate-glucosamine; GlcNp2S3S6S, N-sulfate-3,6-di-O-sulfate-glucosamine; GlcNp 2S6S, N-sulfate-6-O-sulfate-D-glucosamine; IdoAp2S, 2-O-sulfate-iduronic acid; Manp, mannose; Pyr, pyruvate group; Rhap, rhamnose; SO_3H, sulfate group.

(Hirsh et al., 2001; Crowther and Warkentin, 2008), it remains a lead molecule to study the structure-activity relationship. A heparin contamination crisis arose in the early 2008; some Chinese heparin preparations caused severe side effects resulting in the death of more than 100 patients (Liu et al., 2009). The impurity was identified as a variant of chondroitin sulfate, in which sulfate groups exhibited an unusual pattern (Guerrini et al., 2008). Today, despite the risk of contamination during the extraction process or by animal pathogenic agents, heparin from natural sources continues to be widely used clinically. Still new alternative macromolecules are looked for. In this context, marine microbial polysaccharides stand for a promising source of macromolecules with reduced risk of contamination by mammalian pathogenic agents. New emerging products of high added-value from the marine environment, showing structural homology to heparin such as the presence of sulfate groups, offer the promising opportunity of novel biologically active compounds (Pomin, 2014a).

Exopolysaccharides (EPS) are glycopolymers that microorganisms secrete in their surrounding environment (Sutherland et al., 1972). They can be capsular polymers, which are attached to the cell membrane through the lipopolysaccharides (LPS) anchored in the membrane or other specific proteins (Decho, 1990). EPS can also be produced as a slime loosely bound around the cell or dispersed in the environment (Sutherland, 1982). Bacterial polysaccharides are also present within the membrane cell as a major constituent of the LPS and may be involved in pathogenicity. These glycopolymers have mostly a protective role and permit resistance under extreme environmental conditions by participating in the cell membrane integrity, trapping nutrients, allowing adhesion to surfaces, protecting from toxic compounds and adverse conditions such as freezing (Jannasch and Taylor, 1984; Decho, 1990; Finore et al., 2014). EPS production requires energy from cells representing a carbon investment for microorganisms but the benefits to growth and survival are higher than the production cost (Poli et al., 2011).

THE MARINE BIOSPHERE AS A SOURCE OF NEW GLYCOPOLYMERS

Representing more than 70% of our planet, ocean is an under-explored and under-exploited vast reservoir for biological organisms and chemical compounds. France owns an expanded maritime domain as well as oceanographic ships, submarine and robots allowing to explore the marine biosphere, and especially deep seas. The marine biosphere is heterogeneous because a large range of ecosystems exist such as microbial mats, Antarctic sea ice, hypersaline marine environments, shallow and deep-sea hydrothermal vents. They are characterized by physical and chemical parameters such as pressure, temperature, pH, chemical compounds, usually toxic. Within the deep-sea hydrothermal vents, large physico-chemical gradients exist; for example, the temperature varies from 2°C, the temperature of the surrounding sea water to the hot temperature of the hydrothermal plume, which can reach 350°C (Baross and Hoffman, 1985). Due to their microbial diversity, these ecosystems might offer promising new biomolecules (Deming, 1998).

MARINE ECOSYSTEMS WITH A FOCUS ON DEEP-SEA HYDROTHERMAL VENTS

Deep-sea hydrothermal vents result from oceanic plate tectonic and submarine volcanic activities. They appear at the sea ridges or on subduction back-arc areas, at a depth of 500 to 4000 m (**Figure 1**). Seawater at high temperature (up to 350°C) and charged with metals and other compounds such as hydrogen sulfide, hydrogen, ammonia, carbon dioxide flows out of structures built from precipitates called chimneys. Depending on the composition of the fluid, the plume appears with different intensity of white or black color (white or black smokers) (Burgaud et al., 2014). Due to volcanic activities of the crust, these ecosystems are ephemeral (Van Dover et al., 2002). Some other active areas with a diffuse emission of warm or cold water also exist. Deep-sea ecosystems also include cold seeps and sediments or microbial mats (Jannasch and Taylor, 1984). The first hydrothermal vent was discovered in 1977 in the Galapagos area at a depth of 2600 m (Jannasch and Taylor, 1984), although deep-sea biology started with the Challenger expedition (1873–1876). The presence of bacteria in the open ocean seabed is known since the 1880s from the Travaillier and Talisman expeditions (1882–1883) (Zobell and Morita, 1959), when the first microorganisms were found at 5000 m deep (Jannasch and Taylor, 1984). In the 1950s, Galathea expedition collected bacteria-containing sediment samples from 10,000 m deep (Zobell and Morita, 1959).

Deep-sea hydrothermal ecosystems, the most productive ecosystems from the deep ocean, are based on a primary chimiosynthetic production of organic matter sustaining an abundant fauna which is mainly composed of endemic species (Jannasch and Taylor, 1984). These oases of life contrast to the vast surrounding desertic seafloors. A large variety of microbial lifestyles has been described in these ecosystems based on the respiratory type (aerobic-anaerobic), energy and carbon sources. It is adapted to extreme conditions (barophiles needing high pressure, acidophiles-alcalophiles, psychrophiles, mesophiles and extremophiles -growing at temperatures from around 3°C, the temperature of seawater, to 105°C-) (Jannasch and Taylor, 1984). New microorganisms with very diverse metabolisms have then been isolated from marine environment especially in deep-sea habitats (Miroshnichenko and Bonch-Osmolovskaya, 2006). Halophiles are also encountered in some marine ecosystems. The high biodiversity potentially offers a large chimiodiversity. Besides their fundamental interest in basic studies for life origin understanding, chimiosynthetic metabolisms and mechanisms of resistance to high temperatures, these microorganisms arise a great biotechnological interest for the isolation of new enzymes and biomolecules with new or enhanced activities.

FROM MARINE MICROBIAL BIODIVERSITY TO NEW BIOACTIVE MOLECULES

Marine biodiversity is not known currently because only a very small fraction of the microorganisms can be cultivated (Hugenholtz, 2002; Delong et al., 2006). There are reports on particular marine environments with high microbial diversity; this is the case for deep-sea hydrothermal vents in North Atlantic (Sogin et al., 2006). The next generation high throughput DNA sequencing (NGS) methodologies can provide a way to estimate

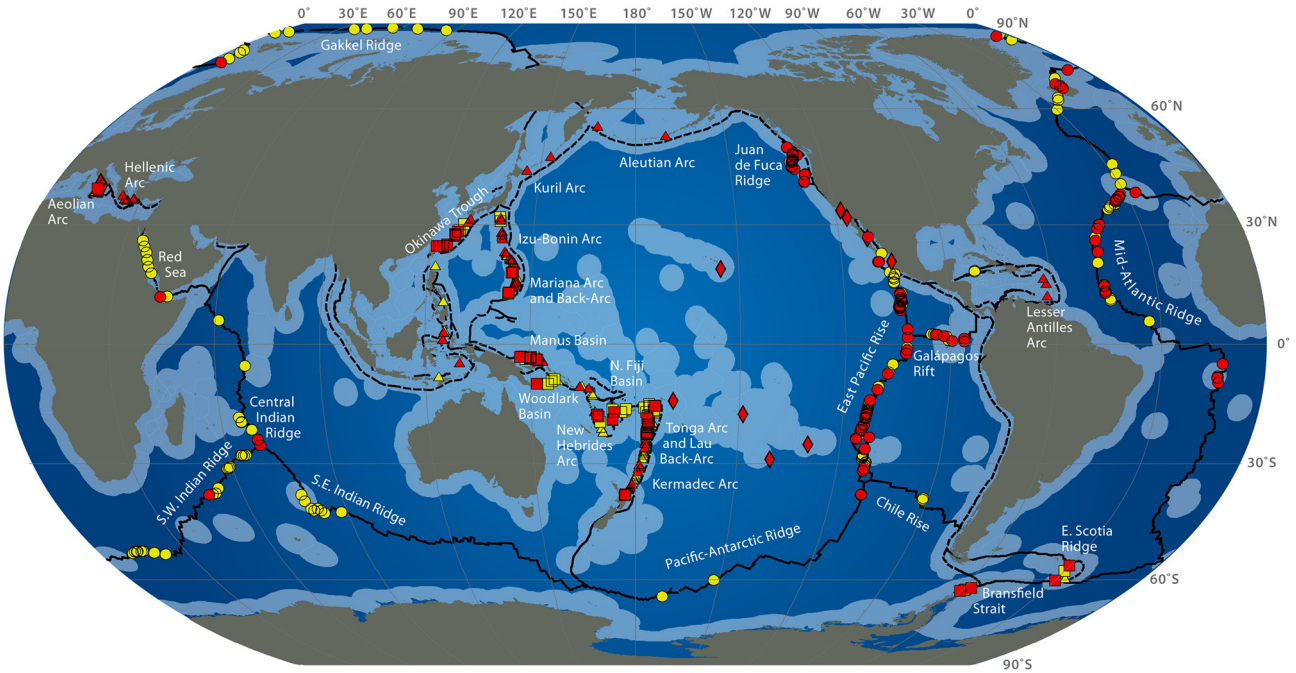


FIGURE 1 | Global distribution of hydrothermal vent fields (2009) from http://www.interridge.org/irvents/files/Ventmap_2009.jpg, accessed 2014, September 9. Hydrothermal vent sites from Mid-Ocean Ridge (circles), Arc volcano (triangles), Back-arc spreading center (squares), other (diamonds) are shown (active sites are red, unconfirmed are yellow). Ridges are in plain line, trench are in dashed line. Light blue indicates exclusive economic zones.

the biodiversity and can be useful to drive some cultivation experiments (Rocha-Martin et al., 2014): when the analysis of metagenomic data sets reveals incomplete pathway for a given compound essential for growth, this compound has to be added as an exogenous source. For example, because SAR11 marine alpha-proteobacteria clade has been shown to be deficient in assimilatory sulfate reduction genes, addition of reduced sulfur compounds in the cultivation medium was tested and was revealed necessary for growth (Tripp et al., 2008).

When the biodiversity increases, the chimiodiversity also increases. Therefore, the chemical study of the marine organisms will most probably result in the discovery of new therapeutic compounds. In particular, it is well recognized that the chemical diversity of marine and microbial compounds is the broadest one (Grabowski et al., 2008). Although microorganisms from the sea have been studied for several decades, their development for biotechnology started only recently (see Imhoff et al., 2011). Today, only a small fraction of the known species has been studied for their biochemical metabolites. Marine microorganisms together with sponges, molluscs, algae, echinoderms are rich source of polysaccharides. Chitin, the second most abundant polysaccharide after cellulose, is found particularly in the shell of crustaceans. Alginates, another marine-derived polysaccharide, are extracted from the cell wall of brown algae (Phaecophyta). Marine-derived polysaccharides naturally bearing sulfate groups are also industrially relevant: carrageenan (red algae), fucoidans (brown algae) and fucans (marine invertebrates) (Berteau and Mulloy, 2003; Kusaykin et al., 2008), ulvan from green seaweeds (Chlorophyta) (Lahaye and Robic, 2007). Microorganisms producing polysaccharides have also been isolated from marine ecosystems especially from deep-sea hydrothermal vents.

Marine chimiodiversity finds applications in human health as therapeutic agents for innovative biological activities. New compounds with new chemical structure can also enable a better understanding of the cellular processes (pathological or not): inhibitors of enzymes involved in inflammatory diseases (manoalide) and phosphorylation processes (okadaic acid), both extracted from sponge, are available for biochemical research (Fenical, 1997). Some examples of marine molecules are already commercialized as analgesic (the peptidic Prialt®), anti-viral (mainly from corals and sponges), anti-cancer (the alkaloid Yondelis® by PharmaMar), or as biomaterials (the calcium carbonate chemical compound Biocoral® by Biocoral from coral). Only a few of them are carbohydrates: alginate of plant origin and chitosan obtained from shrimp extracted chitin have been successfully integrated within wound dressings (Stop Hemo® by Brothier Laboratory in France, Nu-Derm® by Johnson & Johnson in USA) and are also used as encapsulation matrices (geniaBeads® by geniaLab in Germany). Most of the marine molecules are still in research or clinical development, among which four are from marine microorganisms (Imhoff et al., 2011); this leaves the marine biotechnology still at a very promising phase; great efforts are still necessary to get advances in clinical applications.

Bioactive compound prospecting starts with the sampling of organisms. The choices of methods to prepare extracts and to screen for the production of bioactive metabolites are critical steps since they can result in different molecules or even in difficulties to find the activity. The diversification of sources by the

exploration of new ecological niches can maintain an effective process leading to innovative results. Compounds should then be isolated, structurally characterized especially including the stereochemistry which can be the main issue for its activity. Biological properties have to be studied in detail, *in vitro* and subsequently *in vivo* before clinical development stages. Alternatively, synthetic analogs can be studied instead of natural molecules. Chemical synthesis would offer access to structurally defined oligosaccharides in sufficient purity and quantity to carry out biological studies. In addition, the synthesis can bring a library of analog compounds slightly differing in the chemical structure as well as in the activity. This library would be a useful tool to establish the structure-activity relationship. Synthetic analogs of the active pentasaccharide in heparin have been well studied due to the high value of this drug and its side effects (Avci et al., 2003). However, synthetic products are sometimes hard to make because of the complexity of the molecule. This is particularly the case for polysaccharides for which only small oligosaccharides have been obtained to date (Boltje et al., 2009). This issue still limits the progress of glycobiology research and much effort are made to develop oligosaccharide synthesis methodologies. Recently, Galan et al. have shown that ionic liquids could be very useful as solvents and promoters of synthetic reactions (Galan et al., 2013).

MARINE EXOPOLYSACCHARIDES

MICROBIAL POLYSACCHARIDE DIVERSITY

Several EPS-producing marine strains have been studied, which led to the discovery and isolation of novel macromolecules (Finore et al., 2014). Marine bacterial polysaccharides have been the subject of several reviews (Guezennec, 2002; Nazarenko et al., 2003; Mancuso Nichols et al., 2005b; Laurienzo, 2010; Satpute et al., 2010; Freitas et al., 2011; Senni et al., 2011; Colliec-Jouault and Delbarre-Ladrat, 2014; Pomin, 2014b). Most of the marine derived EPS are bacterial (classical bacteria i.e., mesophilic and heterotrophic; extremophilic microorganisms i.e., psychrophilic, thermophilic and halophilic); archaea have also been shown to produce EPS (Rinker and Kelly, 2000; Nicolaus et al., 2010; Poli et al., 2011; Finore et al., 2014; Sinquin and Colliec-Jouault, 2014). In the present manuscript, the main focus was put on the extremophilic prokaryotic marine microorganisms that were shown to produce EPS, with an emphasis on bacteria isolated from deep-sea hydrothermal vents.

Mancuso Nichols et al. (2004) described the production of EPS by the marine strains *Pseudoalteromonas* CAM025 and CAM036 isolated in Antarctica sea water and sea ice. Some other strains from Arctic sea ice have been shown to produce EPS with cryoprotective effect (Liu et al., 2013).

The halophilic strains *Halomonas maura* (Arias et al., 2003), *Halomonas ventosae* (Martinez-Canovas et al., 2004), *Halomonas alkaliantarctica* (Poli et al., 2007), *Hahella chejuensis* (Poli et al., 2010) and the archaeal halophilic *Haloferax mediterranei* (Anton et al., 1988; Parolis et al., 1996) isolated from hypersaline environments were also shown to produce EPS; some of them are sulfated (Poli et al., 2010).

Marine thermophilic anaerobes *Sulfolobus*, *Thermococcus* and *Thermotoga* were described to produce EPS (Vanfossen et al., 2008). *Thermococcus litoralis* produces an EPS which contains sulfate and phosphorus substituents (Rinker and Kelly, 2000); the archaea *Sulfolobus solfataricus* has also been shown to produce a sulfated polysaccharide (Nicolaus et al., 1993). *Geobacillus* sp., *Bacillus thermodenitrificans* and *B. licheniformis* thermophilic strains have been isolated from shallow marine hydrothermal vents of Vulcano Island (Italy) and the polysaccharide they produce has been analyzed (Poli et al., 2010).

Several bacteria belonging to gamma-proteobacteria Alteromonadales or Vibrionales orders and isolated from the deep-sea polychaete annelids *Alvinella pompejana* and *A. caudata* tissues have been shown to produce different EPS: HYD1545 (Vincent et al., 1994), HYD1644 (Dubreucq et al., 1996), HYD721 (Rougeaux et al., 1999a), HYD657 produced by *A. macleodii* subsp. *fijiensis* biovar deepsane (Cambon-Bonavita et al., 2002) and HE800 produced by *Vibrio diabolicus* (Raguenes et al., 1997a; Rougeaux et al., 1999b). This latter polysaccharide possesses a particular hyaluronic acid-like chemical structure and contains both hexosamines and uronic acids alternating in the repeating unit sequence (**Table 2**) (Sinquin and Colliec-Jouault, 2014).

Another gamma-proteobacterium isolated from a deep-sea hydrothermal vent shrimp (*A. macleodii* subsp. *fijiensis* biovar *medioatlantica*) was shown to produce the EPS MS907 (Raguenes et al., 2003) whereas *Alteromonas macleodii* subsp. *fijiensis* producing the pyruvated EPS ST716 (Raguenes et al., 1996; Rougeaux et al., 1998) and *Alteromonas infernus* producing the sulfated EPS GY785 were isolated from hydrothermal fluids (Raguenes et al., 1997b; Roger et al., 2004) (**Table 2**).

Other *Pseudoalteromonas* strains produce diverse EPS: SM9913 isolated from deep-sea sediments produces an acetylated EPS (Qin et al., 2007), SM20310 from the arctic sea ice produces a complex α-mannan (Liu et al., 2013).

Pseudomonas, *Alteromonas*, *Paracoccus* and *Vibrio* sp. bacteria producing EPS under laboratory conditions have also been isolated from microbial mats in French Polynesia, another marine ecosystem considered as extreme. Among them, *Paracoccus zeaxanthinificiens* subsp. *Payriae* and *Vibrio* sp. RA 29 are described to produce sulfated polysaccharides and *Vibrio* sp. MO 245 would produce a polymer very similar to the *Vibrio diabolicus* one (Guézennec et al., 2011).

When known, the chemical and structural diversity of these few molecules confirms the high value of the marine environment as a source of exciting chimiodiversity.

ALGINATE AND CHITOSAN, TWO MAJOR MARINE POLYSACCHARIDES

Alginate traditionally extracted from brown algae and the most important polysaccharide from this type of seaweeds is also produced by soil bacteria *Azotobacter* and *Pseudomonas* species as an exopolysaccharide (Gomez D'ayala et al., 2008). Although they are not marine bacteria, the polysaccharide is usually refered as marine derived. A gene cluster for alginate biosynthesis have been identified in *Vibrio* sp. QY102 suggesting this polymer would be involved in the formation of biofilm by *Vibrio* sp. QY102 (Shi et al., 2008).

The bacterial alginates are all composed of mannuronic (M) and guluronic (G) acids arranged in homo-blocks (M or G) and hetero-blocks (MG) as algal alginates; but that from *Pseudomonas*

does not have block G; in addition many of bacterial alginates are acetylated. G-rich alginates and M-rich alginates have different physico-chemical and biological properties (Colliec-Jouault and Delbarre-Ladrat, 2014). Although their osidic composition is similar, bacterial alginates vary considerably in their structure depending on the producing strain and in the case of alginate produced by seaweeds, on seasonal and environment variations (Gomez D'ayala et al., 2008; Bertagnolli et al., 2014). Industrial production of alginate reaches 30,000 tons annually and it is used as a viscosifier, stabilizer and gelling agent (Hay et al., 2013).

Chitin and chitosan, its partially N-deacetylated derivative, are the most abundant marine polysaccharides; they have great interest for biotechnological applications. They are mainly extracted from shellfish waste. To date no bacterium has been described to produce either chitin or chitosan. Nonetheless, some bacteria have been shown to produce PNAG, poly-*N*-acetyl-glucosamine, a β-1,6-linked *N*-acetyl-D-glucosamine homopolymer, structurally close to chitin, β-1,4-linked *N*-acetyl-D-glucosamine homopolymer (Whitney and Howell, 2013; Ye et al., 2014).

PRODUCTION AND PHYSIOLOGICAL CONDITIONS INFLUENCE

Production of polysaccharides by bacteria is a physiological process that takes place under a diversity of environmental conditions (Kumar et al., 2007), usually in response to a stress including the adaptation to extreme environment (Lapaglia and Hartzell, 1997). In the marine environment, bacteria are usually attached to biotic or abiotic surfaces and are embedded in a slime, an exopolymeric matrix composed of proteins, polysaccharides, DNA and small organic molecules; they form biofilms as a protective response to a stress (Lapaglia and Hartzell, 1997; Guezennec, 2002; Liu et al., 2013). Therefore, it is important to identify any relationship between the biosynthesis of EPS and biofilm formation regulation in order to better control the production process (Rehm, 2010). Limitations in the availability of nutrients such as nitrogen, phosphorous, sulfur, potassium (Kumar et al., 2007; Sabra and Hassan, 2008), as well as temperature, osmotic or pH shifts (Gorret et al., 2001; Kumar et al., 2007) are stressing factors enhancing EPS production. Moreover, a suitable carbon source to be added to the production or growth medium is also a main factor for the yield and features of the produced molecule (Donot et al., 2012). Its excess, in fact, is concomitant with another component limitation such as nitrogen (Freitas et al., 2011). In some cases, the osidic composition of the molecule can change with the carbon substrate (Grobben et al., 1996; Poli et al., 2007; Freitas et al., 2011).

Production yield of marine EPS is usually around 1 g L^{-1} (Decho, 1990). Industrial development is conceivable for a production yield around 10 g L^{-1}. Production costs are driven by the yield of polysaccharide, the amount and cost of carbon source, and by the downstream processes needed for molecule separation. Therefore, studies on production optimization are needed even if some bacterial strains are naturally able to produce EPS at a high yield (e.g., 50 g L^{-1} or over) such as *Agrobacterium* sp. (curdlan), *Xanthomonas campestris* (xanthan), *Zymomonas mobilis* (levan), *Alcaligenes faecalis* (curdlan), *Bacillus* sp. (levan) (Donot et al., 2012). Factors such as medium composition and fermentation conditions i.e., temperature, pH and aeration stand for the main studied variables to optimize the fermentation process (Mancuso Nichols et al., 2005a; Sabra and Hassan, 2008; Finore et al., 2014). The nutritional conditions can also affect the molecular weight as well as the osidic composition of the EPS in some cases (Mancuso Nichols et al., 2005a; Poli et al., 2009; Donot et al., 2012; Finore et al., 2014). A novel *Alteromonas macleodii* strain has been shown to produce 23.4 g L^{-1} exopolysaccharide when grown on 15% lactose which is the highest yield obtained for marine EPS (Mehta et al., 2014).

Fermentation is usually performed in batch or fed-batch. Heat transfer and oxygen supply limitations are however encountered when EPS are highly viscous (Kreyenschulte et al., 2014). Depending on the producing bacterium, the EPS may be produced during the growth (cellulose, gellan or alginate by *Azotobacter vinelandii*), when the growth has ended (curdlan by *Alcaligenes faecalis*), during the stationary phase (Raguenes et al., 1996) or both (xanthan by *X. campestris*) (Decho, 1990; Freitas et al., 2011). The marine EPS HYD1644 is produced only after the initial exponential growth phase (Samain et al., 1997). In *Pseudomonas* sp., an EPS is produced during the exponential phase and another one with a different chemical structure is produced during the stationary phase (Christensen et al., 1985). Some reports emphasize the benefit of a growth-production uncoupling by using a bacteriostatic to stop the growth together with the addition of the carbonated substrate and/or shift in temperature or pH (Looijesteijn and Hugenholtz, 1999).

DOWNSTREAM PROCESS FOR EPS RECOVERY

After fermentation, the downtream process for the recovery of EPS is an important step for the production costs and commercial value of the molecule. Process steps will depend on the molecule, on other undesired molecules produced, but also on the level of purity required.

Downstream process, consisting of the removal of insoluble particles, isolation of the product and further purification, is based on classical steps of extraction and purification. Each method has its advantages and disadvantages (Sinquin and Colliec-Jouault, 2014). Various molecules are usually associated with polysaccharides that act as sponges for some proteins, amino acids, DNA, RNA, salts, metals, fatty acids either dissolved in seawater based medium or released after cell lysis (Decho, 1990; Freitas et al., 2011; Donot et al., 2012). The presence of degrading enzymes or of a second biopolymer must also be taken into account for a good purification process (Kreyenschulte et al., 2014). Therefore some isolation procedures may be better adapted than others as reviewed by Donot et al. (2012).

Centrifugation or filtration step is usually used to remove cells from the culture broth without lysis and EPS isolation (Kreyenschulte et al., 2014). Culture broth is sometimes subjected to heating at the end of the fermentation to kill the bacteria and denature potential polymer-degrading enzymes (Freitas et al., 2011) but this may result in some cell lysis and release of compounds in the soluble medium. The deactivation of the cells is alternatively carried out by chemical, enzymatic or mechanical treatment (Kreyenschulte et al., 2014).

Separation and purification of the biopolymer can be obtained by precipitation with the addition of alcohol or by complexing

metal ions (Kreyenschulte et al., 2014). However, ethanol precipitation may trap and co-precipitate proteins and ions (Kumar et al., 2007) and needs a large amount of alcohol. Filtration or ultrafiltration is recognized as a good method to separate high molecular weight from other small adsorbed compounds and has been used since a long time (Wilkie et al., 1957; Kreyenschulte et al., 2014). Some additional extractions to remove contaminating compounds may be appropriate such as new precipitation, chemical extraction or enzymatic treatment but they may decrease the recovery yield (Decho, 1990; Freitas et al., 2011). These separation steps can in turn be hindered when polysaccharide is highly viscous; higher temperature or dilution in water may facilitate the process however increasing the costs (Freitas et al., 2011). The choice of the whole procedure has to be adapted to polymer characteristics as well as to the desired recovery yield, purity and integrity degrees. After isolation, the polysaccharide is freeze dried for a better conservation. Polysaccharides are highly hydrophilic due to hydroxyl groups, especially when they are polyanionic (carboxyl groups), a widespread feature in marine environment, or when they bear sulfate groups; therefore they always conserve a content of water (De Angelis et al., 2013).

PUTATIVE PATHWAYS OF BIOSYNTHESIS

The genetics of the EPS biosynthesis begins to be better understood; however, information appears disparate because it depends on the concerned microorganism and the polysaccharide that it produces.

However, depending on the type of polysaccharide, some general mechanisms can be described (Rehm, 2010). An extracellular glycosyltransferase (GT) is responsible for the biosynthesis outside the cell of homopolysaccharides such as dextran, levan, mutan. This particular GT (usually sucrase) cleaves a disaccharide substrate (usually sucrose) and transfers one of the two obtained monomers to the polymer chain (Rehm, 2010). The occurrence of these enzymes is however limited in marine microorganisms (Decho, 1990).

Except for mechanisms involving sucrase, the biosynthesis starts with the production of nucleotide sugars which will be linked in the repeating unit of the molecule. They are biosynthesized within the central cellular metabolism with usually known enzymes (Rehm, 2010). The repeating unit is then synthesized by appropriate GTs (Whitfield, 2006; Rehm, 2010). After completion, the repeating unit is exported outside the cell and polymerized on the growing EPS chain. Based on biosynthesis and export mechanisms, three pathways have been described.

Some simple heteropolysaccharides (two different residue types maximum such as hyaluronic acid) as well as some homopolysaccharides (cellulose, chitin) are synthesized by a synthase enzyme which polymerizes nucleotide sugars while exporting the growing polymer chain outside the cell (Weigel and De Angelis, 2007; Rehm, 2010). The second pathway relies on the ABC transporter for exportation of the entire polysaccharide synthesized on a lipid carrier (Whitney and Howell, 2013). The third mechanism depends on Wzx-Wzy proteins.

The Wzx-Wzy dependent mechanism has been widely studied in Gram negative bacteria especially for heteropolysaccharide production. Biosynthesis is catalyzed by a membrane-spanning multiprotein complex (Rehm, 2010). A particular GT involved in the initiation step, the phosphoglycosyltransferase (pGT), anchors the first osidic residue to a membrane lipid carrier (undecaprenyl phosphate) through a phosphoryl bond (De Vuyst et al., 2001; Whitfield, 2006). After completion of the repeating unit by successive GTs, it is exported outside the cell across the inner membrane by Wzx and subsequently polymerized by the Wzy protein (Whitfield, 2006). The lipid carrier anchor is also recognized for the translocation of the repeating unit across the inner membrane (Rehm, 2010). The final translocation across the outer membrane involves a member of the outer membrane polysaccharide export protein family such as Wza (Reid and Whitfield, 2005). Heteropolysaccharides from the Gram-positive lactic acid bacteria are synthesized by a very similar mechanism (De Vuyst et al., 2001).

Besides these four types of biosynthesis mechanisms (extracellular, synthase-, Wzx-Wzy- and ABC-transporter), some other gene clusters showing a peculiar mechanism have been described. In general, the overall regulation of the biosynthesis is not completely understood as well.

Sphingans are heteropolysaccharides produced by *Sphingomonas* bacteria, some of whom are of marine origin (Cavicchioli et al., 1999). These polymers are characterized by a tetrasaccharide backbone structure containing rhamnose or mannose (1), glucose (2) and glucuronic acid (1). Gellan, diutan, welan, rhamsan, and sphingan S-88 are examples of them differing by their side chain and substituents (Harding et al., 2004; Freitas et al., 2011). The biosynthesis of sphingans has been recently reviewed by Schmid et al. (2014). The organization of genes required for diutan, welan, gellan, S-88 and S-7 biosynthesis shows similarities; genes for polysaccharide and protein secretion, as well as an operon for the synthesis of dTDP-rhamnose are conserved suggesting a well-conserved mechanism for polysaccharide biosynthesis and secretion (Yamazaki et al., 1996; Harding et al., 2004; Coleman et al., 2008). The mechanism starts by the transfer of glucose-1-phosphate on the isoprenylphosphate lipid. Successive GTs transfer the other sugar nucleotide to the repeating unit similarly to the first steps of Wzx-Wzy dependent mechanisms (Coleman et al., 2008; Schmid et al., 2014). A putative gene for lyase is only present in diutan gene cluster. Comparison of the three gene clusters allowed also the identification of a candidate gene encoding the protein responsible for the addition of rhamnosyl side chain.

Alginate biosynthesis genes in *Azotobacter* and *Pseudomonas* bacteria are similar (Rehm and Valla, 1997), the 12-core genes are clustered in a single operon. Only slight differences exist especially in the regulation (Donot et al., 2012; Hay et al., 2013). Alginate is synthesized as polymannuronnic acid in an undecaprenol-independent manner (Remminghorst and Rehm, 2006) and is *O*-acetylated in the periplasm. The gene cluster includes genes for the precursor synthesis (GDP-mannuronic acid), the polymerization, the translocation across the inner membrane and the periplasm where alginate encounters some modifications (*O*-acetylation and C5-epimerization of mannuronic acid to guluronic acid), and the alginate secretion (Remminghorst and Rehm, 2006; Hay et al., 2013). In the case of alginate biosynthesis by *Azotobacter vinelandii* and *Pseudomonas fluorescens*, the

molecular mechanisms of polymerization and export are not fully understood (Rehm, 2010), but are based on a synthase-dependent mechanism (Whitney and Howell, 2013).

The groups of genes necessary for the biosynthesis of an heteropolysaccharide are typically clustered at one genetic locus of 12–25 kb including genes for the synthesis of the repeating unit (GTs), genes for export, polymerization and regulation (Laws et al., 2001). These clusters are also well known in lactic acid bacteria (LAB) such as *Streptococcus* spp. (Wu et al., 2014) and *Bifidobacterium* spp. (Hidalgo-Cantabrana et al., 2014); in LAB, EPS biosynthesis gene clusters are often located on plasmids (Kumar et al., 2007; Donot et al., 2012). Furthermore, *Escherichia coli* polysaccharide biosynthesis pathways have become a reference model in these studies (Whitfield, 1995, 2006; Willis and Whitfield, 2013).

The activated precursors are also needed for the synthesis of some other cellular components such as peptidoglycan for membranes (Merritt et al., 2013). Therefore, it is of high importance to understand the fluxes of carbon, nitrogen and energy leading both to cells and bioactive molecules or, at least, to modulate them by physiological optimizations or genetic engineering (Rehm, 2010). It is highly probable, due to their respective role, that cell wall biosynthesis has priority, LPS and finally EPS synthesis (Decho, 1990).

With the post-genomic era, more clusters involved in biosynthesis of polysaccharides are described together with some regulations issues. An understanding of how the high-molecular weight polymers are biosynthesized may lead to better efficiencies in EPS production at an industrial level and a better comprehension of how varies the composition upon production conditions. New strategies like genetic engineering of producing microorganisms are being developed to enhance polysaccharide yield and allow an economically effective production (Ates et al., 2011; Finore et al., 2014). On the other hand, the prediction of metabolic network can allow the identification of key factors for an enhanced production: from the genome sequence, mannitol was identified as a stimulator for levan biosynthesis by *Chromohalobacter salexigens* (Ates et al., 2011), experimental evidence was achieved later in another halophilic bacterium *Halomonas smyrnensis* (Ates et al., 2013). Metabolic engineering approaches can complement ongoing efforts on fermentation process engineering with the aim to optimize EPS production (Merritt et al., 2013). In this strategy, research on K5 EPS biosynthesis is a good example as reviewed by Wang et al. (2011). Fermentation process has been optimized for a better yield and productivity of heparosan. Conversion of UDP-glucose to UDP-glucuronic acid by the UDP-glucose dehydrogenase together with the UDP-*N*-Acetyl-glucosamine pathway have been identified as limiting steps to keep balanced supply of nucleotide sugars both for heparosan biosynthesis and cell wall synthesis. Genetic engineering targeting these metabolic reactions has revealed the necessity of a balanced over-expression of KfiA and KfiC GTs. Since a part of the K5 polysaccharide remains linked to the cell membrane, the gene of the K5 lyase capable of breaking this linkage has been genetically modified to increase the amount of K5 released in the supernatant (Wang et al., 2011). K5 lyase gene can also be genetically engineered to control the chain length (Wang et al., 2011).

NOVEL STRATEGIES FOR CHEMICAL AND ENZYMATIC MODIFICATIONS

Overall, physical and bioactive features of polysaccharides are based on molecular chemical structure: the osidic sequence and linkages in the repeating unit, but also the substituents, influence the conformation and geometry of polysaccharide chains as well as polysaccharide-polysaccharide and polysaccharide-protein interactions (Powell et al., 2004; Pomin, 2009). The chemical structure determines the physical properties such as solubility in water or interactions with ions (Geddie and Sutherland, 1993; Kumar et al., 2007). For examples, the acetyl content in chitosan sulfate influences the inhibition of propyl endopeptidase which is involved in progressive memory deficits and cognitive dysfunctions (Je et al., 2005). The sulfate groups in heparin participate in the molecular conformation and influence the binding with calcium cations (Chevalier et al., 2004). They also have a great effect on the anticoagulant activity (Franz and Alban, 1995; Garg et al., 2002; Huang et al., 2003; Liu and Pedersen, 2007). The antiproliferative activity of the heparin molecule depends on the molecular size but not on 3-*O*-sulfo group (Garg et al., 2002) and requires both *N*-acetylation and *N*-sulfation (Longas et al., 2003). As found for heparin structure-function relationships, the amount of sulfate groups, their distribution pattern and the molecular weight are of great importance for GAG-like activities. Therefore, marine polysaccharides may be structurally modified e.g., depolymerized and (over-)sulfated to render them active or to enhance already existing activities (Chopin et al., 2014).

Chemical modifications are widely used for this purpose (Gomez D'ayala et al., 2008; Laurienzo, 2010; Senni et al., 2011): acid hydrolysis (Colliec et al., 1994), radical depolymerization (Nardella et al., 1996), *N*-deacetylation (Zou et al., 1998), sulfation (Nishino and Nagumo, 1992; Guezennec et al., 1998). However, several drawbacks such as lack of control and regioselectivity, use of organic solvents, non-homogeneous conditions are identified (Al-Horani and Desai, 2010). More recently, ionic liquids have been used for cellulose sulfation in homogeneous media (El Seoud et al., 2007; Gericke et al., 2011). Only few reports deal with chemical modifications on marine EPS (Gomez D'ayala et al., 2008). Low-molecular weight oversulfated derivatives of the EPS GY785 from the deep-sea bacterium *A. infernus* and of the EPS HE800 from *V. diabolicus* have been obtained by depolymerization by acid hydrolysis or free-radical reaction followed by sulfation with sulfur trioxide pyridine complex (Colliec-Jouault et al., 2001; Senni et al., 2011). The low-molecular weight and oversulfated derivatives thus obtained exhibit biological activities similar to heparin and other GAGs (Ruiz Velasco et al., 2011; Senni et al., 2011, 2013; Sinquin and Colliec-Jouault, 2014).

Target compound yield by chemical process is low after purification steps and sulfation lacks specificity giving undesirable by-products or uncontrolled final product chemical structure resulting in non-homogeneous products (Chopin et al., 2014). Enzymes, because of their specificity, may allow a better control of the reactions catalyzed. Moreover, enzymatic reactions are more friendly to the environment without the need of solvent or toxic chemicals.

To date, several strategies combining chemical and enzymatic methods for the synthesis of GAGs have been developed (De Angelis et al., 2013). After sugar backbone isolation or synthesis,

modifications, usually using enzymes cloned from vertebrates up to now, are performed *in vitro*.

Escherichia coli K5 polysaccharide has the same chemical structure as the biosynthetic precursor of heparin (heparosan); it has been modified by combinations of chemical and enzymatic methods and converted to "biotechnological heparin" (Kusche et al., 1991; Naggi et al., 2001; Urbinati et al., 2004; Lindahl et al., 2005). *N*-deacetylation and *N*-sulfation were achieved by chemical reactions, and C5-epimerization of glucuronic acid (GlcA) to iduronic acid (IdoA) was carried out using the enzyme epimerase. The sulfation reaction was not regioselective enough and was improved in 2005 to yield the desired 2-*O*-sulfated IdoA (Lindahl et al., 2005); at that time, even if the chemical structure of this neoheparin was still not identical to the heparin AT-binding sequence, biological properties were similar to those of heparin. Other similar chemoenzymatic processes have been patented (Zopetti et al., 2006).

The sulfation step is crucial in these biotechnological processes. Although chemical sulfation is not specific enough, only a few biotechnological processes involve sulfotransferases (STs). In particular, a selectivity toward the sulfation state of the substrate exists (De Angelis et al., 2013). Enzymatic sulfations are however limited by the need of the expensive sulfate donor 3′-phosphoadenosine 5′-phosphosulfate (PAPS). Chondroitin sulfate E has been synthesized from chondroitin sulfate A using a sulfotransferase extracted from squid cartilage (Habuchi et al., 2002). In this study, dermatan sulfate has also been oversulfated with the same enzyme. Enzymatic sulfation has been used to prepare heparan sulfate from chemically desulfated *N*-sulfated heparin (Chen et al., 2005). Using heparan sulfotransferases (2-OST, 6-OST, 3-OST) expressed in *E. coli* and immobilized, as well as PAPS regeneration system, the polysaccharide substrate was subjected to different enzymatic modifications resulting in heparan sulfates with distinct biological activities.

However, although promising, chemoenzymatic methods still need more research efforts to allow the synthesis of GAG-like macromolecules. These multiple step processes are expensive due to the PAPS cost and the production capacity remains limited for a demand in heparin of about 100 tons per year (De Angelis, 2012).

Chitin and chitosan have been extensively studied for their biotechnological applications and have been subjected to a large range of modifications to modulate their biological properties, hence determining their applications (Gomez D'ayala et al., 2008). Removal of acetyl groups (deacetylation) from chitin is the first of the studied modifications among others including carboxymethylation, sulfation, acylation (Gomez D'ayala et al., 2008).

A new alternative to chemical and enzymatic modifications to obtain targeted polysaccharide chemical structure could also be the genetic engineering. This will be feasible if more EPS structures are known together with biosynthetic genetic clusters as well as relationship between structure and bioactivity. EPS biosynthesis pathway reconstruction is feasible with genome sequence and functional annotation and it can be engineered to obtain tailor-made polymers (Schmid et al., 2014). If biosynthesis mechanisms are known, the over-expression or inhibition of targeted genes may lead to enhanced EPS production. It may also allow a better insight into protein function as well as the biosynthesis of tailored molecules with desired features in terms of molecular weight, osidic structure and functional substituents. The activity of GT GumK involved into the biosynthesis of xanthan has been modified by protein engineering; this resulted in a variation in the xanthan production yield highlighting the possibility to obtain tailor-made xanthan molecules by GT engineering (Barreras et al., 2008). Attempts to determine genes involved in the molecular weight control of diutan in *Sphingomonas* sp. were not successful (Coleman et al., 2008), suggesting that the chain length regulation is very complex whatever the biosynthesis mechanism considered (Whitfield, 2010). In *Pseudomonas aeruginosa*, the GDP-mannose dehydrogenase has been identified by over-expression studies as a key regulator protein in alginate biosynthesis (Tatnell et al., 1994).

On the other hand, genomic data would also allow the identification of new enzymatic tools to modify glycopolymers. Enzymes would become promising biotechnological tools for *in vitro* synthesis or *in vivo* biosynthesis engineering. The marine biodiversity has already shown a great potential in providing new biocatalysts (Trincone, 2010). Depolymerising enzymes can be used in *in vitro* depolymerization process but also as tools to study the chemical structure. Carbohydrate sulfotransferases and other enzymes grafting substituents such as acetate, as well as enzymes catalyzing their removal, may be used *in vitro* for binding or for elimination of substituents which are of great importance for the final bioactivity of the molecule. GTs specificity are difficult to characterize especially when they belong to the polyspecific family 4 or 2 (http://www.cazy.org/). Identifying both the polysaccharide chemical structure and the genetic cluster for the biosynthesis would allow the knowledge of the GT enzymatic function in the biosynthetic pathway by genetic knockout. It would also provide useful directions to determine enzymatic specificity and characterize the enzymes.

Recombinant production can be envisioned when polysaccharide biosynthesis cluster is known: it consists in the cloning of the entire biosynthesis cluster in an appropriate heterologous host. This is a new strategy developed together with the expansion of synthetic biology (Winter and Tang, 2012; Cameron et al., 2014; Church et al., 2014). Up to now, only less complex polymers have been studied in recombinant production such as hyaluronic acid (Chien and Lee, 2007; Cimini et al., 2011), chondroitin (Ninomiya et al., 2002) and heparosan (Roman et al., 2003).

CONCLUDING REMARKS

Natural bioactive molecules attract many interests in the search for new therapeutic drugs. Marine environment shields a high diversity of natural products and can be indeed a treasure chest for industrial and pharmaceutical purposes. Due to their functions in survival and competitiveness of marine bacteria in low nutrients and adverse environments, the EPS are ubiquitous in the marine environment.

In addition to various marine origins (animals, seaweeds, invertebrates), microorganisms provide glycopolymers with original chemical structure and promising biological activities. The

marine biotechnology has not yet reached an economically significant field but is a promising field for sustaining convenient macromolecules.

It is also assumed that new isolated bioactive molecules are also the matter for bioinspired new molecules obtained by synthesis. For this purpose, both the discovery of new natural bioactive molecules and their structural characterization are still of considerable importance to obtain well characterized molecules with truly identified mechanism of action. Basic and applied research efforts in this field require a close collaboration between biologists and chemists and expertise in marine microbiology, biochemistry, biology, chemistry and computational sciences to fulfill screenings, structural characterization, bioactivity studies and biosynthesis understanding and metabolic engineering issues. With the synthetic biology approach, a new era is available for the polysaccharide production field by simple ways.

ACKNOWLEDGMENTS

The authors acknowledge the International Cooperation in Ridge-Crest Studies Interridge (Beaulieu, 2013) for map of hydrothermal vent sites (**Figure 1**).

REFERENCES

Al-Horani, R. A., and Desai, U. R. (2010). Chemical sulfation of small molecules - advances and challenges. *Tetrahedron* 66, 2907–2918. doi: 10.1016/j.tet.2010.02.015

Anton, J., Meseguer, I., and Rodriguez-Valera, F. (1988). Production of an extracellular polysaccharide by *Haloferax mediterranei*. *Appl. Environ. Microbiol.* 54, 2381–2386.

Arias, S., Del Moral, A., Ferrer, M. R., Tallon, R., Quesada, E., and Bejar, V. (2003). Mauran, an exopolysaccharide produced by the halophilic bacterium *Halomonas maura*, with a novel composition and interesting properties for biotechnology. *Extremophiles* 7, 319–326. doi: 10.1007/s00792-003-0325-8

Ates, O., Arga, K. Y., and Oner, E. T. (2013). The stimulatory effect of mannitol on levan biosynthesis: lessons from metabolic systems analysis of *Halomonas smyrnensis* AAD6^T. *Biotechnol. Prog.* 29, 1386–1397. doi: 10.1002/btpr.1823

Ates, O., Oner, E., and Arga, K. (2011). Genome-scale reconstruction of metabolic network for a halophilic extremophile, *Chromohalobacter salexigens* DSM 3043. *BMC Syst. Biol.* 5:12. doi: 10.1186/1752-0509-5-12

Avci, F. Y., Karst, N. A., and Linhardt, R. J. (2003). Synthetic oligosaccharides as heparin-mimetics displaying anticoagulant properties. *Curr. Pharm. Des.* 9, 2323–2335. doi: 10.2174/1381612033453929

Baross, J. A., and Hoffman, S. E. (1985). Submarine hydrothermal vents and associated gradient environments as sites for the origin and evolution of life. *Origins Life Evol. B* 15, 327–345. doi: 10.1007/BF01808177

Barreras, M. X., Salinas, S. R., Abdian, P. L., Kampel, M. A., and Ielpi, L. (2008). Structure and mechanism of GumK, a membrane-associated glucuronosyltransferase. *J. Biol. Chem.* 283, 25027–25035. doi: 10.1074/jbc.M801227200

Beaulieu, S. E. (2013). *InterRidge Global Database of Active Submarine Hydrothermal Vent Fields: Prepared for InterRidge, Version 3.1*. World Wide Web electronic publication. Version 3.2. Available online at: http://vents-data.interridge.org (Accessed July 15, 2014).

Bertagnolli, C., Espindola, A. P. D. M., Kleinübing, S. J., Tasic, L., and Silva, M. G. C. D. (2014). *Sargassum filipendula* alginate from Brazil: seasonal influence and characteristics. *Carbohydr. Polym.* 111, 619–623. doi: 10.1016/j.carbpol.2014.05.024

Berteau, O., and Mulloy, B. (2003). Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology* 13, 29R–40R. doi: 10.1093/glycob/cwg058

Boltje, T. J., Buskas, T., and Boons, G.-J. (2009). Opportunities and challenges in synthetic oligosaccharide and glycoconjugate research. *Nat. Chem.* 1, 611–622. doi: 10.1038/nchem.399

Burgaud, G., Meslet-Cladière, L., Barbier, G., and Edgcomb, V. P. (2014). "Astonishing fungal diversity in deep-sea hydrothermal ecosystems: an untapped resource of biotechnological potential?" in *Outstanding Marine Molecules*, eds S. La Barre and J. M. Kornprobst (Weinheim: Wiley-VCH), 85–98. doi: 10.1002/9783527681501

Cambon-Bonavita, M. A., Raguenes, G., Jean, J., Vincent, P., and Guezennec, J. (2002). A novel polymer produced by a bacterium isolated from a deep-sea hydrothermal vent polychaete annelid. *J. Appl. Microbiol.* 93, 310–315. doi: 10.1046/j.1365-2672.2002.01689.x

Cameron, D. E., Bashor, C. J., and Collins, J. J. (2014). A brief history of synthetic biology. *Nat. Rev. Microbiol.* 12, 381–390. doi: 10.1038/nrmicro3239

Cavicchioli, R., Fegatella, F., Ostrowski, M., Eguchi, M., and Gottschal, J. (1999). Sphingomonads from marine environments. *J. Ind. Microbiol. Biotechnol.* 23, 268–272. doi: 10.1038/sj.jim.2900732

Chen, J., Avci, F. Y., Munoz, E. M., McDowell, L. M., Chen, M., Pedersen, L. C., et al. (2005). Enzymatic redesigning of biologically active heparan sulfate. *J. Biol. Chem.* 280, 42817–42825. doi: 10.1074/jbc.M504338200

Chevalier, F., Lucas, R., Angulo, J., Martin-Lomas, M., and Nieto, P. M. (2004). The heparin-Ca^{2+} interaction: the influence of the *O*-sulfation pattern on binding. *Carbohydr. Res.* 339, 975–983. doi: 10.1016/j.carres.2003.12.023

Chien, L.-J., and Lee, C.-K. (2007). Hyaluronic production by recombinant *Lactococcus lactis*. *Appl. Microbiol. Biotechnol.* 77, 339–346. doi: 10.1007/s00253-007-1153-z

Choay, J., Petitou, M., Lormeau, J. C., Sinaÿ, P., Casu, B., and Gatti, G. (1983). Structure-activity relationship in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor Xa activity. *Biochem. Biophys. Res. Commun.* 116, 492–499. doi: 10.1016/0006-291X(83)90550-8

Chopin, N., Guillory, X., Weiss, P., Le Bideau, J., and Colliec-Jouault, S. (2014). Design polysaccharides of marine origin: chemical modifications to reach advanced versatile compounds. *Curr. Org. Chem.* 18, 867–895. doi: 10.2174/1385272818071405151523334

Christensen, B. E., Kjosbakken, J., and Smidsrod, O. (1985). Partial chemical and physical characterization of two extracellular polysaccharides produced by marine, periphytic *Pseudomonas* sp. strain NCMB 2021. *Appl. Environ. Microbiol.* 50, 837–845.

Church, G. M., Elowitz, M. B., Smolke, C. D., Voigt, C. A., and Weiss, R. (2014). Realizing the potential of synthetic biology. *Nat. Rev. Mol. Cell Biol.* 15, 289–294. doi: 10.1038/nrm3767

Cimini, D., De Rosa, M., and Schiraldi, C. (2011). Production of glucuronic acid-based polysaccharides by microbial fermentation for biomedical applications. *Biotechnol. J.* 7, 237–250. doi: 10.1002/biot.201100242

Coleman, R., Patel, Y., and Harding, N. (2008). Identification and organization of genes for diutan polysaccharide synthesis from *Sphingomonas* sp. ATCC 53159. *J. Ind. Microbiol. Biotechnol.* 35, 263–274. doi: 10.1007/s10295-008-0303-3

Colliec, S., Boisson-Vidal, C., and Jozefonvicz, J. (1994). A low molecular weight fucoidan fraction from the brown seaweed *Pelvetia canaliculata*. *Phytochemistry* 35, 697–700. doi: 10.1016/S0031-9422(00)90590-9

Colliec-Jouault, S., Chevolot, L., Helley, D., Ratiskol, J., Bros, A., Sinquin, C., et al. (2001). Characterization, chemical modifications and *in vitro* anticoagulant properties of an exopolysaccharide produced by *Alteromonas infernus*. *Biochim. Biophys. Acta* 1528, 141–151. doi: 10.1016/S0304-4165(01)00185-4

Colliec-Jouault, S., and Delbarre-Ladrat, C. (2014). "Marine-derived bioactive polysaccharides from microorganisms," in *Natural Bioactive Molecules. Impacts and Prospects,* ed G. Brahmachari (New Dehli: Narosa Publishing House), 5.1–5.21.

Crowther, M. A., and Warkentin, T. E. (2008). Bleeding risk and the management of bleeding complications in patients undergoing anticoagulant therapy: focus on new anticoagulant agents. *Blood* 111, 4871–4879. doi: 10.1182/blood-2007-10-120543

De Angelis, P. L. (2012). Glycosaminoglycan polysaccharide biosynthesis and production: today and tomorrow. *Appl. Microbiol. Biotechnol.* 94, 295–305. doi: 10.1007/s00253-011-3801-6

De Angelis, P. L., Liu, J., and Linhardt, R. J. (2013). Chemoenzymatic synthesis of glycosaminoglycans: re-creating, re-modeling and re-designing nature's longest or most complex carbohydrate chains. *Glycobiology* 23, 764–777. doi: 10.1093/glycob/cwt016

Decho, A. (1990). Microbial exopolymer secretions in ocean enviroments: their role(s) in food webs and marine processes. *Oceanogr. Mar. Biol. Annu. Rev.* 28, 73–153.

Delong, E. F., Preston, C. M., Mincer, T., Rich, V., Hallam, S. J., Frigaard, N.-U., et al. (2006). Community genomics among stratified microbial assemblages in the ocean's interior. *Science* 311, 496–503. doi: 10.1126/science.1120250

Deming, J. W. (1998). Deep ocean environmental biotechnology. *Curre. Opin. Biotechnol.* 9, 283–287. doi: 10.1016/S0958-1669(98)80060-8

De Vuyst, L., De Vin, F., Vaningelgem, F., and Degeest, B. (2001). Recent developments in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. *Int. Dairy J.* 11, 687–707. doi: 10.1016/S0958-6946(01)00114-5

Donot, F., Fontana, A., Baccou, J. C., and Schorr-Galindo, S. (2012). Microbial exopolysaccharides: main examples of synthesis, excretion, genetics and extraction. *Carbohydr. Polym.* 87, 951–962. doi: 10.1016/j.carbpol.2011.08.083

Dubreucq, G., Domon, B., and Fournet, B. (1996). Structure determination of a novel uronic acid residue isolated from the exopolysaccharide produced by a bacterium originating from deep sea hydrothermal vents. *Carbohydr. Res.* 290, 175–181. doi: 10.1016/0008-6215(96)00155-3

El Seoud, O. A., Koschella, A., Fidale, L. C., Dorn, S., and Heinze, T. (2007). Applications of ionic liquids in carbohydrate chemistry: a window of opportunities. *Biomacromolecules* 8, 2629–2647. doi: 10.1021/bm070062i

Fenical, W. (1997). New pharmaceuticals from marine organisms. *Trends Biotechnol.* 15, 339–341. doi: 10.1016/S0167-7799(97)01081-0

Finore, I., Di Donato, P., Mastascusa, V., Nicolaus, B., and Poli, A. (2014). Fermentation technologies for the optimization of marine microbial exopolysaccharide production. *Mar. Drugs* 12, 3005–3024. doi: 10.3390/md12053005

Franz, G., and Alban, S. (1995). Structure-activity relationship of antithrombotic polysaccharide derivatives. *Int. J. Biol. Macromol.* 17, 311–314. doi: 10.1016/0141-8130(96)81837-X

Freitas, F., Alves, V. D., and Reis, M. A. M. (2011). Advances in bacterial exopolysaccharides: from production to biotechnological applications. *Trends Biotechnol.* 29, 388–398. doi: 10.1016/j.tibtech.2011.03.008

Galan, M. C., Jones, R. A., and Tran, A.-T. (2013). Recent developments of ionic liquids in oligosaccharide synthesis: the sweet side of ionic liquids. *Carbohydr. Res.* 375, 35–46. doi: 10.1016/j.carres.2013.04.011

Garg, H. G., Cindhuchao, N., Quinn, D. A., Hales, C. A., Thanawiroon, C., Capila, I., et al. (2002). Heparin oligosaccharide sequence and size essential for inhibition of pulmonary artery smooth muscle cell proliferation. *Carbohydr. Res.* 337, 2359–2364. doi: 10.1016/S0008-6215(02)00190-8

Geddie, J. L., and Sutherland, I. W. (1993). Uptake of metals by bacterial polysaccharides. *J. Appl. Bacteriol.* 74, 467–472. doi: 10.1111/j.1365-2672.1993.tb05155.x

Gericke, M., Liebert, T., Seoud, O. A. E., and Heinze, T. (2011). Tailored media for homogeneous cellulose chemistry: ionic liquid/co-solvent mixtures. *Macromol. Mater. Eng.* 296, 483–493. doi: 10.1002/mame.201000330

Gomez D'ayala, G., Maklinconico, M., and Laurienzo, P. (2008). Marine derived polysaccharides for biomedical applications: chemical modification approaches. *Molecules* 13, 2069–2106. doi: 10.3390/molecules13092069

Gorret, N., Maubois, J. L., Engasser, J. M., and Ghoul, M. (2001). Study of the effects of temperature, pH and yeast extract on growth and exopolysaccharides production by *Propionibacterium acidi-propionici* on milk microfiltrate using a response surface methodology. *J. Appl. Microbiol.* 90, 788–796. doi: 10.1046/j.1365-2672.2001.01310.x

Grabowski, K., Baringhaus, K.-H., and Schneider, G. (2008). Scaffold diversity of natural products: inspiration for combinatorial library design. *Nat. Prod. Rep.* 25, 892–904. doi: 10.1039/b715668p

Grice, I. D., and Wilson, J. C. (2010). "Chapter 13 - Analytical approaches towards the structural characterization of microbial wall glycopolymers," in *Microbial Glycobiology,* eds A. P. Moran, P. J. Brennan, and M. Von Itzstein (San Diego, CA: Academic Press), 233–252.

Grobben, G. J., Smith, M. R., Sikkema, J., and De Bont, J. A. M. (1996). Influence of fructose and glucose on the production of exopolysaccharides and the activities of enzymes involved in the sugar metabolism and the synthesis of sugar nucleotides in *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2772. *Appl. Microbiol. Biotechnol.* 46, 279–284. doi: 10.1007/s002530050817

Guerrini, M., Beccati, D., Shriver, Z., Naggi, A., Viswanathan, K., Bisio, A., et al. (2008). Oversulfated chondroitin sulfate is a contaminant in heparin associated with adverse clinical events. *Nat. Biotech.* 26, 669–675. doi: 10.1038/nbt1407

Guezennec, J. (2002). Deep-sea hydrothermal vents: a new source of innovative bacterial exopolysaccharides of biotechnological interest? *J. Ind. Microbiol. Biotechnol.* 29, 204–208. doi: 10.1038/sj.jim.7000298

Guézennec, J., Moppert, X., Raguénès, G., Richert, L., Costa, B., and Simon-Colin, C. (2011). Microbial mats in French Polynesia and their biotechnological applications. *Process Biochem.* 46, 16–22. doi: 10.1016/j.procbio.2010.09.001

Guezennec, J., Pignet, P., Lijour, Y., Gentric, E., Ratiskol, J., and Colliec-Jouault, S. (1998). Sulfation and depolymerization of a bacterial exopolysaccharide of hydrothermal origin. *Carbohydr. Polym.* 37, 19–24. doi: 10.1016/S0144-8617(98)00006-X

Habuchi, O., Moroi, R., and Ohtake, S. (2002). Enzymatic synthesis of chondroitin sulfate E by N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase purified from squid cartilage. *Anal. Biochem.* 310, 129–136. doi: 10.1016/S0003-2697(02)00277-4

Harding, N., Patel, Y., and Coleman, R. (2004). Organization of genes required for gellan polysaccharide biosynthesis in *Sphingomonas elodea* ATCC 31461. *J. Ind. Microbiol. Biotechnol.* 31, 70–82. doi: 10.1007/s10295-004-0118-9

Hay, I. D., Rehman, Z. U., Moradali, M. F., Wang, Y., and Rehm, B. H. A. (2013). Microbial alginate production, modification and its applications. *Microb. Biotechnol.* 6, 637–650. doi: 10.1111/1751-7915.12076

Hidalgo-Cantabrana, C., Sanchez, B., Milani, C., Ventura, M., Margolles, A., and Ruas-Madiedo, P. (2014). Genomic overview and biological functions of exopolysaccharide biosynthesis in *Bifidobacterium* spp. *Appl. Environ. Microbiol.* 80, 9–18. doi: 10.1128/aem.02977-13

Hirsh, J., Anand, S. S., Halperin, J. L., and Fuster, V. (2001). Guide to anticoagulant therapy: heparin: a statement for healthcare professionals from the American Heart Association. *Circulation* 103, 2994–3018. doi: 10.1161/01.cir.103.24.2994

Huang, R. H., Du, Y. M., Yang, J. H., and Fan, L. H. (2003). Influence of functional groups on the *in vitro* anticoagulant activity of chitosan sulfate. *Carbohydr. Res.* 338, 483–489. doi: 10.1016/S0008-6215(02)00505-0

Hugenholtz, P. (2002). Exploring prokaryotic diversity in the genomic era. *Genome Biol.* 3, reviews0003.0001–reviews0003.0008. doi: 10.1186/gb-2002-3-2-reviews0003

Imhoff, J. F., Labes, A., and Wieses, J. (2011). Bio-mining the microbial treasures of the ocean: new natural products. *Biotechnol. Adv.* 29, 468–482. doi: 10.1016/j.biotechadv.2011.03.001

Jannasch, H. W., and Taylor, C. D. (1984). Deep-sea microbiology. *Annu. Rev. Microbiol.* 38, 487–487. doi: 10.1146/annurev.mi.38.100184.002415

Je, J.-Y., Park, P.-J., and Kim, S.-K. (2005). Prolyl endopeptidase inhibitory activity of chitosan sulfates with different degree of deacetylation. *Carbohydr. Polym.* 60, 553–556. doi: 10.1016/j.carbpol.2005.03.007

Kreyenschulte, D., Krull, R., and Margaritis, A. (2014). Recent advances in microbial biopolymer production and purification. *Crit. Rev. Biotechnol.* 34, 1–15. doi: 10.3109/07388551.2012.743501

Kumar, A. S., Mody, K., and Jha, B. (2007). Bacterial polysaccharides - a perception. *J. Basic Microbiol.* 47, 103–117. doi: 10.1002/jobm.200610203

Kusaykin, M., Bakunina, I., Sova, V., Ermakova, S., Kuznetsova, T., Besednova, N., et al. (2008). Structure, biological activity, and enzymatic transformation of fucoidans from the brown seaweeds. *Biotechnol. J.* 3, 904–915. doi: 10.1002/biot.200700054

Kusche, M., Hannesson, H. H., and Lindahl, U. (1991). Biosynthesis of heparin. Use of *Escherichia coli* K5 capsular polysaccharide as a model substrate in enzymic polymer-modification reactions. *Biochem. J.* 275, 151–158.

Lahaye, M., and Robic, A. (2007). Structure and functional properties of ulvan, a polysaccharide from green seaweeds. *Biomacromolecules* 8, 1765–1774. doi: 10.1021/bm061185q

Lapaglia, C., and Hartzell, P. L. (1997). Stress-induced production of biofilm in the hyperthermophile *Archaeoglobus fulgidus*. *Appl. Environ. Microbiol.* 63, 3158–3163.

Laurienzo, P. (2010). Marine polysaccharides in pharmaceutical applications: an overview. *Mar. Drugs* 8, 2435–2465. doi: 10.3390/md8092435

Laws, A., Gu, Y., and Marshall, V. (2001). Biosynthesis, characterisation, and design of bacterial exopolysaccharides from lactic acid bacteria. *Biotechnol. Adv.* 19, 597–625. doi: 10.1016/S0734-9750(01)00084-2

Lindahl, U., Li, J. P., Kusche-Gullberg, M., Salmivirta, M., Alaranta, S., Veromaa, T., et al. (2005). Generation of "neoheparin" from *E. coli* K5 capsular polysaccharide. *J. Med. Chem.* 48, 349–352. doi: 10.1021/jm049812m

Liu, H., Zhang, Z., and Linhardt, R. J. (2009). Lessons learned from the contamination of heparin. *Nat. Prod. Rep.* 26, 313–321. doi: 10.1039/b819896a

Liu, J., and Pedersen, L. C. (2007). Anticoagulant heparan sulfate: structural specificity and biosynthesis. *Appl. Microbiol. Biotechnol.* 74, 263–272. doi: 10.1007/s00253-006-0722-x

Liu, S.-B., Chen, X.-L., He, H.-L., Zhang, X.-Y., Xie, B.-B., Yu, Y., et al. (2013). Structure and ecological roles of a novel exopolysaccharide from the Arctic Sea Ice bacterium *Pseudoalteromonas* sp. strain SM20310. *Appl. Environ. Microbiol.* 79, 224–230. doi: 10.1128/aem.01801-12

Longas, M. O., Garg, H. G., Trinkle-Pereira, J. M., and Hales, C. A. (2003). Heparin antiproliferative activity on bovine pulmonary artery smooth muscle cells requires both N-acetylation and N-sulfonation. *Carbohydr. Res.* 338, 251–256. doi: 10.1016/S0008-6215(02)00441-X

Looijesteijn, P. J., and Hugenholtz, J. (1999). Uncoupling of growth and exopolysaccharide production by *Lactococcus lactis* subsp. *cremoris* NIZO B40 and optimization of its synthesis. *J. Biosci. Bioeng.* 88, 178–182. doi: 10.1016/S1389-1723(99)80198-4

Mahapatra, S., and Banerjee, D. (2013). Fungal exopolysaccharide: production, composition and applications. *Microbiol. Insights* 6, 1–16. doi: 10.4137/mbi.s10957

Mancuso Nichols, C. A., Bowman, J. P., and Guezennec, J. (2005a). Effects of incubation temperature on growth and production of exopolysaccharides by an Antarctic Sea ice bacterium grown in batch culture. *Appl. Environ. Microbiol.* 71, 3519–3523. doi: 10.1128/AEM.71.7.3519-3523.2005

Mancuso Nichols, C. A., Garon, S., Bowman, J. P., Raguenes, G., and Guezennec, J. (2004). Production of exopolysaccharides by Antarctic marine bacterial isolates. *J. Appl. Microbiol.* 96, 1057–1066. doi: 10.1111/j.1365-2672.2004.02216.x

Mancuso Nichols, C. A., Guezennec, J., and Bowman, J. P. (2005b). Bacterial exopolysaccharides from extreme marine environments with special consideration of the Southern Ocean, sea ice, and deep-sea hydrothermal vents: a review. *Mar. Biotechnol.* 7, 253–271. doi: 10.1007/2Fs10126-004-5118-2

Martinez-Canovas, M. J., Quesada, E., Llamas, I., and Béjar, V. (2004). *Halomonas ventosae* sp. nov., a moderately halophilic, denitrifying, exopolysaccharide-producing bacterium. *Int. J. Syst. Evol. Microbiol.* 54, 733–737. doi: 10.1099/ijs.0.02942-0

Mehta, A., Sidhu, C., Pinnaka, A. K., and Roy Choudhury, A. (2014). Extracellular polysaccharide production by a novel osmotolerant marine strain of *Alteromonas macleodii* and its application towards biomineralization of silver. *PLoS ONE* 9:e98798. doi: 10.1371/journal.pone.0098798

Merritt, J. H., Ollis, A. A., Fisher, A. C., and Delisa, M. P. (2013). Glycans-by-design: engineering bacteria for the biosynthesis of complex glycans and glycoconjugates. *Biotechnol. Bioeng.* 110, 1550–1564. doi: 10.1002/bit.24885

Miroshnichenko, M., and Bonch-Osmolovskaya, E. (2006). Recent developments in the thermophilic microbiology of deep-sea hydrothermal vents. *Extremophiles* 10, 85–96. doi: 10.1007/s00792-005-0489-5

Naggi, A., Torri, G., Casu, B., Oreste, P., Zopetti, G., Li, J. P., et al. (2001). Toward a biotechnological heparin through combined chemical and enzymatic modification of the *Escherichia coli K5* polysaccharide. *Semin. Thromb. Hemost.* 27, 437–443. doi: 10.1055/s-2001-17954

Nardella, A., Chaubet, F., Boisson-Vidal, C., Blondin, C., Durand, P., and Jozefonvicz, J. (1996). Anticoagualant low molecular weight fucans produced by radical process and ion exchange chromatography of high molecular weight fucans extracted from the brown seaweed Ascophyllum nodosum. *Carbohydr. Res.* 289, 201–208. doi: 10.1016/0008-6215(96)00110-3

Nazarenko, E. L., Komandrova, N. A., Gorshkova, R. P., Tomshich, S. V., Zubkov, V. A., Kilcoyne, M., et al. (2003). Structures of polysaccharides and oligosaccharides of some Gram-negative marine Proteobacteria. *Carbohydr. Res.* 338, 2449–2457. doi: 10.1016/j.carres.2003.06.004

Nicolaus, B., Kambourova, M., and Oner, E. T. (2010). Exopolysaccharides from extremophiles: from fundamentals to biotechnology. *Environ. Technol.* 31, 1145–1158. doi: 10.1080/09593330903552094

Nicolaus, B., Manca, M. C., Ramano, I., and Lama, L. (1993). Production of an exopolysaccharide from two thermophilic archaea belonging to the genus *Sulfolobus*. *FEMS Microbiol. Lett.* 109, 203–206. doi: 10.1111/j.1574-6968.1993.tb06168.x

Ninomiya, T., Sugiura, N., Tawada, A., Sugimoto, K., Watanabe, H., and Kimata, K. (2002). Molecular cloning and characterization of chondroitin polymerase from *Escherichia coli* strain K4. *J. Biol. Chem.* 277, 21567–21575. doi: 10.1074/jbc.M201719200

Nishino, T., and Nagumo, T. (1992). Anticoagulant and antithrombin activities of oversulfated fucans. *Carbohydr. Res.* 229, 355–362. doi: 10.1016/S0008-6215(00)90581-0

Parolis, H., Parolis, L. A. S., Boan, I. F., Rodriguez-Valera, F., Widmalm, G., Manca, M. C., et al. (1996). The structure of the exopolysaccharide produced by the halophilic archaeon *Haloferax mediterranei* strain R4 (ATCC 33500). *Carbohydr. Res.* 295, 147–156. doi: 10.1016/S0008-6215(96)90134-2

Petitou, M., Duchaussoy, P., Herbert, J.-M., Duc, G., El Hajji, M., Branellec, J.-F., et al. (2002). The synthetic pentasaccharide Fondaparinux: first in the class of antithrombotic agents that selectively inhibit coagulation Factor Xa. *Semin. Thromb. Hemost.* 28, 393–402. doi: 10.1055/s-2002-34309

Poli, A., Anzelmo, G., and Nicolaus, B. (2010). Bacterial exopolysaccharides from extreme marine habitats: production, characterization and biological activities. *Mar. Drugs* 8, 1779–1802. doi: 10.3390/md8061779

Poli, A., Di Donato, P., Abbamondi, G. R., and Nicolaus, B. (2011). Synthesis, production, and biotechnological applications of exopolysaccharides and polyhydroxyalkanoates by Archaea. *Archaea* 2011:693253. doi: 10.1155/2011/693253

Poli, A., Esposito, E., Orlando, P., Lama, L., Giordano, A., De Appolonia, F., et al. (2007). *Halomonas alkaliantarctica* sp. nov., isolated from saline lake Cape Russell in Antarctica, an alkalophilic moderately halophilic, exopolysaccharide-producing bacterium. *Syst. Appl. Microbiol.* 30, 31–38. doi: 10.1016/j.syapm.2006.03.003

Poli, A., Kazak, H., Gürleyendağ, B., Tommonaro, G., Pieretti, G., Öner, E. T., et al. (2009). High level synthesis of levan by a novel *Halomonas* species growing on defined media. *Carbohydr. Polym.* 78, 651–657. doi: 10.1016/j.carbpol.2009.05.031

Pomin, V. H. (2009). Review: an overview about the structure-function relationship of marine sulfated homopolysaccharides with regular chemical structures. *Biopolymers* 91, 601–609. doi: 10.1002/bip.21200

Pomin, V. H. (2014a). Anticoagulant motifs of marine sulfated glycans. *Glycoconjugate J.* 31, 341–344. doi: 10.1007/s10719-014-9530-1

Pomin, V. H. (2014b). Marine medicinal glycomics. *Front. Cell. Infect. Microbiol.* 4:5. doi: 10.3389/fcimb.2014.00005

Powell, A. K., Yates, E. A., Fernig, D. G., and Turnbull, J. E. (2004). Interactions of heparin/heparan sulfate with proteins: appraisal of structural factors and experimental approaches. *Glycobiology* 14, 17R–30R. doi: 10.1093/glycob/cwh051

Qin, G., Zhu, L., Chen, X., Wang, P. G., and Zhang, Y. (2007). Structural characterization and ecological roles of a novel exopolysaccharide from the deep-sea psychrotolerant bacterium *Pseudoalteromonas* sp. SM9913. *Microbiology* 153, 1566–1572. doi: 10.1099/mic.0.2006/003327-0

Raguenes, G., Cambon-Bonavita, M. A., Lohier, J. F., Boisset, C., and Guezennec, J. (2003). A novel, highly viscous polysaccharide excreted by an *Alteromonas* isolated from a deep-sea hydrothermal vent shrimp. *Curr. Microbiol.* 46, 448–452. doi: 10.1007/s00284-002-3922-3

Raguenes, G., Christen, R., Guezennec, J., Pignet, P., and Barbier, G. (1997a). *Vibrio diabolicus* sp. nov., a new polysaccharide-secreting organism isolated from a deep-sea hydrothermal vent polychaete annelid, Alvinella pompejana. *Int. J. Syst. Bacteriol.* 47, 989–995. doi: 10.1099/00207713-47-4-989

Raguenes, G. H., Peres, A., Ruimy, R., Pignet, P., Christen, R., Loaec, M., et al. (1997b). *Alteromonas infernus* sp. nov., a new polysaccharide-producing bacterium isolated from a deep-sea hydrothermal vent. *J. Appl. Microbiol.* 82, 422–430. doi: 10.1046/j.1365-2672.1997.00125.x

Raguenes, G., Pignet, P., Gauthier, G., Peres, A., Christen, R., Rougeaux, H., et al. (1996). Description of a new polymer-secreting bacterium from a deep-sea hydrothermal vent, *Alteromonas macleodii* subsp. *fijiensis*, and preliminary characterization of the polymer. *Appl. Environ. Microbiol.* 62, 67–73.

Rehm, B. H. A. (2010). Bacterial polymers: biosynthesis, modifications and applications. *Nat. Rev. Microbiol.* 8, 578–592. doi: 10.1038/nrmicro2354

Rehm, B. H. A., and Valla, S. (1997). Bacterial alginates: biosynthesis and applications. *Appl. Microbiol. Biotechnol.* 48, 281–288. doi: 10.1007/s002530051051

Reid, A. N., and Whitfield, C. (2005). Functional analysis of conserved gene products involved in assembly of *Escherichia coli* capsules and exopolysaccharides: evidence for molecular recognition between Wza and Wzc for colanic acid biosynthesis. *J. Bacteriol.* 187, 5470–5481. doi: 10.1128/jb.187.15.5470-5481.2005

Remminghorst, U., and Rehm, B. (2006). Bacterial alginates: from biosynthesis to applications. *Biotechnol. Lett.* 28, 1701–1712. doi: 10.1007/2Fs10529-006-9156-x

Rinker, K. D., and Kelly, R. M. (2000). Effect of carbon and nitrogen sources on growth dynamics and exopolysaccharide production for the hyperthermophilic archaeon *Thermococcus litoralis* and bacterium *Thermotoga maritima*. *Biotechnol. Bioeng.* 69, 537–547. doi: 10.1002/1097-0290(20000905)69:5<537::aid-bit8>3.0.co;2-7

Rocha-Martin, J., Harrington, C., Dobson, A., and O'gara, F. (2014). Emerging strategies and integrated systems microbiology technologies for biodiscovery of marine bioactive compounds. *Mar. Drugs* 12, 3516–3559. doi: 10.3390/md12063516

Roger, O., Kervarec, N., Ratiskol, J., Colliec-Jouault, S., and Chevolot, L. (2004). Structural studies of the main exopolysaccharide produced by the deep-sea bacterium *Alteromonas infernus*. *Carbohydr. Res.* 339, 2371–2380. doi: 10.1016/j.carres.2004.07.021

Roman, E., Roberts, I., Lidholt, K., and Kusche-Gullberg, M. (2003). Overexpression of UDP-glucose dehydrogenase in *Escherichia coli* results in decreased biosynthesis of K5 polysaccharide. *Biochem. J.* 374, 767–772. doi: 10.1042/BJ20030365

Rougeaux, H., Guezennec, J., Carlson, R. W., Kervarec, N., Pichon, R., and Talaga, P. (1999a). Structural determination of the exopolysaccharide of *Pseudoalteromonas* strain HYD 721 isolated from a deep-sea hydrothermal vent. *Carbohydr. Res.* 315, 273–285. doi: 10.1016/S0008-6215(99)00019-1

Rougeaux, H., Kervarec, N., Pichon, R., and Guezennec, J. (1999b). Structure of the exopolysaccharide of *Vibrio diabolicus* isolated from a deep-sea hydrothermal vent. *Carbohydr. Res.* 322, 40–45. doi: 10.1016/S0008-6215(99)00214-1

Rougeaux, H., Talaga, P., Carlson, R. W., and Guezennec, J. (1998). Structural studies of an exopolysaccharide produced by *Alteromonas macleodii* subsp. *fijiensis* originating from a deep-sea hydrothermal vent. *Carbohydr. Res.* 312, 53–59. doi: 10.1016/S0008-6215(97)10061-1

Ruiz Velasco, C., Baud'huin, M., Sinquin, C., Maillasson, M., Heymann, D., Colliec-Jouault, S., et al. (2011). Effects of a sulfated exopolysaccharide produced by *Altermonas infernus* on bone biology. *Glycobiology* 21, 781–795. doi: 10.1093/glycob/cwr002

Sabra, W., and Hassan, M. (2008). Exopolysaccharide yield as a kinetic parameter for the statistical optimization of EPS production by *Klebsiella pneumoniae*. *Biotechnol. Adv.* 7, 27–34. doi: 10.3923/biotech.2008.27.34

Samain, E., Milas, M., Bozzi, L., Dubreucq, G., and Rinaudo, M. (1997). Simultaneous production of two different gel-forming exopolysaccharides by an *Alteromonas* strain originating from deep sea hydrothermal vents. *Carbohydr. Polym.* 34, 235–241. doi: 10.1016/S0144-8617(97)00129-X

Satpute, S. K., Banat, I. M., Dhakephalkar, P. K., Banpurkar, A. G., and Chopade, B. A. (2010). Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. *Biotechnol. Adv.* 28, 436–450. doi: 10.1016/j.biotechadv.2010.02.006

Schmid, J., Sperl, N., and Sieber, V. (2014). A comparison of genes involved in sphingan biosynthesis brought up to date. *Appl. Microbiol. Biotechnol.* 98, 7719–7733. doi: 10.1007/s00253-014-5940-z

Senni, K., Gueniche, F., Changotade, S., Septier, D., Sinquin, C., Ratiskol, J., et al. (2013). Unusual glycosaminoglycans from a deep sea hydrothermal bacterium improve fibrillar collagen structuring and fibroblast activities in engineered connective tissues. *Mar. Drugs* 11, 1351–1369. doi: 10.3390/md11041351

Senni, K., Pereira, J., Gueniche, F., Delbarre-Ladrat, C., Sinquin, C., Ratiskol, J., et al. (2011). Marine polysaccharides: a source of bioactive molecules for cell therapy and tissue engineering. *Mar. Drugs* 9, 1664–1681. doi: 10.3390/md9091664

Shi, X.-C., Gong, Q.-H., and Cheng, R.-B. (2008). Detection of the key enzyme of alginate biosynthesis in *Vibrio* sp. QY102. *World J. Microbiol. Biotechnol.* 24, 1613–1615. doi: 10.1007/s11274-007-9632-z

Sinquin, C., and Colliec-Jouault, S. (2014). Les polysaccharides marins et leurs applications dans le domaine de la santé. Bioprocédés dans les domaines de la santé, de l'agroalimentaire et de la chimie. *Techniques de l'Ingénieur BIO6250* 1–5. Available online at: http://www.techniques-ingenieur.fr/base-documentaire/biomedical-pharma-th15/chimie-pharmaceutique-42609210/les-polysaccharides-marins-et-leurs-applications-dans-le-domaine-de-la-sante-bio6250/

Sogin, M. L., Morrison, H. G., Huber, J. A., Welch, D. M., Huse, S. M., Neal, P. R., et al. (2006). Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proc. Natl. Acad. Sci. U.S.A.* 103, 12115–12120. doi: 10.1073/pnas.0605127103

Survase, S. A., Saudagar, P. S., Bajaj, I. B., and Singhal, R. S. (2007). Scleroglucan: fermentative production, downstream processing and applications. *Food Technol. Biotechnol.* 45, 107–118. Available online at: http://hrcak.srce.hr/index.php?show=clanak&id_clanak_jezik=42928&lang=en

Sutherland, I. W. (1982). Biosynthesis of microbial polysaccharides. *Adv. Microb. Physiol.* 23, 79–150. doi: 10.1016/S0065-2911(08)60336-7

Sutherland, I. W., Rose, A. H., and Tempest, D. W. (1972). Bacterial Exopolysaccharides. *Adv. Microb. Physiol.* 8, 143–213. doi: 10.1016/S0065-2911(08)60190-3

Tatnell, P. J., Russell, N. J., and Gacesa, P. (1994). GDP-mannose dehydrogenase is the key regulatory enzyme in alginate biosynthesis in *Pseudomonas aeruginosa*: evidence from metabolite studies. *Microbiology* 140, 1745–1754. doi: 10.1099/13500872-140-7-1745

Trincone, A. (2010). Potential biocatalysts originating from sea environments. *J. Mol. Catal. B Enzym.* 66, 241–256. doi: 10.1016/j.molcatb.2010.06.004

Tripp, H. J., Kitner, J. B., Schwalbach, M. S., Dacey, J. W. H., Wilhelm, L. J., and Giovannoni, S. J. (2008). SAR11 marine bacteria require exogenous reduced sulphur for growth. *Nature* 452, 741–744. doi: 10.1038/nature06776

Turnbull, J. E., and Field, R. A. (2007). Emerging glycomics technologies. *Nat. Chem. Biol.* 3, 74–77. doi: 10.1038/nchembio0207-74

Urbinati, C., Bugatti, A., Oreste, P., Zoppetti, G., Waltenberger, J., Mitola, S., et al. (2004). Chemically sulfated *Escherichia coli* K5 polysaccharide derivatives as extracellular HIV-1 Tat protein antagonists. *FEBS Lett.* 568, 171–177. doi: 10.1016/j.febslet.2004.05.033

Van Dover, C. L., German, C. R., Speer, K. G., Parson, L. M., and Vrijenhoek, R. C. (2002). Evolution and biogeography of deep-sea vent and seep invertebrates. *Science* 295, 1253–1257. doi: 10.1126/science.1067361

Vanfossen, A. L., Lewis, D. L., Nichols, J. D., and Kelly, R. M. (2008). Polysaccharide degradation and synthesis by extremely thermophilic anaerobes. *Ann. N.Y. Acad. Sci.* 1125, 322–337. doi: 10.1196/annals.1419.017

Vincent, P., Pignet, P., Talmont, F., Bozzi, L., Fournet, B., and Guezennec, J. (1994). Production and characterization of an exopolysaccharide excreted by a deep-sea hydrothermal vent bacterium isolated from the polychaete annelid *Alvinella pompejana*. *Appl. Environ. Microbiol.* 60, 4134–4141.

Wang, Z., Dordick, J. S., and Linhardt, R. J. (2011). *Escherichia coli* K5 heparosan fermentation and improvement by genetic engineering. *Bioeng. Bugs* 2, 63–67. doi: 10.4161/bbug.2.1.14201

Weigel, P. H., and De Angelis, P. L. (2007). Hyaluronan synthases: a decade-plus of novel glycosyltransferases. *J. Biol. Chem.* 282, 36777–36781. doi: 10.1074/jbc.R700036200

Whitfield, C. (1995). Biosynthesis of lipopolysaccharide O-antigens. *Trends Microbiol.* 3, 178–185. doi: 10.1016/S0966-842X(00)88917-9

Whitfield, C. (2006). Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. *Ann. Rev. Biochem.* 75, 39–68. doi: 10.1146/annurev.biochem.75.103004.142545

Whitfield, C. (2010). Polymerases: glycan chain-length control. *Nat. Chem. Biol.* 6, 403–404. doi: 10.1038/nchembio.376

Whitney, J. C., and Howell, P. L. (2013). Synthase-dependent exopolysaccharide secretion in Gram-negative bacteria. *Trends Microbiol.* 21, 63–72. doi: 10.1016/j.tim.2012.10.001

Wilkie, K. C. B., Jones, J. K. N., Excell, B. J., and Semple, R. E. (1957). The fractionation of polysaccharides by the method of ultrafiltration. *Rev. Can. Chim.* 35, 795–798. doi: 10.1139/v57-112

Willis, L., and Whitfield, C. (2013). Structure, biosynthesis, and function of bacterial capsular polysaccharides synthesized by ABC transporter-dependent pathways. *Carbohydr. Res.* 378, 35–44. doi: 10.1016/j.carres.2013.05.007

Winter, J. M., and Tang, Y. (2012). Synthetic biological approaches to natural product biosynthesis. *Curr. Opin. Biotechnol.* 23, 736–743. doi: 10.1016/j.copbio.2011.12.016

Wu, Q., Tun, H. M., Leung, F. C.-C., and Shah, N. P. (2014). Genomic insights into high exopolysaccharide-producing dairy starter bacterium *Streptococcus thermophilus* ASCC 1275. *Sci. Rep.* 4:4974. doi: 10.1038/srep04974

Yamazaki, M., Thorne, L., Mikolajczak, M., Armentrout, R. W., and Pollock, T. J. (1996). Linkage of genes essential for synthesis of a polysaccharide capsule in *Sphingomonas* strain S88. *J. Bacteriol.* 178, 2676–2687.

Ye, L., Zheng, X., and Zheng, H. (2014). Effect of *sypQ* gene on poly-*N*-acetylglucosamine biosynthesis in *Vibrio parahaemolyticus* and its role in infection process. *Glycobiology* 24, 351–358. doi: 10.1093/glycob/cwu001

Zobell, C. E., and Morita, R. Y. (1959). Deep-sea bacteria. *Galathea Rep.* 1, 139–154.

Zopetti, G., Oreste, P., and Cipolletti, G. (2006). *Glycosaminoglycans derived from the K5 polysaccharide having high anticoagulant and anti-thrombotic activity and process for their preparation*. U.S. Patent No 2006281152.

Zou, W., Laferriere, C. A., and Jennings, H. J. (1998). Oligosaccharide fragments of the type III group B streptococcal polysaccharide derived from S. *pneumoniae* type 14 capsular polysaccharide by a chemoenzymatic method. *Carbohydr. Res.* 309, 297–301. doi: 10.1016/S0008-6215(98)00134-7

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 July 2014; accepted: 20 September 2014; published online: 08 October 2014.

Citation: Delbarre-Ladrat C, Sinquin C, Lebellenger L, Zykwinska A and Colliec-Jouault S (2014) Exopolysaccharides produced by marine bacteria and their applications as glycosaminoglycan-like molecules. Front. Chem. ***2****:85. doi: 10.3389/fchem.2014.00085*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

ORIGINAL RESEARCH ARTICLE
published: 13 October 2014
doi: 10.3389/fchem.2014.00089

Stereochemical course of hydrolytic reaction catalyzed by alpha-galactosidase from cold adaptable marine bacterium of genus *Pseudoalteromonas*

Irina Y. Bakunina*, Larissa A. Balabanova, Vasiliy A. Golotin, Lyubov V. Slepchenko, Vladimir V. Isakov and Valeriy A. Rasskazov

G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far Eastern Branch, Russian Academy of Sciences, Vladivostok, Russia

Edited by:
Ali Tavassoli, University of Southampton, UK

Reviewed by:
Rahman M. Mizanur, US Army Medical Research Institute of Infectious Diseases, USA
Rui Zhao, Chinese Academy of Sciences, China

****Correspondence:***
Irina Y. Bakunina, Laboratory of Enzyme Chemistry, G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far Eastern Branch, Russian Academy of Sciences, Prospect 100 let Vladivostoku, 159, Vladivostok 690022, Russia
e-mail: bakun@list.ru

The recombinant α-galactosidase of the marine bacterium (α-*Ps*Gal) was synthesized with the use of the plasmid 40Gal, consisting of plasmid pET-40b (+) (Novagen) and the gene corresponding to the open reading frame of the mature α-galactosidase of marine bacterium *Pseudoalteromonas* sp. KMM 701, transformed into the *Escherichia coli* Rosetta(DE3) cells. In order to understand the mechanism of action, the stereochemistry of hydrolysis of 4-nitrophenyl α-D-galactopyranoside (4-NPGP) by α-*Ps*Gal was measured by ^{1}H NMR spectroscopy. The kinetics of formation of α- and β-anomer of galactose showed that α-anomer initially formed and accumulated, and then an appreciable amount of β-anomer appeared as a result of mutarotation. The data clearly show that the enzymatic hydrolysis of 4-NPGP proceeds with the retention of anomeric configuration, probably, due to a double displacement mechanism of reaction.

Keywords: Recombinant retaining α-galactosidase, marine bacterium, ^{1}H NMR spectroscopy

INTRODUCTION

α-Galactosidase (α-D-galactoside galactohydrolase; EC 3.2.1.22) catalyzes the hydrolysis of terminal α-linked galactose residues in oligosaccharides and polymeric galactomannans. They have found a number of useful potential biotechnological and medical applications. An interesting application of α-galactosidase in biomedicine is the processing of B-red blood cells for conversion into universal O-red blood cells (Olsson et al., 2004). In medicine, it plays a crucial role in the treatment of Fabry's disease (Breunig et al., 2003) and for overcoming xenorejection for xenotransplantation (Ezzelarab and Cooper, 2005). α-Galactosidases are used in the food and feed industry to improve the quality of products containing oligosaccharides of raffinose series (Anisha and Prema, 2008).

α-Galactosidases are wide distributed in marine bacteria, especially among γ-proteobacteria (Ivanova et al., 1998) and *Bacteroidetes* (Bakunina et al., 2012), however, an α-galactosidase isolated from the marine bacterium *Pseudoalteromonas* sp. KMM 701 was the first biochemicaly characterized marine α-galactosidase. It catalyzes the hydrolysis of α-galactose residues from non-reducing end of B-trisaccharides and is capable for reducing a serological activity of B-red blood cells at neutral pH. Furthermore, the α-galactosidase is able to interrupt the adhesion of pathogens to human buccal epithelium (Balabanova et al., 2010). These properties showed the great therapeutic potential and opened up broad prospects for application of the enzyme in medicine. The α-galactosidase gene was isolated (GenBank DQ530422) and the amino acid sequence was reconstructed (UniProt Q19AX0). The marine bacterium *Pseudoalteromonas* sp. KMM 701 α-galactosidase has been found to belong to GH36 family in CAZy classification, according to its amino acids sequence (Balabanova et al., 2010).

The question of stereochemical outcome of enzymatic hydrolysis of glycosides is principal in the understanding of a catalytic mechanism and in classification of glycoside hydrolases. ^{1}H NMR spectroscopy is a direct way for determining the stereochemical configuration of the product anomers.

The paper aims to produce the recombinant α-galactosidase of marine bacterium *Pseudoalteromonas* sp. KMM 701 (α-*Ps*Gal) and investigate stereochemistry of the glycoside hydrolysis for understanding the enzymatic mechanism of action.

MATERIALS AND METHODS

RCOMBINANT α-GALACTOSIDASE CONSTRUCTION

For the α-*Ps*Gal gene amplification, Encyclo Taq Polymerase (Eurogene), genomic DNA of the marine bacterium *Pseudoalteromonas* sp. KMM 701 and the gene-specific the upstream primer, G2-NcoI-for40: 5′-ATTACCATGGATGACG ACGACAAGGCCGACACTAAATCATTTTATCGATTAGACA-3′, and the downstream primer, G3-SalI-rev40: 5′-ACACGTCGAC TTACGCTTTGTTGAGCTCAAATATAAGC-3′ were used. The resultant PCR products were purified with agarose gel (Helicon). PCR was carried out in automatic amplifier "Eppendorf." Restriction endonucleases and T4 DNA ligase were purchased from "Thermo Scientific." The restricted PCR product and plasmid pET-40b (+) were purified from agarose gel with the use

of a "Qiagen" column. The recombinant insert of the resultant plasmid 40Gal was sequenced using the automated PE/ABI 310 DNA sequencer and the PE/ABI-ABI PRISM BigDye Terminator cycle sequencing Ready Reaction Kit (PE Applied Biosystems). Preparation of *Escherichia coli* competent cells and heat shock transformation were carried out according to the standard methods (Sambrook et al., 1989).

For producing α-*Ps*Gal of marine bacterium *Pseudoalteromonas* sp. KMM 701 the recombinant plasmid 40Gal was transformed in to the *E. coli* Rosetta(DE3) cells. The obtained recombinant cells were grown on LB plate containing 25 μg/mL of kanamycin overnight at 37°C. A single colony was picked and grown at 200 rpt in 20 mL of LB, with 25 μg/mL of kanamycin at 37°C for 12 h. Overnight culture was transferred to 1 L of fresh LB with 25 μg/mL of kanamycin. When the cell density reached an OD_{600} of 0.6–0.8, 0.2 mM IPTG was added to induce the expression and the incubation was continued at 16°C up to 12 h at 200 rpt. The *E. coli* Rosetta(DE3) cells were transformed with the pET-40b (+) plasmid as a control.

RECOMBINANT α-GALACTOSIDASE PURIFICATION

All purification steps were carried out at +6°C. After harvesting, the transgenic *E. coli* Rosetta (DE3)/40Gal cells were resuspended in 200 mL of buffer A (0.01 M NaH_2PO_4, pH 7.7, 0.01% NaN_3) and sonificated by ultrasonic treatment, then centrifuged at 10,000 g for 30 min. The supernatant was applied to a column (2.5 × 30 cm) of Macro-Prep (BioRad). Elution of the protein was performed with the use a linear gradient of NaCl concentration (0.05–0.3 M) in the buffer A. The enzymatic active fractions were collected and applied to a column (1 × 2.5 cm) of Ni-NTA agarose (Qiagen). Elution of the protein was carried out in buffer B (0.01 M NaH_2PO_4, pH 7.7, 0.01% NaN_3, 0.04 M EDTA). The active fractions were collected and desalted with the use of Bio-Scale™ Mini Macro-Prep® High Q 1 mL cartridge, then incubated with enterokinase (Invitrogen) at 21°C for 15 h to cleave N-terminal plasmid overhang from the chimeric protein (32.5 kDa). Then, the protein solution is applied to a gel filtration column (1.5 × 170 cm) of Superdex 200 (Sigma) equilibrated with the buffer C (0.01 M NaH_2PO_4, pH 7.7, 0.01% NaN_3, 0.1 M NaCl). All purification steps were examined by SDS-PAGE. The concentration of the protein was determined according to Bredford (1976). The obtained recombinant polypeptide is determined by the first 10 amino acids on an automatic sequencer Procise model 492 (Applied Biosystems, USA). All biochemicals and reagents were from "Thermo scientific" and "Sigma-Aldrich."

ENZYME ESSAY

Enzyme activity of the α-*Ps*Gal was determined with 3.3 mM of 4-NPGP in 50 mM sodium phosphate buffer pH 7.5, at 20°C. One unit of the α-galactosidase activity was defined as amount of the enzyme that hydrolyzed 1 μmol of 4-NPGP per min. Total quantity of p-nitrophenol was determined spectrophotometrically at 400 nm ($\varepsilon_{400} = 18300\ M^{-1}\ cm^{-1}$).

MOLECULAR MASS DETERMINATION

The molecular size of the active α-*Ps*Gal after treatment of enterokinase was determined by gel filtration on the column of Superdex 200 (Sigma) (1.5 × 170 cm) in the buffer A at a flow of 0.16 mL/min at 6°C and calibrated using Bio-Rad standard molecular weight markers: Thyroglobulin bovine (670 kDa), γ-globulin bovine (158 kDa), Ovalbumin chicken (44 kDa), Mioglobin horse (17 kDa), Vitamin B_{12} (1.35 kDa). The molecular mass of the enzyme was determined by 12.5 % polyacrylamide gel electrophoresis (PAGE). The protein preparation was mixed with Laemmli sample buffer with heat treatment at 95°C, and then applied to PAGE. The SDS-PAGE gels were stained according to Laemmli (1970).

^{1}H NMR SPECTRA RECORDING AND ANALYSIS

Determination of the anomeric center configuration of the product of the hydrolysis reaction was monitored by ^{1}H NMR spectroscopy. The experiment was performed at 20°C with the use of NMR DRX-500 spectrometer (Bruker, Germany). ^{1}H NMR spectra were acquired over 32,000 data points using a spectral width 5000 Hz.

Prior to analysis by NMR, 0.6 mL 50 mM sodium phosphate buffer, pH 7.5, and substrate were dried on rotary evaporator and dissolved in 0.6 mL D_2O. The deuterium-exchanged α-*Ps*Gal was obtained on vivaspin turbo 10 K MWCO (Sartorius, Germany). Chemical shifts were measured relative to an external standard—acetone (trace) in D_2O. CH_3—signal acetone was set to $\delta = 2.22$ ppm. After recording of the original spectrum at $\tau = 0$ min 6.0 mmol of the deuterium-exchanged p-nitrophenyl galactopyranoside in 0.6 mL of D_2O, 0.1 mL of the deuterium-exchanged α-*Ps*Gal containing 1.14 U was added for initiation of the reaction. ^{1}H NMR spectra were recorded automatically at intervals of 3 min for 60 min from the start of the reaction.

^{1}H NMR anomer signals (αH-1) of α-4-NPGP, α-galactopyranose, and (βH-1) of β-galactopyranose, as well as proton signals of free 4-nitrophenol ring were analyzed and integrated by the standard software "TopSpin 3.2." Integral intensities of signals of each anomer were calculated as a percentage of the total integral intensities of all signals of anomers and were plotted depend on time. The degree of substrate hydrolysis (4-NPGP) was defined as a percent ratio of the ^{1}H NMR integral intensities of proton signals of free 4-nitrophenol ring at 8.109 ppm to proton signals of 4-NPGP at 8.275 ppm and also was plotted depend on time.

RESULTS

RECOMBINANT α-GALACTOSIDASE PRODUCTION AND CHARACTERIZATION

The recombinant plasmid 40Gal of 8306 base pairs (bp) for the synthesis of the recombinant protein α-*Ps*Gal consisted of the NcoI/SalI-fragment of plasmid pET-40b (+) (Novagen) and the gene of 2130 bp corresponding to the open reading frame of the mature α-galactosidase of marine bacterium *Pseudoalteromonas* sp. KMM 701 (**Figure 1**).

The IPTG concentration of 0.2 mM, strain cultivation temperature of 16°C and duration of the cultivation of 12 h at 200 rpt were optimal conditions for the α-*Ps*Gal expression. The induced α-*Ps*Gal expression in 1 L culture of *E.coli* Rosetta(DE3)/40Gal cells yielded up to 10 mg of the functionally active protein with

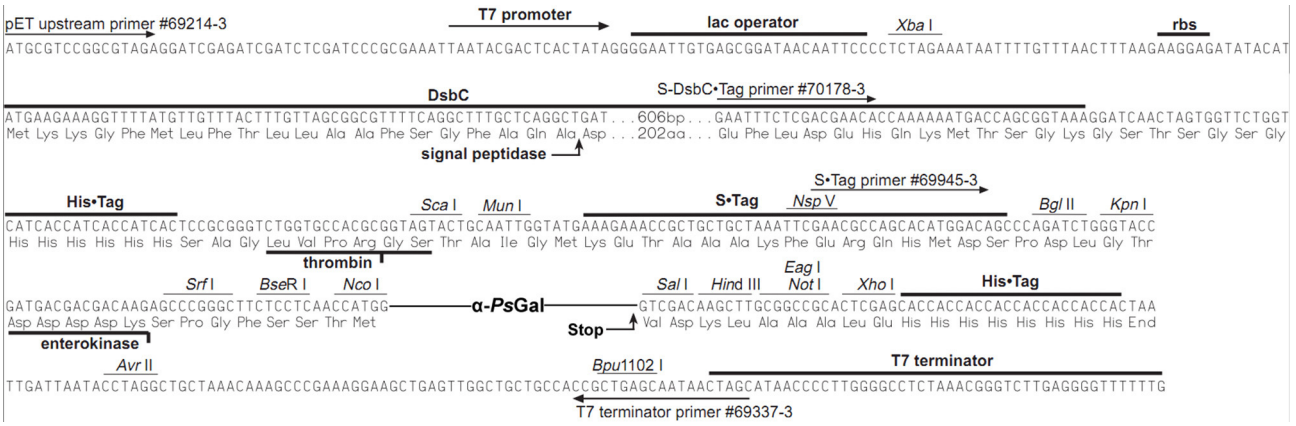


FIGURE 1 | The nucleotide and amino acid sequences of the α-*Ps*Gal expression region.

the specific activity 160 U/mg at the final purification step. α-*Ps*Gal has been found to provide the complete inactivation of serological activity of B-red blood cells and its conversion into the O-red blood cells in the same condition as for the native enzyme (data not shown).

PAGE-SDS showed a single band of the purified recombinant protein with a molecular mass of approximately 80 kDa corresponding to the one subunit of α-*Ps*Gal (**Figure 2**).

The molecular mass of the mature protein determined by gel filtration was to be 160 kDa, suggesting the two-subunit molecular organization of the active α-*Ps*Gal.

^{1}H NMR SPECTRA RECORDING AND ANALYSIS

^{1}H NMR spectra of deuterium-exchanged 4-NPGP and products of it's hydrolysis under the action of the recombinant α-*Ps*Gal are shown on **Figure 3**.

In ^{1}H NMR spectrum 4-NPGP (**Figure 3**, spectrum 1, formula of the compound is shown on the right) one signal was a doublet at 5.84 ppm [$J_{(1,\ 2)} = 3.43$ Hz] and two groups signals at 8.28 and 7.31 ppm corresponded to αH-1 anomer and 4-nitrophenol ring protons in 4-NPGP, respectively. Moreover, signals in region of resonance from 4.13 to 3.96 ppm are observed. These signals corresponded to H-2, H-3, H-4, H-5, and H-6 protons of α-galactopyranose in 4-NPGP (**Figure 3**, spectrum 1).

After addition of the enzyme, the doublet signal was appeared at 5.26 ppm [$J_{(1,\ 2)} = 3.8$ Hz] during the first 3 min (**Figure 3**, spectrum 2, reaction equation is shown on the right). The doublet was identical in chemical shift to the αH-1 resonance of free α-D-galactopyranose (Angyal and Pickles, 1972). Signals arising at 8.11 and 6.68 ppm related to protons of free 4-nitrophenol formed through the reaction. Decrease in the amplitude of signals at 8.28 and 7.31 ppm indicates disappearance the substrate. A doublet signal at 4.57 ppm [$J_{(1,\ 2)} = 7.8$ Hz], which was identical in chemical shift to the H-1 resonance of free β-D-galactopyranose (βH-1) (Angyal and Pickles, 1972), appeared after 10 min of the reaction in accordance with the time of spontaneous mutarotation (**Figure 3**, spectrum 4). Signals of protons H-2, H-3, H-4, H-5, and H-6 of free α- and β-D-galactopyranose occurred at 3.40–4.20 ppm, minor signals at

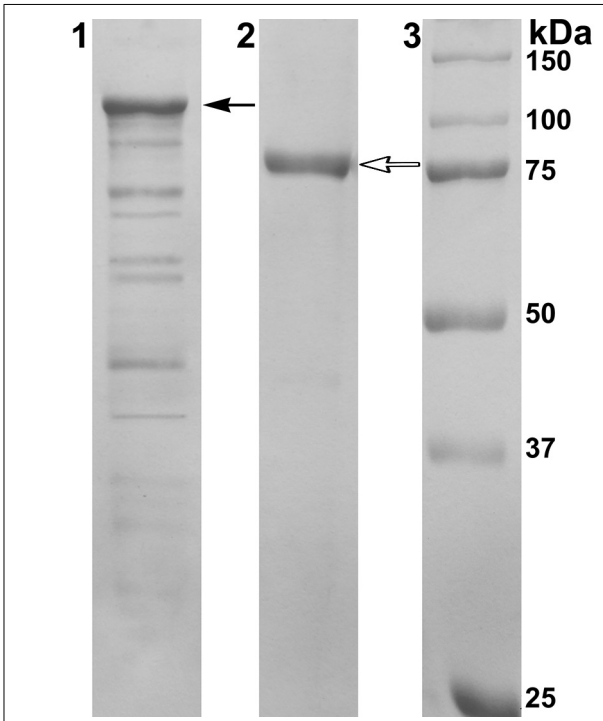


FIGURE 2 | Results of 12.5% SDS-PAGE electrophoresis. Line 1: Cell extract of *E. coli* Rosetta(DE3)/40Gal; Line 2: **α**-*Ps*Gal after final purification step; Line 3: MW-standards. Filled arrow indicates the band corresponding to the chimeric protein with DsbC overhang (112.5 kDa); empty arrow indicates the band corresponding to the mature protein after enterokinase treatment (80 kDa).

5.21 ppm [$J_{(1,\ 2)} = 3.30$] rising at late time of reaction corresponded to β-D-galactofuranose (Angyal and Pickles, 1972) (**Figure 3**, spectra 4 and 5, equation of equilibrium of D-galactose is shown on the right Zhu et al., 2001). Signals in the anomeric region, which could indicate the emergence of new products of the reaction or formation of O-glycosidic bond in the spectra were not found.

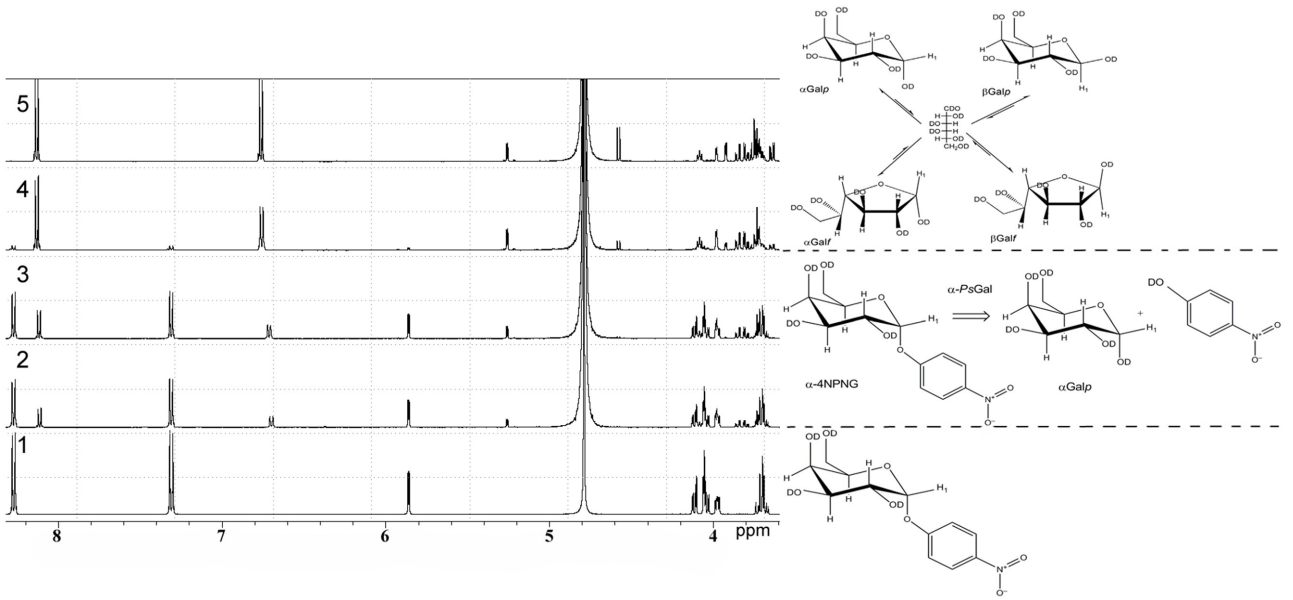


FIGURE 3 | ^{1}H NMR analysis of the stereo selectivity for the recombinant α-*Ps*Gal catalyzed reaction. One-dimensional ^{1}H NMR spectra of the deuterium-exchanged 4-NPGP prior to enzyme addition, at $\tau = 0$ min (spectrum 1), at $\tau = 3$ min (spectrum 2); at $\tau = 6$ min (spectrum 3); at $\tau = 24$ min (spectrum 4); at $\tau = 60$ min (spectrum 5) after initiation of the reaction. (in 50 mM sodium phosphate buffer pH 7.5, at 20°C, D_2O).

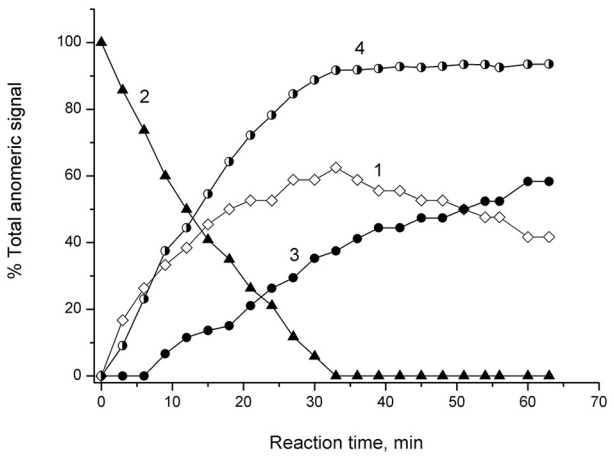


FIGURE 4 | ^{1}H NMR Monitoring of reaction of α-4-NPGP hydrolysis catalyzed by α-*Ps*Gal in 50 mM sodium phosphate buffer pH 7.5, at 20°C, D_2O. Experimental time-dependent changes in integral intensity of anomer signals. Curve 1: αH-1 of reaction product of galatopyranose at 5.26 ppm; Curve 2: αH-1 of 4-NPGP at 5.84 ppm; Curve 3: βH-1 mutarotation product of α-galatopyranose at 4.57 ppm; Curve 4: shows the change in the degree of hydrolysis of the substrate vs. time.

NMR spectroscopic monitoring of the hydrolysis of 4-NPGP by α-*Ps*Gal is shown on **Figure 4**.

Signal at 5.26 ppm the α-D-galactopyranose αH-1 appeared at the first minutes after the addition of α-*Ps*Gal (**Figure 4**, curve 1). Disappearance of doublet at 5.84 ppm of αH-1 of 4-NPGP (**Figure 4**, curve 2) simultaneously with appearance of the signal at 5.26 ppm of αH-1 of α-D-galactopyranose increasing in intensity (**Figure 4**, curve 1) were observed within next 30 min. On curve of the time-dependent changes in signal amplitude of βH-1 at 4.57 ppm lag-period were observed during 9 min (**Figure 4**, curve 3), when degree of the substrate hydrolysis reached up to ∼30% (**Figure 4**, curve 4). After 33 min, the degree of substrate hydrolysis reached up to 98–100% (**Figure 4**, curve 4). After 60 min, the ratio of integral intensities of αH-1 and βH-1 signal was 40:60. Typical equilibrium ratio 31:69 between αH-1 and βH-1 D-galactopyranose anomers (Angyal and Pickles, 1972; Zhu et al., 2001) was formed for 180 min from the start of the reaction in experimental conditions pH 7.5 and 20°C.

DISCUSSION

Marine bacteria have been found to be a good source for α-galatosidases, which are stable at low temperatures and hence find use in medical and food industry applications. These enzymes appear to be also distinct from their terrestrial counterparts in substrate preference and catalytic efficiency. The first example was the enzyme from the marine bacterium *Pseudoalteromonas* sp. KMM 701 that displayed a good stability at low temperatures (20°C for 24 h) and neutral pH (pH 6.7–7.7) and was 4-fold more efficient than the α-galactosidase from green coffee beans for the B-red blood cells conversion into O-red blood cells (Bakunina et al., 1998). Furthermore, a single cold-active α-galactosidase isolated from the Antarctic *Bacillus* sp. LX-1 was reported to be a promising biocatalyst for soybean processing in the food and feed industry (Lee et al., 2012).

Here, we have firstly developed a method for the production of the completely soluble highly active recombinant α-galactosidase of the psychrotrophic marine bacterium *Pseudoalteromonas* sp. KMM 701 (α-*Ps*Gal) in *E. coli*. The use of *E. coli* signal peptide

included in the expression plasmid pET40b(+) resulted in overproduction of α-*Ps*Gal. It should be noted that the step of the hybrid protein 6xHis-Dsb-α-*Ps*Gal incubation with enterokinase could be optionally due to the absence of any effect of 32.5 kDa plasmid fusion protein including N-terminal His-tag on the α-*Ps*Gal activity (**Figures 1**, **2** plasmid scheme and electrophoregram, respectively).

The configuration of the anomeric center of galactose, which was a reaction product of the of 4-NPGP hydrolysed of by α-*Ps*Gal, was monitored by ^{1}H NMR spectroscopy. Upon occurrence of signals of anomeric protons at the initial time of reaction one can be appreciate stereochemical configuration of the anomeric center of the formed product. In ^{1}H NMR spectra of the initial time of the reaction the resonance of the α-anomeric center of galactose is clearly observed (**Figure 3**). β-Anomeric as a result of galactose mutarotation appeared only after 10 min from the beginning of hydrolysis reaction of 4-NPGP under the action of α-*Ps*Gal (**Figure 4**). Mutarotation proceeded until the anomer ratio of 40% α- and 60% β-galactopyranose was established after 60 min, when the reaction was stopped (**Figure 4**). These observations indicate that the primary product must be α-D-galactopyranose, and the enzyme acts on a mechanism leading to retaining of the anomer configuration of the substrate. Based on these data, we conclude that α-*Ps*Gal catalyzes the hydrolysis of glycosidic bound with double displacement mechanism. This mechanism involves two catalytic residues; one responsible for the protonation of the glycosidic oxygen, and the other for stabilization of a carbocationic intermediate. The glycosyl-enzyme intermediate is decomposed by the transfer of a glycosyl moiety to an acceptor molecule, which is in the case of hydrolysis a molecule of water (Sinnott, 1990). The absence of signals corresponding to the anomeric protons involved in the formation of O-glycosidic bonds indicates the lack of transglycosylation in the conditions of this experiment. It is known that initiation of transglycosylation reaction of galactosidases requires high concentrations of substrates (donors and acceptors) and enzymes, low temperatures and the presence of special solvents (Matsuo et al., 1997; Nieder et al., 2003; Goulas et al., 2009).

Considering the postulation that glycoside hydrolases grouped into the same family shared a common catalytic mechanism (Henrissat and Bairoch, 1993, 1996), all enzymes of GH36 family are retaining glycoside hydrolases. At first the technique of ^{1}H NMR experiments was applied for investigation of GH36 enzyme reaction stereochemistry of α-galactosidase from thermopile bacterium *Thermotoga maritima* (Comfort et al., 2007). It was unequivocally shown, that α-galactosidase from *T. maritima* was a retaining glycoside hydrolase. However, this thermophile enzyme has low identity (19.1%) of amino acids sequence with psychrophillic marine *Pseudoalteromonas* sp. KMM 701 (Balabanova et al., 2006). The related catalytic mechanism was shown for GH27 α-galactosidases from *Streptomyces griseoalbus* (Anisha et al., 2011), fungus *Thermomyces lanuginosus* (Puchart et al., 2000), *Phanerochaete chrysosporium* (Brumer et al., 1999), *Trichoderma reesei* (Shabalin et al., 2002). Catalytic mechanism of human α-galactosidase was also studied with ^{1}H NMR experiments (Guce et al., 2010).

^{1}H NMR experiments were successfully applied for direct detecting of catalytic mechanism of novel inverting GH110 family α-galactosidase from *Bacteroides fragilis* (Liu et al., 2008), as well as for detecting a retaining and inverting properties of GH97 α-galactosidase and α-glucosidase from *Bacteroides thetaiotaomicron* (Gloster et al., 2008).

CONCLUSION

For the first time the completely soluble and functional recombinant cold-active α-galactosidase α-*Ps*Gal of marine bacterium was successfully produced in *E. coli* with a high level of yield and specific activity. Based on the results of ^{1}H NMR experiments, it was demonstrated that α-*Ps*Gal catalyzes the hydrolysis of substrate with retaining of the anomeric configuration. It is evident that the marine α-galactosidase catalyzes the substrate hydrolyses with double displacement mechanism as all classical glycoside hydrolases of GH36 family of clan-D GH (Comfort et al., 2007; Cantarel et al., 2009).

ACKNOWLEDGMENT

This work was supported by grants from RFBR 13-04-00806, FEB RAS, and the RAS Presidium under the project "Molecular and Cellular Biology" 12-II6-10.

REFERENCES

Angyal, S. J., and Pickles, V. A. (1972). Equilibria between pyranoses and furanoses. II. *Aust. J. Chem.* 25, 1695–1710. doi: 10.1071/CH9721695

Anisha, G. S., John, R. P., and Prema, P. (2011). Substrate specificities and mechanism of action of multiple α-galactosidases from *Streptomyces griseoloalbus*. *Food Chem.* 124, 349–353. doi: 10.1016/j.foodchem.2010.06.001

Anisha, G. S., and Prema, P. (2008). Reduction of non-digestible oligosaccharides in horse gram and green gram flours using crude α-galactosidase from *Streptomyces griseoloalbus*. *Food Chem.* 106, 1175–1179. doi: 10.1016/j.foodchem.2007.07.058

Bakunina, I. Y., Nedashkovskaya, O. I., Kim, S. B., Zvyagintseva, T. N., and Mikhailov, V. V. (2012). Diversity of glycosidase activities in the bacteria of the phylum *bacteroidetes* isolated from marine algae. *Microbiology* 81, 688–695. doi: 10.1134/S0026261712060033

Bakunina, I. Y., Sova, V. V., Nedashkovskaya, O. I., Kuhlmann, R. A., Likhosherstov, L. M., Martynova, M. D., et al. (1998). α-galactosidase of the marine bacterium *Pseudoalteromonas* sp. KMM 701. *Biochemistry (Mosc)* 63, 1209–1215.

Balabanova, L. A., Bakunina, I. Y., Nedashkovskaya, O. I., Makarenkova, I. D., Zaporozhets, T. S., Besednova, N. N., et al. (2010). Molecular characterization and therapeutic potential of a marine bacterium *Pseudoalteromonas* sp. KMM 701 α-galactosidase. *Mar. Biotechnol.* 12, 111–120. doi: 10.1007/s10126-009-9205-2

Balabanova, L. A., Likshatskaya, G. N., Bakunina, I. Y., Petruk, S. V., Kozhemyako, V. B., and Rasskazov, V. A. (2006). Primary structure and homology modeling of the novel α-galactosidase from marine bacterium. *FEBS J.* 273, 262–263. doi: 10.1111/j.1742-4658.2006.05277.x

Bredford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3

Breunig, F., Knoll, A., and Wanner, C. (2003). Enzyme replacement therapy in Fabry disease: clinical implications. *Curr. Opin. Nephrol. Hypertens.* 12, 491–495. doi: 10.1097/00041552-200309000-00002

Brumer, H., Sims, P. F. G., and Sinnott, M. L. (1999). Lignocellulose degradation by *Phanerochaete chrysosporium*: purification and characterization of the main α-galactosidase. *Biochem. J.* 339, 43–53. doi: 10.1042/0264-6021:3390043

Cantarel, B. L., Coutinho, P. M., Rancurel, C., Bernard, T., Lombard, V., and Henrissat, B. (2009). The carbohydrate-active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res.* 37, D233–D238. doi: 10.1093/nar/gkn663

Comfort, D. A., Bobrov, K. S., Ivanen, D. R., Shabalin, K. A., Harris, J. M., Kulminskaya, A. A., et al. (2007). Biochemical analysis of *Thermotoga maritima* GH-36 α-galactosidase (*Tm*GalA) confirms the mechanistic commonality of clan GH-D glycoside hydrolases. *Biochemistry* 46, 3319–3330. doi: 10.1021/bi061521n

Ezzelarab, M., and Cooper, D. K. C. (2005). Reducing Gal expression on the pig organ – a retrospective review. *Xenotransplantation* 12, 278–285. doi: 10.1111/j.1399-3089.2005.00236.x

Gloster, T. M., Turkenburg, J. P., Potts, J. R., Henrissat, B., and Davies, G. J. (2008). Divergence of catalytic mechanism within a glycosidase family provides insight into evolution of carbohydrate metabolism by human gut flora. *Chem. Biol.* 15, 1058–1067. doi: 10.1016/j.chembiol.2008.09.005

Goulas, T., Goulas, A., Tzortzis, G., and Gibson, G. R. (2009). A novel α-galactosidase from B*ifidobacterium bifidum* with transgalactosylating properties: gene molecular cloning and heterologous expression. *Appl. Microbiol. Biotechnol.* 82, 471–477. doi: 10.1007/s00253-008-1750-5

Guce, A. I., Clark, N. E., Salgado, E. N., Ivanen, D. R., Kulminskaya, A. A., Brumer, H., et al. (2010). Catalytic mechanism of human α-galactosidase. *J. Biol. Chem.* 285, 3625–3632. doi: 10.1074/jbc.M109.060145

Henrissat, B., and Bairoch, A. (1993). New families in the classification of glycosyl hydrolases based on amino-acid sequence similarities. *Biochem. J.* 293, 781–788.

Henrissat, B., and Bairoch, A. (1996). Updating the sequence-based classification of glycosyl hydrolases. *Biochem. J.* 316, 695–696.

Ivanova, E. P., Bakunina, I. Y., Nedashkovskaya, O. I., Gorshkova, N. M., Mikhailov, V. V., and Elyakova, L. A. (1998). Incidence of marine microorganisms producing β-N-acetylglucosaminidases, α-galactosidases and α-N-acetylgalactosaminidases. *Russ. J. Mar. Biol.* 24, 365–372.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685. doi: 10.1038/227680a0

Lee, J., Park, I., and Cho, J. (2012). Production and partial characterization of α-galactosidase activity from an Antarctic bacterial isolate, *Bacillus* sp. LX-1. *Afr. J. Biotechnol.* 11, 12396–12405. doi: 10.5897/AJB12.1219

Liu, Q. P., Yuan, H., Bennett, E. P., Levery, S. B., Nudelman, E., Spence, J., et al. (2008). Identification of a GH110 Subfamily of α-1,3-galactosidases novel enzymes for removal of the α3Gal xenotransplantation antigen. *J. Biol. Chem.* 283, 8545–8554. doi: 10.1074/jbc.M709020200

Matsuo, I., Fujimoto, H., Isomura, M., and Katsumi, A. (1997). Chemoenzymatic synthesis of Gal-α–1,3-gal, Gal-α-1,3-β1,4GlcNAc, and their PEG-conjugates. *Bioorg. Med. Chem.* 7, 255–258. doi: 10.1016/S0960-894X(97)00017-6

Nieder, V., Marx, S. P., Gutiérrez Gallego, R., Kamerling, J. P., Johannes, F. G., Vliegenthart, J. F. G., et al. (2003). Synthesis of nucleotide-activated disaccharides with β-galactosidase from *Bacillus circulans* and α-galactosidase from *Bifidobacterium adolescentis*. *J Mol. Cat. B: Enzym.* 21, 157–166. doi: 10.1016/S1381-1177(02)00123-6

Olsson, M. L., Hilla, C. A., de laVega, H., Liu, P. Q., Stroud, M. R., Valdinocci, J., et al. (2004). Universal red blood cells-enzymatic conversion of blood group A and B antigens. *Transfus. Clin. Biol.* 11, 33–39. doi: 10.1016/j.tracli.2003.12.002

Puchart, V., Vrsanska, M., Bhat, M. K., and Biely, P. (2000). Purification and characterization of α-galactosidase from a thermophilic fungus *Thermomyces lanuginosus*. *Biochim. Biophys. Acta* 1524, 27–37. doi: 10.1016/S0304-4165(00)00138-0

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual, 2nd Edn*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 253.

Shabalin, K. A., Kulminskaya, A. A., Savel'ev, A. N., Shishlyannikov, S. M., and Neustroev, K. N. (2002). Enzymatic properties of α-galactosidase from *Trichoderma reesei* in the hydrolysis of galactooligosaccharides. *Enzyme Microb. Technol.* 30, 231–239. doi: 10.1016/S0141-0229(01)00482-3

Sinnott, M. L. (1990). Catalytic mechanisms of enzymatic glycosyl transfer. *Chem. Rev.* 90, 1171–1202. doi: 10.1021/cr00105a006

Zhu, Y., Zajicek, J., and Serianni, A. S. (2001). Acyclic forms of [1-^{13}C] aldohexoses in aqueous solution: quantitation by ^{13}C NMR and deuterium isotope effects on tautomeric equilibria. *J. Org. Chem.* 66, 6244–6251. doi: 10.1021/jo010541m

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 30 June 2014; accepted: 25 September 2014; published online: 13 October 2014.

*Citation: Bakunina IY, Balabanova LA, Golotin VA, Slepchenko LV, Isakov VV and Rasskazov VA (2014) Stereochemical course of hydrolytic reaction catalyzed by alpha-galactosidase from cold adaptable marine bacterium of genus Pseudoalteromonas. Front. Chem. **2**:89. doi: 10.3389/fchem.2014.00089*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

PERSPECTIVE ARTICLE
published: 16 October 2014
doi: 10.3389/fchem.2014.00092

Sensing marine biomolecules: smell, taste, and the evolutionary transition from aquatic to terrestrial life

Ernesto Mollo[1]*, Angelo Fontana[1], Vassilios Roussis[2], Gianluca Polese[3], Pietro Amodeo[1] and Michael T. Ghiselin[4]

[1] Institute of Biomolecular Chemistry, National Research Council of Italy, Pozzuoli, Italy
[2] Department of Pharmacy, University of Athens, Athens, Greece
[3] Department of Biology, University of Naples "Federico II," Naples, Italy
[4] Department of Invertebrate Zoology and Geology, California Academy of Sciences, San Francisco, CA, USA

Edited by:
Mikhail Kusaykin, G. B. Elyakov Pacific Institute of Bioorganic Chemistry, Russia

Reviewed by:
Peizhong Mao, Oregon Health and Science University, USA
Christian W. Gruber, Medical University of Vienna, Austria

***Correspondence:**
Ernesto Mollo, Institute of Biomolecular Chemistry, National Research Council of Italy, Via Campi Flegrei 34, I-80078 Pozzuoli, Naples, Italy
e-mail: emollo@icb.cnr.it

The usual definition of smell and taste as distance and contact forms of chemoreception, respectively, has resulted in the belief that, during the shift from aquatic to terrestrial life, odorant receptors (ORs) were selected mainly to recognize airborne hydrophobic ligands, instead of the hydrophilic molecules involved in marine remote-sensing. This post-adaptive evolutionary scenario, however, neglects the fact that marine organisms 1) produce and detect a wide range of small hydrophobic and volatile molecules, especially terpenoids, and 2) contain genes coding for ORs that are able to bind those compounds. These apparent anomalies can be resolved by adopting an alternative, pre-adaptive scenario. Before becoming airborne on land, small molecules, almost insoluble in water, already played a key role in aquatic communication, but acting in "contact" forms of olfaction that did not require major molecular innovations to become effective at a distance in air. Rather, when air was "invaded" by volatile marine terpenoids, an expansion of the spatial range of olfaction was an incidental consequence rather than an adaptation.

Keywords: marine natural products, terpenoids, olfaction, gustation, volatility, solubility, odorant receptors, GPCRs

Olfaction (the sense of smell) is generally defined as the ability of terrestrial organisms to detect volatile molecules coming from a distance in the air, whereas in aquatic habitats waterborne signaling is considered the counterpart of airborne signaling. This definition is based on criteria that are "spatial" (the distance between the emitter and the receiver of the signal) rather than "molecular" (interactions between ligands and receptors). The different criteria, however, are not in conflict with each other when only organisms living in the aerial medium are considered. It is widely accepted that the first step in odor perception takes place when odorant airborne molecules—generally compounds with a molecular weight (MW) smaller than ∼300 Da (Mori et al., 2006; Touhara and Vosshall, 2009)—are transported by air, and finally bind to specific sites on odorant receptors (ORs) expressed in olfactory sensory neurons that transmit signals to the brain (Buck, 2000). Difficulties with the above definition of olfaction based on signal range emerge, however, when considering aquatic environments, where solubility, instead of volatility, is the crucial necessary condition for the long-distance transport of biomolecules. Many marine organisms, in fact, have a strong smell but only if they are taken out of the water, because their odorant molecules are hydrophobic and therefore cannot be effective in any form of remote sensing based on diffusion in water. They are mainly small representatives of the largest class of natural products, the terpenoids (isoprenoids), which are widespread both in marine and terrestrial organisms. Although it may seem curious, these marine metabolites (**Figure 1**, yellow spots) should be included in the group of biogenic volatile organic compounds (BVOCs) acting as mediators of growth, development, reproduction and, especially, defense, of many land plants and animals. Remarkably, some of the volatile terpenoids that have been found in marine sponges and nudibranchs (e.g., the odorant furanosesquiterpenes longifolin and dendrolasin) have also been found in terrestrial plants and insects (Pietra, 1995). As a further example, but with a special interest in pharmacology, the strong-smelling liposoluble terpenoid furanodiene showing important pharmacological properties, including anticancer activity (Dolara et al., 1996; Ba et al., 2009; Zhong et al., 2012a,b,c; Buccioni et al., 2014; Xu et al., 2014), is a component of both terrestrial plants and marine benthic invertebrates (Bowden et al., 1980; McPhail et al., 2001; Gavagnin et al., 2003). Therefore, given that olfaction (the sense of smell) is generally regarded as a distance sense, while gustation (the sense of taste) is a contact sense (Smith, 2008), exactly the same volatile molecules, almost insoluble in water, would be considered at the same time as being smelled on land, and tasted by contact at sea. Strangely enough, it has been emphasized that the "gustatory" perceptions of terrestrial tetrapod vertebrates, and the "olfactory" perceptions of fish provided with a sense of smell, are both mediated by stimulating molecules in solution (Smith, 2008). The above incongruities are certainly among the main reasons why evolutionary biologists have not yet been able to write a satisfactory historical narrative on chemoreception, which should consistently relate sequences of contingent

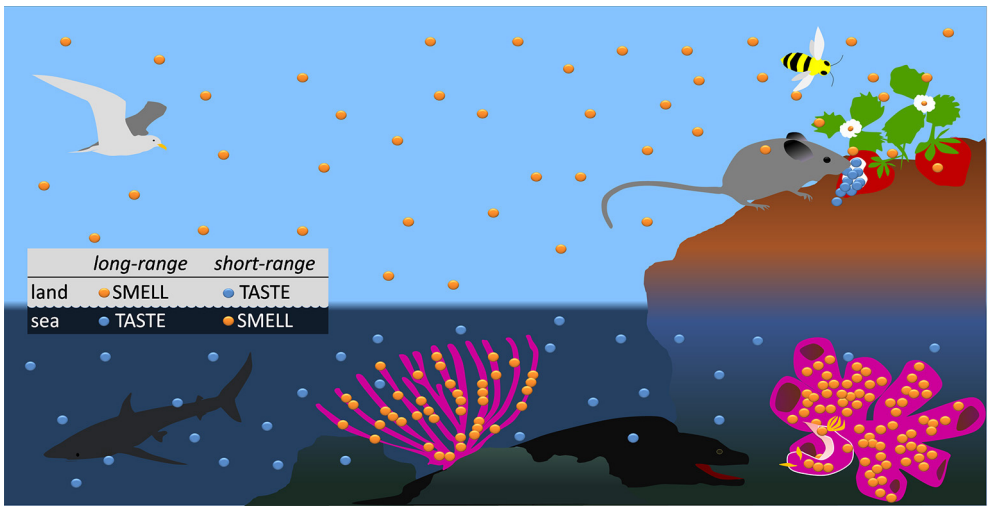


FIGURE 1 | Schematic distribution of airborne/hydrophobic (yellow spots) and non-volatile/waterborne (blue spots) biomolecules in terrestrial and marine environments. The box summarizes the range of the chemical senses in the different environments, when mediated by the above chemical cues.

historical events to laws of nature (Ghiselin, 1997; Cimino and Ghiselin, 2001).

Particularly unpersuasive is the current representation of what happened during the conquest of the land by aquatic organisms. It has been proposed that a successful transition to terrestrial life should have raised dramatically new demands on the chemosensory system, due to the fact that the olfactory stimuli changed from hydrophilic to mainly hydrophobic and airborne molecules (Krång et al., 2012; Tuchina et al., 2014). On the other hand, the traditional notion that olfaction is a "distance sense" led many authors to believe that under water the sense of smell is mediated almost exclusively by waterborne signaling molecules (Ache and Young, 2005; Eisthen and Polese, 2006; Smith, 2008; Shi and Zhang, 2009; Brönmark and Hansson, 2012; Krång et al., 2012; Tuchina et al., 2014). Accordingly, we should consider odorants to be those compounds that are easily dissolved in water. This would certainly embrace dissolved gasses, peptides, proteins, and functionalized hydrocarbons, which are known to function as chemical signals within pelagic interactions (Pohnert et al., 2007), including the non-volatile highly water soluble osmolyte dimethylsulfoniopropionate (DMSP), the volatile and hydrophobic enzymatic breakdown product of which, dimethyl sulfide (DMS), is emitted to the atmosphere and provides a foraging cue for seabirds (Savoca and Nevitt, 2014). Even though the structural differences between DMSP and DMS suggest that they are detected by different receptors, both compounds are evidently involved in long-range forms of chemical communication, pertaining, by definition, to olfaction. However, one would ask what is the actual meaning of olfactory waterborne remote-signaling (**Figure 1**, blue spots) in the majority of sessile marine benthic organisms, such as sponges and soft corals. Their interactions with predators or conspecifics occur, in fact, at extremely close range, and are often mediated by lipophilic compounds. Moreover, the chemoreception of chemical signals is evidently the main system for navigation in slow-moving animals such as nudibranch gastropods. According to the current view, therefore, the tactile detection of lipophilic odorants by nudibranchs is "taste," even when the chemical signals are exactly the same as those "smelled" by land animals.

Biased interpretations of related results in molecular genetics do not help to shed light on the issue. To overcome a sort of molecular "impasse" in chemoreception, it has been proposed that OR genes found in marine organisms encode ORs that detect water-soluble odorants (Niimura and Nei, 2005; Niimura, 2012). Therefore, in spite of our very limited knowledge of the actual specificity of ORs for ligands, subjective categories in molecular genetics have been built up to integrate the phyletic distribution of chemosensory genes, especially in marine organisms, with the traditional definition of the chemical senses based on the distance between the emitter and the detector. Different classes of receptors have been thus hypothesized on the basis of the type of odorous ligands supposedly recognized in the different environments, class I for water soluble, class II for airborne odorants. Moreover, OR genes in teleost fishes and tetrapods have been classified in the seven groups α-η, of which groups α and γ, on the basis of their common occurrence in terrestrial animals, have been assumed to detect airborne odorants (Niimura, 2012). Accordingly, the unexpected presence of α and γ genes in the coelacanth fish *Latimeria chalumnae*, whose genome was recently sequenced (Amemiya et al., 2013), has been explained by the hypothesis that an ancestral coelacanth lineage once inhabited shallow water and then returned to greater depths (Nikaido et al., 2013). But, how could this hypothesis be reasonably extended to explain the finding of an intact functional γ OR gene in zebrafish too (Niimura and Nei, 2005; Picone et al., 2014), for which an "aerial past" has not yet been suggested? However, even leaving aside the evident contradictions originating from the application of this kind of conjecture to totally aquatic species, once again, it would seem, they are evidently based on the unfounded assumption that aquatic fishes can only sense waterborne chemicals

by olfaction, whereas terrestrial vertebrates mainly sense volatile airborne chemicals. This is a patently obvious error, given the implausible "sudden" appearance, during the conquest of land by aquatic organisms, of both the volatile compounds, and the extremely complex chemoreceptorial machinery able to bind and decode such chemical signals (Nara et al., 2011). What we have here would seem to be tradition masquerading as facts, and anomaly being explained away by *ad hoc* hypotheses.

ORs are G protein coupled receptors (GPCRs) belonging to the rhodopsin-like gene family, the first representatives of which seem to have appeared between 800 and 580 million years ago, being present in marine cnidarians, placozoans, and sponges (Römpler et al., 2007; Srivastava et al., 2008, 2010; Churcher and Taylor, 2010). In particular, the antiquity of ORs is clear from the presence of their orthologs both in the cnidarian *Nematostella vectensis*, and in the cephalochordate *Branchiostoma floridae* (Churcher and Taylor, 2009, 2010). This demonstrates that ORs evolved at least 550 million years ago in marine invertebrates. On the other hand, marine natural product chemists have found massive evidence of marine organisms communicating by liposoluble secondary metabolites, most of which play critical ecological roles (Cimino and Ghiselin, 2001, 2009; Mollo et al., 2008). In particular, marine animals that are unable to escape predators by rapid locomotion provide some of the best documented examples of defensive strategies based on the use of allomones. Nonpolar terpenoids are commonly released on the body surface of these animals or in their tracks, but can also be accumulated in sacrificial parts of the body, to be locally detected by predators at extremely high concentrations (Carbone et al., 2013). Remarkably, considerable structural variability among the protective compounds, which include both polar and nonpolar metabolites, suggests that solubility in water did not play a critical role in the evolution of chemical defense in marine invertebrates (Pawlik, 2012). Marine liposoluble terpenoids have also been studied for their ability to indicate a food source for the receiver (kairomones), stimulating feeding once prey have been contacted (Hay, 2009), while volatile terpenes have been also found to act as gamete attractants (pheromones) in brown algae and cnidarians (Jaenicke and Boland, 1982; Coll et al., 1995). Notably, the subset with MW <300 Da of water-insoluble molecules that enable many marine benthic invertebrates to repel predators, and reproduce successfully, also impart a characteristic smell to those organisms when exposed to air. According to the current view, however, the aquatic detection of those small molecules, which are both odorants on land and insoluble in water, should be called "gustation," a sense requiring physical contact with the emitter (Smith, 2008). But, given the premise on the nature of the odorant molecules and their macromolecular counterparts, this argument doesn't make sense; actually it is a *non sequitur*. That the molecules in question must be in contact with the receptors is true of both olfaction and gustation. Volatile terpenoids, however, are among the odorants recognized by ORs on land. How can we accept, then, the idea that both those ligands and the related ligand-receptor complexes lose their sensorial specificity just by moving to the sea? This is unacceptable, unless one changes the name of the receptors when they operate underwater. We should, instead, consider that there is no "action at a distance" in the specific ligand-receptor recognition step.

But there are other arguments against the traditional definition of the chemical senses. Solubility and volatility are affected by local physico-chemical parameters. Thus changes in the local conditions could switch the perception of the same chemical message from "gustative" to "olfactive," and vice versa. In addition, lipophilic compounds can move long distances in water in the form of micellae, much as nonvolatile compounds can be transported in the atmosphere in the form of aerosol particles.

Overall, we have logical reasons to believe that the persistent habit of giving priority to the detection of distant objects in defining the sense of smell in different environments is not really consistent with the recent discovery of the molecular basis for odor recognition (Buck and Axel, 1991), especially for its evolutionary implications. In fact, it would have required an abrupt, extensive and concerted change in the complex patterns of affinity of the ORs for ligands during the transition from water to land, something that would be highly unlikely given the highly combinatorial character of the molecular mechanisms of olfaction (Nara et al., 2011). Moreover, it generates a contradiction in terms, which, not being merely a semantic problem seems to invoke a simplistic unifying theory for chemoreception where taste and smell lose their distinctive features. In this report we propose, instead, a new perspective aiming at a radical solution to this problem, at the same time preserving the usual taste-smell dichotomy.

From our perspective, the transition from aquatic, to semi-aquatic, and terrestrial olfaction certainly required physiological and anatomical adaptations in the chemosensory systems, but did not result in dramatic changes in the complex patterns of affinity of the ORs for ligands. Thus, by assigning a central role to both ligands and receptors involved in this process, and given the evident "molecular continuity" of terpenoid ligands across the transition from aquatic to terrestrial life, we reinterpret the aquatic detection of volatile compounds as the ancient precursor of terrestrial olfaction. Low-solubility in water and volatility in air, common features of the odorant terpenoids, determine opposite communication ranges in the two media, the former enhancing efficacy of short-range or contact communication in water by preventing, or strongly limiting, the dilution of the signal in the medium, the latter allowing long-range communication by dispersion of the signal in air (**Figure 1**). However, the kinds of messages are preserved in both environments, thus making the present perspective sound from an evolutionary point of view. Within this framework, many other important changes observed in the anatomical features of chemosensory organs of terrestrial animals, and in the cellular, sub-cellular, and molecular mechanisms that mediate sensing and processing of chemical stimuli, have to be rather considered post-adaptive phenomena. They have allowed the detection of odorants at astonishingly low concentrations in air, where the chemical signal is extremely diluted and covers long distances, and, together with other neurophysiological and anatomical adaptations, the correct spatial and temporal resolution of the signals. Examples of these adaptations possibly include the enormous increase of the number of OR genes in the terrestrial tetrapods, relative to marine fish (Niimura, 2012),

and the appearance of odorant receptor-coreceptor complexes in insects exhibiting different signal transduction mechanisms, in which G-proteins are only partially or not at all involved (Sato et al., 2008; Wicher et al., 2008).

Unlike that based on signal range, our interpretation of chemoreception can be extended even to very peculiar forms of chemical communication. For instance, at the interface of the aquatic and aerial environments, some marine insects can communicate by detecting pheromones that, being partly hydrophobic and partly hydrophilic, are neither airborne nor waterborne and can disperse in two dimensions on the sea surface, allowing mate location (Tsoukatou et al., 2001). In this case discrimination between smell and taste based of the signal spatial range would certainly be confusing without considering specific molecular interactions of the ligands with chemosensory receptors. This latter approach, however, calls for a rigorous classification of the chemoreceptors and their ligands fully based on molecular and genetic criteria, which will require a better knowledge of both genomes, and interaction patterns. Such studies are certainly desirable both for a better understanding of all forms of chemoreception, and to shed more light on the related anatomical, functional, and physiological adaptations. There can be no doubt, however, that the transition from an aquatic to terrestrial life did not involve dramatic changes in the chemoreception systems, even in those involved in both the perception by contact of non-volatile lipophilic compounds (e.g., terpenoids with MW >300 Da), and the detection at a distance of odorants that are both volatile and waterborne (e.g., amines). In these cases both receptors, and their range, have evidently been conserved. The fact nonetheless remains that the shift from a contact (at sea) to a remote (on land) form of olfaction is logically sound for the detection of the most abundant group of BVOCs (Blanch et al., 2009), namely the small terpenoids detected by ORs.

These considerations also suggest that we ought to reconsider what we call "smell" and "taste" in marine environments, where species live immersed in water, and distance chemoreception of hydrophilic substances is a straightforward matter. Thus, based on molecular criteria, what is currently called "aquatic olfaction" of waterborne molecules should be reasonably considered the real aquatic sense of taste, exploiting molecular mechanisms similar to those involved in terrestrial gustatory perception, where "the stimulating molecules have to be in solution and in

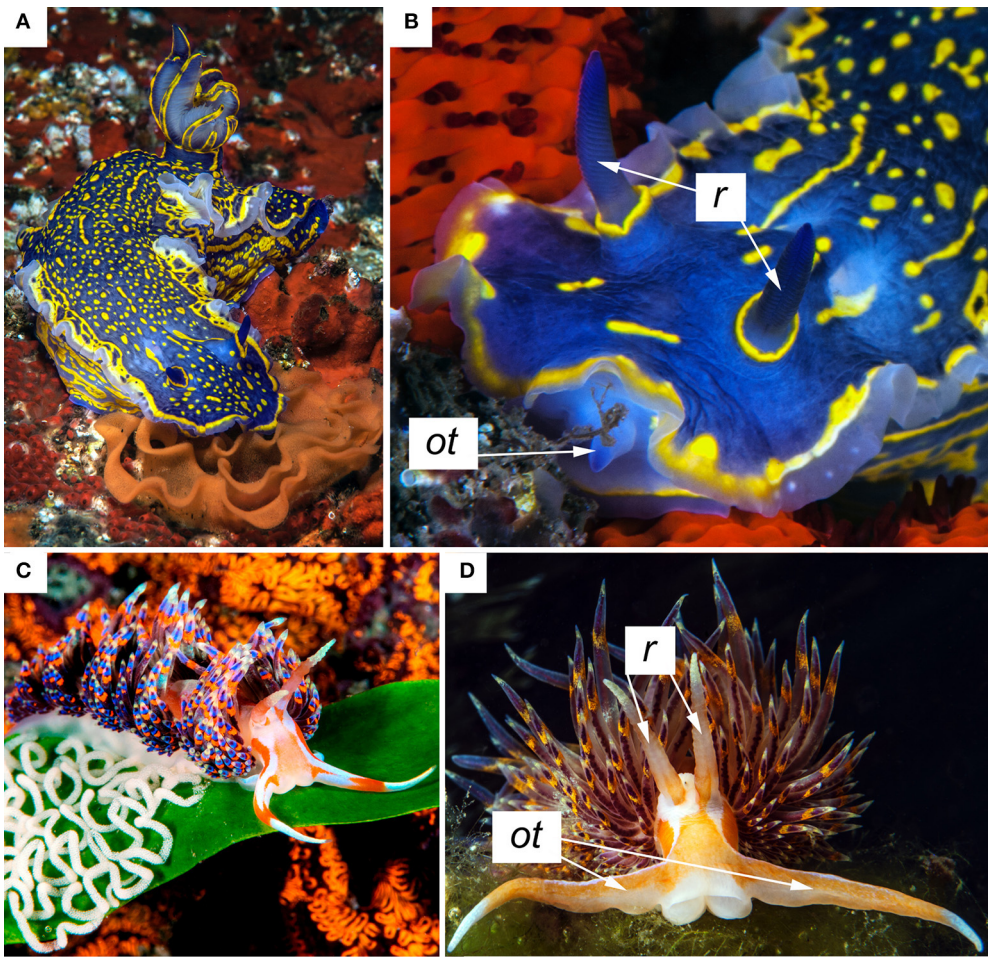


FIGURE 2 | Rhinophores (*r*) and oral tentacles (*ot*) in the nudibranchs *Felimare picta* (A,B), and *Godiva quadricolor* (C,D). Photos are courtesy of G. Villani.

contact with the receptor" (Smith, 2008). The rationale is compelling given that neither sugars nor glutamate, for instance, may be regarded as olfactory molecules by the mere fact that they can be perceived in their dissolved form in water, knowing, *inter alia*, that the existence of sweet and umami taste receptors (TRs) specifically binding the mentioned class of compounds is already supported by sufficiently strong evidence. Thus, the variety of aquatic "noses," defined as the organs that analyse odors in the external fluid medium (Atema, 2012), could be reasonably reinterpreted as aquatic "tongues." As a striking example, the nudibranchs' "rhinophores" (the name of which means nose-bearing) that protrude into the water above the dorsal surface, could be accordingly renamed as "glossophores" (tongue-bearing). Conversely the nudibranchs' "oral tentacles" constantly touching the substrate and sensing liposoluble odorant molecules, could be regarded as the true aquatic noses (**Figure 2**). Similarly, the mouthpart chemosensors of crustaceans, which are used to assess food palatability (Derby and Sorensen, 2008), must be able to detect the insoluble odorant molecules too. Furthermore, although fish "nostrils" that have no connection with the mouth are used to detect waterborne molecules, many fish repeatedly take food into their oral cavity and then reject it, before swallowing or refusing it. According to our perspective, this behavior is due to the crucial need for "smelling" by contact substances that cannot be perceived at a distance. Otherwise, how could those aquatic predators avoid getting poisoned by the small liposoluble molecules contained by many benthic organisms? How can the producer organism defend itself?

In summary, our perspective rejects the widespread misconception that the aquatic sense of smell can be mediated only by waterborne signaling molecules. In fact, ORs-mediated aquatic chemoreception of biomolecules that combine high volatility in air and insolubility (or very low solubility) occurs, either by direct contact with the emitter, or by short-range sensing traces adherent to the substrate. On the other hand, reception of waterborne but non volatile compounds responsible for salty, sweet, bitter, sour, and umami taste perceptions, are mediated by typical TRs both at sea and on land. Thus, for signals of this kind, a "reversal of senses" in their spatial range occurs in the different environments (**Figure 1**).

According to this synchronic view, the terrestrial sense of smell thus derives, to a major extent, from the ORs-mediated ability of aquatic organisms to detect small hydrophobic molecules by contact, but with the expansion of its spatial range on land. Overall, our perspective suggests that during the transition from aquatic to terrestrial life, when the intrinsic "airborne" character of pre-existing water insoluble molecules appeared with their first exposure to air, the pre-existing aquatic ORs acting in contact-communication had a pre-adaptive value, predisposing the lineage to evolve remote forms of communication and all subsequent physiological, behavioral and anatomical adaptations that allow extant land plants to reduce herbivory, flowers to attract pollinators, female insects to attract the males, and humans to exploit a large variety of flavorings, drugs, poisons and perfumes.

ACKNOWLEDGMENT

We thank G. Villani for providing photos of *F. picta* and *G. quadricolor*.

REFERENCES

Ache, B. W., and Young, J. M. (2005). Olfaction: diverse species, conserved principles. *Neuron* 48, 417–430. doi: 10.1016/j.neuron.2005.10.022

Amemiya, C. T., Alföldi, J., Lee, A. P., Fan, S., Philippe, H., MacCallum, I., et al. (2013). The African coelacanth genome provides insights into tetrapod evolution. *Nature* 496, 311–316. doi: 10.1038/nature12027

Atema, J. (2012). "Aquatic odour dispersal fields: opportunities and limits of detection, communication, and navigation," in *Chemical Ecology in Aquatic Systems*, eds C. Brönmark and L. A. Hansson (Oxford: Oxford University Press), 1–18.

Ba, Z., Zheng, Y., Zhang, H., Sun, X., and Lin, D. (2009). Potential anti-cancer activity of furanodiene. *Chin. J. Cancer Res.* 21, 154–158. doi: 10.1007/s11670-009-0154-0

Blanch, J.-S., Peñuelas, J., Sardans, J., and Llusià, J. (2009). Drought, warming and soil fertilization effects on leaf volatile terpene concentrations in *Pinus halepensis* and *Quercus ilex*. *Acta Physiol. Plant.* 31, 207–218. doi: 10.1007/s11738-008-0221-z

Bowden, B. F., Braekman, J., and Mitchell, S. J. (1980). Studies of Australian soft corals. XX. A new sesquiterpene furan from soft corals of the family Xeniidae and an examination of *Clavularia inflata* from north Queensland waters. *Aust. J. Chem.* 33, 927–932. doi: 10.1071/CH9800927

Brönmark, C., and Hansson, L. A. (2012). *Chemical Ecology in Aquatic Systems*. Oxford UK: Oxford University Press.

Buccioni, M., Dal Ben, D., Lambertucci, C., Maggi, F., Papa, F., Thomas, A., et al. (2014). Antiproliferative evaluation of isofuranodiene on breast and prostate cancer cell lines. *Sci. World J.* 2014:264829. doi: 10.1155/2014/264829

Buck, L. (2000). "Smell and taste: the chemical senses," in *Principles of Neural Science*, eds E. R. Kandel, J. H. Schwartz, and T. M. Jessell (New York, NY: McGraw-Hill), 625–652.

Buck, L., and Axel, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* 65, 175–187. doi: 10.1016/0092-8674(91)90418-X

Carbone, M., Gavagnin, M., Haber, M., Guo, Y.-W., Fontana, A., Manzo, E., et al. (2013). Packaging and delivery of chemical weapons: a defensive Trojan horse stratagem in chromodorid nudibranchs. *PLoS ONE* 8:e62075. doi: 10.1371/journal.pone.0062075

Churcher, A. M., and Taylor, J. S. (2009). Amphioxus (*Branchiostoma floridae*) has orthologs of vertebrate odorant receptors. *BMC Evol. Biol.* 9, 242–252. doi: 10.1186/1471-2148-9-242

Churcher, A. M., and Taylor, J. S. (2010). The antiquity of chordate odorant receptors is revealed by the discovery of orthologs in the cnidarian *Nematostella vectensis*. *Genome Biol. Evol.* 3, 36–43. doi: 10.1093/gbe/evq079

Cimino, G., and Ghiselin, M. T. (2001). "Marine natural products chemistry as an evolutionary narrative," in *Marine Chemical Ecology*, eds J. B. McClintock and B. J. Baker (Boca Raton: CRC Press), 115–154.

Cimino, G., and Ghiselin, M. T. (2009). Chemical defense and evolution of opisthobranch gastropods. *Proc. Calif. Acad. Sci.* 60, 175–422.

Coll, J. C., Leone, P. A., Bowden, B. F., Carroll, A. R., Konig, G. M., Heaton, A., et al. (1995). Chemical aspects of Mass spawning in corals. II. Epi-thunbergol, the sperm attractant in the eggs of the soft coral *Lobophytum crassum* (Cnidaria, Octocorallia). *Mar. Biol.* 123, 137–143. doi: 10.1007/BF00350332

Derby, C. D., and Sorensen, P. W. (2008). Neural processing, perception, and behavioral responses to natural chemical stimuli by fish and crustaceans. *J. Chem. Ecol.* 34, 898–914. doi: 10.1007/s10886-008-9489-0

Dolara, P., Luceri, C., Ghelardini, C., Monserrat, C., Aiolli, S., Luceri, F., et al. (1996). Analgesic effects of myrrh. *Nature* 379, 29. doi: 10.1038/379029a0

Eisthen, H. L., and Polese, G. (2006). "Evolution of vertebrate olfactory subsystems," in *Evolution of Nervous Systems*, Vol. 2, ed J. H. Kaas (Oxford: Academic Press), 355–406.

Gavagnin, M., Mollo, E., Castelluccio, F., Crispino, A., and Cimino, G. (2003). Sesquiterpene metabolites of the antarctic gorgonian *Dasystenella acanthina*. *J. Nat. Prod.* 66, 1517–1519. doi: 10.1021/np030201r

Ghiselin, M. T. (1997). *Metaphysics and the Origin of Species*. Albany: State University of New York Press.

Hay, M. E. (2009). Marine chemical ecology: chemical signals and cues structure marine populations, communities, and ecosystems. *Ann. Rev. Mar. Sci.* 1, 193–212. doi: 10.1146/annurev.marine.010908.163708

Jaenicke, L., and Boland, W. (1982). Signal substances and their reception in the sexual cycle of marine brown-algae. *Angew. Chem. Int. Ed.* 21, 643–653. doi: 10.1002/anie.198206433

Krång, A. S., Knaden, M., Steck, K., and Hansson, B. S. (2012). Transition from sea to land: olfactory function and constraints in the terrestrial hermit crab *Coenobita clypeatus*. *Proc. R. Soc. Lond. B Biol. Sci.* 279, 3510–3519. doi: 10.1098/rspb.2012.0596

McPhail, K. L., Davies-Coleman, M. T., and Starmer, J. (2001). Sequestered chemistry of the Arminacean nudibranch *Leminda millecra* in Algoa Bay, South Africa. *J. Nat. Prod.* 64, 1183–1190. doi: 10.1021/np010085x

Mollo, E., Gavagnin, M., Carbone, M., Castelluccio, F., Pozone, F., Roussis, V., et al. (2008). Factors promoting marine invasions: a chemoecological approach. *Proc. Natl. Acad. Sci. U.S.A.* 105, 4582–4586. doi: 10.1073/pnas.0709355105

Mori, K., Takahashi, Y. K., Igarashi, K. M., and Yamaguchi, M. (2006). Maps of odorant molecular features in the mammalian olfactory bulb. *Physiol. Rev.* 86, 409–433. doi: 10.1152/physrev.00021.2005

Nara, K., Saraiva, L. R., Ye, X., and Buck, L. B. (2011). A large-scale analysis of odor coding in the olfactory epithelium. *J. Neurosci.* 31, 9179–9191. doi: 10.1523/JNEUROSCI.1282-11.2011

Niimura, Y. (2012). Olfactory receptor multigene family in vertebrates: from the viewpoint of evolutionary genomics. *Curr. Genomics* 13, 103–114. doi: 10.2174/138920212799860706

Niimura, Y., and Nei, M. (2005). Evolutionary dynamics of olfactory receptor genes in fishes and tetrapods. *Proc. Natl. Acad. Sci. U.S.A.* 102, 6039–6044. doi: 10.1073/pnas.0501922102

Nikaido, M., Noguchi, H., Nishihara, H., Toyoda, A., Suzuki, Y., Kajitani, R., et al. (2013). Coelacanth genomes reveal signatures for evolutionary transition from water to land. *Genome Res.* 23, 1740–1748. doi: 10.1101/gr.158105.113

Pawlik, J. R. (2012). "Antipredatory defensive roles of natural products from marine invertebrates," in *Handbook of Marine Natural Products,* eds E. Fattorusso, W. H. Gerwick, and O. Taglialatela-Scarfati (New York, NY: Springer), 677–710. doi: 10.1007/978-90-481-3834-0_12

Picone, B., Hesse, U., Panji, S., Van Heusden, P., Jonas, M., and Christoffels, A. (2014). Taste and odorant receptors of the coelacanth—a gene repertoire in transition. *J. Exp. Zool. Mol. Dev. Evol.* 322B, 403–414. doi: 10.1002/jez.b.22531

Pietra, F. (1995). Structurally similar natural products in phylogenetically distant marine organisms, and a comparison with terrestrial species. *Chem. Soc. Rev.* 24, 65–71. doi: 10.1039/cs9952400065

Pohnert, G., Steinke, M., and Tollrian, R. (2007). Chemical cues, defence metabolites and the shaping of pelagic interspecific interactions. *TREE* 22, 198–204. doi: 10.1016/j.tree.2007.01.005

Römpler, H., Stäubert, C., Thor, D., Schulz, A., Hofreiter, M., and Schöneberg, T. (2007). G protein-coupled time travel: evolutionary aspects of GPCR research. *Mol. Interv.* 7, 17–25. doi: 10.1124/mi.7.1.5

Sato, K., Pellegrino, M., Nakagawa, T., Nakagawa, T., Vosshall, L. B., and Touhara, K. (2008). Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature* 452, 1002–1006. doi: 10.1038/nature06850

Savoca, M. S., and Nevitt, G. A. (2014). Evidence that dimethyl sulfide facilitates a tritrophic mutualism between primary producers and top predators. *Proc. Natl. Acad. Sci. U.S.A.* 111, 4157–4161. doi: 10.1073/pnas.1317120111

Shi, P., and Zhang, J. (2009). Extraordinary diversity of chemosensory receptor gene repertoires among vertebrates. *Results Probl. Cell Differ.* 47, 1–23. doi: 10.1007/400_2008_4

Smith, C. U. M. (2008). *Biology of Sensory Systems, Second Edition*. Chichester, UK: John Wiley & Sons, Ltd.

Srivastava, M., Begovic, E., Chapman, J., Putnam, N. H., Hellsten, U., Kawashima, T., et al. (2008). The *Trichoplax* genome and the nature of placozoans. *Nature* 454, 955–960. doi: 10.1038/nature07191

Srivastava, M., Simakov, O., Chapman, J., Fahey, B., Gauthier, M. E. A., Mitros, T., et al. (2010). The *Amphimedon queenslandica* genome and the evolution of animal complexity. *Nature* 466, 720–727. doi: 10.1038/nature09201

Touhara, K., and Vosshall, L. B. (2009). Sensing odorants and pheromones with chemosensory receptors. *Annu. Rev. Physiol.* 71, 307–332. doi: 10.1146/annurev.physiol.010908.163209

Tsoukatou, M., Cheng, L., Vagias, C., and Roussis, V. (2001). Chemical composition and behavioural responses of the marine insect *Halobates hawaiiensis* (Heteroptera: Gerridae). *Z. Naturforsch.* 56c, 597–602.

Tuchina, O., Groh, K. C., Talarico, G., Muller, C. H. G., Wielsch, N., Hupfer, Y., et al. (2014). Morphology and histochemistry of the aesthetasc-associated epidermal glands in terrestrial hermit crabs of the genus *Coenobita* (Decapoda: Paguroidea). *PLoS ONE* 9:e96430. doi: 10.1371/journal.pone.0096430

Wicher, D., Schäfer, R., Bauernfeind, R., Stensmyr, M. C., Heller, R., Heinemann, S. H., et al. (2008). Drosophila odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature* 452, 1007–1011. doi: 10.1038/nature06861

Xu, W. S., Dang, Y. Y., Chen, X. P., Lu, J. J., and Wang, Y. T. (2014). Furanodiene presents synergistic anti-proliferative activity with paclitaxel via altering cell cycle and integrin signaling in 95-D lung cancer cells. *Phytother. Res.* 28, 296–299. doi: 10.1002/ptr.4984

Zhong, Z., Dang, Y., Yuan, X., Guo, W., Li, Y., Tan, W., et al. (2012a). Furanodiene, a natural product, inhibits breast cancer growth both *in vitro* and *in vivo*. *Cell. Physiol. Biochem.* 30, 778–790. doi: 10.1159/000341457

Zhong, Z. F., Hoi, P. M., Wu, G. S., Xu, Z. T., Tan, W., Chen, X. P., et al. (2012b). Anti-angiogenic effect of furanodiene on HUVECs *in vitro* and on zebrafish *in vivo*. *J. Ethnopharmacol.* 141, 721–727. doi: 10.1016/j.jep.2011.08.052

Zhong, Z. F., Li, Y. B., Wang, S. P., Tan, W., Chen, X. P., Chen, M. W., et al. (2012c). Furanodiene enhances tamoxifen-induced growth inhibitory activity of ERa-positive breast cancer cells in a PPARγ independent manner. *J. Cell. Biochem.* 113, 2643–2651. doi: 10.1002/jcb.24139

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 26 July 2014; paper pending published: 16 September 2014; accepted: 29 September 2014; published online: 16 October 2014.

Citation: Mollo E, Fontana A, Roussis V, Polese G, Amodeo P and Ghiselin MT (2014) Sensing marine biomolecules: smell, taste, and the evolutionary transition from aquatic to terrestrial life. Front. Chem. ***2****:92. doi: 10.3389/fchem.2014.00092*

This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

frontiers in CHEMISTRY

GENERAL COMMENTARY
published: 13 November 2014
doi: 10.3389/fchem.2014.00103

Corrigendum: Relationships between chemical structures and functions of triterpene glycosides isolated from sea cucumbers

Jong-Young Kwak*

Department of Biochemistry, School of Medicine, Dong-A University, Busan, South Korea
**Correspondence: jykwak@dau.ac.kr*

Edited and reviewed by:
Antonio Trincone, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Italy

Keywords: anticancer activity, cucumarioside, frondoside A, membrane transporters, stichoposides, triterpene glycosides

A corrigendum on

Relationships between chemical structures and functions of triterpene glycosides isolated from sea cucumbers
by Park, J,-I., Bae, H,-R., Kim, C. G., Stonik, V. A., and Kwak, J.-Y. (2014). Front. Chem. 2:77. doi: 10.3389/fchem.2014.00077

The authors corrected structures of compounds in **Figures 2**, **4**, **6**, and **9**.

1. The structures of compounds (3, 4, 5, 6, 10, 15, 20, and 21) are corrected.

2. Compounds 17 and 18 (in Figure 7) are same as compounds 20 and 21 (in **Figure 9**).

Conflict of Interest Statement: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 13 October 2014; accepted: 30 October 2014; published online: 13 November 2014.

*Citation: Kwak J-Y (2014) Corrigendum: Relationships between chemical structures and functions of triterpene glycosides isolated from sea cucumbers. Front. Chem. **2**:103. doi: 10.3389/fchem.2014.00103*
This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry.

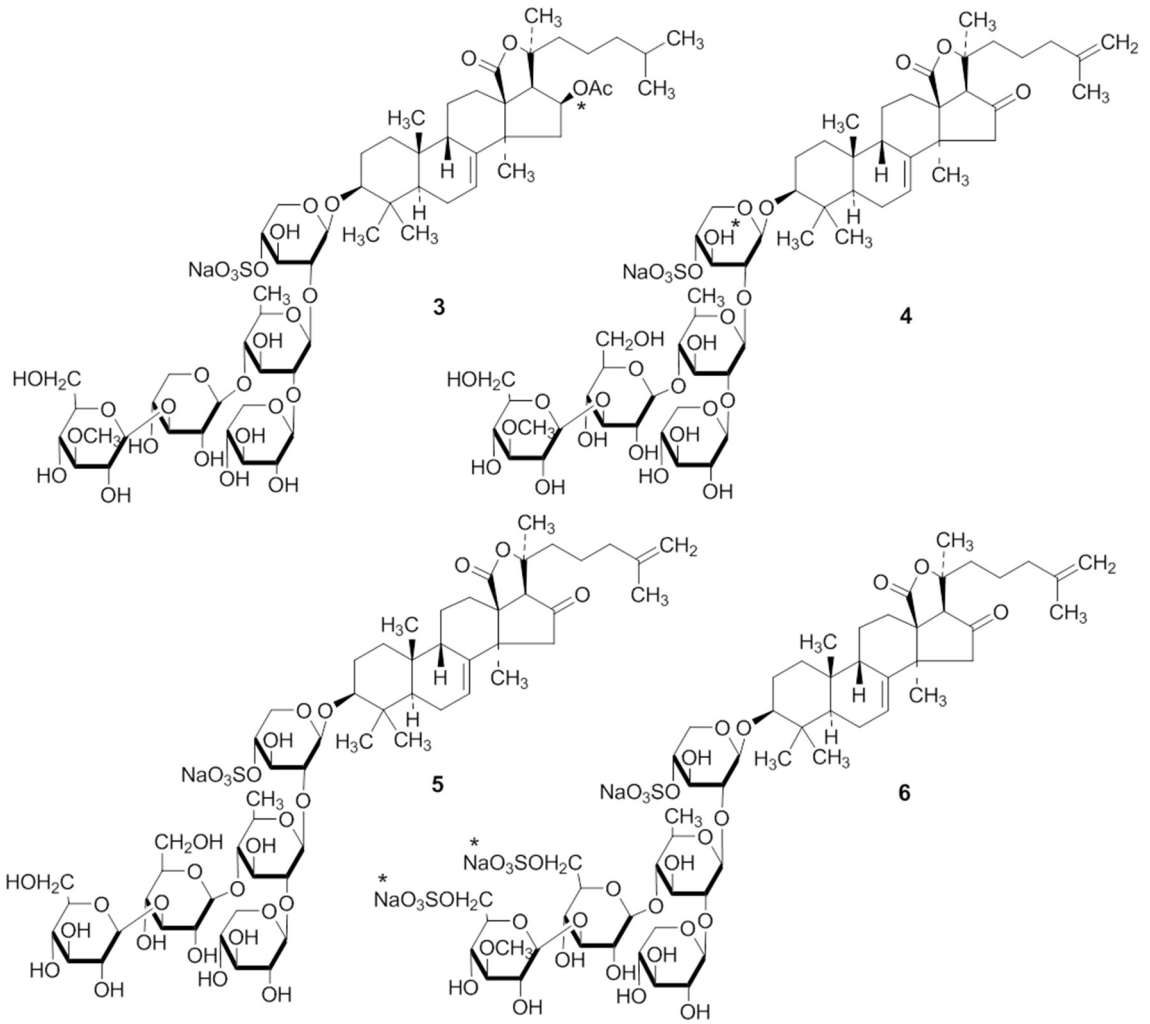


FIGURE 2 | The structures of 3, 4, 5 and 6 are corrected.

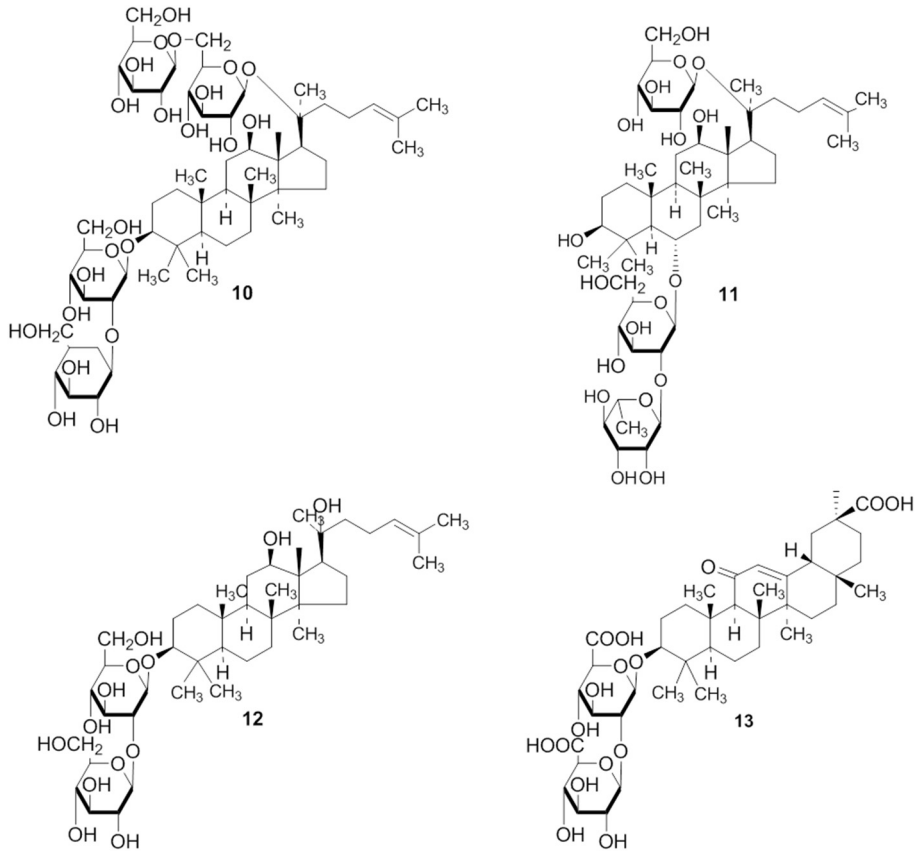


FIGURE 4 | The structure of 10 is corrected.

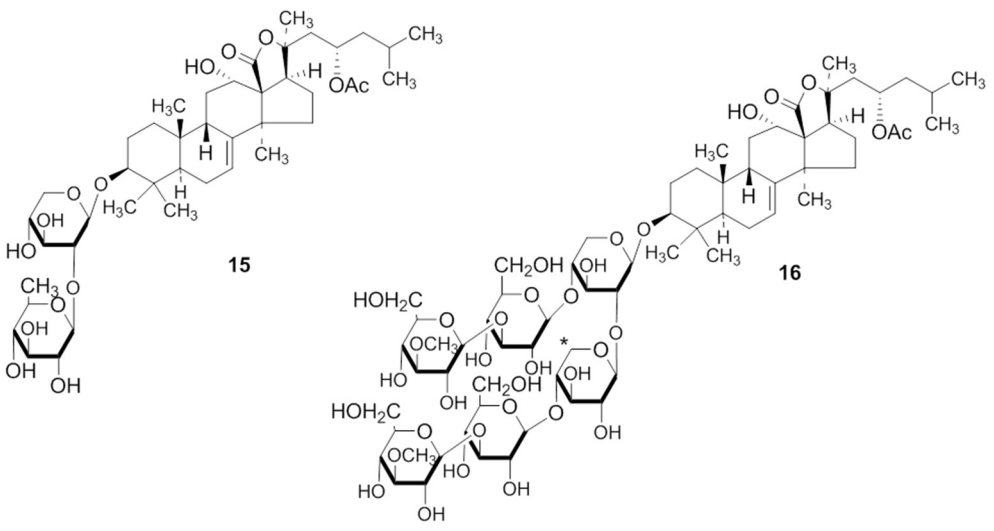


FIGURE 6 | The structure of 15 is corrected.

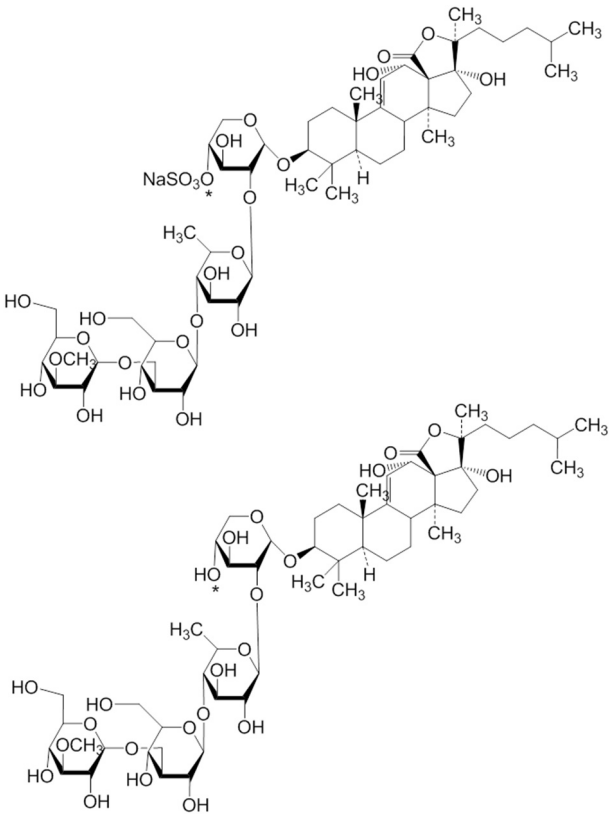


FIGURE 9 | The structures of 20 and 21 are corrected.

frontiers in
BIOENGINEERING AND BIOTECHNOLOGY

REVIEW ARTICLE
published: 11 February 2015
doi: 10.3389/fbioe.2014.00090

Scope of algae as third generation biofuels

Shuvashish Behera, Richa Singh, Richa Arora, Nilesh Kumar Sharma, Madhulika Shukla and Sachin Kumar*

Biochemical Conversion Division, Sardar Swaran Singh National Institute of Renewable Energy, Kapurthala, Punjab, India

Edited by:
Antonio Trincone, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Italy

Reviewed by:
Franz Goecke, Institute of Microbiology A.S.C.R, Czech Republic
Sankar Prasad Bhattacharya, Monash University, Australia
Antonio Trincone, Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Italy

***Correspondence:**
Sachin Kumar, Biochemical Conversion Division, Sardar Swaran Singh National Institute of Renewable Energy, Jalandhar-Kapurthala Road, Wadala Kalan, Kapurthala 144601, Punjab, India
e-mail: sachin.biotech@gmail.com

An initiative has been taken to develop different solid, liquid, and gaseous biofuels as the alternative energy resources. The current research and technology based on the third generation biofuels derived from algal biomass have been considered as the best alternative bioresource that avoids the disadvantages of first and second generation biofuels. Algal biomass has been investigated for the implementation of economic conversion processes producing different biofuels such as biodiesel, bioethanol, biogas, biohydrogen, and other valuable co-products. In the present review, the recent findings and advance developments in algal biomass for improved biofuel production have been explored. This review discusses about the importance of the algal cell contents, various strategies for product formation through various conversion technologies, and its future scope as an energy security.

Keywords: algae, microalgae, biofuels, bioethanol, biogas, biodiesel, biohydrogen

INTRODUCTION

The requirement of energy for the mankind is increasing day by day. The major source of energy is based on fossil fuels only. Thus, the scarcity of fossil fuels, rising price of petroleum based fuels, energy protection, and increased global warming resulted in focusing on renewable energy sources such as solar, wind, hydro, tidal, and biomass worldwide (Goldemberg and Guardabassi, 2009; Dragone et al., 2010; Rajkumar et al., 2014).

Different biomass from various sources like agricultural, forestry, and aquatic have been taken into consideration as the feedstocks for the production of several biofuels such as biodiesel (Boyce et al., 2008; Yanqun et al., 2008), bioethanol (Behera et al., 2014), biohydrogen (Marques et al., 2011), bio-oil (Shuping et al., 2010), and biogas (Hughes et al., 2012; Singh et al., 2014). However, the environmental impact raised from burning of fuels has a great impact on carbon cycle (carbon balance), which is related to the combustion of fossil fuels. Besides, exhaustion of different existing biomass without appropriate compensation resulted in huge biomass scarcity, emerging environmental problems such as deforestation and loss of biodiversity (Goldemberg, 2007; Li et al., 2008; Saqib et al., 2013).

Recently, researchers and entrepreneurs have focused their interest, especially on the algal biomass as the alternative feedstock for the production of biofuels. Moreover, algal biomass has no competition with agricultural food and feed production (Demirbas, 2007). The photosynthetic microorganisms like microalgae require mainly light, carbon dioxide, and some nutrients (nitrogen, phosphorus, and potassium) for its growth, and to produce large amount of lipids and carbohydrates, which can be further processed into different biofuels and other valuable co-products (Brennan and Owende, 2010; Nigam and Singh, 2011). Interestingly, the low content of hemicelluloses and about zero content of lignin in algal biomass results in an increased hydrolysis and/or fermentation efficiency (Saqib et al., 2013). Other than biofuels, algae have applications in human nutrition, animal feed, pollution control, biofertilizer, and waste water treatment (Thomas, 2002; Tamer et al., 2006; Crutzen et al., 2007; Hsueh et al., 2007; Choi et al., 2012). Therefore, the aim of the current review is to explore the scope of algae for the production of different biofuels and evaluation of its potential as an alternative feedstock.

ALGAE: SOURCE OF BIOFUELS

Generally, algae are a diverse group of prokaryotic and eukaryotic organisms ranging from unicellular genera such as *Chlorella* and diatoms to multicellular forms such as the giant kelp, a large brown alga that may grow up to 50 m in length (Li et al., 2008). Algae can either be autotrophic or heterotrophic. The autotrophic algae require only inorganic compounds such as CO_2, salts, and a light energy source for their growth, while the heterotrophs are non-photosynthetic, which require an external source of organic compounds as well as nutrients as energy sources (Brennan and Owende, 2010). Microalgae are very small in sizes usually measured in micrometers, which normally grow in water bodies or ponds. Microalgae contain more lipids than macroalgae and have the faster growth in nature (Lee et al., 2014a). There are about more than 50,000 microalgal species out of which only about 30,000 species have been taken for the research study (Surendhiran and Vijay, 2012; Richmond and Qiang, 2013; Rajkumar et al., 2014). The short harvesting cycle of algae is the key advantage for its importance, which is better than other conventional crops having harvesting cycle of once or twice in a year (Chisti, 2007; Schenk

et al., 2008). Therefore, the main focus has been carried out on algal biomass for its application in biofuel area.

There are several advantages of algal biomass for biofuels production: (a) ability to grow throughout the year, therefore, algal oil productivity is higher in comparison to the conventional oil seed crops; (b) higher tolerance to high carbon dioxide content; (c) the consumption rate of water is very less in algae cultivation; (d) no requirement of herbicides or pesticides in algal cultivation; (e) the growth potential of algal species is very high in comparison to others; (f) different sources of wastewater containing nutrients like nitrogen and phosphorus can be utilized for algal cultivation apart from providing any additional nutrient; and (g) the ability to grow under harsh conditions like saline, brackish water, coastal seawater, which does not affect any conventional agriculture (Spolaore et al., 2006; Dismukes et al., 2008; Dragone et al., 2010). However, there are several disadvantages of algal biomass as feedstock such as the higher cultivation cost as compared to conventional crops. Similarly, harvesting of algae require high energy input, which is approximately about 20–30% of the total cost of production. Several techniques such as centrifugation, flocculation, floatation, sedimentation, and filtration are usually used for harvesting and concentrating the algal biomass (Demirbas, 2010; Ho et al., 2011).

The algae can be converted into various types of renewable biofuels including bioethanol, biodiesel, biogas, photobiologically produced biohydrogen, and further processing for bio-oil and syngas production through liquefaction and gasification, respectively (Kraan, 2013). The conversion technologies for utilizing algal biomass to energy sources can be categorized into three different ways, i.e., biochemical, chemical, and thermochemical conversion and make an algal biorefinery, which has been depicted in **Figure 1**. The biofuel products derived from algal biomass using these conversion routes have been explored in detail in the subsequent sections.

BIODIESEL PRODUCTION

Biodiesel is a mixture of monoalkyl esters of long chain fatty acids [fatty acid methyl esters (FAME)], which can be obtained from different renewable lipid feedstocks and biomass. It can be directly used in different diesel engines (Clark and Deswarte, 2008; Demirbas, 2009). Studies to explore the microalgae as feedstock for the production of liquid fuels had been started for the mid-1980s. In order to solve the energy crisis, the extraction of lipids from diatoms was attempted by some German scientists during the period of World War-II (Cohen et al., 1995). The higher oil yield in algal biomass as compared to oil seed crops makes the possibility to convert into the biodiesel economically using different technologies. A comparative study between algal biomass and terrestrial plants for the production

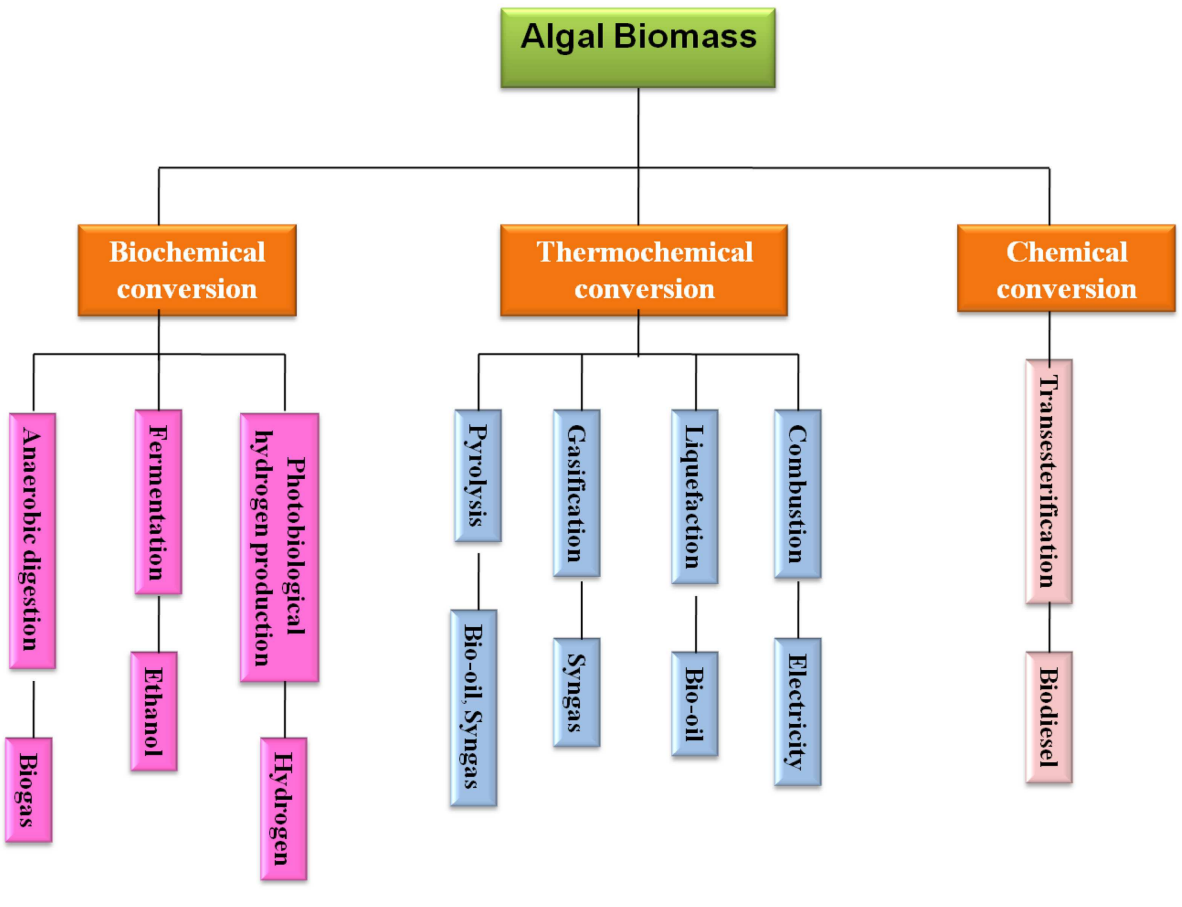


FIGURE 1 | Algal biomass conversion process for biofuel production.

of biodiesel has been depicted in **Table 1**. The oil productivity (mass of oil produced per unit volume of the microalgal broth per day) depends on the algal growth rate and the biomass content of the species. The species of microalgae such as *Kirchneriella lunaris*, *Ankistrodesmus fusiformis*, *Chlamydocapsa bacillus*, and *Ankistrodesmus falcatus* with high levels of polyunsaturated FAME are generally preferred for the production of biodiesel (Nascimento et al., 2013). They commonly multiply their biomass with doubling time of 24 h during exponential growth. Oil content of microalgae is generally found to be very high, which exceed up to 80% by weight of its dry biomass. About 5,000–15,000 gal of biodiesel can be produced from algal biomass per acre per year, which reflects its potentiality (Spolaore et al., 2006; Chisti, 2007).

However, there are some standards such as International Biodiesel Standard for Vehicles (EN14214) and American Society for Testing and Materials (ASTM), which are required to comply with the algal based biodiesel on the physical and chemical properties for its acceptance as substitute to fossil fuels (Brennan and Owende, 2010). The higher degree of polyunsaturated fatty acids of algal oils as compared to vegetable oils make susceptible for oxidation in the storage and further limits its utilization (Chisti, 2007). Some researchers have reported the different advantages of the algal biomass for the biodiesel production due to its high biomass growth and oil productivity in comparison to best oil crops (Chisti, 2007; Hossain et al., 2008; Hu et al., 2008; Rosenberg et al., 2008; Schenk et al., 2008; Rodolfi et al., 2009; Mutanda et al., 2011).

Algal biodiesel production involves biomass harvesting, drying, oil extraction, and further transesterification of oil, which have been described as below.

HARVESTING AND DRYING OF ALGAL BIOMASS

Unicellular microalgae produce a cell wall containing lipids and fatty acids, which differ them from higher animals and plants. Harvesting of algal biomass and further drying is important prior to mechanical and solvent extraction for the recovery of oil. Macroalgae can be harvested using nets, which require less energy while microalgae can be harvested by some conventional processes, which include filtration (Rossignol et al., 1999) flocculation (Liu et al., 2013; Prochazkova et al., 2013), centrifugation (Heasman et al., 2008), foam fractionation (Csordas and Wang, 2004), sedimentation, froth floatation, and ultrasonic separation (Bosma et al., 2003). Selection of harvesting method depends on the type of algal species.

Drying is an important method to extend shelf-life of algal biomass before storage, which avoids post-harvest spoilage (Munir et al., 2013). Most of the efficient drying methods like spray-drying, drum-drying, freeze drying or lyophilization, and sun-drying have been applied on microalgal biomass (Leach et al., 1998; Richmond, 2004; Williams and Laurens, 2010). Sun-drying is not considered as a very effective method due to presence of high water content in the biomass (Mata et al., 2010). However, Prakash et al. (2007) used simple solar drying device and succeed in drying *Spirulina* and *Scenedesmus* having 90% of moisture content. Widjaja et al. (2009) showed the effectiveness of drying temperature during lipid extraction of algal biomass, which affects both concentration of triglycerides and lipid yield. Further, all these processes possess safety and health issues (Singh and Gu, 2010).

EXTRACTION OF OIL FROM ALGAL BIOMASS

Unicellular microalgae produce a cell wall containing lipids and fatty acids, which differ them from higher animals and plants. In

Table 1 | Comparative study between algal biomass and terrestrial plants for biodiesel production.

Feedstock	Conditions	Biodiesel	Reference
ALGAE			
Spirulina platensis	Reaction temperature 55°C, 60% catalyst concentration, 1:4 algae biomass to methanol ratio, 450 rpm stirring intensity	60 g/kg lipid	Nautiyal et al. (2014)
Nannochloropsis sp.	Oil extraction with n-hexane, acidic transesterification	99 g/kg lipid	Susilaningsih et al. (2009)
Scenedesmus sp.	Alkaline (NaOH), temperature of 70°C	321.06 g/kg lipid	Kim et al. (2014)
	Acidic (H_2SO_4) catalyst, temperature of 70°C	282.23 g/kg lipid	
Nannochloropsis salina	Freeze drying of biomass, extraction with chloroform–methanol (1:1 ratio), alkali transesterification	180.78 g/kg lipid	Muthukumar et al. (2012)
Chlorella marina		100 g/kg lipid	
TERRESTRIAL PLANTS			
Madhuca indica	0.30–0.35 (v/v) methanol-to-oil ratio, 1% (v/v) H_2SO_4 as acid catalyst, 0.25 (v/v) methanol, 0.7% (w/v) KOH as alkaline catalyst	186.2 g/kg lipid	Ghadge and Raheman (2005)
Pongamia pinnata	Transesterification with methanol, NaOH as catalyst, temp. 60°C	253 g/kg lipid	Mamilla et al. (2011)
	Acid-catalyzed esterification by using 0.5% H_2SO_4, alkali-catalyzed transesterification	193.2 g/kg lipid	Naik et al. (2008)
Azadirachta indica	Reaction time of 60 min, 0.7% H_2SO_4 as acid catalyst, reaction temperature of 50°C, and methanol: oil ratio of 3:1	170 g/kg lipid	Awolu and Layokun (2013)
Soybean	Hydrotalcite as basic catalyst, methanol/oil molar ratio of 20:1, reaction time of 10 h	189.6 g/kg lipid	Martin et al. (2013)

the literature, there are different methods of oil extraction from algae, such as mechanical and solvent extraction (Li et al., 2014). However, the extraction of lipids from microalgae is costly and energy intensive process.

Mechanical oil extraction

The oil from nuts and seeds is extracted mechanically using presses or expellers, which can also be used for microalgae. The algal biomass should be dried prior to this process. The cells are just broken down with a press to leach out the oil. About 75% of oil can be recovered through this method and no special skill is required (Munir et al., 2013). Topare et al. (2011) extracted oil through screw expeller by mechanical pressing (by piston) and osmotic shock method and recovered about 75% of oil from the algae. However, more extraction time is required as compared to other methods, which make the process unfavorable and less effective (Popoola and Yangomodou, 2006).

Solvent based oil extraction

Oil extraction using solvent usually recovers almost all the oil leaving only 0.5–0.7% residual oil in the biomass. Therefore, the solvent extraction method has been found to be suitable method rather than the mechanical extraction of oil and fats (Topare et al., 2011). Solvent extraction is another method of lipid extraction from microalgae, which involves two stage solvent extraction systems. The amount of lipid extracted from microalgal biomass and further yield of highest biodiesel depends mainly on the solvent used. Several organic solvents such as chloroform, hexane, cyclohexane, acetone, and benzene are used either solely or in mixed form (Afify et al., 2010). The solvent reacts on algal cells releasing oil, which is recovered from the aqueous medium. This occurs due to the nature of higher solubility of oil in organic solvents rather than water. Further, the oil can be separated from the solvent extract. The solvent can be recycled for next extraction. Out of different organic solvents, hexane is found to be most effective due to its low toxicity and cost (Rajvanshi and Sharma, 2012; Ryckebosch et al., 2012).

In case of using mixed solvents for oil extraction, a known quantity of the solvent mixture is used, for example, chloroform/methanol in the ratio 2:1 (v/v) for 20 min using a shaker and followed by the addition of mixture, i.e., chloroform/water in the ratio of 1:1 (v/v) for 10 min (Shalaby, 2011). Similarly, Pratoomyot et al. (2005) extracted oil from different algal species using the solvent system chloroform/methanol in the ratio of 2:1 (v/v) and found different fatty acid content. Ryckebosch et al. (2012) optimized an analytical procedure and found chloroform/methanol in the ratio 1:1 as the best solvent mixture for the extraction of total lipids. Similarly, Lee et al. (1998) extracted lipid from the green alga *Botryococcus braunii* using different solvent system and obtained the maximum lipid content with chloroform/methanol in the ratio of 2:1. Hossain et al., 2008 used hexane/ether in the ratio 1:1 (v/v) for oil extraction and allowed to settle for 24 h. Using a two-step process, Fajardo et al. (2007) reported about 80% of lipid recovery using ethanol and hexane in the two steps for the extraction and purification of lipids. Therefore, a selection of a most suitable solvent system is required for the maximum extraction of oil for an economically viable process.

Lee et al. (2009) compared the performance of various disruption methods, including autoclaving, bead-beating, microwaves, sonication, and using 10% NaCl solution in the extraction of *Botryococcus* sp., *Chlorella vulgaris*, and *Scenedesmus* sp, using a mixture of chloroform and methanol (1:1).

TRANSESTERIFICATION

This is a process to convert algal oil to biodiesel, which involves multiple steps of reactions between triglycerides or fatty acids and alcohol. Different alcohols such as ethanol, butanol, methanol, propanol, and amyl alcohol can be used for this reaction. However, ethanol and methanol are used frequently for the commercial development due to its low cost and its physical and chemical advantages (Bisen et al., 2010; Surendhiran and Vijay, 2012). The reaction can be performed in the presence of an inorganic catalyst (acids and alkalies) or lipase enzyme. In this method, about 3 mol of alcohol are required for each mole of triglyceride to produce 3 mol of methyl esters (biodiesel) and 1 mol of glycerol (by-product) (Meher et al., 2006; Chisti, 2007; Sharma and Singh, 2009; Surendhiran and Vijay, 2012; Stergiou et al., 2013) (**Figure 2**). Glycerol is denser than biodiesel and can be periodically or continuously removed from the reactor in order to drive the equilibrium reaction. The presence of methanol, the co-solvent that keeps glycerol and soap suspended in the oil, is known to cause engine failure (Munir et al., 2013). Thus, the biodiesel is recovered by repeated washing with water to remove glycerol and methanol (Chisti, 2007).

The reaction rate is very slow by using the acid catalysts for the conversion of triglycerides to methyl esters, whereas the alkali-catalyzed transesterification reaction has been reported to be 4000 times faster than the acid-catalyzed reaction (Mazubert et al., 2013). Sodium and potassium hydroxides are the two commercial alkali catalysts used at a concentration of about 1% of oil.

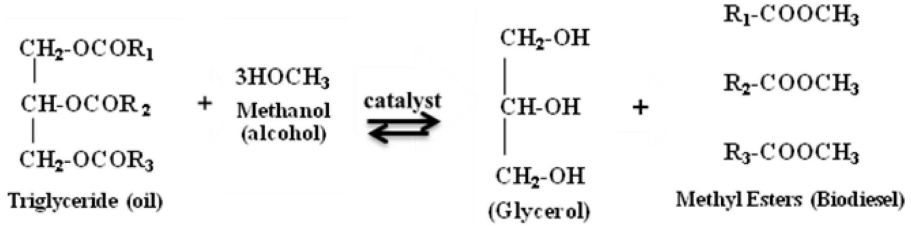


FIGURE 2 | Transesterification of oil to biodiesel. R_{1-3} are hydrocarbon groups.

However, sodium methoxide has become the better catalyst rather than sodium hydroxide (Singh et al., 2006).

Kim et al. (2014) used *Scenedesmus* sp. for the biodiesel production through acid and alkali transesterification process. They reported $55.07 \pm 2.18\%$, based on lipid by wt of biodiesel conversion using NaOH as an alkaline catalyst than using H_2SO_4 as $48.41 \pm 0.21\%$ of biodiesel production. In comparison to acid and alkalies, lipases as biocatalyst have different advantages as the catalysts due to its versatility, substrate selectivity, regioselectivity, enantioselectivity, and high catalytic activity at ambient temperature and pressure (Knezevic et al., 2004). It is not possible by some lipases to hydrolyze ester bonds at secondary positions, while some other group of enzymes hydrolyzes both primary and secondary esters. Another group of lipases exhibits fatty acids selectivity, and allow to cleave ester bonds at particular type of fatty acids. Luo et al. (2006) cloned the lipase gene lipB68 and expressed in *Escherichia coli* BL21 and further used it as a catalyst for biodiesel production. LipB68 could catalyze the transesterification reaction and produce biodiesel with a yield of 92% after 12 h, at a temperature of 20°C. The activity of the lipase enzyme with such a low temperature could provide substantial savings in energy consumption. However, it is rarely used due to its high cost (Sharma et al., 2001).

Extractive transesterification

It involves several steps to produce biodiesel such as drying, cell disruption, oils extraction, transesterification, and biodiesel refining (Hidalgo et al., 2013). The main problems are related with the high water content of the biomass (over 80%), which overall increases the cost of whole process.

In situ transesterification

This method skips the oil extraction step. The alcohol acts as an extraction solvent and an esterification reagent as well, which enhances the porosity of the cell membrane. Yields found are higher than via the conventional route, and waste is also reduced. Industrial biodiesel production involves release of extraction solvent, which contributes to the production of atmospheric smog and to global warming. Thus, simplification of the esterification processes can reduce the disadvantages of this attractive bio-based fuel. The single-step methods can be attractive solutions to reduce chemical and energy consumption in the overall biodiesel production process (Patil et al., 2012). A comparison of direct and extractive transesterification is given in **Table 2**.

Table 2 | Comparison of extractive transesterification and *in situ* methods (Haas and Wagner, 2011).

Sl. no.	Extractive transesterification	*In situ* transesterification
1	Low heating value	Heating value is high
2	Product yield is low	Higher product yield
3	Process is complex and time taking	Quick and simple operation process
4	Lipid loss during process	Avoided potential lipid loss
5	Waste water pollutes the environment	Reduced waste water pollutants
6	Production cost is high	Absence of harvesting and dewatering lowers the cost

BIOETHANOL PRODUCTION

Several researchers have been reported bioethanol production from certain species of algae, which produce high levels of carbohydrates as reserve polymers. Owing to the presence of low lignin and hemicelluloses content in algae in comparison to lignocellulosic biomass, the algal biomass have been considered more suitable for the bioethanol production (Chen et al., 2013). Recently, attempts have been made (for the bioethanol production) through the fermentation process using algae as the feedstocks to make it as an alternative to conventional crops such as corn and soyabean (Singh et al., 2011; Nguyen and Vu, 2012; Chaudhary et al., 2014). A comparative study of algal biomass and terrestrial plants for the production of bioethanol has been given in **Table 3**. There are different micro and macroalgae such as *Chlorococcum* sp., *Prymnesium parvum*, *Gelidium amansii*, *Gracilaria* sp., *Laminaria* sp., *Sargassum* sp., and *Spirogyra* sp., which have been used for the bioethanol production (Eshaq et al., 2011; Rajkumar et al., 2014). These algae usually require light, nutrients, and carbon dioxide, to produce high levels of polysaccharides such as starch and cellulose. These polysaccharides can be extracted to fermentable sugars through hydrolysis and further fermentation to bioethanol and separated through distillation as shown in **Figure 3**.

PRE-TREATMENT AND SACCHARIFICATION

It has been reported that, the cell wall of some species of green algae like *Spirogyra* and *Chlorococcum* contain high level of polysaccharides. Microalgae such as *C. vulgaris* contains about 37% of starch on dry weight basis, which is the best source for bioethanol with 65% conversion efficiency (Eshaq et al., 2010; Lam and Lee, 2012). Such polysaccharide based biomass requires additional processing like pre-treatment and saccharification before fermentation (Harun et al., 2010). Saccharification and fermentation can also be carried out simultaneously using an amylase enzyme producing strain for the production of ethanol in a single step. Bioethanol from microalgae can be produced through the process, which is similar to the first generation technologies involving corn based feedstocks. However, there is limited literature available on the fermentation process of microalgae biomass for the production of bioethanol (Schenk et al., 2008; John et al., 2011).

The pre-treatment is an important process, which facilitates accessibility of biomass to enzymes to release the monosaccharides. Acid pre-treatment is widely used for the conversion of polymers present in the cell wall to simple forms. The energy consumption in the pre-treatment is very low and also it is an efficient process (Harun and Danquah, 2011a,b). Yazdani et al. (2011) found 7% (w/w) H_2SO_4 as the promising concentration for the pre-treatment of the brown macroalgae *Nizimuddinia zanardini* to obtain high yield of sugars without formation of any inhibitors. Candra and Sarinah (2011) studied the bioethanol production using red seaweed *Eucheuma cottonii* through acid hydrolysis. In this study, 5% H_2SO_4 concentration was used for 2 h at 100°C, which yielded 15.8 g/L of sugars. However, there are other alternatives to chemical hydrolysis such as enzymatic digestion and gamma radiation to make it more sustainable (Chen et al., 2012; Yoon et al., 2012; Schneider et al., 2013).

Table 3 | Comparative study between algal biomass and terrestrial plants for bioethanol production.

Feedstock	Conditions	Bioethanol	Reference
ALGAE			
Chlorococcum infusionum	Alkaline pre-treatment, temp. 120°C, *S. cerevisiae*	260 g ethanol/kg algae	Harun et al. (2011)
Spirogyra	Alkaline pre-treatment, synthetic media growth, saccharification of biomass by *Aspergillus niger*, fermentation by *S. cerevisiae*	80 g ethanol/kg algae	Eshaq et al. (2010)
Chlorococcum humicola	Acid pre-treatment, temp. 160°C, *S. cerevisiae*	520 g ethanol/kg microalgae	Harun and Danquah (2011a)
TERRESTRIAL PLANTS			
Madhuca latifolia	Strain *Zymomonas mobilis* MTCC 92, immobilized in *Luffa cylindrical* sponge disks, temp. 30°C	251.1 ± 0.012 g ethanol/kg flowers	Behera et al. (2011)
Manihot esculenta	Enzyme termamyl and amyloglucosidase, 1 N HCl, *Saccharomyces cerevisiae*, ca-alginate immobilization	189 ± 3.1 g ethanol/kg flour cassava	Behera et al. (2014)
Sugarcane bagasse	Acid (H_2SO_4) hydrolysis, *Kluyveromyces* sp. IIPE453, Fermentation at 50°C	165 g ethanol/kg bagasse	Kumar et al., 2014
Rice straw	Cellulase, β-glucosidase, solid state fermentation, strain *Trichoderma reesei* RUT C30, and *Aspergillus niger* MTCC 7956	93 g ethanol/kg pretreated rice straw	Sukumaran et al. (2008)

Similar to starch, there are certain polymers such as alginate, mannitol, and fucoidan present in the cell wall of various algae, which requires additional processing like pre-treatment and saccharification before fermentation. Another form of storage carbohydrate found in various brown seaweeds and microalgae is laminarin, which can be hydrolyzed by β-1,3-glucanases or laminarinases (Kumagai and Ojima, 2010). Laminarinases can be categorized into two groups such as exo- and endo-glucanases based on the mode of hydrolysis, which usually produces glucose and smaller oligosaccharides as the end product. Both the enzymes are necessary for the complete digestion of laminarin polymer (Lee et al., 2014b).

Markou et al. (2013) saccharified the biomass of *Spirulina* (*Arthrospira platensis*), fermented the hydrolyzate and obtained the maximum ethanol yield of 16.32 and 16.27% ($g_{ethanol}/g_{biomass}$) produced after pre-treatment with 0.5 N HNO_3 and H_2SO_4, respectively. Yanagisawa et al. (2011) investigated the content of polysaccharide materials present in three types of seaweeds such as sea lettuce (*Ulva pertusa*), chigaiso (*Alaria crassifolia*), and agar weed (*Gelidium elegans*). These seaweeds contain no lignin, which is a positive signal for the hydrolysis of polysaccharides without any pre-treatment. Singh and Trivedi (2013) used *Spirogyra* biomass for the production of bioethanol using *Saccharomyces cerevisiae* and *Zymomonas mobilis*. In a method, they followed acid pre-treatment of algal biomass and further saccharified using α-amylase producing *Aspergillus niger*. In another method, they directly saccharified the biomass without any pre-treatment. The direct saccharification process resulted in 2% (w/w) more alcohol in comparison to pretreated and saccharified algal biomass. This study revealed that the pre-treatment with different chemicals are not required in case of *Spyrogyra*, which reflects its economic importance for the production of ethanol. Also, cellulase enzyme has been used for the saccharification of algal biomass containing cellulose. However, this enzyme system is more expensive than amylases and glucoamylases, and doses required for effective cellulose saccharification are usually very high. Trivedi et al. (2013) applied different cellulases on green alga *Ulva* for saccharification and found highest conversion efficiency of biomass into reducing sugars by using cellulase 22119 rather than viscozyme L, cellulase 22086 and 22128. In this experiment, they found a maximum yield of sugar 206.82 ± 14.96 mg/g with 2% (v/v) enzyme loading for 36 h at a temperature of 45°C.

FERMENTATION

There are different groups of microorganisms like yeast, bacteria, and fungi, which can be used for the fermentation of the pretreated and saccharified algal biomass under anaerobic process for the production of bioethanol (Nguyen and Vu, 2012). Nowadays, *S. cerevisiae* and *Z. mobilis* have been considered as the bioethanol fermenting microorganisms. However, fermentation of mannitol, a polymer present in certain algae is not possible in anaerobic condition using these well known microorganisms and requires supply of oxygen during fermentation, which is possible only by *Zymobacter palmae* (Horn et al., 2000).

Certain marine red algae contain agar, a polymer of galactose and galactopyranose, which can be used for the production of bioethanol (Yoon et al., 2010). The biomass of red algae can be depolymerized into different monomeric sugars like glucose and galactose. In addition to mannitol and glucose, brown seaweeds contain about 14% of extra carbohydrates in the form of alginate (Wargacki et al., 2012). Horn et al. (2000) reported the presence of alginate, laminaran, mannitol, fucoidan, and cellulose in some brown seaweeds, which are good source of sugars. They fermented brown seaweed extract having mannitol using bacteria *Z. palmae* and obtained an ethanol yield of about 0.38 g ethanol/g mannitol.

In the literature, there are many advantages supporting microalgae as the promising substrate for bioethanol production. Hon-Nami (2006) used *Chlamydomonas perigranulata* algal culture and obtained different by-products such as ethanol and butanediol. Similarly, Yanagisawa et al. (2011) obtained glucose

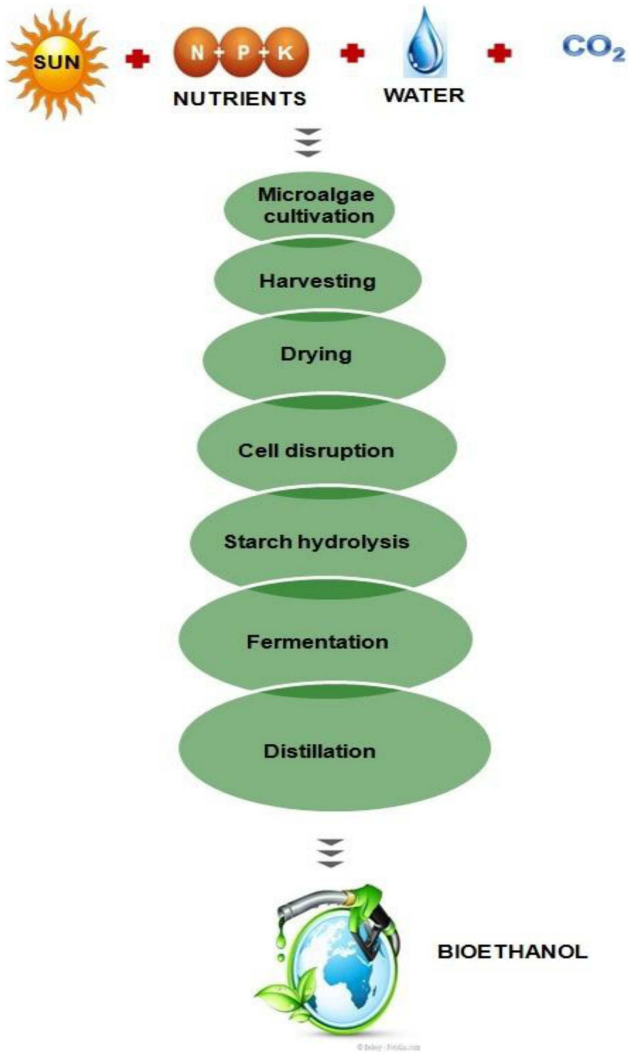


FIGURE 3 | Process for bioethanol production from microalgae.

and galactose through the saccharification of agar weed (red seaweed) containing glucan and galactan and obtained 5.5% of ethanol concentration through fermentation using *S. cerevisiae* IAM 4178. Harun et al. (2010) obtained 60% more ethanol in case of lipid extracted microalgal biomass rather than intact algal biomass of *Chlorococcum* sp. This shows the importance of algal biomass for the production of both biodiesel and bioethanol.

BIOGAS PRODUCTION

Recently, biogas production from algae through anaerobic digestion has received a remarkable attention due to the presence of high polysaccharides (agar, alginate, carrageenan, laminaran, and mannitol) with zero lignin and low cellulose content. Mostly, seaweeds are considered as the excellent feedstock for the production of biogas. Several workers have demonstrated the fermentation of various species of algae like *Scenedesmus*, *Spirulina*, *Euglena*, and *Ulva* for biogas production (Samson and Leduy, 1986; Yen and Brune, 2007; Ras et al., 2011; Zhong et al., 2012; Saqib et al., 2013). The production of biogas using algal biomass in comparison to some terrestrial plants is shown in **Table 4**.

Biogas is produced through the anaerobic transformation of organic matter present in the biodegradable feedstock such as marine algae, which releases certain gases like methane, carbon dioxide, and traces of hydrogen sulfide. The anaerobic conversion process involves basically four main steps. In the first step, the insoluble organic material and higher molecular mass compounds such as lipids, carbohydrates, and proteins are hydrolyzed into soluble organic material with the help of enzyme released by some obligate anaerobes such as *Clostridia* and *Streptococci*. The second step is called as acidogenesis, which releases volatile fatty acids (VFAs) and alcohols through the conversion of soluble organics with the involvement of enzymes secreted by the acidogenic bacteria. Further, these VFAs and alcohols are converted into acetic acid and hydrogen using acetogenic bacteria through the process of acetogenesis, which finally metabolize to methane and carbon dioxide by the methanogens (Cantrell et al., 2008; Vergara-Fernandez et al., 2008; Brennan and Owende, 2010; Romagnoli et al., 2011).

Sangeetha et al. (2011) reported the anaerobic digestion of green alga *Chaetomorpha litorea* with generation of 80.5 L of biogas/kg of dry biomass under 299 psi pressure. Vergara-Fernandez et al. (2008) evaluated digestion of the marine algae *Macrocystis pyrifera* and *Durvillaea antarctica* marine algae in a two-phase anaerobic digestion system and reported similar biogas productions of 180.4 ($\pm$1.5) mL/g dry algae/day with a methane concentration around 65%. However, in case of algae blend, same methane content was observed with low biogas yield. Mussgnug et al. (2010) reported biogas production from some selected green algal species like *Chlamydomonas reinhardtii* and *Scenedesmus obliquus* and obtained 587 and 287 mL biogas/g of volatile solids, respectively. Further, there are few studies, which have been conducted with microalgae showing the effect of different pretreatment like thermal, ultrasound, and microwave for the high production of biogas (Gonzalez-Fernandez et al., 2012a,b; Passos et al., 2013).

However, there are different factors, which limit the biogas production such as requirement of larger land area, infrastructure, and heat for the digesters (Collet et al., 2011; Jones and Mayfield, 2012). The proteins present in algal cells increases the ammonium production resulting in low carbon to nitrogen ratio, which affects biogas production through the inhibition of growth of anaerobic microorganisms. Also, anaerobic microorganisms are inhibited by the sodium ions. Therefore, it is recommended to use the salt tolerating microorganisms for the anaerobic digestion of algal biomass (Yen and Brune, 2007; Brennan and Owende, 2010; Jones and Mayfield, 2012).

BIOHYDROGEN PRODUCTION

Recently, algal biohydrogen production has been considered to be a common commodity to be used as the gaseous fuels or electricity generation. Biohydrogen can be produced through different processes like biophotolysis and photo fermentation (Shaishav et al., 2013). Biohydrogen production using algal biomass is comparative to that of terrestrial plants (**Table 5**). Park et al. (2011) found *Gelidium amansii* (red alga) as the potential source of biomass for the production of biohydrogen through anaerobic

Table 4 | Comparative study between algal biomass and terrestrial plants for biogas production.

Feedstock	Conditions	Biogas	Reference
ALGAE			
Blue algae	pH-6.8, microcystin (MC) biodegradation	189.89 mL/g of VS	Yuan et al. (2011)
Chlamydomonas reinhardtii	Drying as the pre-treatment, batch fermentation, temp. 38°C	587 mL/g of VS	Mussgnug et al. (2010)
Scenedesmus obliquus		287 mL/g of VS	
Ulva sp.	Batch reactor, Co-digestion with bovine slurry, temp. 35°C	191 mL/g of VS	Vanegas and Bartlett (2013)
Laminaria digitata		246 mL/g of VS	
Saccorhiza polyschides		255 mL/g of VS	
Saccharina latissima		235 mL/g of VS	
TERRESTRIAL PLANTS			
Banana stem	Pre-treatment: 6% NaOH in 55°C for 54 h. 37 ± 1°C for 40 days, batch	357.9 mL/g of VS	Zhang (2013)
Saline creeping wild ryegrass	35°C for 33 days, batch	251 mL/g of VS	Zheng (2009)
Rice straw	Pre-treatment: ammonia conc. 4% and moisture content 70%, temp. 35 ± 2°C, 65 days, 120 rpm, batch	341.35 mL/g of VS	Yuan (2014)
Date palm tree wastes	Pre-treatment: alkaline, particle size 2–5 mm, temp. 40°C	342.2 mL/g of VS	Al-Juhaimi (2014)

Table 5 | Comparative study between algal biomass and terrestrial plants for biohydrogen production.

Feedstock	Conditions	Biohydrogen	Reference
ALGAE			
Gelidium amansii	Hydrolysis at 150°C	53.5 mL of H_2/g of dry algae	Park et al. (2011)
Laminaria japonica	Mesophilic condition (35 ± 1°C), pH of 7.5, anaerobic sequencing batch reactor, hydraulic retention time (HRT) of 6 days	71.4 mL H_2/g of dry algae	Shi et al. (2011)
TERRESTRIAL PLANTS			
Bagasse	Strain *Klebsiella oxytoca* HP1, temp. 37.5°C, pH-7	107.8 ± 7.5 mL H_2/g bagasse	Wu et al. (2010)
Corn stalk	Temp. 55°C, pH-7.4	61.4 mL/g of cornstalk	Cheng and Liu (2011)
Pretreated wheat straw	Strain *Caldicellulosiruptor saccharolyticus*, Temp. 70°C, pH-7.2	44.7 mL/g of dry wheat straw	Ivanova et al. (2009)
Wheat straw	Acid pre-treatment, simultaneous saccharification and fermentation (SSF)	141 mL/g VS	Nasirian et al. (2011)

fermentation. Nevertheless, they found 53.5 mL of H_2 from 1 g of dry algae with a hydrogen production rate of 0.518 L H_2/g VSS/day. The authors found an inhibitor, namely, 5-hydroxymethylfurfural (HMF) produced through the acid hydrolysis of *G. amansii* that decreases about 50% of hydrogen production due to the inhibition. Thus, optimization of the pre-treatment method is an important step to maximize biohydrogen production, which will be useful for the future direction (Park et al., 2011; Shi et al., 2011). Saleem et al. (2012) reduced the lag time for hydrogen production using microalgae *Chlamydomonas reinhardtii* by the use of optical fiber as an internal light source. In this study, the maximum rate of hydrogen production in the presence of exogenic glucose and optical fiber was reported to be 6 mL/L culture/h, which is higher than other reported values.

Some of microalgae like blue green algae have glycogen instead of starch in their cells. This is an exception, which involves oxidation of ferrodoxin by the hydrogenase enzyme activity for the production of hydrogen in anaerobic condition. However, another function of this enzyme is to be involved in the detachment of electrons. Therefore, different researchers have focused for the identification of these enzyme activities having interactions with ferrodoxin and the other metabolic functions for microalgal photobiohydrogen production. They are also involved with the change of these interactions genetically to enhance the biohydrogen production (Gavrilescu and Chisti, 2005; Hankamer et al., 2007; Wecker et al., 2011; Yacoby et al., 2011; Rajkumar et al., 2014).

BIO-OIL AND SYNGAS PRODUCTION

Bio-oil is formed in the liquid phase from algal biomass in anaerobic condition at high temperature. The composition of bio-oil varies according to different feedstocks and processing conditions, which is called as pyrolysis (Iliopoulou et al., 2007; Yanqun et al., 2008). There are several parameters such as water, ash content, biomass composition, pyrolysis temperature, and vapor residence time, which affect the bio-oil productivity (Fahmi et al., 2008). However, due to the presence of water, oxygen content, unsaturated and phenolic moieties, crude bio-oil cannot be used as fuel. Therefore, certain treatments are required to improve its quality

(Bae et al., 2011). Bio-oils can be processed for power generation with the help of external combustion through steam and organic rankine cycles, and stirling engines. However, power can also be generated through internal combustion using diesel and gas-turbine engines (Chiaramonti et al., 2007). In literature, there are limited studies on algae pyrolysis compared to lignocellulosic biomass. Although, high yields of bio-oil occur through fluidized-bed fast pyrolysis processes, there are several other pyrolysis modes, which have been introduced to overcome their inherent disadvantages of a high level of carrier gas flow and excessive energy inputs (Oyedun et al., 2012). Demirbas (2006) investigated suitability of the microalgal biomass for bio-oil production and found the superior quality than the wood. Porphy and Farid (2012) produced bio-oil from pyrolysis of algae (*Nannochloropsis* sp.) at 300°C after lipid extraction, which composed of 50 wt% acetone, 30 wt% methyl ethyl ketone, and 19 wt% aromatics such as pyrazine and pyrrole. Similarly, Choi et al. (2014) carried out pyrolysis study on a species of brown algae *Saccharina japonica* at a temperature of 450°C and obtained about 47% of bio-oil yield.

Gasification is usually performed at high temperatures (800–1000°C), which converts biomass into the combustible gas mixture through partial oxidation process, called syngas or producer gas. Syngas is a mixture of different gases like CO, CO_2, CH_4, H_2, and N_2, which can also be produced through normal gasification of woody biomass. In this process, biomass reacts with oxygen and water (steam) to generate syngas. It is a low calorific gas, which can be utilized in the gas turbines or used directly as fuel. Different variety of biomass feedstocks can be utilized for the production of energy through the gasification process, which is an added advantage (Carvalho et al., 2006; Prins et al., 2006; Lv et al., 2007).

CONCLUSION AND FUTURE PERSPECTIVES

Recently, it is a challenge for finding different alternative resources, which can replace fossil fuels. Due to presence of several advantages in algal biofuels like low land requirement for biomass production and high oil content with high productivity, it has been considered as the best resource, which can replace the liquid petroleum fuel. However, one of its bottlenecks is the low biomass production, which is a barrier for industrial production. Also, another disadvantage includes harvesting of biomass, which possesses high energy inputs. For an economic process development in comparison to others, a cost-effective and energy efficient harvesting methods are required with low energy input. Producing low-cost microalgal biofuels requires better biomass harvesting methods, high biomass production with high oil productivity through genetic modification, which will be the future of algal biology. Therefore, use of the standard algal harvesting technique, biorefinery concept, advances in photobioreactor design and other downstream technologies will further reduce the cost of algal biofuel production, which will be a competitive resource in the near future.

ACKNOWLEDGMENTS

The authors are thankful to Prof. Y. K. Yadav, Director, NIRE, Kapurthala for his consistent support to write this review paper. The authors greatly acknowledge the Ministry of New and Renewable Energy, New Delhi, Govt. of India, for providing funds to carry out research work.

REFERENCES

Afify, A. M. M., Shanab, S. M., and Shalaby, E. A. (2010). Enhancement of biodiesel production from different species of algae. *Grasas y Aceites* 61, 416–422. doi:10.3989/gya.021610

Al-Juhaimi, F. Y. (2014). Biogas production through the anaerobic digestion of date palm tree wastes – process optimization. *Bioresources* 9, 3323–3333. doi:10.15376/biores.9.2

Awolu, O. O., and Layokun, S. K. (2013). Optimization of two-step transesterification production of biodiesel from neem (*Azadirachta indica*) oil. *Int. J. Energy Environ.* 4, 39. doi:10.1186/2251-6832-4-39

Bae, Y. J., Ryu, C., Jeon, J. K., Park, J., Suh, D. J., Suh, Y. W., et al. (2011). The characteristics of bio-oil produced from the pyrolysis of three marine macroalgae. *Bioresour. Technol.* 102, 3512–3520. doi:10.1016/j.biortech.2010.11.023

Behera, S., Mohanty, R. C., and Ray, R. C. (2011). Ethanol production from mahula (*Madhuca latifolia* L.) flowers using free and immobilized (in *Luffa cylindrical* L. sponge discs) cells of *Zymomonas mobilis* MTCC 92. *Ann. Microbiol.* 61, 469–474. doi:10.1007/s13213-010-0160-y

Behera, S., Mohanty, R. C., and Ray, R. C. (2014). Batch ethanol production from cassava (*Manihot esculenta* Crantz.) flour using *Saccharomyces cerevisiae* cells immobilized in calcium alginate. *Ann. Microbiol.* doi:10.1007/s13213-014-0918-8

Bisen, P. S., Sanodiya, B. S., Thakur, G. S., Baghel, R. K., and Prasad, G. B. K. S. (2010). Biodiesel production with special emphasis on lipase-catalyzed transesterification. *Biotechnol. Lett.* 32, 1019–1030. doi:10.1007/s10529-010-0275-z

Bosma, R., Van Spronsen, W. A., Tramper, J., and Wijffels, R. H. (2003). Ultrasound a new s separation technique to harvest microalgae. *J. Appl. Phycol.* 15, 143–153. doi:10.1023/A:1023807011027

Boyce, A. N., Chowd-Hury, P., and Naqiuddin, M. (2008). Biodiesel fuel production from algae as renewable energy. *Am. J. Biochem. Biotechnol.* 4, 250–254. doi:10.3844/ajbbsp.2008.250.254

Brennan, L., and Owende, P. (2010). Biofuels from microalgae-a review of technologies for production, processing, and extractions of biofuels and co-products. *Renew. Sustain. Energ. Rev.* 14, 557–577. doi:10.1016/j.rser.2009.10.009

Candra, K. P., and Sarinah, S. (2011). Study on bioethanol production using red seaweed *Eucheuma cottonii* from Bontang sea water. *J. Coastal Dev.* 15, 45–50. doi:10.4172/1410-5217.10000328

Cantrell, K. B., Ducey, T., Ro, K. S., and Hunt, P. G. (2008). Livestock waste-to-bioenergy generation opportunities. *Bioresour. Technol.* 99, 7941–7953. doi:10.1016/j.biortech.2008.02.061

Carvalho, A. P., Meireles, L. A., and Malcata, F. X. (2006). Microalgal reactors: a review of enclosed system designs and performances. *Biotechnol. Prog.* 22, 1490–1506. doi:10.1002/bp060065r

Chaudhary, L., Pradhan, P., Soni, N., Singh, P., and Tiwari, A. (2014). Algae as a feedstock for bioethanol production: new entrance in biofuel world. *Int. J. Chem. Technol. Res.* 6, 1381–1389.

Chen, C. Y., Zhao, X. Q., Yen, H. W., Ho, S. H., Cheng, C. L., Bai, F., et al. (2013). Microalgae-based carbohydrates for biofuel production. *Biochem. Eng. J.* 78, 1–10. doi:10.1016/j.bej.2013.03.006

Chen, R., Yue, Z., Deitz, L., Liu, Y., and Liao, W. (2012). Use of an algal hydrolysate to improve enzymatic hydrolysis of lignocellulose. *Bioresour. Technol.* 108, 149–154. doi:10.1016/j.biortech.2011.12.143

Cheng, X. Y., and Liu, C. Z. (2011). Hydrogen production via thermophilic fermentation of cornstalk by *Clostridium thermocellum*. *Energy Fuel* 25, 1714–1720. doi:10.1021/ef2000344

Chiaramonti, D., Oasmaa, A., and Solantausta, Y. (2007). Power generation using fast pyrolysis liquids from biomass. *Renew. Sustain. Energ. Rev.* 11, 1056–1086. doi:10.1016/j.rser.2005.07.008

Chisti, Y. (2007). Biodiesel from microalgae. *Biotechnol. Adv.* 25, 294–306. doi:10.1016/j.biotechadv.2007.02.001

Choi, J. H., Woo, H. C., and Suh, D. J. (2014). Pyrolysis of seaweeds for bio-oil and bio-char production. *Chem. Eng. Trans.* 37, 121–126. doi:10.1016/j.biortech.2014.09.068

Choi, W., Han, J., Lee, C., Song, C., Kim, J., Seo, Y., et al. (2012). Bioethanol production from *Ulva pertusa* kjellman by high-temperature liquefaction. *Chem. Biochem. Eng.* 26, 15–21.

Clark, J., and Deswarte, F. (2008). *Introduction to Chemicals from Biomass.* Wiley series in renewable resources. Hoboken: John Wiley & Sons.

Cohen, Z., Norman, H. A., and Heimer, Y. M. (1995). Microalgae as a source of x-3 fatty acids. *World Rev. Nutr. Diet.* 77, 1–31.

Collet, P., Helias, A., Lardon, L., Ras, M., Goy, R. A., and Steyer, J. P. (2011). Life-cycle assessment of microalgae culture coupled to biogas production. *Bioresour. Technol.* 102, 207–214. doi:10.1016/j.biortech.2010.06.154

Crutzen, P. J., Mosier, A. R., Smith, K. A., and Winiwarter, W. (2007). N_2O release from agro-biofuel production negates global warming reduction by replacing fossil fuels. *Atmos. Chem. Phys.* 7, 11191–11205. doi:10.5194/acpd-7-11191-2007

Csordas, A., and Wang, J. (2004). An integrated photobioreactor and foam fractionation unit for growth and harvest of *Chaetoceros* sp in open systems. *Aquac. Eng.* 30, 15–30. doi:10.1016/j.aquaeng.2003.07.001

Demirbas, A. (2006). Oily products from mosses and algae via pyrolysis. *Energy Source* 28, 933–940. doi:10.1080/009083190910389

Demirbas, A. (2007). Progress and recent trends in biofuels. *Prog. Energy Combust. Sci.* 33, 1–18. doi:10.1016/j.pecs.2006.06.001

Demirbas, A. (2009). Progress and recent trends in biodiesel fuels. *Energy Convers. Manag.* 50, 14–34. doi:10.1016/j.enconman.2008.09.001

Demirbas, A. (2010). Use of algae as biofuel sources. *Energy Convers. Manag.* 51, 2738–2749. doi:10.1016/j.enconman.2010.06.010

Dismukes, G. C., Carrieri, D., Bennette, N., Ananyev, G. M., and Posewitz, M. C. (2008). Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. *Curr. Opin. Biotechnol.* 19, 235–240. doi:10.1016/j.copbio.2008.05.007

Dragone, G., Fernandes, B., Vicente, A. A., and Teixeira, J. A. (2010). "Third generation biofuels from microalgae," in *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*, ed. A. Mendez-Vilas (Madrid: Formatex), 1315–1366

Eshaq, F. S., Ali, M. N., and Mohd, M. K. (2010). *Spirogyra* biomass a renewable source for biofuel (bioethanol) production. *Int. J. Eng. Sci. Technol.* 2, 7045–7054.

Eshaq, F. S., Ali, M. N., and Mohd, M. K. (2011). Production of bioethanol from next generation feed-stock alga *Spirogyra* species. *Int. J. Eng. Sci. Technol.* 3, 1749–1755.

Fahmi, R., Bridgwater, A. V., Donnison, I., and Yates, N. (2008). The effect of lignin and inorganic species in biomass on pyrolysis oil yield, quality and stability. *Fuel* 87, 1230–1240. doi:10.1016/j.fuel.2007.07.026

Fajardo, A. R., Cerdan, L. E., Medina, A. R., Fernandez, F. G. A., Moreno, P. A. G., and Grima, E. M. (2007). Lipid extraction from the microalga *Phaeodactylum tricornutum. Eur. J. Lipid Sci. Technol.* 109, 120–126. doi:10.1002/ejlt.200600216

Gavrilescu, M., and Chisti, Y. (2005). Biotechnology – a sustainable alternative for chemical industry. *Biotechnol. Adv.* 23, 471–499. doi:10.1016/j.biotechadv.2005.03.004

Ghadge, S. V., and Raheman, H. (2005). Biodiesel production from mahua (*Madhuca indica*) oil having high free fatty acids. *Biomass Bioenergy* 28, 601–605. doi:10.1016/j.biombioe.2004.11.009

Goldemberg, J. (2007). Ethanol for a sustainable energy future. *Science* 315, 808–810. doi:10.1126/science.1137013

Goldemberg, J., and Guardabassi, P. (2009). Are biofuels a feasible option? *Energy Policy* 37, 10–14. doi:10.1016/j.enpol.2008.08.031

Gonzalez-Fernandez, C., Sialve, B., Bernet, N., and Steyer, J. P. (2012a). Comparison of ultrasound and thermal pretreatment of *Scenedesmus* biomass on methane production. *Bioresour. Technol.* 110, 610–616. doi:10.1016/j.biortech.2012.01.043

Gonzalez-Fernandez, C., Sialve, B., Bernet, N., and Steyer, J. P. (2012b). Thermal pretreatment to improve methane production of *Scenedesmus* biomass. *Biomass Energy* 40, 105–111. doi:10.1016/j.biombioe.2012.02.008

Haas, M. I., and Wagner, K. (2011). Simplifying biodiesel production: the direct or in situ transesterification of algal biomass. *Eur. J. Lipid Sci. Technol.* 113, 1219–1229. doi:10.1002/ejlt.201100106

Hankamer, B., Lehr, F., Rupprecht, J., Mussgnug, J. H., Posten, C., and Kruse, O. (2007). Photosynthetic biomass and H_2 production by green algae: from bioengineering to bioreactor scale up. *Physiol. Plant.* 131, 10–21. doi:10.1111/j.1399-3054.2007.00924.x

Harun, R., and Danquah, M. K. (2011a). Influence of acid pre-treatment on microalgal biomass for bioethanol production. *Process Biochem.* 46, 304–309. doi:10.1016/j.procbio.2010.08.027

Harun, R., and Danquah, M. K. (2011b). Enzymatic hydrolysis of microalgae biomass for bioethanol production. *Chem. Eng. J.* 168, 1079–1084. doi:10.1016/j.cej.2011.01.088

Harun, R., Danquah, M. K., and Forde-Gareth, M. (2010). Microalgal biomass as a fermentation feedstock for bioethanol production. *J. Chem. Technol. Biotechnol.* 85, 199–203. doi:10.1002/jctb.2287

Harun, R., Jason, W. S. Y., Cherrington, T., and Danquah, M. K. (2011). Exploring alkaline pretreatment of microalgal biomass for bioethanol production. *Appl. Energy* 88, 3464–3467. doi:10.1016/j.apenergy.2010.10.048

Heasman, M., Diemar, J. O., Connor, W., Shushames, T., Foulkes, L., and Nell, J. (2008). Development of extended shelf-life microalgae concentrate diets harvested by centrifugation for bivalve molluscs – a summary. *Aquac. Res.* 31, 637–659. doi:10.1046/j.1365-2109.2000.318492.x

Hidalgo, P., Toro, C., Ciudad, G., and Navia, R. (2013). Advances in direct transesterification microalgal biomass for biodiesel production. *Rev. Environ. Sci. Biotechnol.* 12, 179–199. doi:10.1007/s11157-013-9308-0

Ho, S. H., Chen, C. Y., Lee, D. J., and Chang, J. S. (2011). Perspectives on microalgal Co_2-emission mitigation systems-a review. *Biotechnol. Adv.* 29, 189–198. doi:10.1016/j.biotechadv.2010.11.001

Hon-Nami, K. (2006). A unique feature of hydrogen recovery in endogenous starch to alcohol fermentation of the marine microalga *Chlamydomonas perigranulata. Appl. Biochem. Biotechnol.* 131, 808–828. doi:10.1385/ABAB:131:1:808

Horn, H. J., Aasen, I. M., and Østgaard, M. (2000). Production of ethanol from mannitol by *Zymobacter palmae. J. Ind. Microbiol. Biotechnol.* 24, 51–57. doi:10.1038/sj.jim.2900771

Hossain, A. B. M. S., Salleh, A., Boyce, A. N., Chowd-Hury, P., and Naqiuddin, M. (2008). Biodiesel fuel production from algae as renewable energy. *Am. J. Biochem. Biotechnol.* 4, 250–254. doi:10.3844/ajbbsp.2008.250.254

Hsueh, H. T., Chu, H., and Yu, S. T. (2007). A batch study on the bio-fixation of carbon dioxide in the absorbed solution from a chemical wet scrubber by hot spring and marine algae. *Chemosphere* 66, 878–886. doi:10.1016/j.chemosphere.2006.06.022

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., et al. (2008). Microalgal triacylglycerols as feedstocks for biofuels production: perspectives and advances. *Plant J.* 54, 621–639. doi:10.1111/j.1365-313X.2008.03492.x

Hughes, A. D., Kelly, M. S., Black, K. D., and Stanley, M. S. (2012). Biogas from macroalgae: is it time to revisit the idea? *Biotechnol. Biofuels* 5, 1–7. doi:10.1186/1754-6834-5-86

Iliopoulou, E. F., Antonakou, E. V., Karakoulia, S. A., Vasalos, I. A., Lappas, A. A., and Triantafyllidis, K. S. (2007). Catalytic conversion of biomass pyrolysis products by mesoporous materials: effect of steam stability and acidity of Al-MCM-41 catalysts. *Chem. Eng. J.* 134, 51–57. doi:10.1016/j.cej.2007.03.066

Ivanova, G., Rakhely, G., and Kovacs, K. (2009). Thermophilic biohydrogen production from energy plants by *Caldicellulosiruptor saccharolyticus* and comparison with related studies. *Int. J. Hydrogen Energy* 34, 3659–3670. doi:10.1016/j.ijhydene.2009.02.082

John, R. P., Anisha, G. S., Nampoothiri, K. M., and Pandey, A. (2011). Micro and macroalgal biomass: a renewable source for bioethanol. *Bioresour. Technol.* 102, 186–193. doi:10.1016/j.biortech.2010.06.139

Jones, C. S., and Mayfield, S. P. (2012). Algae biofuels: versatility for the future of bioenergy. *Curr. Opin. Biotechnol.* 23, 346–351. doi:10.1016/j.copbio.2011.10.013

Kim, G. V., Choi, W. Y., Kang, D. H., Lee, S. Y., and Lee, H. Y. (2014). Enhancement of biodiesel production from marine alga, *Scenedesmus* sp. through in situ transesterification process associated with acidic catalyst. *BioMed. Res. Int.* 2014:391542 doi:10.1155/2014/391542

Knezevic, Z. D., Siler-Marinkovic, S. S., and Mojovic, L. V. (2004). Immobilized lipases as practical catalysts. *Acta Period. Technol.* 35, 151–164. doi:10.2298/APT0435151K

Kraan, S. (2013). Mass cultivation of carbohydrate rich microalgae, a possible solution for sustainable biofuel production. *Mitig. Adapt. Strateg. Glob. Change.* 18, 27–46. doi:10.1007/s11027-010-9275-5

Kumagai, Y., and Ojima, T. (2010). Isolation and characterization of two types of β-1,3-glucanases from the common sea hare *Aplysia kurodai. Comp. Biochem. Physiol. Biochem. Mol. Biol.* 155, 138–144. doi:10.1016/j.cbpb.2009.10.013

Kumar, S., Dheeran, P., Singh, S. P., Mishra, I. M., and Adhikari, D. K. (2014). Bioprocessing for bagasse hydrolysate for ethanol and xylitol production using thermotolerant yeast. *Biprocess Biosyst. Eng.* 38, 39–47. doi:10.1007/s00449-014-1241-2

Lam, M. K., and Lee, K. T. (2012). Microalgae biofuels: a critical review of issues, problems and the way forward. *Biotechnol. Adv.* 30, 630–690. doi:10.1016/j.biotechadv.2011.11.008

Leach, G., Oliveira, G., and Morais, R. (1998). Spray-drying of *Dunaliella salina* to produce a b-carotene rich powder. *J. Ind. Microbiol. Biotechnol.* 20, 82–85. doi:10.1038/sj.jim.2900485

Lee, A., Lewis, D., and Ashman, P. (2009). Microbial flocculation, a potentially low-cost harvesting technique for marine microalgae for the production of biodiesel. *J. Appl. Phycol.* 21, 559–567. doi:10.1007/s10811-008-9391-8

Lee, K., Eisterhold, M. L., Rindi, F., Palanisami, S., and Nam, P. K. (2014a). Isolation and screening of microalgae from natural habitats in the Midwestern United States of America for biomass and biodiesel sources. *J. Nat. Sci. Biol. Med.* 5, 333–339. doi:10.4103/0976-9668.136178

Lee, K. C., Takamitsu, A., Ibrahim, D., Kosugi, A., Prawitwong, P., Lan, D., et al. (2014b). Purification and characterization of a thermostable laminarinase from *Penicillium rolfsii* c3-2(1) IBRL. *Bioresource* 9, 1072–1084. doi:10.15376/biores.9.1

Lee, S. J., Yoon, B. D., and Oh, H. M. (1998). Rapid method for the determination of lipid from the green alga *Botryococcus braunii*. *Biotechnol. Tech.* 12, 553–556. doi:10.1023/A:1008811716448

Li, Y., Horsman, M., Wu, N., Lan, C. Q., and Dubois-Calero, N. (2008). Biofuels from microalgae. *Biotechnol. Progr.* 24, 815–820. doi:10.1021/bp070371k

Li, Y., Naghdi, F. G., Garg, G., Adarme-Vega, T. C., Thurecht, K. J., Ghafor, W. A., et al. (2014). A comparative study: the impact of different lipid extraction methods on current microalgal lipid research. *Microb. Cell Fact.* 13, 1–9. doi:10.1186/1475-2859-13-14

Liu, J., Zhu, Y., Tao, Y., Zhang, Y., Li, A., Li, T., et al. (2013). Freshwater microalgae harvested via flocculation induced by pH decrease. *Biotechnol. Biofuels* 6, 98. doi:10.1186/1754-6834-6-98

Luo, Y., Zheng, Y., Jiang, Z., Ma, Y., and Wei, D. (2006). A novel psychrophilic lipase from *Pseudomonas fluorescens* with unique property in chiral resolution and biodiesel production via transesterification. *Appl. Microbiol. Biotechnol.* 73, 349–355. doi:10.1007/s00253-006-0478-3

Lv, P., Yuan, Z., Wu, C., Ma, L., Chen, Y., and Tsubaki, N. (2007). Biosyngas production from biomass catalytic gasification. *Energy. Convers. Manag.* 48, 1132–1139. doi:10.1016/j.enconman.2006.10.014

Mamilla, V. R., Mallikarjun, M. V., and Rao, G. L. N. (2011). Preparation of biodiesel from Karanja oil. *Int. J. Energy Eng.* 1, 94–100. doi:10.5963/IJEE0102008

Markou, G., Angelidaki, I., Nerantzis, E., and Georgakakis, D. (2013). Bioethanol production by carbohydrate-enriched biomass of *Arthrospira* (*Spirulina*) *platensis*. *Energies* 6, 3937–3950. doi:10.3390/en6083937

Marques, A. E., Barbosa, A. T., Jotta, J., Coelho, M. C., Tamagnini, P., and Gouveia, L. (2011). Biohydrogen production by *Anabaena* sp. PCC 7120 wild-type and mutants under different conditions: light, nickel, propane, carbon dioxide and nitrogen. *Biomass Bioenergy* 35, 4426–4434. doi:10.1016/j.biombioe.2011.08.014

Martin, M., Pires, R. F., Alves, M. J., Hori, C. E., Reis, M. H. M., and Cardoso, V. L. (2013). Transesterification of soybean oil for biodiesel production using hydrotalcite as basic catalyst. *Chem. Eng. Trans.* 32, 817–822. doi:10.3303/CET1332137

Mata, T. M., Martins, A., and Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: a review. *Ren. Sust. Energy. Rev.* 14, 217–232. doi:10.1016/j.rser.2009.07.020

Mazubert, A., Poux, M., and Aubin, J. (2013). Intensified processes for FAME production from waste cooking oil: a technological review. *Chem. Eng. J.* 233, 201–223. doi:10.1016/j.cej.2013.07.063

Meher, L. C., Vidya, S. D., and Naik, S. N. (2006). Technical aspects of biodiesel production by transesterification – a review. *Renew. Sustain. Energ. Rev.* 10, 248–268. doi:10.1016/j.biotechadv.2014.10.003

Munir, N., Sharif, N., Naz, S., Saleem, F., and Manzoor, F. (2013). Harvesting and processing of microalgae biomass fractions for biodiesel production (a review). *Sci. Tech. Dev.* 32, 235–243.

Mussgnug, J. H., Klassen, V., Schluter, A., and Kruse, O. (2010). Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *J. Biotechnol.* 150, 51–56. doi:10.1016/j.jbiotec.2010.07.030

Mutanda, T., Ramesh, D., Karthikeyan, S., Kumari, S., Anandraj, A., and Bux, F. (2011). Bioprospecting for hyper-lipid producing microalgal strains for sustainable biofuel production. *Bioresour. Technol.* 102, 57–70. doi:10.1016/j.biortech.2010.06.077

Muthukumar, A., Elayaraja, S., Ajithkumar, T. T., Kumaresan, S., and Balasubramanian, T. (2012). Biodiesel production from marine microalgae *Chlorella marina* and *Nannochloropsis salina*. *J. Petrol. Technol Altern. Fuels* 3, 58–62. doi:10.5897/JPTAF12.010

Naik, M., Meher, L. C., Waik, S. N., and Das, L. M. (2008). Production of biodiesel from high free fatty acid Karanja (*Pongamia pinnata*) oil. *Biomass Bioenergy* 32, 354–357. doi:10.1016/j.biombioe.2007.10.006

Nascimento, I. A., Marques, S. S. I., Cabanelas, I. T. D., Pereira, S. A., Druzian, J. I., de Souza, C. O., etal. (2013). Screening microalgae strains for biodiesel production: lipid productivity and estimation of fuel quality based on fatty acids profiles as selective criteria. *Bioenerg. Res.* 6, 1–13. doi:10.1007/s12155-012-9222-2

Nasirian, N., Almassi, M., Minaei, S., and Widmann, R. (2011). Development of a method for biohydrogen production from wheat straw by dark fermentation. *Int. J. Hydrogen Energy* 36, 411–420. doi:10.1016/j.ijhydene.2010.09.073

Nautiyal, P., Subramanian, K. A., and Dastidar, M. G. (2014). Kinetic and thermodynamic studies on biodiesel production from *Spirulina platensis* algae biomass using single stage extraction-transesterification process. *Fuel* 135, 228–234. doi:10.1016/j.fuel.2014.06.063

Nguyen, T. H. M., and Vu, V. H. (2012). Bioethanol production from marine algae biomass: prospect and troubles. *J. Viet. Env.* 3, 25–29. doi:10.13141/jve.vol3.no1

Nigam, P. S., and Singh, A. (2011). Production of liquid biofuels from renewable resources. *Prog. Energy Combust. Sci.* 37, 52–68. doi:10.1016/j.pecs.2010.01.003

Oyedun, A. O., Lam, K. L., Gebreegziabher, T., Lee, H. K. M., and Hui, C. W. (2012). Optimisation of operating parameters in multi-stage pyrolysis. *Chem. Engg. Trans.* 29, 655–660. doi:10.3303/CET1229110

Park, J. H., Yoon, J. J., Park, H. D., Kim, Y. J., Lim, D. J., and Kim, S. H. (2011). Feasibility of biohydrogen production from *Gelidium amansii*. *Int. J. Hydrogen Energy* 36, 13997–14003. doi:10.1016/j.ijhydene.2011.04.003

Passos, F., Sole, M., Garcia, J., and Ferrer, I. (2013). Biogas production from microalgae grown in wastewater: effect of microwave pretreatment. *Appl. Energy* 108, 168–175. doi:10.1016/j.watres.2013.10.013

Patil, P. D., Gude, V. G., Mannarswamy, A., Cooke, P., Nirmalakhandan, N., Lammers, P., et al. (2012). Comparison of direct transesterification of algal biomass under supercritical methanol and microwave irradiation conditions. *Fuel* 97, 822–831. doi:10.1016/j.fuel.2012.02.037

Popoola, T. O. S., and Yangomodou, O. D. (2006). Extraction, properties and utilization potentials of cassava seed oil. *Biotechnology* 5, 38–41. doi:10.3923/biotech.2006.38.41

Porphy, S. J., and Farid, M. M. (2012). Feasibility study for production of biofuel and chemicals from marine microalgae *Nannochloropsis* sp. based on basic mass and energy analysis. *ISRN Renew. Energ.* 2012:156824. doi:10.5402/2012/156824

Prakash, J., Pushparaj, B., Carlozzi, P., Torzillo, G., Montaini, E., and Materassi, R. (2007). Microalgal biomass drying by a simple solar device. *Int. J. Solar Energy* 18, 303–311. doi:10.1080/01425919708914325

Pratoomyot, J., Srivilas, P., and Noiraksar, T. (2005). Fatty acids composition of 10 microalgal species. *J. Sci. Technol.* 27, 1179–1187.

Prins, M. J., Ptasinski, K. J., and Janssen, F. J. J. G. (2006). More efficient biomass gasification via torrefaction. *Energy* 31, 3458–3470. doi:10.1016/j.energy.2006.03.008

Prochazkova, G., Safarik, I., and Branyik, T. (2013). Harvesting microalgae with microwave synthesized magnetic microparticles. *Bioresour. Technol.* 130, 472–477. doi:10.1016/j.biortech.2012.12.060

Rajkumar, R., Yaakob, Z., and Takriff, M. S. (2014). Potential of the micro and macro algae for biofuel production: a brief review. *Bioresour.* 9, 1606–1633. doi:10.15376/biores.9.1

Rajvanshi, S., and Sharma, M. P. (2012). Microalgae: a potential source of biodiesel. *J. Sustain. Bioenergy Syst.* 2, 49–59. doi:10.4236/jsbs.2012.23008

Ras, M., Lardon, L., Bruno, S., Bernet, N., and Steyer, J. P. (2011). Experimental study on a coupled process of production and anaerobic digestion of *Chlorella vulgaris*. *Bioresour. Technol.* 102, 200–206. doi:10.1016/j.biortech.2010.06.146

Richmond, A. (2004). *Handbook of Microalgal Culture: Biotechnology and Applied Phycology*. Osney Mead, Oxford: Blackwell Science Ltd.

Richmond, A., and Qiang, H. (2013). *Handbook of Microalgal Culture: Applied Phycology and Biotechnology*, Second Edn. Hoboken, NJ: Wiley-Blackwell.

Rodolfi, L., Zittelli, G. C., Bassi, N., Padovani, G., Biondi, N., Bonini, G., et al. (2009). Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnol. Bioeng.* 102, 100–112. doi:10.1002/bit.22033

Romagnoli, F., Blumberga, D., and Gigli, E. (2011). Biogas from marine macroalgae: a new environmental technology-life cycle inventory for a further LCA. *Environ. Climate Technol.* 4, 97–108. doi:10.2478/v10145-010-0024-5

Rosenberg, J. N., Oyler, G. A., Wilkinson, L., and Betenbaugh, M. J. (2008). A green light for engineered algae: redirecting metabolism to fuel a biotechnology

revolution. *Curr. Opin. Biotechnol.* 19, 430–436. doi:10.1016/j.copbio.2008.07.008

Rossignol, N., Vandanjon, L., Jaoue, P., and Quemeneur, F. (1999). Membrane technology for the continuous separation of microalgae/culture medium: compared performances of cross-flow microfiltration and ultrafiltration. *Aquac. Eng.* 20, 191–208. doi:10.1016/S0144-8609(99)00018-7

Ryckebosch, E., Muylaert, K., and Foubert, I. (2012). Optimization of an analytical procedure for extraction of lipids from microalgae. *J. Am. Oil Chem. Soc.* 89, 189–198. doi:10.1007/s11746-011-1903-z

Saleem, M., Chakrabarti, M. H., Raman, A. A. A., Hasan, D. B., Daud, W. M. A. W., and Mustafa, A. (2012). Hydrogen production by *Chlamydomonas reinhardtii* in a two-stage process with and without illumination at alkaline pH. *Int. J. Hydrogen Energy* 37, 4930–4934. doi:10.1016/j.ijhydene.2011.12.115

Samson, R., and Leduy, A. (1986). Detailed study of anaerobic digestion of *Spirulina maxima* algal biomass. *Biotechnol. Bioeng.* 28, 1014–1023. doi:10.1002/bit.260280712

Sangeetha, P., Babu, S., and Rangasamy, R. (2011). Potential of green alga *Chaetomorpha litorea* (Harvey) for biogas production. *Int. J. Curr. Sci.* 1, 24–29.

Saqib, A., Tabbssum, M. R., Rashid, U., Ibrahim, M., Gill, S. S., and Mehmood, M. A. (2013). Marine macroalgae *Ulva*: a potential feed-stock for bioethanol and biogas production. *Asian J. Agri. Biol.* 1, 155–163.

Schenk, P., Thomas-Hall, S., Stephens, E., Marx, U., Mussgnug, J., Posten, C., et al. (2008). Second generation biofuels: high efficiency microalgae for biodiesel production. *Bioenergy Res.* 1, 20–43. doi:10.1007/s12155-008-9008-8

Schneider, R. C. S., Bjerk, T. R., Gressler, P. D., Souza, M. P., Corbelline, V. A., and Lobo, E. A. (2013). "Potential production of biofuel from microalgae biomass produced in wastewater," in *Biodiesel – Feedstocks, Production and Applications*, ed. Z. Fang (Rijeka: InTech). doi:10.5772/52439

Shaishav, S., Singh, R. N., and Satyendra, T. (2013). Biohydrogen from algae: fuel of the future. *Int. Res. J. Env. Sci.* 2, 44–47.

Shalaby, E. A. (2011). "Algal biomass and biodiesel production," in *Biodiesel – Feedstocks and Processing Technologies*, ed. S. Margarita (Rijeka: InTech), 111–132. ISBN: 978-953-307-713-0.

Sharma, R., Chisti, Y., and Banerjee, U. C. (2001). Production, purification, characterization, and applications of lipases. *Biotechnol. Adv.* 19, 627–662. doi:10.1016/S0734-9750(01)00086-6

Sharma, Y. C., and Singh, B. (2009). *Development* of biodiesel: current scenario. *Renew. Sustain. Energ. Rev.* 13, 1646–1651. doi:10.1016/j.biortech.2010.06.057

Shi, X., Jung, K. W., Kim, H., Ahn, Y. T., and Shin, H. S. (2011). Direct fermentation of *Laminaria japonica* for biohydrogen production by anaerobic mixed cultures. *Int. J. Hydrogen Energy* 36, 5857–5864. doi:10.1016/j.ijhydene.2011.01.125

Shuping, Z., Yulong, W., Mingde, Y., Kaleem, I., Chun, L., and Tong, J. (2010). Production and characterization of bio-oil from hydrothermal liquefaction of microalgae *Dunaliella tertiolecta* cake. *Energy* 35, 5406–5411. doi:10.1016/j.energy.2010.07.013

Singh, A., He, B., Thompson, J., and Gerpen, J. V. (2006). Process optimization of biodiesel production using alkaline catalysts. *Appl. Eng. Agric.* 22, 597–600. doi:10.13031/2013.21213

Singh, A., Nigam, P. S., and Murphy, J. D. (2011). Mechanism and challenges in commercialisation of algal biofuels. *Bioresour. Technol.* 102, 26–34. doi:10.1016/j.biortech.2010.06.057

Singh, D. P., and Trivedi, R. K. (2013). Production of biofuel from algae: an economic and eco-friendly resource. *Int. J. Sci. Res.* 2, 352–357.

Singh, J., and Gu, S. (2010). Commercialization potential of microalgae for biofuels production. *Renew. Sustain. Energ. Rev.* 14, 2596–2610. doi:10.1016/j.rser.2010.06.014

Singh, R., Behera, S., Yadav, Y. K., and Kumar, S. (2014). "Potential of wheat straw for biogas production using thermophiles," in *Recent Advances in Bio-Energy Research*, eds S. Kumar, A. K. Sarma, S. K. Tyagi, and Y. K. Yadav (Kapurthala: SSS-National Institute of Renewable Energy), 242–249.

Spolaore, P., Joannis-Cassan, C., Duran, E., and Isambert, A. (2006). Commercial applications of microalgae. *J. Biosci. Bioeng.* 101, 87–96. doi:10.1263/jbb.101.87

Stergiou, P. Y., Foukis, A., Filippou, M., Koukouritaki, M., Parapouli, M., Theodorou, L. G., et al. (2013). Advances in lipase-catalyzed esterification reactions. *Biotechnol. Adv.* 31, 1846–1859. doi:10.1016/j.biotechadv.2013.08.006

Sukumaran, R. K., Singhania, R., Mathew, G. M., and Pandey, A. (2008). Cellulase production using biomass feed stock and its application in lignocellulose saccharification for bio-ethanol production. *Renew. Energ.* 34, 421–424. doi:10.1016/j.renene.2008.05.008

Surendhiran, D., and Vijay, M. (2012). Microalgal biodiesel-acomprehensive review on the potential and alternative biofuel. *Res. J. Chem. Sci.* 2, 71–82.

Susilaningsih, D., Djohan, A. C., Widyaningrum, D. N., and Anam, K. (2009). Biodiesel from indigenous Indonesian marine microalgae *Nanochloropsis* sp. *J. Biotechnol. Res. Trop. Reg.* 2, 1–4.

Tamer, E., Amin, M. A., Ossama, E. T., Bo, M., and Benoit, G. (2006). Biological treatment of industrial wastes in a photobioreactor. *Water Sci. Technol.* 53, 117–125. doi:10.2166/wst.2006.344

Thomas, D. N. (2002). *Seaweeds*. Washington, DC: Smithsonian Books, Natural History Museum.

Topare, N., Rout, S. J., Renge, V. C., Khedkar, S. V., Chavan, Y. P., and Bhagat, S. L. (2011). Extraction of oil from algae by solvent extraction and oil expeller method. *Int. J. Chem.* 9, 1746–1750.

Trivedi, N., Gupta, V., Reddy, C. R., and Jha, B. (2013). Enzymatic hydrolysis and production of bioethanol from common macrophytic green alga *Ulva fasciata* Delile. *Bioresour. Technol.* 150, 106–112. doi:10.1016/j.biortech.2013.09.103

Vanegas, C. H., and Bartlett, J. (2013). Green energy from marine algae: biogas production and composition from the anaerobic digestion of Irish seaweed species. *Environ. Technol.* 34, 2277–2283. doi:10.1080/09593330.2013.765922

Vergara-Fernandez, A., Vargas, G., Alarcon, N., and Velasco, A. (2008). Evaluation of marine algae as a source of biogas in a two-stage anaerobic reactor system. *Biomass Bioenergy* 32, 338–344. doi:10.1016/j.biombioe.2007.10.005

Wargacki, A. J., Leonard, E., Win, M. N., Regitsky, D. D., Santos, C. N., Kim, P. B., et al. (2012). An engineered microbial platform for direct biofuel production from brown macroalgae. *Science* 335, 308–313. doi:10.1126/science.1214547

Wecker, M. S. A., Meuser, J. E., Posewitz, M. C., and Ghirardi, M. L. (2011). Design of a new biosensor for algal H_2 production based on the H_2-sensing system of *Rhodobacter capsulatus*. *Int. J. Hydrogen Energy* 36, 11229–11237. doi:10.1016/j.ijhydene.2011.05.121

Widjaja, A., Chien, C. C., and Ju, Y. H. (2009). Study of increasing lipid production from fresh water microalgae *Chlorella vulgaris*. *J. Taiwan Inst. Chem. Eng.* 40, 13–20. doi:10.1016/j.jtice.2008.07.007

Williams, P. J. L. B., and Laurens, L. M. L. (2010). Microalgae as biodiesel and biomass feedstocks: review and analysis of the biochemistry, energetics and economics. *Energy Environ. Sci* 3, 554–590. doi:10.1039/b924978h

Wu, X., Li, Q., Dieudonne, M., Cong, Y., Zhou, J., and Long, M. (2010). Enhanced H_2 gas production from bagasse using adhE inactivated *Klebsiella oxytoca* HP1 by sequential dark-photo fermentations. *Bioresour. Technol.* 101, 9605–9611. doi:10.1016/j.biortech.2010.07.095

Yacoby, I., Pochekailov, S., Topotrik, H., Ghirardi, M. L., King, P. W., and Zhang, S. (2011). Photosynthetic electron partitioning between (FeFe)-hydrogenase and ferrodoxin: NADP+-oxidoreductase (FNR) enzymes in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 108, 9396–9401. doi:10.1073/pnas.1103659108

Yanagisawa, M., Nakamura, K., Ariga, O., and Nakasaki, K. (2011). Production of high concentrations of bioethanol from seaweeds that contain easily hydrolysable polysaccharides. *Process Biochem.* 46, 2111–2116. doi:10.4161/bioe.23396

Yanqun, L., Mark, H., Nan, W., Christopher, Q. L., and Nathalie, D. C. (2008). Biofuels from microalgae. *Biotechnol. Progress.* 24, 815–820.

Yazdani, P., Karimi, K., and Taherzadeh, M. J. (2011). "Improvement of enzymatic hydrolysis of a marine macro-alga by dilute acid hydrolysis pretreatment," in *World Renewable Energy Congress (Bioenergy Technology), 8-13 May*, ed. B. Moshfegh (Linkoping, Sweden), 186–191.

Yen, H. W., and Brune, D. E. (2007). Anaerobic co-digestion of algal sludge and waste paper to produce methane. *Bioresour. Technol.* 98, 130–134. doi:10.1016/j.biortech.2005.11.010

Yoon, J. J., Kim, Y. J., Kim, S. H., Ryu, H. J., Choi, J. Y., Kim, G. S., et al. (2010). Production of polysaccharides and corresponding sugars from red seaweed. *Adv. Mater. Res.* 9, 463–466. doi:10.1016/j.biortech.2008.11.053

Yoon, M., Choi, J. I., Lee, J. W., and Park, D. H. (2012). Improvement of saccharification process for bioethanol production from *Undaria* sp. by gamma irradiation. *Radiat. Phys. Chem.* 81, 999–1002. doi:10.1016/j.radphyschem.2011.11.035

Yuan, H. (2014). Effect of ammoniation pretreatment at low moisture content on anaerobic digestion performance of rice straw. *Bioresources* 9, 6707–6718. doi:10.15376/biores.9.4

Yuan, X., Shi, X., Zhang, D., Qiu, Y., Guo, R., and Wang, L. (2011). Biogas production and microcystin biodegradation in anaerobic digestion of blue algae. *Energy Environ. Sci.* 4, 1511–1515. doi:10.1039/c0ee00452a

Zhang, C. (2013). Alkaline pretreatment for enhancement of biogas production. *Bioresour. Technol.* 149, 353–358. doi:10.1016/j.biortech.2013.09.070

Zheng, Y. (2009). Anaerobic digestion of saline creeping wild ryegrass for biogas production and pretreatment of particleboard material. *Bioresour. Technol.* 100, 1582–1588. doi:10.1016/j.biortech.2008.08.048

Zhong, W., Zhang, Z., Luo, Y., Qiao, W., Xiao, M., and Zhang, M. (2012). Biogas productivity by co-digesting Taihu blue algae with corn straw as an external carbon source. *Bioresour. Technol.* 114, 181–186. doi:10.1016/j.biortech.2012.02.111

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 31 July 2014; accepted: 29 December 2014; published online: 11 February 2015.

Citation: Behera S, Singh R, Arora R, Sharma NK, Shukla M and Kumar S (2015) Scope of algae as third generation biofuels. Front. Bioeng. Biotechnol. ***2****:90. doi: 10.3389/fbioe.2014.00090*

This article was submitted to Marine Biotechnology, a section of the journal Frontiers in Bioengineering and Biotechnology.

frontiers in Chemistry

OPINION
published: 30 June 2015
doi: 10.3389/fchem.2015.00039

Are multifunctional marine polysaccharides a myth or reality?

Svetlana Ermakova[1]*, *Mikhail Kusaykin*[1], *Antonio Trincone*[2] and *Zvyagintseva Tatiana*[1]

[1] G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far Eastern Branch of the Russian Academy of Sciences, Vladivostok, Russia, [2] Istituto di Chimica Biomolecolare, Consiglio Nazionale delle Ricerche, Pozzuoli, Italy

Keywords: brown algae, polysaccharide, fucoidan, fucoidanase, food supplement, biological activities

Marine algae are ancient photosynthetic organisms that constitute the largest group in the plant kingdom. They are used for functional food, cosmetic additives, supplements productions, and in traditional medicine due to taste, prophylactic, and therapeutic effects. Algae contain microelements and iodine-containing organic compounds, as well as vitamins, mannitol more than terrestrial plants.

Polysaccharides of algae are especially valuable substances. Some of them (for example agarose, carrageenans, and alginates) have found widespread application. Information about them was published a long time ago and described in detail in books. At the moment polysaccharides synthesized by brown algae (laminarans and especially fucoidans) are of greatest interest. A laminarans were found in both marine and terrestrial organisms. It should be noticed that fucoidans are truly marine polysaccharides. The general term "fucoidan" is used to integrate the molecules, differenced in composition, structure, and in degree of sulfation, acetylation, etc. (Berteau and Mulloy, 2003; Kusaykin et al., 2008). Content of fucoidans depends on the species and on the stage of development of algae and may vary from 0.1 to 20% of dry weight of algae (Mabeau et al., 1990; Zvyagintseva et al., 2003). Huge amount of reserves of fucoidans accumulate in brown algae, which grow in the seas at temperate and northern latitudes (Ermakova et al., 2011; Sokolova et al., 2011; Men'shova et al., 2012; Thinh et al., 2013). Analogs of these polysaccharides have not been found on the land till now. Fucoidans long since are attracted attention due to diverse biological activity, low toxicity, and plant origin (Berteau and Mulloy, 2003; Kusaykin et al., 2008). Last is important because of contamination and side effects of the preparation produced from animals (for example, heparin).

A large number of publications are devoted to the study of antitumor, anticoagulant, antimutagenic activities, and immunostimulatory, antiinfective and antioxidant properties of these polysaccharides. However, despite the obvious prospects for exploitation in medicine, none of fucoidan is declared yet as a drug. The reason is that the structural diversity of fucoidans is extremely large. Structural investigation of fucoidans is of great difficulties because of varieties of monosaccharide compositions, different types of glycosidic linkages, presence of large numbers of non-carbohydrate substituents. There are only a small number of fucoidans with established basic elements of the chemical structure (Chizhov et al., 1999; Bilan et al., 2002, 2004, 2008; Zvyagintseva et al., 2003; Shevchenko et al., 2007; Anastyuk et al., 2009, 2010, 2014; Kuznetsova, 2009; Ale et al., 2011; Vishchuk et al., 2011, 2013; Thinh et al., 2013). Unfortunately in the study of biological properties and enzymatic transformations of these molecules, fucoidans with unidentifiable structure are often used, thus reducing the generalization of the results obtained. Over the past 15–20 years there has been an increase in the number of structural studies of fucoidans. It became obvious that the study of their biological action, without regard to the structure does not allow to create drugs based on these polysaccharides.

It is now considered that fucoidans are species-specific polysaccharides. This means that each alga synthesizes fucoidan or set of fucoidans characteristic only for it. In monosaccharide composition of fucoidans necessarily there are sulfated residues of fucose and often galactose.

OPEN ACCESS

Edited by:
Xuechen Li,
The University of Hong Kong, Hong Kong

Reviewed by:
Paola Laurienzo, Consiglio Nazionale delle Ricerche, Italy
Yannis Karamanos, Université d'Artois, France

***Correspondence:**
Svetlana Ermakova, svetlana_ermakova@hotmail.com

Specialty section:
This article was submitted to Chemical Biology, a section of the journal Frontiers in Chemistry

Received: *30 April 2015*
Accepted: *08 June 2015*
Published: *30 June 2015*

Citation:
Ermakova S, Kusaykin M, Trincone A and Tatiana Z (2015) Are multifunctional marine polysaccharides a myth or reality? Front. Chem. 3:39. doi: 10.3389/fchem.2015.00039

As minor components residues of mannose, glucuronic acid, xylose, and other more rare monosaccharides, are present (Kusaykin et al., 2008).

1,3-α-L-Fucans are most often found in algae (Zvyagintseva et al., 2003; Anastyuk et al., 2010). α-1,4-Glycosidic linkage between L-fucose residues is less common and is present mainly as a 1,3;1,4-α-L-fucans. Brown algae also often synthesize galactofucans. The position and content of galactose residues in various galactofucans depend on the type of algae; content is frequently comparable to the that of fucose (Shevchenko et al., 2007; Anastyuk et al., 2009; Thinh et al., 2013). This is the most structurally diverse group of fucoidans. A smallest group of fucoidans is represented by fucomannuronans (Imbs et al., 2011). Furthermore, there are fucoidans, containing more heterogeneous monosaccharide composition.

In order to establish the structure of polysaccharides the most promising approach is based on the use of enzymes. Enzymatic transformation of polysaccharides can be extremely useful not only for the establishment of structural features, but also for the access to biologically active fragments (Silchenko et al., 2013; Menshova et al., 2014; Trincone, 2014). Reports about producers and properties of the enzymes (fucoidanases) are rare despite the growing interest in the fucoidans (Kusaykin et al., 2008). No more than 20 producers of fucoidanases are known, mainly isolated from marine fungi and bacteria (Sakai et al., 2003; Descamps et al., 2006; Rodriguez-Jasso et al., 2010; Silchenko et al., 2013, 2014). This rareness is due to the absence of quantitative simple methods for determination of the activity of fucoidanases. Precise assessment of enzymatic features is also hampered by the use of structurally uncharacterized substrates. So, in their transformation enzymes with different specificities should be involved.

Few sources of fucoidanases were found among marine invertebrates (Kitamura et al., 1992; Giordano et al., 2006; Silchenko et al., 2014). Fucoidanase in *Patinopecten yessoensis* was discovered in 1992 by the action on fucoidan from *Nemacystus decipiens* (Kitamura et al., 1992). Information about the structure of substrate reported in the article, consisting of L-fucose residues and small amounts of D-galactose residues, is quite scarce. Data about the type of glycosidic linkages are absent (Tako et al., 1999). High molecular weight products (about 50 kDa) formed sufficiently under the action of fucoidanase from *P. yessoensis*. Information about their structures is not available.

We found new sources of fucoidanases: the vietnamese mollusk *Lambis* sp. and the marine bacteria *Formosa algae* KMM 3553 (Khanh et al., 2011; Silchenko et al., 2014). Analysis of the hydrolysis products of fucoidans with established structure from collection of our laboratory, showed that both fucoidanases are endo-enzymes hydrolyzing α-1,4-glycosidic linkages in fucans (Silchenko et al., 2014).

Purification grade of fucoidans is also important for the investigation of biological properties. Unfortunately, uncharacterized crude preparations are often used even in scientific research. Methods for isolation and purification of fucoidan may be different. The most universal scheme includes preprocessing of algae by organic solvents extracting most secondary metabolites, such as polyphenols and other UV absorbing compounds (Shevchenko et al., 2005). These substances, usually powerful antioxidants, often are strongly associated with fucoidans and removal of them entails great difficulties. We show that the purification of fucoidans from impurities results in a loss of antioxidant activity (Imbs et al., 2015). Not only antioxidant, but also antibacterial activity of fucoidans can be completely or partially due to impurities. Separation of fucoidans from them is not always possible, as polysaccharides often form strong complexes with polyphenols, which cannot be destroyed without affecting the integrity of the fucoidan molecules. Nevertheless the evidence of antioxidant activity due to impurities of fucoidans were studied quite intensively (Wang et al., 2008; Hu et al., 2010; Costa et al., 2011). However the data on the purity of fucoidans is often absent.

It is interesting to note that specific biological activities of fucoidans are associated with their structures. So, the formation and growth of the colony of breast cancer cells are suppressed by galactofucans from *Saccharina japonica* and *Undaria pinnatifida.* Human colon cancer cells are more sensitive to fucoidan from *Saccharina cichorioides* (consisted of (1→3)-α-L-fucose residues), human melanoma cells—to fucoidan from *Fucus evanescens* (Moon et al., 2009; Vishchuk et al., 2011, 2013).

Thus, the intensification of structural studies of fucoidans and the use of highly purified preparations will help to dispel some myths about the effect of fucoidans on organisms and to outline the range of biological properties only related to polysaccharides. The first is immunomodulatory (Khil'chenko et al., 2011), antibacterial, antiviral (Prokofjeva et al., 2013), and antitumor activities (Ermakova et al., 2011; Vishchuk et al., 2011, 2013).

In Russia in 2006 the suplement "Fucolam®" (No 77.99.23.3.y.739.1.06, Russia), based on structurally characterized fucoidan from the brown alga *Fucus evanescens*, synthesizing from 12 to 15% of the polysaccharide, was registered. The biological effects of the "Fucolam®" are studied in detail. It was established that the "Fucolam®" in addition to the immunomodulatory, antibacterial, antiviral, and antitumoral activities has probiotic, hepatoprotective, glucose, and cholesterol lowering effects (Drozd et al., 2006, 2011; Kuznetsova, 2009; Khil'chenko et al., 2011; Lapikova et al., 2012; Besednova et al., 2014, 2015; Zaporozhets et al., 2014). It is a prominent representative of multifunctional agent and can serve as the base for drug development.

According to known data from the studies above mentioned, the spectrum of biological properties of fucoidans is wide enough. These natural substances are outstanding representatives of multifunctional compounds, and this is not a myth but a reality.

Acknowledgments

This work was supported by grants from REBR (14-04-93003, 15-04-01004_a) and Program of FEB RAS "P-42."

References

Ale, M. T., Mikkelsen, J. D., and Meyer, A. S. (2011). Important determinants for fucoidan bioactivity: a critical review of structure-function relations and extraction methods for fucose-containing sulfated polysaccharides from brown seaweeds. *Mar. Drugs.* 9, 2106–30. doi: 10.3390/md9102106

Anastyuk, S. D., Imbs, T. I., Dmitrenok, P. S., and Zvyagintseva, T. N. (2014). Rapid mass spectrometric analysis of a novel fucoidan, extracted from the brown alga *Coccophora langsdorfii*. *ScientificWorldJournal.* 2014:972450. doi: 10.1155/2014/972450

Anastyuk, S. D., Shevchenko, N. M., Nazarenko, E. L., Dmitrenok, P. S., and Zvyagintseva, T. N. (2009). Structural analysis of a fucoidan from the brown alga *Fucus evanescens* by MALDI-TOF and tandem ESI mass spectrometry. *Carbohydr. Res.* 344, 779–787. doi: 10.1016/j.carres.2009.01.023

Anastyuk, S. D., Shevchenko, N. M., Nazarenko, E. L., Imbs, T. I., Gorbach, V. I., Dmitrenok, P. S., et al. (2010). Structural analysis of a highly sulfated fucan from the brown alga *Laminaria cichorioides* by tandem MALDI and ESI mass spectrometry. *Carbohydr. Res.* 345, 2206–2212. doi: 10.1016/j.carres.2010.07.043

Berteau, O., and Mulloy, B. (2003). Sulfated fucans, fresh perspectives: structures, functions, and biological properties of sulfated fucans and an overview of enzymes active toward this class of polysaccharide. *Glycobiology* 13, 29R–40R. doi: 10.1093/glycob/cwg058

Besednova, N. N., Zaporozhets, T. S., Kuznetsova, T. A., Kryzhanovskii, S. P., Kovalev, N. N., and Zviagintseva, T. N. (2014). Hepatoprotective effects of extracts and polysaccharides from seaweed. *Antibiot. Khimioter.* 59, 30–37.

Besednova, N. N., Zaporozhets, T. S., Somova, L. M., and Kuznetsova, T. A. (2015). Prospects for the use of extracts and polysaccharides from marine algae to prevent and treat the diseases caused by *Helicobacter pylori*. *Helicobacter* 20, 89–97. doi: 10.1111/hel.12177

Bilan, M. I., Grachev, A. A., Ustuzhanina, N. E., Shashkov, A. S., Nifantiev, N. E., and Usov, A. I. (2002). Structure of a fucoidan from the brown seaweed *Fucus evanescens* C. Ag. *Carbohydr. Res.* 337, 719–730. doi: 10.1016/S0008-6215(02)00053-8

Bilan, M. I., Grachev, A. A., Ustuzhanina, N. E., Shashkov, A. S., Nifantiev, N. E., and Usov, A. I. (2004). A highly regular fraction of a fucoidan from the brown seaweed *Fucus distichus* L. *Carbohydr. Res.* 339, 511–517. doi: 10.1016/j.carres.2003.10.028

Bilan, M. I., Vinogradova, E. V., Tsvetkova, E. A., Grachev, A. A., Shashkov, A. S., Nifantiev, N. E., et al. (2008). A sulfated glucuronofucan containing both fucofuranose and fucopyranose residues from the brown alga *Chordaria flagelliformis*. *Carbohydr. Res.* 343, 2605–2612. doi: 10.1016/j.carres.2008.06.001

Chizhov, A. O., Dell, A., Morris, H. R., Haslam, S. M., McDowell, R. A., Shashkov, A. S., et al. (1999). A study of fucoidan from the brown seaweed *Chorda filum*. *Carbohydr. Res.* 320, 108–219. doi: 10.1016/S0008-6215(99)00148-2

Costa, L. S., Fidelis, G. P., Telles, C. B., Dantas-Santos, N., Camara, R. B., Cordeiro, S. L., et al. (2011). Antioxidant and antiproliferative activities of heterofucans from the seaweed *Sargassum filipendula*. *Mar. Drugs.* 9, 952–966. doi: 10.3390/md9060952

Descamps, V., Colin, S., Lahaye, M., Jam, M., Richard, C., Potin, P., et al. (2006). Isolation and culture of a marine bacterium degrading the sulfated fucans from marine brown algae. *Mar. Biotechnol.* 8, 27–39. doi: 10.1007/s10126-005-5107-0

Drozd, N. N., Miftakhova, N. T., Savchik, E., Kalinina, T. B., Makarov, V. A., Imbs, T. I., et al. (2011). Antithrombotic and hemorrhagic activities of fucoidan isolated from *Fucus evanescens* brown algae. *Eksp. Klin. Farmakol.* 136, 471–473.

Drozd, N. N., Tolstenkov, A. S., Makarov, V. A., Kuznetsova, T. A., Besednova, N. N., Shevchenko, N. M., et al. (2006). Pharmacodynamic parameters of anticoagulants based on sulfated polysaccharides from marine algae. *Bull. Exp. Biol. Med.* 142, 591–593. doi: 10.1007/s10517-006-0426-3

Ermakova, S., Sokolova, R., Kim, S. M., Um, B. H., Isakov, V., and Zvyagintseva, T. (2011). Fucoidans from brown seaweeds *Sargassum hornery, Eclonia cava*, *Costaria costata*: structural characteristics and anticancer activity. *Appl. Biochem. Biotechnol.* 164, 841–850. doi: 10.1007/s12010-011-9178-2

Giordano, A., Andreotti, G., Tramice, A., and Trincone, A. (2006). Marine glycosyl hydrolases in the hydrolysis and synthesis of oligosaccharides. *Biotechnol. J.* 1, 511–530. doi: 10.1002/biot.200500036

Hu, T., Liu, D., Chen, Y., Wu, J., and Wang, S. (2010). Antioxidant activity of sulfated polysaccharide fractions extracted from *Undaria pinnitafida in vitro*. *Int. J. Biol. Macromol.* 46, 193–198. doi: 10.1016/j.ijbiomac.2009.12.004

Imbs, T. I., Shevchenko, N. M., Semenova, T. L., Sukhoverkhov, S. V., and Zvyagintseva, T. N. (2011). Compositional heterogeneity of sulfated polysaccharides synthesized by the brown alga *Costaria costata*. *Chem. Nat. Comp.* 47, 96–97. doi: 10.1007/s10600-011-9839-y

Imbs, T. I., Skriptsova, A. V., and Zvyagintseva, T. N. (2015). Antioxidant activity of fucose-containing sulfated polysaccharide obtained from *Fucus evanescens* using different extraction methods. *J. Appl. Phycol.* 27, 545–553. doi: 10.1007/s10811-014-0293-7

Khanh, H. H. N., Kusaykin, M., Zakharenko, A., Ly, B. M., Hang, C. T. T., Ngoc, N. T. D., et al. (2011). Tinh sach va khao sat dac tinh cua enzyme thuy phan alginate tu gan tuy oc ban tay *Lambis* sp. (Purification and properties of an alginate lyase from the hepatopancreas of Lambis sp.). *J. Biotechnol.* 9, 1–9.

Khil'chenko, S. R., Zaporozhets, T. S., Shevchenko, N. M., Zvyagintseva, T. N., Vogel, U., Seeberger, P., et al. (2011). Immunostimulatory activity of fucoidan from the brown alga *Fucus evanescens*: role of sulfates and acetates. *J. Carbohydr. Chem.* 30, 291–305. doi: 10.1080/07328303.2011.604456

Kitamura, K., Masaru, M., and Yasui, T. (1992). Enzymic degradation of fucoidan by fucoidanase from the hepatopancreas of *Patinopecten yessoensis*. *Biosci. Biotechnol. Biochem.* 56, 490–494. doi: 10.1271/bbb.56.490

Kusaykin, M., Bakunina, I., Sova, V., Ermakova, S., Kuznetsova, T., Besednova, N., et al. (2008). Structure, biological activity, and enzymatic transformation of fucoidans from the brown seaweeds. *Biotechnol. J.* 3, 904–915. doi: 10.1002/biot.200700054

Kuznetsova, T. A. (2009). Fucoidan extracted from *Fucus evanescens* brown algae corrects immunity and hemostasis disorders in experimental endotoxemia. *Bull. Exp. Biol. Med.* 147, 66–69. doi: 10.1007/s10517-009-0445-y

Lapikova, E. S., Drozd, N. N., Makarov, V. A., Zviagintseva, T. N., Shevchenko, N. M., Kuznetsova, T. A., et al. (2012). Influence of intravenous injection of fucoidan from brown seaweed *Fucus evanescens* by plasma rabbits anticoagulant activity and neutralisation by sulphate protamin of fucoidans antithrombin activity *in vitro*. *Patol. Fiziol. Eksp. Ter.* 2, 42–44.

Mabeau, S., Kloareg, B., and Joseleau, J. P. (1990). Fractionation and analysis of fucans from brown algae. *Phytochemistry* 29, 2441–2445. doi: 10.1016/0031-9422(90)85163-A

Menshova, R. V., Ermakova, S. P., Anasyuk, S. D., Isakov, V. V., Dubrovskaya, Y. V., Kusaikin, M. I., et al. (2014). Structure, enzymatic transformation and anticancer activity of branched high molecular weight laminaran from brown alga *Eisenia bicyclis*. *Carbohydr. Polym.* 99, 101–109. doi: 10.1016/j.carbpol.2013.08.037

Men'shova, R. V., Ermakova, S. P., Rachidi, S. M., Al-Hajje, A. H., Zvyagintseva, T. N., and Kanaan, H. M. (2012). Seasonal variations of the composition, structural features, and antitumor properties of polysaccharides from *Padina pavonica* (Lebanon) as a function of composition. *Chem. Nat. Comp.* 47, 870–875. doi: 10.1007/s10600-012-0091-x

Moon, H. J., Lee, S. H., Ku, M. J., Yu, B. C., Jeon, M. J., Jeong, S. H., et al. (2009). Fucoidan inhibits UVB-induced MMP-1 promoter expression and down regulation of type I procollagen synthesis in human skin fibroblasts. *Eur. J. Dermatol.* 19, 129–134. doi: 10.1684/ejd.2008.0611

Prokofjeva, M. M., Imbs, T. I., Shevchenko, N. M., Spirin, P. V., Horn, S., Fehse, B., et al. (2013). Fucoidans as potential inhibitors of HIV-1. *Mar. Drugs.* 11, 3000–3014. doi: 10.3390/md11083000

Rodriguez-Jasso, R. M., Mussatto, S. I., Pastrana, L., Aguilar, C. N., and Teixeira, J. A. (2010). Fucoidan-degrading fungal strains: screening, morphometric evaluation, and influence of medium composition. *Appl. Biochem. Biotechnol.* 162, 2177–2188. doi: 10.1007/s12010-010-8992-2

Sakai, T., Ishizuka, K., and Kato, I. (2003). Isolation and characterization of a fucoidan-degrading marine bacterium. *Mar. Biotechnol.* 5, 409–416. doi: 10.1007/s10126-002-0118-6

Shevchenko, N. M., Anastiuk, S. D., Gerasimenko, N. I., Dmitrenok, P. S., Isakov, V. V., and Zviagintseva, T. N. (2007). Polysaccharide and lipid composition of the brown seaweed *Laminaria gurjanovae*. *Russ. J. Bioorganic Chem.* 3, 96–107. doi: 10.1134/s1068162007010116

Shevchenko, N. M., Imbs, T. I., Urvantseva, A. I., Kusaykin, M. I., Kornienko, V. G., Zvyagintseva, T. N., et al. (2005). *Method of Processing Seaweed*. European Patent No. WO2005/014657.

Silchenko, A. S., Kusaykin, M. I., Kurilenko, V. V., Zakharenko, A. M., Isakov, V. V., Zaporozhets, T. S., et al. (2013). Hydrolysis of fucoidan by fucoidanase isolated from the marine bacterium Formosa algae. *Mar. Drugs.* 11, 2413–2430. doi: 10.3390/md11072413

Silchenko, A. S., Kusaykin, M. I., Zakharenko, A. M., Menshova, R. V., Khanh, H. H. N., Dmitrenok, P. S., et al. (2014). Endo-1,4-fucoidanase from Vietnamese marine mollusk *Lambis* sp. which producing sulphated fucooligosaccharides. *J. Mol. Cat. B Enzym.* 102, 154–160. doi: 10.1016/j.molcatb.2014.02.007

Sokolova, R. V., Ermakova, S. P., Awada, S. M., Zvyagintseva, T. N., and Kanaan, H. M. (2011). Composition, structural characteristics, and antitumor properties of polysaccharides from the brown algae *Dictyopteris polypodioides* and *Sargassum* sp. *Chem. Nat. Compd.* 47, 329–334. doi: 10.1007/s10600-011-9925-1

Tako, M., Nakada, T., and Hongou, F. (1999). Chemical characterization of fucoidan from commercially cultured *Nemacystus decipiens* (Itomozuku). *Biosci. Biotechnol. Biochem.* 63, 1813–1815. doi: 10.1271/bbb.63.1813

Thinh, P. D., Menshova, R. V., Ermakova, S. P., Anastyuk, S. D., Ly, B. M., and Zvyagintseva, T. N. (2013). Structural characteristics and anticancer activity of fucoidan from the brown alga *Sargassum mcclurei*. *Mar. Drugs* 11, 1456–1476. doi: 10.3390/md11051456

Trincone, A. (2014). Molecular fishing: marine oligosaccharides. *Front. Mar. Sci.* 1:26. doi: 10.3389/fmars.2014.00026

Vishchuk, O. S., Ermakova, S. P., and Zvyagintseva, T. N. (2011). Sulfated polysaccharides from brown seaweeds *Saccharina japonica* and *Undaria pinnatifida*: isolation, structural characteristics, and antitumor activity. *Carbohydr. Res.* 346, 2769–2776. doi: 10.1016/j.carres.2011.09.034

Vishchuk, O. S., Ermakova, S. P., and Zvyagintseva, T. N. (2013). The effect of sulfated (1→3)-alpha-l-fucan from the brown alga S*accharina cichorioides* Miyabe on resveratrol-induced apoptosis in colon carcinoma cells. *Mar. Drugs.* 11, 194–212. doi: 10.3390/md11010194

Wang, J., Zhang, Q., Zhang, Z., and Li, Z. (2008). Antioxidant activity of sulfated polysaccharide fractions extracted from *Laminaria japonica*. *Int. J. Biol. Macromol.* 42, 127–132. doi: 10.1016/j.ijbiomac.2007.10.003

Zaporozhets, T. S., Besednova, N. N., Kuznetsova, T. A., Zviagintseva, T. N., Makarenkova, I. D., Kryzhanovskii, S. P., et al. (2014). The prebiotic potential of polysaccharides and extracts of seaweeds. *Russ. J. Mar. Biol.* 40, 1–9. doi: 10.1134/S1063074014010106

Zvyagintseva, T. N., Shevchenko, N. M., Chizhov, A. O., Krupnova, T. N., Sundukova, E. V., and Isakov, V. V. (2003). Water-soluble polysaccharides of some far-eastern brown seaweeds. Distribution, structure, and their dependence on the developmental conditions. *J. Exp. Mar. Biol. Ecol.* 294, 1–13. doi: 10.1016/S0022-0981(03)00244-2

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.