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High Molecular Weight λ-Carrageenan Improves the Color Stability of Phycocyanin by Associative Interactions

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Phycocyanin is a protein-chromophore structure present in *Arthrospira platensis* commonly used as a blue-colorant in food. Color losses of phycocyanin can be reduced by electrostatic complexation with λ-carrageenan. The aim of this study was to investigate the effect of molecular weight (M_W) of λ-carrageenan on the color stabilization of electrostatic complexes formed with phycocyanin and λ-carrageenan. Samples were heated to 70 or 90°C at pH 3.0 and stored at 25°C for 14 days. The M_W of λ-carrageenan was reduced by ultrasound treatments for 15, 30, 60, and 90 min. Prolonged ultrasonication had a pronounced effect on the *M*w, which decreased from 2,341 to 228 kDa (0–90 min). Complexes prepared with low M_W λ-carrageenan showed greater color changes compared to complexes prepared with high M_W λ-carrageenan. The M_W had no visible effect on color stability on day 0, but green/yellow shifts were observed during storage and after heating to 70°C. Medium M_W showed less color stabilization effects compared to low M_W when heated to 70°C. Moreover, for solutions prepared with ultrasonicated λ-carrageenan, significant hue shifts toward green/yellow, and precipitation were observed after a heat treatment at 90°C. In addition, the sizes of the complexes were significantly reduced (646–102 nm) by using ultrasonicated λ-carrageenan, except for the lowest M_W λ-carrageenan when heated to 90°C. Overall, these findings demonstrated that decreasing the M_W of λC had adverse effects on the color stability of PC:λC complexes heated to 70 and 90°C.

Keywords: complexation, coloring food, binding affinity, size exclusion chromatography, carbohydrates, sonochemistry, interaction, microalgae

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INTRODUCTION

Phycocyanin (PC) is a blue food-colorant with a low stability against pH-shifts and heat treatments (Berns and MacColl, 1989; Chaiklahan et al., 2012). Concentrations of more than 20% PC are found in the dry mass of *Arthrospira platensis*, therefore the cyanobacterium can be utilized for the industrial production of PC (Vernès et al., 2016; Martínez et al., 2017; Kilimtzidi et al., 2019). *Arthrospira platensis* accumulates allophycocyanin (aPC) and C-phycocyanin (CPC), both of which stabilize at least 1 chromophore per subunit. APC and CPC are structured by α- and β-subunits

with globular folding that build the PC monomers, which assemble into trimers and hexamers (Soulier and Bryant, 2021). Both protein-chromophore complexes are part of the light-harvesting units located on the thylakoid membrane of eukaryote algae and cyanobacteria. While having a similar function, CPC differs from aPC by having an additional chromophore connected to its β-subunit (Böcker et al., 2020; Soulier and Bryant, 2021).

Not only the unique color arising from PC but its high nutritional value have been exploited for a few decades (Amara and Steinbüchel, 2013; Lupatini et al., 2017; Costa et al., 2019). In particular, the phycocyanobilins from CPC were shown to have antioxidant activities and radical scavenging properties. Additionally, the apoprotein from aPC is believed to possess an antioxidative character (Eriksen, 2008). Moreover, PC is the only approved blue coloring food within the European Union with some others currently pending approval (Abiusi et al., 2022). Coloring foods provide an excellent solution for appealing colors while meeting customer demands for natural products and they are generally defined as purely physically and non-selctively extracted coloring additives from foodstuff (Carle and Schweiggert, 2016).

In the near future, the color stability of PC-containing extracts will be of tremendous importance (García et al., 2021). Amino acid residues of the protein interact with the phycocyanobilin chromophore *via* non-covalent interactions, mainly hydrogen bonds and some hydrophobic interactions (Tong et al., 2020). Thereby, the chromophore keeps extended and appears blue (Kupka and Scheer, 2008). These interactions are weakened by structural changes of the protein arising from altering environmental conditions such as changes in pH, temperature, or ionic strength (MacColl and Guard-Friar, 1987; Berns and MacColl, 1989; Chaiklahan et al., 2012).

Typically, the denaturation of PC causes a shift of absorption from 620–650 nm to 346–360 nm (Scheer and Kufer, 1977; Berns and MacColl, 1989) and structural changes of the chromophore can cause bathochromic shifts (Scheer and Kufer, 1977; Soulier and Bryant, 2021). However, the susceptibility of the protein to different treatments is a major drawback because foods are commonly preserved by temperatures ranging from 72 to 121°C and often undergo a change in pH (Heiss and Eichner, 1995; Böcker et al., 2019).

Recently, electrostatic complexation with lambda carrageenan (λC) was suggested as a promising tool for the stabilization of PC in acidic environments and during heat treatments (Li et al., 2021; Buecker et al., 2022a). Carrageenan is commonly found among various red algae (*Rhodophyta*) families where it is located in between the cellulose structure. It is assembled of repeating 2-sulfated 1,3 linked α-D-galactose and 2,6-disulfated 1,4-linked β-D-galactose, having a M_W of 200–800 kDa. λC carries three sulfate groups which result in a highly negative charge (Phillips and Williams, 2009; Wüstenberg, 2015; Zia et al., 2017; McKim et al., 2019). The electrostatic interactions with proteins at pH-values below the isoelectric point (*pI*) showed to be essential for the complex formation of PC and λC (Buecker et al., 2022a). Further, hydrophobic interaction and hydrogen bonding between the sulfate groups and the α-, β-subunits have been proposed to contribute to the color stability of PC:λC complexes (Buecker et al., 2022a).

In general, molecules of high M_W showed to have beneficial effects on complexation by reduced solvent-solute (*i.e.,* the complex is favored) interactions due to three reasons (Pathak et al., 2017; Weiss et al., 2019). First, a higher *M*w results in higher viscosity, slowing down diffusion and thus protein aggregation (La Fuente et al., 2004). Second, increasing the M_W decreases the entropy of mixing. Thus, large molecules tend to form soluble complexes. Third, λC of high M_W has a greater spatial volume which increases the number of anchor points for the protein (Schmitt et al., 1998; Pathak et al., 2017).

The cavitation forces of ultrasound can be used to break down polysaccharides. In particular, high-intensity ultrasound with high power (10–1,000 Wcm^{-2}) and low frequencies (10–100 kHz) is suitable for M_W reduction of carbohydrates (Feng et al., 2011; Zabot et al., 2021). In this process, cavitation bubbles expand to a size where their implosion generates strong shear forces. High molecular weight molecules tend to break in the center, while smaller molecules break at random locations along the chain. Low-intensity ultrasound, which applies high frequencies above 1 MHz and power intensities below 1 Wcm^{-2}, is more commonly used to promote sonochemical reactions or to obtain information about media. Shear forces with low-intensity ultrasound are much lower because the gas bubbles generated do not grow as large (Feng et al., 2011).

Yet, there has been no detailed investigation of how PC stability and thus color stability is affected by complexation of λC with different M_W's. In the present study, an ultrasonication treatment was performed to obtain λC of different M_W, which has been established in previous studies as a technique to reduce the *M*w of carrageenan (Hosseini et al., 2013). The aim of the study was to gain insight into the complexation behavior of PC:λC in order to tailor protein-polysaccharide complexes in food applications with respect to their colloidal and color stability. Our general hypothesis was that a higher M_W would favor color stability.

MATERIALS AND METHODS

Materials

Powders of λC and an *Arthrospira platensis* extract rich in phycocyanin were contributed by GNT International B.V. (Mierlo, Netherlands) and TIC Gums Inc. (White Marsh, USA), respectively. The *Arthrospira platensis* extract will be referred to as PC powder in this study. All chemicals, namely sodium hydroxide (NaOH), hydrochloric acid (HCL), sodium azide (NaN_3), sodium nitrate ($NaNO_3$) were at least of analytical grade and utilized from Carl Roth GmbH & Co. KG (Karlsruhe, Germany).

Methods

Complex Formation and Treatments

Solution Preparation

Stock solutions of λC ($c = 1.0\%$ *w/w*) and PC ($c = 0.1\%$ *w/w*) were prepared with double-distilled water or in 0.1 M $NaNO_3$ for

high pressure size exclusion chromatography (HPSEC) analyses. The solutions were treated with NaN_3 ($c = 0.05\%$ *w/w*) to prevent microbial growth and were stirred for 8 h to ensure complete hydration (Buecker et al., 2022a).

Ultrasonication

To lower the *M*w, stock solutions of λC were treated for 0, 15, 30, 60, and 90 min using a volume of 100 mL. The ultrasound treatment times were chosen based on previous studies (Meunier et al., 2001; Wu et al., 2008). A Bandelin Sonopuls HD2200 with a TT 13 probe, having a diameter of 13 mm, (BANDELIN electronic GmbH & Co. KG, Berlin, Germany) was used for the ultrasound treatment. The system was operated at an amplitude of 100% (149 μm_{ss}) with a frequency of 20 kHz and a constant power of 200 W. The highest possible energy inputs were chosen to be within the range of high-intensity ultrasound treatment (>10 $W \cdot cm^{-2}$, 10–100 kHz) and to efficiently generate λC with different M_W (Feng et al., 2011). Evaporation during the ultrasonic treatment was prevented by the covering the sample with a lid and simultaneously cooling of the samples in ice water. Thereby it was possible to maintain the temperature at 12 ± 2°C for the entire ultrasonic treatment time.

Formation of Electrostatic Complexes

Complex formation was induced by lowering the pH from ∼6.7 to 3.0 using a concentration of 0.15% *w/w* λC and 0.1% *w/w* PC powder at a ratio of 1:4 (PC:λC), which was based on a previous study (Buecker et al., 2022a). The biopolymer solutions were set to pH 3.0 and 6.0 ± 0.02 by HCL or NaOH while being stirred steadily. After initial pH-equilibration, the samples were stored overnight at 4°C. A pH readjustment was performed the next morning prior to the heat treatment.

Heat Treatment

Heat treatment was carried out in polycarbonate centrifuge tubes containing 45 g of the solution. The solutions were heated to 70 and 90°C in a forced-convection water bath (MGW Lauda M20 MS Circulating Bath, Lauda Dr. R. Wobser GmbH & Co. KG, Germany) and the temperature was held constant for 1 min. The samples were subsequently cooled down in a water bath to 25°C. A thermal treatment was carried out to demonstrate the influence of the M_W on the color stabilization during temperatures typically used in thermal food processing. The temperatures were chosen because they are in the upper and lower range of common temperatures utilized for pasteurization of most fruit juices (Ramesh, 2007).

Storage Tests

Zeta potential, size by dynamic light scattering (DLS), absorption, and transmittance (see below) values were analyzed at day 0, 3, 6, and 14. In between measurements all samples were stored at 25°C in a climate cabinet (HCP50, Memmert GmbH & Co. KG, Schwabach, Germany).

Analyses

PC Concentration

The individual concentrations of C-phycocyanin (CPC) and allophycocyanin (aPC) in the *Arthrospira platensis* powder were determined using their absorption maxima of 620 and 650 nm, respectively. According to the two-wavelength method of Yoshikawa and Belay (2008) the total concentration of PC was calculated by summing up the amount of aPC and CPC (Equations 1, 2) (Yoshikawa and Belay, 2008). To obtain the concentration in g/100 g the results were multiplied by 0.1.

$$c_{\mathrm{C-phycocyanin}} = 0.162 \cdot A_{620} - 0.098 \cdot A_{650} \left(\mathrm{g \cdot L^{-1}}\right) \quad (1)$$

$$c_{\mathrm{allophycocyanin}} = 0.180 \cdot A_{650} - 0.042 \cdot A_{620} \left(\mathrm{g \cdot L^{-1}}\right) \quad (2)$$

here, A_{620} and A_{650} refer to the absorption at 620 and 650 nm.

HPSEC Measurement

For the HPSEC measurements, the 0, 15, 30. 60, 90 min ultrasonicated λC solutions were dissolved in the mobile phase (0.1 M $NaNO_3$) (Gómez-Ordóñez et al., 2012). Prior to the measurement, the samples were filtered by Chromafil regenerated cellulose filters RC-20/25 with a pore size of 0.45 µm from Chromafil (Machery-Nagel GmbH & Co. KG, Düren, Germany). The samples were injected into an Agilent HP Series 1100 (Agilent Technologies GmbH & Co. KG, Waldbronn, Germany) with a phase hydroxylated methacrylate packed column TSKgel G5000PW_{XL} (TOSOH Bioscience, Tokyo, Japan) that was equipped with an Agilent 1100 refractive index detector (Agilent Technologies GmbH & Co. KG, Waldbronn, Germany). The injection volume was set to 20 µL and the flow rate to 0.6 mL/min. A six-point calibration curve with dextran standards ranging from 12 to 270 kDa (Fluka Analytical, Buchs, Switzerland) was used to calculate the number average molecular weight (M_n) distribution from the peak of the RID signal according to Equation (3). Herein c_i corresponds to the concentration of component *i* and M_i being the molecular weight of fraction i (Balke et al., 1994; Spichtig and Austin, 2008).

$$M_n = \frac{\sum c_i}{\sum \frac{c_i}{M_i}} \quad (3)$$

Zeta Potential

For the zeta potential measurement, the samples were filled in folded capillary cells. The zeta potential was analyzed by a Zetasizer Nano Series particle size analyzer (Nano-Zs ZEN 3600, Dispersion Technology Software DTS v 5.1, Malvern Instruments Ltd., Worcestershire, UK). Throughout the measurement, the sample temperature was kept constant at 25°C. The instrument utilizes a laser with a 90° scattering angle and an operating wavelength of 633 nm. The zeta potential is calculated from the electrophoretic mobility of the particles in solution. By utilizing the Smoluchowski approximation the random motion of the particles in solution can be estimated. Herein f(κa) equals 1.5 with κa referring to the ratio of the particle radius to its electrostatic double-layer thickness in polar media (>10^{-3} molar salt) (Jiang et al., 2009). The measurement was repeated three times.

Size Measurement

The sample size was determined in folded capillary cells by dynamic light scattering (DLS) at a constant temperature of 25°C.

The laser measurement was carried out by a Zetasizer Nano Series particle size analyzer (Nano-Zs ZEN 3600, Dispersion Technology Software DTS v 5.1, Malvern Instruments Ltd., Worcestershire, UK) (Jiang et al., 2009). The laser operates at a wavelength of 633 nm and a measurement angle of 173°. Results are reported as intensity mean.

Transmittance and Absorption Measurement

Absorption analysis of the samples was carried out by an Ultrospec 2100 *pro* (Biochrom GmbH, Berlin, Germany) spectrophotometer in semi-micro UV cuvettes. The instrument was blanked with double-distilled water. The absorption spectrum was determined from 240 to 750 nm and the transmittance was detected at 780 nm. A turbidity baseline correction was performed using the absorption values at 750 nm. All absorption and transmittance data are shown as relative values to unheated and pure PC powder solutions ($c = 0.1\%$ *w/w*) having a pH of 6.0 (Buecker et al., 2022a).

Color Analysis

The colorimetric values lightness (L^*), a^* (– green/+ red value), b^* (– blue/+ yellow value), chroma (C^*) and $\text{hue}_{a,b}$ (h°) in the CIELAB color space 1931 were determined using the absorption spectra. An absorber angle of 2° and illuminant C were employed for the calculation (Schanda, 2007; Wrolstad and Smith, 2017).

Statistics

Statistical analysis was carried out by SPSS Statistics 27 (IBM Inc., Armonk, USA) and Excel (Microsoft Inc., Redmond, USA). Excel was used for the calculation of means and standard deviations. SPSS was used to analyze for statistically significant differences between means. An ANOVA with a subsequent Tukey *post-hoc* test or double-sided *t*-Tests was performed. The confidence interval was set to 95%. All samples were prepared in duplicate and at least one repetition of the measurements was conducted.

RESULTS AND DISCUSSION

First, the PC content was determined at pH 6.0 (Equations 1, 2). Herein, an aPC content of 5.6% (*w/w*) and a CPC content of 31.3% (*w/w*) were determined, resulting in a total PC content of 36.9% (*w/w*) in the powder. This amount is similar to the values that have been reported previously (Buecker et al., 2022a).

SEC of Ultrasonicated λC

The overall aim of this study was to analyze the effect of molecular weight on the color stabilization properties of λC. Changes in M_W by ultrasonication treatment were analyzed by SEC (**Figure 1**).

During SEC, molecules with a lower M_W can diffuse into a larger number of pores of the column material, which leads to a prolonged elution time through the column (Catherine, 2000). As expected, ultrasonicated λC exhibited a shift to longer elution times with increasing treatment times, indicating a decrease in M_W caused by the ultrasonication treatment. Untreated λC eluted after about 13 min, while λC treated for 90 min eluted after 16 min. Also, the peak of the signal appeared sharper after the ultrasonication treatment indicating a more homogenous size distribution of λC with increasing treatment time.

In addition, with prolonged ultrasonic treatment, the rate of λC hydrolysis decreased, causing the change in M_W to be smaller with increasing treatment time. This is due to the fact that a higher amount of energy is required to break stiffer λC with low M_W, causing the molecular degradation rate constant to decrease (Weiss et al., 2011; Ogutu, 2015; Tecson et al., 2021). Thus, the strongest reduction in size was obtained for the 15 min treatment. These findings were in agreement with the observations made for κ-carrageenan in an earlier study (Meunier et al., 2001). Polymers with lower M_W exhibit increased ultrasonic damping in solution. This increased relaxation behavior leads to sound waves with a lower amplitude and higher frequency which decreases the power of cavitation (Cochran et al., 1974; Lii et al., 1999). Accordingly, only minor *M*w differences were observed for λC ultrasonicated for 60 and 90 min. The M_W in turn determines the intrinsic viscosity, which is also used to measure polymer degradation. Thus, it can be assumed that the viscosity of the samples decreased with increasing duration of ultrasonic treatment (Mohod and Gogate, 2011; Azizi and Farahnaky, 2016).

The number average M_W of λC was calculated from the chromatogram using Equation (3). The native λC had a M_W of ∼2,340 kDa which is about 100 times higher than the M_W of poligeenan, which is known to have adverse health effects when consumed (Younes et al., 2018). The M_W reached around 228 kDa after 90 min treatment time, and thus the ultrasonication treatment resulted in about a 10-fold decrease in M_W. However, as the M_W decrease between 60 and 90 min was only minor, the following experiments were carried out with λC ultrasonicated for 15, 30, and 90 min.

Visual Appearance

After the decrease in M_W was established, the color stability of PC:λC complexes was analyzed at pH 3.0 (strong complexation) and pH 6.0 (no/low complexation) after heat treatment at 70 or 90°C. Initially, the solutions were visually evaluated (**Figure 2**).

The aim of these experiments was to elucidate the effect of different M_W of λC on the stabilization of PC:λC complexes during a thermal treatment. Unheated samples were not considered in this study since this would possess a potential food safety risk and more studies are needed to investigate the long term safety aspect of such solutions. At first glance, the decrease in λC M_W did not influence the color stability at day 0. All PC:λC complexes occurred turquoise at pH of 3.0 and royal blue at a pH of 6.0. Moreover, the color fading became evident for samples heated at 70°Cand was even more pronounced when heated to 90°C, as expected. The change to a turquoise color at pH 3.0 is related to a change in concentration of the individual PC monomers, trimers, and hexamers, the more pronounced stability of the aPC trimer, and possibly the dissociation of the α- and β-subunit causing the chromophore to change its planarity (Hefferle et al., 1984; Berns and MacColl, 1989; Marx and Adir, 2014; Soulier and Bryant, 2021; Buecker et al., 2022a).

With increasing treatment temperature, the color of solutions at pH 6.0 was less stable than at pH 3.0. This was in

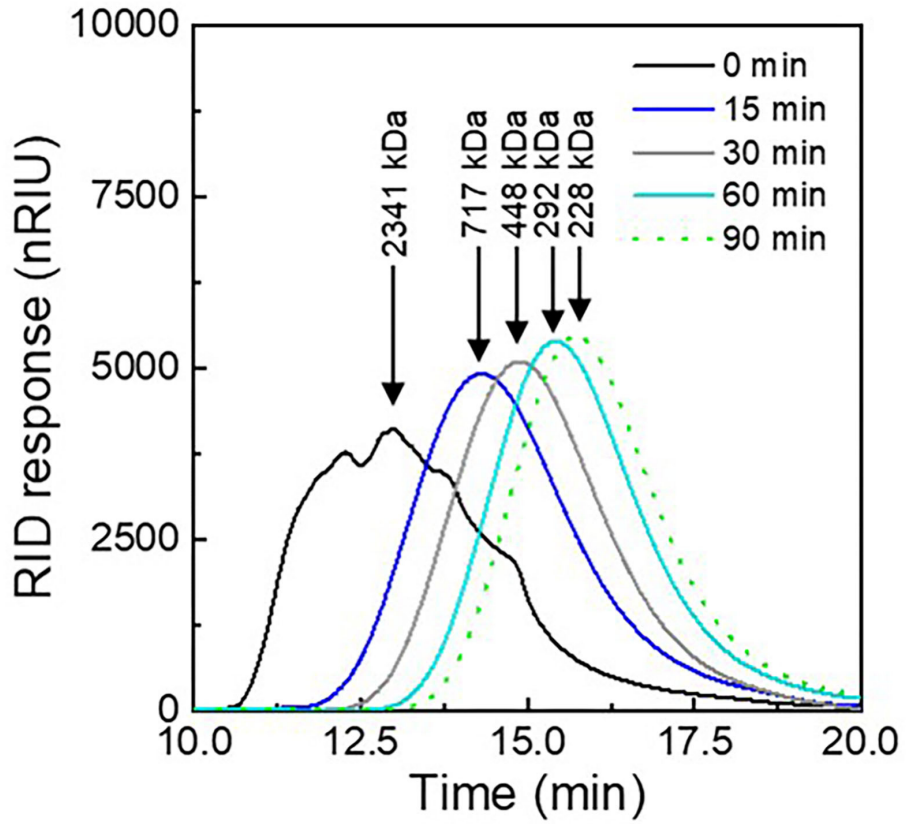

FIGURE 1 | Molecular weight elution profiles of λC solutions prepared in $NaNO_3$ after different ultrasonication treatment times (0–90 min).

agreement with prior experiments and can be related to a stabilizing effect of electrostatic complexation of PC and λC that is not occurring at pH 6.0 (Buecker et al., 2022a). When the samples were stored for the 14 days period, a strong color degradation and green/yellow shift were observed in ultrasonication treated solutions, especially for samples heated to 90°C and to a lesser extent for samples heated to 70°C. At storage conditions of 25°C, the half-life of PC color in solutions without added preservatives is about 18 days, however much shorter at lower pH values (Kannaujiya and Sinha, 2016; Buecker et al., 2022b). The strong time-dependent color degradation was intended to highlight the influence of M_W and could have been reduced by lowering the storage temperature. Moreover, color degradation might be slowed down by inducing complexation with polymers that exhibit a strong electrostatic interaction with the protein. Additionally, samples prepared at pH 3.0 with 90 min ultrasonicated λC and subsequent heating to 90°C exhibited precipitation (**Figure 4**). The aggregation of PC:λC complexes and subsequent precipitation might be associated to a decreased electrostatic repulsion among complexes (Wagoner et al., 2016). Thus, the density of the λC:PC complexes increased. The zeta potential measurements showed that the charge magnitude of the solutions decreased with extended ultrasonication treatment (**Supplementary Figure 1**). This could be related to hydrolysis of λC (∼pKa 2.0) or sonochemical reactions reducing the reactive sites of the backbone (Kardos and Luche, 2001; Al-Zebari et al., 2019). Especially at high temperatures, hydrophobic interactions among PC increase, which might overcome the electrostatic repulsion (Tanford, 1980). Further, an increased charge neutralization upon complexation could lead to a decreased zeta potential (Pillai et al., 2021). Last, according to Stoke's law of sedimentation, the decreased viscosity might additionally favor complex precipitation (Phillips and Williams, 2009).

Expressed in the CIE 1931 $L^*C^*h^\circ$ chromaticity diagram, the green/yellow shift as a function of storage time and M_W becomes very clear for samples heated to 90°C (**Figure 3**).

The differences upon change in M_W became more evident after time and with increasing treatment temperature. Samples prepared with 0 min ultrasonicated λC showed to stabilize the turquoise color at a pH of 3. This was in alignment with prior studies (Li et al., 2021; Buecker et al., 2022a), but ultrasonicated samples showed a destabilization in color over time, especially when heated at 90°C. On the one hand it was suggested that the decreasing M_W lead to the formation of denser complexes and on the other hand the increasing stiffness of the low M_W λC lead to decreased complex stability and thus to a pronounced fading of the color (Ogutu, 2015; Pathak et al., 2017; Weiss et al., 2019).

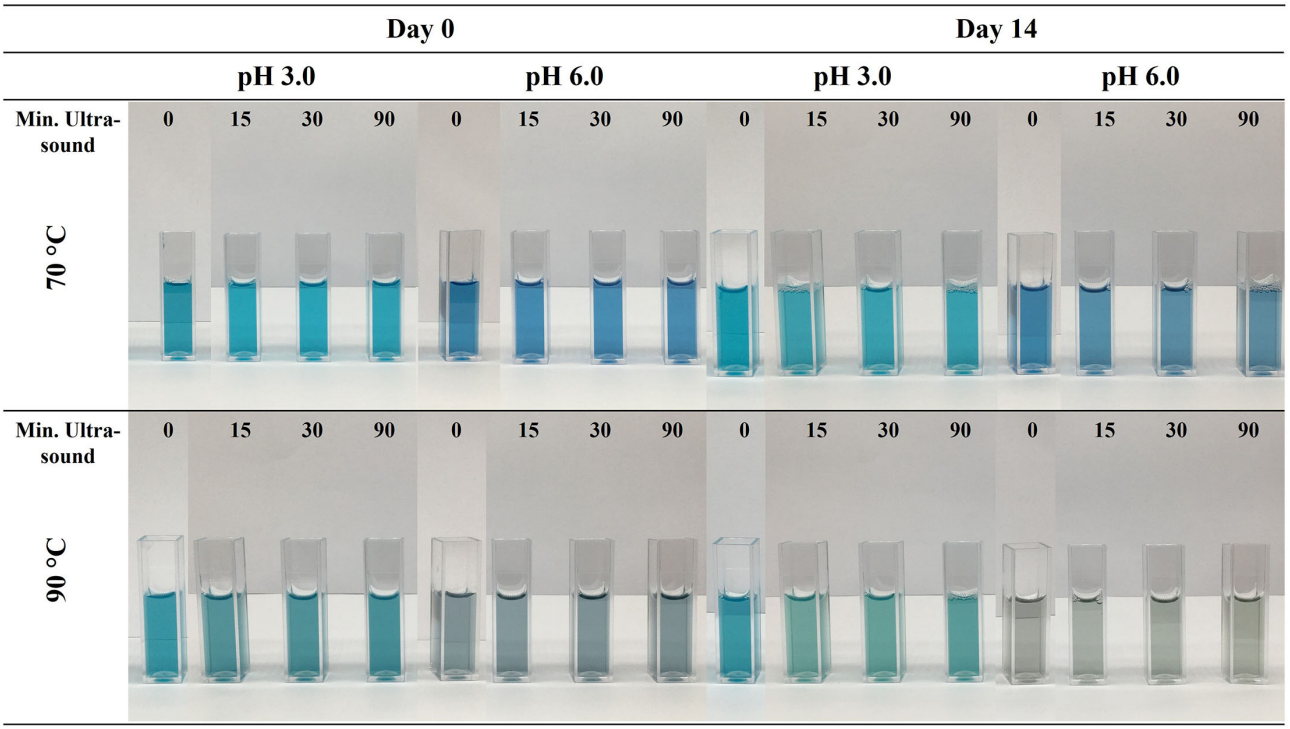

FIGURE 2 | Visual appearance PC:λC (1:4) complexes prepared with λC solutions treated with ultrasonication for 0, 15, 30, and 90 min. Samples were heated to 70 and 90 C at pH 3.0 and 6.0, the solutions were stored for 14 days at 25°C.

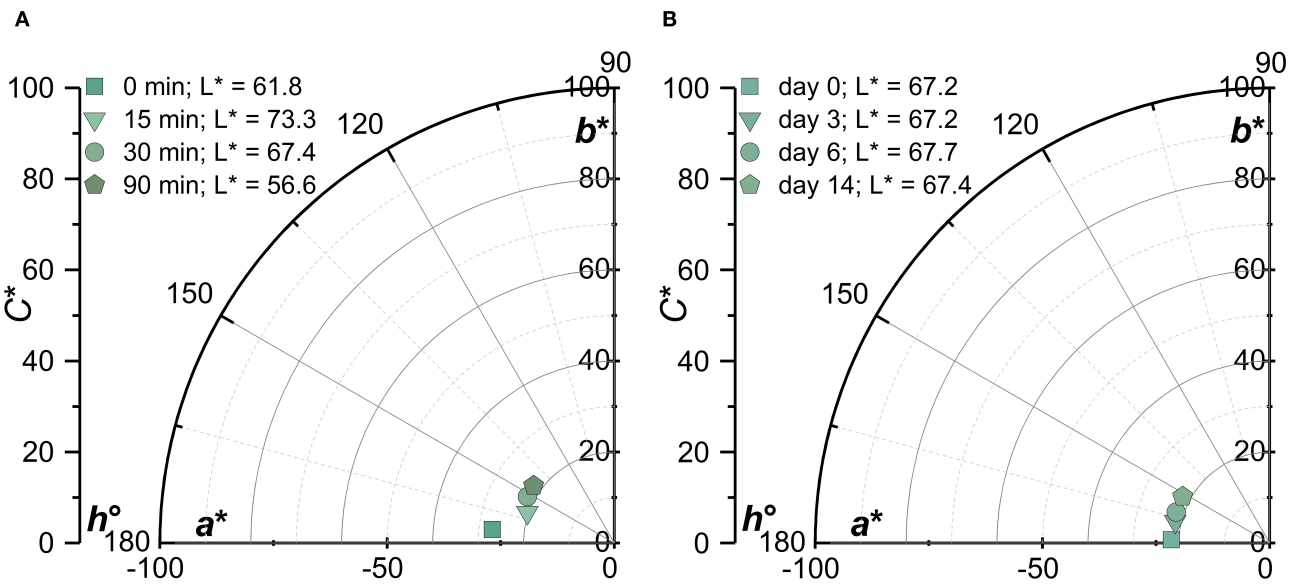

FIGURE 3 | CIE 1931 $L^*C^*h^\circ$ chromaticity diagram of PC:λC (1:4) complexes at a pH of 3 and heated to 90 C as a function of **(A)**: λC ultrasonication treatment time 0–90 min (2,341–228 kDa) at day 14 and **(B)**: storage time (days 0–14).

Effect on Transmittance and Absorption

Color is the first sensory characteristic that consumers perceive in foods, which impacts their purchasing behavior (Carle and Schweiggert, 2016). To quantify the color degradation, transmittance and absorption measurements were carried out (**Figure 4**). In addition, the color differences

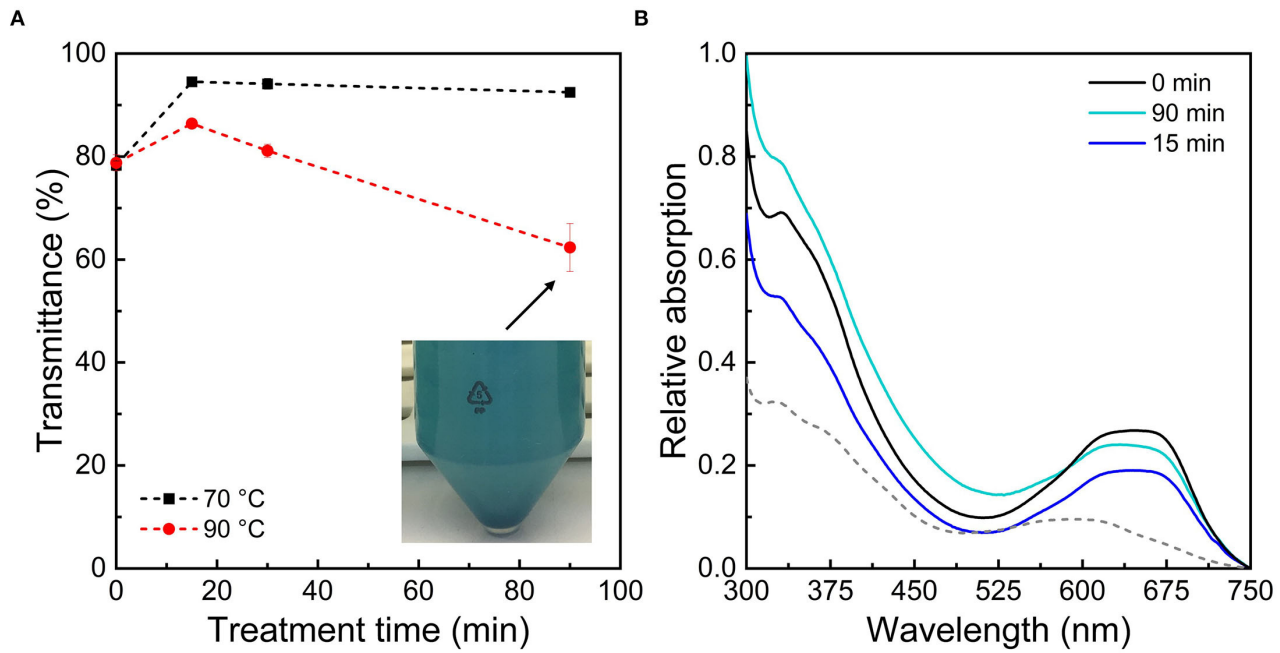

FIGURE 4 | (A) Relative transmittance of PC:λC (1:4) complexes prepared at pH 3.0 stored for 14 days at 25°C after heat treatments to 70 and 90°C plotted over ultrasonication treatment time. **(B)** Relative absorption of PC:λC complexes heated to 90°C at pH 3.0 with a subsequent storage of 14 days at 25°C after different ultrasonication treatment times. The absorption of PC:λC complexes prepared with untreated λC at pH 6.0, heated to 90°C and stored for 14 days (25°C) is plotted as dotted line.

(ΔE) and difference vectors of the colors in the CIE 1931 $L^*C^*h^\circ$ color space were calculated (**Table 1**).

The color was not affected by the λC ultrasonic treatment until the samples were heated and stored (data not shown). As only minor changes have been observed at day 0, values are only shown for day 14 at 90°C.

The transmittance measurements showed that the effect of M_W differed between solutions heated to 70 and 90°C (**Figure 4A**). Transmittance decreases with the formation of larger complex structures because more light is scattered (Kelley and McClements, 2003). The transmittance decreased for PC:λC complexes heated to 70°C only when solutions were prepared with λC that was not ultrasonicated. As samples were prepared with ultrasonicated λC, the transmittance increased. Possibly, smaller and more soluble complexes were formed by the smaller size of carrageenan, which results in less light scattering.

Solutions heated to 90°C prepared with ultrasonicated λC exhibited a decrease in transmittance with increasing treatment time. Complex solutions containing untreated λC as well as λC which was ultrasonicated for 90 min had the lowest transmittance, whereas solutions containing λC which was ultrasonicated for 15 min had higher transmittance values. The complexes formed from 15 min ultrasonicated λC and PC might be smaller and consequently more soluble. It is suggested that λC which was not ultrasonicated forms large complexes with λC surrounding multiple PC molecules. Wang et al. (2000) showed that increasing the M_W of a polymer resulted in larger biopolymer complexes. A critical M_W needs to be exceeded in terms of complex formation (Schmitt et al., 1998). However, as the low M_W λC (∼228 kDa) also stabilized the color of the PC:λC complexes, it can be assumed that the critical M_W was reached (**Figure 4B**). This might be related to the flexibility of λC and a low intrinsic persistence length of 2.7 nm (Berth et al., 2008; Pathak et al., 2017). Our findings are in agreement with Hosseini et al. (2013) who reported decreased affinity of ultrasonicated κ-carrageenan to β-lactoglobulin.

With extended ultrasonication and thereby M_W reduction of λC the transmittance decreased for samples heated to 90°C, which was related to increased light scattering because of an increase in complex size (**Table 2**). This could be related to multiple effects. It was reported that polymers of low M_W form denser complexes, possibly because of less steric hindrance (Liu, 2007; Pathak et al., 2017). Larger λC molecules create a stronger steric hindrance that increases the repulsive interactions between the particles, which keeps them in a distance that is not relevant for attractive interactions (Moore et al., 2015). The radius of gyration is roughly 93 nm for λC not treated with ultrasound (Berth et al., 2008). This can slow down the aggregation and denaturation of PC in the samples (Stone et al., 2013; Zhang et al., 2021). Also, a decreased binding affinity of λC to PC could result in accelerated protein unfolding during heating and thereby exposure of hydrophobic groups (Pathak et al., 2017). Hydrophobic interactions among PC could be beneficial during complex development over time as PC association (i.e., intramolecular interactions) within the complexes might be improved and thereby decrease the hydrophobic surface of PC

TABLE 1 | Color difference (ΔE) and difference vectors of PC:λC (1:4) complexes at a pH of 3.0.

	Ref. = Day 0, unheated, and 0 min ultrasonication time							
	Day 0				**Day 14**			
	0 min	**15 min**	**30 min**	**90 min**	**0 min**	**15 min**	**30 min**	**90 min**
	Heated to 70°C							
ΔE	9.88	12.56	13.14	11.61	11.34	14.10	14.46	13.40
$\vec{D}$	$\begin{pmatrix}0.18\\3.11\\9.38\end{pmatrix}$	$\begin{pmatrix}11.86\\3.12\\-2.69\end{pmatrix}$	$\begin{pmatrix}12.24\\3.63\\-3.12\end{pmatrix}$	$\begin{pmatrix}10.90\\3.70\\-1.55\end{pmatrix}$	$\begin{pmatrix}-0.31\\4.05\\10.59\end{pmatrix}$	$\begin{pmatrix}12.52\\6.41\\0.96\end{pmatrix}$	$\begin{pmatrix}13.03\\6.25\\0.42\end{pmatrix}$	$\begin{pmatrix}11.45\\6.48\\2.53\end{pmatrix}$
	Heated to 90°C							
ΔE	17.31	20.20	20.87	26.00	19.20	28.53	29.72	32.76
$\vec{D}$	$\begin{pmatrix}-0.28\\8.83\\14.89\end{pmatrix}$	$\begin{pmatrix}8.36\\14.76\\10.97\end{pmatrix}$	$\begin{pmatrix}4.24\\14.28\\14.62\end{pmatrix}$	$\begin{pmatrix}-4.33\\15.35\\20.53\end{pmatrix}$	$\begin{pmatrix}-1.11\\9.09\\16.87\end{pmatrix}$	$\begin{pmatrix}10.37\\16.78\\20.61\end{pmatrix}$	$\begin{pmatrix}4.52\\16.76\\24.12\end{pmatrix}$	$\begin{pmatrix}-6.32\\18.18\\26.51\end{pmatrix}$
	Ref. = Day 0, heated to 70°C, and 0 min ultrasonication time							
	Day 0				**Day 14**			
	0 min	**15 min**	**30 min**	**90 min**	**0 min**	**15 min**	**30 min**	**90 min**
	Heated to 70°C							
ΔE	–	16.80	17.38	15.32	1.61	15.30	15.98	13.61
$\vec{D}$	–	$\begin{pmatrix}11.68\\0.01\\-12.07\end{pmatrix}$	$\begin{pmatrix}12.06\\0.52\\-12.50\end{pmatrix}$	$\begin{pmatrix}10.72\\0.59\\-10.93\end{pmatrix}$	$\begin{pmatrix}-0.49\\0.94\\1.21\end{pmatrix}$	$\begin{pmatrix}12.34\\3.30\\-8.42\end{pmatrix}$	$\begin{pmatrix}12.85\\3.14\\-8.96\end{pmatrix}$	$\begin{pmatrix}11.27\\3.37\\-6.85\end{pmatrix}$
	Heated to 90°C							
ΔE	7.96	14.32	12.99	17.16	9.67	20.42	20.55	23.72
$\vec{D}$	$\begin{pmatrix}-0.46\\5.72\\5.51\end{pmatrix}$	$\begin{pmatrix}8.18\\11.65\\1.59\end{pmatrix}$	$\begin{pmatrix}4.06\\11.17\\5.24\end{pmatrix}$	$\begin{pmatrix}-4.51\\12.24\\11.15\end{pmatrix}$	$\begin{pmatrix}-1.29\\5.98\\7.49\end{pmatrix}$	$\begin{pmatrix}10.19\\13.67\\11.23\end{pmatrix}$	$\begin{pmatrix}4.34\\13.65\\14.74\end{pmatrix}$	$\begin{pmatrix}-6.50\\15.07\\17.13\end{pmatrix}$

$\vec{D}$*: Difference vector between the reference (ref.) and sample point* $\begin{pmatrix}L^*\\a^*\\b^*\end{pmatrix}$.

ΔE: Distance between two colors (color 1 and 2) in the CIELAB 1931 color space according to:

$\Delta E = \sqrt{(L^*_1 - L^*_2)^2 + (a^*_1 - a^*_2)^2 + (b^*_1 - b^*_2)^2}$.

The samples were prepared with M_W reduced λC via ultrasonication for 15, 30, and 90° min. Reference samples (Ref.) had a pH of 3.0, were either unheated or heated to 70°C, and the λC was ultrasonicated for 0 min.

aggregates (Tanford, 1980). This might enlarge the binding sites for λC resulting in denser complexes. In emulsions, protein-polysaccharide complexes with high M_W polysaccharides have been shown to prevent droplet aggregation. It was suggested that steric hindrance prevents aggregation processes (Ibanoglu, 2005; Zhao et al., 2018). Our results show that larger polysaacharides are similarly more effective at preventing protein aggregation. Thus, a complex growth over time could be a result of decreased electrostatic and steric stabilization with decreasing λC M_W (Wagoner et al., 2016). As mentioned previously, the magnitude of the zeta potential was decreased which could be associated with either enhanced charge neutralization or dissociation of λC (**Supplementary Figure 1**). Further, the decreased M_W might alter the ideal mixing ratio of the biopolymers and thereby change the surface charge of the complex due to increased charge neutralization (Hosseini et al., 2013). However, clearly more research is necessary to find the exact mechanism of increasing optical density over ultrasonication treatment time using these parameters.

The absorption spectrum of PC:λC solutions which were stored for 14 days at 25°C after a heat treatment to 90°C is shown in **Figure 4B**. To assess the color stabilization a reference line of PC:λC samples prepared at pH 6.0 is shown as well. At pH 3.0, complexation with λC stabilized all solutions against color fading, which is in accordance with the visual observation presented in **Figure 2**. All samples prepared at pH 3.0 exhibited a pronounced shoulder at ∼670 nm, which was expected for a successful complexation with λC and which was responsible for the turquoise color appearance. The bathochromic shift was associated with a planarity changes of the phycocyanobilin (Buecker et al., 2022a). The best color preservation was obtained with high M_W λC (2,341 kDa), which was related to a better

binding affinity (Pathak et al., 2017). However, with prolonged ultrasonication up to 90 min color perception was increasingly influenced by the enhanced scattering of denatured proteins (**Figure 4A**) such as PC. Thus, the absorption peak at 347 nm increased (**Figure 4B**) (Scheer and Kufer, 1977; Wang et al., 2000; Hosseini et al., 2013). Overall, the color perception changed to slightly intenser color values which was reflected by an increased absorption (**Figures 2, 4B**).

Table 1 shows the color differences between unheated and heated (70°C) PC:λC complexes having a pH of 3.0 at day 0. When heated to 70°C, a ΔE below 10 was observed only for complexes prepared with 0 min ultrasonicated λC. Thus, the color difference was perceptible but can still be considered the same color (Karma, 2020). Samples prepared with ultrasonicated λC must be considered to have a different color (ΔE > 10) after the thermal treatment to 70°C. Prolonged ultrasonication time and storage of the samples increased the color difference, especially for samples heated to 90°C. Samples with high M_W λC were chosen as reference. The $L^*a^*b^*$-values of the references were $L^* = 62.92$, $a^* = -35.91$, $b^* = -13.92$ for unheated samples and $L^* = 63.10$, $a^* = -32.80$, $b^* = -4.54$ for samples heated to 70°C (Buecker et al., 2022a). The L^* values increased with storage time and decreased with longer ultrasonic treatment. While the L^* values of the samples prepared with 0 min ultrasonicated λC were hardly affected by heat, heating had a strong influence on the samples prepared with ultrasonicated λC. The a^* and b^* values decreased with increasing storage time and when heated to 90°C. For samples prepared with ultrasound treated λC, the b^* values increased when heated to 70°C and decreased when heated to 90°C. The color differences increased considerably due to the ultrasonic treatment of λC, which was especially evident after heating to 90°C. It is supposed that the M_W reduction had a negative effect on complex formation and thus negatively impacted color stabilization, as explained before. This is consistent with our previous observations and in line with the literature (Hosseini et al., 2013; Pathak et al., 2017).

TABLE 2 | Size (nm) of PC:λC (1:4) complexes was determined by DLS.

Heat-treatment	day	Ultrasonication time			
		0 min	15 min	30 min	90 min
70°C	0	646 ± 59^{aA}	118 ± 2^{aB}	116 ± 26^{aB}	102 ± 10^{aB}
	3	554 ± 10^{abA}	132 ± 21^{aB}	108 ± 9^{aB}	100 ± 5^{aB}
	6	509 ± 50^{abA}	121 ± 9^{aB}	107 ± 3^{aB}	102 ± 2^{aB}
	14	410 ± 3^{bA}	111 ± 5^{aB}	102 ± 9^{aB}	100 ± 2^{aB}
90°C	0	202 ± 11^{aAB}	139 ± 3^{aA}	183 ± 20^{aAB}	419 ± 126^{aB}
	3	202 ± 11^{aA}	143 ± 1^{abA}	186 ± 7^{aA}	467 ± 124^{aB}
	6	202 ± 7^{aA}	150 ± 3^{bcA}	199 ± 3^{aA}	488 ± 104^{aB}
	14	202 ± 14^{aA}	155 ± 1^{cA}	214 ± 16^{aA}	573 ± 151^{aB}

Significant size differences of samples conducted to the same heat-treatment are indicated by lowercase letters in the same column and capital letters within a row.
The M_W of λC was decreased by ultrasonication for 15, 30 and 90 min. Measurements were performed after heating the samples to 70 and 90°C at day 0, 3, 6, and 14 days.

Effect on Complex Size

The last experiments were carried out to measure the size of the formed complexes to shed light on the growth behavior. The size was determined by DLS for PC:λC complexes formed and stored at pH 3.0 heated to 70 and 90°C (**Table 2**).

The size of the complexes formed after heating to 70°C decreased due to the ultrasound treatment. While complexes formed with untreated λC were about 650 nm, complexes formed by λC which was ultrasonicated for 90 min were about 100 nm. The decrease of complex size by lowering the biopolymers M_W is in agreement with previous findings and the transmittance

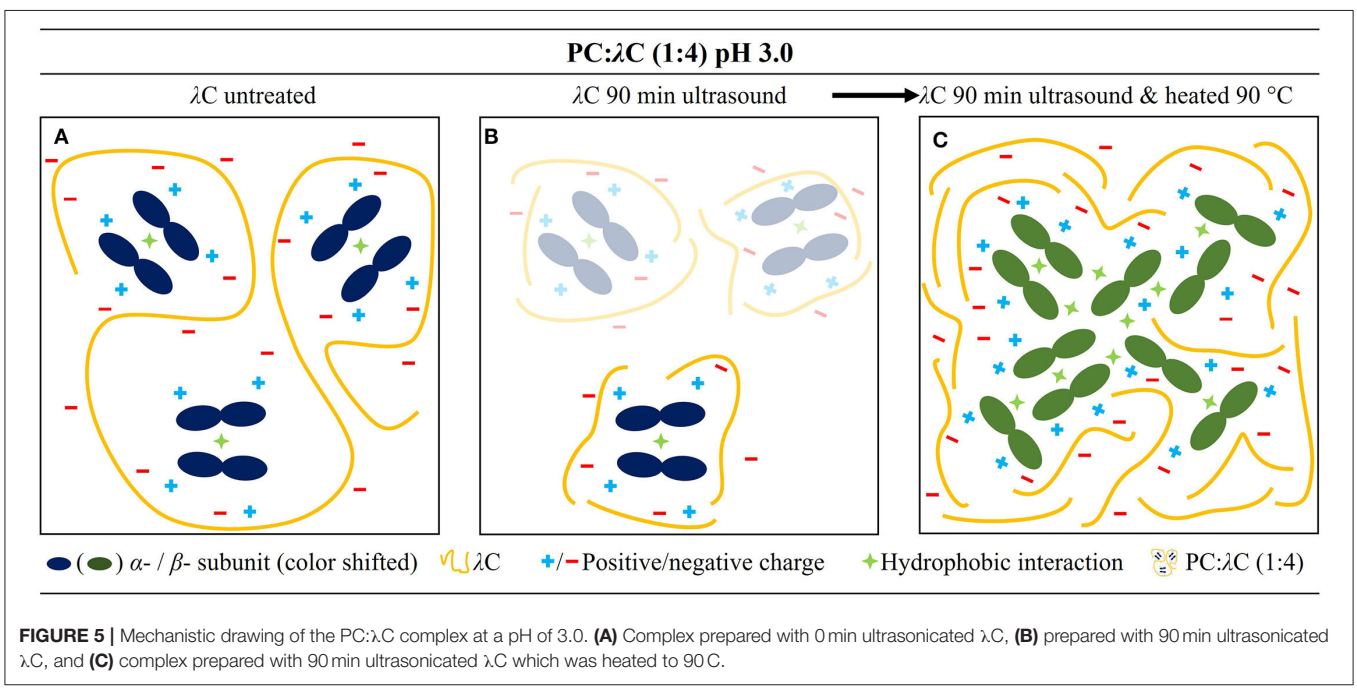

FIGURE 5 | Mechanistic drawing of the PC:λC complex at a pH of 3.0. **(A)** Complex prepared with 0 min ultrasonicated λC, **(B)** prepared with 90 min ultrasonicated λC, and **(C)** complex prepared with 90 min ultrasonicated λC which was heated to 90 C.

measurements presented earlier (Wang et al., 2000; Hosseini et al., 2013). The complex size of the samples prepared with untreated λC decreased over time which could be referred to structural rearrangements driven by an entropy gain as described by the Veis and Aranyi (1960) theory (Veis and Aranyi, 1960; Phillips and Williams, 2009). Intramolecular hydrophobic interactions among proteins change up over time because of internal restructuring processes to obtain the lowest possible free energy and is in agreement with previous findings for PC:λC complexes (Li et al., 2020; Buecker et al., 2022b).

After heating the samples to 90°C the size of the samples prepared with untreated λC and λC treated for 30 and 90 min was similar at day 0. Heated samples with untreated λC had a constant size of 202 nm over the entire storage time whereas solutions with ultrasonicated λC increased in size. The increase in size could be related to multiple effects as mentioned in the previous chapter (Effect on Transmittance and Absorption). We suggest a complex association due to decreased repulsion among complexes. Zeeb et al. (2018) showed that an Ostwald ripening type growth type behavior occurred for biopolymer complexes of whey protein isolate and pectin after heating in the associative regime due to increasing hydrophobic and decreasing electrostatic interactions (Zeeb et al., 2018). This is in accordance with the considerable increase in size of the samples prepared with λC ultrasonicated for 15 min. The increase in size was not significant for the other solutions. However, after 3 days of storage, the samples ultrasonicated for 90 min increased in size. Thus, they were significantly bigger than all the other samples. The increasing size of complexes prepared with ultrasonicated λC is proposed to cause complex sedimentation and storage instability.

CONCLUSION

This study demonstrated that the decreasing M_W of λC had adverse effects on the color stability of PC:λC complexes heated to 70 and 90°C. Herein samples heated to 90°C showed best performances with high M_W (2,341 kDa) λC, which was related to an increased binding affinity. The green/yellow shift of the complexes' during storage was promoted by lower M_W λC. However, low M_W polymers are also suitable for complexation of PC, but to a slightly lesser extent. The size of the complexes was decreased which could be beneficial in terms of storage stabilization and transmittance of food products. Large and dense complexes formed upon heating to low M_W λC:PC-complexes to 90°C which could be more suitable for cloudy foods. Moreover, a secondary aggregation by decreased electrostatic repulsion was proposed to result in complex growth of samples prepared with ultrasonicated λC. Hence, precipitation needs to be prevented in products with a high thermal load and λC of lower M_W (below 717 kDa) (**Figure 5**). Yet, prolonged ultrasonication enhanced color intensity.

The results of the study are important for, among others, carrageenan manufacturers with regard to tailor-made product solutions and food companies stabilizing proteins by complexation with polysaccharides.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

SB: writing—original draft, software, investigation, formal analysis, validation, data curation, and visualization. LG: supervision, conceptualization, writing—original draft, and writing—review and editing. ML: methodology and writing—review and editing. EL: supervision and writing—review and editing. JW: conceptualization, project administration, supervision, writing—review and editing, resources, and funding acquisition. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fsufs.2022.915194/full#supplementary-material

Supplementary Figure 1 | Zeta potential (mV) of PC:λC (1:4) solutions heated to 70 or 90°C at pH 3.0 or 6.0 and unheated λC at pH 3.0 plotted over ultrasonication treatment time.

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Conflict of Interest: EL is employed by GNT Europa Gmbh.

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