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Isolation, identification, molecular docking analysis, and cytoprotection of seven novel angiotensin I-converting enzyme inhibitory peptides from miiuy croaker byproducts-swim bladders

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For efficiently utilizing the processing byproducts of miiuy croaker to prepare novel angiotensin I-converting enzyme (ACE) inhibitory (ACEi) peptides, *in vitro* gastrointestinal (GI) digestion method was screened and employed to prepare swim bladder hydrolysate with the highest ACEi activity. Subsequently, seven novel ACEi peptides were isolated from the hydrolysate and identified as DEGPE, EVGIQ, SHGEY, GPWGPA, GPFGTD, SPYGF, and VIGPF with molecular weights of 545.49, 544.58, 591.55, 583.63, 592.59, 569.60, and 531.63 Da, respectively. SHGEY and SPYGF exhibited remarkable ACEi activity with IC_{50} values of 0.86 ± 0.12 and 0.37 ± 0.06 mg/mL. Molecular docking experiment illustrated that the significant ACEi activity of SHGEY and SPYGF with the affinity of -8.7 and -9.7 kcal/mol mainly attributed to effectively combining with the ACEi active sites by hydrophobic interaction, electrostatic force and hydrogen bonding. Moreover, SHGEY and SPYGF could significantly up-regulate the nitric oxide (NO) production and decrease the endothelin-1 (ET-1) secretion in human umbilical vein endothelial cells (HUVECs), but also abolished the negative impacting of norepinephrine to the levels of NO and ET-1. Furthermore, SHGEY and SPYGF showed significant protection to HUVECs against H_2O_2 damage by increasing superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) activity to lower the contents of reactive oxide species and malondialdehyde. Consequently, ACEi peptides derived from miiuy croaker swim bladders, especially SHGEY and SPYGF, are health-promoting ingredients for functional products as a supplementary treatment to hypertension and cardiovascular diseases.

KEYWORDS

miiuy croaker (*Miichthys miiuy*), swim bladder, angiotensin I converting enzyme, peptide, molecular docking experiment, cytoprotection

Introduction

Hypertension seriously affects the morbidity and mortality of cardiovascular and renal diseases (Kharazmi-Khorassani et al., 2019; Abedin et al., 2022). Because of the different lifestyles and genetic factors, the number of people with high blood pressure is now about 1.3×10^6, but this number is expected to rise to 1.56×10^6 by 2030 (Mabhida et al., 2021). The current and future situation will have a serious implication on human health and place a great strain on the finances of countries around the world (Abdelhedi and Nasri, 2019; Mabhida et al., 2021). The renin-angiotensin system bears crucial roles in maintaining blood pressure homeostasis and keeping the balance between fluid and salt (Zheng et al., 2022). Angiotensin (Ang) I-converting enzyme (ACE) is the key protease in participating the regulating of blood pressure through the renin-angiotensin system (Xu et al., 2021; Qiao et al., 2022), and its function is converting Ang I to Ang II for the inactivation of vasodilator bradykinin, which further induce the increase of blood pressure (BP). Then, inhibiting ACE activity is an ideal method in hypertension treatment (Fan et al., 2019; Chakraborty and Roy, 2022). Therefore, the chemosynthetic ACE inhibitors (captopril (Cap), lisinopril, enalapril, *etc.*) have been widely used in treating heart failure and hypertension in the clinical (Fan et al., 2019; Chakraborty and Roy, 2022), but the disturbing thing is those synthetic ACE inhibitors display some serious adverse symptoms, such as headache, impaired taste perception, dizziness, chronic dry cough, nausea, and hyperkalemia (Lee et al., 2010; Lee and Hur, 2017; Abedin et al., 2022). Therefore, researchers are focusing on searching safer ACE inhibitors from natural resources as the alternatives of synthetic ACE inhibitors to control hypertension (Abedin et al., 2022).

Presently, some natural ACE inhibitors have been isolated from diversified biological resources, and ACE inhibitory (ACEi) peptides drew great public attention due to their safe and effective therapeutic properties on hypertension (Pujiastuti et al., 2019; Abedin et al., 2022; Chakraborty and Roy, 2022). In the experiments on spontaneously hypertensive rats (SHRs), corn germ ACEi peptide with IC_{50} of 0.83 mg/mL could markedly lower the systolic BP, adjust the level of relaxing and contracting factors derived by endothelium in serum, and suppress the ACE activities in different organs (Guo et al., 2020). PPLLFAAL with IC_{50} of 28 μmmol/L could remarkably reduce the systolic BP and diastolic BP of SHRs after intravenous administration (Su et al., 2021). Similarly, LGF and GLFF from leaf hydrolysate of *Moringa oleifera* exhibited double inhibitory activity of ACE and renin and significantly reduced the systolic and diastolic BP of SHRs (Ma et al., 2021). VIPVPFF from yeast hydrolysate with IC_{50} of 10.27 μM could increase the nitric oxide (NO) levels, upregulate approximately 15-fold expression of GUCY1A1 gene, and activate several hypertension-related pathways in human umbilical vein endothelial cells (HUVECs) (Huang et al., 2021). Moreover, many ACEi peptides, such as LY, RALP, GHS (He et al., 2019a), EMFGTSSET (Pei et al., 2022), IWHHT (Gu et al., 2019), and VPP (Chakrabarti et al., 2017), show multi-activities, especially antioxidant and antiphlogistic activities. The multiple activities of those ACEi peptides should play synergistic roles with their ACEi activity in the control of hypertension (Majumder and Wu, 2015; Fan et al., 2019).

Globle fish production reached around 179 million tons and approximately 50% of these catches become byproducts during factory processing (Manikkam et al., 2016, Sila and Bougatef, 2016; Sheng et al., 2022). Those fish byproducts will lead to serious environmental problems if they can't be handled properly (Zhang et al., 2019; Yang et al., 2019a). To make full use of these fish byproducts, many ACEi peptides were prepared from different processing byproducts, such as tuna black muscle and bone (Lee et al., 2010; Qiao et al., 2022), smooth-hound viscera (Abdelhedi et al., 2017; Abdelhedi et al., 2018), cuttlefish muscle (Balti et al., 2015), Nile tilapia skeleton (Borges-Contreras et al., 2019), rainbow trout viscera (Vásquez et al., 2022) [34], and Alaska pollack skins (Yang et al., 2021). Those sea-food derived ACEi peptides exhibit high potential application value in diet or clinical treatment on anti-hypertension (Abdelhedi and Nasri, 2019; Fan et al., 2019).

Miiuy croaker (*Miichthys miiuy*) is an important species of the Sciaenidae family, which is a popular marine economic fish (He et al., 2019b; Geng et al., 2021). In China, miiuy croaker is the most common materials used to produce dried croaker, but fish scales and viscus are discarded as byproducts in the process. Therefore, collagen and antioxidant peptides were prepared from those byproducts (Li et al., 2018; Zhao et al., 2018a; Zhao et al., 2018b). Moreover, FPYLRH from miiuy croaker showed notably cytoprotective effect

Abbreviations: ACE, angiotensin I-converting enzyme; ACEi, angiotensin-I-converting enzyme inhibitory; GI, gastrointestinal; MMP1, Asp-Glu-Gly-Pro-Glu (DEGPE); MMP2, Glu-Val-Gly-Ile-Gln (EVGIQ); MMP3, Ser-His-Gly-Glu-Tyr (SHGEY); MMP4, Gly-Pro-Trp-Gly-Pro-Ala (GPWGPA); MMP5, Gly-Pro-Phe-Gly-Thr-Asp (GPFGTD); MMP6, Ser-Pro-Tyr-Gly-Phe (SPYGF); MMP7, Val-Ile-Gly-Pro-Phe (VIGPF); ET-1, endothelin-1; NO, nitric oxide; BP, blood pressure; Ang, angiotensin; Cap, captopril; SHRs, spontaneously hypertensive rats; HUVECs, human umbilical vein endothelial cells; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; ROS, reactive oxygen species; GSH, glutathione; TNBS, 2,4,6-Trinitrobenzene sulfonic acid; FAPGG, N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly; NE, norepinephrine; SD, standard deviation; GPC, gel permeation chromatography; MTT, 3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide; CAN, acetonitrile; MSCH, protein hydrolysate of miiuy croaker swim bladder prepared using *in vitro* GI digestion processing; RP-HPLC, reversed-phase high performance liquid chromatography; MW, molecular weight; Q-TOF, quadrupole time-of-flight; DH, degree of hydrolysis; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Nrf2, nuclear factor erythroid 2-related factor 2; HO-1, heme oxygenase-1; PI3K, phosphatidylinositol-3-kinase; Akt, protein kinase B; eNOS, endothelial NO synthase.

against H_2O_2-injuried HUVECs by activating the intracellular antioxidant system to remove excess reactive oxygen species (ROS) (Cai et al., 2019). However, there was no literature on ACEi peptides produced using miiuy croaker swim bladders. Therefore, to make more efficient use of this resource, the objectives of this study were to (i) isolate ACEi peptides from swim bladder hydrolysate of miiuy croaker, (ii) identify the sequences and characterize the properties of isolated ACEi peptides, and (iii) evaluate the cytoprotective activity of the isolated ACEi peptides.

Materials and methods

Materials and chemical reagents

Miiuy croaker (*M. miiuy*) was provided by Zhejiang Hailisheng Group Co. Ltd. (China). HUVECs were purchased from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). The assay kits of NO (Product no. A013-2-1), endothelin-1 (ET-1) (Product no. H093), superoxide dismutase (SOD) (Product no. A001-3-2), glutathione peroxidase (GSH-Px) (Product no. A005-1-2), and malondialdehyde (MDA) (Product no. A003-4-1) were purchased from Nanjing Jiancheng Bioengineering Institute Co., Ltd. (China). 2,4,6-Trinitrobenzene sulfonic acid (TNBS), Sephadex G-25 resin, Alcalase, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) (CAS no. 298-93-1), Neutrase, Cap, DMSO, acetonitrile (ACN), norepinephrine (NE), Glutathione (GSH), trypsin (enzyme activity ≥200000 U/g, CAS no. 9002-07-7), pepsin (enzyme activity ≥200000 U/g, CAS no. 9001-75-6), papain, N-[3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG) (CAS no. 64967-39-1), Dulbecco's Modified Eagle Medium (DMEM) (Product no. 11995), and ACE were purchased from Sigma-Aldrich (Shanghai) Trading Co., Ltd. (China). ACEi peptides of MMP3-MMP7 (purity > 95%) were synthesized in Shanghai Apeptide Co. Ltd. (China).

Preparation of swim bladder hydrolysate of miiuy croaker

The degreasing process of miiuy croaker swim bladder was performed according to the described method (Chi et al., 2014; Zhao et al., 2018b). The swim bladders were homogenized and mixed with isopropanol at a material/ratio of 1:4 (w/v) for 6 h, and the solution was replaced each 2.0 h. Lastly, the solution was centrifuged at 6 000 g for 20 min and the resulted solid was air-dried at 35°C.

The hydrolytic process was performed on the previous methods (Chi et al., 2014). The dispersions of degreased swim bladders (1%, w/v) was treated by supersonic processing for 30 min and hydrolyzed respectively using four proteases under optimal conditions (Table 1).

The *in vitro* GI digestion method (pepsin-trypsin system) was carried out on the described method (Zhao et al., 2019a). The dispersions of degreased swim bladders (pH 1.5, 1%) were treated by supersonic processing for 30 min and firstly degraded by pepsin for 2 h, and the pH was adjusted to 7.0 and further degraded by trypsin for 2 h.

After digestion, the solutions were placed in 95°C for 15 min and centrifuged at 8 000 g for 20 min. The supernatant was lyophilized and stored in -20°C freezer. The protein hydrolysate prepared using *in vitro* GI digestion processing was referred to as MSCH.

Purification of APs from SCH

MSCH (100.0 mg/mL) was fractionated by 1, 5 and 10 kDa ultrafiltration membrane and four fractions including MSCH-1 (< 1 kDa), MSCH-2 (1-5 kDa), MSCH-3 (5-10 kDa), and MSCH-4 (> 10 kDa) were prepared.

MSCH-1 solution (5 mL, 50.0 mg/mL) was fractionated with Sephadex G-25 column (3.6 × 150 cm) eluted with ultrapure water under the flow rate of 0.8 mL/min. Each eluate (2.4 mL) was collected by monitoring absorbance at 220 nm. Three subfractions (MSCH-1a, MSCH-1b, and MSCH-1c) were isolated from MSCH-I.

MSCH-1b solution (20 μL, 100.0 μg/mL) was finally separated by RP-HPLC on a Zorbax 300SB-C18 column (4.6 × 250 mm, 5 μm) with a linear gradient of ACN (5% ACN in 5 min; 5-25% ACN in 10 min; 25-50% ACN in 10 min; 50-100% ACN in 10 min; 100% ACN in 5 min) containing 0.06% trifluoroacetic acid at 1.5 mL/min flow rate. The eluate absorbance was monitored at 220 and 280 nm. Seven ACEi peptides (MMP1 to MMP7) were collected and lyophilized.

TABLE 1 Hydrolysis parameters of different proteases and *in vitro* GI digestion method.

Protease	Temperature (°C)	Enzyme dosage (g enzyme/100 g defatted swim bladder)	Time (h)	pH value
Alcalase	50	2	4	2.0
Trypsin	37	2	4	7.0
Papain	50	2	4	6.0
Pepsin	37	2	4	7.0
Neutrase	60	2	4	8.0
in vitro GI digestion	37	Trypsin 1+ Pepsin 1	2	2.0

Identification of sequences and molecular weights (MWs) of ACEi peptides

The sequences of MMP1 to MMP7 were analyzed using an Applied Biosystems 494 protein sequencer (Perkin Elmer, USA) (Zhao et al., 2018b). A quadrupole time-of-flight (Q-TOF) mass spectrometric device (Micromass, Waters, USA) in the combination of an ESI source were employed to determine the MWs of MMP1 to MMP7 (Chi et al., 2014).

Determination of ACEi activity

The ACEi activity was determined according to the previous protocol by Zhao et al. (Zhao et al., 2019b). In brief, 50 μL FAPGG solution as substrate (1 mM) in HEPES-HCl buffer (0.5 mM, pH 8.3, containing 300 mM salt) were mixed with 40 μL sample (5, 10, 20, 40mg/mL) and 10 μL of ACE solution. The mixture was pre-incubated at 37°C for 5 min. Then, 50 μL of 1.0 mol/L FPAGG solution were added into the mixture to initiate the reaction and incubated at 37°C for 30 min. The control was prepared using 80 mM HEPES-HCl buffer containing 300 mM NaCl (pH 8.3) instead of the sample. The sample group and control group were run in the same manner. After that, measure the absorbance of the sample solution at 340 nm. All samples were measured as described above, respectively. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity. The ACEi activity was calculated by the following equation:

$$ACEi \quad activity \quad (\%) = (1 - B_0 - B_{30}/A_0 - A_{30}) \times 100$$

A_0 and B_0 represent the initial absorbance of the control group and the sample group; A_{30} and B_{30} represent the absorbance after 30 minutes for the control group and the sample group.

Determination of degree of hydrolysis (DH)

DH (%) was measure according to the previous method (Wang et al., 2013a). The hydrolysate (50 μL) was mixed with 0.5 mL of 0.2 M phosphate buffer, pH 8.2 and 0.5 mL of 0.05% TNBS reagent. TNBS was freshly prepared before use by diluting with deionized water. The mixture was incubated at 50°C for 1 h in a water bath. The reaction was stopped by adding 1 mL of 0.1 M HCl and incubating at room temperature for 30 min. The absorbance was monitored at 420 nm. L-Leucine was used as a standard. To determine the total amino acid content, hydrolysate was completely hydrolyzed with 6 M HCl with a sample to acid ratio of 1:100 at 120°C for 24 h. DH (%) was calculated using the following equation:

$$DH \quad (\%) = [(At - A0)/(Amax - A0)] \times 100$$

where A_t was the amount of a-amino acids released at time t, A_0 was the amount of a-amino acids in the supernatant at 0 h, and A_{max} was the total amount of a-amino acids obtained after acid hydrolysis at 120°C for 24 h.

Molecular docking experiment of MMP3 and MMP6

This assay of MMP3 and MMP6 was performed on the previous method (Zheng et al., 2022; Suo et al., 2022a) and commissioned to Shanghai NovoPro Biotechnology Co., Ltd (China). The crystal structures of human ACE-lisinopril complex (1O8A.pdb) and captopril were acquired from the RCSB PDB Protein Data Bank (PDB code: 1UZF). The interaction between ACE and MCO was analyzed to determine the position and size of the binding pocket using Chimera software. All non-standard residues in the 1UZF model were deleted, and AutodockTools were used to convert PDB files into PDBQT files (adding Gasteiger charge and setting key distortion). Peptide molecules were converted into SMILES format by PepSMI tool, and 3D models were drawn by Discovery Studio program and energy minimization was done using steepest descent and conjugate gradient techniques. Molecular docking and free energy calculation were carried out using flexible docking tool of Autodock Vina. Finally, the interaction between ACE and peptide molecules was analyzed by Chimera software. According to the binding-energy value and scores of MMP3 and MMP6, their best ranked docking poses in the active site of ACE were acquired.

Effects of MMP3 and MMP6 on HUVECs

HUVEC culture and cytotoxic assessment using MTT assay

The cytotoxic assay was carried out according to the previous method (Zhao et al., 2019b). HUVECs were cultured at a density of 1×10^4 cells/cm^2 to confluence in DMEM at 37°C in a humidified 5% CO_2 atmosphere. The cytotoxicity of MMP1-MMP7 on HUVECs was measured using MTT assay (Zhao et al., 2019b). In short, HUVECs in 96-well plates at a density of 1×10^4 cells/cm^2 were separately treated with 20 μL samples at 50 and 200 μM, respectively, and cultured for 24 h. Then, 20 μL MTT solution (5 mg/mL) were put in and incubated for 4 h. In the end, DMSO was joined in each well plate and the absorbance (A) at 490 nm was measured. The cell viability was calculated: Cell viability (%) = $(A_{sample}/A_{control}) \times 100$.

Evaluation of NO and ET-1 production

HUVECs were cultured in 96 well-plates at a density of 1 × 10^4 cells/cm^2 and treated with Cap (1 μM), NE (0.5 μM) or ACEi peptides (100 and 200 μM for MMP3 and MMP6) for 24 h, or incubated with both 200 μM ACEi peptide (MMP3 and MMP6) and NE (0.5 μM) for 24 h. The NO and ET-1 contents of HUVECs were determined after 24 h according to their assay kits as manufactures' protocol (Zhao et al., 2019b; Suo et al., 2022b).

Cytoprotection of MMP3 and MMP6 on H_2O_2-damaged HUVECs

Protection on H_2O_2-damaged HUVECs

The cytoprotective assay was carried out on the described methods (Cai et al., 2019; Wang et al., 2021b). In short, HUVECs were cultured in a 96-well plate at a density of 1 × 104 cells/cm^2 for 24 h. Soon afterwards, the supernatant was cleared away and 20 μL of peptide sample (MMP3 or MMP6) with the final concentrations of 100 and 200 μM were joined in the sample groups, respectively. ACEi peptides (MMP3 and MMP6) were removed after 8 h and H_2O_2 with the final concentration of 400 μM was added in and incubated for 24 h.

Measurement of levels of ROS, MDA, SOD, and GSH-Px

The ROS content was detected on the previous method and indicated as % of blank control (Cai et al., 2019); the levels of MDA, SOD, and GSH-Px were detected using their assay kits in accordance with the manufacturer' protocols and expressed as U/mg prot (Cai et al., 2019).

Data analysis

All data are expressed as the mean ± standard deviation (SD) (n = 3) and analyzed by SPSS 19.0. Significant difference analysis was employed ANOVA test with Dunnett or Tukey Test ($P < 0.05$, $P < 0.01$, or $P < 0.001$).

Results and discussion

Preparation of swim bladder hydrolysate of miiuy croaker (MSCH)

The proteins of miiuy croaker swim bladder were separately hydrolyzed using four proteases and *in vitro* GI digestion method. Figure 1A manifested that the DH of hydrolysate produced using *in vitro* GI digestion method was 27.26 ± 1.38%, which was remarkedly higher than those of other hydrolysates produced using Alcalase, Neutrase, pepsin, and trypsin, respectively ($P < 0.05$). In addition, the ACEi activity of five hydrolysates showed a similar trend (Figure 1B). At 3.0 mg/mL, the hydrolysate produced using *in vitro* GI digestion method showed the highest ACEi activity (51.37± 2.06%), followed by the hydrolysates prepared using Alcalase, Neutrase, trypsin, and pepsin, respectively ($P < 0.05$).

Enzymatic hydrolysis method is a popular process to produce protein hydrolysates because of its multiple significant properties, such as easy to control, environmentally friendly, and no residual chemical reagents (Chi et al., 2015a; Chi et al., 2015b; Sila and Bougatef, 2016). The biological functions of protein hydrolysates are closely contacted with the chemical structures and composition of bio-peptides, which is dramatically affected

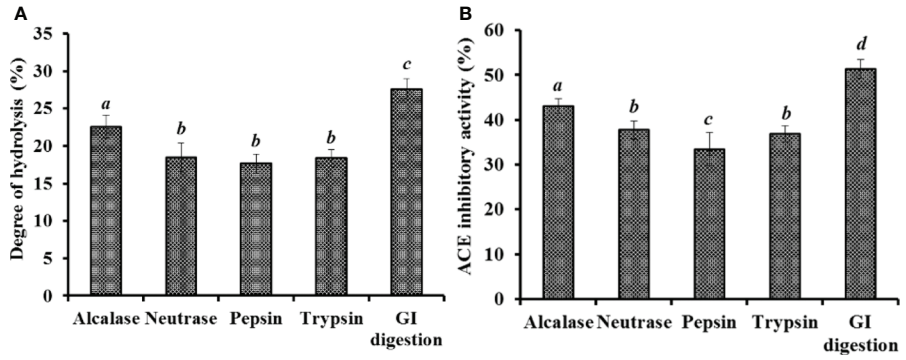

FIGURE 1
Degree of hydrolysis (%) **(A)** and ACEi activity (%)**(B)** of swim bladder hydrolysates of miiuy croaker using four proteases and *in vitro* GI digestion method. All values are means ± SD (n = 3). $^{a-d}$ Same letters indicate no significant difference ($p < 0.05$).

by the specificity of protease (Wang et al., 2013b; Chi et al., 2015c). Therefore, single and multiplex enzyme hydrolysis are frequently applied to generate hydrolysates from diverse protein sources (Lan et al., 2019; Yang et al., 2019b). Then, *in vitro* GI digestion method is mostly employed as an inexpensive, effective, easy to operate and safe method to prepare the protein hydrolysates (Vieira et al., 2016; Zhao et al., 2019a). Therefore, the swim bladder hydrolysate produced by *in vitro* GI digestion method was named as MSCH and chosen for further purification.

Preparing ACEi peptides from MSCH

MSCH was fractionated into MSCH-1, MSCH-2, MSCH-3, and MSCH-4 using ultrafiltration method. The ACEi activity of MSCH-I was 62.97 ± 2.39% at 3.0 mg/mL, and its inhibiting ability was prominently higher than those of MSCH (51.37± 2.06%), MSCH-2 (49.13 ± 2.35%), MSCH-3 (46.74 ± 1.82%), and MSCH-4 (21.96 ± 1.09%) ($P < 0.05$). Proteins and large MW peptides are difficult to approach and combine with the active site of ACE, leading to decreased inhibitory activity (Fan et al., 2019). Therefore, ultrafiltration process often serves to collect small MW bioactive peptides from protein hydrolysates (Abdelhedi et al., 2017). The current findings agreed with the reports that the lowest MW fractions of hydrolysates from *Moringa oleifera* leaf (Ma et al., 2021), tuna black muscle (Qiao et al., 2022) and frame (Lee et al., 2010), smooth-hound viscera (Abdelhedi and Nasri, 2019), Antarctic krill (Zhao et al., 2019b), *Cyclina sinensis* (Yu et al., 2018), and *Takifugu flavidus* skin (Su et al., 2021) presented the highest ACEi activities. Then, MSCH-I was selected for the next step isolation.

Using gel permeation chromatography of Sephadex G-25, MSCH-1 was divided into three peptide fractions (MSCH-1a, MSCH-1b, and MSCH-1c) and their ACEi rates were displayed at Figure 2. At 3.0 mg/mL, the ACEi rate of MSCH-1b was 76.25 ± 3.61%, which was markedly stronger than those of MSCH-1 (62.97 ± 2.39%), MSCH-1a (43.57 ± 1.59%), and MSCH-1c (35.24 ± 2.21%), respectively (Figure 2B) ($P < 0.05$). Gel filtration is a popular method to collect fractions with a particular MW dimension from a complex mixture and is generally applied in group isolation of protein hydrolysates from different sea food, such as by-catch shrimp waste (Joshi et al., 2020), miiuy croaker muscle (He et al., 2019b), skipjack tuna byproducts (Yang et al., 2019a; Wang et al., 2022a), red stingray (*Dasyatis akajei*) cartilages (Pan et al., 2019), rainbow trout viscera (Vásquez et al., 2022), Antarctic krill (Zhao et al., 2019b), and three-spot seahorse (Shi et al., 2020). In the experiment, MSCH-1b displayed the best ACEi activity, but it does not obtain the lowest MW. These finding suggested that some other influence factors besides MW, such as amino acid composition and linking sequences, also significantly affect the ACEi ability of peptides (Sila and Bougatef, 2016; Abdelhedi et al., 2017; Fan et al., 2019).

Finally, MSCH-1b was purified by RP-HPLC. According to the RP-HPLC profiles of MSCH-1b at 220 and 280 nm (Figure 3), seven ACEi peptides with retention time of 8.49 min (MMP1), 14.47 min (MMP2), 17.75 min (MMP3), 21.52 min (MMP4), 24.50 min (MMP5), 28.41 min (MMP6), and 33.25 min (MMP7) were concentrated on their HPLC peaks and freeze-dried (Table 2).

Peptide sequences and MWs determination (MMP1- MMP7)

By employing Protein/Peptide Sequencer, the sequences of seven ACEi peptides (MMP1- MMP7) were identified as Asp-Glu-Gly-Pro-Glu (DEGPE, MMP1), Glu-Val-Gly-Ile-Gln (EVGIQ, MMP2), Ser-His-Gly-Glu-Tyr (SHGEY, MMP3), Gly-Pro-Trp-Gly-Pro-Ala (GPWGPA, MMP4), Gly-Pro-Phe-Gly-Thr-Asp (GPFGTD, MMP5), Ser-Pro-Tyr-Gly-Phe (SPYGF, MMP6), and Val-Ile-Gly-Pro-Phe (VIGPF, MMP7),

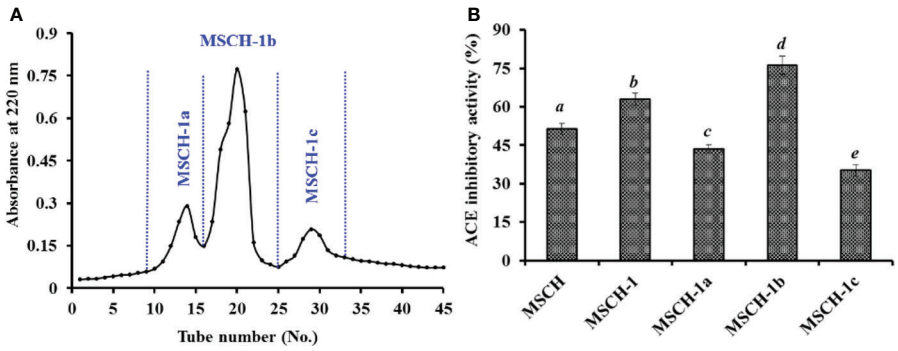

FIGURE 2
Chromatogram profiles of MSCH-1 isolated by Sephadex G-25 **(A)** and ACEi activity of prepared subfractions (MSCH-1a to MSCH-1c) from MSCH-I at 3.0 mg/mL **(B)**. All values are means ± SD (n = 3). $^{a-e}$ Same letters indicated no significant difference ($p < 0.05$).

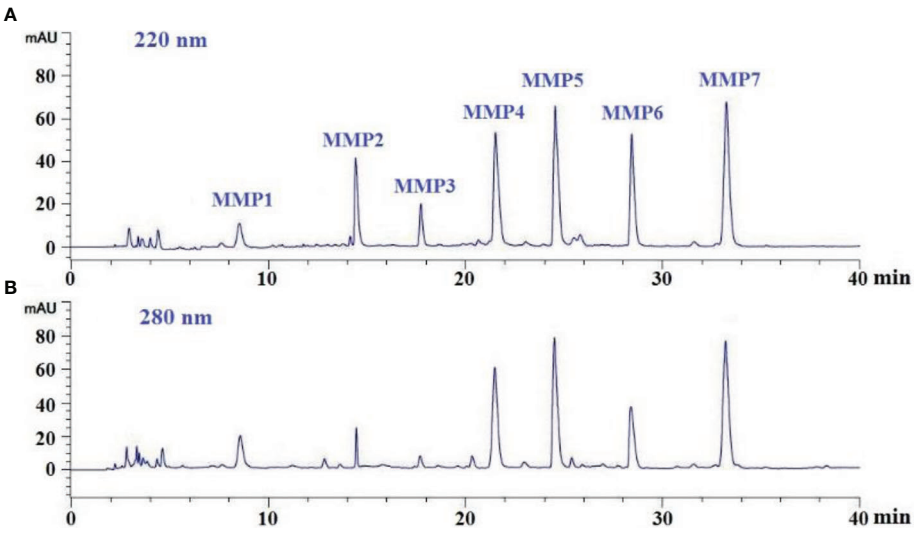

FIGURE 3
Elution profiles of sub-fraction MSCH-1b by RP-HPLC at 220 nm **(A)** and 280 nm **(B)**.

respectively, and their MWs were determined as 545.49, 544.58, 591.55, 583.63, 592.59, 569.60, and 531.63 Da, respectively (Figure 4), which agreed well with their theoretical MWs (Table 2).

ACEi activity and molecular docking analysis

Table 2 indicated that the IC_{50} values of MMP3 and MMP6 on ACE were 0.86 ± 0.12 and 0.37 ± 0.06 mg/mL, which were notably lower than those of other five ACEi peptides ($P < 0.05$). Additionally, the IC_{50} vales of MMP3 and MMP5 were lower than those of ACEi peptides from protein hydrolysates of *Arthrospira platensis* (PTGNPLSP: 1.54 mg/mL) (Wang et al., 2021a), *Sepia officinalisn* (VGLYP: 3.23 mg/mL) (Balti et al., 2015), *Oncorhynchus keta* (GLP: 2.91 mg/mL) (Lee et al., 2014), *Salmo salar* (YP: 1.54 mg/mL) (Neves et al., 2017), *Katsuwonus pelamis* muscle (IPK: 2.47 mg/mL; FEM: 2.18 mg/mL) (Qiao et al., 2022; Zheng et al., 2022), *Okamejei kenojei* (MVGSAPGVL 3.09 mg/ml) (Ngo et al., 2015), *Ctenopharyngodon idella* (VAP: 1.71 mg/ml) (Chen et al., 2012), and stone fish (EVLIQ: 1.44 mg/mL) (Auwal et al., 2019). The ACEi capabilities of MMP3 and MMP6 indicated that they might serve as active ingredients added in BP lowering products.

Molecular docking experiment of MMP3 and MMP6 was carried out to analyze their ACEi mechanisms (Figure 5). Figure 5A indicated that MMP3 (SHGEY) formed hydrogen bonds with Ala354, His353, Glu376, Ser284, His383, His387, and Tyr523 residues of ACE, therein, MMP3 (SHGEY) formed hydrogen bonds with S1 pocket (Ala354) and S2 pocket (His353). Additionally, MMP3 (SHGEY) interacted with Glu384, Glu411, Lys454, and Lys511 residues through hydrophobic effect, and contacted with Val380 and Val379

TABLE 2 Amino acid sequences, molecular weights (MWs), and ACEi activity (IC_{50} value) of seven isolated ACEi peptides (MMP1- MMP7) from swim bladder hydrolysate of miiuy croaker (MSCH).

	Retention time (min)	Amino acid sequence	Observed MW/Theoretical MW (Da)	ACEi activity(IC_{50}, mg/mL)
MMP1	8.49	Asp-Glu-Gly-Pro-Glu (DEGPE)	545.49/545.50	2.79 ± 0.42 [a]
MMP2	14.47	Glu-Val-Gly-Ile-Gln (EVGIQ)	544.58/544.60	4.06 ± 0.37 [b]
MMP3	17.75	Ser-His-Gly-Glu-Tyr (SHGEY)	591.55/591.57	0.86 ± 0.12 [c]
MMP4	21.52	Gly-Pro-Trp-Gly-Pro -Ala (GPWGPA)	583.63/583.64	2.18 ± 0.24 [d]
MMP5	24.50	Gly-Pro-Phe-Gly-Thr-Asp (GPFGTD)	592.59/592.60	1.65 ± 0.13 [e]
MMP6	28.41	Ser-Pro-Tyr-Gly-Phe (SPYGF)	569.60/569.61	0.37 ± 0.06 [f]
MMP7	33.25	Val-Ile-Gly-Pro-Phe (VIGPF)	531.63/531.64	1.32 ± 0.17 [g]

All values are means ± SD (n= 3). [a-g] Same letters indicated no significant difference ($P > 0.05$).

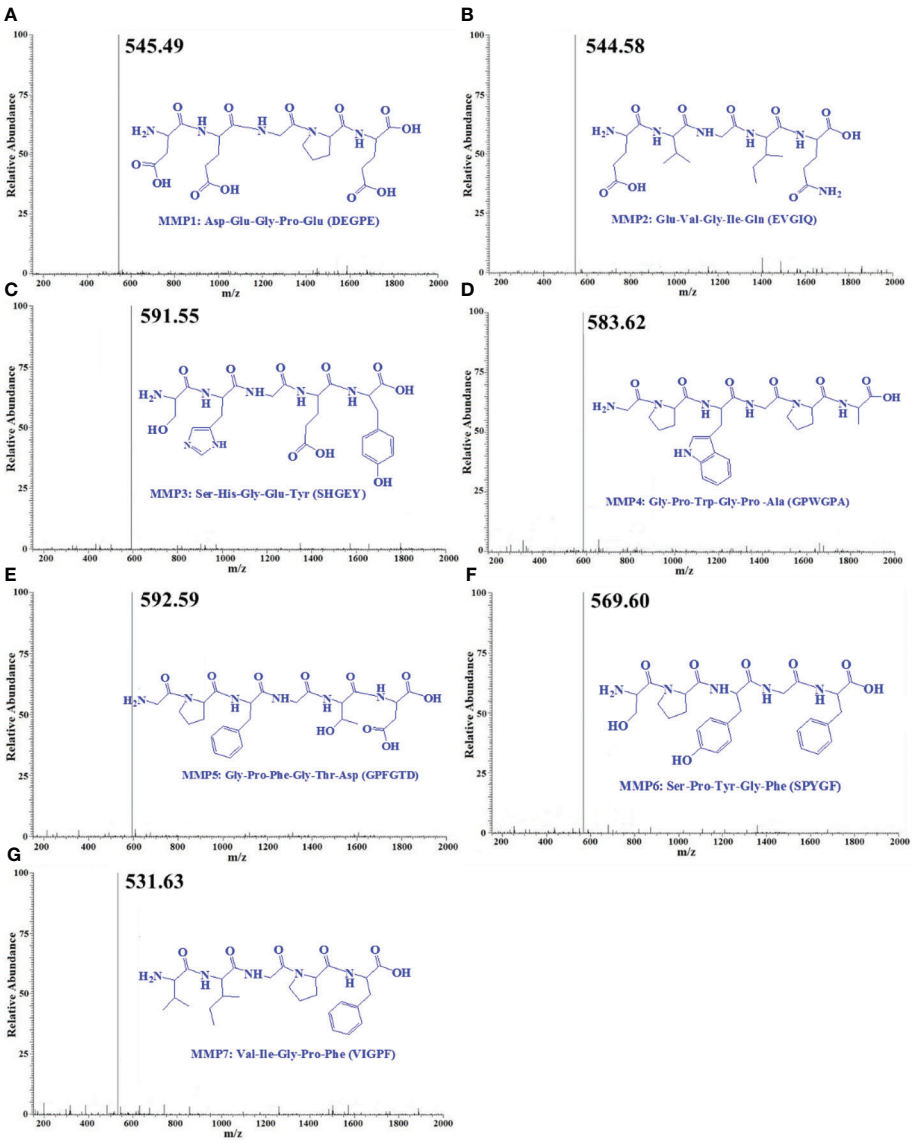

FIGURE 4
Mass spectrogram of seven ACEi peptides (MMP1- MMP7) from protein hydrolysate of miiuy croaker swim bladders (MSCH). **(A)** MMP1; **(B)** MMP2; **(C)** MMP3; **(D)** MMP4; **(E)** MMP5; **(F)** MMP6; **(G)** MMP7.

residues through electrostatic force. Figure 5B confirmed that MMP6 (SPYGF) formed hydrogen bonds with Tyr523(S1), His513(S2), Glu411, and Arg522 residues of ACE, therein, MMP6 (SPYGF) established hydrogen bonds with S1 (Tyr523) and S2 (His513) pockets. Furthermore, MMP6 (SPYGF) interacted with His387 residue through hydrophobic effect, and contacted with Trp357, Pro407, and His410 residues through electrostatic force. Molecular docking assay proved that MMP3 and MMP6 exhibited better ACEi activity attributing to the effectively interacting with the active sites of ACE by different forces, especially hydrophobic interaction, electrostatic force, and hydrogen bonding.

In addition, the affinity of MMP3 and MMP6 with ACE was -8.3 and -9.2 kcal/mol, which was close to those of YLLLK (-8.2 kcal/mol), GVQEGAGHYALL (-7.0 kcal/mol) (Zarei et al., 2019), YSK (-7.9 kcal/mol) (Wang et al., 2017), SP (-5.7 kcal/mol), VDRYF (-9.7 kcal/mol) (Zheng et al., 2022), PMHIR (-10.37 kcal/mol), and PQVSTPTL (-6.64 kcal/mol) (Li et al., 2022).

Molecular size greatly affects the affinity between peptide and ACE, which further remarkedly influences ACEi ability of antihypertensive peptides (Abdelhedi and Nasri, 2019; Fan et al., 2019). Crystallographic studies proved that potent ACEi peptides with 2 to 12 amino acid residues are easy to link to

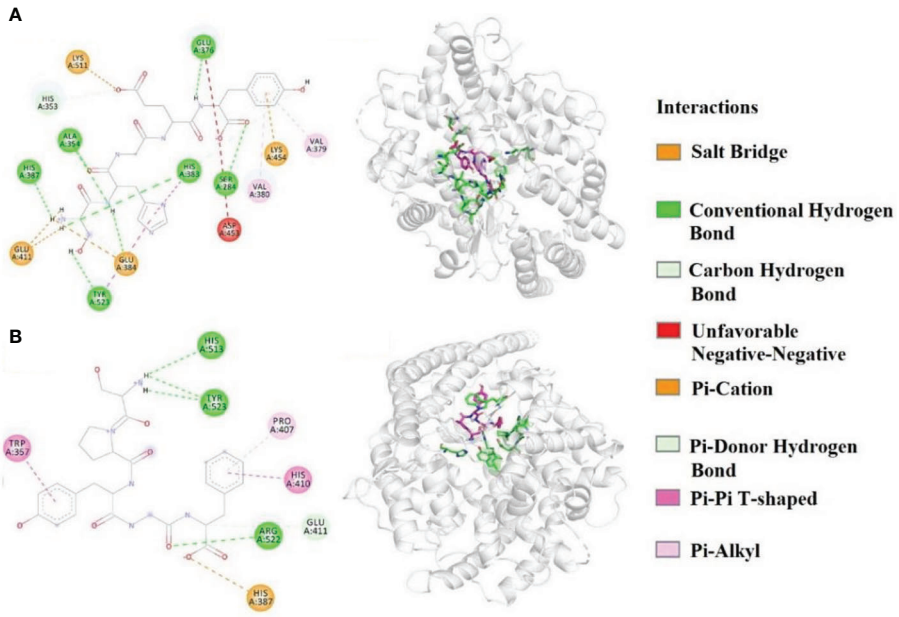

FIGURE 5
Molecular docking result of MMP3 **(A)** and MMP6 **(B)** with ACE (A1) 2-D details of ACE and MMP3 interaction. (A2) 3-D interaction details for MMP3; (B1) 2-D details of ACE and MMP6 interaction; (B2) 3-D interaction details for MMP6.

the acting site of ACE (Chen et al., 2012). Similarly, VPP and IPP could be easier access the ACE channel and effectively coordinate with Zn^{2+} with higher interaction scores than larger peptides (Abdelhedi et al., 2018). In addition, Su et al. proved that peptides with four to nine amino acid residues could passively pass through cell membranes to play their functions in cells (Su et al., 2021). In the experiment, MMP3 (SHGEY) and MMP6 (SPYGF) are pentapeptides, and the MWs increase their access to the active site of ACE, which was proved by their affinities with ACE (-8.7 and -9.7 kcal/mol for MMP3 and MMP6, respectively).

The amino acid composition and sequence could be more relevant with the ACEi activity of peptides than the MWs (Auwal et al., 2019). The C-terminal amino acids have been wildly discussed because of their crucial functions on the antihypertensive peptides (Fan et al., 2019). The aromatic (Tyr, Phe, and Trp) and branched-chain (Val, Leu and Ile) amino acids were in favor of combining with the C-terminal active site of ACE (Sun et al., 2017). Therefore, Tyr and Phe at the C-terminus of MMP3 and MMP6 are critical for their affinities with ACE. Ser was regarded as conducing to the high ACEi activity of EMFGTSSET because it could establish three hydrogen bonds with ACE residues Ala-356 and Tyr-523 (Pei et al., 2022). Also, molecular docking was found hydroxyl group of Ser residue played vital effects in the ACEi capability of SP because it could form hydrogen bond with the His383 residue of ACE (Zheng et al., 2022). Therefore, Ser residues of MMP3 and MMP6 could significantly strengthen their ACEi activity. Furthermore, ACEi peptides with reasonable proportion of hydrophilic and hydrophobic amino acids can helpfully bind with the active center of ACE to control its function (Yea et al., 2014; Auwal et al., 2019). In MMP3 and MMP6, MMP6 has a more reasonable ratio of hydrophobic and hydrophilic amino acids, and this should be the main reason that MMP6 showed more ACEi activity than MMP3.

Effects of MMP3 and MMP6 on HUVECs

The effects of MMP3 and MMP6 on the viability of HUVECs at 100-400 μM were shown in Figure 6A. At the designed concentration, the viability of MMP3 group was ranged from 98.67 ± 2.08% to 101.25 ± 0.98%, and the viability of MMP6 group was ranged from 98.81 ± 1.67% to 102.18 ± 1.52%. In normal tissues, the cell proliferation and death generally keep an appropriate balance, and the active substances with strong inhibiting ability on cell proliferation indicate their possible toxicity risk to the body, and are deemed to be inadequacy to developing healthy products with non-antitumor functions (Zhao et al., 2019b; Qiao et al., 2022). These data suggested that MMP3 and MMP6 didn't show significant cell toxicity in HUVECs and should suite to developing anti-blood pressure health products.

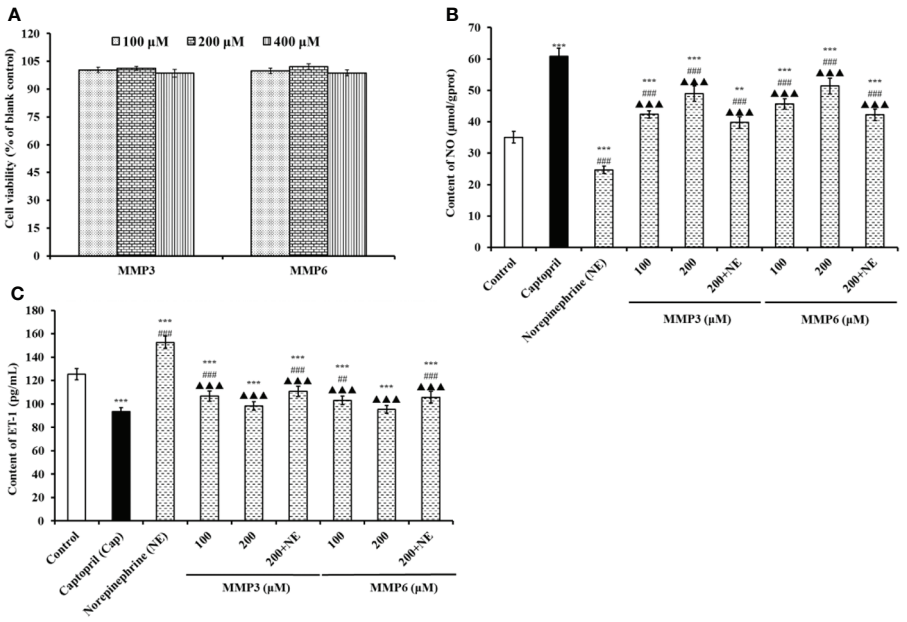

FIGURE 6
The effects of MMP3 and MMP6 on the cell viability **(A)**, NO production **(B)**, and ET-1secretion **(C)** of HUVECs, respectively. Captopril (Cap) was designed as a positive control. All values are means ± SD (n= 3). ***$P < 0.001$ VS Control; $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ VS Cap; ▲▲▲$P < 0.001$ VS Norepinephrine (NE).

According to Figure 6B, the NO contents in MMP3 and MMP6 groups were significantly increased than control group (35.06±1.86 μmol/gprot) ($P < 0.001$), and the NO contents of MMP3 and MMP6 groups increased to 48.91 ± 2.46 and 51.38 ± 2.56 μmol/gprot at 200 μM. However, the NO contents in HUVECs treated with MMP3 and MMP6 were lower than that (60.82±2.67 μmol/gprot) of Cap treated group ($P < 0.001$). Moreover, NE could significantly decrease the content of NO (24.67±1.15 μmol/gprot) than control group ($P < 0.001$), but the NO content negative decreased by NE was separately compensated to 39.75 ± 1.83 and 42.26 ± 1.85 μmol/gprot in MMP3 and MMP6 group at 200 μM ($P < 0.001$).

Figure 6C indicated that the ET-1 secretion of HUVECs was negatively affected by MMP3 and MMP6, and the ET-1 levels of MMP3 and MMP6 groups reduced to 98.39 ± 3.65 and 95.38 ± 3.36 pg/mL at 200 μM. On the contrary, NE could significantly increase the ET-1 secretion (152.69 ± 5.38 pg/mL) than control group (125.39 ± 4.69 pg/mL) ($P < 0.001$), but the decrease of ET-1 secretion influenced by NE was partially restored by MMP3 and MMP6 treatment and decreased to 110.79 ± 4.28 and 105.72 ± 4.92 pg/mL at 200 μM ($P < 0.001$). The ET-1 secretion of HUVECs was significantly decreased to 93.54 ± 3.15 pg/mL by 0.5 μM Cap treatment than control group (125.39 ± 4.69 pg/mL) ($P < 0.001$).

Hypertension is a complicated chronic disease which leads to endothelial dysfunction by influencing the production of NO and ET-1 in endothelial cells, which further causes apoptosis and arterial vasoconstriction. Therefore, HUVECs are widely applied to analyze vascular endothelium characters and the key biological pathways on endothelium function and cardiovascular diseases. In pathologic situations, NO deficiency will give rise to the risk of cardiovascular, and improving the production of endothelial NO represents a good therapeutic approach for atherosclerosis (Abdelhedi and Nasri, 2019; Fan et al., 2019). Therefore, some ACEi peptides, such as KYIPIQ (Lin et al., 2020), IPIPATKT (Chen et al., 2021), WF (Zhao et al., 2019b), GRVSNCAA, TYLPVH (Zhang et al., 2021), IVTNWDDMEK, VGPAGPRG (Wang et al., 2022b), SP (Zheng et al., 2022), MKKS and LPRS (Qiao et al., 2022), play their hypotensive activity by enhancing the production of NO in HUVECs.

Literatures reported that increasing in Ang-II and ET-1 could cause endothelial abnormalities, which are closely correlated with hypertension and coronary heart disease (Jiang et al., 2021). Decreased production of ET-1 in the renal medulla can elevate systemic blood pressure (Kohan, 2008). ACEi oligopeptides of SP, YRK, MKKS, FQK, FAS, and LPRS from tuna muscle and Antarctic krill displayed same function of bringing down the ET-1 level (Zhao et al., 2019b; Qiao et alo, 2022). VIEPR and VVLYK from oil palm kernel expeller could dose-dependently inhibit the secretion of intracellular ET-1 in EA.hy926 cells (Zheng et al., 2017). GRVSNCAA and TYLPVH from *Ruditapes philippinarum* gave play to their function of lowering blood pressure through markedly lowering ET-1 generation (Zhang et al., 2021). Umami Peptide IPIPATKT from Sanhuang chicken hydrolysate could reduce the ET-1content in the insulin-resistant-HepG2 (IR-HepG2) and

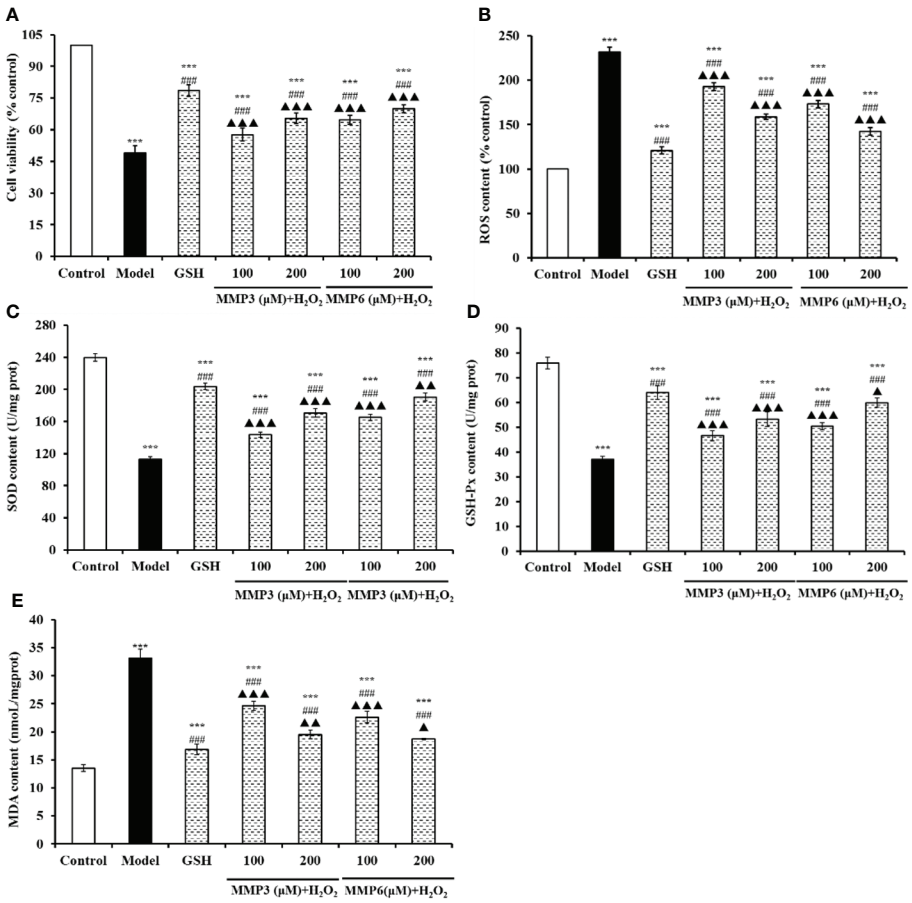

FIGURE 7
Effects of MMP3 and MMP6 on the cell viability **(A)**, ROS **(B)**, SOD **(C)**, GSH-Px **(D)**, and MDA **(E)** of H_2O_2-induced HUVECs. All values are means ± SD (n= 3). GSH was designed as a positive control. *** $P < 0.001 < 0.01$ VS control; ### $P < 0.001$ VS model; ▲▲▲ $P < 0.001$ VS GSH.

HUVEC models (Jiang et al., 2021). Moreover, IPIPATKT displayed hypotensive and decreased glucose level effects in SHRs and C57BL/6N mice. According to the finding, ACEi peptides of MMP3 and MMP6 prominently promote NO production while restrict ET-1 secretion in HUVECs. Moreover, MMP3 and MMP6 can reverse the negative effects of NE on NO and ET-1 production in HUVECs.

Cytoprotective effects of MMP3 and MMP6 on H_2O_2-induced HUVECs

Figure 7A presented the cytoprotective effects of MMP3 and MMP6 on the H_2O_2-induced HUVECs at 100 and 200 μM. MMP3 and MMP6 revealed the dose-dependently protective effects on the H_2O_2-damaged HUVECs, and the cell viability of MMP3 and MMP6 groups at 200 μM were increased to 65.37 ± 2.49% and 70.03 ± 1.82%, respectively, which were significantly higher than that of model group (48.96 ± 3.46%) ($P < 0.001$). However, the cell viability of MMP3 and MMP6 groups was lower than that of the GSH group (78.63 ± 2.68%) ($P < 0.001$). Then, MMP3 and MMP6 could give a strong protection to H_2O_2-induced HUVECs by increasing their viability.

Figure 7B indicated that the ROS levels were markedly lowered after pretreating with MMP3 and MMP6 compared with model group (231.85 ± 5.62%) ($P < 0.001$). At 200 μM, the ROS levels of MMP3 and MMP6 groups were observably dropped to 158.92 ± 3.16% and 142.37 ± 4.36% of the control group, respectively. However, the ROS levels of MMP3 and MMP6 groups were higher than that of the GSH group (120.75 ± 3.98%) ($P < 0.001$). In addition, MMP6 showed the higher ability on scavenging ROS than MMP3 at the determined concentrations.

Figure 7C and Figure 7D showed the activity of SOD and GSH-Px incubated with MMP3 and MMP6 at 100 and 200 μM was gradually increased. At the concentrations of 100 and 200 μM, SOD activity in MMP3 group was 143.59 ± 3.08 and 170.66 ± 5.32 U/mg prot, and the SOD activity in MMP6 groups was 165.38 ± 3.96 and 190.53 ± 4.67 U/mg prot. Moreover, the SOD activity in MMP3 and

MMP6 groups was markedly higher than that of the model group (112.69 ± 3.57 U/mg prot) ($P < 0.001$). The changes of GSH-Px activity showed the same trend with the levels of SOD (Figure 7D). At the concentrations of 100 and 200 μM, the GSH-Px activity in MMP3 group was 46.58 ± 2.01 and 53.27 ± 2.85 U/mg prot, and the activity in MMP6 group was 50.39 ± 1.39 and 59.87 ± 1.88 U/mg prot. GSH-Px activity of peptide groups were observably higher than that of the model group (36.98 ± 1.23 U/mg prot) ($P < 0.001$).

Figure 7E revealed that the MDA levels were markedly lowered after pretreating with MMP3 and MMP6 compared with model group (33.19 ± 1.52 nmol/mg prot) ($P < 0.001$). At 200 μM, the MDA levels of MMP3 and MMP6 groups were dramatically decreased to 19.53 ± 0.76 and 18.75 ± 0.13 nmol/mg prot, respectively. MMP6 showed stronger ability on decreasing MDA content than MMP3, but it ability was still lower to that of GSH (16.85 ± 0.96 nmol/mg prot).

Oxidative stress is bound up with apoptosis, cell migration, hypertrophy, inflammation, angiogenesis, and endothelial dysfunction with reference to vascular remodeling of hypertension-related diseases (Sinha and Dabla, 2015). ROS is known as the mediums of angiotensin II-induced blood pressure, and inhibition of ROS content is contributed to normalizing endothelium function and decreased vascular inflammation and reaction, which further reduce blood pressure and prevent the hypertension development (Sinha and Dabla, 2015). For another, MDA is a key peroxidation product of the cell membrane lipid and serves as a well-known indicator for estimating the oxidative damage degree (Wang et al., 2021b). Presently, some food-derived peptides showed ACEi and antioxidant activities, such as. TPCPPQ, YSKA, and VLSTSFPPK from Pixian broad bean (Li et al., 2021), rice bran (Wang et al., 2017), and *Kluyveromyces marxianus* (Mirzaei et al., 2018), respectively. Moreover, some bioactive peptides showed significant protective function in Angiotensin II or H_2O_2-induced cell models. Zheng et al. found that ACEi peptides of VIEPR, LPILR, ADVFNPR and VVLYK from Oil palm kernel expeller could exert antihypertensive effect through scavenging excessive ROS and protect vascular endothelial cells from excessive ROS-induced damage (Zheng et al., 2017). Umami peptides of CC, CCNK, and HCHT played their cytoprotective effects by reducing the ROS content (Hao et al., 2020). ACEi peptides IVTNWDDMEK and VGPAGPRG could protect HUVECs against H_2O_2 damage by up-regulating the expression of nuclear factor erythroid 2-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) to decrease the production of ROS and MDA (Wang et al., 2022b). ACEi peptide EMFGTSSET (IC_{50} 15.08 μM) from *Isochrysis zhanjiangensis* has excellent effect in regulating hypertension by reducing the contents of ROS, cytokines, and adhesion factors in Ang II-induced HUVECs (Pei et al., 2022). FNLRMQ from *Takifugu bimaculatus* skin could alleviated the viability and facilitated apoptosis of Ang-II-induced HUVECs by regulating phosphatidylinositol-3-kinase (PI3K)/protein kinase B (Akt)/endothelial NO synthase (eNOS) and Nrf2/HO-1 signaling pathways (Cai et al., 2021). FPYLRH, FWKVV, and FMPLH could promote the bioactivity of SOD/GSH-Px to bring down the oxidative damage of DNA and the production of ROS and MDA in H_2O_2-induced HUVECs (Wang et al., 2020). Moreover, the antioxidant mechanisms of bioactive peptides, such as FEIHCC (Chen et al., 2020), IVTNWDDMEK, VGPAGPRG (Wang et al., 2022b), KVLPVPEK (Tonolo et al., 2020), MHQPHQPLPPTVMF (Qin et al., 2021), APKGVQGPNG (Rahman et al., 2018), ICRD, LCGEC (Han et al., 2020), and VHVV (Tsai et al., 2020), indicated that they could regulate the Nrf2 pathway in cell model to induce the overexpression of antioxidases to decrease the damage of ROS. The current results suggested that the protective activities to H_2O_2-damaged HUVECs of MMP3 and MMP6 were similar to those previous reported peptides, and the acting mechanism should be connected with activating Nrf2 pathway to improve intracellular antioxidase bioactive.

Conclusion

In a conclusion, the swim bladder hydrolysate of miiuy croaker with high ACEi activity was produced using *in vitro* GI digestion processing, and seven novel ACEi peptides were isolated from the hydrolysate and identified as DEGPE, EVGIQ, SHGEY, GPWGPA, GPFGTD, SPYGF, and VIGPF, respectively. SHGEY and SPYGF displayed noticeable hypotensive activity through inhibiting ACE activity, increasing NO production and decreasing ET-1 secretion in HUVECs, and protecting HUVECs from H_2O_2-induced oxidative damage. Moreover, SHGEY and SPYGF exhibited significant ACEi activity attributing to their effective interaction with the active sites of ACE by hydrogen bonding, electrostatic force and hydrophobic interaction. Therefore, this study not only develops technical support for utilizing miiuy croaker swim bladders to produce novel ACEi peptides, but also contributes to dispose the environmental pollution problems of fish byproducts. More importantly, seven novel ACEi peptides, especially SHGEY and SPYGF, might be used as natural functional ingredients for developing noticeable hypotensive products. However, the antihypertensive activities and mechanisms of SHGEY and SPYGF in animal models should be performed in future experiments, which will provide better insight into their potential in the management of hypertension.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors

Author contributions

W-YZ: investigation, methodology, data analysis, and validation. Y-MW: methodology, data analysis, and writing-original draft. S-KS and S-LZ: data curation, investigation, and methodology. BW: resources, funding acquisition, supervision, and writing-review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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.

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