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Identification of Novel Nonapeptides from *Sipunculus nudus* L. and Comparing Its ACEI Activities Mechanism by Molecular Docking

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Abstract

In this study, the sea food *Sipunculus nudus* L. was hydrolyzed by trypsin, and peptides were isolated and purified from the hydrolysates. As a result, two novel nonapeptides were identified by LC–MS–MS with amino sequences of GFAGDDAPR and GLGGLSPEK. The ACEI activity were determined and the IC_{50} values of the peptides for ACE inhibition activity were 0.76 mmol/L and 0.91 mmol/L, respectively. The results showed that both peptides had ACE inhibitory activity. Analysis of the Lineweaver–Burk plot demonstrated that these peptides served as non-competitive ACE inhibitors. Molecular docking study showed that these peptides could interact with the active site of ACE mainly through hydrogen bonding and electrostatic force. The amino acid residue that plays a key role in ACE inhibitory activity was its C-terminal Arg. It is therefore suggested that *S. nudus* may be a useful raw material for the production of antihypertensive peptides which can offer therapeutic and commercial benefits as an ingredient in functional foods.

Keywords *Sipunculus nudus* L. · ACE inhibitory peptide · Preparation · Identification

Introduction

Hypertension is a clinical syndrome characterized by increased systemic arterial blood pressure (systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg), which may be accompanied by functional or organic damage of heart, brain, kidney and other organs (Doyle 1991; Ralston et al. 2016). According to the World Health Organization, 9.4 million people die from high blood pressure every year in the world, and this number is far larger than that of other risk factors. Based on the current data, it is predicted by some studies that the number of adults with hypertension will reach 1.56 billion by 2025 (Kearney et al. 2005).The renin angiotensin aldosterone system (RAAS) is considered to be one of the main pressor systems in the pathogenesis of hypertension. Angiotensin I converting enzyme (ACE) plays an important role in renin angiotensin and kallikrein system by increasing the production of angiotensin II and decreasing the formation of bradykinin (Ame et al. 2019; Natesh et al. 2003). At present, a variety of ACE inhibitors (ACEI) have been developed, such as captopril, enalapril, lisinopril, fosinopril and so on (Overlack 1996; Wilin et al. 2018). These drugs have a strong antihypertensive effect in clinic, but there are many adverse reactions such as irritative dry cough, rash, hyperkalemia, angioneurotic edema and so on. These adverse reactions may reduce the compliance of patients. Therefore, it is important to find substances with fewer side effects but improved safety and enhanced ACE inhibitory activity. It is generally believed that ACE inhibitory peptides derived from natural food have high safety and fewer side effects, and may assist or even replace ACE inhibitors in the treatment of hypertension. More than one hundred ACE inhibitory peptides have

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been isolated and identified from marine organisms with enzymatic hydrolysis method (Ryan et al. 2011; Stadnik et al. 2015). For example, some ACE inhibitory peptides have been successfully isolated from Rhopilema esculentum, marine macroalga (*Ulva intestinalis*) and the cuttlefish (*Sepia officinalis*) muscle(Balti et al. 2015; Li et al. 2014; Sun et al. 2019).

Sipunculus nudus L. (Shachong, called in Chinese) mainly lives along the coast of the pacific ocean, Indian ocean and Atlantic ocean (Hsu et al. 2013), and it is a popular seafood in Asian countries owing to its high nutritional and pharmacological values. The majority of studies about *S. nudus* focused on its polysaccharide content. Li et al. reported the radiation protection of *S. nudus* polysaccharide, Sun and Zhang studied the immune repair and anti-hypoxia bioactivities of *S. nudus* polysaccharide, respectively (Li et al. 2016; Su et al. 2018; Zhang et al. 2011). Unlike polysaccharide, the studies of polypeptide of *S. nudus* mainly concentrated on the anti-inflammatory, antithrombotic and other activities (Ge et al. 2018; Sangtanoo et al. 2020). However, to the best of our knowledge, an enzymatic hydrolysis method to prepare *S. nudus* peptides with ACE inhibitory activity has not been reported yet.

Therefore, in the present study, we attempted to hydrolyze *S. nudus* for the preparation of ACE inhibitory peptides. The main ACE inhibitory peptides were isolated and identified to investigate their inhibitory activity. In addition, the peptide with the highest ACE inhibitory activity was characterized and synthesized for the prediction of its potential binding sites with ACE simulated by molecular docking.

Materials and Methods

Materials

Fresh *S. nudus*, which was originally identified by Professor Li Sidong, Guangdong Ocean University, was purchased from an aquaculture base in Zhanjiang (N 21° 12′; E 110° 4′), a coastal city in southern China. Before enzymatic hydrolysis, the *S. nudus* were removed viscera and stored at − 20℃ until use.

ACE (0.25 U, from rabbit lung), Hippuryl-histidine-leucine (HHL, HPLC, purity ≥ 98%) and captopril (purity > 99%) were purchased from Sigma-Aldrich Co. (USA). Glacial acetic acid, sodium chloride and sodium hydroxide were purchased from Guangdong Guanghua Sci-Tech Co., Ltd (Guangdong, China). Pancreatin was purchased from Nanning Pangbo Bioen-gineering Co., Ltd (Guangxi, China). Acetonitrile was purchased from Beijing DIKMA Technology Co., Ltd (Beijing, China). Tris (hydroxymethyl) aminomethane was provided by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). All of the chemical reagents were analytical grade, except for acetonitrile which was chromatographic grade.

Enzyme Hydrolysis and Ultrafiltration of *S. nudus*

Fresh *S. nudus* (200 g) was washed and viscera with cold pure water, and then homogenized at 4℃. The homogenate was diluted with 2.0 L pure water and adjusted the pH to 7.5 with 0.1 mol/L NaOH solution after adding 2 g trypsin powder (6000 IU/g). Enzymatic hydrolysis was performed according to Luntown with slight modifications: hydrolysis was carried out at 45 ℃ for 4 h and stirred slightly(Lunow et al. 2013). At the end of the hydrolysis, the temperature was raised to 90℃ and kept for 5 min. The hydrolysate was extracted with n-hexane (4:1 water/oil, v/v) to remove the lipid before centrifugal separation at 12,000 × g for 30 min, and then the supernatant was collected.

The supernatant was ultrafiltrated into fractions using membranes with different pore sizes to obtain peptide fragments with various molecular weights: < 2.5 kDa, 2.5–5 kDa, 5–10 kDa, and 10–100 kDa. The ACE inhibitory activity of the peptide fragments were measured for further purifications.

Determination of ACE inhibition activity

The ACE inhibitory assay was conducted according to Cushman and Cheung et al. (1971) (Wu et al. 2002; Zhao et al. 2019). with some modifications. HHL and ACE were dissolved in 50 mmol/L Tris-CH3COOH buffer (containing 300 mmol/L NaCl, pH 8.3), resulting in the concentrations of 5 mmol/L and 0.1 U/mL, respectively. The samples were dissolved in 50 mmol/L Tris-CH_3COOH buffer (containing 300 mmol/L NaCl, pH 8.3) to different concentrations. The above solutions were filtered through 0.22 μm membrane. 100 μL sample was mixed with 10 μL ACE and incubated at 37 ℃ for 10 min. HHL was incubated for 10 min with the same conditions. 100 μL HHL was mixed with sample-ACE mixture and incubated at 37 ℃ for 60 min. 150 μL CH_3COOH was added to terminate the reaction. The production of hippuric acid (HA) in 10 μL reaction solution was determined by HPLC (1260 Infinity II, Agilent Technologies Co., Ltd.). BDS hypersil C18 column (4.6 mm × 250 mm, 5 μm, Agilent Technologies Inc., St. Clara, CA, USA) was used. Mobile phase A (0.5% acetic acid aqueous solution) and 10% mobile phase B (100% ACN) were used at the flow rate of 1.0 mL/min. The HA in the reaction solution was eluted and detected at the wavelength of 228 nm. All tests were repeated for three times. ACE inhibitory activity was calculated as follows:

$$\text{ACE inhibitory rate } (\%) = (A_{control} - Ai_{inhibitor})/A_{control} \times 100$$

where $A_{inhibitor}$ was the relative area of the hippuric acid (HA) peak obtained from the reaction of ACE and HHL with inhibitor. $A_{control}$ was the relative area of the hippuric acid (HA) peak obtained from the reaction of ACE and HHL without inhibitor. IC_{50} is defined as the peptide concentration that can inhibit half of the ACE activity.

Purification and Identification of ACE Inhibitory Peptides

The peptide fragment with highest ACE inhibition activity was further separated with Task-gel G3000SW column (7.5 mm × 600 mm, 10 µm), eluting with deionized water at the flow rate of 1.0 mL/min. The elution peak at 280 nm was collected, and it was further purified and identified by RP-HPLC coupled with an ESI-TOF–MS/MS (electrospray-ionization time of flight mass-mass spectrometry) according to the method of Lunow et al. (2013). The obtained amino acid sequences were then mapped through the PepBank, EROP-Moscow, and BIOPEP-UWM database.

Properties of ACE Inhibitory Peptides

The related properties of the target peptide can be retrieved through the online database. The peptideranker activity score server can predict the bioactivity of the target peptide. The higher the score, the higher the probability of bioactivity, (http://bioware.ucd.ie/). Innovagen online server can be used to evaluate the dissolution characteristics of target peptides (http://www.innovagen.com/proteomics-tools). Swiss target prediction can be used to predict the most likely macromolecular target of bioactive small molecules (http://www.swisstargetprediction.ch/).

Characterization of the Interaction Pattern with ACE

The peptides were dissolved in Tris-CH3COOH buffer (50 mmol/L, containing 300 mmol/L NaCl, and pH 8.3) to the concentrations of 0.078 and 0.16 mmol/L. HHL was dissolved in the buffer system to the concentration of 0.31, 0.63, 1.25, 2.5 and 5 mmol/L. The ACE inhibitory activity was detected according to the method in 2.3. The interaction pattern with ACE in the presence of inhibitory peptides was obtained by Lineweaver–Burk diagram (Seibert et al. 2014; Singh et al. 1984).

Molecular Docking

The molecular docking work was completed using discovery studio 3.5 (DS 3.5, ac-celerations, San Diego, CA, USA). The X-ray structure of human tACE was downloaded from RCSB protein database (PDB ID: 1O8A, resolution: 2.00 Å, http://www.rcsb.org/pdb/explore/explore.do?structureId=1O8A). The structure of ACE was dehydrated and hydrotreated with discover studio 3.5. Discovery studio 3.5 was used to design the three-dimensional structure and minimize the energy. The binding sites were X: 44.8452, Y: 26.7921 and Z: 41.7074. The affinity was evaluated by molecular dynamics (MD) method based on CHARMM using CDOCKER module. High temperature MD was used to generate random ligand conformations, which were then transformed into binding sites. Then random rigid body rotation and simulated annealing are used to generate candidate structures. The final energy minimization was used to improve the structure of the ligand. -CDOCKER ENERGY and -CDOCKER interaction energy are two criteria to evaluate the results of CDOCKER. -CDOCKER ENERGY was a negative value of the total energy. -CDOCKER interaction energy was negative. Finally, PyMOL and discovery studio 3.5 were used to process and generate images(Auwal et al. 2019; Gagnon et al. 2016; Guo et al. 2017).

Statistical Analysis

SPSS statistics 19.0 (IBM SPSS statistics for windows, IBM Corporation, Armonk, NY, USA) and the Graphpad prism version 6.01 (Graphpad Software Inc., San Diego, CA, USA) were used for data analysis. One way ANOVA was used to compare the differences among the groups. Results were showed as the mean value ± SD, $p < 0.05$ was considered as statistically significant.

Results

Hydrolysate and Ultrafiltration Fragment

As a main proteolytic enzyme during gastro-intestinal digestion, trypsin was used to hydrolyse *S. nudus* proteins. The hydrolysis time and enzyme/substrates ratio were determined as two relevant factors that influence the ACE inhibitory activity (data not shown). The hydrolysate was further separated based on their molecular weight by ultrafiltration, and four size fractions were obtained: FI (MW < 2.5 kDa), FII (MW 2.5–5 kDa), FIII (MW5–10 kDa), FIV (MW > 10 kDa). The fractions were lyophilized and weigh with the yield of 20.1%, 12.6%, 3.3% and 64.0%, respectively; and their ACEI rate (%) were: 65.69 ± 11.09, 50.29 ± 11.73, 46.24 ± 10.26, and 46.62 ± 5.40, respectively. By contrast, The ACEI rate (%) for the unhydrolyzed homogenate was 3 ± 1.48, the hydrolysate was 48 ± 3.62; these results indicated that the peptides with ACE inhibition activity were released from their proteins undergoing enzymic hydrolysis process. Among all of the fractions, FI

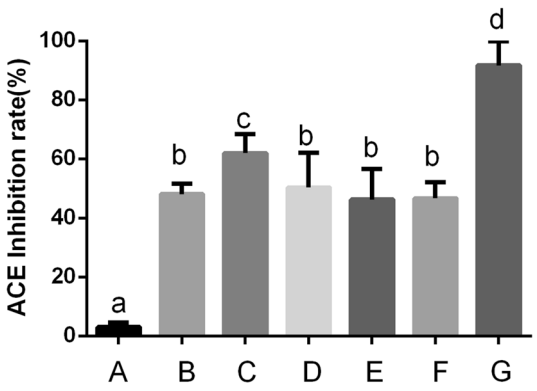

Fig. 1 ACE inhibition rate of each component (%).the unhydrolyzed homogenate group (**A**), the hydrolyzed homogenate group (**B**), FI MW < 2.5 kDa (**C**), FII MW2.5–5 kDa (**D**), FIII MW5–10 kDa (**E**), FIV MW > 10 kDa (**F**), the captopril represent positive group (**G**). Different letters on the bars indicate significant difference ($p < 0.05$), the bars are expressed as the mean ± SD (n = 3)

(MW < 2.5 kDa) showed the highest ACEI activity with an inhibition rate of 65.69%. The results also indicated that the ACEI of decreased with the increase of the molecular weight of the peptide (Fig. 1).

Purification and Identification of the Peptides' Structure

As for the fraction < 2.5 kDa with the highest ACE inhibition rate, it was further separated by gel chromatography. The chromatogram was shown in (Fig. 2). Since the compound with a smaller molecular weight has a longer retention time in the gel chromatography, the eluted peak with retain time 26.3 min was collected and lyophilized, further purified by high-performance liquid chromatography (Fig. 3). The sub-fraction was then subjected to peptide amino acid sequencing by ESI-TOF–MS/MS. Two novel nonapeptides were identified with the following amino acid sequences: Gly-Phe-Ala-Gly-Asp-Asp-Ala-pro-Arg (GFAGDDAPR; m/z = 904.92 Da), and

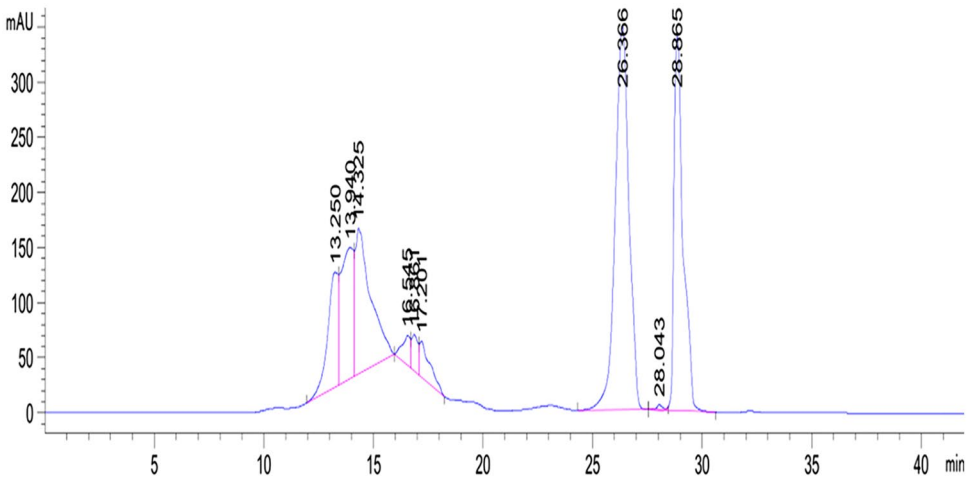

Fig. 2 The elution peaks separated by Taskgel G3000SW gel chromatography

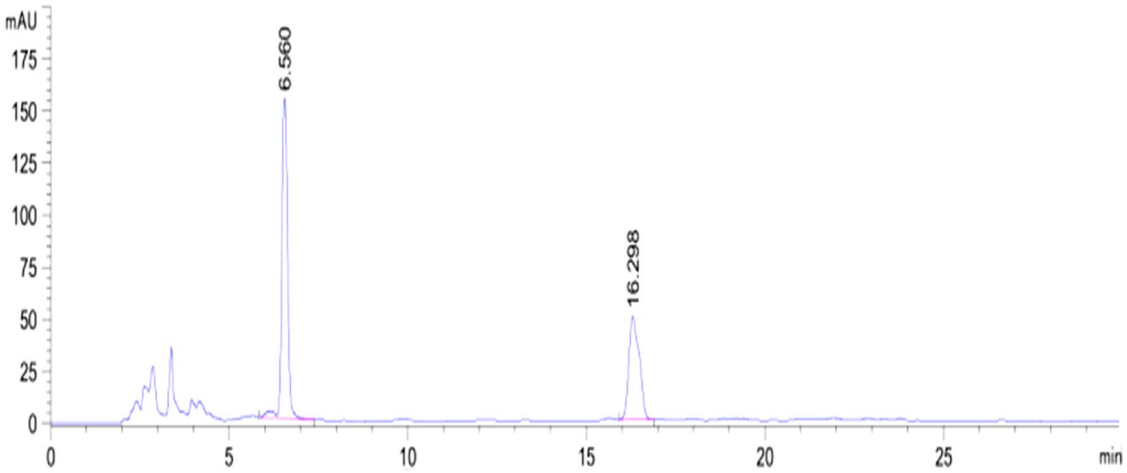

Fig. 3 The 28–30 min elution peak in gel chromatography was further purified by C18 column

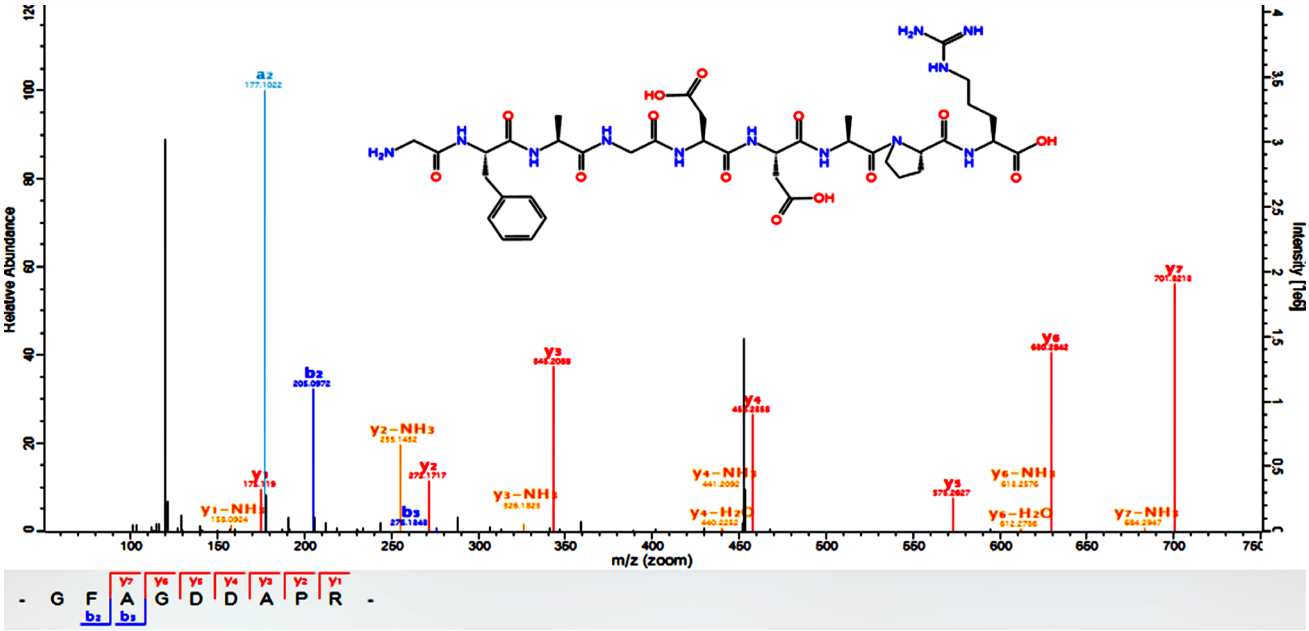

Fig.4 The structure identification of GFAGDDAPR by LC–MS/MS

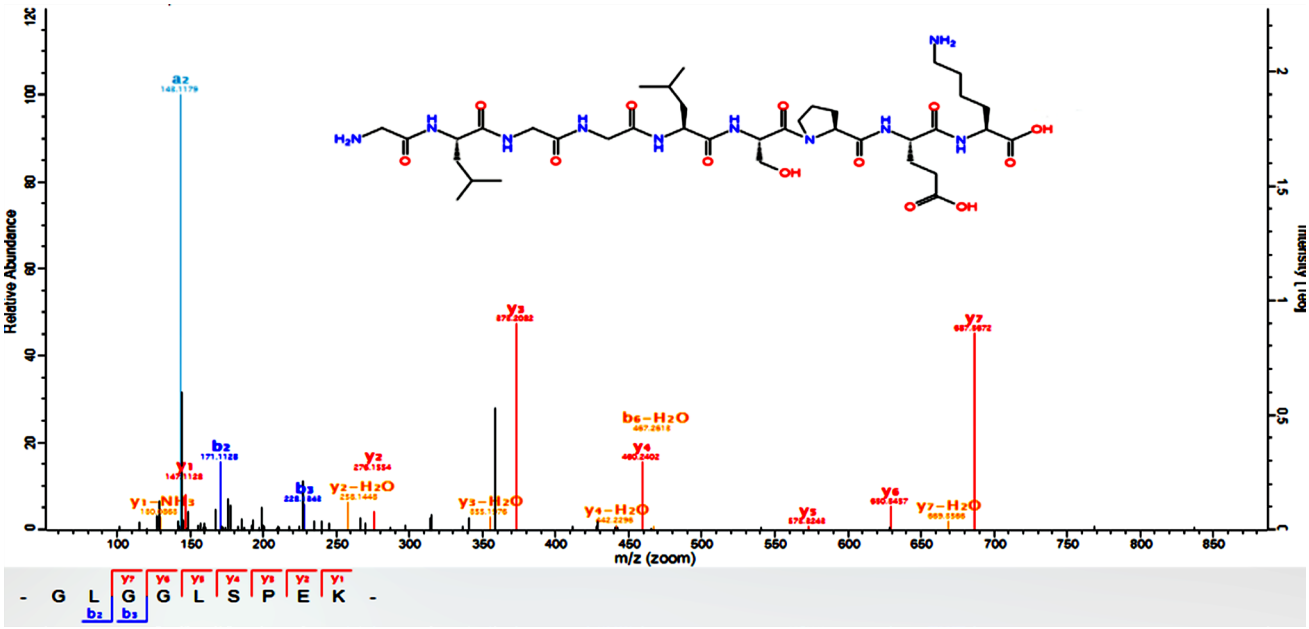

Fig.5 The structure identification of GLGGLSPEK by LC–MS/MS

Gly-Lue-Gly-Gly-Lue-Ser-Pro-Glu-Lys (GLGGLSPEK; m/z = 856.96) (Figs. 4 and 5), which were novel peptides that have not been reported elsewhere. The SwissProt database (https://www.uniprot.org/uniprot/) was used to align the peptide fragments to locate the homologous protein region. It was found that the peptide GFAGDDAPR was originated from the protein R7TPD2, the GLGGLSPEK was a fragment of the protein R7URV6, which of the proteins were submitted from *Capitella teleta* (Polychaete worm). We also searched for antihypertensive peptides in different databases such as BIOPEP, ACE-pepDB and EROP-Moscow database, but found no result (last search on 29th April 2021). Through the literature retrieval, both of nanopeptides were the first time

purified from *S. nudus* and identified their amino acid subsequences as ACE inhibitors.

ACE Inhibition IC_{50} and Properties of the Peptide

The IC_{50} value of the two nanopeptides was 0.76 mM and 0.91 mM, respectively. The physical and chemical properties of the two peptides were searched through online databases. It was shown in Table 1 that the PI values of the two peptides were 3.71 and 6.85, respectively, which suggested a good water solubility. GFAGDDAPR is more likely to have biological activity because of its higher biological activity prediction score as well as a lower IC_{50} value (Table 1).

Hydrolysate and Ultrafiltration Fragment

The relative peak area of HA was used as the evaluation index of ACE inhibitory activity. Lineweaver–Burk plot was drawn with 1/V as the y-axis and 1/C (HHL) as the x-axis (Fig. 6).

According to the plot, the intersection of the Lineweaver–Burk plot of different concentrations of GFAGDDAPR and GLGGLSPEK were neither on the x-axis nor on the y-axis. In addition, the slopes of the three lines were different from the intercepts of the x-axis or the y-axis. It showed that the two peptides may be a mixed non-competitive ACE inhibition mode (Chen et al. 2020; Heo et al. 2017). Calculated according to the Lineweaver–Burk plots (Table 2), the Km values of GFAGDDAPR and GLGGLSPEK were statistically different and not constant. With the increase of the peptide concentration, the maximum response rate increased, and the difference was statistically significant ($p < 0.05$). The maximum reaction rate of GLGGLSPEK did not show a concentration-dependent manner, and the difference was not statistically significant ($p > 0.05$).

Molecular Docking

CDOCKER was used to study the interaction mechanism between ligand and receptor. The -CDOCKER_ENERGY and -CDOCKER_INTERACTION_ENERGY of GFAGDDAPR were 132.844 kcal/mol and 115.766 kcal/mol, respectively. The -CDOCKER_ENERGY and -CDOCKER_INTERACTION_ENERGY of GLGGLSPEK were 97.3387 kcal/mol and 104.204 kcal/mol, respectively (Table 3). Both peptides can form a stable docking structure with ACE. GFAGDDAPR had a higher CDOCKER score than GLGGLSPEK, suggesting that GFAGDDAPR had a stronger interaction with ACE and may have a better inhibitory effect (Alnabulsi et al. 2019)..

There are three main active pockets in the ACE molecule: S1, S2, and S10. The amino acid residues ALA354, GLU384

Table 1 Properties of the two peptides

Peptides	Number of Residues	Molecular Weight (Da)	Iso-Electric Point (PI)	Solubility in Water	Net charge at PH = 7	Predictable probability of biological activity	IC_{50} (mmol/L)
GFAGDDAPR	9	904.92	3.71	Good	− 1	0.599	0.76 ± 0.056
GLGGLSPEK	9	856.96	6.85	Good	0	0.244	0.91 ± 0.020

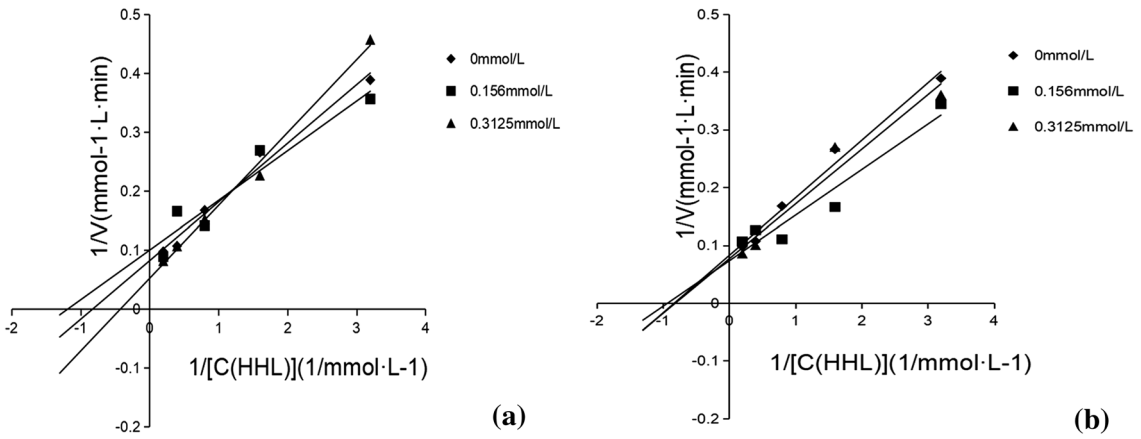

Fig. 6 Lineweaver–Burk plots are used to determine the inhibitory mode of peptides on ACE. 1/V and 1/C(HHL) represent the reciprocal of reaction rate and substrate concentration, respectively. **a** GFAGDDAPR; **b** GLGGLSPEK

Table 2 Kinetic parameters of ACE catalytic reaction under different peptide concentrations

Kinetics Parameters	0	GFAGDDAPR		GLGGLSPEK	
		0.078 mmol/L	0.16 mmol/L	0.078 mmol/L	0.16 mmol/L
km (mmol/L)	1.48 ± 0.67	0.93 ± 0.43	3.70 ± 1.20	3.40 ± 0.35	1.48 ± 0.09
Vmax (mmol/L/min)	14.18 ± 2.68	10.41 ± 2.28	25.66 ± 3.55	14.82 ± 2.97	14.62 ± 2.13

Table 3 Scoring of molecular docking

Compound	-CDOCKER_ENERGY(kcal/mol)	-CDOCKER_INTERACTION_ENERGY(kcal/mol)
GFAGDDAPR	132.84	115.77
GLGGLSPEK	97.34	104.20

and TYR523 in the S1 pocket, the residues GLN281, HIS353, LYS511, HIS513 and TYR520 in the S2 pocket, and the residue GLU162 in the S10 pocket are the active sites that interact with ACE inhibitors such as lisinopril and are useful for inhibiting ACE (Natesh et al. 2003; Sturrock et al. 2004). The binding modes of GFAGDDAPR and GLGGLSPEK with ACE were shown in Figs. 7 and 8.

The active pocket of ACE is a hydrophobic pocket. GFAGDDAPR and GLGGLSPEK formed a wide range of hydrophobic interactions with the surrounding amino acid residues. GFAGDDAPR and GLGGLSPEK interacted with the active pocket of ACE mainly through hydrogen bonding, electrostatic force and van der Waals force. Table 4 showed that GFAGDDAPR forms 13 hydrogen bonds with ASN277, GLN281, ASN285, ALA170, THR372, CYS370, ALA354, GLU384, ASP415 of ACE, including 3 hydrogen bonds formed between ALA354 and GLU384 of the active site S1 pocket. GLGGLSPEK formed 7 hydrogen bonds with ACE residues ALA170, ASN285, GLU162, ASP377, and GLU376. Among them, GLGGLSPEK formed a hydrogen bond with GLU162 belonging to the S10 pocket. The above results indicate that GFAGDDAPR had stronger ACE affinity than GLGGLSPEK and can form a tighter binding with ACE. In the detection of ACE inhibitory activity, GFAGDDAPR had a lower IC_{50} value (GFAGDDAPR IC_{50}: 0.76 mmol/L; GLGGLSPEK IC50: 0.91 mmol/L).

Discussion

The ACEI activity of *S. nudus* homogenate before hydrolysis was too low to be detected. By contrast the *S. nudus* hydrolysate, which merely consisted of soluble peptides, demonstrated significant ACEI activity, and all of the peptide fragments obtained by ultrafiltration possessed obvious

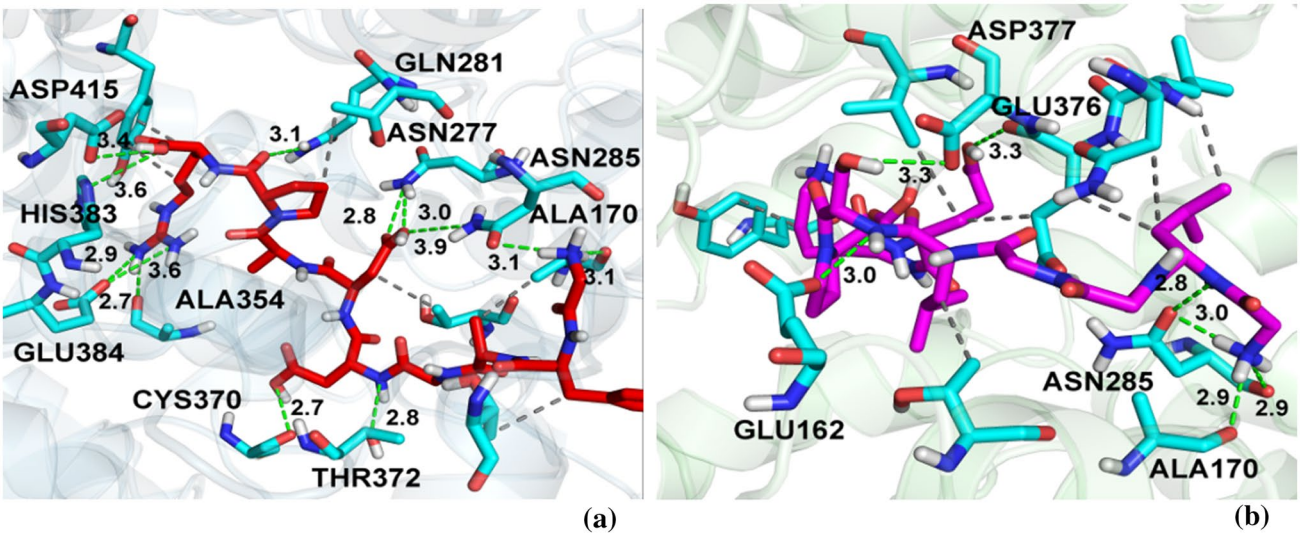

Fig. 7 ACE combined with GFAGDDAPR (**a**) (red sticks) and GLGGLSPEK (**b**) (magenta sticks). The secondary structure of the protein was shown in palegreen cartoons, key residues were shown in cyan sticks, and GFAGDDAPR and GLGGLSPEK were shown in red and magenta, respectively. The green dashed line represented Hydrogen Bonds and the gray dashed line represented Hydrophobic Interactions

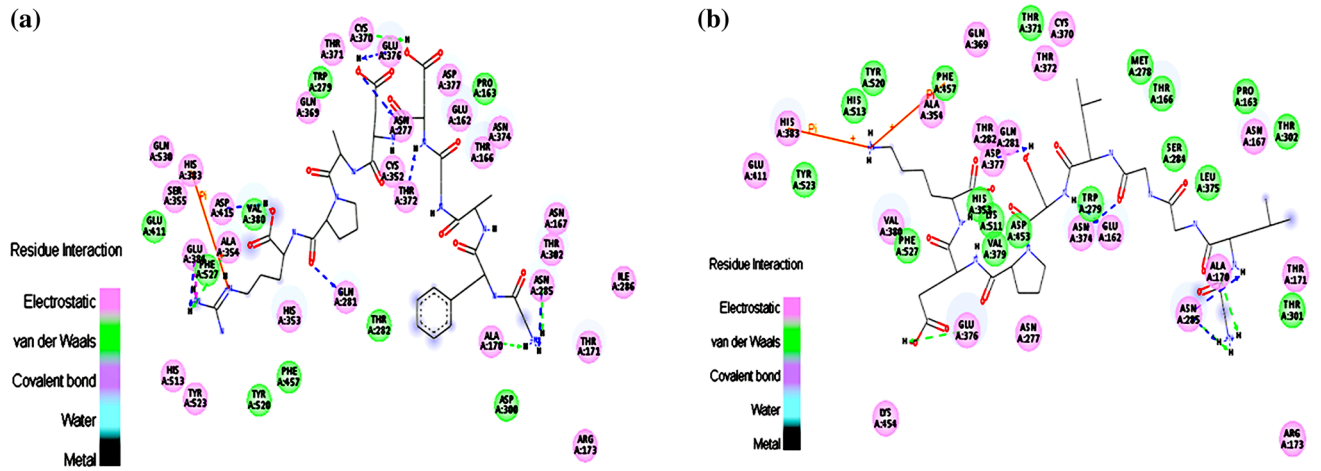

Fig. 8 Two-dimensional (2D) images of ACE combined with GFAGDDAPR (**a**) and GLGGLSPEKV (**b**)

ACE inhibition activity, which is consistent with previous reports of some plant animal proteins, such as longan seed protein and sea cucumber protein (Nuchprapha et al. 2020; Zhong et al. 2018).

Peptides with less amino acid residues or lower molecular weight have a higher chance to bind to the ACE enzyme active site, thereby blocking ACE enzyme activity and preventing angiotensin I from undergoing conversion to angiotensin II (Luna-Vital et al. 2015). In ACE spatial structure, there is a small groove which serves to block protein with high molecular weight access to the active side. The unhydrolyzed protein possessing high molecular weight cannot bind to the ACE active site due to its steric hindrance, so it does not have ACE inhibitory activity. Once the protein macromolecule was hydrolyzed, small peptide fragments were released and these small peptides can pass through the groove and bind to the ACE active site, which demonstrate a potent inhibitory activity. Moreover, the lower the molecular

Table 4 Hydrogen bond formed between peptide and ACE

Peptides	Distance (Å)	From	To	Active pocket
GFAGDDAPR	3.0	A:ASN277:HD21	(a):O41	
	2.8	A:ASN277:HD22	(a):O42	
	3.1	A:GLN281:HE22	(a):O58	
	3.0	(a):H72	A:ASN285:O	
	3.9	(a):H73	A:ASN285:OD1	
	3.1	(a):H74	A:ALA170:O	
	2.8	(a):H92	A:THR372:OG1	
	2.7	(a):H95	A:CYS370:O	
	2.7	(a):H118	A:ALA354:O	S1
	2.9	(a):H118	A:GLU384:OE2	S1
	3.6	(a):H120	A:GLU384:OE2	S1
	3.6	(a):H122	A:HIS383:ND1	
	3.4	(a):H122	A:ASP415:OD2	
GLGGLSPEK	2.9	(b):H67	A:ALA170:O	
	2.8	(b):H68	A:ASN285:O	
	2.9	(b):H69	A:ASN285:OD1	
	3.0	(b):H72	A:ASN285:OD1	
	3.0	(b):H98	A:GLU162:OE2	S10
	3.3	(b):H101	A:ASP377:OD2	
	3.3	(b):H113	A:GLU376:O	

weight, the higher the inhibitory activity. This is consistent with earlier research which found that ACE inhibitory activity was greater when peptides of low molecular weight were used in comparison to higher molecular weights. Nuchprapha et al. (2020) reported the highest level of ACE inhibitory activity from the low-molecular-weight peptide derived from longan seed proteins hydrolysate (< 3 kDa). Kheeree et al. (2020) found that peptides derived from lemon basil seeds retained their ACE inhibitory activity even with molecular weights as low as 0.65 kDa.

The identified peptide sequences in this study were then commercially synthesized and their ACEI activity was evaluated. This finding was consistent with previous conclusion that ACE inhibitory activity was stronger when peptides of low molecular weight were used in comparison to higher molecular weights. In the case of those peptides derived from longan seed protein hydrolysate, the relevant sequences were quite short, with just 10 amino acids. These findings matched those reported by Natesh et al. (2003), who showed by crystallography that large peptide molecules cannot access the active ACE sites.

The PI of a protein plays an important role in the charge change of weakly acidic and basic side chain groups. Generally, the solubility of the hydrolysate at the isoelectric point is low, which will limit its use in foods or medicines with specific PH (Yu et al. 2018). The PI value of GFAGDDAPR is slightly acidic. The solubility of GFAGDDAPR may be reduced under acidic conditions, which limits its use in acidic foods or drugs. In addition, the net charge of the peptide is the cause of the change in the solubility of the peptide. The net charge of a peptide is determined by the number of ionizable carboxyl and amino groups exposed by the peptide. When the number of ionizable groups of a peptide increases, the net charge on the surface of the peptide will increase, which has the effect of improving intra- and intermolecular elec-trostatic repulsion. Thus, not only the aggregation between the peptide and the peptide is reduced, but the interaction between the peptide and water is increased, and the solubility of the peptide is improved (Achouri et al. 1998). GLGGLSPEK and GFAGDDAPR have high water solubility, which is conducive to their further development during commercialization.

ACE inhibitory peptides have some similarities in molecular weight and structure. It is not yet, however, fully understood how peptide structures affects the relationship with ACEI activity, as the structure–activity relationship of ACEI peptides has yet to be fully established. It has been widely accepted that there is some correlation between the structure and ACEI activity of peptides.

For ACEI peptides, their structure–activity relationship with ACE binding could be strongly affected by the three factors including MW, the C-terminal/N-terminal amino acid residue. In addition to molecular weight, the N-terminal and C-terminal tripeptide sequences are also an important factor affecting the ACE inhibitory activity. Previous studies have shown that hydrophobic amino acids (Pro, Phe, Trp, Met) and aliphatic amino acids (Ile or Leu) at the N-terminus (Achouri et al. 1998; Yu et al. 2018), hydrophobic amino acids, aliphatic amino acids (Ala, Val, Ile, Leu, Met) and aromatic amino acids (Tyr, Phe, Trp) residues at the C-terminus with tripeptide sequence have enhanced ACE inhibitory activity (Cheung et al. 1980; Lin et al. 2018; Zhao et al. 2019). If both hydrophobic amino acids and positively charged amino acids (such as Lys and Arg) were present, the inhibitory effect of the peptide can be improved. It is reported that the ACE preferred substrates contain Arg residue at the C-terminal position due to the positive charge of guanidine group in side chain of Arg, and this structural property contributes substantially to its inhibitory potency. This was supported by the data that the IC_{50} of peptide LHLPLR was 1.8 μM, which was twofold than that of LHLPLP (Zhong et al. 2018). Hence, the presence of Arg residue in the C-terminal of GFAGDDAPR might contribute to its potent ACE inhibitory activity.

These amino acid residues have a variety of molecular functions, like the formation of hydrogen bonds and other intermolecular forces, which enable the inhibitory peptide to form a stable combination with ACE and exert its inhibitory activity (Andrews et al. 1985). GFAGDDAPR had Phe at the N-terminal and Ala, Pro, Arg at the C-terminal. Among them, the C-terminal Arg may be the key amino acid for ACE inhibitory activity and plays an important role in the ACE inhibitory activity(Deng et al. 2018). The results of molecular docking showed that among the 13 hydrogen bonds formed between GFAGDDAPR and ACE, 5 of them were formed by C-terminal Arg (Table 4, Figs. 7a and 8a). Specifically, the C-terminal Arg formed 3 hydrogen bonds with the amino acid residues ALA354 and GLU384 in the S1 pocket of the ACE active site, and 2 hydrogen bonds with the amino acid residues HIS383 and ASP415 around the active pocket. These 5 hydrogen bonds could facilitate GFAGDDAPR and ACE to form a stable structure with an enhanced interaction.

Lue is at the N-terminus of GLGGLSPEK, and Pro and Lys are at the C-terminus. These hydrophobic groups can form a variety of interaction forces with ACE. In the sequence of GLGGLSPEK, there was no amino acid residue that can form multiple hydrogen bonds with the active site of ACE, such as Arg, but we found that Ser in the middle of GLGGLSPEK forms a hydrogen bond with the amino acid residue GLU162 of ACE active pocket S10, and it formed a hydrogen bond with the residue ASP377 outside the active site (Table 4, Figs. 7b and 8b). This suggests that Ser may play a key role in the ACE inhibitory activity of GLGGLSPEK.

The hydrophilic-hydrophobic ratio of the peptide also affects the peptide's inhibitory activity on ACE (Asoodeh et al. 2016; Mirzaei et al. 2018). In general, polypeptides with moderate hydrophobicity have higher ACE inhibitory activity (Kheeree et al. 2020). Because ACE inhibitory peptides with suitable hydrophobicity have higher tissue affinity and lipophilicity, they not only enable the peptide to pass through the cell membrane to reach the hydrophobic active site on ACE in the blood, but also prolong the action time of the peptide in the body, which is helpful to maintain the stability of blood pressure (Laurent 2017; Lin et al. 2019; Zhao et al. 2019). The hydrophobic group content of GFAGDDAPR was higher than that of GLGGLSPEK. Therefore, it is speculated that GFAGDDAPR may have better antihypertensive activity in vivo. Although some structure–activity relationships of ACE inhibitory peptides have been explained through existing studies, more in-depth research is still limited, and further elucidation is needed.

Conclusions

Two novel nonapeptides with in vitro ACE inhibitory activity, GFAGDDAPR and GLGGLSPEK, were isolated and identified from *S. nudus*. Both of the peptides could interact with ACE in a mixed non-competitive inhibition mode. The results of molecular docking showed that the two peptides mainly interacted with ACE by hydrogen bonding and other forces. Arg and Ser may be the amino acid residues that play a key role in inhibiting ACE activity. These peptides have the potential to become pro-drugs for the treatment of hypertension. Taken together, as a kind of seafood, *S. nudus* has the potential application to be developed into functional food with the activity of anti-hypertension.

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Author Contributions XT and YQ performed experiments, HL and QL collected and analyzed data, XT and HL wrote the manuscript. HL and YL designed the experiments and supervised the work.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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References

Achouri A, Zhang W, Shiying X (1998) Enzymatic hydrolysis of soy protein isolate and effect of succinylation on the functional properties of resulting protein hydrolysates. Food Res Int 31(9):617–623. https://doi.org/10.1016/S0963-9969(98)00104-5

Alnabulsi SM, Al-Shar'i NA (2019) Hit identification of SMYD3 enzyme inhibitors using structure-based pharmacophore modeling. Future Med Chem 11(10):1107–1117. https://doi.org/10.4155/fmc-2018-0462

Ames MK, Atkins CE, Pitt B (2019) The renin-angiotensin-aldosterone system and its suppression. J Vet Intern Med 33(2):363–382. https://doi.org/10.1111/jvim.15454

Andrews PR, Carson JM, Caselli A, Spark MJ, Woods R (1985) Conformational analysis and active site modelling of angiotensin-converting enzyme inhibitors. J Med Chem 28(3):393–399. https://doi.org/10.1021/jm00381a021

Asoodeh A, Homayouni-Tabrizi M, Shabestarian H, Emtenani S, Emtenani S (2016) Biochemical characterization of a novel antioxidant and angiotensin I-converting enzyme inhibitory peptide from *Struthio camelus* egg white protein hydrolysis. J Food Drug Anal 24(2):332–342. https://doi.org/10.1016/j.jfda.2015.11.010

Auwal SM, Zainal Abidin N, Zarei M, Tan CP, Saari N (2019) Identification, structure-activity relationship and in silico molecular docking analyses of five novel angiotensin I-converting enzyme (ACE)-inhibitory peptides from stone fish (*Actinopyga lecanora*) hydrolysates. PLoS ONE 14(5):e0197644. https://doi.org/10.1371/journal.pone.0197644

Balti R, Bougatef A, Sila A, Guillochon D, Dhulster P, Nedjar-Arroume N (2015) Nine novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) muscle protein hydrolysates and antihypertensive effect of the potent active peptide in spontaneously hypertensive rats. Food Chem 170:519–525. https://doi.org/10.1016/j.foodchem.2013.03.091

Chen J, Ryu B, Zhang Y, Liang P, Li C, Zhou C, Yang P, Hong P, Qian ZJ (2020) Comparison of an angiotensin-I-converting enzyme inhibitory peptide from tilapia (*Oreochromis niloticus*) with captopril: inhibition kinetics, in vivo effect, simulated gastrointestinal digestion and a molecular docking study. J Sci Food Agric 100(1):315–324. https://doi.org/10.1002/jsfa.10041

Cheung HS, Wang FL, Ondetti MA, Sabo EF, Cushman DW (1980) Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. J Biol Chem 255(2):401–407

Cushman DW, Cheung HS, (1971) Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem Pharmocol 20(7):1637–1648. https://doi.org/10.1016/0006-2952(71)90292-9

Deng Z, Liu Y, Wang J, Wu S, Geng L, Sui Z, Zhang Q (2018) Antihypertensive effects of two novel angiotensin I-converting enzyme (ACE) inhibitory peptides from *Gracilariopsis lemaneiformis*

(Rhodophyta) in spontaneously hypertensive rats (SHRs). Mar Drugs 16(9):299–315. https://doi.org/10.3390/md16090299
Doyle AE (1991) Hypertension and vascular disease. Am J Hypertens 4(2 Pt 2):103s–106s. https://doi.org/10.1093/ajh/4.2.103s
Gagnon JK, Law SM, Brooks CL 3rd (2016) Flexible CDOCKER: development and application of a pseudo-explicit structure-based docking method within CHARMM. J Comput Chem 37(8):753–762. https://doi.org/10.1002/jcc.24259
Ge YH, Chen YY, Zhou GS, Liu X, Tang YP, Liu R, Liu P, Li N, Yang J, Wang J, Yue SJ, Zhou H, Duan JA (2018) A novel antithrombotic protease from marine worm *Sipunculus nudus*. Int J Mol Sci 19(10):2023–2040. https://doi.org/10.3390/ijms19103023
Guo M, Chen X, Wu Y, Zhang L, Huang W, Yuan Y, Fang M, Xie J, Wei D (2017) Angiotensin I-converting enzyme inhibitory peptides from Sipuncula (*Phascolosoma esculenta*): purification, identification, molecular docking and antihypertensive effects on spontaneously hypertensive rats. Process Biochem 63:84–95. https://doi.org/10.1016/j.procbio.2017.08.009
Heo SY, Ko SC, Kim CS, Oh GW, Ryu B, Qian ZJ, Kim G, Park WS, Choi IW, Phan TT, Heo SJ, Kang DH, Yi M, Jung WK (2017) A heptameric peptide purified from *Spirulina* sp. gastrointestinal hydrolysate inhibits angiotensin I-converting enzyme- and angiotensin II-induced vascular dysfunction in human endothelial cells. Int J Mol Med 39(5):1072–1082. https://doi.org/10.3892/ijmm.2017.2941
Hsu TH, Ning Y, Gwo JC, Zeng ZN (2013) DNA barcoding reveals cryptic diversity in the peanut worm *Sipunculus nudus*. Mol Ecol Resour 13(4):596–606. https://doi.org/10.1111/1755-0998.12097
Kearney PM, Whelton M, Reynolds K, Muntner P, Whelton PK, He J (2005) Global burden of hypertension: analysis of worldwide data. Lancet 365(9455):217–223. https://doi.org/10.1016/s0140-6736(05)17741-1
Kheeree N, Sangtanoo P, Srimongkol P, Saisavoey T, Reamtong O, Choowongkomon K, Karnchanatat A (2020) ACE inhibitory peptides derived from de-fatted lemon basil seeds: optimization, purification, identification, structure-activity relationship and molecular docking analysis. Food Funct 11(9):8161–8178. https://doi.org/10.1039/d0fo01240h
Laurent S (2017) Antihypertensive drugs. Pharmacol Res 124:116–125. https://doi.org/10.1016/j.phrs.2017.07.026
Li J, Li Q, Li J, Zhou B (2014) Peptides derived from *Rhopilema esculentum* hydrolysate exhibit angiotensin converting enzyme (ACE) inhibitory and antioxidant abilities. Molecules 19(9):13587–13602. https://doi.org/10.3390/molecules190913587
Li N, Shen X, Liu Y, Zhang J, He Y, Liu Q, Jiang D, Zong J, Li J, Hou D, Chen W, Wang Q, Luo Q, Li K (2016) Isolation, characterization, and radiation protection of *Sipunculus nudus* L polysaccharide. Int J Biol Macromol 83:288–296. https://doi.org/10.1016/j.ijbiomac.2015.11.071
Lin YH, Chen CA, Tsai JS, Chen GW (2019) Preparation and identification of novel antihypertensive peptides from the in vitro gastrointestinal digestion of marine cobia skin hydrolysates. Nutrients 11(6):1351–1366. https://doi.org/10.3390/nu11061351
Lin YH, Chen GW, Yeh CH, Song H, Tsai JS (2018) Purification and identification of angiotensin I-converting enzyme inhibitory peptides and the antihypertensive effect of *Chlorella sorokiniana* protein hydrolysates. Nutrients 10(10):1397–1411. https://doi.org/10.3390/nu10101397
Luna-Vital DA, González de Mejía E, Mendoza S, Loarca-Piña G (2015) Peptides present in the non-digestible fraction of common beans (*Phaseolus vulgaris* L.) inhibit the angiotensin-I converting enzyme by interacting with its catalytic cavity independent of their antioxidant capacity. Food Funct 6(5):1470–1479. https://doi.org/10.1039/c5fo00190k
Lunow D, Kaiser S, Brückner S, Gotsch A, Henle T (2013) Selective release of ACE-inhibiting tryptophan-containing dipeptides from food proteins by enzymatic hydrolysis. Eur Food Res Technol 237(1):27–37. https://doi.org/10.1007/s00217-013-2014-x
Mirzaei M, Mirdamadi S, Ehsani MR, Aminlari M (2018) Production of antioxidant and ACE-inhibitory peptides from *Kluyveromyces marxianus* protein hydrolysates: purification and molecular docking. J Food Drug Anal 26(2):696–705. https://doi.org/10.1016/j.jfda.2017.07.008
Natesh R, Schwager SL, Sturrock ED, Acharya KR (2003) Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. Nature 421(6922):551–554. https://doi.org/10.1038/nature01370
Nuchprapha A, Paisansak S, Sangtanoo P, Srimongkol P, Saisavoey T, Reamtong O, Choowongkomon K, Karnchanatat A (2020) Two novel ACE inhibitory peptides isolated from longan seeds: purification, inhibitory kinetics and mechanisms. J RSC Adv. https://doi.org/10.1039/d0ra00093k
Overlack A (1996) ACE inhibitor-induced cough and bronchospasm. Incidence, mechanisms and management. Drug Saf 15(1):72–78. https://doi.org/10.2165/00002018-199615010-00006
Ralston J, Reddy KS, Fuster V, Narula J (2016) Cardiovascular diseases on the global agenda: the United Nations high level meeting, sustainable development goals, and the way forward. Glob Heart 11(4):375–379. https://doi.org/10.1016/j.gheart.2016.10.029
Ryan JT, Ross RP, Bolton D, Fitzgerald GF, Stanton C (2011) Bioactive peptides from muscle sources: meat and fish. Nutrients 3(9):765–791. https://doi.org/10.3390/nu3090765
Sangtanoo P, Srimongkol P, Saisavoey T, Reamtong O, Karnchanatat A (2020) Anti-inflammatory action of two novel peptides derived from peanut worms (*Sipunculus nudus*) in lipopolysaccharide-induced RAW264.7 macrophages. Food Funct 11(1):552–560. https://doi.org/10.1039/c9fo02178g
Seibert E, Tracy TS (2014) Fundamentals of enzyme kinetics. Methods Mol Biol 1113:9–22. https://doi.org/10.1007/978-1-62703-758-7_2
Singh PD, Johnson JH (1984) Muraceins–muramyl peptides produced by *Nocardia orientalis* as angiotensin-converting enzyme inhibitors II Isolation and structure determination. J Antibiot 37(4):336–343. https://doi.org/10.7164/antibiotics.37.336
Stadnik J, Kęska P (2015) Meat and fermented meat products as a source of bioactive peptides. Acta Sci Pol Technol Aliment 14(3):181–190. https://doi.org/10.17306/j.Afs.2015.3.19
Sturrock ED, Natesh R, van Rooyen JM, Acharya KR (2004) Structure of angiotensin I-converting enzyme. Cell Mol Life Sci 61(21):2677–2686. https://doi.org/10.1007/s00018-004-4239-0
Su J, Jiang L, Wu J, Liu Z, Wu Y (2018) Effect of polysaccharides extracted from *Sipunculus nudus* (SNP) on the lifespan and immune damage repair of *Drosophila melanogaster* exposed to Cd (VI). Nat Prod Res 32(11):1329–1332. https://doi.org/10.1080/14786419.2017.1342088
Sun S, Xu X, Sun X, Zhang X, Chen X, Xu N (2019) Preparation and identification of ACE inhibitory peptides from the marine macroalga *Ulva intestinalis*. Mar Drugs 17(3):179–196. https://doi.org/10.3390/md17030179
Wilin KL, Czupryn MJ, Mui R, Renno A, Murphy JA (2018) ACE inhibitor-induced angioedema of the small bowel: a case report and review of the literature. J Pharm Pract 31(1):99–103. https://doi.org/10.1177/0897190017690641
Wu J, Aluko RE, Muir AD (2002) Improved method for direct high-performance liquid chromatography assay of angiotensin-converting enzyme-catalyzed reactions. J Chromatogr A 950(1–2):125–130. https://doi.org/10.1016/s0021-9673(02)00052-3
Yu F, Zhang Z, Luo L, Zhu J, Huang F, Yang Z, Tang Y, Ding G (2018) Identification and molecular docking study of a nvel angiotensin-I

converting enzyme inhibitory peptide derived from enzymatic hydrolysates of *Cyclina sinensis*. Mar Drugs 16(11):411–427. https://doi.org/10.3390/md16110411

Zhang CX, Dai ZR (2011) Anti-hypoxia activity of a polysaccharide extracted from the *Sipunculus nudus* L. Int J Biol Macromol 49(4):523–526. https://doi.org/10.1016/j.ijbiomac.2011.06.018

Zhao YQ, Zhang L, Tao J, Chi CF, Wang B (2019) Eight antihypertensive peptides from the protein hydrolysate of Antarctic krill (*Euphausia superba*): Isolation, identification, and activity evaluation on human umbilical vein endothelial cells (HUVECs). Food Res Int 121:197–204. https://doi.org/10.1016/j.foodres.2019.03.035

Zhong C, Sun LC, Yan LJ, Lin YC, Liu GM, Cao MJ (2018) Production, optimisation and characterisation of angiotensin converting enzyme inhibitory peptides from sea cucumber (*Stichopus japonicus*) gonad. Food Funct 9(1):594–603. https://doi.org/10.1039/c7fo01388d