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A pivotal peptide (Ile-Leu-Lys-Pro) with high ACE- inhibitory activity from duck egg white: identification and molecular docking

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Abstract

Salted duck egg white was desalted and hydrolyzed to produce angiotensin I-converting enzyme (ACE) inhibitory peptides. Single factor test and response surface design were performed to determine the best hydrolysis conditions: enzyme dosage 11875.99 U/g, substrate concentration 33.04 g/L and hydrolysis time 4 h. The fraction V (M_W < 1 kDa), which exhibited the strongest ACE inhibitory activity, was characterized by HPLC-ESI-MS/MS. Eighty-three peptides were identified, and among them Ile-Leu-Lys-Pro, Ile-Asn-Ser-Trp, Ile-Arg and His-Pro-Ala were synthesized for further research. Ile-Leu-Lys-Pro exhibited the highest ACE inhibitory activity (IC_{50}: 0.355 mM). The molecular docking studies revealed that nine amino acids contributed to stabilize the docking complex. The ACE inhibition of Ile-Leu-Lys-Pro and Ile-Asn-Ser-Trp were mainly attributed to Ile in N-terminal. The residues Glu362 and Ala332 were the important binding sites in molecular docking. This research expands the understanding of ACE inhibitory peptides from duck egg white as well as highlights an opportunity for recycling an otherwise discarded byproduct.

Keywords: desalted duck egg white peptides (DPs); enzymatic hydrolysis; angiotensin I-converting enzyme (ACE) inhibitory peptide; electrospray ionization mass spectrometry (ESI-MS); molecular docking.

Practical Application: Results in this research can be used for the intensive processing of duck egg white, especially for the recycling of salted duck egg white. The obtained polypeptide with antihypertensive activity can be used for the development of functional food and improvement of food industry.

1 Introduction

Angiotensin converting enzyme (ACE) has a significant impact on the regulation of blood pressure and it promotes the transformation of angiotensin I to angiotensin II by inactivating the potent vasodilator bradykinin in the circulatory or endocrine of human renin-angiotensin system (Jimsheena & Gowda, 2010). A high level of ACE inhibitory activity was effective in antihypertensive activities (Mirdhayati et al., 2016). Thus, ACE is identified as a potential target for synthetic pharmaceuticals, which inhibited ACE activity to reduce blood pressure. Compared with pharmacological agents, peptides derived from natural food with high ACE inhibitory activity were preferred for their safety (Ngo et al., 2016). In consideration of this, ACE inhibitory peptides are proposed as ideal functional food supplements for human health (Feng et al., 2016).

It has been reported that bovine collagen was a potential precursor of ACE inhibitory peptides based on in silico and in vitro protein digestions and two promising in silico peptides (Tyr-Trp and Leu-Arg-Tyr) were confirmed as novel ACE inhibitors (Fu et al., 2016). Porcine skin gelatin was also hydrolyzed with a targeted enzymatic approach (Aspergillus niger prolyl endoproteinase) to produce hydrolysate with potent ACE inhibitory activity after 4-h hydrolysis and the sequence was identified as Met-Gly-Pro (O'Keeffe et al., 2017). Besides animal source, plant protein like soybean and grain is also an abundant source of ACE inhibitory peptides. Leu-Ser-Trp was previously identified from thermolysin-digested soy protein hydrolysate as a potent ACE-inhibitory peptide (Lin et al., 2017). Sweet sorghum grain protein hydrolysate was fractionated to four fractions (>10, 5-10, 1-5 and <1 kDa) by ultrafiltration, with the < 1kDa fraction exhibited the strongest ACE inhibitory activity (Wu et al., 2016).

Salted duck egg is one of the most popular and traditional preserved egg products in both Southeast Asia and China, and it contains all essential amino acids for human beings (Kaewmanee et al., 2011). Due to the content of 7-10% sodium chloride, large amounts of salted duck egg white are discarded every year (Zhao et al., 2014). After hydrolysis by enzyme, hen egg white is reported to possess many functions such as anti-inflammatory, antimicrobial, ACE-inhibitory and antidiabetic activities (Abeyrathne et al., 2018; Grootaert et al., 2017; Yu et al., 2012). Meanwhile, the ACE inhibitory activity was explored by hydrolyzing different proteins, such as grain and milk protein (Connolly et al., 2015; Vukic et al., 2017). However, the utilization of desalted duck egg white to produce the ACE inhibitory peptides have not been reported.

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Therefore, in the present study, we attempted to optimize the hydrolysis conditions of desalted duck egg white for the preparation of ACE inhibitory peptides. The main ACE inhibitory peptides were isolated and identified to investigate their ACE-inhibitory activity. Additionally, the peptide with the highest ACE inhibitory activity was synthesized to characterize its stability during the simulation in molecular docking process.

2 Materials and methods

2.1 Materials and chemicals

Salted duck egg white with a sodium chloride content of 7% and a protein content of 10% was donated by Hubei Shendan Healthy Food Co., Ltd. (Wuhan, China). Neutrase 0.8 L, alcalase 2.4L, papain, protamex, flavourzyme, trypsin and pepsin were purchased from Novozymes (Copenhagen, Denmark). ACE were isolated from fresh hog lung. Angiotensin I-converting enzyme (ACE) Elisa kits were purchased from the PLA navy hospital (Beijing, China). Polymeric-Cellulose spiral-wound membranes, including molecular weight cut-off (MWCO) at 1 kDa, 3 kDa, 5 kDa and 6 kDa, were purchased from Millipore Inc. (Billerica, Massachusetts, USA). HPLC-grade acetonitrile was purchased from Fisher Chemical (Fair Lawn, NJ, USA), and HPLC-grade water was prepared using a Milli-Q system (Millipore Iberica, Madrid, Spain). Trifluoroacetic acid (TFA) of HPLC grade was purchased from Sigma Chemical Co. (Saint Louis, MO, USA). All other chemicals and solvents were of analytical grade.

2.2 Preparation of desalted duck egg white and Angiotensin I-Converting Enzyme (ACE)

Salted duck egg white was desalted by the electro-dialysis equipment (QQX, Zhejiang Qianqiu Environmental Water Treatment Co., Ltd, Hangzhou, China) as reported in our previous work (Zhao et al., 2014). The angiotensin I-converting enzyme (ACE) was prepared as described previously (Cushman & Cheung, 1971) with minor modification.

2.3 Enzymatic hydrolysis and assay of Degree of Hydrolysis (DH)

The desalted duck egg white was hydrolyzed by seven enzymes: Neutrase 0.8L (50 °C, pH 7.0), alcalase 2.4L (55 °C, pH 8.0), papain (55 °C, pH 6.0), protamex (50 °C, pH 6.5), flavourzyme (50 °C, pH 6.5), trypsin (40 °C, pH 7.8) and pepsin (37 °C, pH 2.0). After water bath at 70 °C for 40 min, the hydrolysis process was conducted. The supernatant was lyophilized after centrifugation and used for the determination of ACE inhibitory activity and the degree of hydrolysis (DH).

As for the single factor experiments, the independent variables contain Substrate concentrations (pure protein concentrations) of 20,30,40,50 and 60 g/L, Enzyme dosages of 5000, 10000,15000, 20000 and 25000 U/g, and Hydrolysis time of 20000 U/g for 2,3,4,5 and 6 h. The other hydrolysis conditions were: hydrolysis temperature 50 °C, pH 6.5. The degree of hydrolysis (DH) and ACE inhibitory activity were investigated after enzymatic reaction. The DH was determined by the pH-stat method and calculated using the following Equation 1:

$$DH\ (\%) = B \times Nb\ /\ (\alpha \times Mp\ \times\ htot)\ \times 100 \quad (1)$$

Where htot is the total number of peptide bonds in the protein substrate (11.1 mmol/g protein).

2.4 Response Surface Design (RDS)

A Box-Behnken experimental design (BBD) was used to optimize the hydrolysis conditions. The following independent variables were selected: hydrolysis time (X_1, 2, 3, 4 h), enzyme dosage (X_2, 10000, 15000, 20000 U/g) and substrate concentration (X_3, 20, 30, 40 g/L). A three-level three-factor BBD design was employed.

2.5 Assay of ACE inhibitory activity

ACE inhibitory activity was analyzed by spectrophotometry using hippuryl-histidyl-leucine (HHL) as substrate, according to the method of Cushman & Cheung (Cushman & Cheung, 1971) with minor modifications. The details were the same as previous reports (Huang et al., 2011). The IC_{50} value was defined as the concentration of ACE inhibitor namely protein hydrolysate needed to reduce 50% of ACE activity and determined by regression analysis of ACE inhibitory activity (%) versus protein concentration. The IC_{50} value was identified as mg protein/mL. The ACE inhibitory activity (%) was expressed as follow (Equation 2):

$$ACE\ inhibitory\ activity\ (\%) = (A-S)\ /\ (A-C)\ \times 100 \quad (2)$$

Where A is the absorbance at 228 nm without protein hydrolysate; S is the absorbance with protein hydrolysate; and C is the absorbance without protein hydrolysate and ACE.

2.6 Fractionation of ACE inhibitory peptides

The desalted duck egg white hydrolysate was filtered sequentially through 1 kDa, 3 kDa, 5 kDa and 6 kDa Mw cutoff membranes (Millipore Co., Billerica, Massachusetts, USA) and separated into five fractions: fraction I (Mw > 6 kDa), fraction II (Mw5 -6 kDa), fraction III (Mw 3-5 kDa), fraction IV (Mw 1-3 kDa), fraction V (Mw < 1 kDa). Each fraction was assayed for ACE inhibitory activity. The highest ACE inhibitory fraction was pooled, lyophilized and stored at -20 °C until use.

2.7 HPLC analysis instrumentation

The HPLC analysis was performed on a Waters e2695 HPLC-DAD system (Waters, Milford, MA, USA), equipped with a diode array detector, a vacuum degasser, an auto-sampler, a binary pump, and a column compartment. The highest ACE inhibitory fraction (Mw < 1 kDa) at a concentration of 10 mg/mL were analyzed on a ZORBAX SB-C18 column (250 mm × 4.6 mm, 5 μM, Agilent Co., Santa Clara, CA, USA) at a flow rate of 1 mL/min, with the temperature of the column maintained at 25 °C. A binary gradient elution system, comprising water (with 0.1% TFA, A) and acetonitrile (with 0.1% TFA, B), was applied as follows: 0.0-15.0 min, 5-12% B; 15.0-45.0 min, 12-35% B. The volume of injection was 20 μL, and the DAD detector was set at 220 nM for analysis.

2.8 Peptide identification by HPLC-ESI-MS/MS

For HPLC-ESI-MS/MS analysis, the HPLC system described above was replaced by an Agilent1100 series HPLC-ESI-MS system (Agilent Technologies) but with the same column and gradient elution as in the HPLC analysis. Mass spectrometry conditions were set as follows: ESI^+ ion source; drying gas temperature, 365 °C; nebulizer, 40.00 psi; dry gas flow rate, 10.0 L/min; capillary voltage, 3500 V; scan spectra from m/z 50 to 1000. The sequences were identified using LCMSD-Trap Data Analysis software and confirmed by searching the online MS database on the UCSF Mass Spectrometry Facility (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=mspattern; NCBInr. 2015. 3. 10, SwissProt. 2015. 3. 5, UniProtKB.2015.3. 5).

2.9 Molecular docking on ACE binding site

The structure of four peptides were constructed by using MOE software. The structure was energy minimized by using steepest descent and conjugate gradient techniques (Force field: MMFF94x). The crystal structure of human ACE-lisinopril complex (PDB: 5amb) was derived from the Protein Data Bank (PDB). Before the docking, water molecules and the inhibitor lisinopril were removed in the ACE model. The polar hydrogens were then added to the ACE model. The flexible docking of peptide at the ACE-binding site was performed using the docking tool of MOE software. The docking runs were carried out on parameters (Placement: Triangle Matcher; Rescoring 1: Alpha HB; Refinement: Force field; Rescoring 2: ASE; Retain: 30) with a radius of 10 Å. Evaluation of the molecular docking was performed according to the scores of several scoring functions, including E_conf, E_place, E_score1, E_refine and E_score2. According to the scores and binding-energy, the best pose for each peptide was obtained.

2.10 Statistical analysis

Experimental data were presented as mean values ± SD. The mean values were compared by Duncan's multiple range test at $P < 0.05$ using SPSS software version 17.0.

3 Result and discussion

3.1 Hydrolysis of desalted duck egg white

As shown in Figure 1D, there was no significant difference of ACE inhibitory activity in neutrase, alcalase, promamex

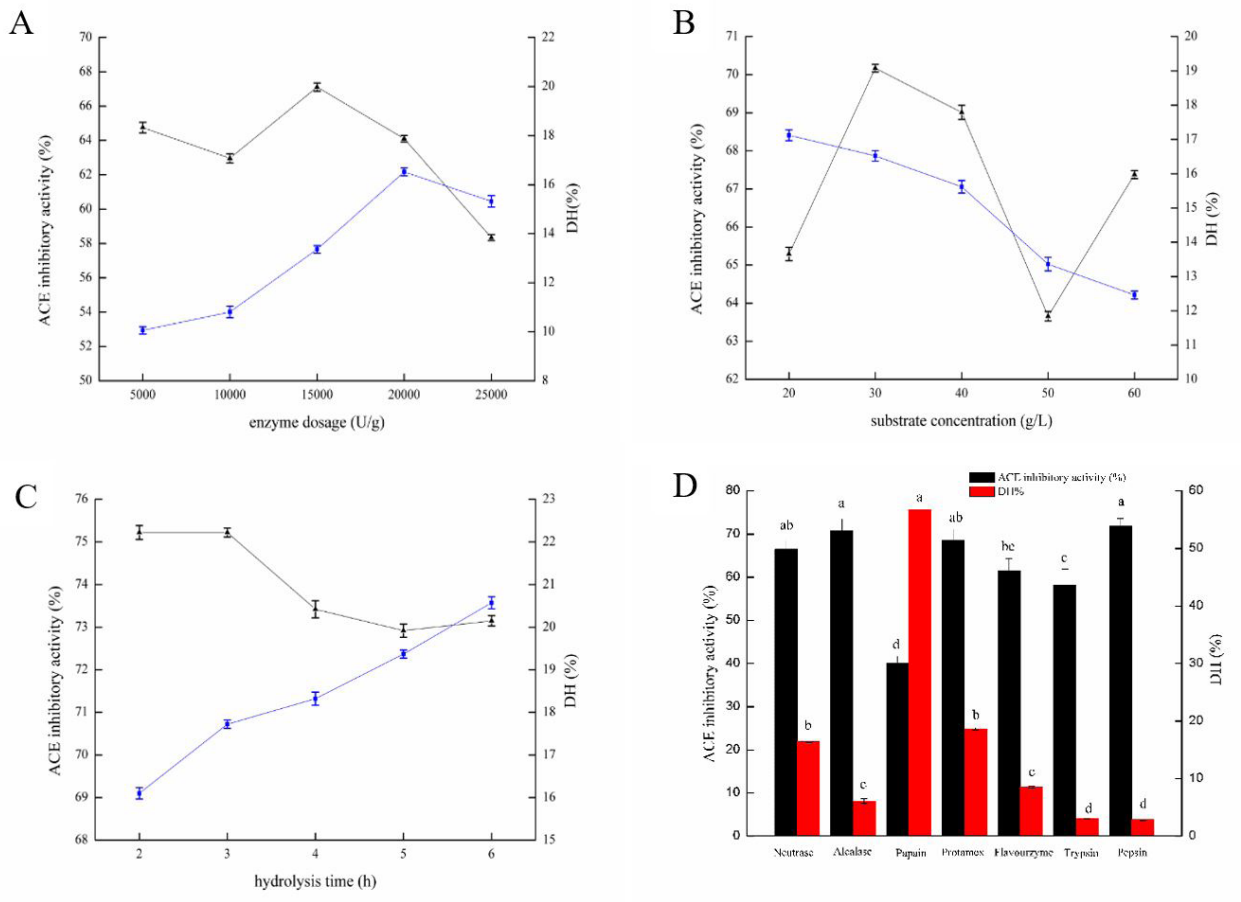

Figure 1. ACE inhibitory activity (▲ACE and ▪DH) of different enzyme dosage, substrate concentration and hydrolysis time. (A) Effect of the enzyme dosages on ACE inhibitory activity and DH; (B) Effect of substrate concentrations on ACE inhibitory activity and DH; (C) Effect of enzymatic hydrolysis on ACE inhibitory activity and DH; (D) ACE inhibitory activity (%) and DH (%) of desalted duck egg white hydrolysates by using seven kinds of proteases. Results are expressed as mean values at three replicates.

and pepsin treatment groups, which reached 66.45%, 70.74%, 68.45% and 71.95%, respectively. The degree of hydrolysis by using promamex was slightly higher than that of neutrase and significantly higher than that of alcalase and pepsin. Therefore, promamex was chosen as the best protease. It was reported that proteases in enzymatic hydrolysis resourced from plant, animals and microorganism might have high ACE inhibitory activity relatively (Forghani et al., 2016; Pandey et al., 2018). Some previous studies found that the production of egg white enzymatic hydrolysates with alcalase and pepsin had high ACE inhibitory activity (Miguel et al., 2006; Miguel et al., 2004; Yu et al., 2011b; Liu et al., 2010). It was also demonstrated that hen egg white hydrolysate with antioxidant and antithrombin activity were prepared by using alcalase (Yu et al., 2011a). In the present study, the protamex present the characteristics of both endopeptidase and exopeptidase, constructing more enzyme cutting sites (Zhang et al., 2018).

As shown in Figure 1A, there was a constantly increase of DH from 5000 U/g to 20000 U/g, followed a slightly decrease from 20000 U/g to 25000 U/g. The ACE inhibitory activity reached the highest point of 67.11% when the enzyme dosage was 15000 U/g, which then decreased to 58.33% till 25000 U/g. It may be due to the fact that enzyme substrate ratio came much higher with the increase of enzyme dosage after 15000 U/g. The high ACE inhibitory activity peptides further hydrolyzed may result in the cleavage at sites which do not facilitate ACE inhibition activity. Similar opinion was obtained by Cinq-Mars & Li-Chan (2007), who reported a maximum of ACE inhibition at 3.0% E/S for hydrolysates of hake fillets obtained with Protamex (Cinq-Mars & Li-Chan, 2007). In order to obtain higher ACE inhibitory activity peptides, 15000 U/g was chosen as the enzyme dosage.

Additionally, the DH value decreased from 20 g/L to 60 g/L, with a sharp decrease from 40 g/L to 50 g/L (Figure 1B). The highest DH of 17.12% appeared in substrate concentration 20 g/L, which was a little higher than DH of 16.52% on the substrate concentration 30 g/L. It was observed that the ACE inhibitory activity significantly increased with the increasing substrate concentration to 30 g/L, and decreased with successional increasing substrate concentration to 50 g/L. This fact was attributed to low enzyme substrate ratio when substrate concentrate exceeded 30 g/L. The lack of enzyme led to low DH, and the most protein was hydrolyzed to long peptides with low ACE inhibitory activity. It was reported that the effective ACE inhibitory peptides always contained 2-15 amino acid residues (Li et al., 2015). Therefore, the desalted duck egg white was not hydrolyzed adequately when substrate concentrate was higher than 30 g/L.

Figure 1C showed almost converse trends of ACE inhibitory activity and DH with the change of hydrolysis time. It was found that the DH increased from 2 to 6 hours, while the ACE inhibitory activity decreased from 3 to 5 hours. The ACE inhibitory activity kept the highest of 75.22% from 2 to 3 hours. This result illustrated that the appropriate hydrolysis time was key indicator in enzyme hydrolysis reaction. Though the DH was constantly higher, the ACE inhibitory activity declined because of the over hydrolysis. It was reported that DH increased in the hydrolysis of small-spotted catshark hydrolysate with ACE inhibitory activity by using subtilisin and trypsin in the first 30 min. However, the ACE inhibition activity was not observed to increase when the DH was over 14% (Garcia-Moreno et al., 2015). Overall, the single indicator reaction condition was optimized to the hydrolysis time 3 h, the substrate concentration 30 g/L and the enzyme dosage 15000 U/g.

3.2 Optimization of enzymatic hydrolysis by response surface design

The response surface was drawn from the Box-Behnken experimental results. The influence of different hydrolysis factors on ACE inhibitory activity of desalted duck egg white protein hydrolysate was analyzed. Table 1 showed that the enzyme dosage was the most significant factor followed by the hydrolysis time. However, the substrate concentration had no significant influence on the ACE inhibitory activity. The quadratic terms of X_2X_2, X_3X_3 and the interaction term of X_1X_2 were significant in ACE inhibitory activity. According to the multiple regression analysis, the following second-order polynomial regression equation (Equation 3) could explain the experimental data:

Table1. Variance analysis of the experimental results of BBD.

Source	Sum of squares	d.f.	Mean square	F-value	P-value	Note
Model	141.94	9	15.77	10.62	0.0090	significant
X_1	17.23	1	17.23	11.60	0.0191	
X_2	0.74	1	0.74	0.50	0.5111	
X_3	27.4	1	27.4	18.45	0.0077	
X_1X_2	19.55	1	19.55	13.17	0.0151	
X_1X_3	3.67	1	3.67	2.47	0.1768	
X_2X_3	0.05	1	0.05	0.034	0.8615	
X_1^2	0.69	1	0.69	0.47	0.5248	
X_2^2	59.77	1	59.77	40.25	0.0014	
X_3^2	18.16	1	18.16	12.23	0.0173	
Residual	7.42	5	1.48			
Lack of fit	7.02	3	2.34	11.61	0.0803	Non-significant
Pure error	0.4	2	0.2			
Cor.total	149.36	14				

$$Y = 34.02590 + 0.30807 \cdot X_1 + 1.74779 \cdot X_2 + 2.79826E - 0.03 \cdot X_3 + 0.22108 \cdot X_1X_2 - 1.91525E - 0.04 \cdot X_1X_3 + 2.23818E - 0.06 \cdot X_2X_3 - 0.4330 \cdot X_1^2 - 0.040235 \cdot X_2^2 - 8.86989E \cdot X_3^2 \quad (3)$$

The determination coefficient indicated that the model was adequate for prediction within the range of the experimental variables. The low P-value ($P < 0.001$) suggested that this model was statistically significant. Meanwhile, the response surface and contour plots (Figure 2) showed the interactive effects of three hydrolysis parameters on ACE inhibitory activity of hydrolysate. These plots displayed the responses to two independent variables and the point of maximum activity. It was observed that ACE inhibitory activity increased until enzyme dosage, substrate concentration and hydrolysis time reach an optimum point and then started to decrease. The optimal conditions were predicted by numerical optimization of the Design Expert software. The optimum conditions of the three different factors were as follows: enzyme dosage 11875.99 U/g, substrate concentration 33.04 g/L and hydrolysis time 4 h. Under these conditions, the predicted ACE inhibitory activity was 83.87%. In order to confirm the accuracy of the model, the experiment was conducted under optimal conditions and the ACE inhibitory activity was 84.94%, which was in agreement with the predicted value. These results indicated that the employed model was adequate to predict the hydrolysis condition and confirmed that the deduced conditions are optimal for ACE inhibitory peptides from desalted duck egg white.

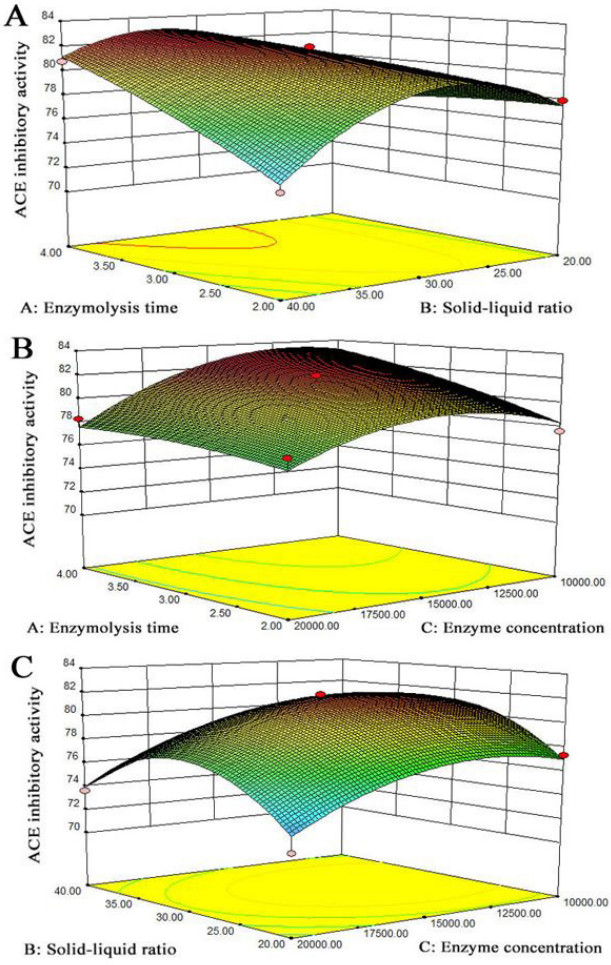

Figure 2. Response surface and contour plots showing the interactive effects of three hydrolysis parameters on ACE inhibitory activity of duck egg white peptides.

3.3 Ultrafiltration and identification of ACE inhibitory peptides

Ultrafiltration was performed to separate desalted duck egg white peptides from the optimal hydrolysis conditions into five fractions: fraction I ($M_W > 6$ kDa), fraction II (6 kDa > M_W >5 kDa), fraction III (5 kDa > M_W > 3 kDa), fraction IV (3 kDa > MW > 1 kDa) and fraction V (MW < 1 kDa). The ACE inhibitory activity of all fractions were exhibited in Figure 3. It was found that the fractions III to V were significantly higher than fractions I and II in ACE inhibitory activity. The fraction V revealed the highest ACE inhibitory activity of 68.2%, while fraction II showed the lowest activity of 24.33%. Similar result was also reported that the ACE inhibitory activities of the corn hydrolysate with $M_W < 1$ kDa showed the most potent ACE inhibitory activity (Huang et al., 2011). The results suggested that peptide with lower M_W exhibited stronger ACE inhibitory activity than peptides with higher M_W. This tendency was also observed in peptides derived from sea cucumber that the fraction with $M_W < 3$ kDa showed stronger ACE inhibitory activity than that of other fractions with higher M_W (Zhong et al., 2018). This may be explained that peptide with lower M_W can preferentially pass through the gap and bind with the active sites of ACE (Wilson et al., 2011). In addition, as an orally administered peptide, it was proposed that the lower M_W peptide was more resistant to hydrolysis by various proteinases and was more readily absorbed into the intestinal tissue and transported to the site of action in vivo

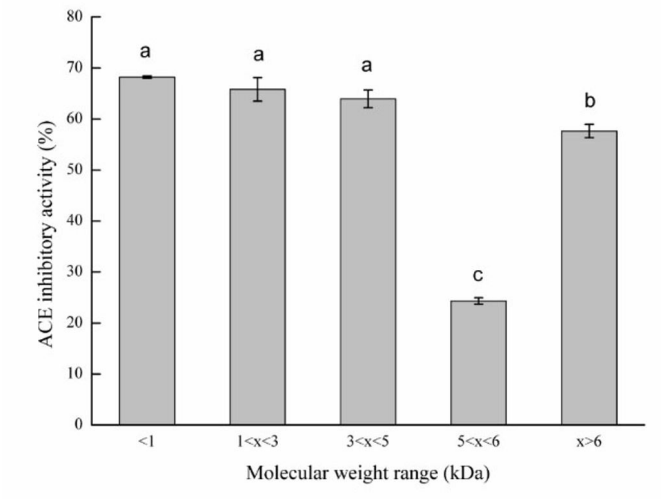

Figure 3. ACE inhibitory activity of different ultrafiltration fractions. Results are mean values of at least three replicates. The concentration of peptides is 1mg/ml. a, b, c within column, means without a common letter are significantly different, $P < 0.05$.

(Ding et al., 2014). Therefore, the fraction V (< 1 kDa) was selected for further research.

The fraction V (< 1 kDa) was then analyzed by HPLC-ESI-MS/MS, with an electro-spray ionization (ESI) source. Over 40 molecular ion peaks were chosen for further analysis due to the high response values. Through analysis of these molecular ion peaks, 83 kinds of the peptides were obtained with average polymerization degree of 2-5. The overall results of database comparison of peptides were listed in the Supplementary Material.

According to the previous reports on the relationship between structure and activity of ACE inhibitory peptides, ACE inhibitory activity was strongly influenced by the C-terminal amino hydrophobic or aromatic residues such as Trp, Tyr, Pro and Phe. When the N-terminal contains long chain or branched chain of hydrophobic amino acids, including Ile (I), Leu (L) and Val (V), the ACE inhibitory activity is higher (Harrison & Acharya, 2014). Meanwhile, when the third amino acid residue from C-terminal is Ile (I), Leu (L) or Trp (W), it can enhance the ACE inhibitory activity (Majumder & Wu, 2011). Thus, four most typical peptides, Ile-Arg (IR), His-Pro-Ala (HPA), Ile-Leu-Lys-Pro (ILKP) and Ile-Asn-Ser-Trp (INSW), based on the theory were chosen and then synthesized. The ACE inhibitory activity of peptides ILKP, INSW and fraction (<1kDa) were 69.8%, 55.16% and 50.83% (Figure 4). It was obvious that the ACE inhibitory activity of ILKP and INSW were significantly higher than fraction (< 1kDa). ILKP was further studied on the relationship of concentration and ACE inhibitory activity. The IC_{50} value of ILKP was 0.355mM from the regression equation $y = -0.0007x^2 + 0.4169x$ ($R^2 = 0.9959$).

In this study, the amino acid Ile (I) and Leu (L), which have the same molecular weight, appeared in IR, ILKP and INSW. These two typical amino acids came in pairs in the ILKP. It was also reported in other ACE inhibitory peptides, such as the Leu-Tyr (IC_{50} = 1.87 mM), Arg-Ala-Leu-Pro (IC_{50} = 0.97 mM) from rapeseed protein (He et al., 2013), Ala-Ser-Leu (IC_{50} = 102.15 mM) from the silkworm chrysalis protein (Wu et al., 2015), and Met-Ile-Leu-Leu-Phe-Arg (IC_{50}=0.12mM) from tilapia (Toopcham et al., 2015). These results showed that the branched chain amino acid Leu (L) had great influence on the activity of ACE inhibitory both in the C-terminal and N-terminal, especially when Ile (I) and Leu (L) repeated in a peptide sequence, which could produce synergistic effect and improved the activity of the ACE inhibitory peptides.

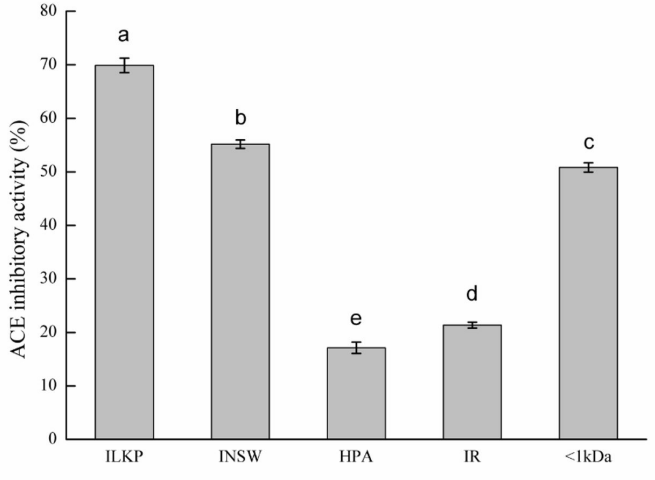

Figure 4. ACE inhibitory activity of synthetic peptides and fraction (< 1kDa). Results are mean values of at least three replicates. The concentration of peptides is 1 mg/mL. a, b, c, d, e within column, means without a common letter are significantly different, $P < 0.05$.

The present study also showed that the amino acid Pro (P) had different effects depending on the location in the sequence of peptides. As for the synthesized peptides HPA and ILKP, the ACE inhibitory activity of ILKP was significantly higher than HPA. It may be reasoned that the hydrophobic amino acids Pro (P) had positive influence on the ACE inhibitory activity as C-terminal residue. The Pro-Val-Asn-Asn-Pro-Gln-Ile-His in the small red bean with the IC_{50} value of 206.7 μM (Rui et al., 2013), and Val-Glu-Leu-Tyr-Pro with the IC_{50} value of 5.22 μM from squid muscle (Balti et al., 2015) were consistent with the findings in our study.

3.4 Molecular docking simulation

The docking results for the interaction of ILKP and INSW with ACE were shown in Figure 5. Through the analysis of scoring functions, the best pose for ILKP and INSE were obtained (Figure 5A, C) with binding energy value of -32.040 kJ/mol and -28.510 kJ/mol. As for the interaction forces, the binding mode between ACE residues and ILKP were hydrogen bonds, electrostatic, hydrophobic and hydrophilic force (Figure 5B), which was the same with INSW (Figure 5D) except for the presence of Zn at the ACE active site. The binding mode was in coincidence with most of ACE inhibitory peptides, such as the Lys-His-Val (IC_{50} = 12.82 μM) from silkworm pupa (Jia et al., 2015), Val-His-Trp (IC_{50} = 0.91 μM) from chlorella vulgaris (Xie et al., 2018) and IGPR (IC_{50} = 0.43 mM) from salmo salar (Yu et al., 2018).

The peptides ILKP and INSW contacted with both hydrophobic and hydrophilic residues of ACE. ILKP interacted with three hydrophobic residues and nineteen hydrophilic residues of ACE (Figure 5B). INSW interacted with two hydrophobic residues and eighteen hydrophilic residues of ACE (Figure 5D). Accordingly, ILKP contacts with ACE by more residues and gets more stable conjunction. The previous studies reported that hydrogen bonds played the most important role in stabilizing the docking complex (Chaudhary et al., 2009). In this study, hydrogen bonds were also found to be necessary in binding mode. ILKP formed three hydrogen bonds with residues Glu362, Ala332 and Lys489 of ACE. INSW generated five hydrogen bonds with residues Glu362, His491, His331 and Ala332 of ACE (Figure 5B, 5D).

ACE has three main active site pockets (S1, S2 and S1'). S1 pocket includes residues Ala354, Glu384 and Tyr523, and S2 pocket includes residues Gln281, His353, Lys511, His513 and Tyr520, while S1' contains residue Glu162 (Abdelhedi et al., 2018). However, the suggested active residues were not involved in the contact between ILKP and ACE or INSW and ACE, which was responsible for the lower inhibitory activity than ACE inhibitory

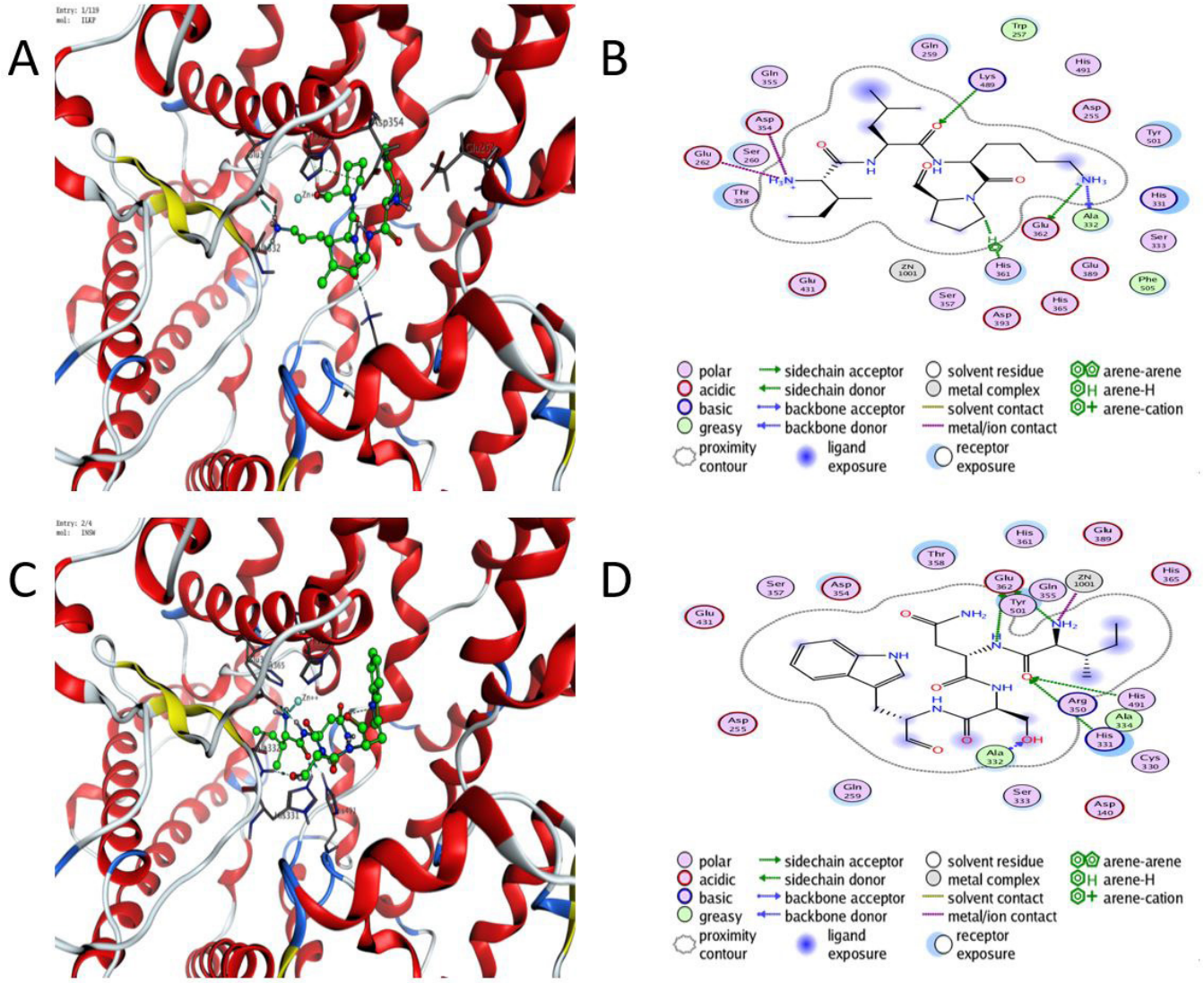

Figure 5. The molecular docking results for the interaction of ILKP and INSW with ACE (PDB: 5amb). (A) General overview of docking pose of ILKP (green stick model) at the ACE active site; (B) The binding mode between ACE residues and ILKP (green stick model) after docking at the ACE active site; (C) General overview of docking pose of INSW (green stick model) at the ACE active site; (D) The binding mode between ACE residues and INSW (green stick model) after docking at the ACE active site. Zn is represented in ball. Green and blue dotted lines indicate hydrogen bond formation while magenta dotted lines indicate electrostatic force formation.

peptide Gly-Asn-Gly- Ser-Gly-Tyr-Val-Ser-Arg (IC_{50} = 14.5 μM) explored from sipuncula (Guo et al., 2017), binding with Glu384 and Tyr523 of S1 pocket. It was also the reason why identified Leu-Leu established higher inhibitory activity confirmed to be bind with His353, Ala354 and Glu384 (Pan et al., 2012).

Nevertheless, other active residues were found in our research. It was observed that Histidine (His491, His331, His365, His361), Glutamate (Glu362, Glu262, Glu431, Glu389), and Alanine (Ala332) are the main amino acid sites in docking process. Glu362 and Ala332 were involved in hydrogen bonds interaction with ILKP and INSW respectively. Thus, the residues Glu362 and Ala332 may be important sites in molecular docking between ACE and ACE inhibitory peptides (Lan et al., 2015; Liu et al., 2016). Meanwhile, the N-terminal amino of Ile (I) in ILKP and INSW was demonstrated to create hydrogen bonds and electrostatic force on Zn, which was in agreement with the previous prediction that the N-terminal Ile(I) or Leu (L) could increase the ACE inhibitory activity of peptides (Ferreira et al., 2007; Balti et al., 2015).

4 Conclusion

The promamex was chosen as the protease to hydrolyze the desalted duck egg white. Eighty-three peptides were successfully identified to exhibit high ACE inhibitory activity relatively. The most typical peptide sequence was ILKP. The molecular docking studies revealed that nine amino acids in the ACE active site greatly contributed to stabilize the docking complex. This study suggested that the ACE inhibitory peptide derived from

desalted duck egg white could be utilized to develop functional foods for prevention of hypertension. Further work needs to be conducted to demonstrate in vivo antihypertensive activity of ILKP.

Conflict of interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author contributions

Haitao Li and Tao Hou proposed and designed the experiment. Haitao Li, Xiaoyan Chen, Yan Guo, and Tao Hou all participated in the experiment. Haitao Li and Jun Hu analyzed the data and wrote the manuscript. Tao Hou and Jun Hu revised the manuscript and were responsible for the supervision of the whole research.

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Supplementary Material

Supplementary material accompanies this paper.

Data summary of MW < 1 kDa fractions.

This material is available as part of the online article from https://www.scielo.br/j/CTA