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Research Article

Purification of Angiotensin-I-Converting Enzyme Inhibitory Peptides Derived from *Camellia oleifera* Abel Seed Meal Hydrolysate

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China is a large country that produces *Camellia oleifera* Abel seed meal (COASM), a by-product of tea-seed oil, which is only used as an organic fertilizer, resulting in a serious waste of high-quality resources. The preparation of the ACE inhibitory peptide from COASM and the study of its functional properties are of practical importance in improving the comprehensive utilization of COASM. Our manuscript presents an optimized preparation of ACE inhibitory peptides with alkaline protease and enzyme kinetics parameters. Ultrafiltration, gel chromatography, and RP-HPLC purification were conducted for ACE inhibitory peptides, and peptide molecular weight distribution and amino acid composition were analyzed in the enzymolysis liquid. The following were the conditions of the optimized enzymatic hydrolysis to obtain ACE inhibitory peptides from COASM: 15 times of hydrolysis in distilled water for 3.5 h at 50°C, pH = 8.5, substrate concentration of 17 mg/g, and addition of 6% (w/w) alkaline protease. Under this condition, the peptides produced exhibited an ACE inhibition rate of 79.24%, and the reaction kinetics parameters are as follows: K_m = 0.152 mg/mL and V_{max} = 0.130 mg/mL·min. The majority of ACE inhibitory peptides from COASM have molecular weight below 1 kDa, and a high ACE inhibitory rate was achieved after dextran gel chromatography separation and purification (whose IC_{50} was 0.678 mg/mL). The hydrophobic amino acid content in this fraction reached 51.21%.

1. Introduction

Hypertension has become a global killer. Patients with high blood pressure also gradually appear younger [1, 2]. Thus, a safe and effective treatment for high blood pressure must be developed. Angiotensin-I-converting enzyme (ACE) is a dipeptide carboxyl protease that plays an important role in blood pressure regulation. Effectively inhibiting the ACE rate can help reduce blood pressure [3, 4]. With the development in biological active peptide research, lowering the blood pressure and obtaining functional peptides from natural resources have become a hot spot in the study of medicine [4–6].

The methods of preparing ACE inhibitory peptides mainly include enzyme hydrolysis and microbial fermentation. Enzymatic hydrolysis refers to the hydrolysis by proteases to obtain all kinds of bioactive peptides, some of which can inhibit ACE [2, 7, 8]. This method is simple and can be used to easily control digestion conditions. The obtained ACE inhibitory peptides still require ultrafiltration, macroporous resin adsorption, gel chromatography, RP-HPLC, and preparative chromatography purification [9, 10]. Otte was the first to study the effects of using five kinds of protease hydrolysis and nine kinds of milk protein to produce ACE inhibitory peptides. Gel chromatography and RP-HPLC were applied to separate and purify the ACE inhibitory peptides from the lactalbumin protease hydrolysate. Five high rates of ACE inhibitory peptides with IC_{50} values of 1–5 μM were achieved [11]. Minervini used *Lactobacillus helveticus PR4* for the protease enzymolysis of casein from six species of animal (e.g., cattle, sheep, goats, pigs, water buffalo, and people) and thus produced a variety of ACE inhibitory peptides [12].

C. oleifera, which belongs to the Theaceae family, is regarded as one of the four largest woody oil species in the world and has high economic values and ecological benefits [13, 14]. COASM is a by-product of oil extraction, and its utilization ratio is relatively low. For a long time, COASM has been used as a fertilizer and fuel, resulting in a serious waste of resources [15]. *C. oleifera* contains 10%–20% protein, and some small peptides derived from food proteins are easily digested and absorbed by the human body. COASM can provide nutrients for human growth and development and exhibit strong antioxidant and lipid-lowering properties and other health care functions. Thus, it is a promising functional factor worldwide [16, 17].

In this study, COASM was used as the raw material, and the response surface method was used to determine the best enzymolysis conditions for alkaline protease. The enzymolysis kinetic parameters were obtained according to the Michaelis–Menten equation. Ultrafiltration, gel chromatography, and RP-HPLC were conducted to produce a component with high ACE inhibitory rate. The amino acid composition and in vitro digestion of this component were subsequently analyzed. Our experiment can provide the basis for further research to increase the value of *C. oleifera*.

2. Materials and Methods

2.1. Materials and Reagents. COASM samples were provided by Lv Yuan *C. oleifera* processing Co. (Qionghai city, Hainan, China). Angiotensin-I-converting enzyme (ACE), *N*-[3-(2-furylacryloyl)]-L-phenyalanyl-glycyl-glycine (FAPGG), and Sephadex G-25 glucan gel were purchased from Sigma-Aldrich Co. (USA). Trypsin (250 U/mg), neutral protease (50 U/mg), alkaline protease (2,000 U/mg), and papain (6,000 U/mg) were purchased from Novozymes Biological Technology Co. (Beijing, China). Tris-HCl buffer, NaCl, NaOH, formic acid, ethanol, and phenol reagents were of analytical grade and purchased from Beijing Chemical Reagent Co. (Beijing, China). Deionized water was used throughout the experiment.

2.2. Preparation of ACE Inhibitory Peptides from COASM. COASM samples underwent lyophilization, crushing, screening with 40-mesh size using petroleum ether as solvent with a S/L ratio of 1 : 8 (g/ml), and 65°C extraction for 4 h to remove fat. In brief, 1.5 g of defatted COASM powder was accurately weighted in a beaker. The optimal pH and temperatures for four kinds of protease were as follows: trypsin (pH 8.0, 37°C), neutral protease (pH 7.0, 50°C), alkaline protease (pH 9.5, 60°C), and papain (pH 5.7, 60°C). These proteases were set to digest for 4 h. Enzymatic hydrolysis conditions are as follows: a S/L ratio of 1 : 15 (g/ml) and enzyme amount for 5.0% (w/w). The enzymes were inactivated via a boiling water bath for 10 min. After centrifugation for 10 min at 4°C and 10,000 rpm, the supernatant fluid was subjected to evaporation and freeze drying. Finally, the ACE inhibitory peptides were obtained [18, 19].

2.3. Determination of the Degree of Hydrolysis (DH) from COASM Enzymolysis. DH was determined according to the pH-stat method, which is based on the protein hydrolysis process related to the pH value of the solution. The pH system was maintained by adding acid or alkaline, whose quantity must be based on the calculated DH from the enzymolysis of COASM using the following equation [18, 20, 21]:

$$\mathrm{DH}(\%) = \left(\frac{V_b \times N_b}{Z_p \times 7.3223}\right) \times 100, \quad (1)$$

where V_b is the NaOH volume (mL), N_b is the sodium hydroxide concentration (0.1 mol/L), Z_p is the protein weight (g), and 7.23 is the number of peptide bonds in per g proteins (mmol) and that of COASM was 7.23.

2.4. Determination of ACE Inhibitory Rate. ACE inhibition rate of COASM hydrolysate was evaluated by the method established in our laboratory [22]. In brief, 10 μL of ACE aqueous solution and 10 μL of ACE inhibitory peptides (concentration of 1 mg/mL) were added to an enzyme microplate. The substrate (1.0 mmol/L FAPGG dissolved in 50 mmol/L Tris-HCl containing 0.3 mol/L·NaCl, pH 7.5) was preheated for 37°C for 5 min, added to the microplate, and allowed to react for 2 min. The absorbance at 340 nm was recorded and labeled as A_1. After a reaction time of 30 min, the absorbance at 340 nm was determined and labeled as A_2. The control group comprised 10 μL of substrates instead of ACE inhibitory peptides. The initial absorbance of the blank solution was recorded as A_{01} and that after the reaction was recorded as A_{02}. Changes in the absorbance value (ΔA Inhibitor $= A_1 - A_2$, ΔBlank $= A_{01} - A_{02}$) were calculated to obtain the ACE inhibitory rate:

$$\text{ACE inhibitory rate}/\% = \left(1 - \frac{\Delta A \text{ inhibitor}}{\Delta \text{blank}}\right) \times 100. \quad (2)$$

2.5. Response Surface Design Experiment under Enzymatic Hydrolysis Conditions. According to the results of the single-factor experiment, the experimental design was optimized using response surface methodology [23]. The Box–Behnken center combination experimental principle was also applied [24, 25]. In this principle, we selected five factors, namely, substrate concentration, pH value, reaction temperature, enzyme quantity, and reaction time, which affect the ACE inhibitory rate. Three levels per factor were used to optimize the test, and the ACE inhibitory rate was used as the response variable.

2.6. Determination of the Maximum Reaction Rate of MI Constant and Enzyme-Promoting Reaction. The substrate concentration of COASM protein solution with 0.02, 0.04, 0.06, 0.08, and 0.1 mg/mL was prepared, respectively; alkaline protease was added and hydrolysated for pH 9.5 at 60°C. 0.05 mol/L·NaOH solution was used to maintain a constant pH value in COASM protein solution. The average

velocity of the generated peptides speed was calculated instead of the initial velocity. MI constant and maximum reaction rate were obtained according to the following formula [1, 10]:

$$\left(\frac{1}{V_o}\right) = \left(\frac{K_m}{V_{max}[S]} + \frac{1}{V_{max}}\right), \quad (3)$$

where V_o is the average velocity of the generated peptides speed, [S] is the substrate concentration, K_m is the MI constant, and V_{max} is the maximum reaction rate.

2.7. Separation and Identification of ACE Inhibitory Peptides

2.7.1. Ultrafiltration of ACE Inhibitory Peptides. The COASM protein hydrolytic liquid was filtered using three (10, 5, and 1 kDa) ultrafiltration membranes and separated by a Vivaflow ultrafiltration system [22, 26]. The fractions were collected and freeze-dried, and their IC_{50} values were measured. The fraction with a highest ACE inhibition rate and minimum mean IC_{50} value was selected for subsequent purification.

2.7.2. Gel Chromatography of ACE Inhibitory Peptides. The desalination Sephadex G-15 resin was loaded in a 1.6 cm × 60 cm glass chromatographic column. Samples of 10, 25, and 50 mg/mL solution were prepared with ultrapure water, and 3 mL was eluted each time at a flow rate of 1 mL/min ultrapure water. Samples were collected by using a tube every 10 min, and 50 samples were finally obtained. The absorbance of these 50 samples was measured at 280 nm. The obtained fractions were collected and then freeze-dried and preserved at 4°C [27, 28].

2.7.3. Preparation of RP-HPLC Method to Purify ACE Inhibitory Peptides. After isolating Sephadex G-25, the component with highest ACE inhibitory rate was dissolved in ultrapure water. After 0.22 μm membrane filtering, RP-HPLC was conducted for further purification with the following conditions: chromatographic column: C_{18} column (standard: 4.6 × 100 mm, 5 μm), detection wavelength of 215 nm, and mobile phases A (acetonitrile) and B (ultrapure water containing 0.10% trifluoroacetic acid); elution conditions were 0% A for 5 min and 0–40% A for 5–30 min; sample quantity of 10 μl; and column temperature at 35°C [21, 29].

2.7.4. Amino Acid Analysis of ACE Inhibitory Peptides. Type and content of amino acids in ACE inhibitory peptides from COASM was determined by the method established in our laboratory [22]. 1 mL of the filtrate was taken into a 5 mL volumetric flask and dried in a vacuum drier. The residue was dissolved in deionized water, then dried, repeated for two times, and dissolved in 1 mL of pH 2.2 in the Tris-HCl buffer. Accurately absorbing 0.2 mL mixed standard amino acids, diluting to 5 mL with pH2.2 Tris-HCl buffer to the concentration of 1.0 nmol/10 μl, which as the standard amino acid for determination, the amino acid automatic analyzer with the external standard method to determine the type and content of amino acids in ACE inhibitory peptides from COASM.

2.8. Statistical Analysis. Final data were expressed as the mean ± standard deviation ($\overline{x} \pm s$). ANOVA was employed to assess the differences among groups. Statistical analysis was performed using SPSS 21.0. Significant differences were denoted by $P < 0.05$.

3. Results and Discussion

3.1. Screening of Protease for COASM. Figure 1(b) shows that, during the different protein hydrolyses of COASM, the ACE inhibition rates all showed a trend of an initial increase and then decrease with the increasing hydrolysis time. The ACE inhibitory rate in alkaline protease hydrolysate was the highest with a maximum of 61.5% at 2.5 h hydrolysis time, and the DH was also the largest but after 3.5 h of hydrolysis. By comparing the DH and corresponding hydrolysate of ACE inhibition rate, the results showed that the DH was not positively correlated with ACE inhibition rate. The DH was the third for papain protease hydrolysate (Figure 1(a)), but this hydrolysate exhibited the lowest ACE inhibition rate (Figure 1(b)). This phenomenon occurred because the ACE inhibition rate was associated with enzyme loci. The more the ACE inhibitory peptides are cut down from COASM proteins, the higher their ACE inhibition rate. Hydrolysates with a high DH does not necessarily exhibit a high ACE inhibitory activity [2, 30]. Alkaline protease is a kind of serine enzyme that tends to form the peptide with hydrophobic amino acids at the end [9, 31]. Furthermore, alkaline protease can cut -CO-NH internal peptide, which makes protein converted into peptides with a smaller molecular weight [7, 29]. Vasquez-Villanueva et al. used alkaline protease to enzymolyze corn protein and found that the alkaline protease hydrolysate has a high ACE inhibitory rate [30]. Minervini et al. selected alkaline protease to hydrolyse whey protein and produce ACE inhibitory peptides. This selection is due to the high amount of alkaline protease hydrolysis locus, which has particularity for selecting hydrophobic amino acids on the protein carboxyl terminal [12]. Therefore, based on the above data reported and our results shown in Figure 1, our study selected alkaline protease hydrolysate for further purification.

3.2. Various Factors on Preparation of ACE Inhibitory Peptides by Alkaline Protease Hydrolysis COASM Proteins. The basis of parameters for alkaline protease hydrolysis solution was set as follows: COASM protein concentration was 9%, the ratio of the enzyme and substrate (E : S) was 5%, and pH was 9.50 and hydrolysed at 55°C for 3 h. A single-factor experiment was used to study the temperature (40°C, 45°C, 50°C, 55°C, and 60°C), pH (8.5, 9.0, 9.5, 10, and 10.5), adding amounts of E/S (4%, 5%, 6%, 7%, and 8%), COASM protein concentrations (3%, 5%, 7%, 9%, and 11%), and hydrolysis time (1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 h), which verify

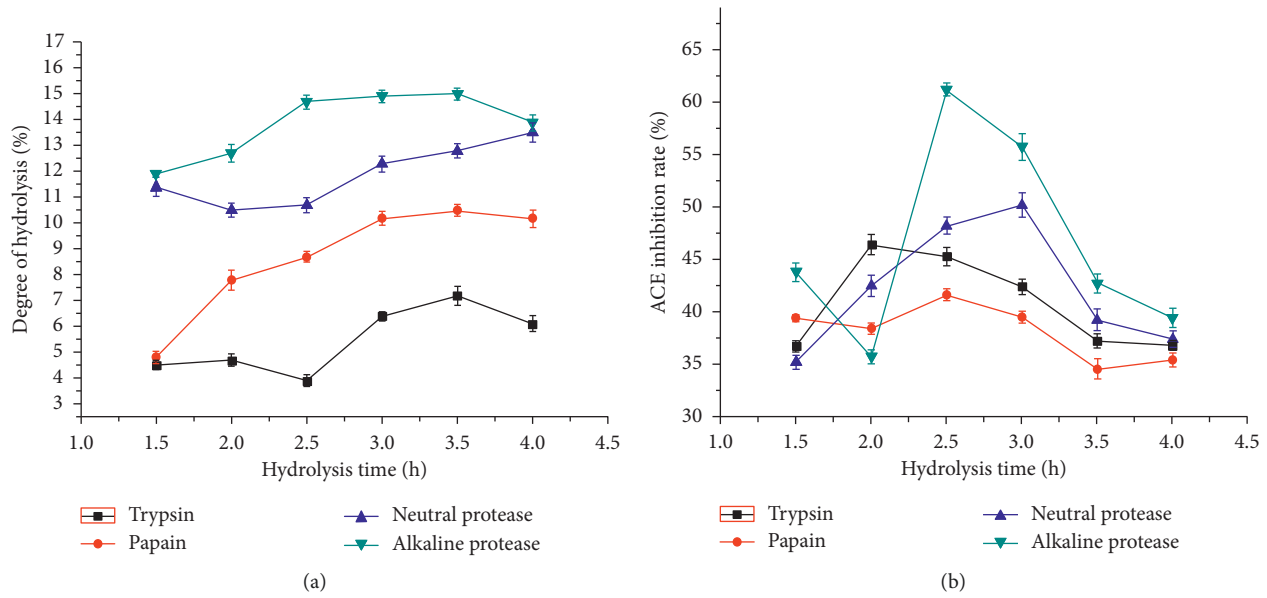

Figure 1: Effect of enzymes and hydrolysis time on properties of protein from COASM: (a) DH; (b) ACE inhibition rate.

the effect of these factors on the ACE inhibitory peptides of alkaline protease for COASM protein. The results are shown in Figure 2.

Figures 2(a) and 2(b) show that, with the DH and ACE inhibitory rate of COASM, hydrolysates initially increased and then decreased with the increase in the hydrolysis temperature. With the increase in the hydrolysis temperature and pH, the alkaline protease activity was also gradually enhanced; hence, the DH and ACE inhibitory rate increased until reaching the maximum. The continuous increase in the temperature and pH led to the change in the enzyme structure, influenced the combination of the substrate, gradually inactivated the enzyme, and reduced the DH and ACE inhibition rate.

According to Figures 2(c) and 2(d), with the increasing hydrolysis time and alkaline protease amount, the DH of COASM hydrolysates gradually increased, whereas the ACE inhibition rate initially increased and then decreased. These results confirmed that the DH and ACE inhibition rate were not necessarily related. Figure 2(e) shows that, with the increasing substrate concentration, the DH and ACE inhibitory rate of COASM hydrolysates were all rapidly increased at the substrate concentration before 9% and then were remained in a stable state.

3.3. Optimization of ACE Inhibitory Peptide Preparation for Alkaline Protease Enzymolysis of COASM Protein. According to the screening test results of the main factors, the response surface method optimized the preparation of ACE inhibitory peptides for the enzymolysis of alkaline protease from COASM protein. The Box–Behnken design and results are shown in Table 1; the response value was ACE inhibition rate.

Table 1 shows the multiple regression analysis data from SAS software. The quadratic polynomial fitting equation was

$$\mathrm{DH} = 12.48 + 0.17\mathrm{A} + 0.28\mathrm{B} - 0.026\mathrm{C} + 0.062\mathrm{AB} - 0.0025\mathrm{AC} - 0.0005\mathrm{BC} - 0.55\mathrm{A}^2 - 0.53\mathrm{B}^2 - 0.64\mathrm{C}^2, \quad (4)$$

where A, B, and C are the hydrolysis temperature, pH, and time, respectively. The quadratic polynomial regression equation prediction model was significant ($p < 0.05$), whereas the lost item was not significant ($p = 0.073 > 0.05$). All these results showed the reliability of the model. Determination coefficient, $R^2 = 91.48\%$, showed that the experimental data were well-fitted on the equation. Thus, this model could be used to simulate the optimization of the enzymatic hydrolysis of COASM powder.

3.4. Reaction Kinetics in the Preparation of ACE Inhibitory Peptides from COASM Protein Using Alkaline Protease. COASM protein was added into different concentration solutions for the hydrolysis using alkaline protease under the optimum conditions. The reciprocal of the substrate concentration is the abscissa, whereas that of the generated peptides speed is the ordinate. The double bottom diagram is shown in Figure 3. Based on the Lineweaver–Burk method, K_m and V_{max} were 0.152 mg/mL and 0.130 mg/mL·min, respectively, as calculated using the formulation in Figure 3.

3.5. Analysis of ACE Inhibitory Rate from COASM Peptide after Ultrafiltration Membrane Filtration. COASM peptides were prepared using the optimized process and were ultrafiltrated by molecular weights of 10, 5, and 1 kDa ultrafiltration membrane. The fractions were obtained and lyophilized to determine the ACE inhibitory rate and IC_{50} value concentration. The results are shown in Table 2.

Table 2 shows that, with the gradual decrease in the molecular weight of the ultrafiltration membrane, the ACE

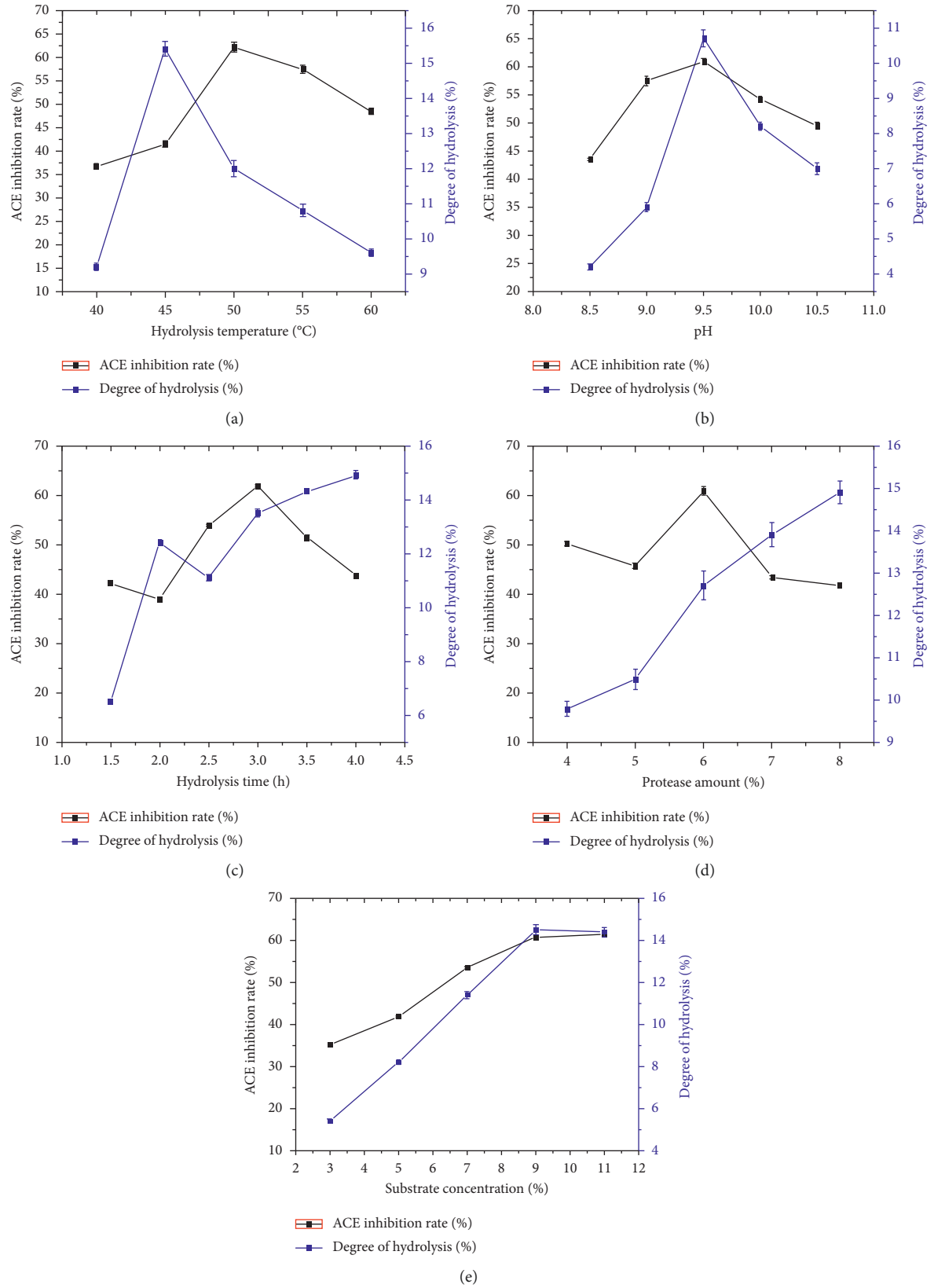

Figure 2: Influence of the selected factors on the DH and ACE inhibitory rate hydrolysate by alkaline protease: (a) hydrolysis temperature; (b) pH; (c) hydrolysis time; (d) protease amount; (e) substrate concentration.

Table 1: Box–Behnken design and results for ACE inhibitory peptides by alkaline protease.

Number	Hydrolysis temperature (°C)	pH	Hydrolysis time (h)	ACE inhibitory peptides
1	45	9.5	3.0	57.07
2	45	10	3.0	59.47
3	55	9.5	3.0	54.14
4	55	10.0	3.0	60.64
5	50	9.5	2.5	56.55
6	50	9.0	3.5	61.21
7	50	10.0	2.5	60.05
8	50	10.0	3.5	58.58
9	50	9.0	2.5	59.01
10	55	10.0	3.0	57.88
11	55	9.5	3.5	60.53
12	50	9.0	3.5	58.03
13	50	9.5	3.0	61.65
14	55	9.5	2.5	56.45
15	50	10.0	3.5	58.62
16	50	9.5	3.0	59.58
17	50	9.5	3.0	60.98

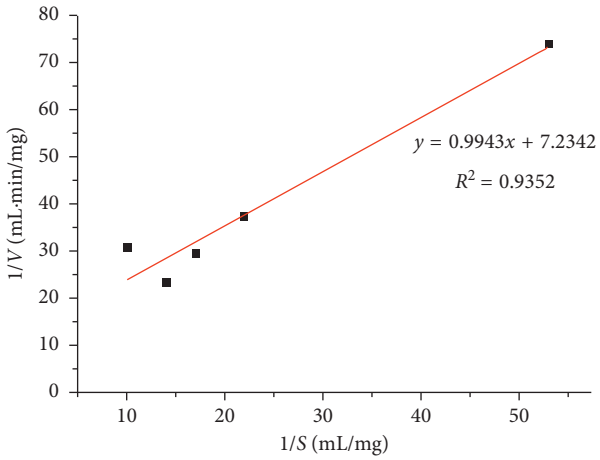

Figure 3: Equation curve of 1/V to 1/S for Lineweaver–Burk (alkaline protease).

Table 2: ACE inhibition rate and IC_{50} of the ultrafiltration fractions in the sample.

Molecular weight	ACE inhibition rate (%)	IC_{50} (mg/mL)
$M < 10$ kDa	73.78 ± 1.14	0.874
$M < 5$ kDa	77.27 ± 0.89	0.728
$M < 1$ kDa	79.24 ± 1.14	0.678

inhibition rate of samples also gradually increased, and the IC_{50} value concentrations gradually reduced. These results suggest that ultrafiltration allowed to obtain a fraction with molecular weight peptides lower than 1 kDa, exhibiting higher ACE inhibitory activity. Our results are similar to those of Korhonen et al., who used ultrafiltration to separate ACE inhibitory peptides from whey protein hydrolysate. They found that the IC_{50} value is the minimum for the peptide in 1 kDa membrane ultrafiltration [20]. Our laboratory also demonstrates that the cashew nut peptides exhibiting ACE inhibitory activity had molecular weight lower than 3000 Da [22]. The above results also verified the conclusion that ACE inhibitory peptides are mainly composed of peptides below 1 kDa.

3.6. COASM Peptide Purified by Gel Chromatography. Figure 4 shows the peptide profile of hydrolysate solutions with three different concentrations. The separation effect for each peak was ineffective for 10 mg/mL concentration. Given the low and diluted concentration of the sample, the separation effect between the components was not ideal. When the sample concentration was 25 mg/mL, the separation of the three peaks became apparent and it was more effective.

When the sample concentration was increased to 50 mg/mL, the chromatographic column separation effect was significantly reduced. Samples were continuously diluted during gel chromatography separation. When the separation efficiency is affected, the sample concentration should be increased as soon as possible. However, the viscosity of samples was also increased when the sample concentration was extremely high, which would affect the flow of components in the gel and finally reduce the resolution of the gel chromatography. Therefore, 25 mg/mL was selected as the best sample concentration.

Peaks I, II, and III from the 25 mg/mL COASM peptide after Sephadex G-15 separation were collected and freeze-dried, and then their IC_{50} value concentrations were determined. The results were in the following order: I (0.145 mg/mL) < II (0.202 mg/mL) < III (0.213 mg/mL). The IC_{50} value concentration of peak I was lower than those of the other two components, which might be because peak I contains free amino acids. Je et al. used gel chromatography to purify ACE inhibitory peptides from Alaska pollock (*Theragra chalcogramma*) frame protein hydrolysate; the IC_{50} value reached 0.066 mg/mL [18]. Therefore, peak I was collected as the subsequent test samples after freeze drying.

3.7. COASM Peptide Purified by RP-HPLC. The component I of the COASM peptide was solubilized in ultrapure water and

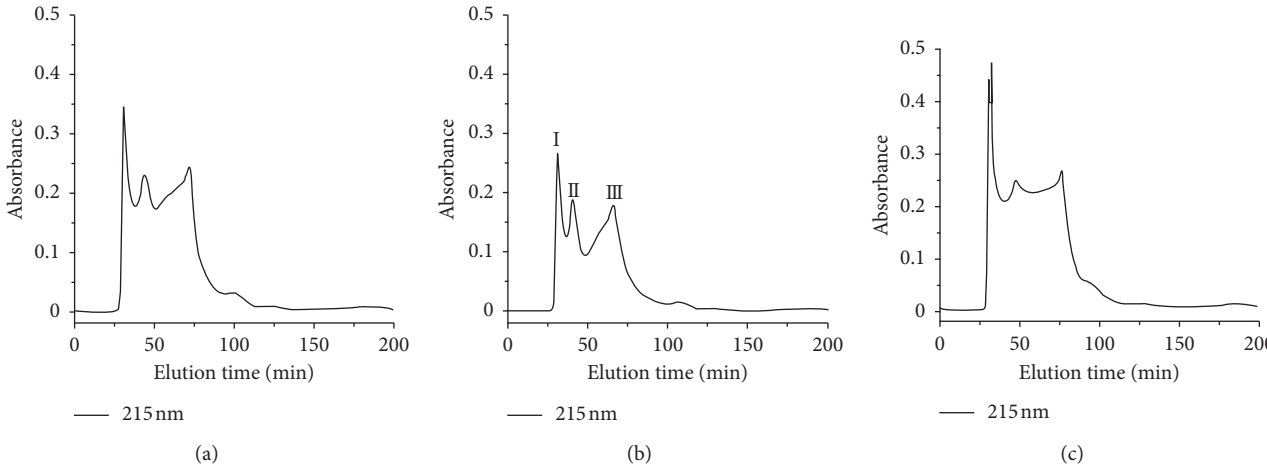

Figure 4: Separation of Sephadex G-15 at sample concentrations of 10 mg/mL (a), 25 mg/mL (b), and 50 mg/mL (c).

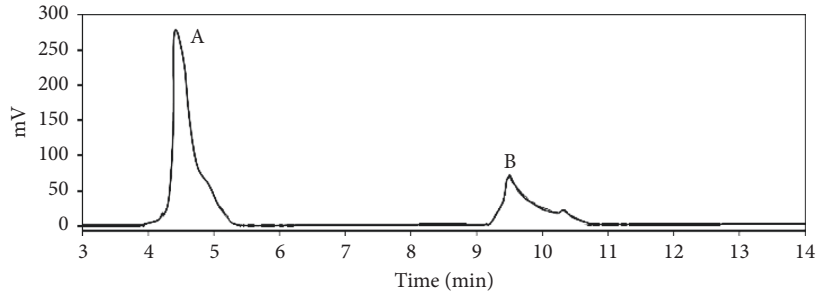

Figure 5: Peptide mapping of fraction I from COASM separated by RP-HPLC.

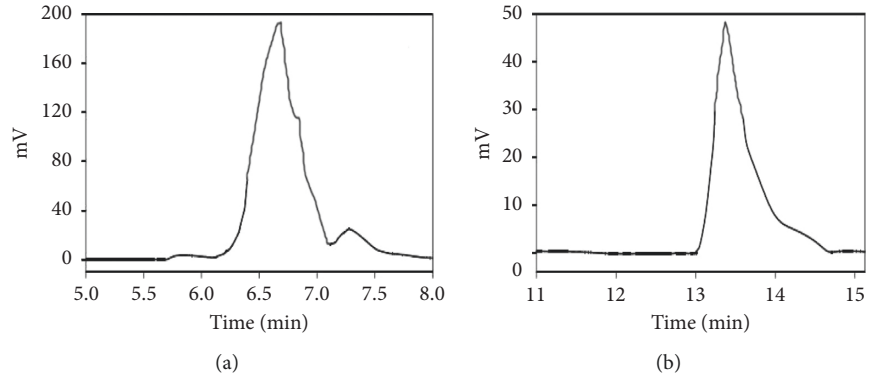

Figure 6: Purity analysis of fractions (a) and (b) from COASM hydrolysate.

injected into the RP-HPLC after 0.22 μm filter membrane treatment. Peak I purity was determined after RP-HPLC purification, and the results are shown in Figures 5 and 6.

Figure 5 shows that component I of COASM ACE inhibitory peptides has two main peaks, and its purity was determined by analytical RP-HPLC after collection. The results are shown in Figure 6.

According to Figure 6(a) and 6(b), the chromatogram had two peaks. Based on calculations, the peak concentrations of A were 82.17% and 17.83%, whereas those of B were 90.24% and 9.76%.

3.8. Amino Acid Composition Analysis of COASM Peptide. It has been reported that ACE inhibitory peptides contain hydrophobic amino acids residues and also that the three amino acid sequence of C-terminal in these peptides can affect the ability of peptides to combine with ACE. If these sequences contain hydrophobic amino acids, it is highly likely that they had high ACE inhibitory activity [16, 26]. Table 3 shows that the hydrophobic and aromatic amino acids in peak I account for 51.21% and 9.59% of the total amino acid, respectively. This finding agrees with the study by Aleman et al. who isolated the highest ACE inhibitory

Table 3: Amino acid compositions of fraction I (%) (g/100 g protein).

Amino acid	Content
Asp	3.14
Thr	3.17
Ser	1.89
Glu	3.48
Gly	9.43
Ala	7.34
Val	3.78
Met	2.78
Iso	2.75
Leu	5.99
Tyr	2.28
Phe	3.56
Lys	3.78
His	1.78
Arg	3.01
Pro	2.87
Trp	0.04
Cys	0.28

rate component (molecular weight was less than 1,000 Da) from squid skin collagen with the hydrophobic amino acid content of up to 54.6% [31]. Table 3 also indicates that peak I had high glycine, alanine, leucine, and valine contents. Several studies have found that the ACE inhibitory peptides from sea stings, sea cucumbers, and shrimps contain glutamic acid, glycine, alanine, and leucine [29, 31].

4. Conclusions

DH was not positively correlated with the ACE inhibition rate in COASM protein hydrolysates. After conducting the response surface method to optimize the hydrolytic process, alkaline protease enzymolysis of COASM protein was found to exhibit the highest ACE inhibitory rate. The molecular weight of the ACE inhibitory peptide was less than 1 kDa after ultrafiltration. The components of ACE inhibitory peptides in COASM protein could be separated by Sephadex G-15. Component I had the strongest inhibitory rate that reached 79.24%, and its IC_{50} was 0.678 mg/mL. RP-HPLC can be used to prepare ACE inhibitory peptides from COASM. Purity analysis of the single component revealed that the separated component I has high alanine, glutamic acid, glycine, and leucine contents.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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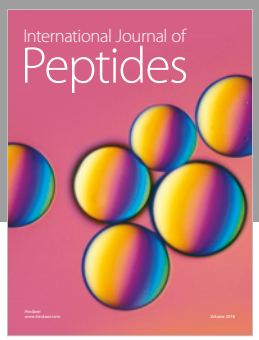

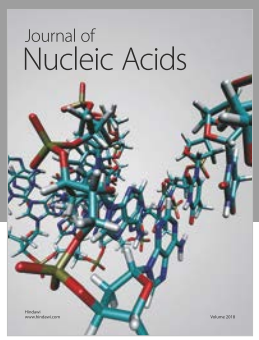

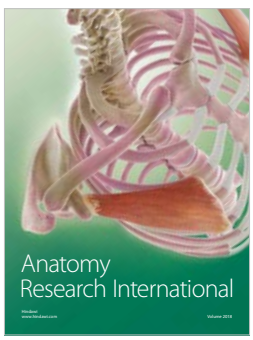

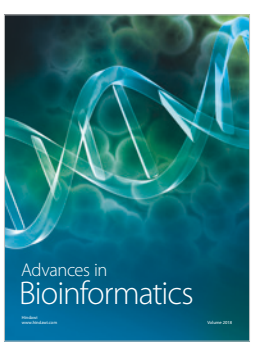

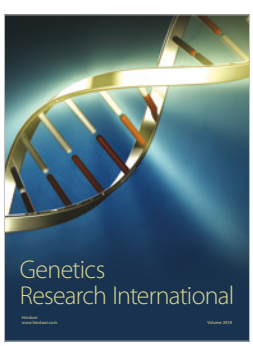

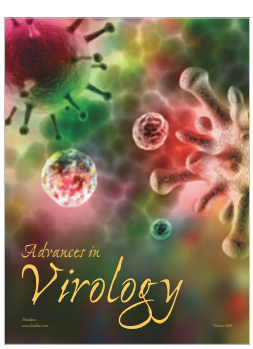

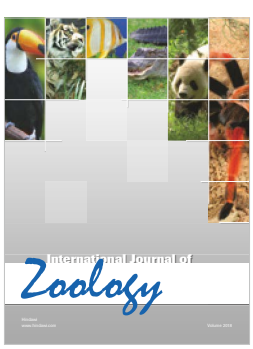

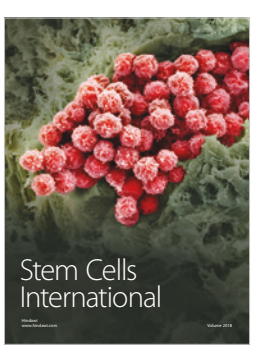

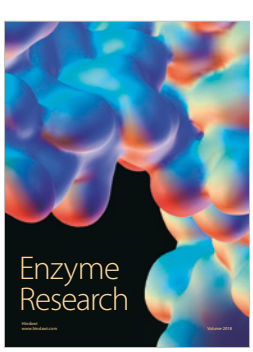

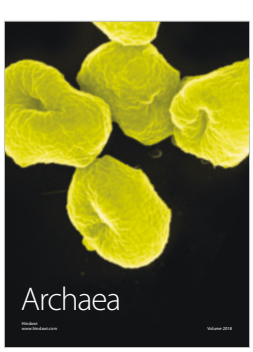


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