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Angiotensin I Converting Enzyme Inhibitory Peptides from Simulated *in Vitro* Gastrointestinal Digestion of Cooked Eggs

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Egg proteins are an excellent source of bioactive peptides. The purpose of this work was to study the effect of cooking methods on the production of angiotensin converting enzyme (ACE) inhibitory peptides. Boiled or fried eggs (in the forms of whites, yolks, and whole eggs) were digested by gastrointestinal tract proteases at simulated gut conditions. Fried egg digests showed more potent activity than those of boiled egg digests; the fried whole egg digest had an IC_{50} value of 0.009 mg protein/mL. This hydrolysate was further purified by cation exchange chromatography and gel filtration chromatography. Seven peptides, Val-Asp-Phe (IC_{50}: 6.59 μM), Leu-Pro-Phe (10.59 μM), Met-Pro-Phe (17.98 μM), Tyr-Thr-Ala-Gly-Val (23.38 μM), Glu-Arg-Tyr-Pro-Ile (8.76 μM), Ile-Pro-Phe (8.78 μM), and Thr-Thr-Ile (24.94 μM), were identified by liquid chromatography−mass spectrometry (LC-MS/MS), and their IC_{50} values were predicted by using our previously reported structure and activity models. The presence of several tripeptides from *in vitro* simulated gastrointestinal egg digest indicates that these peptides may be absorbed into the body and exert an *in vivo* antihypertensive activity, although *in vivo* study is needed to confirm this assumption. Our results showed that *in vitro* digestion of cooked eggs could generate a number of potent ACE inhibitory peptides which may have implications for cardiovascular disease prevention, including hypertension.

KEYWORDS: Eggs; *in vitro* digestion; boiled eggs; fried eggs; peptide; angiotensin converting enzyme (ACE).

INTRODUCTION

Egg is an important part of human diet. The main components of eggs are water (75%), proteins (12%), lipids (12%), vitamins, carbohydrates, and minerals (*1*). Despite the fact that egg contains all necessary nutrients for new life, consumption of table eggs has decreased in many developed countries in the last four decades due to the controversial cholesterol perception. Nevertheless, current evidence has suggested that there is no direct relationship between egg intake and the incidence of heart disease (*2*). Heart disease is the number one leading cause of death and represents a major health concern in North American (*3*). High blood pressure, defined as blood pressure greater than 140 mmHgsystolic and/or 90 mmHgdiastolic pressure, is one of the identified major risk factors for heart disease (*4*, *5*).

Egg is a rich source of various bioactive proteins and bioactive peptides. It is now well established that during gastrointestinal digestion or processing of food proteins, small peptides may be released and could act as regulatory compounds that may have certain physiological benefits for human health (*6*). These so-called bioactive peptides either can be absorbed through the intestine to enter the blood circulation and exert systemic effects or can produce local effects within the gastrointestinal tract (*7*). Various bioactive peptides, such as antimicrobial peptides, anticancer peptides, antihypertensive peptides or angiotensin converting enzyme (ACE) inhibitory peptides and protease inhibitors have been reported from eggs (*8*).

ACE is the key enzyme which is responsible for regulation of blood pressure through the rennin−angiotensin system. ACE catalyzes the formation of angiotensin II, a potent vasoconstrictor, from angiotensin I and inactivates bradykinin, a vasodilator (*9*). Elevated activity of ACE could lead to a higher level of angiotensin II and therefore result in high blood pressure or hypertension. ACE is the target enzyme for antihypertensive drug development; currently, ACE inhibitory drugs, such as captopril, benazepril, and enalapril, are the first-line therapy for hypertension (*10*). Due to the presence of those inevitable adverse side effects associated with the use of synthetic drugs (*11*), development of ACE inhibitory peptides from food proteins is under extensive research as the alternatives for the prevention and treatment of hypertension (*12*−*14*).

Recent studies have shown that ACE inhibitory peptides derived from egg proteins can significantly reduce blood pressure in both hypertensive animals and human subjects (*15*). Table egg consumption accounts for about 70% of total egg

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Table 1. IC_{50} Values of the Digested Egg Samples[a]

treatment	IC_{50} value mg/mL (total dry matter)	IC_{50} value mg/mL (protein conc)
pepsin digested boiled egg whites	0.19 (0.02) d,e	0.142 c,d
pepsin and pancreatin digested boiled egg whites	0.14 (0.01) d,e,f	0.098 d,e
pepsin digested boiled egg yolk	3.55 (0.28) a	1.118 a
pepsin and pancreatin digested boiled egg yolk	2.50 (0.26) b	0.846 a,b
pepsin digested boiled whole egg	0.05 (0.01) f	0.019 e,f
pepsin and pancreatin digested boiled whole egg	0.05 (0.01) f	0.025 e
pepsin digested fried egg white	0.09 (0.17) e, f	0.063 e
pepsin and pancreatin digested fried egg white	0.11 (0.02) e, f	0.076 e
pepsin digested fried egg yolk	1.65 (0.36) c	0.459 b,c
pepsin and pancreatin digested fried egg yolk	0.59 (0.09) d	0.168 c
pepsin digested fried whole egg	0.05 (0.02) f	0.025 e
pepsin and pancreatin digested fried whole egg	0.02 (0.02) f	0.009 f

[a] Given values are means of duplicate trials of four independent determinations; values presented in parenthesis are standard deviations; means in the same column with different letters are significantly different ($p < 0.05$).

uses in Northern American; however, there is limited information available if consumption of eggs may have health implications due to the production of bioactive peptides in the gut-tract. Several ACE inhibitory peptides were identified from crude egg white after digestion with pepsin (*16*), but the effects of cooking methods on the production of peptides under simulated gastrointestinal conditions have not been reported. Therefore, the objectives of this study were to investigate the effects of cooking methods (boiling and frying) on the production of ACE inhibitory peptides in a simulated digestion model system and to characterize the potent peptides from the digested egg samples.

MATERIALS AND METHODS

Materials. Fresh white-shell eggs were obtained from Poultry Research Centre farm of the University of Alberta (Edmonton, AB, Canada). To make boiled eggs, fresh eggs were placed in a single layer in a saucepan and were covered with at least one inch of water over the top of the shells; after boiling for 10 min, the eggs were placed under running water for 5 min and peeled; egg white and egg yolk were separated or used as whole egg. To make fried egg samples, homogenized whole egg, manually separated fresh egg white and yolk were prepared individually in the frying pan (preheated to 350 °F) for 80 s, 40 s for each side. After cooling, all egg samples were frozen immediately at −20 °C till further *in vitro* digestion. Angiotensin converting enzyme (ACE, from rabbit lung), pepsin (porcrine gastric mucosa), pancreatin (porcine pancreas), and hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma (Oakville, ON, Canada).

Digestion of Egg Samples. Each individual egg sample was prepared into a 5% (w/v, dry weight) slurry in distilled water. After heating the sample slurry at 80 °C for 10 min, the temperature was adjusted to 37 °C by putting it into an ice bucket, and the pH was adjusted to 2 by adding 1 N HCl or 1 N NaOH. After stabilization, the sample slurries were first digested by pepsin (4%, w/w of protein) for 3 h and then the pH was increased to 7.5 to inactivate the enzyme by adding 1N NaOH solution; half of the sample was withdrawn from the slurry as the pepsin digest, and another half was subjected to further pancreatin (2%, w/w of protein) digestion for another 3 h. The hydrolysis was terminated by raising the temperature to 95 °C and maintaining it for 10 min; the digests were then freeze-dried without further centrifugation separation to mimic the conditions in the gut. All the digestions were carried out through Titrando (Metrohm, Herisan, Switzerland) for maintaining constant pH during the course of the hydrolysis. The temperature of the sample during digestion was maintained with a circulating water bath. Each digest was prepared individually in duplicate.

ACE Inhibitory Activity. Lipids in the egg yolk and whole egg digests were removed by hexane extraction prior to ACE determination. Hexane was added at a ratio of (4:1), and then the sample was shaken for 1 h. Then the extract was filtered by using Watman no. 5 filter paper, and the process was repeated one more time. Then the residues were dried overnight in a fume hood. ACE inhibitory activity was measured according to Cushman and Cheung (1971) (*17*). For each assay, 25 μL of sample hydrolysate and 125 μL of 5 mM HHL solution (all prepared in 100 mM potassium phosphate buffer containing 300 mM NaCl) were incubated at 37 °C for 5 min. Then, 10 μL of ACE (prepared in buffer) was added and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 0.35 mL of 1 N HCl and mixed. To each tube, 1.5 mL of ethyl acetate was added and mixed thoroughly; after centrifugation at 3000*g* for 5 min at room temperature, 1 mL aliquot of each ethyl acetate layer was transferred to a tube and evaporated in a hot air oven at 120 °C for 30 min. The dried hippuric acid was redissolved in 1 mL of pure water, and the absorbance was determined at 228 nm. The IC_{50} value was defined as the concentration of the peptides that can inhibit 50% of the enzyme activity. Each assay was performed in duplicate of four independent determinations.

Proximate Analysis. The moisture and ash contents of the digests were determined by the AOAC method (*18*). Fat content was estimated using the Goldfish extraction method and expressed as grams per 100 grams (dry weight basis) of sample. Protein content was determined using a TruSpec CN carbon/nitrogen determinator (Leco Corp., St. Joseph, MI) and multiplying the nitrogen content by a factor of 6.25.

Purification of ACE Inhibitory Peptides. Defatted, fried whole egg digest with pepsin and pancreatin was dissolved in 10 mM ammonium acetate (pH 4) buffer. After passing through the molecular weight cutoff 3000 ultrafiltration membrane, 2 mL of the permeate was loaded onto a HiPreP 16/10 SP FF cation exchange column (16 mm × 100 mm, GE Healthcare Sweden) coupled to an ÄKTA explorer 10XT system. The column was equilibrated with 10 mM ammonium acetate (pH 4) and eluted with 0.5 M ammonium carbonate buffer at a flow rate of 5 mL/min. Fractions were collected and freeze-dried for the ACE inhibitory assay. The most active fractions from cation exchange column were dissolved in 20% ethanol and further purified by size exclusion chromatography using a Superdex peptide 10/300GL column (10 mm × 300−310 mm, GE Health care Sweden). The sample injection volume was 500 μL and eluted over 1.5 column volumes. The fractions were collected, lyophilized, and analyzed for ACE inhibitory activity; the highest ACE inhibitory fraction peak was analyzed by liquid chromatography mass spectrometry for sequence identification.

Liquid Chromatography−Mass Spectrometry (LC-MS). Identification of the peptides present in the fraction was carried out by liquid chromatography mass spectrometry (LC-MS). The analysis was carried out in a Waters (Micromass) Q-TOF Premier instrument (Milford, MA), and the sample was separated by a Waters Atlantis dC18 (75 μm × 150 mm, 3 μm) UPLC column (Milford, MA). The system solvents were as follows: solvent A = 0.1% formic acid in optima LC/MS grade water, and solvent B = 0.1% formic acid in optima grade acetonitrile. Samples were dissolved in solvent A; then 5 μL of sample was injected to the 5 μm trapping column. Sample was trapped for 2 min at a flow rate of 10 μL/min using 99% solvent A, followed by a gradient from 99% A to 90% A over 5 min, to 70% A over 30 min, to 60% A over 3 min and to 5% A over 1 min at a constant flow rate of 0.350 μL/min. Then the flow rate was increased to 0.500 μL/min, held at 5% A for 2 min, increased to 98% A over 1 min, held for another 27 min, and then decreased to 0.350 μL/min over 1min. Ionization was performed by the electrospray ionization technique (ESI) with a nanoLockspray ionization source in a positive ion mode (capillary voltage 3.80 kV and a source temperature of 100 °C). Peptide mass was detected with a Q-TOF analyzer operated in a positive ion MS/MS mode. A full-scan MS/MS was performed for each sample with an acquisition range *m*/*z* 50−1990 Da. The peptides were then characterized by using a Peaks viewer 4.5 (Bioinformatics Solutions Inc., Waterloo, ON, Canada) and the peptide sequences were identified

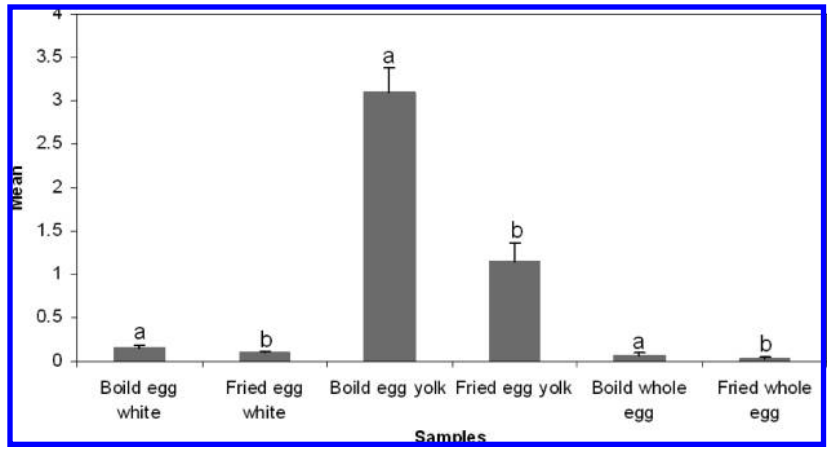


Figure 1. Effects of cooking methods on the IC_{50} values; bars with different letters are significantly different within the same type of egg sample (e.g., egg white, egg yolk, or whole egg) ($p < 0.05$).

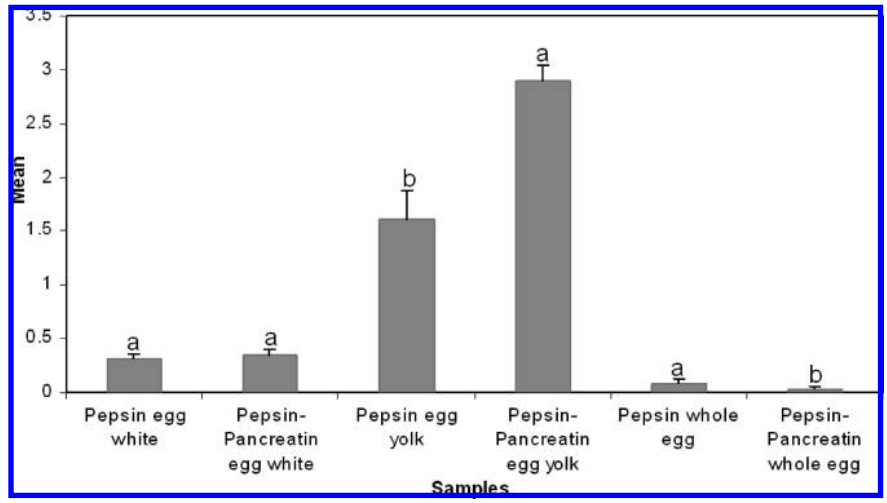


Figure 2. Effects of proteases on the IC_{50} values; bars with different letters are significantly different within the same type of egg sample (e.g., egg white, egg yolk, or whole egg) ($p < 0.05$).

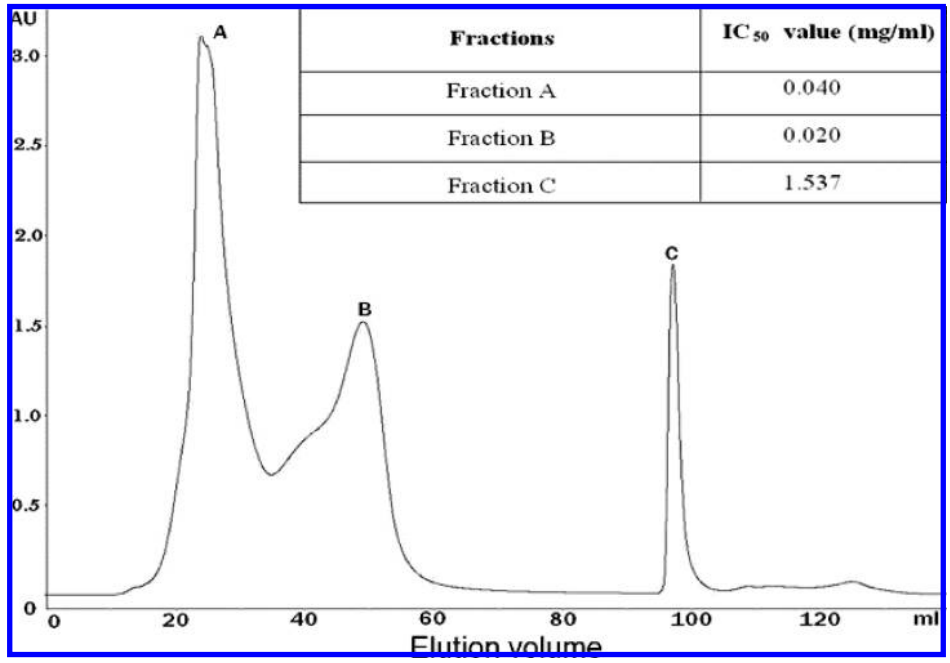
from respective monoisotopic mass. The IC_{50} values of the identified peptides were predicted by recently developed QSAR models (*19, 20*) using the SIMCA-P version +11 (Umetrics Inc., Kinnelon, NJ).

Statistical Analysis. A pdmix model was used to analyze the differences in different cooking methods for all the data using Statistical Analysis System Software, SAS version 9.0 (SAS Institute, Cary, NC); alpha (α) was taken as 0.05, and the p value <0.05 was taken as an indicator of significant difference. One way ANOVA was used to detect the differences among the various treatments.

RESULTS AND DISCUSSION

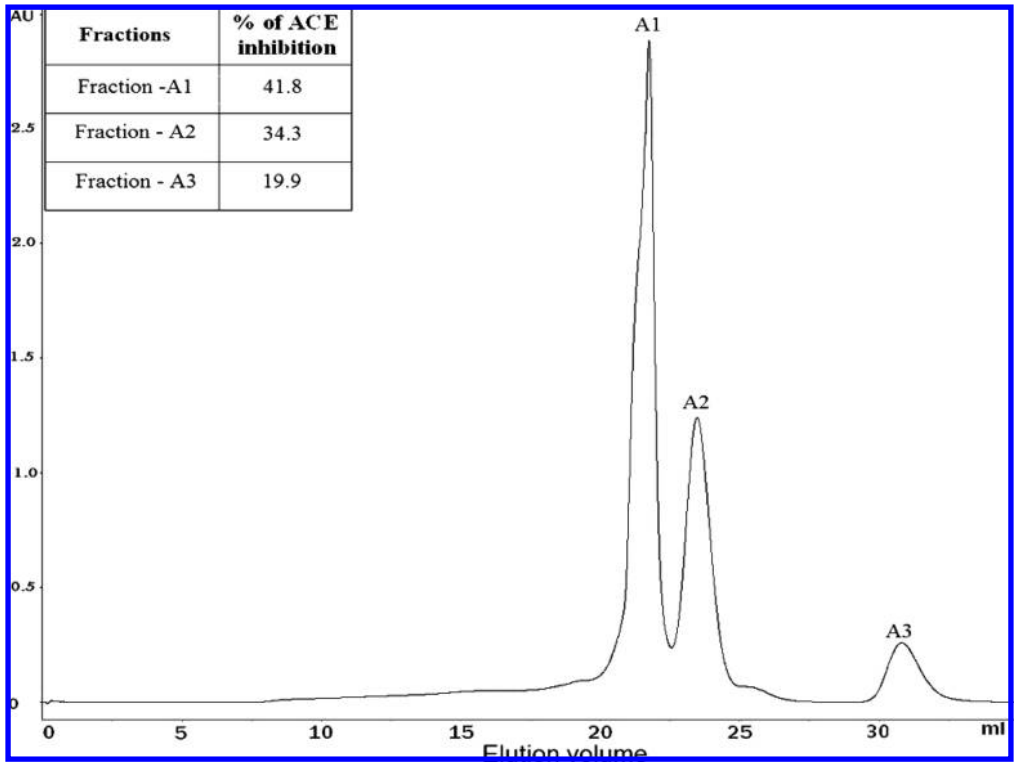
ACE Inhibitory Activities of Digests. The ACE inhibitory activity of various samples was shown in **Table 1**. To simulate the condition in the gut, our digests were prepared without further centrifugation, although centrifugation is widely used in preparing protein hydrolysates to remove large peptides or undigested proteins. Our results showed that IC_{50} values ranged from 0.02 to 3.55 mg dry weight/mL or 0.009−1.118 mg protein/mL. Boiled egg yolk showed the weakest activity whereas the fried whole egg showed the strongest inhibitory activity; as shown in **Figure 1**, fried egg samples had significantly lower IC_{50} values than those of boiled egg samples in all the treatments. Fried eggs were prepared at a higher temperature (170 °C) than that of boiled eggs (100 °C), which could affect the release of bioactive peptides; moreover, proteins in the frying process experienced nearly identical thermal treatment whereas, in the boiling process, there is a thermal gradient from the outside to the core of the egg where the yolk proteins or the white protein part close to the yolk may have not been extensively denatured, resultant in a lower digestibility and weaker ACE inhibitory activity of boiled egg samples. Our results showed that both whole egg samples showed lower IC_{50} values than those of egg white or yolk digests; possible explanations for this observation are that (*1*) there are synergistic interactions between peptides from egg white and peptides from yolk or (*2*) a mixture of white and yolk would facilitate the digestion of proteins and therefore release more potent peptides than either in white or in yolk. The effects of enzymes on activity are related to the types of substrates. There is no significant difference in the egg white digests; in whole eggs, pepsin−pancreatic digested samples showed significantly lower IC_{50} values than that of pepsin digested samples. Whereas in egg yolk, pepsin−pancreatin digested samples had significantly higher IC_{50} values than those of pepsin digested samples (**Figure 2**); this result implies that potent ACE inhibitory peptides from pepsin digest may be further digested by pancreatin.

Miguel et al. (*16*) reported that crude egg white after hydrolysis with pepsin had an IC_{50} value of 55.3 μg/mL (0.05 mg/mL), compared with the value of 0.142 mg protein/mL in boiled egg white and 0.063 mg protein/mL in fried egg white. The IC_{50} value of 0.009 mg protein/mL in fried whole eggs was compared favorably to previous reported results, typically ranging from 0.015 to 2.53 mg protein/mL (*21*−*23*). *In vitro*



Fractions	IC_{50} value (mg/ml)
Fraction A	0.040
Fraction B	0.020
Fraction C	1.537

Figure 3. Cation exchange chromatogram of whole egg pepsin−pancreatin digest. The inset table is the IC_{50} values of the collected fractions which were denoted as fractions A, B, and C.



Fractions	% of ACE inhibition
Fraction -A1	41.8
Fraction - A2	34.3
Fraction - A3	19.9

Figure 4. Gel filtration chromatogram of fraction A from the cation exchange chromatogram in Figure 3. The inset table is the ACE inhibitory percentage values of the collected fractions which were denoted as fractions A1, A2, and A3.

digestion of soybean protein with pepsin and pancreatin was reported to have an IC_{50} value of 0.28 mg/mL, which was almost 30 times less potent than those for pepsin and pancreatin digested whole egg (*3*). Our results were also favorably comparable to in vitro pepsin−pancreatin stimulated gastrointestinal digestion of fish (Pacific hake fillet) protein with a reported IC_{50} value of 90 μg/mL (0.09 mg/mL) (*24*).

Purification and Identification of ACE Inhibitory Peptide. Fried whole egg pepsin−pancreatin digest, showing the most potent ACE inhibitory activity, was selected for further purification (**Table 1**). Cation exchange chromatography of the sample resulted in three (A, B, C) fractions, as illustrated in **Figure 3**; fraction C showed the weakest activity compared to fractions A and B. The two most potent fractions (A and B) were further fractionated by gel filtration chromatography using a Superdex peptide 10/300GL column; three major fractions were obtained from both fractions A (**Figure 4**) and B (**Figure 5**). The most potent fractions, A1 (41.78% inhibition) and B2 (44.10% inhibition), were analyzed by LC-MS/MS to identify the peptides sequences.

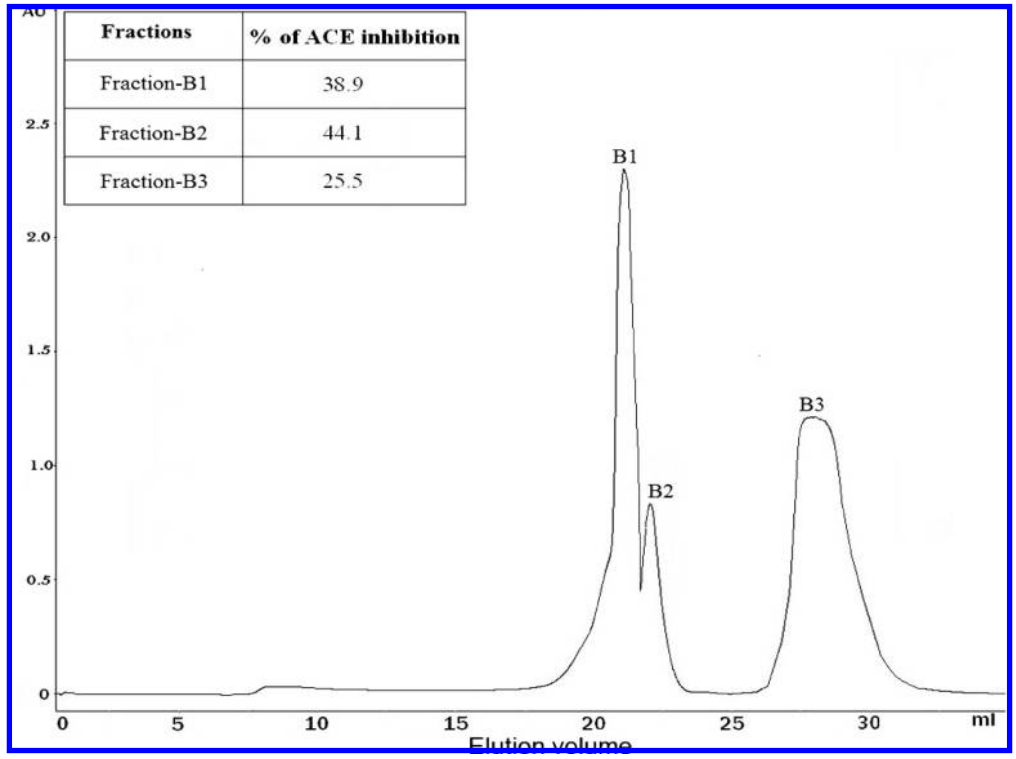


Figure 5. Gel filtration chromatogram of fraction B from the cation exchange chromatogram in Figure 3. The inset table is the ACE inhibitory percentage values of the collected fractions which were denoted as fractions B1, B2, and B3.

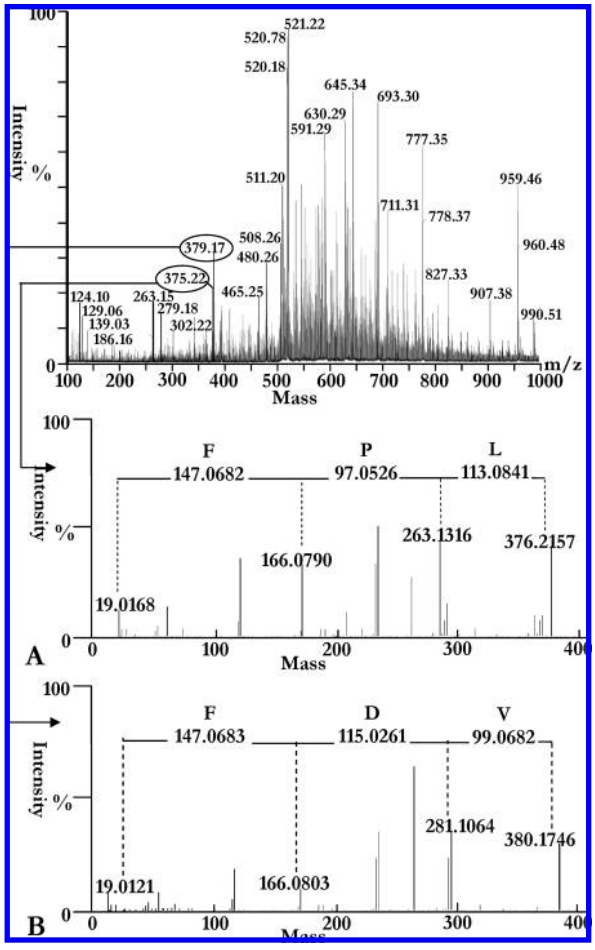


Figure 6. MS spectra of fraction A1. Two representative peptides, LPF (A) and VDF (B), were *de novo* sequenced by using their MS-MS spectra by monoisotopic mass of the amino acids.

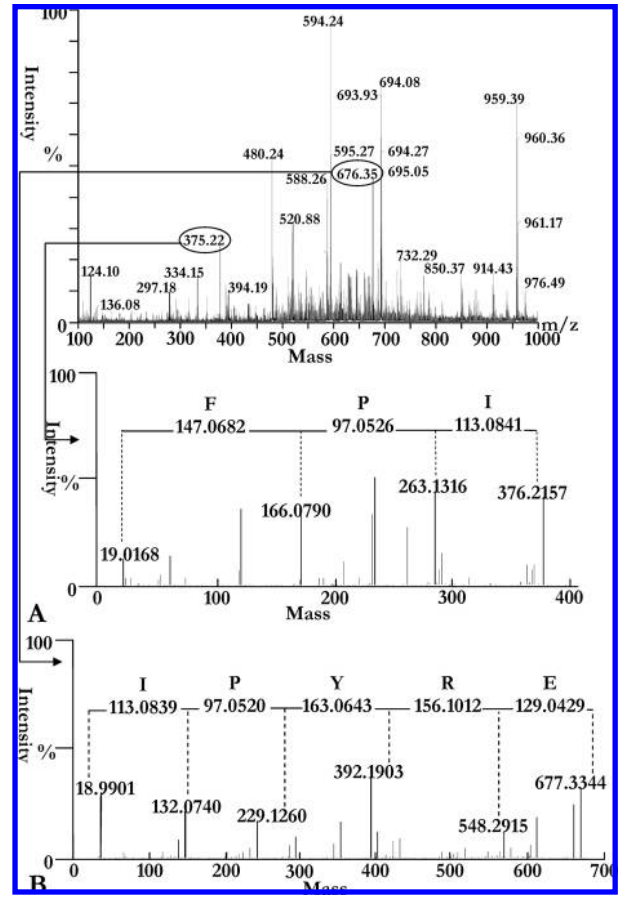


Figure 7. MS spectra of fraction B2. Two representative peptides IPF (A) and ERYPI (B) were *de novo* sequenced by using their MS-MS spectra by monisotopic mass of the amino acids.

Even after two chromatographic separations, the composition of each fraction is very complicated; 52 individual peptides were

Table 2. Representative ACE Inhibitory Peptides Were Identified by LC-MS/MS, and Their Activities Were Predicted by Previously Reported Structure and Activity Models (*19, 20*)

fractions	sequence[a]	calc mass	obs mass M + $[H]^+$	predicted IC_{50} (μM)	parent protein	position
fraction A1	VDF	379.41	380.17	6.59	phosvitin	f105−107
	LPF	375.46	376.21	10.59	ovalbumin	f233−235
	MPF	393.50	397.17	17.98	ovalbumin	f197−199
	YTAGV	509.55	510.24	27.38	livetin	f419−423
fraction B2	ERYPI	676.77	677.35	8.76	ovalbumin	f110−114
	IPF	375.46	376.21	8.78	phosvitin	f96−98
	TTI	333.38	334.18	24.94	ovomucin	f462−464

[a] Amino acid sequences are denoted by single letter code.

identified from fraction A1 (**Figure 6**), and 64 peptides were identified from fraction B2 (**Figure 7**). According to the MS/MS spectrum, all the peptides were identified by *de novo* sequencing. The total mass of the peptide combination was obtained from the mass detector, and then the monoisotopic mass of an individual amino acid was subtracted to identify the exact sequence with an accurate mass. MS/MS spectra of potent peptides were displayed using a single positively charged ion (M + $[H]^+$); the ACE inhibitory activities of these identified peptides were predicted by using our previously reported ACE inhibitory peptide models (*19*, *20*). Representative ACE inhibitory peptides were reported (**Table 2**). It should be noted that both egg white proteins and egg yolk proteins contributed to the production of potent ACE inhibitory peptides. The activities of these new peptides were lower than those of previously identified peptides from ovalbumin, Phe-Phe-Gly-Arg-Cys-Val-Ser-Pro (0.4 μM) and Glu-Arg-Lys-Ile-Lys-Val-Tyr-Leu (1.2 μM) (*6, 25*), but they were comparable to those of two previously identified peptides from egg white pepsin hydrolysate, Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu (4.7 μM) and Arg-Ala-Asp-His-Pro-Phe-Leu (6.2 μM) (*16*). These previously reported peptides have not been identified in our digests, which may be due to the difference in sample preparations, but Glu-Arg-Tyr-Pro-Ile was found to be a fragment of Tyr-Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu reported (*16*). The exceptionally potent ACE inhibitory activity in the fried whole egg digest indicates a possible synergistic effect among various peptides; a peptide synergistic effect has been reported (*30*).

Most of the peptides identified were tripeptides; two tripeptides, Val-Asp-Phe and Ile-Pro-Phe, have IC_{50} values of 6.59 μM and 8.78 μM, respectively. Three tripeptides identified, Leu-Pro-Phe, Met-Pro-Phe, and Ile-Pro-Phe, all contain Pro-Phe at their COOH terminus. Aromatic or branched-chain amino acids contribute to potent ACE inhibition (*26*); isoleucine, alanine, leucine, and methionine residues in the ultimate position were supposed to play an important role in binding to ACE (*27*). Phe, an aromatic amino acid, contributes to the inhibition of the activity of ACE. Our previous structure and activity study of ACE inhibitory peptides has shown the importance of aromatic amino acids at the carboxyl terminus, positively charged amino acids at the middle position, and hydrophobic amino acids at the amino terminus in determining the potency of ACE inhibitory tripeptides (*20*). The identified tripeptides contain aromatic amino acid residues at the carboxyl terminus and hydrophobic amino acid residues at the amino terminus but lack positive amino acid residues in the middle. Therefore, the potency of these peptides is less than that of the previously predicted tripeptide, Leu-Arg-Trp, with the determined IC_{50} value of 0.23 μM (*28*). Our activities were comparable to those of Val-Arg-Pro and Met-Tyr-Tyr, which were reported to have IC_{50} values of 2.2 and 9.6 μM, respectively (*29*). Leu-Pro-Phe was previously reported (*30*) with an IC_{50} value of 40 μM from corn gluten, which was weaker than our predicted value.

It is well accepted that small peptides such as di- and tripeptides have lower osmolarities than free amino acids and may be absorbed more efficiently than either amino acids or intact proteins (*31*). The characterization of several tripeptides from *in vitro* mimic gastrointestinal egg digest indicates a possible high absorption of these peptides to exert *in vivo* antihypertensive activity, although *in vivo* study is needed to confirm this assumption. Our results showed that *in vitro* digestion of cooked eggs could generate a number of potent ACE inhibitory peptides which may have implications for cardiovascular disease prevention, including hypertension. Findings from this study provided further evidence that eggs are an excellent source of health-promoting food. Further research is needed to determine clinically if the consumption of cooked eggs may have blood pressure lowering activity in human subjects.

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