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Angiotensin I Converting Enzyme Inhibitory Peptides from In Vitro Pepsin−Pancreatin Digestion of Soy Protein

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Angiotensin I converting enzyme (ACE) inhibitory activity was determined in the soy protein isolate (SPI) digest produced by in vitro pepsin−pancreatin sequential digestion. The inhibitory activity was highest within the first 20 min of pepsin digestion and decreased upon subsequent digestion with pancreatin. An IC_{50} value of 0.28 ± 0.04 mg/mL was determined after 180 min of digestion, while no ACE inhibitory activity was measured for the undigested SPI at 0.73 mg/mL. Chromatographic fractionation of the SPI digest resulted in IC_{50} values of active fractions ranging from 0.13 ± 0.03 to 0.93 ± 0.08 mg/mL. Although many of the fractions showed ACE inhibition, peptides with lower molecular masses and higher hydrophobicities were most active. The findings show that many different peptides with ACE inhibitory activities were produced after in vitro pepsin−pancreatin digestion of SPI and lead to the speculation that physiological gastrointestinal digestion could also yield ACE inhibitory peptides from SPI.

KEYWORDS: Soy protein isolate; soy peptide; angiotensin I-converting enzyme inhibitory activity; in vitro digestion; pepsin; pancreatin

INTRODUCTION

Soybean is a traditional food in Asia and has been a part of the Asian diet for many years. In 1999, the U.S. Food and Drug Administration approved a health claim stating "diets low in saturated fat and cholesterol that include 25 g of soy protein per day may reduce the risk of heart disease" (*1*). Heart disease is a major health concern in Canada and the United States, because it is the number one leading cause of death (*1*, *2*). Heart diseases, such as arteriosclerosis, coronary heart disease, stroke, peripheral arterial disease, and heart failure, may be caused by hypertension or high blood pressure greater than 140 mm Hg systolic and/or 90 mm Hg diastolic pressure (*3*, *4*).

Hypertension is commonly treated with antihypertensive or blood pressure lowering drugs, such as captopril, benazepril, enalapril, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, and trandolapril (*5*). These drugs are angiotensin I converting enzyme (ACE) inhibitors; they inhibit ACE and subsequently prevent conversion of inactive hormone angiotensin I (DRVYIHPFHL) to active hormone angiotensin II (DRVYIHPF) (*5*−*7*). Angiotensin II raises blood pressure by vasoconstriction as well as stimulation of synthesis and release of aldosterone, a hormone that promotes sodium and water retention in the kidneys and thus increases blood volume in blood vessels. Furthermore, inhibition of ACE prevents degradation of bradykinin, a vasodilator. In addition to ACE inhibitors, other antihypertensive drugs include diuretics, β-blockers, calcium channel blockers, and angiotensin receptor blockers (*8*). However, ACE inhibitors are the most commonly prescribed drugs, because they cause fewer adverse side effects than other antihypertensive drugs (*9*).

Recent research studies have reported discoveries of soy peptides that inhibit ACE in chemical assays as well as rat studies (*10*−*20*). These peptides were produced by fermentation or enzyme digestion of soy protein. Enzymes, such as alcalase, papain, trypsin, pancreatin, *Bacillus subtilis* protease, and pepsin, have been used individually for the hydrolysis of soy (*12*, *13*, *15*, *17*−*19*). However, a combination of pepsin and pancreatin has not been used to investigate if ACE inhibitory peptides will be produced in an in vitro digestion model system with enzymes similar to those in the gastrointestinal digestive system of humans.

Therefore, the objective of this study was to investigate if ACE inhibitory soy peptides would be produced in an in vitro digestion model system using enzymes similar to digestive enzymes in humans. In addition to monitoring the ACE inhibitory activity in the total soy protein digest, the possibility of generating soy peptide fractions with more potent activity than the unfractionated digest was investigated by measuring the activity of fractions obtained after ultrafiltration, anion exchange, reversed-phase, gel filtration, and immobilized metal affinity chromatography (IMAC).

MATERIALS AND METHODS

Materials. Soy protein isolate (SPI, ADM Protein Specialities Division, Decatur, IL) was donated by Hain Celestial Canada Yves Veggie Cuisine (Delta, BC, Canada). Pepsin (2500−3500 units/mg

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protein, catalog no. P-7012), pancreatin (8 × USP, catalog no. P-7545), hippuryl-histidyl-leucine (HHL, catalog no. H-1635), ACE (from rabbit lung, 3.1 units/mg protein, catalog no. A-6778), ACE inhibitor (pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, catalog no. A-0773), captopril (catalog no. C-8856), and L-leucine (catalog no. L-8912) were purchased from Sigma-Aldrich (St. Louis, MO). 2,4,6-Trinitrobenzenesulfonic acid (TNBS, product no. 8746) was purchased from Eastman Kodak Co. (Rochester, NY). Purified bovine lactoferricin (3196 Da) was purchased from the Centre for Food Technology (Hamilton, Australia).

In Vitro Digestion. In vitro digestion was carried out in triplicate according to the method of Garrett et al. (*21*). SPI solution (5% w/v, in distilled, deionized water containing 0.02% sodium azide) was adjusted to pH 2.0 with 1 N HCl, and pepsin (4% w/w, protein basis) was added. The solution was incubated at 37 °C for 1 h before the pH was adjusted to 5.3 with 0.9 M $NaHCO_3$. Pancreatin (4% w/w, protein basis) was added, and the pH was adjusted to 7.5 with 1 N NaOH. The solution was incubated at 37 °C for 2 h and then submerged in a boiling water bath for 10 min to terminate the digestion. The SPI peptide digest was centrifuged at 16000*g* for 10 min, and the supernatant containing soy peptides (58% yield) was collected and stored at −25 °C until used.

For the monitoring of % ACE inhibition at intervals during digestion, aliquots of SPI digest were removed at 0, 20, 40, 60, 90, 120, and 180 min during in vitro digestion. The aliquots were submerged in a boiling water bath for 3 min to inactivate pepsin and pancreatin. The aliquots were cooled and stored at −25°C until used to carry out the ACE inhibitory assay.

Degree of Hydrolysis (DH). DH was analyzed in triplicate according to the method of Adler-Nissen (*22*) and Kwan et al. (*23*) with modifications by Liceaga-Gesualdo and Li-Chan (*24*). Aliquots (1.0 mL) of SPI peptide digest were removed after 30, 60, 120, and 180 min of in vitro digestion. The aliquot was mixed with trichloroacetic acid (1.0 mL, 24%) and centrifuged at 12350*g* for 5 min. The supernatant (0.2 mL) was added to sodium borate buffer (2.0 mL, 0.05 M, pH 9.2) and 2,4,6-trinitrobenzenesulfonic acid (1.0 mL, 4.0 mM) and incubated at room temperature for 30 min in the dark. An aliquot of NaH_2PO_4 (1.0 mL, 2.0 M) containing Na_2SO_3 (18 mM) was added, and the absorbance was measured at 420 nm using a spectrophotometer. DH was calculated as % DH = $(h/h_{tot}) \times 100$, where DH = percent ratio of the number of peptide bonds broken (h) to the total number of bonds per unit weight (h_{tot}) and h_{tot} = 7.75 mequiv/g of soy protein (*22*). L-Leucine was used as a standard in the DH assays.

ACE Inhibitory Activity Assay. ACE inhibitory activity assay was carried out in triplicate according to the method of Cushman and Cheung (*25*) with modifications by Wu et al. (*18*). SPI or digest sample (30 μL), HHL (150 μL, 6.5 mM), and ACE (25 μL, 2.5 mU) were incubated at 37 °C for 1 h. HCl (250 μL, 1 N) and ethyl acetate (1.5 mL) were added, and the mixture was mixed by vortexing and centrifuged at 2000*g* for 5 min. After centrifugation, 1.0 mL of the top layer (containing hippuric acid extracted into ethyl acetate) was taken, and ethyl acetate was evaporated off. The residual hippuric acid was redissolved with distilled, deionized water (1 mL) prior to measurement of the absorbance at 228 nm. The IC_{50} value was defined as the amount of peptide required to inhibit ACE activity by 50%. The % ACE inhibition was defined as the percentage of ACE activity inhibited by a specific amount of peptide. Results were reported as means ± SD. The ACE inhibitory activity of SPI digest during digestion and after 3 h of sequential digestion was based on triplicate in vitro digestion experiments, each assayed in triplicate. Triplicate ACE inhibitory assays were carried out on a single SPI digest that was subjected to various chromatographic fractionation steps. Captopril and ACE inhibitor (pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) were used as standards.

Anion Exchange Chromatography (AEC). The supernatant (45 mL) from in vitro digestion was loaded onto a column (2.5 cm × 20 cm, i.d., Bio-Rad Laboratories Inc., Hercules, CA) packed with DEAE Sephacel anion exchange resin (Amersham Bioscience Piscataway, NJ) and equilibrated in phosphate buffer (0.05 M, pH 7) (Amersham Bioscience). Fractions were eluted using the equilibration buffer containing NaCl (0.2, 0.5, 1, 1.5, and 2 M) at a flow rate of 2.5 mL/min, and the elution was monitored at 280 nm. SPI peptide fractions (1, 1.1, 1.2, 1.3, 2, and 3) were collected. Corresponding fractions from two replicate chromatography runs were pooled. The pooled fractions were lyophilized and stored at −25 °C until used.

Ultrafiltration. Each of the lyophilized soy peptide fractions from AEC was ultrafiltered sequentially using an ultrafiltration unit (Amicon, Millipore Corporation, model 8050, Beverly, MA) through membranes (Amicon) of 10000 and 3000 nominal molecular weight limit (NMWL) under the conditions of 40 psi nitrogen gas and 4 °C. The filtrate was collected, lyophilized, and stored at −25 °C until used.

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC). The lyophilized SPI peptide fraction (1.1) collected from AEC was redissolved into 6 mL of distilled, deionized water, and 50 μL of the fraction was loaded onto a Jupiter300 C-18 reversed-phase column (4.6 nm × 250 nm, i.d., Phenomenex, United States) that was equilibrated in 2% acetonitrile containing trifluoroacetic acid (TFA) (0.05%, v/v) and connected to a HPLC system (Hewlett-Packard Series 1050, Waldbrunn, Germany). SPI peptide fractions were eluted using a gradient of acetonitrile (2−16.5%, v/v, over 60 min, followed by 16.5−100%, v/v, over 20 min) containing TFA (0.05%, v/v) at a flow rate of 0.5 mL/min, and elution was monitored at 214 nm. SPI peptide fractions (1.1.1, 1.1.2, 1.1.3, 1.1.4, and 1.1.5) were collected, and corresponding fractions from 36 replicate chromatography runs were pooled. The pooled fractions were lyophilized and stored at −25 °C until used.

Gel Filtration Fast Protein Liquid Chromatography (GF-FPLC). Lyophilized SPI peptide fractions (1.1.2, 1.1.3, 1.1.4, and 1.1.5) collected from HPLC were redissolved in distilled, deionized water, and 30−250 μL of each fraction was loaded onto a Superdex Peptide 10/300 GL gel filtration column (10 nm × 300−310 nm, i.d., Amersham Bioscience) that was equilibrated in 30% acetonitrile containing TFA (0.05%, v/v) and connected to a FPLC system (Amersham Bioscience). SPI peptide fractions were eluted using acetonitrile (30%, v/v) containing TFA (0.05%, v/v) at a flow rate of 0.2 mL/min, and elution was monitored at 214 nm. SPI peptide fractions were collected, and corresponding fractions from four replicate chromatography runs were pooled. The pooled fractions were lyophilized and stored at −25 °C until used. Phenylalanine (FW 165), lactoferricin (FW 3196 and 3124), and ACE inhibitor (pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro, FW 1101.3) were used as molecular mass standards.

IMAC. Chelating Sepharose Fast Flow resin (Amersham Bioscience) was packed into a column (1.5 cm × 10 cm, i.d., Bio-Rad Laboratories Inc.) and charged with 0.05 M $CuCl_2$ solution. SPI peptide supernatant (17.5−35 mL) from in vitro digestion was loaded onto the column that was equilibrated using 0.02 M phosphate buffer (at pH 7) containing 1.0 M NaCl. SPI peptide fractions were eluted using the equilibration buffer at pH 4 and 3. Elution was monitored at 280 nm. SPI peptide fractions were collected, and corresponding fractions from four replicate chromatography runs were pooled. The pooled fractions were lyophilized and stored at −25 °C until used.

Amino Acid (AA) Composition and Peptide Concentration. Lyophilized SPI peptide fractions were sent to The Advanced Protein Technology Centre at The Hospital for Sick Children (Toronto) for the analysis of AA content after precolumn derivatization based on Waters Pico-Tag HPLC System. The peptide concentration was calculated based on AA analysis results.

Statistical Analysis. Using Minitab Statistical Software (version 13.30), analysis of variance using general linear model and pairwise comparisons with Tukey's method were carried out to compare the IC_{50} values of SPI samples.

RESULTS AND DISCUSSION

DH and ACE Inhibition during in Vitro Digestion. DH increased most rapidly from 60 to 120 min of digestion with pancreatin (**Figure 1**). The hydrolysis tended to slow down during 120−180 min of digestion with pancreatin, as shown by the leveling off effect in DH.

In contrast to the time course of hydrolysis, the inhibitory activity against ACE increased most rapidly from 0 to 20 min of digestion with pepsin and remained at a plateau during the next 20−60 min of digestion with pepsin (**Figure 1**). The

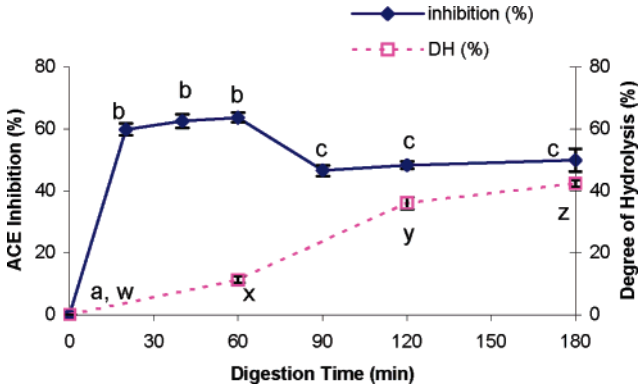

Figure 1. DH (- - -) and ACE inhibition (—) as a function of time course of digestion of SPI with pepsin (0–60 min) and pancreatin (60–180 min). Aliquots of SPI taken during digestion were adjusted to 0.29 mg/mL for the assay for % ACE inhibition. Data points with different letters (a–c for ACE inhibition or w–z for DH) are significantly different ($p < 0.05$).

inhibitory activity against ACE decreased during 60−90 min of digestion with pancreatin and remained at that level upon further incubation until the termination of digestion at 180 min.

These results indicate that the soy peptides generated during pepsin digestion had a greater ACE inhibitory activity than soy peptides after subsequent digestion with pancreatin. The study by Ahn et al. (*12*) observed similar findings with respect to the time course of digestion of defatted soybean peptides with *Bacillus subtilis* protease (40 °C, 6 h). The ACE inhibitory activity of defatted soybean peptides increased most rapidly at the start of digestion from 0 to 1 h, followed by leveling off to a plateau during the remaining 1−6 h. Besides soy protein, in vitro digestion of other food proteins also revealed a similar pattern, in which peptides produced during the initial stages of digestion had a greater ACE inhibitory activity than peptides produced during the later stages of digestion. Moreover, peptides produced during pepsin digestion had a greater ACE inhibitory activity than peptides produced during pancreatin digestion (*26*−*28*).

In the study by Megias et al. (*26*), sunflower protein was digested sequentially with pepsin (37 °C, 3 h) and pancreatin (37 °C, 2.5 h). The sunflower peptides produced after 10 min of digestion with pepsin had a greater ACE inhibitory activity than peptides produced during the remaining pepsin digestion and pancreatin digestion. In another study, commercial fermented bovine milk was digested with pepsin (37 °C, 1.5 h) and then with corolase (porcine pancreatic enzymes containing trypsin, chymotrypsin, aminopeptidase, and carboxylpeptidase) for 2.5 h (*27*). The milk peptides produced after 1.5 h of digestion with pepsin had a greater ACE inhibitory activity than the peptides produced during digestion with corolase. Furthermore, Hernàndez-Ledesma et al. (*27*) found that increasing digestion time with corolase led to production of peptides with decreasing ACE inhibitory activity. Another study by Yang et al. (*28*) digested spinach leaf protein at 37 °C using three different methods: digestion with pepsin for 5 h, digestion with pancreatin for 5 h, and digestion with pepsin for 5 h followed by subsequent digestion with pancreatin for 5 h. Yang et al. (*28*) found that spinach leaf peptides produced after pepsin digestion had a greater ACE inhibitory activity than spinach leaf peptides produced after pancreatin digestion or spinach leaf peptides produced after pepsin and pancreatin digestion.

Several studies provide possible reasons for the stronger ACE inhibitory peptides after pepsin digestion. The C-terminal residues of ACE inhibitory peptides play a predominant role in competitive binding to the active site of ACE (*29*). Peptides with hydrophobic and aromatic AAs at the C-terminal are among the most favorable for strong competitive binding to ACE (*29*−*31*). Because pepsin cleaves at the carboxyl end of hydrophobic and aromatic AAs (Phe, Tyr, Trp, and Leu), it results in peptides with hydrophobic and aromatic AAs at the C-terminal (*32*). These peptides bind tightly to ACE at its active site and compete with angiotensin I for occupancy; therefore, ACE cannot bind to angiotensin I to convert it to angiotensin II (*33*). Another study by Gibbs et al. (*34*) found that protease (pepsin) of lower specificity produced more oligopeptides and a higher percentage of bioactive peptides from whey than protease (trypsin) of higher specificity.

Table 1. IC_{50} Values of SPI, SPI Digest, and Collected Fractions[a]

fraction	IC_{50}[b] mg/mL	IC_{50}[b] mM	peptide content[c] (mg)	peptide yield (%)
SPI	ND[d]	ND		
SPI digest	0.28 ± 0.04^{b}	2.5 ± 0.4^{b}	2896	100
	anion exchange			
1 filtrate	0.26 ± 0.02^{b}	2.3 ± 0.2^{b}	2030	70
2 filtrate	ND	ND	738	25
1.1	0.21 ± 0.02^{ab}	1.9 ± 0.2^{ab}	1569	54
1.2	0.93 ± 0.08^{d}	8.3 ± 0.7^{d}	230	8.0
1.3	ND	ND	107	3.7
	RP-HPLC			
1.1.1	0.60 ± 0.02^{c}	4.5 ± 0.2^{c}	764	26
1.1.2	0.24 ± 0.03^{ab}	1.8 ± 0.2^{ab}	220	7.6
1.1.3	0.13 ± 0.03^{a}	1.1 ± 0.3^{a}	67	2.3
1.1.4	0.31 ± 0.02^{b}	2.5 ± 0.2^{b}	69	2.4
1.1.5	0.14 ± 0.01^{a}	1.2 ± 0.1^{a}	368	13
captopril	7.2 ng/mL	33 nM		
ACE inhibitor	455 ng/mL	413 nM		

[a] Values with different letters are significantly different ($p < 0.05$). [b] The IC_{50} value was calculated from total AA content (picomoles or nanograms) from AA analysis. [c] The peptide content was calculated from AA analysis and volume of the digest and collected fractions. [d] ND = not determined, 0% ACE inhibition at 0.73 mg/mL of SPI, 0.66 mM of fraction 2 filtrate, and 1.9 mM of fraction 1.3.

ACE Inhibitory Activity of Soy Peptides. The SPI digest collected after 180 min of digestion had an IC_{50} value of 0.28 ± 0.04 mg/mL (2.5 ± 0.4 mM), whereas 0.73 mg/mL of undigested SPI (collected at 0 min) demonstrated no inhibition against ACE (**Table 1**). The IC_{50} value of SPI digest found in the present study after 180 min of sequential pepsin−pancreatin digestion is comparable to the IC_{50} values of soy digest reported in other studies using other enzymes. For example, IC_{50} values of 0.34, 0.1964, and 0.73 mg/mL were reported for soy digest collected from alcalase digestion (50 °C, 12 h), *B. subtilis* protease digestion (40 °C, 1 h), and pepsin digestion (39 °C, 12 h), respectively (*12*, *17*, *20*), while Shin et al. (*14*) reported that soy peptides in a commercial fermented soybean paste had an IC_{50} value of 0.2763 mg/mL.

The ACE inhibitory activity of captopril and ACE inhibitor (pGlu-Trp-Pro-Arg-Pro-Gln-Ile-Pro-Pro) were determined and compared to that of soy peptides. Captopril and ACE inhibitor had a stronger ACE inhibitory activity than that of soy peptides. The IC_{50} values of captopril and ACE inhibitor were found to be 7.2 and 455 ng/mL, respectively (**Table 1**), which compares closely to the reported IC_{50} values of 1.3−8.9 ng/mL for captopril (*36*−*39*) and 100−300 ng/mL for ACE inhibitor (*40*).

AEC. After 180 min of in vitro digestion, SPI digest was collected and the supernatant containing digested SPI (soy peptides) was collected. The supernatant was loaded onto an anion exchange column to separate soy peptides on the basis

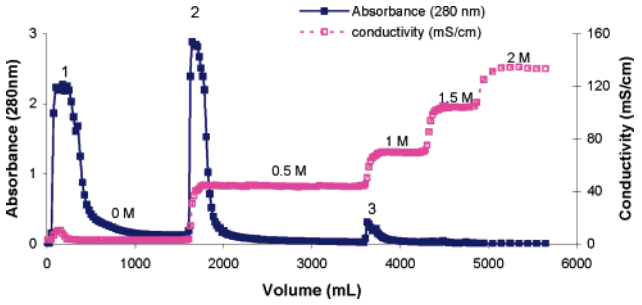

Figure 2. DEAE-AEC profile of SPI digest collected after 180 min of sequential pepsin–pancreatin digestion. Fractions were eluted by stepwise gradient of NaCl (0, 0.5, 1.0, 1.5, and 2.0 M). Fractions 1 and 2 were collected and adjusted to 0.66 mM for ACE inhibition assay. Fraction 1 was also subjected to a second AEC (see **Figure 3**).

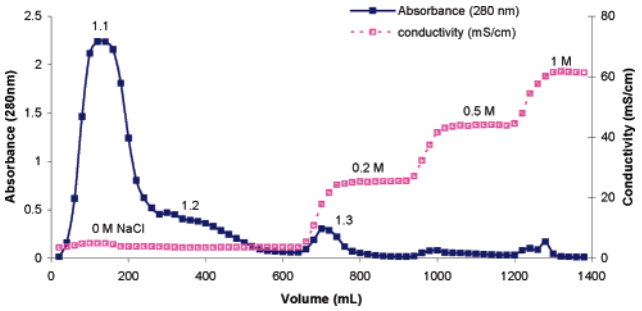

Figure 3. Profile from second AEC of fraction 1.

of charge. Soy peptides eluted from the AEC column as three fractions: fraction 1 eluted at 0 M NaCl, fraction 2 eluted at 0.5 M NaCl, and fraction 3 eluted at 1 M NaCl (**Figure 2**). Each fraction was filtered sequentially through two ultrafiltration membranes with molecular mass cutoffs of 10000 and 3000 NMWL. Each of the fractions passed through the 10000 NMWL membrane, and the collected filtrate also passed through the 3000 NMWL membrane. Subsequently, the filtrate that passed through the 3000 NMWL membrane was collected and adjusted to 0.66 mM and the % ACE inhibitory activity of the filtrate was determined. The fraction 1 filtrate exhibited 30% inhibition against ACE, whereas the fraction 2 filtrate showed 0% inhibition against ACE. The IC_{50} value of the fraction 1 filtrate was 0.26 ± 0.02 mg/mL (2.3 ± 0.2 mM) (**Table 1**). Because of the negligible % ACE inhibitory activity of the fraction 2 filtrate, its IC_{50} value was not investigated.

The fraction 1 filtrate was loaded to the AEC column for a second run through the column, because a large amount of soy peptides eluted in fraction 1. The second run was carried out to confirm that the AEC column capacity had not been exceeded during the first run. As shown in **Figure 3**, the majority of soy peptides in the fraction 1 filtrate eluted at 0 M NaCl, which indicated that the AEC column was not unreasonably overloaded during the first run. From the second run, the fraction 1 filtrate eluted as three fractions: fractions 1.1 (eluted at 0 M NaCl), 1.2 (eluted at 0 M NaCl), and 1.3 (eluted at 0.2 M NaCl) (**Figure 3**). Fractions 1.1 and 1.2 had IC_{50} values of 0.21 ± 0.02 (1.9 ± 0.2 mM) and 0.93 ± 0.08 mg/mL (8.3 ± 0.7 mM), respectively (**Table 1**). Fraction 1.3 at 1.9 mM had no inhibition against ACE; therefore, the IC_{50} value of fraction 1.3 was not investigated.

The IC_{50} values of fractions 1 filtrate, 1.1, and 1.2 were comparable to the IC_{50} values reported in the study by Chen et al. (*19*). In that study, soy peptides from pepsin digestion (37 °C, 24 h) and subsequently eluted from AEC column had IC_{50} values of 0.24 (fraction SP-I) and 1.2 (fraction SP-II) mg/mL.

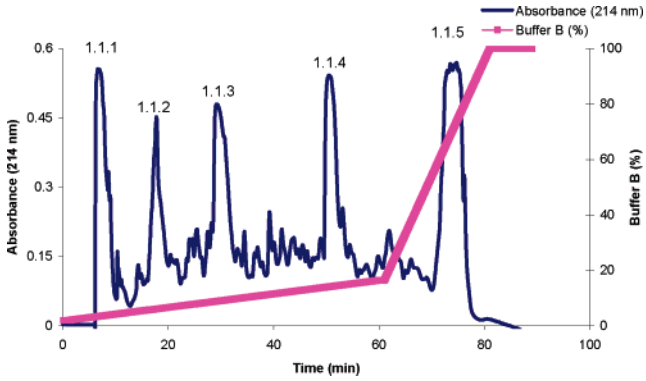

Figure 4. RP-HPLC profile of fraction 1.1 from AEC; fractions were eluted by increasing the concentration of buffer B [acetonitrile containing 0.05% TFA (v/v)].

It is interesting to note that the IC_{50} values of fraction 1 filtrate and fraction 1.1 are similar to the IC_{50} value of the unfractionated SPI digest, while fraction 2 filtrate and fractions 1.2 and 1.3 had no or lower ACE activities than the digest. This indicates that AEC of SPI digest did not yield soy peptide fractions with greater ACE inhibitory activity (lower IC_{50} value) than the digest.

RP-HPLC. Fraction 1.1 from the second AEC was loaded onto a RP-HPLC column to be further separated on the basis of nonpolarity. Fraction 1.1 eluted from RP-HPLC column as five fractions. Fractions 1.1.1, 1.1.2, 1.1.3, 1.1.4, and 1.1.5, eluting at 3.5, 6, 9, 14, and 74% (v/v) acetonitrile, respectively (**Figure 4**), had IC_{50} values of 0.60 ± 0.02 (4.5 ± 0.2 mM), 0.24 ± 0.03 (1.8 ± 0.2 mM), 0.13 ± 0.03 (1.1 ± 0.3 mM), 0.31 ± 0.02 (2.5 ± 0.2 mM), and 0.14 ± 0.01 (1.2 ± 0.1 mM) mg/mL, respectively, as compared to 0.21 ± 0.02 mg/mL (1.9 ± 0.2 mM) for the starting fraction 1.1 (**Table 1**).

The results of this study are contrary to findings reported in other studies, in which fractions with a much greater inhibitory activity were obtained after RP-HPLC. Although fractions 1.1.3 and 1.1.5 had an ACE inhibitory activity that was significantly ($p < 0.05$) greater than that of SPI digest, the activity of these fractions was not significantly greater than the starting fraction 1.1 prior to RP-HPLC, while fraction 1.1.1 had a significantly lower ACE inhibitory activity than the SPI digest as well as the starting fraction 1.1. In comparison, Ahn et al. (*12*) fractionated soy peptides using RP-HPLC and isolated soy peptides (IC_{50} = 26.52 μg/mL) with a greater ACE inhibitory activity than soy peptides (IC_{50} = 58.82 μg/mL) from the preceding hydrophobic interaction chromatography. Shin et al. (*14*) fractionated soy peptides using RP-HPLC and isolated peptides (IC_{50} = 6.8 μg/mL) with a greater ACE inhibitory activity than soy peptides (IC_{50} = 41.8 μg/mL) from the preceding ultrafiltered fraction. Chen et al. (*19*) fractionated a soy peptide fraction using RP-HPLC and isolated four peptides (IC_{50} = 39−153 μM) with a greater ACE inhibitory activity than the preceding fraction (IC_{50} = 0.24 mg/mL) from AEC.

GF-FPLC. Each of the fractions from RP-HPLC, except fraction 1.1.1, was loaded to GF-FPLC column and separated on the basis of size (**Figure 5**). Fraction 1.1.1 was not selected, because it had a lower ACE inhibitory activity than the SPI digest. The eluted fractions were collected; their molecular masses and probable peptide lengths were estimated (**Table 2**). It is interesting to note that peptide fractions from GF-FPLC had low molecular masses, which is consistent with previous studies that also noted peptides with ACE inhibitory activity have low molecular masses (*17*, *41*).

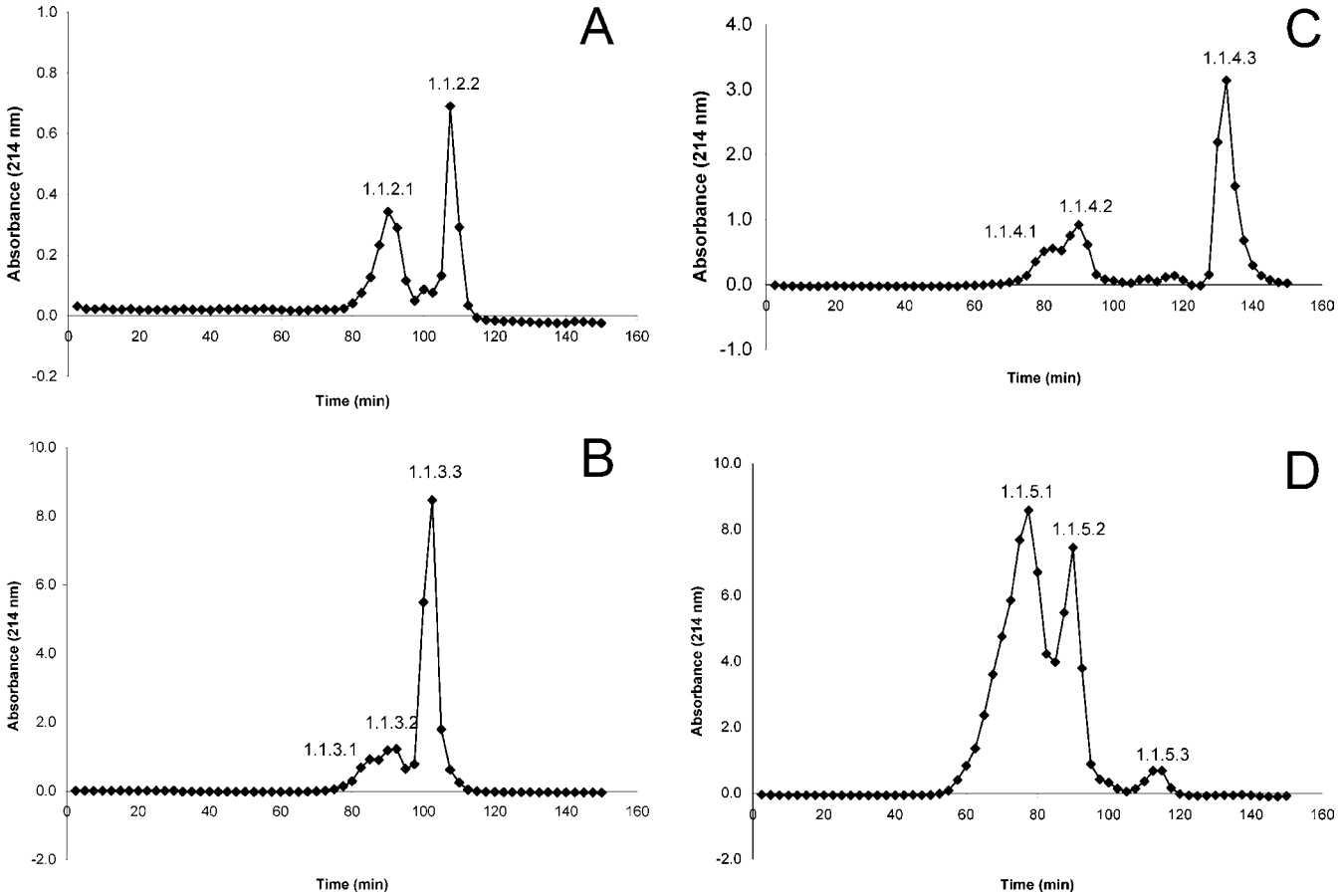

Figure 5. GF-FPLC profiles of fractions 1.1.2 (**A**), 1.1.3 (**B**), 1.1.4 (**C**), and 1.1.5 (**D**) obtained from RP-HPLC of fraction 1. Fractions were eluted by 30% (v/v) acetonitrile containing 0.05% TFA (v/v).

Table 2. Molecular Masses of Fractions Eluted from GF-FPLC

fraction	molecular masses (Da)	probable peptide length (expressed as number of AAs)
1.1.2.1	350 ± 27	2–3
1.1.2.2	<165	1
1.1.3.1	519 ± 0	4–5
1.1.3.2	321 ± 14	2–3
1.1.3.3	<165	1
1.1.4.1	653 ± 27	4–5
1.1.4.2	371 ± 0	2–3
1.1.4.3	<165	1
1.1.5.1	894 ± 53	7–8
1.1.5.2	405 ± 48	3
1.1.5.3	<165	1

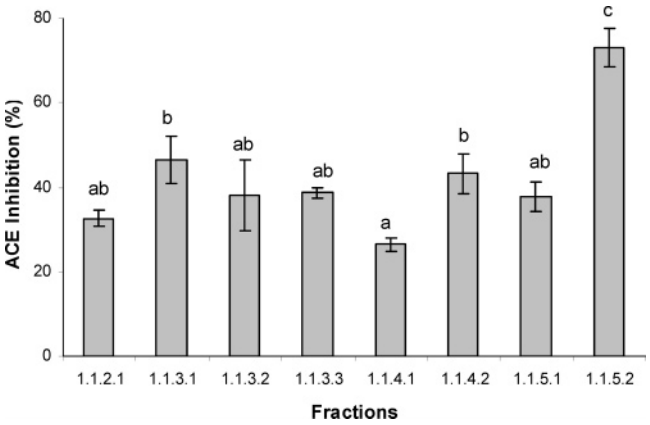

Figure 6. ACE inhibitory activity of fractions from GF-FPLC. (The concentration of soy peptides in each fraction was adjusted to 1.2 mM for the assay.) Bars with different letters are significantly different ($p < 0.05$).

The fractions collected from GF-FPLC were adjusted to 1.2 mM and tested for % inhibition against ACE (**Figure 6**). Fractions 1.1.2.2, 1.1.4.3, and 1.1.5.3 were not tested, because of insufficient peptide concentration; despite the high absorbance at 214 nm of these fractions, their actual peptide content as determined by AA analysis was too low for the ACE inhibition assay. The ACE inhibitory activity of fractions 1.1.4 and 1.1.5, which were collected from RP-HPLC, increased after further separation using GF-FPLC. Fraction 1.1.4.2 had greater ACE inhibition than fraction 1.1.4; the former (at 1.2 mM, collected from GF-FPLC) had 43 ± 5% inhibition against ACE, whereas the latter required 2-fold the amount (2.5 ± 0.2 mM, collected from RP-HPLC) for 50% inhibition against ACE. Fraction 1.1.5.2 also had greater ACE inhibition than fraction 1.1.5; the former (at 1.2 mM, collected from GF-FPLC) inhibited 73 ± 5% of ACE activity, whereas the latter (at 1.2 mM, collected from RP-HPLC) inhibited 50% of ACE activity. The ACE inhibitory activity of GF-FPLC fractions from fraction 1.1.2 and 1.1.3 remained about the same as before GF-FPLC. Overall, out of all of the GF-FPLC fractions, fraction 1.1.5.2 had significantly ($p < 0.05$) the strongest inhibition against ACE and also had greater ACE inhibitory activity than the unfractionated SPI digest. On comparison, fraction 1.1.5.2 had a lower molecular mass than fraction 1.1.5.1 and was more hydrophobic than fractions 1.1.1, 1.1.2, 1.1.3, and 1.1.4. Therefore, the results suggest that peptides with low molecular masses that are hydrophobic may have stronger inhibition against ACE.

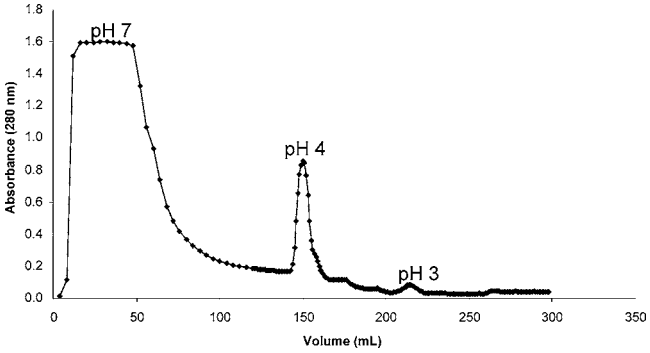

Figure 7. IMAC profile of soy peptide digest collected after 180 min of sequential pepsin–pancreatin digestion. Fractions were collected after elution with phosphate buffer (0.02 M containing 1.0 M NaCl) at pH 7, 4, and 3.

Table 3. AA Composition of SPI Digest and Fractions from AEC

	% mole					
		AEC fractions				
	SPI digest	1	2	1.1	1.2	1.3
Asx (Asp + Asn)	11.80	11.30	20.90	10.10	18.20	12.90
Glx (Glu + Gln)	17.90	16.00	42.10	12.90	29.40	31.60
Ser	6.95	7.09	5.94	7.51	4.46	5.11
Gly	8.43	8.27	7.46	8.60	8.16	8.39
His	2.08	2.10	1.66	2.48	1.61	2.58
Arg	6.04	6.63	2.80	7.44	2.03	3.43
Thr	4.14	4.45	1.55	4.72	4.28	3.96
Ala	6.21	6.44	1.64	7.01	5.19	4.43
Pro	6.50	6.62	6.53	6.45	7.14	8.71
Tyr	2.20	2.33	1.09	2.44	1.79	1.54
Val	5.51	5.67	1.98	6.16	5.03	4.14
Met	1.23	1.20	0.57	1.31	0.97	0.74
Cys	ND[a]	ND	ND	ND	ND	ND
Ile	4.93	4.96	0.49	5.30	4.27	3.98
Leu	7.76	7.96	2.02	8.75	4.77	4.62
Phe	4.11	4.22	0.78	4.18	1.80	2.08
Lys	4.14	4.69	2.53	4.69	0.86	1.79
Trp	ND	ND	ND	ND	ND	ND

[a] ND = not determined.

IMAC. The study by Shin et al. (*14*) reported a soy peptide, His-His-Leu, with strong ACE inhibitory activity of $IC_{50} = 2.2$ μg/mL. Therefore, soy peptide digest from in vitro digestion was loaded onto an IMAC column to isolate histidine-containing soy peptides. Soy digest was collected after 180 min of in vitro digestion and centrifuged to remove undigested SPI. The supernatant containing soy peptides was loaded to the IMAC column to separate soy peptides on the basis of accessible histidine. Soy peptides with available histidine residues would bind to the IMAC column, whereas other soy peptides would pass through in the unbound fraction.

Soy peptides eluted from the IMAC column as three fractions at pH values 7, 4, and 3 (**Figure 7**). The ACE inhibitory assay was carried out on the fractions that eluted at pH 7 and 4, and their IC_{50} values were 0.24 ± 0.03 and 0.53 ± 0.04 mM, respectively. The fraction at pH 7 includes soy peptides that did not bind to the IMAC column. The significantly ($p < 0.05$) lower IC_{50} value of the fraction at pH 7 indicated that soy peptides without accessible histidine had a significantly ($p < 0.05$) greater ACE inhibitory activity than histidine-rich soy peptides (fraction eluted at pH 4). The findings from RP-HPLC and GF-FPLC also showed a similar trend in that the fractions (1.1.3, 1.1.5, and 1.1.5.2) with high ACE inhibitory activity had very low amounts of His, as shown by subsequent AA analysis results in **Tables 3** and **4**. These findings suggested that peptides with accessible histidine may not necessarily have a high ACE inhibitory activity. Other factors, such as AA sequence, may also contribute to the ACE inhibitory activity. For example, the sequence similarity of His-His-Leu to the dipeptide (His-Leu) at the C-terminal of angiotensin I may have aided the binding of His-His-Leu to ACE and thus inhibition against ACE. The ACE inhibitory assay was not carried out for the fraction that eluted at pH 3, because the peak was small and had an insufficient amount of soy peptides.

Table 4. AA Composition of Fractions from RP-HPLC

	% mole				
	RP-HPLC fractions				
	1.1.1	1.1.2	1.1.3	1.1.4	1.1.5
Asx (Asp + Asn)	8.10	3.83	12.10	8.38	9.53
Glx (Glu + Gln)	18.50	8.40	16.00	11.10	10.10
Ser	7.78	4.46	7.67	6.84	6.06
Gly	6.62	5.38	9.59	9.52	10.30
His	3.41	1.35	2.45	2.38	1.61
Arg	19.50	2.52	2.53	2.82	2.44
Thr	3.98	2.88	6.43	5.37	4.84
Ala	10.60	3.92	7.71	5.88	5.39
Pro	1.85	3.15	4.43	11.70	13.90
Tyr	0.08	8.40	1.57	1.59	2.02
Val	6.74	3.48	6.39	10.70	7.19
Met	1.00	0.79	0.70	1.19	0.96
Cys	ND[a]	ND	ND	ND	ND
Ile	0.27	11.50	8.26	7.40	9.61
Leu	0.30	37.18	10.20	6.92	8.85
Phe	0.06	0.10	0.30	3.99	4.78
Lys	11.20	2.64	3.63	4.12	2.41
Trp	ND	ND	ND	ND	ND

[a] ND = not determined.

Table 5. AA Composition of Fractions from GF-FPLC

	% mole							
	GF-FPLC fractions							
	1.1.2.1	1.1.3.1	1.1.3.2	1.1.3.3	1.1.4.1	1.1.4.2	1.1.5.1	1.1.5.2
Asx (Asp + Asn)	3.42	11.20	9.80	0.08	11.00	7.32	9.28	11.10
Glx (Glu + Gln)	8.45	18.60	9.24	0.08	11.60	10.10	10.40	6.77
Ser	5.20	5.31	6.93	0.14	6.90	5.05	5.80	5.44
Gly	5.99	8.94	7.68	0.14	9.82	7.26	10.20	8.44
His	1.60	3.03	1.54	0.00	1.86	2.51	1.84	0.94
Arg	2.55	3.09	1.88	0.02	2.92	2.75	2.49	1.64
Thr	3.13	5.09	5.09	0.09	5.68	4.84	5.08	3.20
Ala	3.74	8.85	4.98	0.06	7.42	5.10	5.41	4.82
Pro	3.18	7.75	1.91	0.03	12.80	10.00	15.50	9.35
Tyr	0.87	0.43	2.13	3.22	0.78	0.10	1.83	2.41
Val	3.92	7.69	3.84	0.00	7.71	14.40	6.59	6.90
Met	1.00	0.81	0.84	0.00	1.01	1.87	1.43	1.30
Cys	ND[a]	ND	ND	ND	ND	ND	ND	ND
Ile	13.40	3.46	13.70	0.43	6.76	8.72	8.72	13.10
Leu	38.80	6.29	22.90	0.74	4.98	9.98	7.76	12.40
Phe	0.51	0.31	4.89	95.00	0.96	7.06	4.01	10.50
Lys	4.24	9.23	2.69	0.00	7.86	2.95	3.67	1.73
Trp	ND	ND	ND	ND	ND	ND	ND	ND

[a] ND = not determined.

AA Composition. SPI digest and soy peptides from AEC, RP-HPLC, and GF-FPLC were analyzed for AA composition. The AA composition of the fractions reflected the type of chromatography used to obtain the fractions (**Tables 3**−**6**). As shown in **Table 3**, much higher amounts of negatively charged AAs (Asp and Glu) were found in the bound fractions (2, 1.2, and 1.3) than the unbound fractions (1 and 1.1) from AEC. **Table 4** showed much higher amounts of hydrophilic AAs (Arg and Lys) in the fraction (1.1.1) that eluted early than the fractions (1.1.2, 1.1.3, 1.1.4, and 1.1.5) that eluted later during RP-HPLC.

Table 6. AA Composition of Fractions from IMAC

	% mole	
	IMAC fractions	
	pH 7	pH 4
Asx (Asp + Asn)	11.10	4.58
Glx (Glu + Gln)	15.80	8.08
Ser	7.48	3.85
Gly	8.93	6.59
His	2.19	16.90
Arg	6.43	13.20
Thr	4.50	3.16
Ala	6.54	3.45
Pro	6.87	9.35
Tyr	2.55	6.11
Val	5.80	3.75
Met	1.19	1.25
Cys	ND[a]	ND
Ile	5.09	3.08
Leu	8.06	7.22
Phe	3.91	6.84
Lys	3.65	2.68
Trp	ND	ND

[a] ND = not determined.

Fraction 1.1.1 also had much lower amounts of certain hydrophobic AAs (Ile and Leu) than fractions 1.1.2, 1.1.3, 1.1.4, and 1.1.5. Moreover, as expected, the bound fraction (pH 4) had much higher amounts of His than the unbound fraction (pH 7) from IMAC (**Table 6**). An interesting finding from **Table 5** showed that although fraction 1.1.3.3 had extremely high amounts of Phe (95%), it had similar ACE inhibitory activity as the other fractions that had much lower amounts of Phe, such as fractions 1.1.2.1 (0.51% Phe), 1.1.3.1 (0.31% Phe), 1.1.3.2 (4.89% Phe), 1.1.4.1 (0.96% Phe), 1.1.4.2 (7.06% Phe), and 1.1.5.1 (4.01% Phe).

In general, the results in **Tables 3−6** show a diverse AA composition among fractions with ACE inhibitory activity, which reflects the findings in the published literature. Other studies that have reported ACE inhibitory peptides have also identified the AA compositions of the peptides as being composed of a variety of AAs (*14*, *17*, *19*, *41*, *43*, *44*). However, the C-terminal residues of ACE inhibitory peptides played a predominant role in competitive binding to the active site of ACE (*29*). Peptides with hydrophobic and aromatic AAs at the C-terminal are among the most favorable for strong competitive binding to ACE (*29−31*).

Overall, in reviewing the literature, soy peptides with ACE inhibitory activity have been reported to contain Gly, nonpolar AA (Ala or Leu), aromatic AA (Phe), polar AA (Gln, Asn or Pro), or negatively charged AA (Asp or Glu) at the carboxyl terminal and Gly, nonpolar AA (Ile or Val), aromatic AA (Tyr or Phe), polar AA (Gln), positively charged AA (His), or negatively charged AA (Asp) at the amino terminal. Val was the most frequently observed AA in ACE inhibitory soy peptides, followed by these AAs in decreasing frequency: Leu, Phe, Asp, Pro, Gln, Gly, Ala, Ile, Asn, Glu, His, Thr, Arg, Met, and Lys.

The results from this study showed that in vitro digestion of SPI with pepsin and pancreatin produced soy peptides with ACE inhibitory activity. This finding suggests the potential production of ACE inhibitory soy peptides upon consumption and digestion of SPI, since the pepsin and pancreatin enzymes used in this study are similar to digestive enzymes in a gastrointestinal digestive system. Inhibitory activity was observed within the first 20 min of pepsin digestion; subsequent digestion with pancreatin resulted in lower activity. The soy peptides produced after 3 h of in vitro sequential digestion with pepsin and pancreatin were all smaller than 3000 Da. Fractionation of SPI digest based on reversed-phase, gel filtration, and IMAC chromatography did result in soy peptide fractions with a significantly greater ACE inhibitory activity than the unfractionated digest. Nevertheless, despite their diversity in AA composition, many of the fractions exhibited ACE inhibitory activity, and no single fraction was isolated with extraordinarily high activity. Nonetheless, peptides with low molecular masses that were hydrophobic had higher inhibition against ACE. In conclusion, these findings suggest that the ACE inhibitory activity after 3 h of in vitro sequential digestion of SPI with pepsin and pancreatin may be attributed to the generation of numerous soy peptides contributing to the overall ACE inhibitory activity of the digest.

ABBREVIATIONS USED

ACE, angiotensin I converting enzyme; SPI, soy protein isolate; HHL, hippuryl-histidyl-leucine; DH, degree of hydrolysis; NMWL, nominal molecular weight limit; RP-HPLC, reversed-phase high-performance liquid chromatography; AEC, anion exchange chromatography; FPLC, fast protein liquid chromatography; GF-FPLC, gel filtration fast protein liquid chromatography; IMAC, immobilized metal affinity chromatography; AA, amino acid.

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