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Identification and Inhibitory Properties of Multifunctional Peptides from Pea Protein Hydrolysate

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Pea protein isolate was hydrolyzed with alcalase, and the hydrolysate passed through a 1 kDa cutoff ultrafiltration membrane. The permeate was freeze-dried and fractionated on a cationic solid-phase extraction (SPE) column. All fractions were tested for their inhibitory activities against angiotensin-converting enzyme (ACE), renin, and calmodulin-dependent phosphodiesterase 1 (CaMPDE). With the exception of the first eluted fraction, inhibitory properties of the SPE fractions against CaMPDE (but not ACE and renin) were directly related to cationic character (residence time on the column). However, the fraction that eluted with 1% ammonium hydroxide (SPE 1%) had the highest peptide yield and was subsequently fractionated using two consecutive rounds of reversed-phase high-performance liquid chromatography to obtain three peaks with major peptides identified as IR, KF, and EF by ultra performance liquid chromatography−tandem mass spectrometry. The three dipeptides showed weak inhibitory properties toward CaMPDE but strong inhibitions (IC_{50} values <25 mM) of ACE and renin. In general, the peptides had higher potency against ACE than against renin. It is indicated from our results that these peptides may be used as potential ingredients to formulate multifunctional food products and nutraceuticals.

KEYWORDS: ACE; bioactive peptides; CaMPDE; pea; renin

INTRODUCTION

In addition to their nutritional contributions, food proteins are increasingly being expected to make contributions in the area of functional foods and nutraceuticals. Bioactive functional properties of proteins determine their performance and effectiveness in the process of influencing human health. Functional properties of proteins can be modified by changing its conformation and structure through physical, chemical, or enzymatic treatment. Particular attention has been devoted to enzymatic hydrolysis because of the more moderate hydrolysis conditions as well as less or no undesirable side reactions and byproducts.

Many studies have demonstrated that enzymatic hydrolysis of food proteins can release peptides exhibiting various bioactivities and lead to improved health status (*1*−*7*). Some of these peptides are even multifunctional (*8*−*10*). Multifunctional peptides can be used to target multiple pathological situations or to offer addictive beneficial effects for one condition. In the human body, blood pressure is regulated by the renin−angiotensin system (RAS), in which renin and angiotensin I-converting enzyme (ACE) are two key enzymes and the final product angiotensin II is the major vasoconstrictor. Many studies have been done to compare the effects of single versus dual blockade of RAS using ACE inhibitors and angiotensin II receptor blockers (ARBs). However, because of incomplete protection provided by ACE inhibitors and ARBs, the most recent emergence of combining renin inhibitors and ACE inhibitors/ARBs may afford a better strategy (*11*, *12*). For example, in a previous study, a flaxseed protein hydrolysate fraction was shown to have in vitro inhibitory activities against ACE and renin (*13*).

Calmodulin (CaM)-dependent cyclic nucleotide phosphodiesterase (CaMPDE) is involved in the degradation of cyclic nucleotides and regulates a large variety of cellular functions. It has been indicated to be involved in many physiological conditions (*14*−*17*). CaMPDE is a good target for therapeutic purposes not only due to its tight connection with various physiological functions at cellular levels but also based on the fact that a larger and quicker change of second messenger concentration can be achieved through regulation of its degradation rather than synthesis (*18*). The aim of this study was to purify and identify multifunctional peptides from pea protein digest with in vitro inhibitory activities against ACE, renin, and CaMPDE.

MATERIALS AND METHODS

Materials. Pea protein isolate (PPI, Propulse) was a gift from Nutri-Pea Ltd. (Portage la Prairie, Manitoba, Canada) and contained 82% protein, <12% carbohydrate, <3% lipid, <4% ash, and 6% moisture, according to the manufacturer's analytical data. Alcalase, ACE (EC 3.4.15.1), hippuryl-histidinyl-leucine (HHL), CaMPDE, and CaM were purchased from Sigma-Aldrich. Renin assay buffer, renin protein, and renin substrate were provided in the Renin Inhibitor Screening Assay Kit purchased from Cayman (Cayman Chemical, Ann Arbor, MI). The cation-exchange system (Bond Elut SCX cartridge, 60 mL column volume) was purchased from Varian (Varian Canada, Mississauga, ON).

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Preparation of Pea Protein Hydrolysate (PPH). PPH was prepared in a reactor with temperature and pH control devices according to a previous method (*19*). PPI was dispersed in Milli-Q water to obtain a 5% (w/v) protein slurry. After the slurry was heated to 50 °C and adjusted to pH 9.0 with 1 M NaOH solution, 4% alcalase (w/w, PPI protein basis) was then added to initiate the hydrolysis. The temperature and pH were maintained at a constant value for 6 h. The hydrolysis was stopped by adjusting the reaction mixture to pH 4.0. The hydrolysate was then centrifuged at 10000*g* for 25 min, the supernatant was further passed through a 1 kDa molecular mass cutoff ultrafiltration membrane, and the resulting permeate (PPH) was collected and freeze-dried.

Solid-Phase Extraction (SPE). SPE was carried out according to the manufacturer's instructions (Varian Canada). The cationic exchange cartridge was activated by adding 40 mL of 99.9% methanol followed by 40 mL of Milli-Q water. PPH was prepared in a 25 mg/mL solution using aqueous 1% phosphoric acid. For peptide separation, first, 40 mL of PPH solution was loaded onto the cartridge and washed with 40 mL of 2% formic acid thrice and then twice with 40 mL of methanol to remove unbound peptides. Second, 40 mL of each elution solvent from lowest to highest gradient (equal amount of 99.9% methanol mixed with 0.5, 1, 1.5, 2, and 5% ammonium hydroxide in Milli-Q water) was loaded sequentially onto the cartridge to elute bound peptides; the eluted fraction from each solvent was collected separately. After the highest gradient (5% ammonium hydroxide), 40 mL of aqueous 30% ammonium hydroxide was added to clean the cartridge. The cartridge was then regenerated for a subsequent run by adding 40 mL of 99.9% methanol followed by 40 mL of Milli-Q water. The five eluted fractions were each collected, solvent evaporated, freeze-dried, and analyzed for their inhibitory activities against ACE, renin, and CaMPDE.

ACE Inhibition Assay. The ACE activity was determined according to the method of Wu et al. (*20*), which was slightly modified as follows. ACE and HHL were prepared with 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl into 20 mU/mL and 4.15 mM, respectively. The final peptide concentration in the reaction mixture was 1 mg/mL. At 37 °C, a 50 μL sample was mixed with 50 μL of ACE and preincubated for 10 min. For the control, 50 μL of borate buffer was used instead of sample. HHL was preincubated at 37 °C for 3 min before 150 μL was added to the reaction mixture and incubated at 37 °C for 30 min. The reaction was terminated after 30 min by adding 250 μL of ethyl acetate and vortexed immediately for 1 min. After centrifugation at 5000*g* for 10 min, 200 μL of the upper layer was transferred into another tube and dried by nitrogen flow. Then, 1 mL of Milli-Q water was added into the tube to dissolve the hippuric acid residue, and absorbance at 228 nm was measured. The percentage of inhibition was calculated as

$$\text{inhibition}\% = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \times 100$$

The inhibitory concentration that reduced ACE activity by 50% (IC_{50}) was determined by nonlinear regression from a plot of peptide concentration versus percentage inhibition.

Renin Inhibition Assay. The renin inhibition assay was performed according to the method of Yuan et al. (*21*) using the Renin Inhibitor Screening Assay Kit. Prior to the assay, renin buffer was diluted with 50 mM Tris-HCl, pH 8.0, containing 100 mM NaCl. The renin protein solution was diluted 20× with assay buffer before use, and the assay buffer was prewarmed to 37 °C before the reaction was initiated in a fluorometric microplate reader (Spectra MAX Gemini, Molecular Devices, Sunnywale, CA) maintained at 37 °C. Before the reaction, (1) 20 μL of substrate, 160 μL of assay buffer, and 10 μL of Milli-Q water were added to the background wells; (2) 20 μL of substrate, 150 μL of assay buffer, and 10 μL of Milli-Q water were added to the control wells; and (3) 20 μL of substrate, 150 μL of assay buffer, and 10 μL of sample were added to the inhibitor wells. The final peptide concentration in the sample reaction mixture was 1 mg/mL. The reaction was initiated by adding 10 μL of renin to the control and sample wells. The microplate was shaken for 10 s to mix and incubated at 37 °C for 15 min, and then, the fluorescence intensity (FI) was recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm. The percentage inhibition was calculated as follows:

$$\text{inhibition}\% = \frac{\text{FI of control well} - \text{FI of sample well}}{\text{FI of control well}} \times 100$$

The inhibitory concentration that reduced renin activity by 50% (IC_{50}) was determined by nonlinear regression from a plot of peptide concentration versus percentage inhibition.

CaMPDE Inhibition Assay. The method of Sharma and Wang (*22*) was used to determine CaMPDE through colorimetric estimation of inorganic phosphate. Because CaM is required for enzyme activation, a mixture of CaM + PPH sample was initially prepared to enhance the inhibitory potential of the peptides. Each of the following reagents was then added to the reaction mixture: 100 μL of buffer (0.36 M Tris-HCl, 0.36 M imidazole, and 0.045 M magnesium acetate, pH 7.5), 20 μL of 4.5 mM $CaCl_2$, 400 μL of Milli-Q water, 30 μL of 10 units/mL 5′-nucleotidase (in 10 mM Tris-HCl containing 0.5 mM magnesium acetate, pH 7.0), 50 μL of 0.31 units/mL CaMPDE, and 200 μL of CaM (50 units/mL)−peptide mixture to give a final peptide concentration of 1 mg/mL. For control, 200 μL of CaM solution (50 U/mL) was added instead of CaM−peptide mixture. All reagents were mixed thoroughly and incubated in water bath at 30 °C for 10 min followed by addition of 0.1 mL of 10.8 mM cyclic adenosine monophosphate to initiate the reaction. The reaction was stopped after 90 min by adding 0.1 mL of 55% TCA. After centrifugation at 5000*g* for 10 min, 0.5 mL of supernatant was transferred into another tube, and 0.5 mL of ammonium molybdate was added followed by 50 μL of reducing agent (12% sodium bisulfate, 1.2% sodium sulfite, and 0.25% 1-amino-2-naphthol-4-sulfonic acid). All components were mixed thoroughly, and absorbance was measured at 660 nm. The percentage of inhibition was calculated as

$$\text{inhibition}\% = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} \times 100$$

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) Purification. In the first step of RP-HPLC purification, the SPE fraction eluted with 1% ammonium hydroxide (SPE 1%) was dissolved in 0.05% (v/v) TFA in water solution at a concentration of 50 mg/mL. The separation was conducted on a Waters Symmetry C18 column (5 μm, 19 mm × 150 mm) coupled with a Waters 7171 plus autosampler, Waters 600 controller, and Waters 2996 photodiode array detector (Waters, Milford, MA). The injection volume was 2 mL, the flow rate was 5 mL/min, and the absorbance was monitored at 210 nm. The fraction was automatically collected every minute by a Waters fraction collector III. After sample injection, the column was eluted with a gradient of 0−60% methanol containing 0.05% TFA within 90 min. HPLC fractions were collected into different pooled fractions, the solvent evaporated, and the aqueous residue was freeze-dried. The eluate was collected into six fractions and analyzed for percent inhibitory activities against ACE, renin, and CaMPDE.

In second round of RP-HPLC, the third fraction from initial RP-HPLC separation (SPE 1%_3) was the most abundant and was dissolved using 0.05% TFA in water solution at a concentration of 10 mg/mL. The second RP-HPLC separation was conducted on the same Waters HPLC system with the same injection volume and flow rate. The column was eluted at a gradient from 15 to 25% methanol containing 0.05% TFA within 40 min. Three pooled peaks (SPE 1%_3-1, -2, and -3) were collected, solvent evaporated, freeze-dried, and analyzed by liquid chromatography/mass spectrometry (LC/MS) to identify the most abundant peptide in each peak followed by determination of amino acid sequence using ultra performance liquid chromatography−tandem mass spectrometry (UPLC-MS/MS).

Identification of Peptide Sequences. Amino acid sequence analysis was done using a Waters Acquity UPLC coupled with a Waters Quattro Micro API MS/MS system (Waters). Samples were dissolved in Milli-Q water at 1 mg/mL and eluted at 0.2 mL/min through a Waters Acuity UPLC HHS T3 1.8 μm column (2.1 mm × 100 mm) with a gradient of 99.9% methanol increased from 10 to 15, 10 to 20, and 15 to 25% within 15 min for sample SPE 1_3-1, -2, and -3, respectively. The eluate was electrosprayed directly into the mass spectrometer with nitrogen used as the collision gas. Spectra were recorded over the 50−1000 m/z range. The capillary voltage was set to 1.5 kV, the cone voltage was 40 V, and the collision energy was 38 V. The amino acid sequence of each sample was analyzed from the mass of daughter ions and compared with pea protein sequence using search tools available on Expasy (www.expasy.ch/tools/). Predicted peptide sequences were synthesized by GenScript USA Inc. (GenScript USA Inc., Piscataway, United States).

Table 1. Inhibitory Activities of Fractions Obtained from Cationic SPE

concentration of NH_4OH (%)	inhibitory properties (%)[a,b]		
	ACE	renin	CaMPDE
0.5	0.00 ± 0.00 e	0.00 ± 0.00 d	46.9 ± 1.03 c
1	27.27 ± 12.16 c	29.25 ± 0.02 b	19.35 ± 2.39 e
1.5	18.18 ± 3.04 d	17.21 ± 0.01 c	36.40 ± 0.32 d
2	33.34 ± 0.01 b	0.00 ± 0.00 d	52.12 ± 3.38 b
5	45.16 ± 0.05 a	68.53 ± 0.02 a	62.22 ± 1.56 a

[a] 1 mg/mL peptide concentration. [b] Mean values with different letters are significantly different at $p < 0.05$.

Statistical Analysis. Data are means of triplicate determinations and expressed as means ± standard errors of the mean. Data were analyzed using Duncan Multiple Range Test by SAS version 9.2; differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

SPE Fractionation. Five fractions were obtained after cation-exchange SPE, and their inhibitory activities are shown in **Table 1**. Fraction SPE 0.5% had the least positively charged peptides, and SPE 5% had the most positively charged peptides because they eluted with the lowest and highest concentrations of ammonium hydroxide, respectively. Peptide yields (protein contents) as a percentage of bound peptides were 0.42 (22.37%), 38.17 (100%), 3.75 (77.26%), 2.25 (49.17%), and 2.83 (55.58%) for fractions eluted with 0.5, 1, 1.5, 2, and 5% ammonium hydroxide, respectively. The fraction that eluted with 1% ammonium hydroxide (SPE 1%) had the highest yield, which was also higher when compared with a cationic flaxseed protein hydrolysate fraction that was reported to have protein yields between 5.4 and 18.5% (*13*). The ACE inhibitory activity was not detected in fraction SPE 0.5%, but inhibition against ACE ranged from 12% to more than 45% for other fractions (**Table 1**). Generally, ACE inhibitory activities of the SPE fractions were higher with fractions 4 and 5 that eluted late from the column (higher net positive charges). This is in agreement with previous structure−activity study reports that suggested the presence of positively charged amino acid residues contribute substantially to the ACE inhibitory potency of peptides, because positively charged ACE inhibitors can interact with a negatively charged binding site on the enzyme protein (*23*, *24*). Fractions SPE 0.5% and 2% exhibited no inhibitory activity against renin, but moderate to high activities were observed in the other fractions (29.24, 17.21, and 68.53% for fraction SPE 1.0, 1.5, and 5%, respectively; **Table 1**). It is difficult to compare our results with others because there is only one study examining renin inhibition from food protein hydrolysate (IC_{50} values ranged from 1.25 to 2.75 mg/mL for flaxseed protein hydrolysate). The significantly high activity of SPE 5% against renin also suggests that the presence of positively charged amino acid residues may contribute to greater peptide inhibition of renin activity.

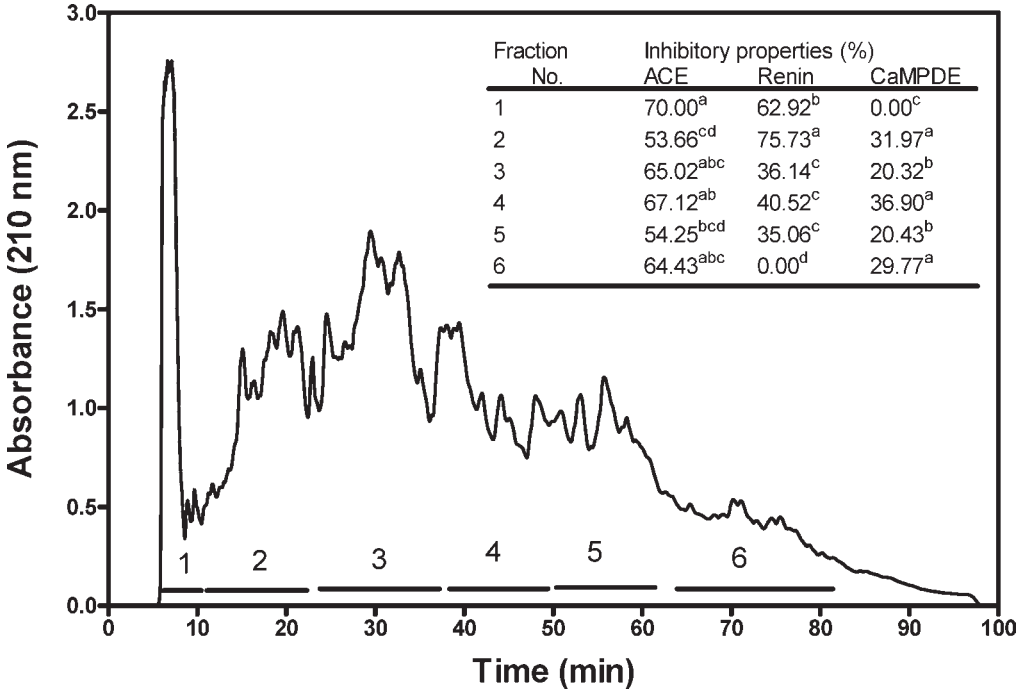
SPE fractions exhibited strong to moderate CaMPDE inhibitory activities (19−62%). With the exception of the first eluted fraction (SPE 0.5%), it seems that inhibitory activity was positively related to residence time of the peptides (high net positive charge density) on the column (**Table 1**). Inhibition of CaMPDE is enhanced through initial interaction of compounds with CaM or Ca^{2+}/CaM complex, which then reduces the level of enzyme activation. A previous study revealed that positively charged compounds could interact with the negative charges on the highly acidic CaM and prevent effective CaM-dependent activation of enzymes (*25*). We have also reported that cationic peptide fractions from pea protein were able to bind CaM and inhibited activity of CaM-dependent protein kinase II (*19*, *26*). A previous report also observed that inhibition of CaM-dependent neuronal nitric oxide synthase was correlated with the degree of positive charge of peptide fractions from flaxseed protein hydrolysate (*27*). Our current results indicate that inhibition of CaMPDE was significantly higher ($p < 0.05$) with the fraction that was eluted last (most cationic property) from the column. Not many studies have reported CaMPDE inhibition by food protein hydrolysate except Kizawa et al. (*28*) who described some peptides isolated from α-casein and shown to inhibit CaMPDE activity. On the basis of the yield and inhibitory properties, SPE fraction 1 (SPE 1%) was used for further peptide isolation. The very low yields of SPE fractions 0.5, 1.5, 2, and 5% made them unfeasible to use for further work.

RP-HPLC Separation of Fraction SPE 1%. Figure 1 shows the RP-HPLC chromatogram of fraction SPE 1% separation. It is shown that most peptides were eluted out around 30 min, and were then separated into six pooled fractions, namely, SPE 1%_1−6. The third fraction, SPE 1%_3, was the most abundant and had high ACE inhibition but moderate inhibitions of renin and CaMPDE. Because the separation was based on hydrophobicity, the later the fractions were eluted from the column, the stronger their hydrophobic properties. Inhibitory activities of SPE 1% subfractions against ACE, renin, and CaMPDE are displayed as an insert in **Figure 1**. Strong ACE inhibitory activities with more than 50% inhibition were observed in all of the fractions. SPE 1%_1 had the highest activity against ACE with 70% inhibition, which was significantly higher ($p < 0.05$) than the value for SPE 1%_2 and _5. Our results are similar to fractions (0.5 mg/mL) separated from mungbean alcalase hydrolysate with 5.36−56.19% inhibition of ACE activity (*29*) and better than HPLC-separated fractions from caprine kefir with 50% inhibition at concentration from 21.8 to 416 mg/mL (*30*). Higher ACE inhibitory activities were reported from six RP-HPLC fractions from peanut flour alcalase hydrolysate with results ranging from less than 5% to about 45% at 45.5 μg/mL (*31*), as well as a number of HPLC-separated fractions of alcalase-hydrolyzed chicken bone protein with 50% inhibitions at peptide concentrations that were much less than the 1 mg/mL used in this work (*32*).

All SPE 1% RP-HPLC fractions except SPE 1%_6 showed strong to moderate renin inhibitory activities from 35.06 to 75.73%. As compared with SPE 1% that inhibited 29.4% of renin activity at the same concentration, each subfraction of SPE 1% exhibited higher inhibitory activities. This may be explained by the fact that each subfraction of SPE 1% was more purified (higher levels of renin-binding peptides) than SPE 1% itself and hence showed stronger activities.

Generally, SPE 1% subfractions displayed similar and relatively low inhibitory activities against CaMPDE. This may be due to the fact that separation was based on hydrophobic properties of the peptides, whereas a strong cationic property is required for effective inhibition of CaM-dependent enzymes (*25*). Better CaMPDE inhibition was observed from two cationic fractions from flaxseed protein hydrolysate with 33.5 and 25% inhibition at 0.1 mg/mL (1 mg/mL was used in the present work) and the cationic peptide fractions from alcalase hydrolysis of egg white lysozyme, which had 26.9−43.6% inhibition (*10*). However, different assay systems and enzyme units were used in these studies.

RP-HPLC Purification of Subfraction SPE 1%_3 and Peptide Identification. Figure 2 shows further separation of the third fraction (SPE 1%_3) from the first RP-HPLC. There were two relatively small peaks at 18 and 23 min each, and the biggest one appeared at 28 min. The three peaks were pooled into fractions SPE 1%_3-1, SPE 1%_3-2, and SPE 1%_3-3 and analyzed by UPLC-MS/MS to identify and obtain amino acid sequence of the most abundant peptide in each peak. **Figure 3a**,**c**,**e**, respectively,



Fraction No.	Inhibitory properties (%) ACE	Renin	CaMPDE
1	70.00[a]	62.92[b]	0.00[c]
2	53.66[cd]	75.73[a]	31.97[a]
3	65.02[abc]	36.14[c]	20.32[b]
4	67.12[ab]	40.52[c]	36.90[a]
5	54.25[bcd]	35.06[c]	20.43[b]
6	64.43[abc]	0.00[d]	29.77[a]

Figure 1. RP-HPLC chromatogram of the peptide fraction (SPE 1%) that was eluted by 1% NH_4OH during SPE. Numbers 1−6 refer to pooled peptide fractions, which were collected, freeze-dried, and tested for inhibitory properties. Fraction SPE 1%_3 was used for further peptide identification. Inset is the inhibitory data at 1 mg/mL peptide concentration.

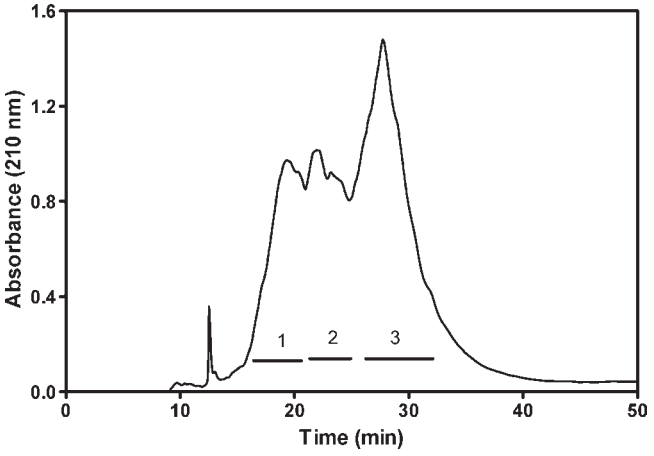


Figure 2. RP-HPLC chromatogram of fraction #3 (SPE 1%_3) from **Figure 1**. For each peak, the most abundant peptide was identified by mass spectrometry followed by chemical synthesis and determination of inhibitory properties that are shown in **Table 2**.

show the UPLC chromatograms obtained for the SPE 1%_3-1, -2, and -3 peaks, respectively. Each peak showed relatively high purity, and the retention times of major peptides in SPE 1%_3-1, -2, and -3 were at 1.63, 2.31, and 3.46 min, respectively. **Figure 3b**, **d**,**f** illustrates the spectrum of three ions with m/z 288.35, 294.31, and 295.34 and amino acid sequences of the identified peptides were IR, KF, and EF, respectively, for SPE 1%_3-1, -2, and -3 as determined by MS/MS.

IC_{50} values of synthesized peptides against ACE and renin are summarized in **Table 2**. IR has the highest inhibitory activity against ACE, and KF showed the lowest activity. As compared with 168 dipeptides with IC_{50} values ranging from 0.002 to 17 mM collected in a database (*33*), IR, KF, and EF showed moderate to strong ACE inhibitory activities. The IC_{50} value for KF in present study is higher than the previously reported values of 0.028 and 0.115 mM (*33*) and, therefore, display lower ACE inhibitory activities. The previous database (*33*) also indicated that IR had an IC_{50} value of 0.696 mM, which is also lower but close to the value obtained in this work. To the best of our knowledge, EF has not been previously reported and was identified for the first time in this work. However, with continuously developed new analytical methods and different experimental conditions in each lab, comparison of IC_{50} values among different studies has become more difficult. Furthermore, the amount of enzyme units used in various laboratories can also considerably affect the ACE inhibitory activities of peptides (*34*). Some reports did not indicate the enzyme units used, and without such information, it is hard to compare peptides in regard to their ACE inhibitory activities.

IR displayed a higher inhibitory activity against renin than KF and EF. There is currently no report of renin inhibitory food protein-derived pure peptides; therefore, it is difficult to obtain data for comparison with findings from this study. Only one study from our group reported flaxseed protein hydrolysates with no to moderate renin inhibitory activities (IC_{50} values ranged from 1.22 to 2.81 mg/mL) (*13*). However, synthesized peptides have been used as renin inhibitors with very low IC_{50} values in nanomolar (*35*−*37*). The amino acid sequence of the three dipeptides suggests that the presence of a bulky or aromatic group at the C-terminal (R or F) and a hydrophobic amino acid at the N-terminal (I) may enhance the potency of the dipeptides against renin activity. However, before any structure−activity relationship of renin inhibitory peptides can be established, more information of the amino acids sequences of other food protein-derived renin inhibitory peptides is needed.

Because inhibitory activities were low (<15%) against CaMPDE, IC_{50} values were not determined. At 1 mg/mL, IR, KF, and EF showed 3.51, 9.52, and 13.33% inhibitions of CaMPDE, respectively. In contrast, some isolated peptides from α-casein pepsin digest and Korean herbs were shown to inhibit CaMPDE activity over the range of 1−50 μM (*28*, *38*, *39*). CaMPDE inhibitory activities of the synthesized dipeptides used in this work were

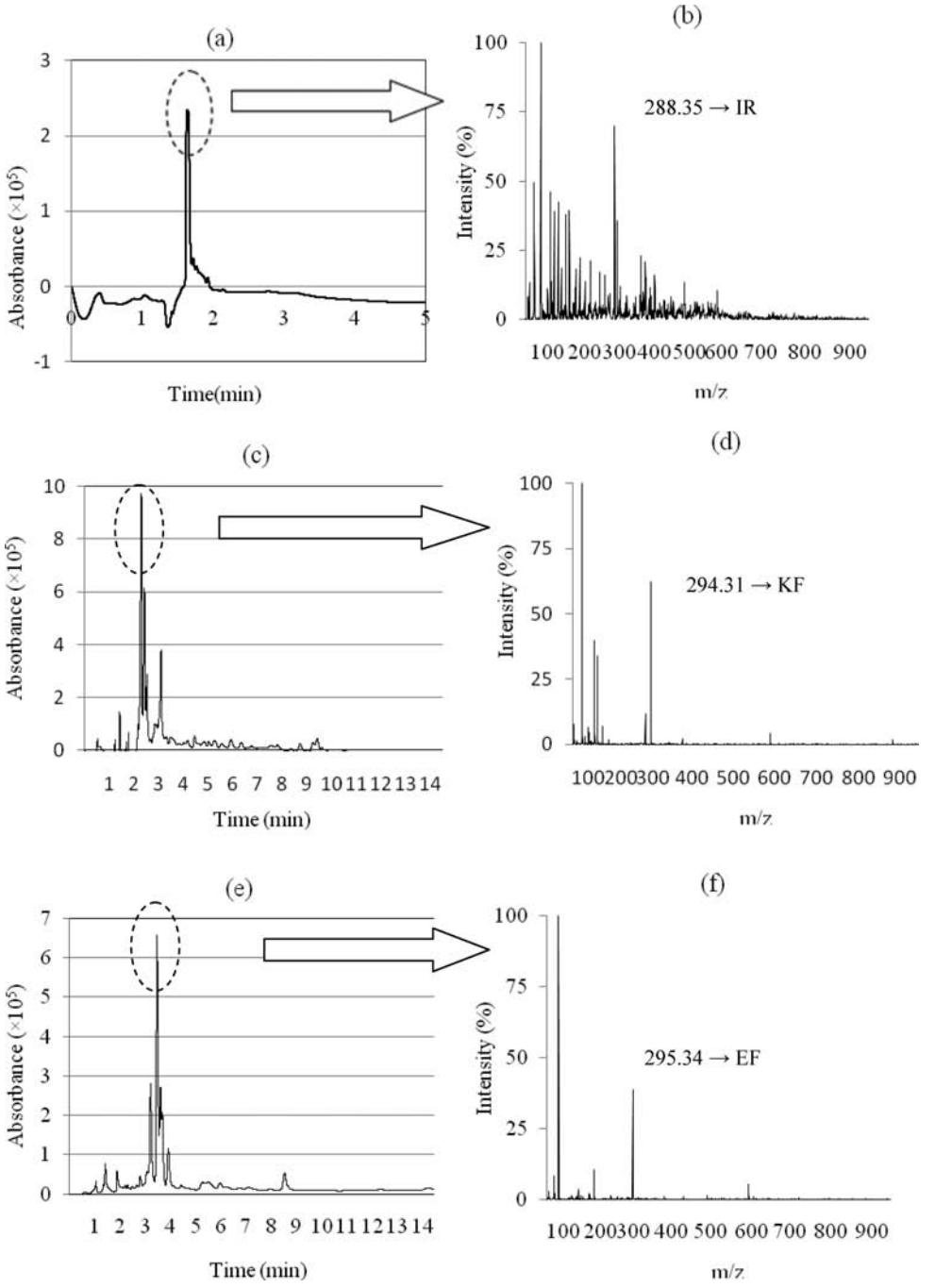


Figure 3. UPLC chromatograms and MS spectra of isolated peptides. (**a**, **c**, and **e**) UV chromatograms of peaks SPE 1%_3-1, -2, and -3, respectively. (**b**, **d**, **f**) MS spectra of three ions with *m*/*z* 288.35, 294.31, and 295.34, found in peaks SPE 1%_3-1, -2, and -3 (**Figure 2**), respectively.

Table 2. IC_{50} (mM) of Synthesized Peptides against ACE and Renin

peptide	ACE	renin
IR	2.25 ± 0.31	9.2 ± 0.18
KF	7.23 ± 0.69	17.84 ± 1.12
EF	2.98 ± 1.24	22.66 ± 1.71

lower when compared with previous results (*28*, *38*, *39*). Comparison between studies can be difficult due to variations in analytical methods and enzyme units that are used for inhibition assays.

This study showed that PPI can be hydrolyzed to produce small peptides with positive charges as evidenced by their ability to bind to a negatively charged SPE matrix. These peptides show various inhibitory activities against ACE, renin, and CaMPDE. Inhibitions of ACE and CaMPDE were positively related to electric charges. Two steps of further separation of fraction SPE 1% by RP-HPLC led to the identification of three multifunctional dipeptides, IR, KF, and EF, with strong inhibitions of ACE and renin but weak inhibition of CaMPDE. It was observed that a hydrophobic residue at the N terminus and a bulky amino acid residue at C terminus was a preferred structural arrangement for renin inhibition by the three synthesized peptides. To the best of our knowledge, this is the first reported identification of renin inhibitory IR and EF from food proteins. The results indicate that these pea protein-derived bioactive peptides could represent potential ingredients for the formulation of multifunctional functional foods and nutraceuticals. Further work is required to determine the in vivo effects of these peptides, especially as positive intervention agents in metabolic disorders.

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