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# Peptides with Angiotensin I-Converting Enzyme (ACE) Inhibitory Activity from Defibrinated, Hydrolyzed Bovine Plasma

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Defibrinated bovine plasma (DBP) was treated with the microbial protease Flavourzyme to obtain protein hydrolysates with various degrees of hydrolysis (DH). The angiotensin I-converting enzyme (ACE) inhibiting activity of the hydrolyzed protein was assessed with hippuryl-His-Leu as the substrate. The amount of hippuric acid released, due to uninhibited ACE activity, was determined by high-performance liquid chromatography. ACE inhibiting (ACEI) activity was found to increase with increasing DH; the 43% DH hydrolysate exhibited the highest activity and had an $IC_{50}$ of 1.08 mg/mL. Peptide fractions with high ACEI activity were isolated using size exclusion chromatography. The fraction that possessed the highest ACEI activity contained peptides with GYP, HL(I), HPY, HPGH, L(I)F, SPY, and YPH sequence motifs, as determined by reversed-phase liquid chromatography−tandem mass spectrometry using a novel immonium precursor-ion scanning technique. Some of these motifs correspond to sequences found in bovine serum albumin, a potential source of ACEI peptides in bovine plasma.

**KEYWORDS: Angiotensin I-converting enzyme inhibitors; bioactive peptides; immonium precursor-ions; LC-MS; MALDI-TOF MS; plasma protein; peptide sequencing; protein hydrolysis**

## INTRODUCTION

Proteins are fundamental and integral food components that provide nutritional and functional properties. Nutritionally they are a source of energy and amino acids that are essential for growth and maintenance. Functionally they affect the physicochemical and sensory properties of protein-containing foods. In addition to the above-mentioned functions, many food proteins possess specific biological activities. These biological activities include antihypertensive, antioxidant, antimicrobial, immune stimulating, mineral binding, and opioid activities (*1*). Such regulatory peptides can be released by enzymatic hydrolysis of proteins in vivo or in vitro and may act as potential physiological modulators that originate from foods. Among the bioactive peptides, the ones possessing antihypertensive activity derived from food proteins have been of particular interest in recent years. This may be partially attributed to the recognition of the relationship between food and health, which is the primary focus of functional foods.

Hypertension is an important modifiable risk factor for cardiovascular disease (*2*) and affects >20% of all Canadian adults (*3*, *4*). Therefore, the search for diet-related preventive measures for hypertension is obviously of interest within the scope of functional foods. Logically, food-protein-derived peptides with such important biological activities have the potential to be used as ingredients in functional or health-promoting foods.

Certain peptides derived from food proteins are capable of inhibiting angiotensin I-converting enzyme (ACE; peptidyl dipeptidase; EC 3.4.15.1), which is part of the renin−angiotensin system (*1*). Inhibition of ACE is considered to be a useful therapeutic approach in the treatment of high blood pressure. Therefore, in the development of drugs to control high blood pressure, the ACE has been an important target for inhibition. Within the enzyme cascade of the renin−angiotensin system, ACE cleaves the dipeptide histidyl−leucine from the C terminus of angiotensin I (an inactive decapeptide) to form the physiologically active octapeptide angiotensin II, one of the most potent vasoconstrictors known (*5*). This potent vasoconstrictor is also involved in the release of a sodium-retaining steroid, aldesterone, from the adrenal cortex, which increases blood pressure. ACE degrades and inactivates bradykinin, a vasoactive

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nonapeptide with antihypertensive properties. Thus, the activity of ACE raises blood pressure by increasing both vascular resistance and fluid volume. Moreover, ACE has been shown to degrade neuropeptides including the enkephalins, neurotensin, and substance P, which may interact with the cardiovascular system (*5*). Accordingly, ACE has a hypertensive effect; thus, it is believed that blood pressure may be suppressed by inhibiting the activity of the enzyme. Most of the oligopeptides that inhibit the ACE have an antihypertensive effect and, as well, may mediate an increase in immunostimulatory and neurotransmitter activities (*6*).

The first publication on peptides with ACE inhibitory activity was from Ferreira and co-workers (*7*). They reported on naturally occurring oligopeptides containing 5−13 amino acid residues per molecule that were isolated from the venom of the snake *Bothorps jararaca*. Most of these peptides had a C-terminal sequence of Ala-Pro or Pro-Pro. Peptide-based ACE inhibitory drugs were developed for clinical use in controlling hypertension since this discovery (*5*). In recent years, other protein-derived ACE inhibitors have been derived from both animal and plant sources. Among these, ACE inhibitors derived from animal proteins, peptides of lactic acid fermented milk (*8*), and hydrolyzed casein (*9*−*12*), whey protein (*6*, *13*−*15*), fish muscle proteins (*16*−*20*), porcine muscle (*21*), and gelatin (*22*, *23*) have been reported. Porcine blood plasma has also been noted to contain ACE inhibiting (ACEI) peptides, both naturally (*24*) and when hydrolyzed (*25*).

In the development of new applications for defibrinated bovine plasma (DBP), hydrolytic products of plasma proteins were studied for ACEI activity. This paper discusses the ACEI activity of hydrolyzed DBP and the amino acid sequences of the ACEI peptides, which have never before been reported. Use of high-performance liquid chromatography (HPLC) to determine ACE activity and a novel liquid chromatography−tandem mass spectrometry (LC-MS/MS) technique to resolve and obtain sequence information for peptides by immonium precursor-ion scanning is also described.

## MATERIALS AND METHODS

**Materials.** Food-grade DBP was obtained from Harimex B.V. (Strathmore, AB, Canada) and used for enzymatic hydrolysis. The microbial enzyme Flavourzyme L was supplied by Novo Nordisk Biochem North America Inc. (Franklinton, NC). All solvents were of HPLC grade and chemicals were of ACS grade or better and were acquired from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada).

**Hydrolysis of Blood Plasma Proteins.** DBP (6.5% crude protein, calculated as %N × 6.25) was thermally treated (90 °C for 20 min) and then hydrolyzed with Flavourzyme (30−110 LAPU/g of protein) for 2−19 h. The number of peptide bonds cleaved was determined spectrophotometrically by using the *o*-phthalaldehyde method and expressed as degree of hydrolysis (DH). Details concerning the hydrolysis procedure and DH determination adapted for DBP have been described elsewhere (*26*). The hydrolyzed plasma so obtained was passed through a 10 kDa molecular weight cutoff membrane (Novocell, Pall Gelman, Mississauga, ON, Canada), and the filtrate was lyophilized to obtain a dry powder.

**ACE Inhibitory Assay.** The activity of angiotensin I-converting enzyme was determined using hippuryl-His-Leu (HHL) as the substrate (*27*) with the following modifications reported by Mehanna and Dowling (*28*). The assay was conducted in a Tris buffer (50 mM, pH 8.3) containing 300 mM NaCl. The same buffer was used to dilute the drug, enzyme, and substrate. The initial assay volume consisted of 50 μL of the substrate (3.0 mM), 50 μL of the enzyme (i.e., ACE from porcine kidney) solution containing 1.25 milliunits of declared enzyme activity, and 50 μL of assay sample. All of these solutions (in a capped glass vial) were incubated for 30 min at 37 °C in a water bath (Fisher Isotemp, Fisher Scientific, Nepean, ON, Canada) without mixing and then for an additional 30 min after mixing. Glacial acetic acid (150 μL) was added to stop ACE activity. The reaction mixture so obtained was used as is to quantitate the hippuric acid produced due to ACE activity on the substrate.

**HPLC Determination of Hippuric Acid Content.** An HPLC method was developed to separate and quantify free hippuric acid in the reaction mixture liberated due to the action of ACE on HHL. A reversed-phase C18 column (Bondclone C18, 5μ, 250 × 4.6 mm) protected by a guard column (Bondclone C18, 10μ, 50 × 1.0 mm; Phenomenex, Torrance, CA) was employed. The mobile phase was an isocratic system consisting of 12.5% (v/v) acetonitrile in deionized water, and its pH was adjusted to pH 3.0 by adding glacial acetic acid. The injection volume used was 10 μL. Elution of hippuric acid was detected by monitoring the absorbance at 228 nm. The HPLC system comprising a solvent delivery system pump (Waters W 600EP, Waters Ltd., Mississauga, ON, Canada), an autosampler (Waters 715 ultra wisp), a system controller (Waters 600) equipped with Millennium software, and a photodiode array UV−vis detector (Waters 996) was used. A series of standard hippuric acid solutions were prepared (25−100 μM) to construct a calibration curve of peak area versus hippuric acid concentration. The control reaction mixture contained 50 μL of buffer instead of the assay sample; the control was expected to liberate the maximum amount of hippuric acid from the substrate due to uninhibited ACE activity. The percent inhibition of enzyme activity was calculated as follows:

$$\%\ \text{inhibition} = \frac{[\text{hippuric acid}]_{\text{control}} - [\text{hippuric acid}]_{\text{sample}}}{[\text{hippuric acid}]_{\text{control}}} \times 100$$

DBP, hydrolyzed to different DH values, and fractions of molecular weight of <10 kDa were assayed for ACE inhibitory activity. Among the hydrolyzed DBP samples the one having the highest percent inhibition was chosen to determine the $IC_{50}$ value. The concentration of hydrolysate needed to inhibit 50% of ACE activity was defined as the $IC_{50}$ value. The clinical inhibitor of ACE, captopril, was used for comparative purposes. All analyses were carried out in triplicate, and results are reported as means ± standard deviation. Data were analyzed using a SPSS program (version 10.05), and the least significant difference (LSD) was used to evaluate significance among means.

**Isolation of ACE Inhibitory Peptides.** The DBP hydrolysate that exhibited the greatest ACEI activity was further studied; its peptides were isolated and sequenced. The selected hydrolyzate was separated on a LC column (20 cm × 2 cm i.d.) packed with Toyopearl HW 40C resin (particle size of 50−100 μm, Tosoh Biosep LLC, Montgomeryville, PA), using distilled water as the mobile phase (flow rate of 3.1 mL/min). The absorbance of the eluent was monitored at 280 and 220 nm (i.e., characteristic UV bands for proteins and peptide bonds, respectively), and fractions were collected over 5 min intervals. On the basis of UV data, fractions were pooled into four major groups (fractions I, II, III, and IV). The ACEI activity of these pooled fractions was determined according to the method previously described. The four fractions were lyophilized for further studies.

**HPLC Separation of Peptide Fractions.** On the basis of the ACE activity assay, those exhibiting the greatest inhibitory activity (fractions I and II) were further separated on a TSKgel G2500PW column (Tosoh Biosep LLC) by employing the HPLC system described in the previous section. A mobile phase of 45% (v/v) acetonitrile containing 0.1% (v/v) trifluoroacetic acid (TFA) was utilized at a flow rate of 0.2 mL/min. The separated peptide bands from the fractions were isolated and further purified by passing through a C18 column to identify their peptide sequence. Molecular mass standards, bovine lung aprotinin (6500 Da), bovine insulin chain B oxidized (3495 Da), human angiotensin II (1040 Da), and Thr-Tyr-Ser (369 Da), were separated on the same column under similar HPLC conditions. From fraction II, the prominent peak, which eluted just after 50 min, was isolated with a preparative column (TSKgel G2500PW, under same HPLC conditions) and used for further studies. The separated peptide bands from the fractions were isolated and further purified by passing them through a C18 column to identify their peptide sequence. Fractions correspond-

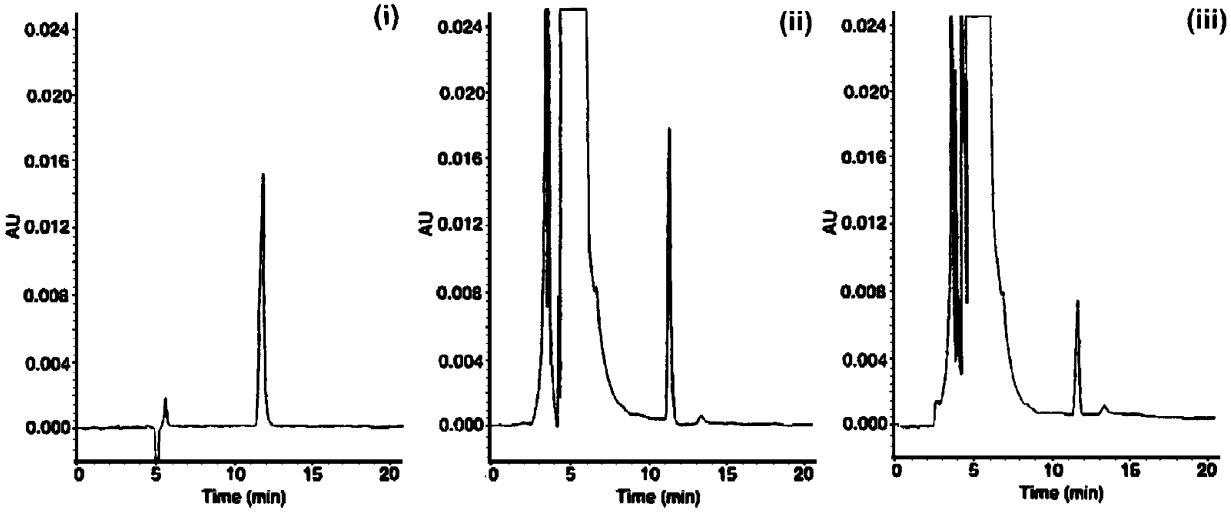



**Figure 1.** HPLC separation of standard hippuric acid (i) and assay mixtures ACE uninhibited (ii) and ACE inhibited with captopril (iii).

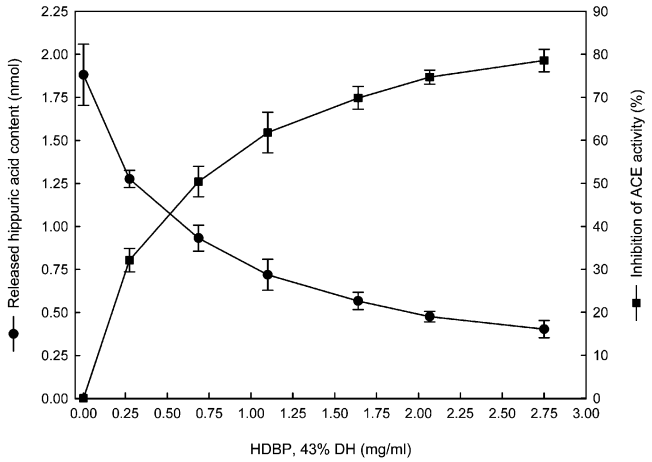



**Figure 2.** Release of hippuric acid and inhibition of ACE activity in the presence of hydrolyzed defibrinated bovine plasma (43% DH, <10 kDa fraction) in the assay mixture.

ing to the resolved LC peaks were collected for subsequent analysis by LC-MS/MS.

**Detection and Sequence Analysis of ACE Inhibitory Peptides.** Fractionation of DBP hydrolysates using size exclusion chromatography (SEC) yielded samples that contained compounds with similar molecular weights but differing chemical properties. To identify peptides in these samples, a novel approach based upon C18 reversed-phase HPLC tandem mass spectrometry (MS/MS) and immonium precursor-ion scanning was employed to provide direct sequence analysis of peptides in complex mixtures (*29*). ACEI fractions were first screened using matrix-assisted laser desorption/ionization-time-of-flight (MALDI-TOF) mass spectrometry to obtain molecular mass information and to identify fractions that may contain target peptides. One microliter of each fraction was mixed with 1 $\mu$L of a UV-absorbing matrix (5 mg/mL $\alpha$-cyano-4-hydroxycinnamic acid in 30:70, v/v, water/acetonitrile) on the MALDI target plate and analyzed using a Voyager-DE STR instrument (Applied Biosystems, Framingham, MA) operating in the positive ion and reflectron modes. Experimental conditions were as follows: accelerating voltage, 20000 V; grid voltage at 72.5%; guide wire voltage at 0.001%; delay, 200 ns; laser power, 2280.

Subsequent LC-MS/MS analysis was performed using an HP1100 solvent delivery system (Hewlett-Packard) and a Quattro LC quadrupole tandem mass spectrometer (Micromass, Manchester, U.K.). Two microliters of sample were loaded onto a C18 column (2.1 $\times$ 100 mm, Genesis, Jones Chromatography, Hengoed, U.K.) using a six-port rotary valve and eluted at 0.25 mL/min using a binary solvent system comprising 12 mM acetic acid in 100% water (solvent A) and 12 mM acetic acid in 90% acetonitrile (solvent B). The solvent program was as follows: 100% A for 0−5 min, 20% A at 25 min, and 0% A at 27 min. The flow rate was increased to 0.35 mL/min at 27.1 min and the solvent composition returned to 100% A by 29 min to recondition the column before the next run.

Compounds eluting from the column were ionized by positive ion electrospray (electrospray ionization; ESI) using the following conditions: ESI capillary, 3.75 kV; HV lens, 200 V; cone voltage, 65 V; skimmer, 5 V; source temperature, 85 °C; nebulizing gas flow, 80 L/h; and drying gas flow, 470 L/h. Use of a high cone voltage induced partial fragmentation of peptides and other compounds in the ESI source. Fragmentation of intact peptides gives rise to b- and y-ions, which correspond to the sequential loss of amino acid residues from the N or C terminus, and immonium ions, which are formed by rearrangement of specific residues cleaved from the peptide backbone. Detection of immonium ions is diagnostic for peptides and for specific amino acid residues within those peptides (*30*). Moreover, coeluting immonium ions provide information on the amino acid composition of individual peptides, provided these are well resolved by HPLC.

Fragment ions can also be generated by collision-induced dissociation (CID) within the tandem mass spectrometer. By using the first analyzer (MS1) as a mass filter, intact molecular (and fragment) ions formed in the ESI source can be transmitted one after another into a collision cell, where CID of these precursor ions results in (further) fragmentation. If the second analyzer (MS2) is set to detect fragment ions of a specific mass-to-charge (*m*/*z*) ratio, the spectrometer will record a mass spectrum containing all precursor ions that produce such fragments. By setting the MS2 to monitor for different immonium ions, it was possible to use this precursor-ion scanning method to resolve peptides containing specific amino acids from other components in the HPLC eluent. This technique also picked out certain peptide fragments formed in the ESI source; moreover, if the immonium ion corresponded to the N- or C-terminal amino acid of the peptide, the precursor-ion spectrum contained predominantly b- or y-ions, respectively. Hence, immonium precursor-ion scanning provided simultaneous detection and sequence analysis for peptides resolved on-line using HPLC. The resulting sequence motifs were compared with the amino acid sequences of bovine plasma proteins recorded in on-line databases (SWISSPROT and PRF) to identify the source of proteins of these peptides.

## RESULTS AND DISCUSSION

Flavourzyme was able to hydrolyze DBP to give different degrees of hydrolysis depending on the enzyme-to-substrate ratio and hydrolysis time employed. Hydrolysis of plasma proteins with Flavourzyme resulted in a maximum of 43% DH at an enzyme concentration of 110 leucine amino peptidase units/g of protein and hydrolysis time of 15.5 h (*26*). At this DH nearly 78% of the molecules had a mass of <1040Da (*26*).

**ACEI Activity of Hydrolysates.** Representative chromatograms of hippuric acid (HA) (i) and uninhibited (ii) and inhibited

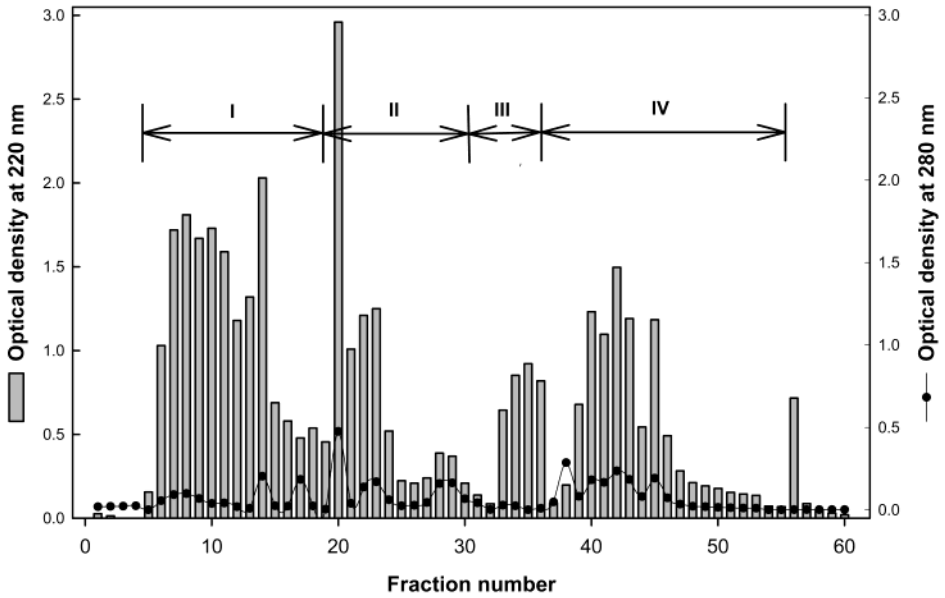



**Figure 3.** SEC of hydrolyzed DBP (43% DH, <10 kDa fraction) on a Toyopearl HW 40C column. Separation was performed at 3.1 mL/min and collected at a fraction volume of 15.5 mL. Solid bars represent optical density (OD) at 220 nm, and the line represents OD at 280 nm. Individual fractions were pooled on the basis of the presence of peptide bonds to obtain four separate groups (fractions I, II, III, and IV).

**Table 1.** Percent Inhibition of Angiotensin I-Converting Enzyme (ACE) Activity by Defibrinated Bovine Plasma (DBP) Proteins and Their Flavourzyme-Hydrolyzed Products

| sample[a] | % inhibition of ACE activity[b] |
|---|---|
| unhydrolyzed DBP | 3.20 ± 1.45a |
| hydrolyzed DBP | |
| 13% DH | 61.50 ± 1.57b |
| 26% DH | 73.07 ± 1.86c |
| 38% DH | 75.72 ± 0.82c |
| 43% DH | 78.93 ± 2.04d |

[a] 2 mg/mL samples were used for the assay, and <10 kDa fraction of hydrolyzed DBP was assayed. [b] Means with different letters are different ($p < 0.05$).

**Table 2.** Percent Inhibition of ACE Activity by Fractions of Hydrolyzed DBP (43% DH, <10 kDa) Separated on a Toyopearl HW 40C Column

| sample[a] | % inhibition of ACE activity[b] | mass recovered from separation[c] (mg) |
|---|---|---|
| fraction I | 62.43 ± 0.45b | 195 |
| fraction II | 63.18 ± 2.73b | 137 |
| fraction III | 70.26 ± 0.78c | 53 |
| fraction IV | 36.59 ± 3.32a | 215 |
| unfractionated (2 mg/mL) | 63.54 ± 1.95b | |
| captopril (5.33 ng/mL) | 57.02 ± 2.92 | |

[a] Separation of fractions refers to *Figure 3*. Mass was determined after lyophilization. [b] Means with different letters are different ($p < 0.05$). [c] 600 mg was applied onto the Toyopearl HW 40C column and eluted with distilled water.

(iii) assay mixtures are provided in **Figure 1**. Under the conditions used for analysis, HA was eluted at 12 min. The assay mixture contained the substrate HHL, which is converted to HA and the dipeptide His-Leu due to the hydrolytic removal of the C-terminal dipeptide by porcine kidney ACE activity. A reduced amount of HA was released and detected when ACE activity was inhibited by the test sample. Separation of HA in the assay mixture using a C18 column with the aforementioned HPLC conditions was achieved without any interferences.

Most of the ACEI studies reported in the literature have utilized the colorimetric method described by Cushman and Cheung (*27*) for quantifying ACE activity. In their method, extraction of released HA (due to ACE activity on HHL) into ethyl acetate and solvent removal to recover HA were necessary steps prior to the determination of the concentration via UV absorbance. Another colorimetric method as described by Matsui et al. (*31*) involves specific binding of 2,4,6-trinitrobenzenesulfonate (TNBS) to the primary amine group of His-Leu; His-Leu is the other hydrolytic product of the substrate HHL. The TNBS−dipeptide derivative has a maximum absorbance at 426 nm. However, if the assay sample is a mixture of peptides, as in the case for the present study, there is a possibility that primary amino moieties of peptides other than those from the His-Leu substrate can react with TNBS. Therefore, the HPLC method described by Mehanna and Dowling (*28*) has a definite advantage over the colorimetric methods by separating one of the products of the enzymatic reaction for direct quantification. The assay is very simple to perform and does not require laborious precolumn separation steps. The standard curve prepared with results of three independent runs at each HA concentration level showed a linear response with a correlation coefficient ($r^2$, $P < 0.05$) of 0.999.

**Table 1** provides percent inhibition of ACE activity by native DBP and its hydrolyzed products of various DH values. According to the values calculated, unhydrolyzed DBP has a minimum or negligible amount of ACEI activity compared to the uninhibited control. According to Hazato and Kase (*24*), ACE inhibitory peptides could be isolated from an 80% (v/v) methanol extract of porcine blood plasma; however, in this study, DBP in its native state exhibited a very low activity against cleavage of the dipeptide by ACE. When DBP was hydrolyzed with Flavourzyme, the ACE inhibitory activity was augmented in the hydrolysate and an increased activity was observed as the DH values improved. At the highest DH (43%) achieved from Flavourzyme hydrolysis (*26*), the greatest ACE inhibitory activity (78.9 ± 2.0%) was detected (**Table 1**). It should be noted that although the study was continued with the 43% DH sample, the other two hydrolysates at 26 and 40% DH also warrant further examination as they exhibited high ACEI activity (**Table 2**). Hydrolysis of proteins by either heat, alkali, acid, or enzyme releases bioactive peptides. Due to the limited reactivity and easily controllable nature, enzymatic

cleavage by proteases has been the most commonly used approach to generate peptides with important bioactivities (*15*).

**Figure 2** presents the ACE inhibitory activity and release of HA as a function of hydrolyzed DBP (43% DH) concentration at a constant substrate concentration of 3 mM. It shows that in the 43% DH, components of the <10 kDa fraction acted as inhibitors of the ACE activity. An $IC_{50}$ value of 1.08 mg/mL was obtained for the 43% DH DBP hydrolysate. This figure also shows that 100% inhibition of the enzyme was not attained even at the highest hydrolysate concentration employed in this study, thereby indicating the possibility of a noncompetitive type of inhibition.

**Peptides with ACEI Activity.** The hydrolysate of 43% DH is predominated with peptides (77.7%) of a molecular mass of <1040 Da (*26*). The Toyopearl HW 40C resin separates molecules on the basis of their size. According to absorbance readings at 220 nm (i.e., for amide bonds), which were stronger than those at 280 nm, four pooled fractions (fractions I, II, III, and IV) were obtained (**Figure 3**). All four fractions exhibited ACE inhibitory activity, and the first two (fractions I and II) exhibited very similar degrees of activity (**Table 2**). Together, these two fractions comprised almost 55% of the recovered mass from the column elution. Fraction III showed a slightly higher inhibitory activity than either fraction I or II but consisted of only a very small mass fraction of the total. Fraction IV had the lowest activity of all.

**Figure 4** depicts the separation of fractions I (chromatogram ii) and II (chromatogram iii) on the TSKgel G2500PW column; this packing material also separates molecules on the basis of their size. Before Toyopearl HW 40C fractionation, the DBP hydrolysate was resolved into several peaks [**Figure 4(i)**]. Fraction I obtained from Toyopearl HW 40C separation was resolved into a similar pattern as the unfractionated hydrolysate during the first 43 min of separation on the TSKgel G2500PW. Fraction II was resolved into two clearly separated bands with one being much more pronounced than the other. The prominent peak (fraction II), which eluted just after 50 min of retention on the column was isolated using a preparative column (TSKgel G2500PW) for further studies. Purification of the peptide fractions was accomplished with a C18 resin before the constituent peptides were sequenced.

**Sequence Analysis and Identification of ACEI Peptides.** Fractions exhibiting in vitro ACE inhibitory activity were first analyzed by MALDI-TOF MS to screen for their peptide content. Molecular ions ($MH^+$) generated by MALDI that have an even *m*/*z* ratio generally contain nitrogen, which has an odd valence (3) but even mass (14). A number of such ions were detected in fraction II (**Figure 5**), thereby suggesting that at least some of the major components (e.g., *m*/*z* 416) were peptides. LC-MS/MS was subsequently performed on this fraction to obtain peptide sequence information. Monitoring the LC eluent for precursors of tyrosyl (Y)-, histidyl (H)-, and prolyl (P)-immonium ions produced chromatograms containing a number of distinct peaks (**Figure 6**). Coelution of H-, Y-, and P-immonium ions at 7.0 min, together with information from the corresponding precursor ion spectra (**Figure 7**), resulted in positive identification of the peptide HPY (*m*/*z* 416). This was also detected in the MALDI spectrum of fraction II (**Figure 5**). Similarly, Y- and P-immonium precursor-ion spectra corresponding to the LC peak at 9.9 min identified GYP (*m*/*z* 336) as a second component (**Figure 8**).

Molecular weight and sequence data for these and other peptides detected in fraction II are summarized in **Table 3**. Peptide **5** (HPY) was sufficiently abundant for its sequence to be confirmed by ESI-MS/MS analysis of the unseparated fraction; however, none of the other peptides could be detected without LC separation. In total, the amino acid sequences of two dipeptides (peptides **2** and **6**), four tripeptides (peptides **3**, **4**, **5**, and and **8**), and one tetrapeptide (peptide **7**) were determined, with molecular masses ranging from 268 to 427 Da. Free histidine was also detected in fraction II from the LC-MS/MS method.

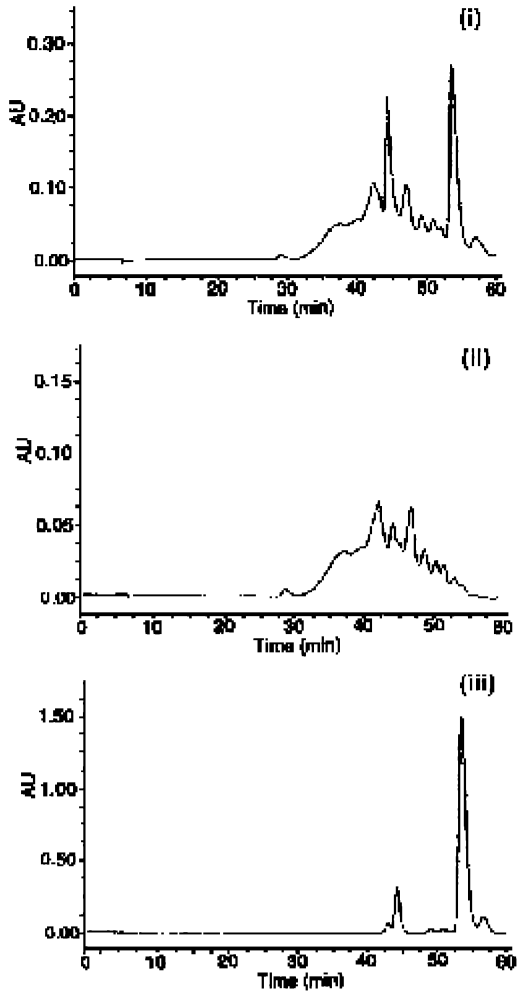
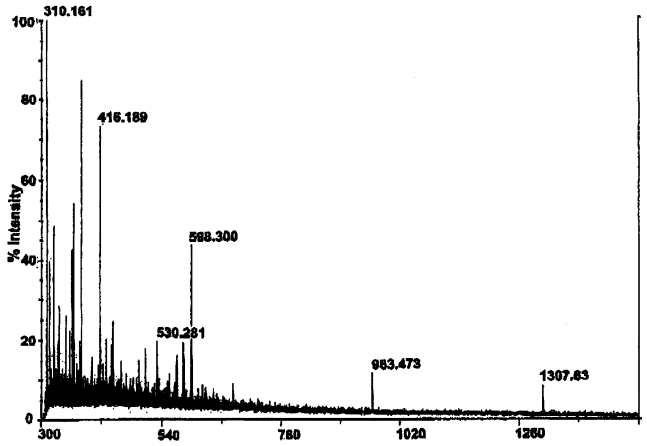



**Figure 4.** HPLC chromatograms for separation of DBP (43% DH, <10 kDa fraction) (i) and pooled fractions from SEC on Toyopearl HW 40C, fraction I (ii) and fraction II (iii) using a TSKgel G2500PW column. Molecular mass standards bovine lung aprotinin (6500 Da, 33 min), insulin chain B oxidized (3495 Da, 35 min), angiotensin II (1040 Da, 45 min), and Thr-Tyr-Ser (369 Da, 49 min) were separated on the same column under similar separation conditions. The *Y* axis represents absorbance at 220 nm.

It is important to note that the particular plasma product DBP is devoid of fibrinogen and thrombin, which were recovered during the process of defibrination. Because DBP consists mainly of albumin and globulins ($\alpha$, $\beta$, and $\gamma$) (*26*), the identified amino acid sequences were traced back to these proteins. Sequences for the dipeptides, LF (**6**), and the tripeptide HPY (**5**) were conserved within the primary structure of bovine serum albumin (BSA) (**Table 3**), whereas those for the other peptides were not. Histidine is abundantly found in serum albumin but could have originated from many other plasma proteins. Although the sequences of other peptides (peptides **2**, **3**, **4**, **7**, and **8**) matched with different proteins as reported from *Bos taurus*, these are not reported here, because a detailed screening




$MH^+$ Ions generated by MALDI of fraction II (*m/z*)[a]

| | |
|---|---|
| 184.1 | 530.3 |
| 255.8 | 570.4 |
| 310.2 | 582.4 |
| 416.2 | 598.3 |
| 441.1 | 963.5 |
| 507.3 | 1307.6 |

[a] excludes ions observed in matrix blank

**Figure 5.** MALDI-TOF mass spectrum of peptide bands separated from fraction II [refer to **Figure 4(iii)**]. $MH^+$ ions of even *m/z* contain nitrogen, which is found in all peptides. Subsequent LC-MS/MS analysis identified the major ion at *m/z* 416 as the tripeptide HPY.

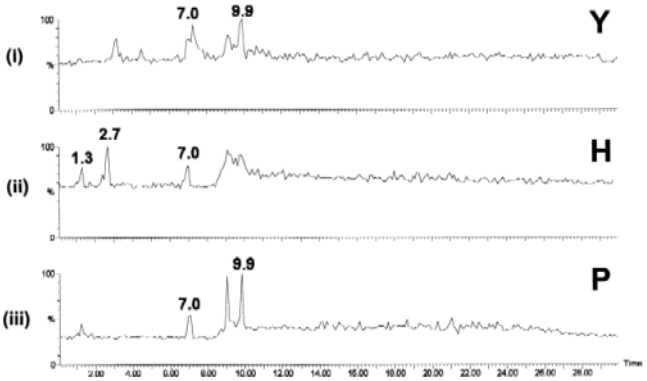



**Figure 6.** Reversed-phase HPLC chromatograms obtained by monitoring the column eluent for precursors of (i) tyrosyl-, (ii) histidyl-, and (iii) prolyl-immonium ions. Peptide sequence information can be obtained from the corresponding precursor-ion spectra at each LC peak and confirmed by coelution of precursors for the component amino acids.

of the types of proteins present in DBP was not carried out for cross-referencing.

A tripeptide LVL, with potent ACEI activity, has been identified from a Flavourzyme digest of porcine blood proteins (*25*). A peptide with the same sequence has been recovered from porcine blood plasma as a naturally present ACE inhibitor (*24*). Tryptic hydrolysis of human serum albumin has produced two peptides (AW and AFKAWAVAR) that have ACEI activity (*32*). None of these peptides were identified in the present study, although this may be due to differences in the substrate and the specificity of the enzymes employed (the study of ref *32* has used trypsin for generating peptides). Flavourzyme is a protease that is obtained from *Aspergillus oryzae* and possesses both exopeptidase and endoprotease (*33*) activities, whereas trypsin

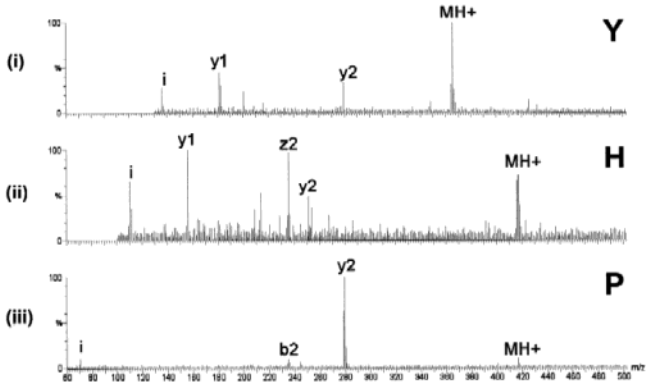



**Figure 7.** Precursor-ion spectra for (i) tyrosyl-, (ii) histidyl-, and (iii) prolyl-immonium ions at an LC retention time of 7.0 min. Complete y-ion series for both tyrosine and histidine identify the coeluting peptides SPY and YPH, respectively. However, the most abundant peptide detected at this retention time is HPY, as shown by the strong precursor-ion spectrum for the immonium ion of proline. The presence of b- and y-ions is consistent with an internal proline residue.

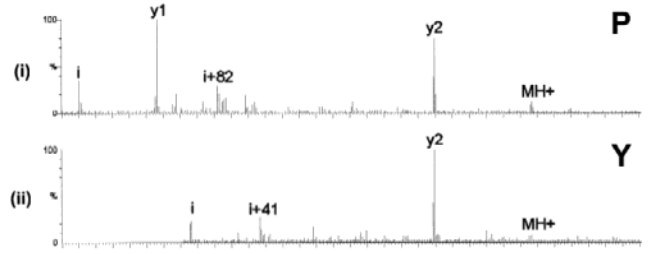



**Figure 8.** Precursor-ion spectra for (i) prolyl- and (ii) tyrosyl-immonium ions at an LC retention time of 9.9 min. A complete y-ion series for the C-terminal proline identifies the peptide as GPY, which is confirmed by precursors for tyrosine (the immonium ion of glycine, the smallest amino acid, does not produce informative precursor-ion spectra). The formation of immonium ions (i, inside the spectra) in the ESI source is confirmed by detection of solvent adducts with acetonitrile (41 Da).

**Table 3.** Proposed Amino Acid Sequence, Molecular Mass, and Relationship to the Primary Sequence of Bovine Serum Albumin of LC-Separated Peptides Identified from Fraction II of Hydrolyzed DBP

| peptide | retention time (min) | estimated molecular mass ($MH^+$, *m/z*) | matching peptide fragment in BSA |
|---|---|---|---|
| 1. H | 1.3 | 156 | |
| 2. H(L/I) | 2.7 | 269 | |
| 3. SPY | 7.0 | 366 | |
| 4. YPH | 7.0 | 416 | |
| 5. HPY[a] | 7.0 | 416 | f(145–147) |
| 6. (L/I)F | 8.8 | 279 | f(69–70),[b] f(503–504)[b] |
| 7. HPGH | 9.1 | 428 | |
| 8. GYP | 9.9 | 336 | |

[a] Sequence confirmed by ESI-MS/MS of the molecular ion in the unseparated fraction. [b] Only the LF sequence matched with these fragments.

is a serine protease and cleaves peptide bonds that are C-terminal to Arg and Lys.

Among the peptides identified in this study, HPY (**5**), LF (**6**), and SPY (**3**) are similar in that their C-terminal amino acid is aromatic. Two others have histidine in the C-terminal position (YPH, **4**; and HPGH, **7**) and another (**8**) has proline there. Mullally and others (*34*) have reported peptide **6** from $\beta$-lactoglobulin f(104−105) as a potent ACE inhibitor. According to Fujita et al. (*35*), longer peptides can undergo further hydrolysis in vivo and might yield small peptide fragments that have higher ACEI activity than the parent ones. The ACEI

peptides YGLF [α-la f(50−53)] (*34*, *36*) and YLLF [β-lg f(102−105)] (*34*), which are also from whey proteins, show the similarity of LF (**6**) in the C terminus and could yield LF on further hydrolysis. The peptides **3**, **4**, **7**, and **8** identified in this study have not been reported in any of the previous work.

The present investigation employed an in vitro assay to determine the ACEI activity of the hydrolysates and peptide fractions. Although they are very small peptides, it is difficult to draw any conclusions about their potent in vivo activity at this point. It is well established that small peptides are more easily absorbed in the large intestinal tract than bigger ones (*37*, *38*). It has also been demonstrated that there is a good possibility ACE inhibitory peptides due to the activity of pepsin or pancreatin when such peptides are orally ingested (*35*). The peptides reported in the present study are very short and are less likely to be cleaved by gastric proteases. The shorter peptides tend to have higher in vivo ACEI activity as observed in fish proteins (39) than the longer ones. According to the study on ACEI peptides of a *Bonito* digest, the peptides LKPNM (prodrug-type) and LKP exhibited remarkably high antihypertensive activities in vivo despite the weaker in vitro ACE inhibitory effects. Therefore, the DBP−peptide fractions warrant in vivo assessment, although they exhibited lower in vitro ACEI activity (when $IC_{50}$ values are compared) than some of the peptides reported from food proteins.

It is of considerable interest in today's food industry to search for and develop functional food ingredients that have specific benefits in modulating certain physiological conditions in general as well as in preventing certain diseases. A good case in point comes from dairy products. For example, the fermented milk beverage (*40*) and protein hydrolysates developed from whey proteins (Davisco Foods International; *41*) are recognized as having antihypertensive activity due to the ACEI activity of the contained peptides and are presently available to the consumer. The findings in this study indicate the possibility of developing ACEI peptides with potential health benefits; these could be obtained from byproducts in the meat industry, such as bovine blood plasma. Such an approach looks very promising in adding value to underutilized protein products.

**Conclusions.** This study demonstrates that proteolysis of bovine plasma proteins can result in peptides with ACE inhibitory activity. The ACEI peptides identified in this study are very short and presumably have originated from serum albumin and globulins, as this particular plasma protein product is devoid of thrombin and fibrinogen. Further development of the LC-MS/MS immonium precursor-ion scanning method may allow larger peptides to be detected and sequenced using this technique. Our study also confirms that HPLC separation of HA provides a rapid and simple method of quantifying ACE activity using the artificial substrate hippuryl-His-Leu.

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