Properties and Human Origin of Two Angiotensin-I-Converting Enzyme Inhibitory Peptides Isolated from a Tryptic Hydrolysate of Human Serum Albumin

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Two angiotensin-I-converting enzyme (ACE) inhibitory peptides were isolated from a tryptic hydrolysate of human serum albumin (HSA). The peptides were identified by sequencing and other analyses as Ala-Trp and the nonapeptide Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg (human albutensin A), corresponding to f(213—214) and f(210—218) of HSA, respectively. Synthetic versions of both peptides had previously been shown to have ACE inhibitory activity. The present results are the first to show that these peptides have a potential natural origin in humans. Additional studies were done to define the inhibitory properties of these peptides, as they had not been previously reported. The dipeptide and nonapeptide showed dose-dependent inhibition of ACE, with IC50 values of 12 and 1.7 μmol/l, respectively. Lineweaver-Burk plots suggested that Ala-Trp is a competitive inhibitor, and that human albutensin A is a noncompetitive inhibitor.

Key words ACE inhibitory peptide; tryptic hydrolysate; albutensin A; bioactive peptide; human serum albumin

Almost all organisms, including animals, plants, and microorganisms, as well as a number of synthetic compounds have been examined in the search for bioactive substances as starting materials for medicines.1—7 However, we believe that the human body might be the best resource for such bioactive peptides because such substances would be expected to be less toxic to humans. Although numerous substances have been isolated from the human body, there are probably many others, especially degradation products of proteins, that have not yet been identified. To demonstrate this idea, we previously isolated Acein-15 and Acein-2.9 Both Acein-1 and Acein-2 are novel peptides that inhibit angiotensin-I-converting enzyme (ACE, peptidyl dipeptidyl hydrolase). Acein-1 was isolated from a tryptic hydrolysate of human plasma with the amino acid sequence of Tyr-Leu-Tyr-Glu-Ile-Ala-Arg, which corresponds to f(138—144) of human serum albumin (HSA). Acein-2 (Leu-Ile-Tyr) was isolated from the same hydrolysate, which corresponds to f(518—520) of human α2-macroglobulin.

In this paper we describe the isolation of two additional ACE inhibitory peptides from a tryptic hydrolysate of HSA. A search of the literature revealed that synthetic versions of both peptides had previously been shown to have ACE inhibitory activity.10,11 However, there is no report yet of either peptide being produced from a human source, and the previous studies did not examine the inhibitory properties of these peptides. Here, in addition to showing that tryptic and chymotryptic digests of HSA can produce these peptides, we describe some of the properties of these peptides, including IC50 values and inhibitory mechanisms based on Lineweaver-Burk plots.

ACE has classically been associated with the renin-angiotensin system, which regulates peripheral blood pressure. ACE raises blood pressure by converting angiotensin I, released from angiotensigenon by renin, into the potent vasoconstrictor angiotensin II. ACE also degrades vasodilative bradykinin in blood vessels and stimulates the release of aldosterone in the adrenal cortex. Consequently, ACE inhibitors may exert an antihypertensive effect.12,13

MATERIALS AND METHODS

Enzymes and Other Reagents HSA (Fraction V) and trypsin (EC 3.4.21.4, from porcine pancreas) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). ACE (EC 3.4.15.1 from rabbit lung) was purchased from Wako Pure Chemical Ind. (Osaka, Japan). Hippuryl-Histidyl-Leucine (HHL), Bradykinin-potentiatior B (BPP) and N-benzoyl-Tyr ethyl ester were obtained from The Peptide Institute (Osaka). All other reagents were of analytical grade unless otherwise specified.

Tryptic Hydrolysis HSA (450 mg) was dissolved in 48.8 ml of 50 mm Tris HCl buffer (pH 8.0). Tryptsin was added to the HSA solution at an enzyme:substrate ratio of 1:50 and the mixture was hydrolyzed at 37 °C for 3 h. The reaction was stopped by heating in boiling water for 10 min, and the hydrolysate was centrifuged for 20 min at 4 °C and 3000 × g. The supernatant was used for subsequent analyses.

Reversed-Phase Gradient HPLC The supernatant was fractionated by reversed-phase high-performance liquid chromatography (HPLC) with gradient elution. A GILSON HPLC system was used with an autogradient set (Model 802, 803C, and 2 Model 302 pumps, GILSON Medical Electronics, Inc., Middleton, WI). Separation was performed at room temperature on a Develosil ODS-5 (Nomura Kagaku Co., Osaka) column (4.0 i.d. × 150 mm). A linear acetonitrile gradient between 0.1% trifluoroacetic acid (TFA) in water (solvent A) and 80% acetonitrile with 0.1% TFA in water (solvent B) was used as follows: 0% B for 20 min, 0—40% B for 60 min, 40% B for 30 min, and 100% B for 10 min. The flow rate was 1.0 ml/min, and the effluent was monitored continuously at 215 nm. All peaks were fractionated and evaporated.
for measuring inhibitory activity.

**Reversed-Phase Isocratic HPLC**  The active fractions were further purified by reversed-phase isocratic HPLC. The HPLC system used was the same as the gradient HPLC system. Separation was performed on a single or a tandem-linked Develosil ODS-5 column (4.0 i.d.×150 mm) at room temperature. Sixteen or twenty percent acetonitrile with 0.1% TFA was used as the eluent. The flow rate was 0.5 ml/min, and the effluent was monitored continuously at 215 nm. The active fractions were collected and evaporated.

**ACE Inhibition Assay**  The activity of ACE was measured spectrophotometrically using HHL as the substrate, by a modification of the method of Cushman and Cheung as described by Maruyama et al. Five millimolar HHL and an appropriate quantity of ACE inhibitor were dissolved in 100 mM sodium borate buffer, pH 8.3, containing 300 mM NaCl, and incubated with 20 munits/ml ACE at 37°C for 30 min. The concentration of ACE inhibitors needed to inhibit 50% of the ACE activity was defined as the IC_{50} value. BPB (IC_{50}=3.3 μm) was used as a positive control for ACE inhibition.

**Amino Acid Sequence Analysis and Mass Spectrometry**  The amino acid sequence was determined using Edman degradation on a pulsed-liquid protein sequencer, equipped with an on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems 477A/120A, Foster City, CA). A mass analysis of the peptides was carried out using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (Kompact MALDI IV, Shimadzu Co., Kyoto, Japan).

**Preparation of Peptides** Peptides were synthesized with the solid phase Fmoc method on a peptide synthesizer (Applied Biosystems 433A, Foster City, CA) and deprotected in the usual manner. Purification was performed by HPLC with a reversed-phase column (Capcell Pak C18, 10 i.d.×250 mm, Shiseido Co., Tokyo, Japan) employing a linear gradient elution system from H_{2}O to 50% acetonitrile containing 0.1% TFA for 30 min. The flow rate was set for 3.0 ml/min. Effluents were monitored by UV absorption at 220 nm, and the peaks with 220 nm absorbency were collected and lyophilized. Some of the peptides were synthesized at Biologica Co., Nagoya, Japan.

**Measurement of Chymotryptic Activity** The chymotrypsin activity was measured by using N-benzoyl-L-tyr ethyl ester (BTEE) as the substrate. The reaction mixture consisted of 1.5 ml trypsin solution in 80 mM Tris HCl buffer containing 100 mM CaCl_{2} (pH 7.8) and 1.5 ml of 1.0 mM BTEE in 50% MeOH. The reaction was started by adding the trypsin solution. The increase in absorbance at 256 nm was measured at precisely 1, 2 and 3 min. The chymotrypsin activity (BTEE units/l) was calculated from the expression 31100×ΔAbs/min.

**RESULTS AND DISCUSSION**

The tryptic hydrolysate of HSA was found to have significant ACE inhibitory activity, while no activity was observed prior to the tryptic digestion. Only two active fractions, Fr. 13 and Fr. 31, were found when the hydrolysate was subjected to reversed-phase gradient HPLC (Fig. 1).

The Fr. 13 was further separated with reversed-phase isocratic HPLC with an eluent of 16% acetonitrile containing 0.1% TFA. The tenth peak in this chromatogram (Fr. 13-10) showed ACE inhibitory activity. Sequencing of the purified peptide F13-10 indicated that it was a dipeptide, Ala-Trp. No other amino acid except Trp was observed on the third and fourth sequencing cycles (data not shown). We attribute the additional Trp residues to a gradual degradation of the purified peptide. In the amino acid analysis of the purified peptide, only Ala was found. This was expected as Trp is degraded under the hydrolysis conditions used (6 mol/l HCl at 110°C for 18 h). The mass number of this peptide as determined by mass spectrometry was 276.20 (M+H)^{+}. This value was consistent with the value calculated from the peptide sequence, 276.31. Moreover, the purified peptide was identical to a synthetic Ala-Trp dipeptide on isocratic HPLC analysis (Fig. 2A). Consequently, the purified peptide Fr. 13-10 was identified as the dipeptide, Ala-Trp. The sequence Ala-Trp was identified as f(213−214) of HSA, as it occurs only at this position in HSA, and it does not occur in trypsin.

Ala-Trp does not appear to be a tryptic fragment because tryptic digestion does not result in a C-terminal Trp residue.
The chymotryptic activity in the trypsin solution was determined to be 2 BTEE units/mg protein. Thus, Ala-Trp must have been a product of chymotryptic activity, which according to the manufacturer of the trypsin, occurs as a contaminant.

Fr. 31 from the reversed-phase gradient HPLC was further separated with reversed-phase isocratic HPLC with 20% acetonitrile containing 0.1% TFA. The eighth peak in this chromatogram (Fr. 31-8) showed ACE inhibitory activity. Sequencing and amino acid analyses showed that the purified peptide F31-8 was a nonapeptide, Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg. A synthetic peptide of the same sequence was made, and was found to have the same retention time as the purified peptide on isocratic HPLC analysis with an eluent of 20% acetonitrile containing 0.1% TFA (Fig. 2B). The amino acid sequence of the nonapeptide was found to be identical to that of f(210-218) in HSA, and thus overlaps the Ala-Trp peptide described above. The nonapeptide is very similar to bovine albutensin A, which was isolated from a tryptic hydrolysate of bovine serum albumin by Chiba and Yoshikawa. Accordingly, we refer to our nonapeptide as human albutensin A.

The fact that both the nonapeptide containing Ala-Trp and Ala-Trp are generated in the trypsin digest indicates that the Ala-Trp sequence is only partially digested by the chymotryptic activity of the trypsin preparation used in this experiment. The reason for this is unknown. Also, the fact that human albutensin A has a Lys residue at position 3 indicates that this residue is only partially digested by trypsin. This must also be the case in the generation of bovine albutensin A, which also has a Lys residue at position 3.

The ACE inhibitory activity of synthetic Ala-Trp and synthetic human albutensin A were found to be dose-dependent with IC$_{50}$ values of 12.1 and 1.7 μmol/l, respectively (Fig. 3). Cheung et al. reported that several synthetic dipeptides including Ala-Trp have ACE inhibitory activity. The present study thus confirms this result and also shows that this Ala-Trp has a potential natural origin in humans, i.e., in a tryptic and chymotryptic hydrolysate of HSA.

Chiba and Yoshikawa, who made a synthetic human albutensin A, briefly mentioned that it showed ACE inhibitory activity, but they did not isolate the natural peptide or determine its IC$_{50}$ value. The IC$_{50}$ value of human albutensin A that we obtained (1.7 μmol/l) appears to be very low among the peptidyl ACE inhibitors derived from enzymatic hydrolysates of proteins. IC$_{50}$ values of other peptides have been reported to range from 0.27 to 171.8 μmol/l (Table 1).

To clarify the inhibition mechanism kinetically, Lineweaver-Burk plots were determined for Ala-Trp and human albutensin A. These plots showed that Ala-Trp is a competitive inhibitor, with an intercept on the 1/[V] axis as shown in Fig. 4. These plots suggested that Ala-Trp could bind to the catalytic site of ACE. Some synthetic dipeptides as well as Ala-Trp were reported as competitive inhibitors of ACE due to their small size, which enable them to enter the catalytic site of ACE. In contrast, human albutensin A, like Acein-1, showed noncompetitive inhibition, with an intercept on the 1/[S] axis (Fig. 4B). These findings suggest that human albutensin A could not be a substrate of, i.e., could not be cleaved by, ACE.

Acein-1 was previously isolated from a tryptic hydrolysate of human plasma corresponding to f(138-144) in HSA. Thus, one would expect that Acein-1 would also be generated in the present study in which the starting material was human serum albumin. Under the same HPLC conditions used in this study, Acein-1 has a retention time different from the retention times of Ala-Trp and human albutensin A, so three peaks with ACE inhibitory activity would be expected. However, only the peaks for Ala-Trp and human albutensin A were found. The reason for the absence of a third peak corresponding to Acein-1 is unknown. One possibility is that the
inhibitory peptides are found in the enzymatic hydrolysates of human proteins: Acein-1, Acein-2, Ala-Trp and human albutensin A.

Other ACE inhibitory peptides isolated from enzymatic hydrolysates of proteins were reported from α-Zein, casein and lactoglobulin (Table 1). Peptides derived from food proteins such as casein hydrolysates have been shown to have significant physiological effects and have been used to prevent hypertension.20

The present results suggest that Ala-Trp and human Albutensin A, as well as Acein-1 and Acein-2, could be used as starting materials for anti-hypertensive drugs against ACE. Further studies will be needed to determine whether these peptides are released from serum albumin by endogenous tryptic and chymotryptic hydrolysis in the human body to inhibit ACE as part of normal physiological regulation.

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