Acein-1, a novel angiotensin-I-converting enzyme inhibitory peptide isolated from tryptic hydrolysate of human plasma

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Abstract A novel angiotensin-I-converting enzyme (ACE) inhibitory peptide, designated acein-1, was isolated from the tryptic hydrolysate of human plasma. Gel filtration and cation exchange chromatography were performed to purify this peptide, followed by reversed-phase gradient and isocratic high-performance liquid chromatography. Acein-1 was found to be a heptapeptide, Tyr-Leu-Tyr-Glu-Ile-Ala-Arg, corresponding to f(138–144) of human serum albumin. The synthetic heptapeptide, hexapeptide (Tyr-Leu-Tyr-Glu-Ile-Ala, des-^{7}R acein-1) and octapeptide (Tyr-Leu-Tyr-Glu-Ile-Ala-Arg-Arg, acein-1R) showed dose-dependent inhibitions of ACE, and their IC_{50} values were 16 μmol/l, 500 μmol/l and 86 μmol/l, respectively. Acein-1 might be a non-competitive inhibitor, while acein-1R may be an uncompetitive inhibitor, as shown by Lineweaver-Burk plots.

Key words: Acein-1; Angiotensin-I-converting enzyme inhibitor; Bioactive peptide; Tryptic hydrolysate; Human serum albumin

1. Introduction

Bioactive substances as starting materials for medicines have been searched for among almost all natural resources, including animals, plants, and microorganisms, as well as synthetic compounds [1–7]. One of the best sources of such bioactive substances is the human body, because substances isolated from the human body can be expected to be non-toxic and safe for use in medicines. Although numerous substances have been isolated from the human body, more as yet unknown bioactive substances are thought to be present in the human body, especially in proteins. We have been searching for novel bioactive peptides in proteins in the human body.

Angiotensin-I-converting enzyme (ACE, peptidyl dipeptide hydrolase, EC 3.4.15.1) has been classically associated with the renin-angiotensin system which regulates peripheral blood pressure. ACE raises blood pressure by converting angiotensin I released from angiotensinogen 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 [8,9].

Several ACE inhibitory peptides and bradykinin-potentiating peptides have been isolated from the enzymatic hydrolysates of several proteins [10–18]. Weyers et al. isolated several tryptic peptides from rabbit serum albumin which enhanced bradykinin activity [12]. Albutensin A, a peptide derived from the tryptic digest of bovine serum albumin, showed an ACE inhibitory effect [13]. Other ACE inhibitory peptides were isolated from enzymatic hydrolysates of casein [14–16] and lactoglobulin [17,18]. It is also suspected that bioactive peptides are present in enzymatic hydrolysates of proteins.

In this article, we report a novel ACE inhibitory peptide, designated acein-1, isolated from the tryptic hydrolysate of human plasma, and we describe the inhibitory activity of acein-1, acein-1R and des-^{7}R acein-1. The inhibitory mechanism of acein-1 is non-competitive, while that of acein-1R is uncompetitive, as shown by Lineweaver-Burk plots.

2. Materials and methods

2.1. Enzymes and other reagents

Freshly frozen plasma for use in transfusion was used as human plasma. ACE (EC 3.4.15.1, from rabbit lung) and trypsin (EC 3.4.21.4, from bovine pancreas) were purchased from Wako Pure Chemicals (Osaka, Japan). Hippuryl-histidyl-leucine (HHL) and bradykinin-potentiator B (BPB) were obtained from The Peptide Institute (Osaka, Japan). Sephadex G-25 and SP-Sephadex C-25 were from Pharmacia Biotech (Uppsala, Sweden), and the SEP-PAK C18 cartridge was from Waters (Milford, MA, USA). All other reagents were of analytical grade unless otherwise specified.

2.2. Inhibitory assay for ACE

The inhibitory activity of ACE was measured spectrometrically using HHL as the substrate, by a modification of the method of Cushman and Cheung [19] as described by Maruyama et al. [15]. 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 mU/ml ACE at 37°C for 30 min. The concentration of ACE inhibitors needed to inhibit 50% of ACE activity was defined as the IC_{50} value. BPB (IC_{50} = 3.3 μM) was used as the positive control for ACE inhibition [20].

2.3. Tryptic hydrolysis

Ten milliliters of human plasma was diluted to 1/8 with 50 mM Tris-HCl buffer (pH 8.0). Trypsin was added to the plasma solution at an enzyme/substrate ratio of 1:50 and hydrolyzed at 37°C for 3 h. The reaction was stopped by heating in boiling water for 10 min, and the hydrolysate was centrifuged at 3000×*g* for 20 min to obtain the supernatant.

2.4. Sephadex G-25 gel filtration

A sample of tryptic hydrolysate was concentrated 5 times by distillation under reduced pressure, and 10 ml of the concentrate was applied directly to a Sephadex G-25 column (30×360 mm i.d.) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). Elution was used with the same solution at a flow rate of 3.6 ml/min. Fractions showing inhibitory activity were combined.

2.5. SP-Sephadex C-25 cation exchange column chromatography

About 100 ml of the combined fractions from the gel filtration was applied to an SP-Sephadex C-25 cation exchange column (30×420

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mm i.d.) equilibrated with 10 mM ammonium acetate (pH 4.0). Materials were eluted with a linear gradient formed with 300 ml of the same solution in the mixing chamber and 300 ml of the same solution containing 500 mM ammonium formate in the reservoir, and then 300 ml of the final solution at a flow rate of 3.0 ml/min. Eluates from 187 to 228 ml, which showed activity, were combined and desalted by an SEP-PAK C18 cartridge and evaporated.

2.6. Reversed-phase gradient HPLC

The desalted sample was purified 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 of Model 302 pumps, Gilson Medical Electronics, Middleton, WI, USA). Separation was performed at room temperature on a Develosil ODS-5 (Nomura Kagaku, Osaka, Japan) column (4.0×150 mm i.d.) packed in our laboratory. A linear acetonitrile gradient between 0.1% trifluoroacetic acid (TFA) in water as solvent A and 80% acetonitrile with 0.1% TFA in water as 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.

2.7. Reversed-phase isocratic HPLC

The active fraction was further purified by reversed-phase isocratic HPLC. The HPLC system used was the same as the gradient HPLC system. Separation was performed on a tandem-linked Develosil ODS-5 column (4.0×150 mm i.d.) at room temperature. Twenty percent of acetonitrile with 0.1% TFA was used as the effluent for the isolation of the peptide. The flow rate was 0.5 ml/min, and the effluent was monitored continuously at 215 nm. An active fraction was collected and evaporated.

2.8. 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, USA). The 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, Kyoto, Japan).

2.9. Peptide synthesis

Some of the peptides were synthesized using Fmoc solid-phase synthesis at Biologica, Nagoya, Japan, and some were kindly provided by Dr. Toshifumi Akizawa, Setsunan University, Osaka, Japan.

3. Results and discussion

With the use of HHL as the substrate, the tryptic hydrolysate of human plasma was found to have significant ACE inhibitory activity. The tryptic hydrolysate for 3 h reached the maximum inhibition when various time periods of hydrolysis were carried out and the activity remained unchanged for at least 24 h, while a plasma sample without tryptic digestion had no activity. An active fraction from a Sephadex G-25 column was eluted at almost the same position as peptides of molecular weight of about 1000 (data not shown). Fractions eluted from SP-Sephadex C-25 cation exchange chromatography which showed activity were collected and concentrated through a SEP-PAK C18 cartridge. The sample was purified by reversed-phase HPLC with gradient elution. The peptic sample eluted at around 20% acetonitrile showed significant inhibitory activity, and a single peak isolation was performed by isocratic HPLC with an eluent of 0.1% TFA containing 20% acetonitrile.

In the amino acid sequencing analysis, the purified peptide was found to be a heptapeptide, the sequence of which was Tyr-Leu-Tyr-Glu-Ile-Ala-Arg. The mass number obtained from this peptide was consistent with the sequence obtained by the mass spectrometric determination (data not shown). In addition, the purified peptide was identical to the synthetic heptapeptide on isocratic HPLC analysis. Consequently, the purified peptide was identified as the heptapeptide, Tyr-Leu-Tyr-Glu-Ile-Ala-Arg, and designated acein-1.

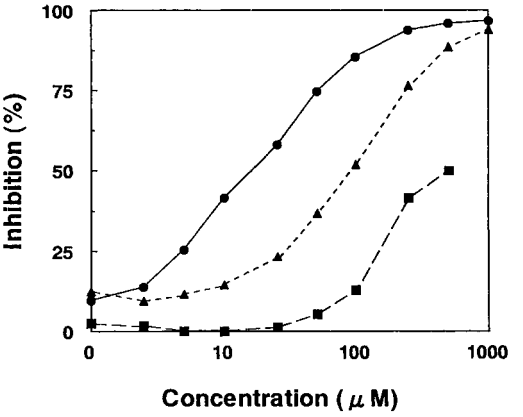

Fig. 1. Dose-dependent inhibition of ACE by acein-1 (●), acein-1R (▲) and des-^{7}R acein-1 (■). Each point represents the mean value of three experiments.

The amino acid sequence of acein-1 was found to be identical to that of f(138–144) in human serum albumin by protein-database analysis. Acein-1 was found to originate from human serum albumin by tryptic digestion because f(137), the former amino acid of acein-1 in it, was Lys and C-terminus of acein-1 was Arg. Bovine, felca and porcine serum albumins have the same amino acid sequence as acein-1. On the other hand rabbit, sheep and horse serum albumins involve Tyr-Leu-Tyr-Glu-Val-Ala-Arg, ^{5}Ile residue in acein-1 was replaced by Val. Synthetic rabbit acein-1 showed the similar ACE inhibitory effect to acein-1 (data not shown).

Several peptides related to ACE inhibition have been isolated from tryptic hydrolysates of serum albumins. One is albutensin A, derived from the tryptic hydrolysate of bovine serum albumin as a contractile peptide for guinea pig ileum; its amino acid sequence was reported as Ala-Leu-Lys-Ala-Trp-Ser-Val-Ala-Arg by Chiba and Yoshikawa [13]. The amino acid sequence of human albutensin A, Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg, corresponds to f(210–218) of human serum albumin. Only human albutensin A has been reported previously to have ACE inhibitory activity among the enzymatic hydrolysates of human proteins. Other such peptides are two bradykinin-potentiating peptides, peptide-AVI (Leu-Val-Glu-Ser-Ser-Lys) and peptide-AVII (Thr-Pro-Val-Ser-Glu-Lys), both of which were isolated from rabbit serum albumin by Weyers et al. [12]. The corresponding sequences of human serum albumin were estimated to be Leu-Val-Ala-Ala-Ser-Gln, f(475–480) for peptide-AVI and Thr-Pro-Val-Ser-Asp-Arg, f(467–472) for peptide-AVII. These corresponding peptides might have inhibitory activity against ACE; this has not yet been examined. Acein-1 is a novel ACE inhibitory peptide derived from the tryptic hydrolysate of human serum albumin.

Acein-1R, the C-terminal extended octapeptide of acein-1 with the amino acid sequence Tyr-Leu-Tyr-Glu-Ile-Ala-Arg-Arg, was estimated to have inhibitory activity because many ACE inhibitors have a C-terminal Arg, as does acein-1 [14,18]. Acein-1 and acein-1R were examined for ACE inhibitory activity and compared with that of des-^{7}R acein-1 (Tyr-Leu-Tyr-Glu-Ile-Ala), which has no C-terminal Arg. The dose-de-

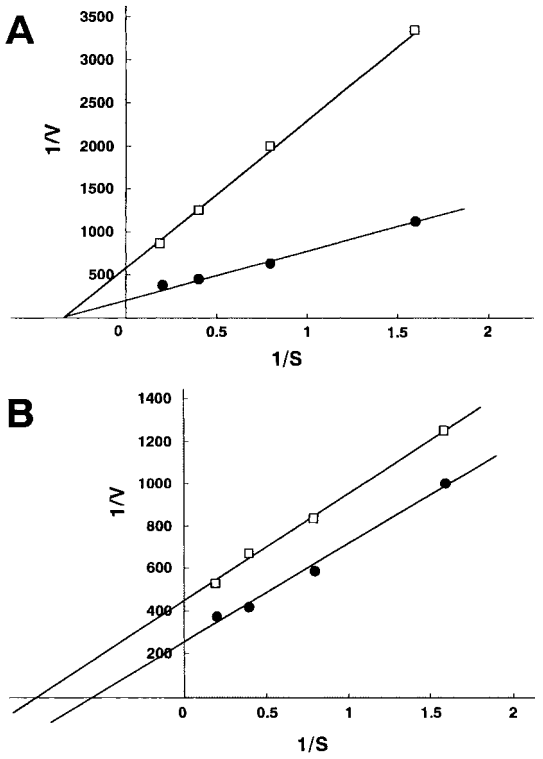

Fig. 2. Lineweaver-Burk plots of the inhibition of ACE by acein-1 (A) and acein-1R (B). Each point represents the mean value of three experiments. ACE activities were measured in the absence (●) or in the presence (□) of 25 μmol/l acein-1 (A) or 50 μmol/l acein-1R (B).

pendent relationship of these three synthetic peptides regarding ACE inhibition is shown in Fig. 1. The positive charge of the C-terminal Arg was indicated to contribute substantially to the ACE inhibitory activity, and an Arg residue was found to be more effective than Arg-Arg on the C-terminus. Acein-1, which originates from the tryptic hydrolysate of human serum albumin, was the most active among the three peptides. The IC_{50} values of acein-1, acein-1R and des-^{7}R acein-1 were 16 μmol/l, 86 μmol/l and 500 μmol/l, respectively.

Acein-1 and acein-1R show the significant IC_{50} values which were similar to those of other ACE inhibitory peptides derived from tryptic hydrolysates of several proteins; the IC_{50} of bovine albutensin A (from bovine serum albumin) was reported as 3.4 μmol/l [13], while that of human albutensin A was not reported; Ala-Leu-Pro-Met-His-Ile-Arg (from bovine β-lactoglobulin) as 42.6 μmol/l [17]; CEI12, Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys (from bovine casein) as 77 μmol/l [15]; and CEIb7, Ala-Val-Pro-Tyr-Pro-Gln-Arg (from bovine casein) as 15 μmol/l [16].

To clarify the inhibition mechanism kinetically, two sets of rate experiments for each peptide were carried out, and the results are plotted double reciprocally in Fig. 2. The Lineweaver-Burk plots of acein-1 (Fig. 2A) showed that it is a non-competitive inhibitor, with an intercept on the 1/[S] axis [21]. These plots suggested that acein-1 could not bind to the catalytic site of ACE and that it could not be hydrolyzed by ACE. In contrast, acein-1R showed uncompetitive inhibition, generating almost parallel lines in Fig. 2B. The reason for the difference between the inhibitory mechanisms that acein-1 is a non-competitive inhibitor and that acein-1R, which only contains an additional C-terminal Arg to acein-1, is an uncompetitive one is yet to be elucidated. However, it was estimated that acein-1R could be a substrate of ACE as well as being an ACE inhibitor, because des-^{7}R acein-1, the ACE hydrolysate of acein-1R, had ACE inhibitory activity. The Lineweaver-Burk plots of acein-1R may therefore indicate the cooperative inhibition of acein-1R with des-^{7}R acein-1. Further experiments are needed to examine the presumption.

It should be noted that peptides derived from food proteins such as casein hydrolysates have been shown to have significant physiological effects and have been used in an attempt to prevent hypertension [10]. The present results suggest that acein-1 could be used as a starting material for anti-hypertensive drugs against ACE, although it is as yet unknown whether acein-1 is released from serum albumin by endogenous tryptic hydrolysis in the human body to inhibit ACE as part of the physiological regulation.

Consequently, acein-1 could represent an example of the human body as a potential resource for starting materials for medicines such as ACE inhibitors.

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