Journal of Biochemistry and Molecular Biology, Vol. 35, No. 2, March 2002, pp. 239-243

JOURNAL OF
Biochemistry and Molecular Biology

Short communication

Structure and Activity of Angiotensin I Converting Enzyme Inhibitory Peptides Derived from Alaskan Pollack Skin

Hee-Guk Byun and Se-Kwon Kim*
Department of Chemistry, Pukyong National University, Pusan 608-737, Korea

Received 27 August 2001, Accepted 1 November 2001

Angiotensin I that converts the enzyme (ACE) inhibitory peptide, Gly-Pro-Leu, previously purified and identified from the Alaskan pollack skin gelatin hydrolysate, were synthesized. In addition, the peptides Gly-Leu-Pro, Leu-Gly-Pro, Leu-Pro-Gly, Pro-Gly-Leu, Pro-Leu-Gly, Gly-Pro, and Pro-Leu, which consisted of glycine, proline, and leucine, were synthesized by the solid-phase method. The IC_{50} values of each tripeptide – namely Leu-Gly-Pro, Gly-Leu-Pro, Gly-Pro-Leu, Pro-Leu-Gly, Leu-Pro-Gly, and Pro-Gly-Leu – were 0.72, 1.62, 2.65, 4.74, 5.73, and 13.93 μM, respectively. The ACE inhibitory activity of these tripeptides was higher than that of dipeptides, such as Gly-Pro and Pro-Leu with IC_{50} values of 252.6 and 337.3 μM, respectively. Among the tripeptides, Leu-Gly-Pro and Gly-Leu-Pro had higher inhibitory activity than Gly-Pro-Leu that was isolated from the Alaskan pollack skin gelatin hydrolysate. Among the different types of tripeptides that were examined, the highest ACE inhibitory activity was observed for Leu-Gly-Pro. It had the leucine residue at the *N*-terminal and proline residue at the *C*-terminal.

Keywords: Alaskan pollack skin, Angiotensin I converting enzyme, Peptide synthesis, Tripeptide

Introduction

The Angiotensin I converting enzyme (ACE, peptidyldipeptide hydrolase, EC 3.4.15.1) belongs to the class of zinc proteases that need zinc and chloride for its activation. ACE generates the powerful vasoconstrictor angiotensin II that removes the *C*-terminal dipeptide from the lysate of the precursor decapeptide angiotensin I (Skggs *et al*., 1954). Furthermore, ACE also inactivates the vasodilator bradykinin (Yang *et al*., 1970).

Since the discovery of an ACE inhibitory peptide in snake venom, many more peptides have been identified from the enzymatic hydrolysates of various natural sources. These include cheese whey (Amhar *et al*., 1996; Abubakar *et al*., 1998), casein (Karaki *et al*., 1990; Yamamoto *et al*., 1994), corn zein (Miyoshi *et al*., 1991), soy sauce (Kinoshita *et al*., 1993), soybean (Okamoto *et al*., 1995), and fish muscle, such as sardine (Ukeda *et al*., 1992), tuna (Kohama *et al*., 1988), and bonito (Matsumura *et al*., 1993).

According to previous studies, many ACE inhibitory peptides consisted of 2~12 amino acid residues. In particular, the IC_{50} values of peptides with proline at the *C*-terminal were around 1~82 μM (Yamamoto, 1997). ACE inhibitory peptides that are derived from proteins recently received a lot of attention due to the development of functional foods that contribute to homeostasis (Ariyoshi, 1993).

The structure and activity of various ACE inhibitory peptides are related to the isolation techniques and sources (Maruyama *et al*., 1989). These peptides, which are inactive within the sequence of the parent protein, may be released due to enzymatic hydrolysis. The structures of ACE inhibitory activity sequences were identified from *in vitro* enzymatic or *in vivo* gastrointestinal digests of the appropriate precursor proteins. Furthermore, chemical synthesis was carried out to confirm the sequence of potential inhibitory activity peptides (Saito *et al*., 1994; Astawan *et al*., 1995). Although the relationship of the structure and activity of ACE inhibitory peptides has not yet been established (Mesel, 1997), these peptides possess certain common features. The relationships between the structure and activity level of various ACE inhibitory peptides indicate that binding to ACE is strongly influenced by the *C*-terminal tripeptide sequence of the substrate. The *C*-terminal tripeptide residues may interact with subsites at the active site of ACE (Ondetti and Cushman, 1982). ACE prefers to have substrates or competitive inhibitors that contain hydrophobic amino acid residues such as proline, phenylalanine, and tyrosine at three positions from the *C*-terminal (Cheung *et al*., 1980). Most of the naturally occurring peptide inhibitors contain proline at their *C*-terminal.

*To whom correspondence should be addressed.
Tel: 82-51-620-6375; Fax: 82-51-628-8147
E-mail: sknkim@mail.pknu.ac.kr

In previous studies, we isolated ACE inhibitory activity peptides from Alaskan pollack skin gelatin hydrolysate. The present study was carried out in order to investigate the relationship between ACE inhibitory activity and the structure of synthetic peptides.

Materials and Methods

Materials The Angiotensin I converting enzyme, Hippuryl-Histidyl-Leucine (Hip-His-Leu), was used as a substrate. Captopril was purchased from the Sigma Chemical Co. (St. Louis, USA). Butyloxycarbonyl amino acids, resins, and other reagents for the solid phase peptide synthesis were acquired from Applied Biosystem Inc. (Branchburg, USA). All of the other reagents used in this study were reagent grade chemicals.

Assay for ACE inhibitory activity The ACE inhibitory activity was assayed by modification of the method of Cushman and Cheung (1971). A sample solution (50 μl) with 50 μl of the ACE solution (25 mU/ml) was pre-incubated at 37°C for 5 min, then incubated with 150 μl of the substrate (8.3 mM Hip-His-Leu in a 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) at 37°C for 60 min. The reaction was stopped by the addition of 250 μl of 1 M HCl. The resulting hippuric acid was extracted with 1.5 ml of ethylacetate. After centrifugation ($800 \times g$, 15 min), 1 ml of the upper layer was transferred into a glass tube, and evaporated at room temperature for 2 h under vacuum. The hippuric acid was dissolved in 3.0 ml of distilled water. The absorbance was measured at 228 nm using a Hitachi Model U-3210 Spectrophotometer (Hitachi, Tokyo, Japan). The IC_{50} value was defined as the concentration of the inhibitor that is required to inhibit 50% of the ACE inhibitory activity.

Synthesis of peptides The peptides were synthesized using the solid phase method with a 430A Peptide Synthesizer (Applied Biosystem Inc., Branchburg, USA). Hydrogen fluoride was used for removing the side chain protecting groups, and for cleaving peptides from their solid support. The synthetic peptides were purified using HPLC (P2000, Sepctra-Physics, San Jose, USA) on a Primesphere ODS C_{18} column ($\phi 0 \times 250$ mm, Phenomenex Co., Cheshire, UK). The column was developed at a flow rate of 2 ml/min by a linear gradient of 70% acetonitrile (0~60%, 40 min) containing 0.1% trifluoroacetic acid

Amino acid analysis of peptides Fifty milligrams of each peptide and 2 ml of 6 M HCl were added in an ampoule, which was allowed to stand at 110°C for 24 h. Then the reaction solution was filtered using a glass-filter. The filtrate was concentrated by evaporation using a rotary evaporator, then diluted with a 0.2 M sodium citrate buffer (pH 2.2) precisely up to 25 ml. An injection volume of 40 μl was used for the analysis by an Automatic amino acid analyzer (Biochrom 20, Pharmacia Biotech, Cambridge, UK).

Results and Discussion

ACE inhibitory activity of synthetic peptides The peptide Gly-Pro-Leu was purified from Alaskan pollack skin gelatin hydrolysates according to a previously reported method from our laboratory (Byun and Kim, 2001). Eight synthetic peptides were prepared in order to study their ACE inhibitory activity and amino acid sequence. The dipeptides, Gly-Pro and Pro-Leu, were synthesized by removing the Leu residue from the *C*-terminal and the Gly residue from the *N*-terminal of the Gly-Pro-Leu, respectively. Furthermore, six tripeptides were chemically synthesized according to the information acquired from a peptide sequence analysis. They were further purified using a reverse-phase HPLC. The results of the IC_{50} values against the synthetic peptides are shown in Table 1. Among the synthetic peptides, the IC_{50} values of Leu-Gly-Pro and Gly-Leu-Pro were 0.72 and 1.62 μM, respectively. Moreover, the IC_{50} values of these peptides had higher inhibitory activities compared to that of the original peptide (Gly-Pro-Leu, IC_{50} 2.65 μM) that was isolated from Alaskan pollack skin gelatin hydrolysates; the peptide, Leu-Gly-Pro showed the strongest inhibitory activity. The amino acid analysis results further revealed that the leucine residue was more prominent than the glycine residue of the *N*-terminal for the ACE inhibitory activity of tripeptides. Cheung *et al.* (1980) studied the ACE inhibitory activity of a series of dipeptides, and indicated that tryptophan, tyrosine, proline, or phenylalanine at the *C*-terminal and branched-chain aliphatic amino acid at the *N*-terminal was suitable for a peptide binding to ACE as a competitive inhibitor. Furthermore, these authors reported that the IC_{50} values of peptides Val-Tyr and Cys-Trp-Leu-Pro-Val-Tyr were 22.0 and 22.2 μM, respectively. In these results, the ACE inhibitory activity of the

Table 1. ACE inhibitory activity of synthetic peptides

Amino acid sequence	Ratio of amino acids in acid hydrolysate	IC_{50} (mM)
Gly-Pro-Leu	Gly 1.40, Pro 0.67, Leu 1.00	2.65
Gly-Pro	Gly 1.00, Pro 0.70	252.63
Pro-Leu	Pro 0.98, Leu 1.00	337.32
Pro-Gly-Leu	Pro 1.00, Gly 1.00, Leu 0.80	13.93
Leu-Gly-Pro	Leu 1.00, Gly 1.00, Pro 1.02	0.72
Gly-Leu-Pro	Gly 0.82, Leu 1.08, Pro 1.00	1.62
Pro-Leu-Gly	Pro 1.02, Leu 0.97, Gly 1.00	4.74
Leu-Pro-Gly	Leu 1.51, Pro 0.91, Gly 1.00	5.73

peptides was mostly dependent on the *C*-terminal dipeptide residue of the peptide.

According to our results, the ACE inhibitory activity of peptides was mostly dependent on the *C*-terminal amino acid residue. However, among the *N*-terminal amino acids of peptides, the branched alpha-chain aliphatic amino acids were most effective for increasing the peptide binding activity of ACE.

Many of the ACE inhibitory peptides that were isolated from snakes were short di- or tripeptides, and according to the *in vivo* studies, some of the di- or tripeptides were absorbed directly (Kiriyama and Arai, 1990). The long-chain peptides or proteins, however, are highly susceptible to proteolytic enzymes in the gastrointestinal track (Walter *et al*., 1996). Short-chain peptides that have ACE inhibitory activity might have some clinical advantages, such as a specific amino acid sequence that corresponds to the peptides of interest in natural proteins. The amount of peptide to be administrated is less than that of long-chain peptides that have equivalent molar activities. Short-chain peptides would be less susceptible to proteolytic enzymes, and could be easily adsorbed after oral administration since the size of the peptide taken up by the absorptive cell in the small intestine is limited to tri- or tetrapeptides (Matthews and Payne, 1980).

Although the ACE inhibitory activity of Gly-Pro-Leu that is purified from Alaskan pollack skin gelatin hydrolysate was lower than that of Leu-Gly-Pro, its sequence could exist in fish proteins that are consumed as food on a daily basis. Thus, the effects of small peptides that are isolated from protein hydrolysates are important in order to investigate their potential health benefits for use in cardiovascular diseases.

Structure and inhibitory activity of peptides ACE inhibitory peptides that are derived from proteins are regarded as competitive substrates for ACE. The inhibitory activity is mainly dependent on a specific peptide structure. In addition, dipeptide that is produced upon enzymatic cleavage may inhibit the ACE activity without being substrates of the enzyme themselves. Although the structure-activity relationship of ACE inhibitory peptides has not yet been established, these peptides show some common features. Structure-activity relationships among different peptide inhibitors of ACE indicate that binding to ACE is strongly influenced by the *C*-terminal tripeptide sequence of the substrate.

Ondetti and Cushman (1982) proposed a binding model for interactions between the substrate and active site of ACE. The *C*-terminal tripeptide residues may interact with the subsites S_1, S_1', and S_2' at the active site of ACE. ACE appears to prefer substrates or competitive inhibitors that contain hydrophobic amino acid residues at the three positions of the *C*-terminal (Maruyama *et al*., 1987). Captopril owes its potency and selectivity to chemical design, guided by a hypothetical active site model that is based on the observed properties of ACE and on an analogy to the known active site of a related zinc-containing peptidase (Ondetti *et al*., 1977; Cushman *et al*., 1977). The zinc ion of ACE was appropriately located between S_1 and S_1' to participate in the hydrolytic cleavage of the peptide bond of the substrate, resulting in the release of the dipeptide product.

The construction of ACE inhibitors was based on the structure-activity relationship. The structure-activity relationship, or pharmacological characterization, has been very successful in generating numerous ACE inhibitors. Experiments of the structure-activity relationship began with the isolation of several peptides from snake venom that inhibited the metabolism of bradykinin and angiotensin I. In fact, all of the ACE inhibitors that have been generated rely to some extent on the C-terminal structure of Glu-Lys-Trp-Ala-Pro, bradykinin potentiating peptide 5 amino acid (BPP_{5a}) (Cushman *et al*., 1987). This is because the BPP_{5a} C-terminal structure, Trp-Ala-Pro, has very strong interactions within the catalytic site of ACE (Cushman *et al*., 1987).

The optimal tripeptide sequence for such competitive binding is found in Glu-Lys-Trp-Ala-Pro, Trp-Ala-Pro, but the less favorable sequence, Ile-Pro-Pro, that is found in teprotide provides much greater biological activity (Cushman *et al*., 1979). The binding of teprotide to the active site of ACE has been studied mainly by testing the inhibitory activities of analogues of this nonapeptide with one or more amino acid substitutions, and of similar analogues of the more potent but short-acting venom pentapeptide, Glu-Lys-Trp-Ala-Pro. A tripeptide with a free terminal carboxyl group is the minimal structural requirement for the binding and cleavage of the substrate by ACE. Also, the analogous tripeptide sequence is of great importance for the competitive binding of venom peptide inhibitors such as teprotide. Cushman *et al*. (1981) reported that the active sites S_1, S_1' and S_2' of ACE had strong affinities for the side chains of tryptophan, alanine, and proline, respectively.

Miyoshi *et al*. (1991) reported that some tripeptides that are isolated from a hydrolysate of *a*-zein, a maize endosperm protein, act as ACE inhibitors. These peptides (Leu-Arg-Pro, Leu-Ser-Pro, Leu-Gln-Pro, and Leu-Ala-Tyr) had IC_{50} values of 0.27, 1.7, 1.9, and 3.9 μM, respectively. It was also found that Leu-Arg-Pro had a hypotensive activity as a 30 mg/kg intravenous injection decreased the blood pressure of spontaneously hypertensive rats by 15 mmHg. Matsumura *et al*. (1993) isolated four ACE inhibitory peptides from an autolysate of bonito bowels. The IC_{50} values of Leu-Arg-Pro, Ile-Arg-Pro, Val-Arg-Pro, and Ile-Lys-Pro were 1.0, 1.8, 2.2, and 2.5 μM, respectively. These results suggest that the proline residue should be placed at the *C*-terminal for exhibiting the ACE inhibitory activity. Fujita and Yoshikawa (1999) reported that Leu-Lys-Pro-Asn-Met that is derived from fish protein may be hydrolyzed further to produce Leu-Lys-Pro, which has an 8-fold higher ACE inhibitory activity relative to the parent peptide. This suggests that a parent peptide can be regarded as a prodrug-type ACE inhibitory peptide.

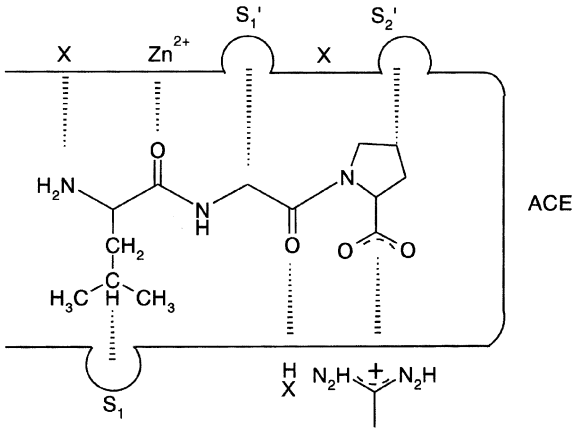

Fig. 1. Binding model for interactions between ACE inhibitory peptide (Leu-Gly-Pro) and the active site of ACE.

In the present results, among all of the peptides, the synthetic peptide Leu-Gly-Pro had the strongest ACE inhibitory activity (IC_{50} value, 0.72 μM). Because of the potent inhibitory activity, peptides should have a branched aliphatic amino acid at the *N*-terminal, such as leucine, and a proline at the *C*-terminal (Fig. 1). Several authors have reported on the relationship between the structure-activity of the ACE inhibitory peptides, and have shown that peptides with high potent inhibitory activity have proline, phenylalanine, or tyrosine at their *C*-terminal. They also contain hydrophobic amino acids in the sequence, such as those of proline-rich peptides.

Acknowledgments This work was supported by the Korea Science Engineering Foundation (KOSEF 961-0609-085-2).

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