J. Fish. Sci. Technol. 8(2), 109-112, 2005

JOURNAL OF
FISHERIES SCIENCE
AND TECHNOLOGY

Notes

Peptide Inhibitor for Angiotensin-Converting Enzyme from Thermolysin Hydrolysate of Manila Clam Proteins

Tae-Gee Lee[1]*, Dong-Min Yeum[2], Young-Sook Kim[2], Saeng-Gyu Yeo[3], Yong-Woo Lee[4], Jin-Soo Kim[5], In-Soo Kim[5] and Seon-Bong Kim[6]

[1]*Department of Hotel Culinary & Baking, Namdo Provincial College of Jeonnam, Damyang 517-802, Korea*
[2]*Department of Food Processing & Baking, Yangsan College, Yangsan 626-740, Korea*
[3]*Department of Hotel Culinary Arts, Busan College of Information Technology, Busan 616-737, Korea*
[4]*Division of Food Science, Dongeui Institute of Technology, Busan, 614-715, Korea*
[5]*Division of Marine Bioscience Institute of Marine Industry, Gyeongsang National University, Tongyeong 650-160, Korea*
[6]*Faculty of Food and Biotechnology, Pukyong National University, Busan 608-737, Korea*

A peptide that inhibits angiotensin-converting enzyme (ACE) was isolated from a hydrolysate of Manila clam (*Ruditapes philippinarum*) proteins prepared with thermolysin. Amino acid sequence of the peptide was determined to be Leu-Leu-Pro. Chemically synthesized Leu-Leu-Pro had an IC_{50} value of 158 μM. Peptides related to the Manila clam-derived peptide were synthesized to study the structure-activity relationships. The tetrapeptide, Leu-Leu-Pro-Pro, had a very weak effect on the enzyme. However, Leu-Leu-Pro-Asn showed no inhibitory activity.

Key words: Angiotensin-converting enzyme (ACE), Manila clam, thermolysin hydrolysate, ACE- inhibitory peptide

Introduction

Among biologically active peptides, antihypertensive peptides have been studied extensively. Angiotensin-converting enzyme (ACE) generates the powerful vasoconstrictor angiotensin II by removing the C-terminal dipeptide from the precursor decapeptide angiotensin I (Skeggs et al., 1954). The enzyme also inactivates the vasodilator bradykinin (Yang et al., 1970). ACE is an unusual zinc-metallopeptidase, in that it is activated by chloride and lacks a narrow *in vitro* substrate specificity.

ACE is predominantly expressed as a membrane-bound endoenzyme in vascular endothelial cells and also in several other types of cells including absorptive epithelial cells, neuroepithelial cells, and male germinal cells (El-Dorry et al., 1982).

The first reported competitive inhibitors of ACE were naturally occurring peptides in snake venom (Ferreira et al., 1970; Ondetti et al., 1971). Subsequently, many other ACE inhibitors have been discovered in enzymatic hydrolysates or related synthetic peptides of bovine casein (Maruyama et al., 1985), zein (Miyoshi et al., 1991), gelatin (Oshima et al., 1979), soy sauce (Kinoshita et al., 1993), and other food proteins. Many studies have also been performed on marine products, such as sardine muscle (Matsui et al., 1993), tuna muscle (Kohara et al., 1988), bonito (Matsumura et al., 1993), and anchovy muscle (Lee et al., 1998). These peptides contain residues with 2 to 12 amino acids, several of them with proline in the C-terminus. During a search for physiologically functional materials from marine food products, we found ACE-inhibitory activity in a thermolysin hydrolysate of Manila clam (*Ruditapes philippinarum)* proteins. This report describes the isolation and identification of an ACE-inhibitory peptide from the hydrolysate of Manila clam.

Materials and Methods

ACE, hippuryl-histidyl-leucine, and thermolysin were purchased from Sigma Chemical Co. (St. Louis, USA). Leu-Leu-Pro, Leu-Leu-Pro-Pro, and Leu-Leu-Pro-Asn were custom-made peptides, purchased from

*Corresponding author: tglee@namdo.ac.kr

the Korea Basic Science Institute (Seoul, Korea). All other reagents were of high performance liquid chromatography (HPLC) or analytical grade. Manila clams were harvested from the southwestern coast of Korea.

An assay mixture containing 15 μL of a prepared sample and 50 μL of a solution of ACE (60 mU/mL) was preincubated for 5 min at 37°C, and then 125 μL of sodium borate buffer (pH 8.3, 400 mM NaCl) dissolved in 5 mM hippuryl-histidyl-leucine were added. The tube containing the mixture was incubated for 30 min at 37°C, and the reaction was stopped by the addition of 20 μL of 10% trifluoroacetic acid (TFA). We then applied 20 μL of the solution to a Zorbax 300SB C_8 column (Agilent Technologies, 4.6×150 mm) for reverse-phase HPLC (Hewlett Packard Co., HP 1100, USA) and eluted it with a linear gradient of acetonitrile (0 to 63%) in 0.1% TFA at a flow rate of 1.0 mL/min. The hippuric acid released was measured by monitoring the absorbance at 228 nm. The IC_{50} value was defined as the concentration that inhibits the ACE-inhibitory activity and the peptide content of each sample after regression analysis.

Fresh raw Manila clams (100 g) were boiled for 10 min in 300 mL of distilled water, then minced and homogenized. Thirty mL of the homogenized sample were added to 50 mL of Tris-HCl buffer (100 mM, pH 8.2, containing 10 mM $CaCl_2$), and then 32 mg of thermolysin were added. After 4.5 hr of digestion at 37°C, the reaction was terminated by boiling for 10 min at 100°C. The precipitate was removed by filtration with Toyo filter paper (Toyo Roshi Co., Ltd.), and the filtrate was then ultrafiltered with a PM-10 membrane (Amicon Co.). The crude peptides were applied to a Sephadex LH-20 column (Pharmacia Fine Chemicals, 26×900 mm), which was eluted with 30% methanol at a flow rate of 20.8 mL/hr. The active fraction was collected and concentrated and then applied to a SP-Toyopearl 650S column (Tosoh Co., Ltd., 16×650 mm) equilibrated with distilled water, which was eluted with a linear gradient of NaCl concentration (0 to 1 M) at a flow rate of 30 mL/hr. The active fraction was collected and concentrated, and then applied to a SuperQ-Toyopearl 650S column (Tosoh Co., Ltd., 16×650 mm) equilibrated with distilled water, which was eluted with a linear gradient of NaCl concentration (0 to 1 M) at a flow rate of 30 mL/hr. The active fraction was purified on a Lichrosphere RP-18 column (Hewlett Packard Co., 4.6×250 mm), which was eluted with a linear gradient of acetonitrile (0 to 63%) in 0.1% TFA at a flow rate of 0.8 mL/min. The active peak from the column was further purified on a μBondasphere C_{18} column (Waters Co., 3.9×150 mm), which was eluted with a linear gradient of acetonitrile (0 to 63%) in 0.1% TFA at a flow rate of 1.0 mL/min. Each chromatography was monitored by absorbance at 210 nm. The amino acid sequence of the purified peptide was analyzed with a protein sequencer (Applied Biosystems, Procise 491, USA).

Results and Discussion

Fig. 1a shows the elution profile of the Sephadex LH-20 chromatogram of the hydrolysate filtered with the PM-10 membrane. The fractions of the most active peak (No. 49-50) were collected, concentrated, and put on the SP-Toyopearl 650S column (Figure not shown). The active fraction (No. 25-26) was obtained at the void volume and was further fractionated by SuperQ-Toyopearl 650S chromatography (Fig. 1b). The fraction (No. 17) with ACE-inhibitory activity was collected and further purified by HPLC on a Lichrosphere RP-18 column (Fig. 1c). Although many peaks were observed by chromatography, only one peak showed inhibitory activity. The peak was collected and purified by another HPLC on a μBondasphere C_{18} column (Figure not shown).

The purified sample was analyzed for amino acid sequence by the automated Edman procedure. The peptide was determined to be a tripeptide, Leu-Leu-Pro, which corresponds to the amino acid sequence 394-396 of rabbit muscle glycogen phosphorylase (Titani et al., 1977), and the sequence 218-220 of human ApoA-I, an apolipoprotein isolated from high density lipoprotein (Brewer et al., 1978). A peptide with an identical amino acid sequence was synthesized, and its HPLC profile was compared to that of the purified one using a μBondasphere C_{18} column. Its retention time was similar to that of the purified sample (Data not shown). The IC_{50} value of the synthetic Leu-Leu-Pro was 158 μM.

Peptides related to the Manila clam–derived peptide were synthesized to study the structure-activity relationships. The ACE-inhibitory activities of these peptides are summarized in Table 1. The tetrapeptide Leu-Leu-Pro-Pro had a weak effect on the enzyme, and Leu-Leu-Pro-Asn had no inhibitory activity.

Table 1. ACE-inhibitory activity of peptides

Peptide	IC_{50} (μM)
Leu-Leu-Pro	158
Leu-Leu-Pro-Pro	1,175
Leu-Leu-Pro-Asn	> 2,000

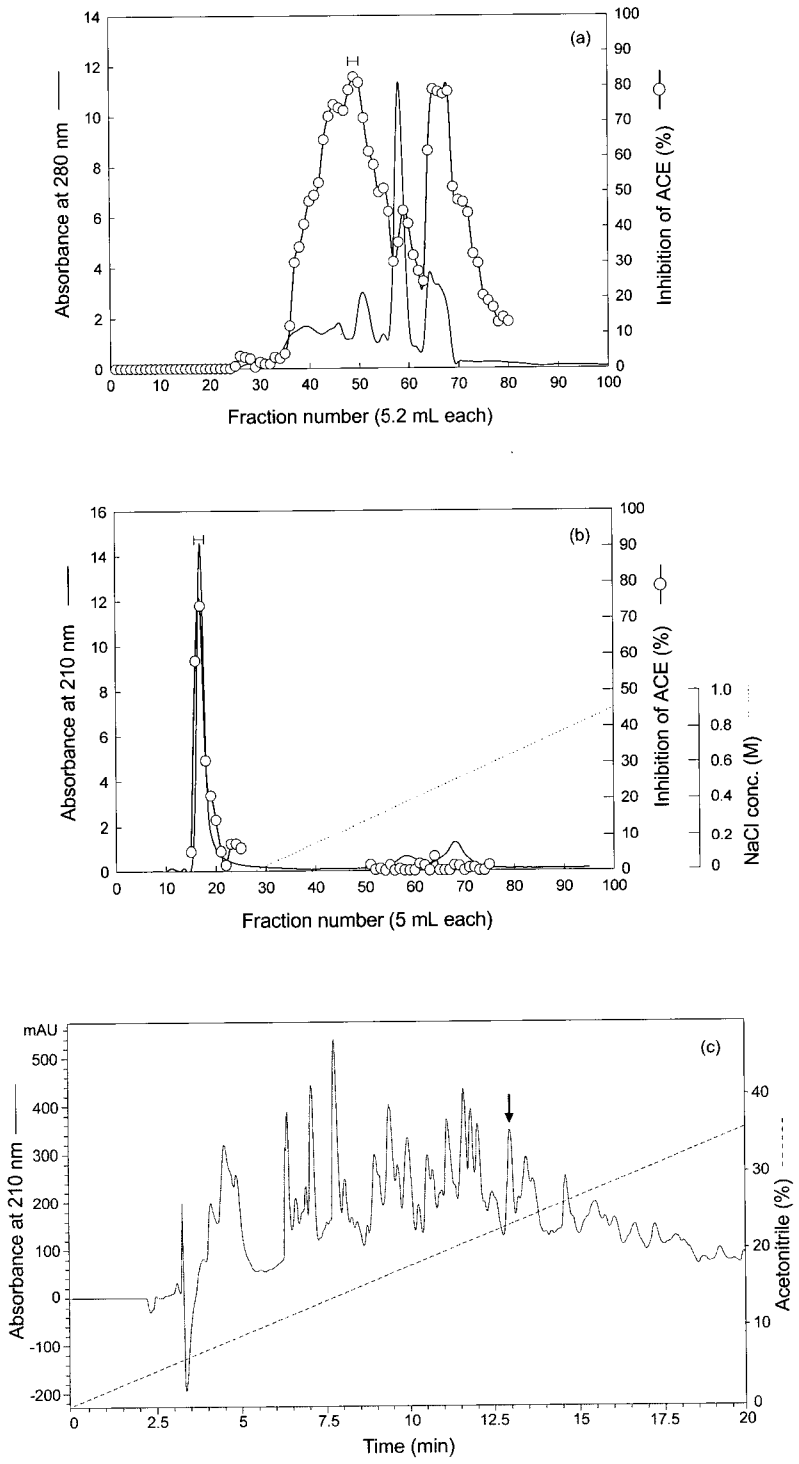

Fig. 1. Purification of Leu-Leu-Pro from a thermolysin hydrolysate of Manila clam proteins. (a) A thermolysin hydrolysate filtered with a PM-10 membrane was chromatographed on a Sephadex LH-20 column. The fractions marked with a horizontal line were collected. (b) Fractions number 25 and number 26 eluted from the SP-Toyopearl 650S column were chromatographed on a SuperQ-Toyopearl 650S column. Fraction number 17 was collected. (c) Reverse-phase HPLC on a Lichrosphere RP-18 HPLC column of the active peak eluted from the SuperQ-Toyopearl 650S column. The peak indicated with the arrow was collected.

and Leu-Leu-Pro-Asn had no inhibitory activity.

Thus, the peptide purified from the thermolysin hydrolysate of Manila clam proteins was identified as a potent inhibitor of ACE. Although the origin of this peptide is unclear, it is interesting that the sequence exists in some biological proteins.

References

Brewer, H.B., Jr., T. Fairwell, A. LaRue, R. Ronan, A. Houser and T.J. Bronzert. 1978. The amino acid sequence of human ApoA-I, an apolipoprotein isolated from high density lipoproteins. Biochem. Biophys. Res. Commun., 80, 623-630.

El-Dorry, H.A., H.G. Bull, K. Iwata, N.A. Thornberry, E.H. Cordes and R.L. Soffer. 1982. Molecular and catalytic properties of rabbit testicular dipeptidyl carboxypeptidase. J. Biol. Chem., 257, 14128-14133.

Ferreira, S.H., D.C. Bartelt and L.J. Greene. 1970. Isolation of bradykinin-potentiating peptides from *Bothrops jararaca* venom. Biochemistry, 9, 2583-2593.

Kinoshita, E., J. Yamakoshi and M. Kikuchi. 1993. Purification and identification of an angiotensin I-converting enzyme inhibitor from soy sauce. Biosci. Biotech. Biochem., 57, 1107-1110.

Kohama, Y., S. Matsumoto, H. Oka, T. Teramoto, M. Okabe and T. Mimura. 1988. Isolation of angiotensin-converting enzyme inhibitor from tuna muscle. Biochem. Biophys. Res. Commun., 155, 332-337.

Lee, T.G., Y.B. Park, D.C. Park, D.M. Yeum, I.S. Kim, Y.S. Gu, Y.H. Park and S.B. Kim. 1998. Angiotensin converting enzyme inhibitory activity in enzymatic hydrolysates of anchovy muscle protein. J. Kor. Fish. Soc., 31, 875-881.

Maruyama, S., K. Nakagomi, N. Tomizuka and H. Suzuki. 1985. Angiotensin-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II. Isolation and bradykinin-potentiating activity on the uterus and the ileum of rats. Agric. Biol. Chem., 49, 1405-1409.

Matsui, T., H. Matsufuji, E. Seki, K. Osajima, M. Nakashima and Y. Osajima. 1993. Inhibition of angiotensin I-converting enzyme by *Bacillus licheniformis* alkaline protease hydrolysates derived from sardine muscle. Biosci. Biotech. Biochem., 57, 922-925.

Matsumura, N., M. Fujii, Y. Takeda, K. Sugita and T. Shimizu. 1993. Angiotensin I-converting enzyme inhibitory peptides derived from bonito bowels autolysate. Biosci. Biotech. Biochem., 57, 695-697.

Miyoshi, S., H. Ishikawa, T. Kaneko, F. Fukui, H. Tanaka and S. Maruyama. 1991. Structures and activity of angiotensin-converting enzyme inhibitors in an α-zein hydrolysate. Agric. Biol. Chem., 55, 1313-1318.

Ondetti, M.A., N.J. Williams, E.F. Sabo, J. Pluscec, E.R. Weaver and O. Kocy. 1971. Angiotensin-converting enzyme inhibitors from the venom of *Bothrops jararaca*. Isolation, elucidation of structure, and synthesis. Biochemistry, 10, 4033-4039.

Oshima, G., H. Shimabukuro and K. Nagasawa. 1979. Peptide inhibitors of angiotensin I-converting enzyme in digests of gelatin by bacterial collagenase. Biochim. Biophys. Acta, 566, 128-137.

Skeggs, L.T., W.H. Marsh, J.R. Kahn and N.P. Shumway. 1954. The existence of two forms of hypertensin. J. Exp. Med., 99, 275-282.

Titani, K., A. Koide, J. Hermann, L.H. Ericsson, S. Kumar, R.D. Wade, K.A. Walsh, H. Neurath and E.H. Fischer. 1977. Complete amino acid sequence of rabbit muscle glycogen phosphorylase. Proc. Natl. Acad. Sci. USA, 74, 4762-4766.

Yang, H.Y.T., E.G. Erdös and Y.A. Levin. 1970. Dipeptidyl carboxypeptidase that converts angiotensin I and inactivates bradykinin. Biochim. Biophys. Acta, 214, 374-376.

(Received March 2005, Accepted June 2005)