PEPTIDE INHIBITOR OF ANGIOTENSIN I-CONVERTING ENZYME DERIVED FROM PORCINE TROPONIN C

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Background

The functions of food may be classified into three categories, namely the primary function (supplying nutrients and energy), the secondary function (taste and palatability) and the tertiary, or physiological, function (biological defense or regulation caused by food). The tertiary functions of many foods have been investigated, but it is not considered that meat has been fully investigated to date. As it so happens, many tertiary functions of foods are caused by peptides. As meats contain significant amounts of protein, one can speculate that they contain many physiologically functional peptides.

Recently, many biologically active peptides from food proteins have been researched. Inhibitors of angiotensin I-converting enzyme (ACE) have attracted particular attention for their ability to prevent hypertension. ACE, a dipeptidylcarboxypeptidase [EC 3.4.15.1], catalyzes angiotensin I to angiotensin II, which both causes hypertension and inactivates bradykinin, an antihypertensive peptide. ACE inhibitory peptides have been researched in foods such as sardine muscle (Kawamura *et al.* 1989), dried bonito (Yokoyama *et al.* 1992), krill (Kawamura *et al.* 1992), and chum salmon (Ohta *et al.* 1999). Arihara *et al.* (2001) have reported on ACE inhibitory peptides derived from porcine water-insoluble proteins, and stated that these peptides were effective as antihypertensive foods. Nevertheless, considerable research on ACE inhibitory peptides from pork remains to be carried out.

Objectives

In this study, we investigated the ACE inhibitory activity of hydrolysate derived from porcine troponin. This hydrolysate was separated to purify and find novel peptides that showed ACE inhibitory activity.

Methods

Preparation and hydrolysis of porcine skeletal muscle troponin

Pork loin (*Longissimus dorsi*) was obtained from Marudai Shimane Farm Co., Ltd. (Shimane, Japan). Troponin was extracted from acetone powder with a high salt buffer; the acetone powder was made from meat washed by a low salt solution (Potter 1982). Hydrochloric acid (HCl) was added to this extract, and a supernatant of the extract was separated using two different concentrations of ammonium sulfate (40% and 60%). Pepsin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Five mg/ml of crude troponin was used as substrate. The ratio of enzyme to substrate was 1/100. Buffer pH was adjusted to 2.0 with 1 M HCl; after digestion it was adjusted to 7.5 with 1 M NaOH. The incubation temperature was 37°C. After incubation, the reaction was terminated by boiling for 10 min at 95°C followed by cooling in ice. The solution was centrifuged for 20 min at 14,000 rpm, and the supernatant was collected for the experiments.

Assay for ACE inhibitory activity

ACE inhibitory assay was performed following the method of Cushman & Cheung (1971), with slight modifications. Briefly, 30 □l of sample was added to 250 □l of 0.1 M borate buffer (pH 8.5) including 7.6 mM hippuryl-L-histidyl-L-leucine (HHL, Nacalai Tesque, Kyoto, Japan) as substrate and 0.608 M NaCl. The reaction was started by the addition of 100 µl of 60 mU/ml bovine lung ACE (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 0.25 M borate buffer (pH 8.5). To terminate the enzyme reaction, 250 µl of 1 M HCl was added. To extract hippuric acid liberated from HHL by ACE, 1.5 ml of ethyl acetate was added and the tubes were vigorously shaken and then centrifuged for 10 min at 2,500 rpm. One ml of the ethyl acetate layer was collected into another tube and dried for 10 min at 100°C. Hippuric acid was dissolved with 5 ml of 1 M NaCl and absorbance at 228 nm was measured. ACE inhibitory activity was calculated as follows:

$$\text{Inhibition (\%)} = [(C - S) / (C - B)] \times 100$$

S: absorbance of sample; C: absorbance of control (buffer for sample); B: absorbance of blank (HCl was added before ACE)

Inhibitory activity was shown as 50% inhibitory protein concentration (IC_{50}) of the sample in assay. An increase of IC_{50} indicates a decrease in ACE inhibitory activity.

Purification of ACE inhibitory peptide from troponin hydrolysate

First, the troponin hydrolysate was separated with anion exchange chromatography. The sample was applied to a DE53 (Whatman International Ltd., ME16 OLS, UK) column (16 x 150 mm) and eluted with a gradient of 0-300 mM NaCl in 20 mM Tris-acetate (pH 7.5) at a flow rate of 1.13 ml/min. Eluted fractions were desalted using a SEP-PAK Plus C_{18} cartridge (Waters Co., Milford, MA, USA) with 50% CH_3CN. Next, the active fraction was separated with reverse phase (RP) HPLC using a Cosmosil $5C_{18}$ AR-II (4.5 x 150mm) (Nacalai Tesque) and eluted with a gradient of 1-80% CH_3CN in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.5 ml/min. Then, the active fraction was applied to the same column and eluted with a 14% CH_3CN in 0.1% TFA at a flow rate of 0.5 ml/min. The active fraction eluted through RP-HPLC was separated with gel-filtration HPLC using a TSK-gel $G2000SW_{XL}$ (7.8 x 300mm) (Tosoh Co., Tokyo, Japan) and eluted with a 20 mM Na-phosphate buffer (pH 7.0) at a flow rate of 0.5 ml/min. Finally, the active fraction was separated with RP-HPLC using Cosmosil 5PE-MS (4.6 x 250mm) (Nacalai Tesque) and eluted with a 12% CH_3CN in 0.1% TFA at a flow rate of 1 ml/min.

Analysis of ACE inhibitory action of peptide

The ACE inhibitory peptides from troponin were reacted to ACE in various concentrations of HHL, and analyzed by Lineweaver-Burk's

reciprocal plot (1934). Then, these peptides (1 mg/ml) were reacted to ACE (20 mU/ml) for 1, 3, 6 and 20 h at 37°C, heated at 95°C for 20 min to terminate ACE reaction, and analyzed by RP-HPLC to discover how well they were digested.

Stability of peptides against digestive proteases

The ACE inhibitory peptides (1 mg/ml) from troponin were reacted with □-chymotrypsin, pepsin or trypsin at a 1:100 ratio of enzyme to substrate. After 6 h at 37°C, the reaction mixture was heated at 95°C for 20 min to terminate enzyme reaction. RP-HPLC analysis and measurement of ACE inhibitory activity was performed.

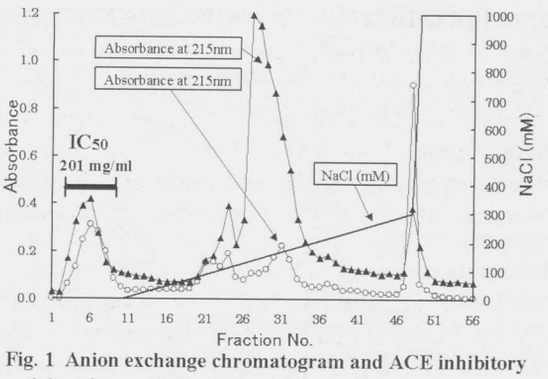

Fig. 1 Anion exchange chromatogram and ACE inhibitory activity of troponin hydrolysate by pepsin.

Results and discussion

Although crude troponin did not show ACE inhibitory activity, peptic hydrolysate of troponin showed relatively strong activity and its IC_{50} was 130 □g protein/ml. We speculated that this activity was due to peptic digestion of troponin.

To search for the ACE inhibitory peptide, this troponin-hydrolysate was purified various chromatographies. First, it was applied to an anion exchange chromatography using DE53 (Fig. 1), and there was a relatively strong activity in the fraction that was eluted early and did not adsorb the filler. After desalting by SEP-PAK Plus C_{18} cartridge, the active fraction showed relatively strong activity and its IC_{50} was 201 □g/ml. ACE inhibitory activity was found in other fractions with broad and weak activity; at this point we speculated that the activity of the whole hydrolysate resulted from the activity of many peptides. The active fraction was separated by RP-HPLC, and the result showed that almost all fractions were eluted at the 10-30% concentration of CH_3CN and the activity was spread out among many fractions. The fraction with the strongest activity (IC_{50} = 78 μg/ml) was re-separated using the same column by isocratic elution (14% CH_3CN). The active fraction from RP-HPLC was separated by the gel-filtration HPLC, and this elute (IC_{50} = 68 μg/ml) was found at the elution volume that peptides with 300-1000 Daltons molecular weight (MW) were eluted. Finally, this fraction was separated with three times of RP-HPLC using Cosmosil 5PE-MS (Fig. 2), and the single peak was separated. The analysis using a protein sequencer (PPSQ-10, Shimadzu Co., Kyoto, Japan) showed this fraction was Arg-Met-Leu-Gly-Gln-Thr-Pro-Thr-Lys (RMLGQTPTK; 9mer, MW = 1031). It was estimated as the sequence at the 44-52 position of skeletal muscle troponin C, and its IC_{50} was 34 μM (35 μg/ml) (Table 1). Also, RMLGQTP (7mer, MW = 802), a part peptide of 9mer, showed activity with 503 μM (404 μg/ml) of IC_{50}. These were recognized the novel ACE-inhibitory peptides that had not previously been reported.

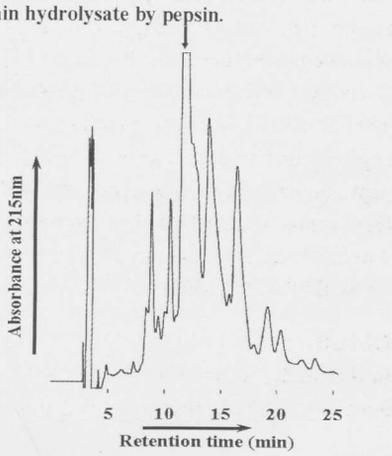

Fig. 2 Chromatogram of RP-HPLC using PE-MS column. Arrow indicates the active peak.

Table 1 ACE inhibitory activity of peptides

Peptide		MW (Da)	IC_{50} μM	IC_{50} μg/ml
RMLGQTP	7mer	802	503	404
RMLGQTPTK	9mer	1031	34	35

To estimate the ACE inhibitory mechanism of these peptides, Lineweaver-Burk's reciprocal plot (1934) was performed. The results showed that 9mer was a non-competitive inhibitor, while 7mer was competitive. The HPLC analysis of 9mer hydrolysate by ACE showed that TK, dipeptide at the carboxyl terminal, was liberated from another peptide relatively slowly. TP of 7mer was rapidly liberated form RMLGQ. Both 9mer and 7mer were rapidly hydrolyzed by ACE after the first dipeptide of the carboxyl terminal was liberated. These results suggested that 7mer showed weak activity because it was easily hydrolyzed by ACE and therefore lost its competitive activity with HHL, although 7mer was competitive with HHL as an ACE substrate because of its high affinity to ACE. Also, we concluded that 9mer showed relatively strong activity because it had a high affinity to ACE and was more difficult for ACE to hydrolyze than 7mer.

To estimate the resistance of 9mer against digestive proteases at oral administration, it was reacted with pepsin, α-chymotrypsin or trypsin. The RP-HPLC analysis of each reaction mixture showed that a significant amount of 9mer remained as substrate after 6h of digestion at 37°C. The rate of residual 9mer was estimated at 75% for pepsin and □-chymotrypsin, and 86% for trypsin (Table 2). ACE inhibitory activities of these reaction mixtures were 33-38 μg/ml as IC_{50}; as they were close to that of 9mer, the products from 9mer by peptic, α -chymotryptic or tryptic digestion were not considered to contribute to the activity. These results suggested that 9mer might not be perfectly digested at oral administration according to its relatively high resistance against digestive proteases. We expected that 9mer worked well *in vivo* as an ACE inhibitor.

Table 2 ACE inhibitory activity of 9mer hydrolysate by digestive proteases

	9mer	9mer+ Pepsin	9mer+ Chymotrypsin	9mer+ Trypsin
9mer peak area (%)	100	75	75	86
IC_{50} (μg/ml)	33	33	38	36

Pertinent literature

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