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Peptide inhibitors for angiotensin I-converting enzyme from enzymatic hydrolysates of porcine skeletal muscle proteins

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Abstract

Inhibitors of angiotensin I-converting enzyme (ACE) have been shown to have antihypertensive effects and have been utilized for pharmaceuticals and physiologically functional foods. In the present study, efforts were directed to find ACE inhibitory activities derived from muscle proteins. Porcine skeletal muscle proteins were hydrolyzed by eight proteases, and the inhibitory activities of the hydrolysates toward ACE were measured. Among the digests of the water-insoluble protein fraction prepared from muscle, thermolysin digest demonstrated the highest activity. Also, among hydrolysates of porcine myosin produced by the same enzymes, thermolysin digest showed the most potent inhibitory activity. Two ACE inhibitory peptides were purified from thermolysin digest of myosin. The sequences of these inhibitory peptides, named myopentapeptides A and B, were Met-Asn-Pro-Pro-Lys and Ile-Thr-Thr-Asn-Pro. These sequences were found in the primary structure of the myosin heavy chain. The concentrations of the peptides showing 50% inhibition values (IC_{50}) of ACE were 945.5 and 549.0 μM, respectively. Also, six tripeptides, Met-Asn-Pro, Asn-Pro-Pro, Pro-Pro-Lys, Ile-Thr-Thr, Thr-Thr-Asn, and Thr-Asn-Pro, which have parts of the sequences of the myopentapeptides, demonstrated activity. Their IC_{50} values were 66.6, 290.5, >1000, 678.2, 672.7, and 207.4 μM, respectively.

Keywords: Angiotensin I-converting enzyme; Myosin; Peptide; Porcine skeletal muscle; Protease digestion

1. Introduction

Angiotensin I-converting enzyme (ACE), which is a dipeptidylcarboxypeptidase, plays an important physiological role in regulating blood pressure (Skeggs, Kahn & Shumway, 1957). ACE converts an inactive form of decapeptide, angiotensin I, to a potent vasoconstrictor, octapeptide angiotensin II, and inactivates bradykinin, which has a depressor action. This enzyme also plays physiological roles in the regulation of local levels of other endogenous peptides, such as enkephalins and substance P. For these reasons, specific inhibitors of ACE are useful for regulating physiological activities associated with ACE in the human body (Ondetti, Rubin & Cushman, 1977).

Several inhibitors of ACE have been found to be effective as antihypertensive pharmaceuticals. ACE inhibitory activity in foods has been also studied (Yamamoto, 1997). ACE inhibitory peptides derived from foods, especially milk proteins (casein and whey proteins), have been reported to show antihypertensive effects in spontaneously hypertensive rats (SHR) by oral administration (Abubakar, Saito, Kitazawa, Kawai & Itoh, 1998; Maruyama, Mitachi, Tanaka, Tomizuka & Suzuki, 1987; Nakamura, Yamamoto, Sakai, Okubo, Yamazaki & Takano, 1995; Yamamoto, Akino & Takano, 1994; Yamamoto, Maeno & Takano, 1999). The antihypertensive effect of sour milk containing two ACE inhibitory peptides (Val-Pro-Pro and Ile-Pro-Pro) derived from casein was demonstrated in SHR (Nakamura, Yamamoto, Sakai & Takano, 1995) and in hypertensive patients (Hata, Yamamoto, Ohni, Nakajima, Nakamura & Takano, 1996). This sour milk product containing bioactive tripeptides has been developed as a new physiologically functional food in Japan (Calpis Food Industry Co., Ltd, Tokyo). Research has also been conducted to characterize the ACE-inhibitory activity derived from other foodstuffs, such as maize (Maruyama, Miyoshi, Kaneko & Tanaka, 1989), eggs (Yoshii, Tachi, Sakamura, Takeyama, Ohba & Itani, 1999), gelatin (Oshima,

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Shimabukuro & Nagasawa, 1979), fish (Seki, Osajima, Matsufuji, Matsui & Osajima, 1995) and fish products (Astawan, Wahyuni, Yasuhara, Yamada, Tadokoro & Maekawa, 1995; Yokoyama, Chiba & Yoshikawa, 1992). However, little is still known about the derivation of ACE-inhibitory activity from muscle proteins of domestic animals (i.e. meat animals).

In the present study, we investigated the ACE inhibitory activity derived from porcine skeletal muscle proteins. Efforts were also made to purify and identify ACE inhibitory peptides from enzymatic hydrolysates of myosin. Such activities and substances could be utilized for producing new healthy meat products, which might open up a new market in the meat industry.

2. Materials and methods

2.1. Materials and reagents

Fresh porcine skeletal muscle (*biceps femoris*) was obtained at a local supermarket. Pepsin (porcine stomach mucosa), trypsin (bovine pancreas) and α-chymotrypsin (bovine pancreas) were purchased from Wako Chemical Co. (Tokyo, Japan). Proteinase K (*Tritirachium album*) and pronase E (*Streptomyces griseus*) were obtained from Merk (Darmstadt, Germany). Ficin (fig tree latex) and papain (*Carica papaya*) were purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Thermolysin (protease type X from *Bacillus thermoproteolyticus*), porcine skeletal muscle myosin, Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) and ACE (from rabbit lung) were obtained from Sigma Chemical Co. (St. Louis, MO). Other chemicals were from Wako Chemicals Co. (Tokyo, Japan).

2.2. Preparation of water-insoluble protein fraction from muscle

Porcine skeletal muscle (*biceps femoris*) was homogenized in a Waring-type blender with two volumes of distilled water. The homogenate was filtered through No. 2 filter paper (Toyo Roshi Kaisha, Tokyo, Japan), and the filtrate was discarded. The homogenate on the filter paper was further washed sufficiently with distilled water to eliminate water-soluble components of muscle. The washed homogenate was lyophilized and used as the water-insoluble protein fraction. Major components of this fraction were confirmed to be myosin, actin and collagen by SDS-polyacrylamide gel electrophoresis (data not shown).

2.3. Digestion of muscle proteins with proteases

Eight kinds of proteases (trypsin, α-chymotrypsin, pronase E, proteinase K, thermolysin, ficin, papain, pepsin) were used for the digestion of muscle proteins. The water-insoluble protein fraction of porcine muscle (10 g) was suspended in distilled water (90 ml) and one of eight proteases (1 mg) was added. After 18 h of digestion at 37°C, each solution was heated at 98°C for 10 min to inactivate the protease. After removal of insoluble materials by centrifugation (3000×***g*** for 20 min), the supernatant solution was used for the measurement of ACE-inhibitory activity. Similarly, porcine skeletal muscle myosin (10 mg) was suspended in distilled water (10 ml) and protease (1 mg) was added. Further procedures were carried out under the same conditions at those for the digestion of the water-insoluble protein fraction.

2.4. Purification of ACE-inhibitory peptides

Porcine skeletal muscle myosin (100 mg) was suspended in distilled water (100 ml), and thermolysin (1 mg) was added. After 18 h of digestion at 37°C, the solution was heated for 10 min at 98°C. The heated solution was centrifuged at 3000×***g*** for 20 min and the precipitate was removed. The supernatant solution was fractionated by high-performance liquid chromatography (HPLC) with reversed-phase mode (column: CAPCELL PAK C18 UG120 4.6×150 mm; Shiseido, Tokyo, Japan). Elution was performed with a linear gradient system from solvent A (0.1% trifluoroacetic acid in distilled water) to solvent B (0.1% trifluoroacetic acid in CH3CN) at a flow rate of 1 ml/min, and absorbance was detected at 215 nm (first HPLC run). The active fraction was lyophilized, dissolved with distilled water, and rechromatographed under the same conditions as described above (second HPLC run). The peptide samples were further purified by HPLC with the same system except for the elution solution (third HPLC run). Elution was performed with a linear gradient (solvent A: 0.015% ammonia in distilled water, solvent B: 0.015% ammonia in CH_3CN).

2.5. Assay of ACE inhibitory activity

The ACE inhibitory activity was measured according to the method of Cushman and Cheung (1971) with modification by Nakamura, Yamamoto, Saki, Okubo et al. (1995). This assay is based on the liberation of hippuric acid from Hip-His-Leu catalyzed by ACE. The method was slightly modified in the present study. A sample solution of peptides (15 μl) was mixed with 125 μl of 100 mM sodium borate buffer (pH 8.3) containing 7.6 mM Hip-His-Leu and 608 mM NaCl and then preincubated for 5 min at 37°C. The reaction was initiated by the addition of 50 μl of ACE dissolved in distilled water (50 m units/ml), and the mixture was incubated for 30 min at 37°C. The reaction was stopped by adding 125 μl of 1N HCl. The hippuric acid liberated by ACE was photometrically determined at 228 nm after ethyl

acetate extraction. The concentration of ACE inhibitors needed to inhibit 50% of ACE activity was defined as the IC_{50} value.

2.6. Analysis of peptides

The molecular formula of each peptide was confirmed from its fast atom bombardment mass spectrum (FAB-MS) obtained using an HX-110 spectrometer (JEOL Ltd, Tokyo, Japan). The sequence of each peptide was analyzed by automated Edman degradation using a 470A Protein Sequencer (Applied Biosystems, Inc., Forster City, CA).

2.7. Synthesis of peptides

The six kinds of tripeptides and two kinds of pentapeptides used in this study were synthesized by the solid phase method with a 430A Peptide Synthesizer (Applied Biosystems, Inc.). Hydrogen fluoride was used for removing the side chain-protecting groups and for cleaving peptides from their solid support. The synthesized peptides were purified by HPLC on a reversed-phase column (CAPCELL PAK C18 UG120 4.6×150 mm; Shiseido, Tokyo) with a linear gradient of CH_3CN (0 to 20%) in 0.1% trifluoroacetic acid.

3. Results

3.1. ACE inhibitory activity of hydrolysates of skeletal muscle proteins

Table 1 shows the ACE inhibitory activity of enzymatic hydrolysates of the water-insoluble protein fraction (mainly myosin and actin) and myosin of porcine skeletal muscle. The results indicated that all enzymes generated ACE inhibitory activities from muscle proteins, but thermolysin was the most suitable for induction of high activity.

3.2. Purification of ACE inhibitory peptides

The digest of myosin produced by thermolysin was fractionated by reversed-phase HPLC (Fig. 1a, first HPLC run). Since ACE inhibitory activities were widely distributed in fractions, it was expected that many ACE inhibitory peptides would be produced from myosin by thermolysin digestion. The fraction with the highest inhibitory activity (25–30 min) was further purified by the same procedure (Fig. 1b, second HPLC run). From the two active fractions shown in Fig. 1b, two peptides with high ACE-inhibitory activity were purified through further chromatography (Fig. 2, third HPLC run). Two peptides purified by three-step HPLC were utilized for sequencing by a protein sequencer with automatic Edman degradation. The purity of these peptides was confirmed by HPLC with several conditions (data not shown).

Table 1
Derivation of the inhibitory activity of angiotensin converting enzyme (ACE) from the water-insoluble protein fraction and myosin of porcine skeletal muscle after digestion with one of eight proteases

Protease	ACE inhibitory activity (%)	
	Water-insoluble protein fraction	Myosin
Thermolysin	86.9	71.8
Proteinase K	82.1	61.7
Pronase E	70.4	35.9
Ficin	62.5	60.6
Papain	74.2	59.2
Trypsin	32.7	60.2
α-Chymotrypsin	65.8	70.1
Pepsin	30.1	61.4

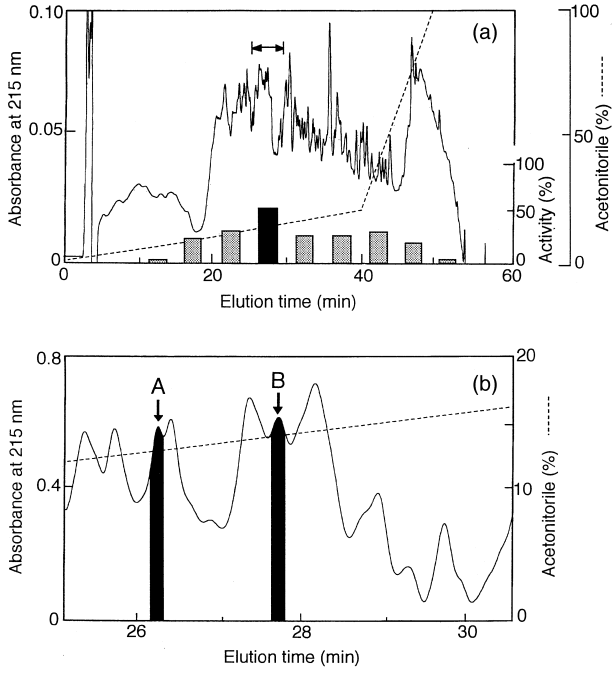

Fig. 1. Fractionation and assay of fractions from the thermolysin hydrolysate of myosin by high performance liquid chromatography (HPLC). (a) Hydrolysate of myosin was loaded on a reversed-phase column (first HPLC run). (b) The fraction of 25–30 min of the first HPLC run was loaded on the same column (second HPLC run). Arrows indicate active fractions. Chromatography and assay of angiotensin converting enzyme (ACE) inhibitory activity were performed as described in Materials and methods.

3.3. Structural analysis of ACE inhibitory peptides

The amino acid sequences of two ACE inhibitory peptides were determined, and the peptides were named myopentapeptides A and B (Table 2). A search for sequence homology in databases revealed that the same sequences existed in the primary structure of the porcine skeletal muscle myosin heavy chain (Chikuni, K., unpublished data [DDBJ/EMBL/GenBank accession nos. AB025260, AB025261 and AB025262]).

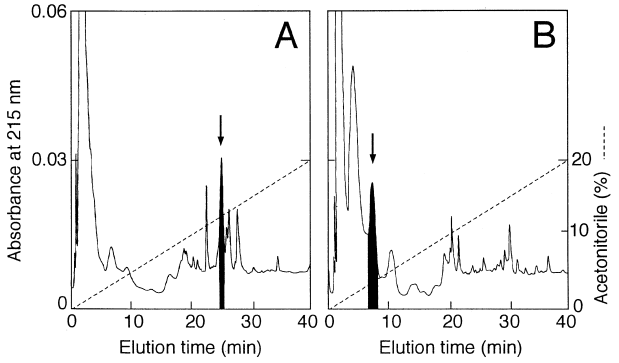

Fig. 2. Purification of angiotensin converting enzyme (ACE) inhibitory peptides derived from myosin by reversed-phase high performance liquid chromatography (HPLC) (third HPLC run). Fig. 2A and 2B show the HPLC run of fractions A and B obtained from the second HPLC run (Fig. 1b), respectively. Arrows indicate active fractions. Chromatography and assay of ACE inhibitory activity were perfomed as described in Materials and methods.

Table 2
Angiotensin converting enzyme (ACE) inhibitory peptides derive from porcine myosin

Myopentapeptide	Sequence	Position in myosin[a]
A	Met-Asn-Pro-Pro-Lys	79–83
B	Ile-Thr-Thr-Asn-Pro	306–310

[a] Position of respective peptides in the seqeunce of the porcine skeletal muscle myosin heavy chain.

3.4. ACE inhibitory activity of synthetic peptides

Myopentapeptides A and B were synthesized according to the amino acid sequences determined in this study. Also, six tripeptides, Met-Asn-Pro, Asn-Pro-Pro, Pro-Pro-Lys, Ile-Thr-Thr, Thr-Thr-Asn, and Thr-Asn-Pro, which have parts of the sequences of the myopentapeptides, were synthesized. The ACE inhibitory activities of eight synthetic peptides are shown in Table 3.

4. Discussion

In the present study, ACE inhibitory activity was detected in the hydrolysates of porcine skeletal muscle proteins. Also, ACE inhibitory peptides (myopentapeptides A and B) from the thermolysin hydrolysate of porcine myosin were purified and identified. To the best of our knowledge, this is the first report of the ACE inhibitory peptides derived from muscle proteins of domestic animals. Since the sequences of myopentapeptides A and B were not only found in the primary structure of the porcine myosin heavy chain but also in those of myosin of the rat (80–84, 304–308), chicken (80–84, 305–309) and human (82–86, 306–310), these sequences are thought to be presence in the myosin of various species, including meat animals.

Table 3
Angoitensin coverting enzyme (ACE) inhibitory activity of synthetic peptides

Sequence	IC_{50} (μM)[a]
Met-Asn-Pro-Pro-Lys	945.5
Met-Asn-Pro	66.6
Asn-Pro-Pro	290.5
Pro-Pro-Lys	> 1000
Ile-Thr-Thr-Asn-Pro	549.0
Ile-Thr-Thr	678.5
Thr-Thr-Asn	672.7
Thr-Asn-Pro	207.4

[a] The concentration of peptide needed to inhibit 50% of the ACE activity.

All of the synthetic peptides used in this study were first found as ACE inhibitors. Several ACE inhibitory peptides derived from fish muscle proteins have been reported (Astawan et al., 1995; Seki et al., 1995; Yamamoto, 1997; Yokoyama et al., 1992). However, the sequences of these peptides are not identical to those of the peptides used in this study. Although the activity of myopentapeptides is relatively low compared with other ACE inhibitory peptides derived from food proteins (Yamamoto, 1997), the activities of synthetic tripeptides, especially Met-Asn-Pro, Asn-Pro-Pro and Thr-Asn-Pro, are higher than those of the respective original myopentapeptides (Table 3). Since small peptides are more easily absorbed in the intestinal tract than larger peptides (Hara, Funabiki, Iwata & Yamazaki, 1984) and since peptides containing proline are generally resistant to the enzymatic digestion (Kim, Bertwhistle & Kim, 1972), it is expected that these tripeptides containing proline would have sufficient antihypertensive activities in vivo. Further studies are needed to measure the activities by using spontaneously hypertensive rats. Such experiments are now in progress in our laboratory.

The results of this study suggest that ACE inhibitory peptides are easily generated from muscle proteins by enzymatic digestion. Thus, in meat products, such as fermented meat products with long-term ripening, ACE inhibitory peptides could be generated. In fact, we have detected ACE inhibitory activity in several commercial fermented meat products and model sausages fermented with some lactic acid bacteria (data not shown). However, we have not confirmed that these activities are from peptides. Naturally occurring ACE inhibitory activity has also been detected in cheese (Ito, Kawata, Yamano, Kakiichi, Hatsuoka & Yokoyama, 1987; Meisel, Goepfert & Gunther, 1997). On the other hand, digestive enzymes (i.e. pepsin, trypsin and α-chymotrypsin) in gastrointestinal tracts generated ACE inhibitory activity from muscle proteins (Table 1). Therefore, it is thought

that ACE inhibitory activity could be generated in the gastrointestinal tract by ingestion of meat.

In the dairy industry, many physiologically functional foods have been developed. Basic studies on the tertiary function of milk components and on physiologically functional dairy products have been extensively conducted (Arai, 1996). However, there have been few such studies on meat products. Although many low-fat and low-salt meat products have been developed, there have been no efforts to introduce physiologically functional properties into meat products. We recently reported that the concept of probiotics (cultures of live microorganisms that benefit the host by improving properties of indigenous microflora) has great potential in the meat industry (Arihara et al., 1998; Sameshima, Magome, Takeshita, Arihara, Itoh & Kondo, 1998). By using bioactive components, properties having potential health benefits can be introduced into meat products, thus improving the value of the products. Utilization of ACE inhibitory activity and substances from muscle proteins could lead to the development of new healthy meat products.

5. Conclusions

This study demonstrated that ACE inhibitory activities can be generated from muscle proteins by enzymatic hydrolysis. Also, protease-digestion of myosin resulted in the generation of ACE inhibitory peptides. The results of this study suggest that ACE inhibitory activities derived from muscle proteins could be utilized to develop physiologically functional foods. Although bioactive peptides, such as ACE inhibitors, have not yet been utilized in the meat industry, meat products with such activity could open up a new market in the near future. It is expected that increasing interest will be shown in basic research and potential applications of bioactive peptides for meat products.

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