Angiotensin I-converting Enzyme Inhibitory Activities of Porcine Skeletal Muscle Proteins Following Enzyme Digestion

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ABSTRACT : Inhibitory activities against angiotensin I-converting enzyme (ACE) of enzymatic hydrolysates of porcine skeletal muscle proteins were investigated. Myosin B, myosin, actin, tropomyosin, troponin and water-soluble proteins extracted from pork loin were digested by eight kinds of proteases, including pepsin, α-chymotrypsin, and trypsin. After digestion, hydrolysates produced from all proteins showed ACE inhibitory activities, and the peptic hydrolysate showed the strongest activity. In the case of myosin B, the molar concentration of peptic hydrolysate required to inhibit 50% of the activity increased gradually as digestion proceeded. The hydrolysates produced by sequential digestion with pepsin and α-chymotrypsin, pepsin and trypsin or pepsin and pancreatin showed weaker activities than those by pepsin alone, suggesting that ACE inhibitory peptides from peptic digestion might lose their active sequences after digestion by the second protease. However, the hydrolysates produced by sequential digestion showed stronger activities than those by α-chymotrypsin, trypsin or pancreatin alone. These results suggested that the hydrolysates of porcine meat were able to show ACE inhibitory activity, even if they were digested *in vivo*, and that pork might be a useful source of physiologically functional factors. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 3 : 417-424)***

Key Words : Angiotensin I-Converting Enzyme, Peptide, Porcine Skeletal Protein, Protease Digestion

INTRODUCTION

The functions of food may be classified into three categories, namely the primary (supplying nutrients and energy), the secondary (taste and palatability) and the tertiary function (biological defense or wholesomeness). The tertiary functions of many foods have been investigated, but meat has not been fully investigated to date. Meat is known to be nutritionally excellent and to taste good, but the harmful or disadvantageous aspects, such as the allergenicity of proteins, are sometimes emphasized. In discussing nutrition, excess intake of animal fat is generally assumed to have a harmful influence on our circulation system. This biased information is one of the major obstacles to increasing meat demand in Japan. Many tertiary functions of proteineous foods are speculated to be caused by peptides. Meats contain significant amounts of protein, and it is therefore possible that physiologically functional peptides will be produced upon digestion.

Pork is the meat with the highest rate of consumption in Japan and it is used as one of the primary materials in many meat products. If it can be demonstrated that pork contains biologically active components, pork consumption will rise and the meat industry will be expected to develop new functional foods incorporating pork components.

Recently, many biologically active peptides from food proteins have been investigated. Inhibitors of angiotensin I-converting enzyme (ACE) have attracted particular attention for their ability to prevent hypertension. ACE catalyzes the conversion of angiotensin I to angiotensin II, which causes hypertension, and inactivates bradykinin, which is antihypertensive. ACE inhibitory peptides have been studied in many other foods, such as dried bonito (Yokoyama et al., 1992), sardine muscle (Suetsuna and Osajima, 1986; Kawamura et al., 1989; Sugiyama et al., 1991; Matsui et al., 1993), krill (Kawamura et al., 1992), tuna muscle (Kohama et al., 1988), casein (Maruyama et al., 1985), sake lees (Saito et al., 1994), oysters (Matsumoto et al., 1994), whey protein (Eto et al., 1999), hen's eggs (Yoshii et al., 1999), and chum salmon (Ohta et al., 1999). ACE inhibitory peptides from chicken breast muscle (Fujita et al., 2000) and porcine skeletal muscle, *biceps femoris*, (Arihara et al., 2001; Nakashima et al., 2002) were reported. They isolated those peptides from the meat hydrolysates with thermolysin, and reported their effectiveness as hypotensive foods. Although Arihara et al. (2001) studied ACE inhibitory activity of hydrolysates of porcine water-insoluble proteins with digestive protease; they used native proteins as substrates and did not report the activities expressed as the protein concentration producing 50% inhibition (IC_{50}) of those hydrolysates in assays. Since meats are mainly taken after cooking, investigations with denatured proteins and their hydrolysates with digestive enzymes are needed for discussion of their usefulness as

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biologically active foods. Moreover, since there is no report on enzymatic hydrolysates of each protein in porcine meat, ACE inhibitory peptides from individual proteins of porcine meat need to be investigated as well as whole porcine meat.

In this study, we investigated the enzymatic digestion profiles of porcine skeletal muscle proteins and ACE inhibitory activities of the hydrolysates derived from some of these proteins. Then, an artificial digestion of proteins with digestive proteases was performed and the usefulness of pork as an ACE inhibitory food was discussed.

MATERIALS AND METHODS

Preparation of porcine skeletal muscle proteins

Porcine skeletal muscle proteins were extracted from a pork loin (*Longissimus dorsi*) purchased from Marudai Shimane Farm Co., Ltd. (Shimane, Japan). The kinds of extracted protein were myosin B (including myosin, actin, tropomyosin, troponin and other myofibrillar structural proteins), myosin, actin, tropomyosin, troponin and two kinds of water-soluble proteins.

Myosin B (Szent-Györgyi, 1951) was extracted with Weber-Edsall solution (0.6 M KCl, 0.04 M $NaHCO_3$, 0.01 M Na_2CO_3) including 1 mM NaN_3 and myosin (Margossian and Lowey, 1982) with Guba-Straub solution (0.3 M KCl, 0.1 M KH_2PO_4, 0.05 M K_2HPO_4) including 2 mM adenosine 5'-triphosphate (ATP). The residual meat after extraction of myosin was defatted with acetone and dried to be an acetone powder. Actin (Joel and James, 1982) was extracted from the acetone powder with a buffer A (2 mM Tris, 0.2 mM ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, 1 mM NaN_3, pH 8.0), and recovered by polymerizing with adding KCl to 50 mM, $MgCl_2$ to 2 mM and $CaCl_2$ to 0.1 mM. Then, KCl was added into this solution to a final concentration of 0.6 M and the obtained pellet was dissolved with the buffer A. Tropomyosin (Smillie, 1982) was extracted with a high salt buffer (1 M KCl, 25 mM Tris, 0.1 mM $CaCl_2$, 0.1 mM dithiothreitol (DTT), 1 mM NaN_3, pH 8.0) from an acetone powder made from meat washed by a physiological saline (145 mM NaCl, 10 mM Na-Phosphate, pH 7.1). Hydrochloride was added to this extract until pH 4.6, and pellets from the extract were dissolved with 0.5 mM DTT at pH 7.0. The pellets were separated using two different concentrations of ammonium sulfate (AS; 50% and 65%). Troponin (Potter, 1982) was extracted with a high salt buffer from an acetone powder made from meat washed by low salt solution (1% Triton X-100, 50 mM KCl, 5 mM Tris, pH 8.0). Hydrochloric acid was added to this extract until pH 4.6, and the supernatant was separated using two different concentrations of AS (40% and 60%) at pH 7.0. Water-soluble proteins (WSP) were extracted with the physiological saline and designated as WSP45 or WSP80, when the concentration of AS used to recover it was 45% or 80%, respectively.

KCl and AS, which interfere the electrophoresis, were removed from the prepared proteins by dialysis; myosin B, myosin, WSP45 and WSP80 against phosphate-buffered saline (0.8% NaCl, 10 mM Na-Phosphate, pH 7.5) before the experiments; tropomyosin and troponin against the high salt buffer; myosin B for the time-course experiment against 2 mM Tris-HCl buffer with 0.6 M NaCl (pH 7.5).

Hydrolysis by enzymes

Endoproteinase Glu-C (Glu-C), endoproteinase Lys-C (Lys-C), endoproteinase Asp-N (Asp-N) and modified trypsin prepared to suppress autolysis were obtained from Böhringer Mannheim Biochemica Co., Ltd. (Mannheim, Germany); pepsin, α-chymotrypsin and trypsin from Sigma Chemical Co. (St. Louis, MO, USA); and pancreatin from Nacalai Tesque Inc. (Kyoto, Japan).

Five mg/ml of each crude protein was suspended in buffer. Every protein was either denatured or not by heating at 98°C for 10 min, and was then hydrolyzed with each enzyme. The ratio of enzyme to substrate was 1/100 (w/w). For pepsin, buffer pH was adjusted to 2.0 with 1 M HCl, and after digestion it was adjusted to 7.5 with 1 M NaOH. The incubation temperature was 25°C for Glu-C and α-chymotrypsin to prevent autolysis, and 37°C for all other enzymes. After incubation, the reaction was terminated by boiling for 10 min followed by cooling in ice. The solution was centrifuged for 20 min at 18,000 g, and the supernatant was collected for the ACE inhibitory experiments.

Electrophoresis

Two kinds of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed. The first, used for myosin, WSP45 and WSP80, was conducted following Laemmli's (1970) method using a gradient (7.5-17.5% acrylamide) slab gel; and the second, used for actin, tropomyosin and troponin, made use of a tricine system (Schägger and Jagow, 1987) that was optimized for low molecular proteins or peptides. Protein bands were stained with Coomassie Brilliant Blue R-250 (CBB).

Assay for angiotensin I-converting enzyme inhibitory activity

The ACE inhibitory assay was performed using a slight modification of the method of Cushman and Cheung (1971), as well as that of Lieberman (1975) as modified by Yamamoto et al. (1980). Briefly, 30 μl of sample was added to 250 μl of 0.1 M borate buffer (pH 8.5) including 7.6 mM hippuryl-L-histidyl-L-leucine (HHL, Nacalai Tesque) as substrate and 0.608 M NaCl. The reaction was started by the addition of 100 μl of 60 mU/ml rabbit lung ACE (Wako Pure Chemical Industries, Ltd., Osaka, Japan) or bovine lung ACE (Wako Pure Chemical Industries) in 0.25 M

borate buffer (pH 8.5). To terminate the enzyme reaction, 250 µl of 1 M hydrochloric acid 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 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 (hydrochloric acid was added before ACE)

Inhibitory activity was expressed as IC_{50} of the sample in an assay. An increase in IC_{50} indicates a decrease in ACE inhibitory activity.

Determination of protein

For myosin B, myosin and the WSPs, the protein concentrations of the crude extracted proteins and their hydrolysates were measured by the biuret method (Gornall et al., 1949) using bovine serum albumin as standard. For actin, tropomyosin and troponin, those of the crude extracted proteins were measured by the method of Bradford (1976) using bovine immunoglobulin G as standard; and those of their hydrolysates by the UV method (Murphy and Kies, 1960) using wavelengths of 215 and 225 nm. Molar concentrations of peptides were expressed in terms of the amino groups of peptides or free amino acids in the supernatant of the hydrolysate, which was measured using the trinitrobenzene sulfonate (TNBS) method (Hazra et al., 1984).

RESULTS AND DISCUSSION

Enzymatic digestion of porcine skeletal muscle proteins

Since meats are mainly eaten after cooking, we first investigated whether there was any difference between native and denatured protein in their digestive profiles with several proteases. For this purpose, crude myosin, which included mainly myosin and other myofibrillar structural proteins, was hydrolyzed by 7 kinds of protease (Glu-C, Lys-C, Asp-N, modified trypsin, pepsin, α-chymotrypsin, and trypsin). Native myosin (unheated) and denatured myosin by heating at 98°C for 10 min (heated) were used as substrates. Hydrolysis was performed for 0.5-24 h and the degree of protein hydrolysis was evaluated by SDS-PAGE (Figure 1). The SDS-PAGE patterns showed that the denatured myosin was more digestible than native myosin by every protease used. The hydrolysis by pepsin, α-

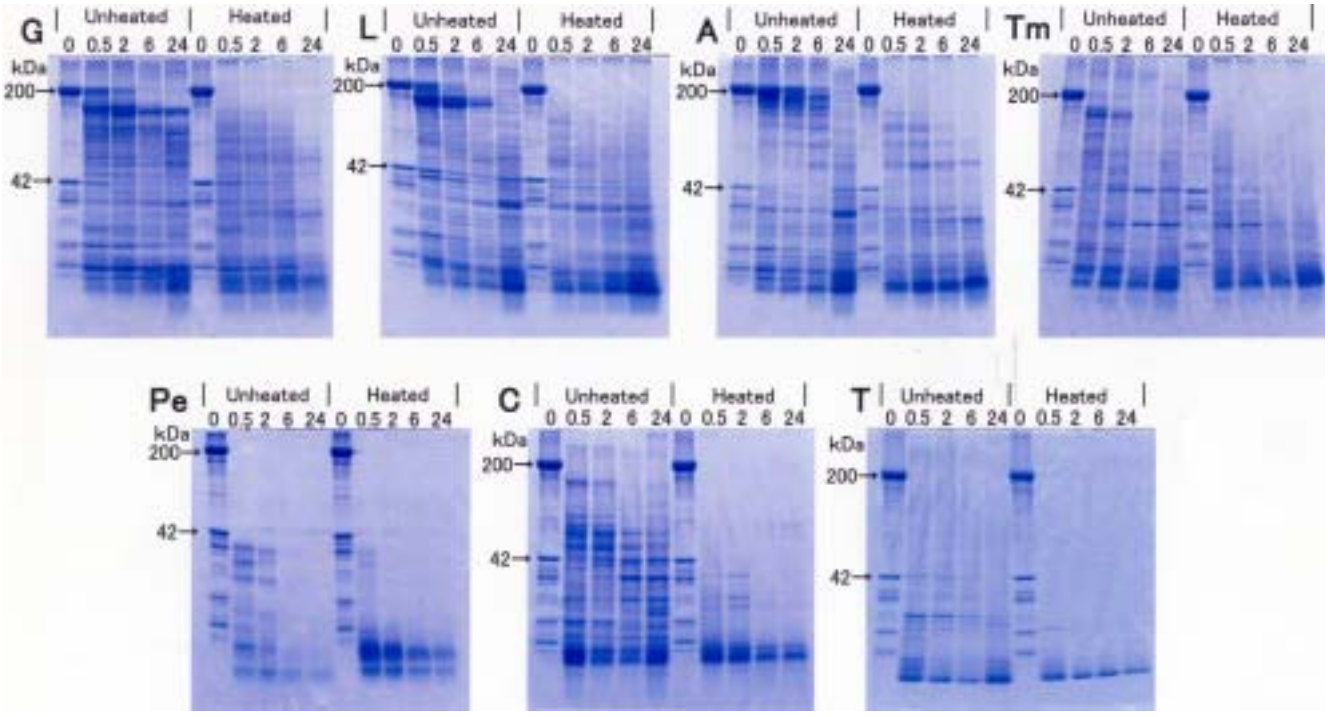

Figure 1. SDS-PAGE profiles of crude myosin hydrolyzed with various enzymes. Native myosin (unheated) and myosin denatured by heating at 98°C for 10 min (heated) were hydrolyzed with 7 kinds of enzyme, respectively. G: endoproteinase Glu-C, L: endoproteinase Lys-C, A: endoproteinase Asp-N, Tm: modified trypsin, Pe: pepsin, C: α-chymotrypsin, T: trypsin, 0-24: incubation time for hydrolysis, 200 kDa: myosin heavy chain, 42 kDa: actin.

chymotrypsin and trypsin proceeded especially quickly. The hydrolysis of denatured myosin was almost complete after 6 h for all proteases. Especially, myosin heavy chain, which was a major component of crude myosin, was quickly digested. This result indicated that cooking of meat protein increased the digestibility of that protein. On the other hand, hydrolysis with modified trypsin was slower than that with native trypsin. The hydrolysates by typical endoproteinases (Glu-C, Lys-C and Asp-N) showed many high molecular-weight proteins, indicating that fewer sites were cleaved by these proteinases than by digestive proteases.

Since the hydrolysis of denatured crude myosin proceeded quickly, the digestibility of some proteins in porcine skeletal muscle was evaluated to estimate the digestibility of the whole muscle. Actin, tropomyosin, troponin, and the two kinds of WSP were either denatured or not by heating at 98°C for 10 min, and were then hydrolyzed by pepsin, α-chymotrypsin or trypsin. The SDS-PAGE patterns showed that almost all denatured substrates were quickly digested (Figure 2, lane 0-24) suggesting that the digestibility of these heat-denatured proteins after oral administration was considered to be excellent. The hydrolysates produced by each protease for 6 h were centrifuged at 18,000 g for 20 min, and their supernatants were collected and subjected to SDS-PAGE analysis (Figure 2, lane S). The SDS-PAGE patterns of the supernatants showed only a small number of bands with relatively low molecular weights, indicating that the whole hydrolysate probably included insoluble substrates in spite of 6 h digestion. Both native WSPs tended to be less digestible than the denatured substrates. On the other hand, native actin, tropomyosin and troponin showed no significant differences from the corresponding denatured substrates (Figure 2, lane 6B), suggesting that the enzymes easily reacted to native proteins due to their conformational changes by reducing agent in buffer, as well as to heat-denatured proteins. In this experiment, there were some lanes with few or no bands, probably because the hydrolysates in these lanes were at concentrations that were too low to be stained by CBB or to remain in the gels.

Angiotensin I-converting enzyme inhibitory activities of denatured protein hydrolysates produced by various proteases

Because the hydrolysis of heat-denatured proteins was almost complete after 6 h (Figures 1 and 2), ACE inhibitory activities of their hydrolysates (supernatants) were measured to estimate their activities *in vivo*. All proteins in Figures 1 and 2 and myosin B, which included whole myofibrillar structural proteins, were used as substrates after heat-denaturation; and all proteases in Figure 1 were used, respectively. First, the inhibitory activities against rabbit ACE are discussed (Table 1). The hydrolysates from every protein hydrolyzed by pepsin or trypsin, which are digestive enzymes, showed relatively strong activity. Some hydrolysates by Glu-C, Lys-C, modified trypsin or α-chymotrypsin also showed strong activity. These activities were similar to or higher than those of the hydrolysates previously reported (Yokoyama et al., 1992; Fujita et al., 2000). These results suggested that every protein extracted from porcine skeletal muscle included some highly active peptide sequences. The activity of myosin B, which amounts to 50% of meat protein (Hattori, 1996), was especially noteworthy, and supports the suggestion that meat is a biologically active food. Since some reports have indicated the hypotensive activities of ACE inhibitory hydrolysates, derived from fish (Sugiyama et al., 1991), whey protein (Eto et al., 1999), hen's eggs (Yoshii et al.,

Table 1. Angiotensin I-converting enzyme (ACE) inhibitory activities of enzymatic hydrolysates from pork protein denatured by heating at 98°C for 10 min

Protein	IC_{50} (μg/ml)							
	Not digested	Glu-C	Lys-C	Asp-N	Trypsin, modified	Pepsin	Chymo-trypsin	Trypsin
Rabbit ACE								
Myosin B	2,000<	333	218	2,000<	839	47	112	107
Myosin		456	103	2,000<	550	112	121	166
Actin						141	182	56
Tropomyosin						201	201	243
Troponin						130	304	104
WSP45	2,000<	1,679	89	2,000<	192	65	116	123
WSP80	2,000<	727	1,274	2,000<	232	48	83	119
Bovine ACE								
Myosin B						115	234	211
Myosin							264	
Actin						139	272	61
Tropomyosin						321	291	327
Troponin						225	480	152

Blank cells: Not tested.

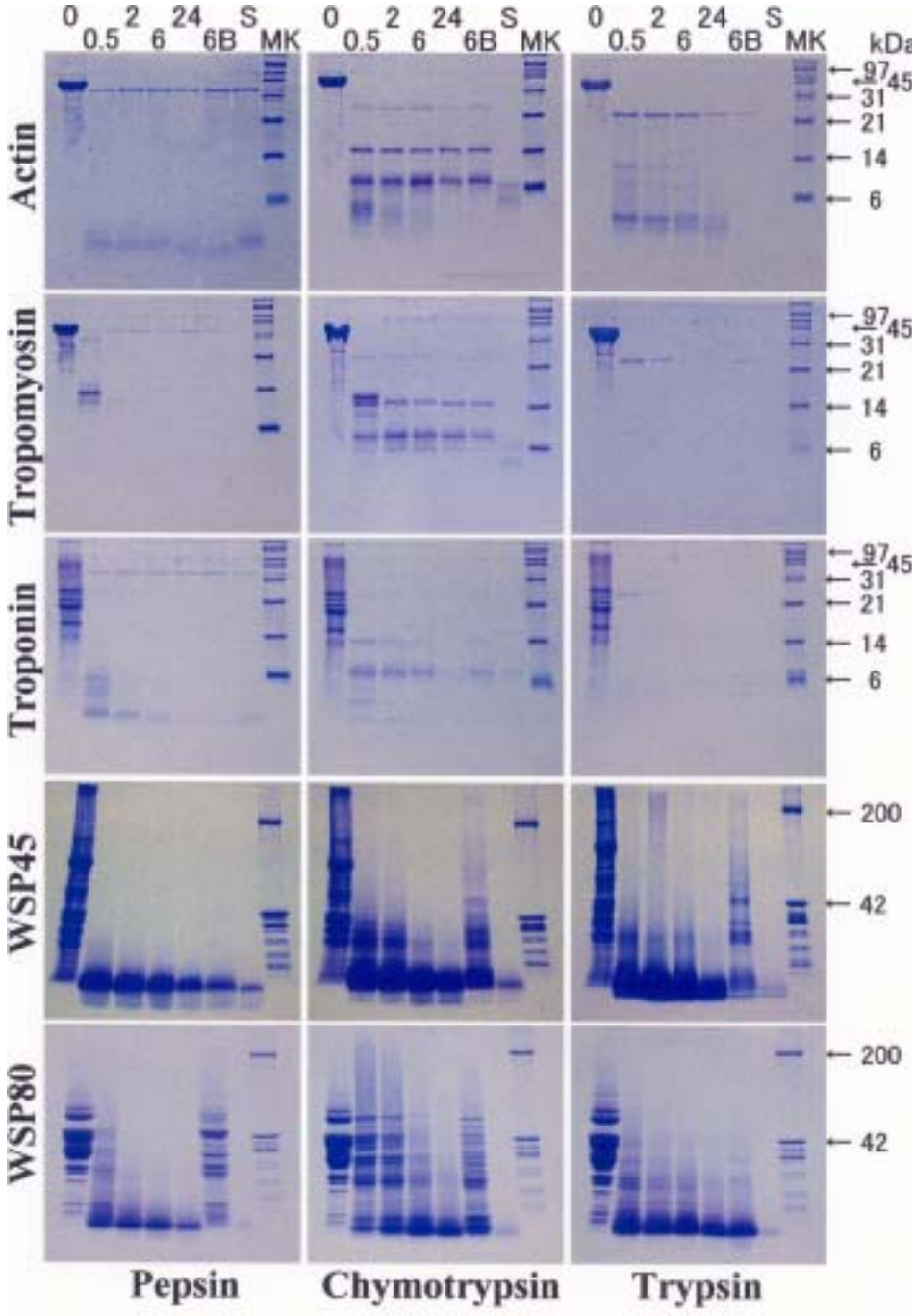

Figure 2. SDS-PAGE profiles of enzymatic hydrolysates of proteins derived form porcine skeletal muscle. Every protein was either denatured or not by heating at 98°C for 10 min, and was then hydrolyzed with each enzyme. 0-24: incubation time for hydrolysis of denatured protein, 6B: hydrolysate of native protein incubated for 6 h, S: supernatant of denatured protein hydrolysates incubated for 6 h, MK: molecular weight marker.

1999), and porcine myosin and water-insoluble protein (Nakashima et al., 2002), after oral administration to spontaneously hypertensive rats, porcine protein hydrolysates prepared here were expected to be useful as hypotensive foods without separation of the active peptides. ACE inhibitory peptides from actin (Yokoyama et al., 1992) and myosin (Arihara et al., 2001) have been reported previously, and our results suggest that other proteins in meat might have active peptide sequences in their primary structures as well.

The inhibitory activities of some hydrolysates against rabbit and bovine ACE were tested, and their activities were found to be quite similar, but with some small differences, as previously reported by Kohama et al. (1988). This result

suggests that these hydrolysates served as ACE inhibitors regardless of the ACE origin. A detailed evaluation of these data indicated that the IC_{50} against bovine ACE tended to be larger than rabbit. The reason for this tendency was not clear, and the difference between both ACEs should be investigated further. The IC_{50} of Pro-Thr-His-Ile-Lys-Trp-Gly-Asp, an ACE inhibitory peptide previously reported by Kohama et al. (1988), was 0.9 μM for bovine ACE and 4.5 μM for rabbit ACE in our experiment. These activities were similar to the results (1 and 2 μM, respectively) of Kohama et al. (1988), and they indicate that the experiment was operating properly.

The hydrolysates made with Lys-C, pepsin, or trypsin showed relatively strong activity, although dipeptides with a basic amino acid at the carboxyl termini did not show very strong activity (Cheung et al., 1980). This result suggests that amino acid sequences other than those of the amino or carboxyl termini contributed to the activity. These sequences appear to participate in the affinity to ACE or the conformation change of ACE.

Time course of angiotensin I-converting enzyme inhibitory activities of protein hydrolysates

As myosin B hydrolysate showed ACE inhibitory activity (Table 1), an experiment to clarify how the activity emerged in the digestive process was performed. Myosin B denatured by heating at 98°C for 10 min was hydrolyzed by pepsin, α-chymotrypsin, trypsin or pancreatin, and both the degree of hydrolysis and the ACE inhibitory activities were evaluated over time (Figure 3). The hydrolysis by all proteases proceeded quickly up to 30 min, and the liberation of peptides from the substrate increased as digestion proceeded (Figure 3). The production of peptides and amino acids by pepsin from 4 to 6 h were more than those by the other proteases, probably because of the broad substrate specificity. The inhibitory activity of the peptic hydrolysate against bovine ACE increased with progress of the digestion, whereas the undigested protein (0 h) showed very weak activity (Figure 3). The IC_{50} (μM) of the peptic hydrolysate was a minimum at 30 min, and gradually increased as hydrolysis proceeded. This suggested that the ACE inhibitory peptides generated at the early stage of hydrolysis were further cleaved a little and decreased. The relatively strong activity, however, remained even after 24 h digestion, suggesting that the ACE inhibitory peptides liberated in the stomach are transferred in a relatively active state to the small intestine.

Angiotensin I-converting enzyme inhibitory activities of peptic hydrolysates of myosin B by further digestion with α-chymotrypsin, trypsin or pancreatin

Because the hydrolysates produced by digestive enzymes, especially pepsin, showed relatively strong ACE inhibitory activities (Table 1, Figure 3), heat-denatured myosin B digested by pepsin for 2 h was further digested by intestinal proteases (α-chymotrypsin, trypsin or pancreatin) for 2 h, and the ACE inhibitory activities of the resulting hydrolysates were measured (Figure 4). The hydrolysates produced by sequential digestion with two proteases showed weaker inhibitory activities than those by peptic digestion alone, possibly because some ACE inhibitory peptides from peptic digestion lost their active sequences after digestion by the other proteases. However, the increase in the IC_{50} value after the sequential digestion was not very large, and the hydrolysates produced by sequential digestion

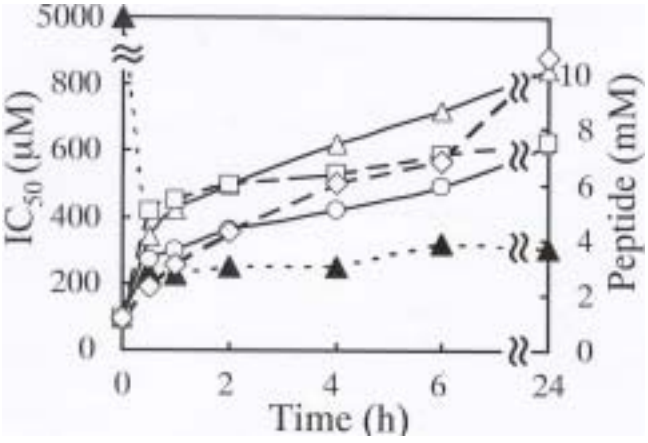

Figure 3. Changes in peptide concentration with increasing time of digestion of myosin B with pepsin (△), α-chymotrypsin (○), trypsin (□), or pancreatin (◇), and changes in the IC_{50} value for a peptic hydrolysate with increasing time (▲). Myosin B was denatured by heating at 98°C for 10 min before digestion.

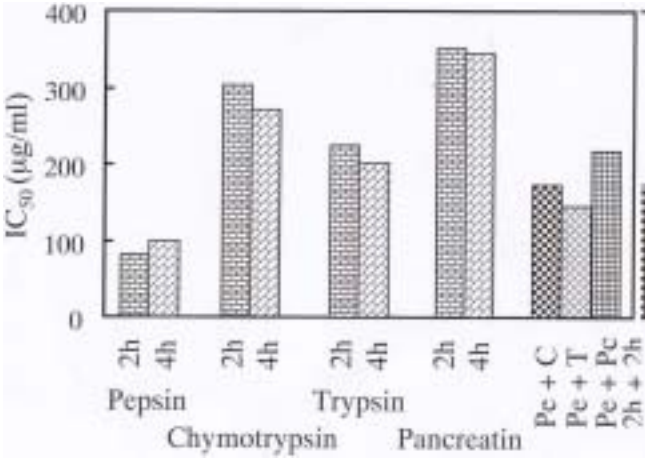

Figure 4. ACE inhibitory activities of enzymatic hydrolysates of porcine myosin B. Myosin B was denatured by heating at 98°C for 10 min and digested for 2 or 4 h by enzyme indicated in figure, respectively. Peptic digested myosin B for 2 h was digested for 2 h by other enzyme indicated in figure, respectively. Pe: pepsin, C: α-chymotrypsin, T: trypsin, Pc: pancreatin.

with two proteases showed stronger activities than those by α-chymotrypsin, trypsin or pancreatin alone (2 h and 4 h). This indicated that sequential digestion by pepsin and α-chymotrypsin, pepsin and trypsin or pepsin and pancreatin in this order effectively produced stronger peptides than that by α-chymotrypsin, trypsin or pancreatin alone.

Pancreatin is a mixture of α-chymotrypsin, trypsin and the other pancreatic enzymes. So, the sequential digestion by pepsin then pancreatin provides a proper model to estimate the fate of ACE inhibitory peptides in the gut system. The relatively high resistance of the peptic hydrolysates of myosin B to the pancreatic enzymes (Figure 4) raises the possibility that ACE inhibitory peptides from porcine myosin B may be absorbed from the intestine *in vivo*. Di- and tripeptides have been reported to be resistant to digestive enzymes and to be absorbed in the intestine (Adibi et al., 1969; Craft et al., 1968; Li et al., 1999). Recently, several studies (Yokoyama et al., 1992; Matsumoto et al., 1994; Eto et al., 1999; Ohta et al., 1999; Yoshii et al., 1999; Fujita et al., 2000; Arihara et al., 2001) have been conducted to design digestive enzyme-resistant peptides made by microbial proteases. Our results indicate that a normal diet like porcine meat might generate ACE inhibitory peptides by the action of digestive enzymes. This is of importance in considering normal eating habits.

Traditionally, biologically functional meat products have been made with the addition of functional materials from other foods (Jimenez-Colmenero et al., 2001). However, our results suggest that meat itself may become a biologically active food through gastric and intestinal digestion. The next step in this research will be to determine whether the present peptides are competitive substrates of ACE or whether they are capable of true inhibitory activities as has been shown by Yokoyama et al. (1992) and Saito et al. (1994). It is also necessary to confirm the *in vivo* action of the peptides by oral administration using the hypertensive rat (Saito et al., 1994; Ohta et al., 1999).

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