# Peptic Hydrolysate of Porcine Crude Myosin Has Many Active Fractions Inhibiting Angiotensin I-converting Enzyme

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**ABSTRACT :** In order to clarify one of the biological functions of pork, we investigated whether a peptic hydrolysate of denatured porcine crude myosin showed inhibitory activity against angiotensin I-converting enzyme (ACE), which contributed to hypertension. Our results indicated that this hydrolysate showed relatively strong activity, and we therefore attempted to separate the involved peptides, which were considered to be active substances. To isolate these active peptides, the hydrolysate was separated using a solid-phase separation, gel filtration high-performance liquid chromatography (HPLC), and two kinds of reverse phase HPLC. In each stage of separation, many fractions were detected, almost all of which showed ACE inhibitory activity. Thus, we suggested that the activity of the hydrolysate as a whole was a result of the activities of the many individual peptides. Six peaks were distinguished, with yields from 34 to 596 ppm of original crude myosin. In addition to the six peaks, many other active fractions were found throughout the separation steps, strongly suggesting that whole porcine crude myosin itself had ACE inhibitory activity. Moreover, pork as food was considered to function as an ACE inhibitory material *in vivo*, because pork proteins consist primarily of crude myosin, which included almost all the myofibrillar structural proteins. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 9 : 1384-1389)***

**Key Words :** Angiotensin I-converting Enzyme, Peptide, Porcine Crude Myosin, Protease Digestion, Pepsin

## INTRODUCTION

The tertiary (physiologically active) function of food, which contributes to the modulation of our physiological systems, has been widely investigated, and certain functional foods have already been developed in Japan (Arai, 1996). Some of these studies have proposed to use food peptides as functional substances generated from foods by digestion with proteases. Among these peptides, peptide inhibitors for angiotensin I-converting enzyme (ACE) have attracted considerable attention. ACE plays an important role in the regulation of blood pressure; it contributes to hypertension by converting angiotensin I to angiotensin II (Skeggs et al., 1956) and by inactivating bradykinin. Although there are other factors involved in high blood pressure, many studies have focused on ACE inhibition and hypotension, because the inhibition of ACE brings about a significant decrease in blood pressure (Ondetti et al., 1977). ACE inhibitory peptides from animal-originated foods have been previously reported by Kawamura et al. (1992), Yokoyama et al. (1992), Seki et al. (1995), Ohta et al. (1999) and Fujita et al. (2000), among others. Since pork contains significant amounts of protein, it is fair to speculate that it contains many physiologically functional peptides. ACE inhibitory peptides from porcine myosin have been reported by Arihara et al. (2001), and the research has been conducted on their hypotensive activity *in vivo* upon oral administration to spontaneously hypertensive rats (Nakashima et al., 2002). Nevertheless, almost all of the researches described above have primarily examined peptides derived from hydrolysates with non-digestive proteases, such as thermolysin and alkalase; a detailed investigation of peptides derived from pork proteins through gastrointestinal protease digestion is important in any discussion of the functions of these peptides in a biologically active food.

In a previous study, we have reported that the enzymatic hydrolysates of a variety of porcine proteins, such as myosin, actin, tropomyosin, troponin, and water-soluble proteins, showed ACE inhibitory activities (Katayama et al., 2003). In the present study, the purpose of this research was to clarify the ACE inhibitory activities of peptides from pork. For this purpose, the peptic hydrolysate of porcine crude myosin, which included almost all myofibrillar structural proteins and was denatured by heating, was separated to isolate ACE inhibitory peptides. We also include a discussion of the activity of the fractions derived through the various stages of separation.

## MATERIALS AND METHODS

### Preparation of porcine crude myosin

Pork loin (*Longissimus dorsi*) was obtained from Marudai Shimane Farm Co., Ltd. (Shimane, Japan). Crude

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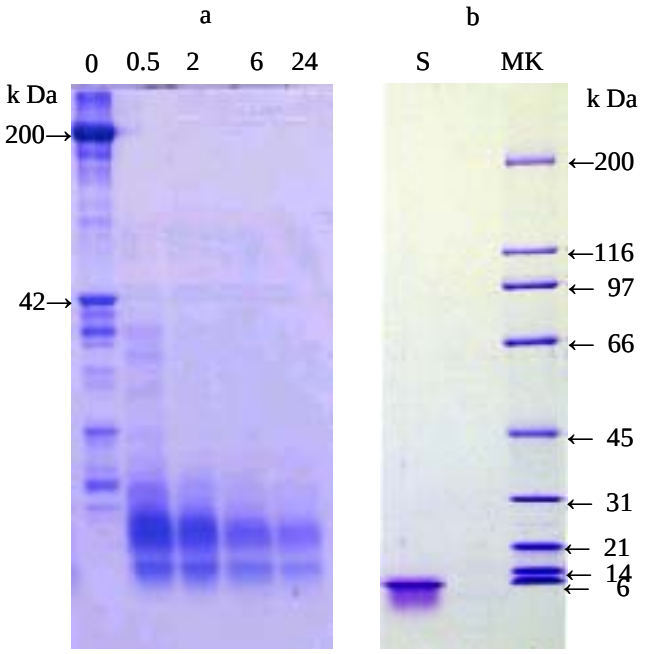


**Figure 1.** SDS-PAGE profiles of porcine crude myosin, which was denatured by heating at 95°C for 10 min, and was digested by pepsin. 0, 0.5, 2, 6, and 24: incubation time (h) for digestion. S: supernatant of peptic hydrolysate incubated for 6 h. MK: molecular weight marker for (b). For (a), myosin heavy chain (200 k Da) and actin (42 k Da) were indicated as molecular weight markers.

myosin was extracted from minced meat using Guba-Straub solution (0.3 M KCl, 0.1 M $KH_2PO_4$, 0.05 M $K_2HPO_4$, 2 mM adenosine triphosphate) following the method described by Margossian and Lowey (1982). The crude myosin extract included myosin, actin, tropomyosin, troponin and other myofibrillar structural proteins. It was dialyzed against phosphate-buffered saline (PBS; 0.8% NaCl, 10 mM Na-phosphate, pH 7.5) before hydrolysis.

**Hydrolysis of crude myosin**

Pepsin was obtained from Sigma Chemical Co. (St. Louis, MO, USA). Crude myosin (5 mg/ml) was suspended in PBS and denatured by heating for 10 min at 95°C. Its pH was adjusted to 2 with 1 M HCl, and pepsin was added at a 1:100 ratio of enzyme to substrate. After 6 h digestion at 37°C, the buffer pH was adjusted to 7.5 with 1 M NaOH, and enzymatic activity was terminated by heating for 10 min at 95°C. The reaction mixture was centrifuged for 10 min at 14,000 rpm, and the resulting supernatant was used for experiments.

**ACE inhibitory assay**

ACE inhibitory activity was measured following the method described by Cushman and Cheung (1971), with slight modifications (Katayama et al., 2003). Briefly, samples were incubated with hippuryl-L-histidyl-L-leucine (HHL, Nacalai Tesque Inc., Kyoto, Japan) as the substrate and rabbit lung ACE (Wako Pure Chemical Industries Ltd., Osaka, Japan) in a buffer containing 0.13 M borate (pH 8.5) and 0.4 M NaCl. The enzyme reaction was terminated by adding 1 M HCl. Hippuric acid liberated from HHL was extracted by ethyl acetate, dried, and dissolved with 1 M NaCl. The absorbance of hippuric acid at 228 nm was measured and the ACE inhibitory activity was calculated. Inhibitory activity was identified as a 50% inhibitory protein concentration ($IC_{50}$) of the sample in assay. One unit of the ACE inhibitory activity was defined as the amount of inhibitor that inhibited 50% of ACE activity in 1 ml of reaction mixture (Seki et al., 1995). The specific activity (activity per weight of protein) of each fraction was calculated (Nakamura et al., 1995).

**Isolation of ACE inhibitory peptides from peptic hydrolysate of porcine crude myosin**

The peptic hydrolysate of porcine crude myosin was separated using a SEP-PAK Plus $C_{18}$ cartridge (Waters Co., Milford, MA, USA) with 10, 20 and 30% $CH_3CN$. The active fractions were then separated by reverse phase (RP) high-performance liquid chromatography (HPLC) using a Cosmosil $5C_{18}$ AR-II (4.5×150 mm) column (Nacalai Tesque), and eluted with a gradient of 10 to 80% $CH_3CN$ in 0.1% trifluoroacetic acid (TFA) at a flow rate of 0.5 ml/min. The active fractions were then applied to the same column and eluted with an isocratic condition of $CH_3CN$ in 0.1% TFA at a flow rate of 0.5 ml/min. The active fractions obtained from RP-HPLC were subjected to gel filtration HPLC using a TSK-gel $G2000SW_{XL}$ (7.8×300 mm) (Tosoh Co., Tokyo, Japan), and eluted with 45% $CH_3CN$ in 0.1% TFA at a flow rate of 0.5 ml/min. Finally, the active fraction was separated through RP-HPLC using a Cosmosil 5PE-MS (4.6×250 mm) column (Nacalai Tesque), and eluted with an isocratic condition of $CH_3CN$ in 0.1% TFA at a flow rate of 1 ml/min. All elutions were monitored using absorbance at 215 nm.

**Electrophoresis**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method described by Laemmli (1970), and using a gradient (7.5 to 17.5% acrylamide) slab gel for separating proteins with a stacking gel (4.5%). Protein bands were stained with Coomassie Brilliant Blue R-250.

**Determination of protein concentrations**

The protein concentrations of the crude myosin and its hydrolysate were measured using the biuret method (Gornall et al., 1949) with bovine serum albumin as a standard. The peptide concentrations of fractions from the

**Table 1.** Angiotensin I-converting enzyme inhibitory activity of digestive protease hydrolysates from various foods

| Origin | Protease | $IC_{50}$ (μg/ml) | Reference |
|---|---|---|---|
| Porcine crude myosin | Pepsin | 112 | This paper |
| | Chymotrypsin | 121 | Katayama et al. (2003) |
| | Trypsin | 166 | |
| Sardine | Pepsin | 620 | Matsui and Kawasaki (2000) |
| | Chymotrypsin | 1,280 | |
| | Trypsin | 2,030 | |
| Dried bonito | Pepsin | 47 | Yokoyama et al. (1992) |
| | Chymotrypsin | 117 | |
| | Trypsin | 161 | |
| Ovalbumin | Pepsin | 45 | Fujita et al. (2000) |
| | Chymotrypsin | >1,000 | |
| | Trypsin | >1,000 | |

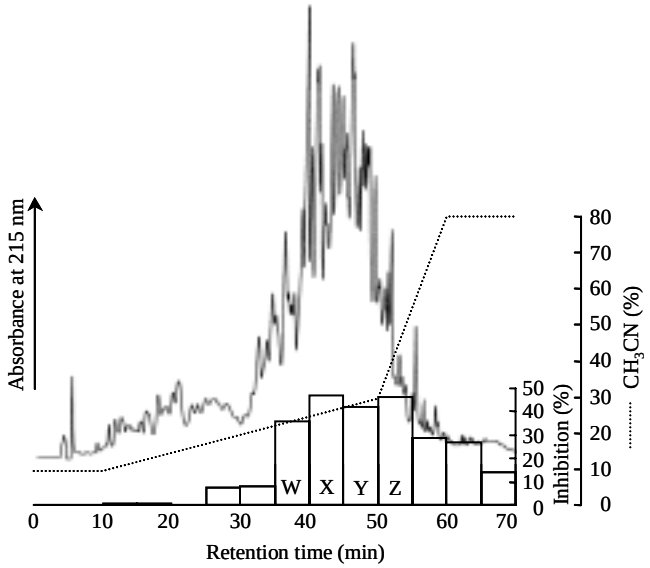


**Figure 2.** First RP-HPLC chromatogram and activity of fraction eluted through SEP-PAK cartridge $C_{18}$ with 20% $CH_3CN$. Eluted fractions were collected every 5 min, and measured their ACE inhibitory activities. W, X, Y and Z were highly active fractions and subjected to the next separation step.

various separation steps were measured by the UV method (Murphy and Kies, 1960) using wavelengths of 215 and 225 nm.

## RESULTS AND DISCUSSION

### ACE inhibitory activity of peptic hydrolysate of porcine crude myosin

The SDS-PAGE pattern of the crude myosin hydrolysate with pepsin showed that many proteins with high molecular weight (MW) decreased as hydrolysis proceeded (Figure 1a); in fact, the supernatant from the peptic hydrolysate after 6 h digestion showed no relatively high MW proteins (Figure 1b). The protein concentration of this supernatant was 3.7 mg/ml, and about 74% of the original crude myosin (5 mg/ml) was recovered as water-soluble proteins, peptides or free amino acids.

The peptic hydrolysate of crude myosin showed ACE inhibitory activity with 112 μg/ml of $IC_{50}$, while the original crude myosin showed no such activity. We suggested that peptides generated from crude myosin by peptic digestion were responsible for this elevated level of inhibition. The activity of this peptic hydrolysate was found to be stronger than that of hydrolysate from sardine and a little weaker than those of hydrolysates from bonito or ovalbumin (Table 1). These data indicated that porcine crude myosin included some peptides with high activity. Crude myosin hydrolyzed with chymotrypsin or trypsin showed weaker activity than the peptic hydrolysate (Katayama et al., 2003), and the activity levels of other hydrolysates (Table 1) showed the same tendencies found with crude myosin. Cheung et al. (1980) stated that dipeptides with hydrophobic amino acid residues showed ACE inhibitory activity. Furthermore, pepsin cleaves protein at hydrophobic amino acid residues such as Phe, Tyr, Leu, and Met, then peptic hydrolysate with hydrophobic amino acid residues could be speculated to include many ACE inhibitory peptides. We therefore carried out the present study to separate and isolate the active peptides from this hydrolysate.

### Separation and activity of ACE inhibitory fractions

The peptic hydrolysate of crude myosin (371 mg) was first separated with a SEP-PAK Plus $C_{18}$ cartridge. Fractions eluted by 10, 20 and 30% $CH_3CN$ showed ACE inhibitory activities, and their $IC_{50}$s were 36, 57 and 46 μg/ml, respectively. The fraction eluted by 20% $CH_3CN$, which had the most protein, was applied to a $C_{18}$ HPLC column and separated with a $CH_3CN$ gradient. Almost all peptides were eluted at $CH_3CN$ concentrations between 20 and 30%, that is, at the concentrations at which relatively hydrophobic peptides were expected to elute (Figure 2). Fractions were collected every 5 min and their ACE inhibitory activities were measured. Four fractions (W, X, Y, and Z), which were eluted between 35 and 55 min, had a relatively high protein concentration and showed strong activity with 43, 49, 44 and 14 μg/ml of $IC_{50}$. These

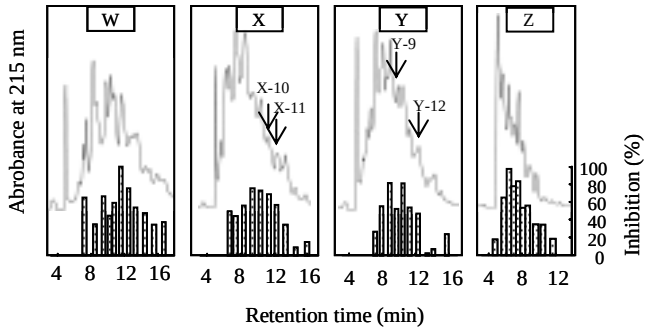


**Figure 3.** Second RP-HPLC chromatograms and activities of fractions (W, X, Y and Z) from the first RP-HPLC. Every peak was collected and its ACE inhibitory activity was measured. ▨ : ACE inhibitory activity of each fraction. X-10, X-11, Y-9 and Y-12 were subjected to the next separation step.

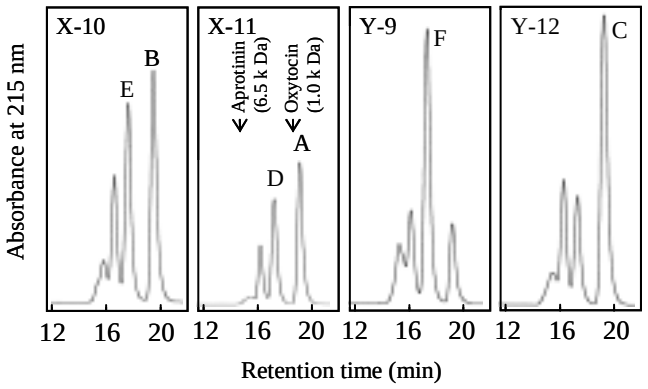


**Figure 4.** Gel filtration HPLC chromatograms of fractions (X-10, X-11, Y-9 and Y-12) from second RP-HPLC. Six fractions (A to F) were subjected to the next separation step. Aprotinin and oxytocin were used as molecular weight standards.

fractions were applied to the same column and separated with an isocratic elution; the fractions W, X, Y, and Z were eluted with 15, 18, 20 and 25% $CH_3CN$, respectively (Figure 3). Every peak was collected, dried and dissolved in 100 μl of $H_2O$, and its ACE inhibitory activities were measured. Almost all peaks showed inhibitory activity, suggesting that the activity of whole crude myosin hydrolysate resulted from the activities of many individual peptides. The peaks with relatively strong activity (over 50% inhibition, see Figure 3) were separated during the next step using gel filtration HPLC. Because many fractions in Figure 3 showed very weak activity after further purification using gel filtration HPLC, only 4 chromatograms of gel filtration HPLC (X-10, X-11, Y-9 and Y-12) are shown in Figure 4. Six fractions (A to F) with strong activity were obtained; their activity levels were 22, 26, 131, 70, 37 and 135 μg/ml of $IC_{50}$, respectively. The molecular weights of these fractions were estimated at less than 6.5 k Da by comparing their retention times to those of molecular weight markers (aprotinin and oxytocin). Finally, these six active fractions were applied to a PE-MS column.

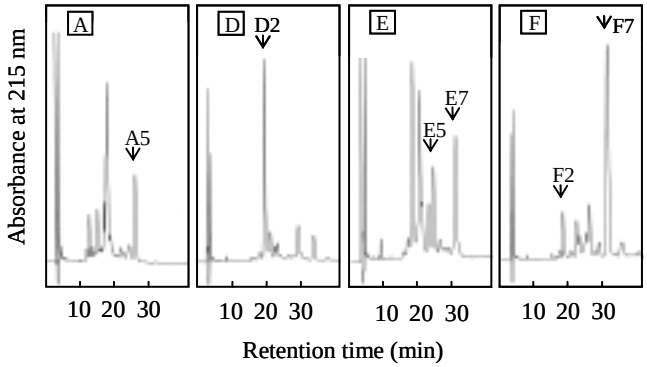


**Figure 5.** RP-HPLC chromatograms using PE-MS column of fractions (A, D, E and F) from gel filtration HPLC. Six fractions (A5, D2, E5, E7, F2 and F7) showed ACE inhibitory activity.

Because B and C showed very weak activity after further purification using a PE-MS column, only 4 chromatograms (A, D, E and F) are shown in Figure 5. Fractions A, D, E and F were eluted with 15, 13, 13 and 14% $CH_3CN$ in 0.1% TFA, respectively. The peaks in Figure 5 (A5, D2, E5, E7, F2 and F7) showed ACE inhibitory activity; their corresponding $IC_{50}$s were 8, 76, 5, 10, 21 and 65 μg/ml. These activities were stronger than those of peptides described by Arihara et al. (2001) and as strong as those of peptides described by Fujita et al. (2000), Matsui and Kawasaki (2000) and Yokoyama et al. (1992).

**Whole activity of porcine crude myosin**

The yield of each fraction (A5, D2, E5, E7, F2 and F7) was between 3.4 and 59.6 mg/100 g of original crude myosin, and totalled about 120 mg/100 g (Table 2). The unit recovery of these fractions to whole hydrolysate was approximately 5,500 ppm and the activity of these fractions was concentrated over 4.5 times. With the exception of the last six fractions, many active fractions were found throughout all separation steps, suggesting that whole porcine crude myosin has ACE inhibitory activity in itself. Moreover, pork as food functions as an ACE inhibitory material *in vivo*, because pork proteins consist primarily of crude myosin, which includes almost all the myofibrillar structural proteins (Hattori, 1996). Although Arihara et al. (2001) have reported previously on ACE inhibitory peptides identified from porcine myosin, the active fractions in the present study were considered to differ from their peptides, because they used another enzyme (thermolysin) to digest myosin, which was not denatured. Furthermore, the ACE inhibitory peptides from chicken muscle reported by Fujita et al. (2000) were also considered to differ from the fractions used here, because they used thermolysin, too.

The role of digestive enzymes in producing biologically active peptides from food is considered to be highly significant in the modern study of foods (Katayama et al., 2003; Li et al., 1999). In the further research, we plan to

**Table 2.** Angiotensin I-converting enzyme inhibitory activity and recovery of fractions from porcine crude myosin

| Separation step | | Fraction | Protein | | $IC_{50}$ | Unit[1] | Unit recovery | Specific activity[2] |
|---|---|---|---|---|---|---|---|---|
| | | | μg | Yeild mg/100 g | μg/ml | | ppm | U/mg |
| | | Intact crude myosin | 500,000 | 100,000 | >2,000 | | | |
| | | Peptic hydrolysate | 371,840 | 74,368 | 112 | 3,320 | 1,000,000 | 8.9 |
| 1 | SEP-PAK $C_{18}$ | 20% $CH_3CN$ | 126,605 | 25,321 | 57 | 2,221 | 669,017 | 17.5 |
| 2 | RP-HPLC | W | 17,242 | 3,448 | 43 | 400 | 120,493 | 23.2 |
| | ($C_{18}$) | X | 20,157 | 4,031 | 49 | 415 | 124,924 | 20.6 |
| | | Y | 18,075 | 3,615 | 44 | 412 | 124,012 | 22.8 |
| | | Z | 6,080 | 1,216 | 14 | 444 | 133,681 | 73.0 |
| | | Subtotal | 61,553 | 12,311 | | 1,670 | 503,111 | |
| 4 | Gel-HPLC | A | 270 | 54 | 22 | 12.6 | 3,781 | 46.7 |
| | | B | 427 | 85 | 26 | 16.2 | 4,878 | 37.9 |
| | | C | 987 | 197 | 131 | 7.5 | 2,262 | 7.6 |
| | | D | 434 | 87 | 70 | 6.2 | 1,858 | 14.3 |
| | | E | 467 | 93 | 37 | 12.7 | 3,838 | 27.2 |
| | | F | 844 | 169 | 135 | 6.3 | 1,887 | 7.5 |
| | | Subtotal | 3,429 | 686 | | 61.4 | 18,504 | |
| 5 | RP-HPLC | A5 | 17 | 3.4 | 8 | 2.1 | 625 | 123.5 |
| | (PE-MS) | D2 | 185 | 37.0 | 76 | 2.4 | 736 | 13.0 |
| | | E5 | 17 | 3.4 | 5 | 3.4 | 1,024 | 200.0 |
| | | E7 | 34 | 6.8 | 10 | 3.5 | 1,045 | 102.9 |
| | | F2 | 48 | 9.7 | 21 | 2.4 | 709 | 50.0 |
| | | F7 | 298 | 59.6 | 65 | 4.6 | 1,376 | 15.4 |
| | | Subtotal | 599 | 119.9 | | 18.3 | 5,515 | |

[1] One unit=protein (μg)/$IC_{50}$ (μg/ml). [2] Units of ACE inhibitory activity per milligram of peptide.

identify these active peptides and to examine the *in vivo* activity of meat after oral administration.

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