Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 90 (2005) 809–814
Food Chemistry
www.elsevier.com/locate/foodchem

Isolation of angiotensin I converting enzyme (ACE) inhibitor from fermented oyster sauce, *Crassostrea gigas*

Jae-Young Je, Ji-Young Park, Won-Kyo Jung, Pyo-Jam Park, Se-Kwon Kim *

Department of Chemistry, Pukyong National University, Busan 608-737, Republic of Korea

Received 15 March 2004; received in revised form 17 May 2004; accepted 17 May 2004

Abstract

Angiotensin I converting enzyme (ACE) inhibitor was isolated from fermented oyster sauce (FOS) and purified. Oyster was fermented with 25% NaCl (w/w) at 20 °C for 6 months. FOS was passed through a 40-mesh sieve, desalted using an electrodialyzer and then lyophilized. ACE inhibitory activity of FOS was investigated, and the IC_{50} value was determined to be 2.45 mg/ml. ACE inhibitor from FOS was purified using SP-Sephadex C-25 ion exchange chromatography, Sephadex G-50 gel chromatography, high-performance liquid chromatography (HPLC) on a gel permeation chromatography (GPC) column and reversed-phase HPLC on a C_{18} column. The purified inhibitor had an IC_{50} value of 0.0874 mg/ml, and it exhibited competitive inhibition against ACE. The purified peptide was evaluated for its antihypertensive effect in spontaneously hypertensive rats (SHRs) following oral administration. Rat blood pressure significantly decreased after inhibitor injection.

Keywords: Fermented oyster; ACE inhibitor; Antihypertensive effect; Competitive inhibition

1. Introduction

Hypertension is one of the most common cardiovascular diseases. It is a worldwide problem of epidemic proportions, which affects 15–20% of all adults. It is the most common serious chronic health problem because it carries a high risk factor for arteriosclerosis, stroke, myocardial infarction and end-stage renal disease. It is suggested that hypertension is closely related to food components, and antihypertensive peptides, controlling hypertension, may be associated with the presence of an antihypertensive peptide motif.

Angiotensin I converting enzyme (EC 3.4.15.1; ACE) plays an important physiological role in regulating blood pressure (Skeggs, Kahn, Kahn, & Shumway, 1957). ACE belongs to the class of zinc proteases and is located in the vascular endothelial lining of lungs. ACE acts as an exopeptidase that cleaves dipeptides from the C-terminus of various oligopeptides (Curtiss, Chon, Vrobel, & Francious, 1978; Yang, Erdös, & Levin, 1971). ACE catalyzes the hydrolysis of angiotensin I to generate a potent vasoconstrictor, angiotensin II, and inactivates bradykinin, which has a depressor action. Since the discovery of ACE inhibitors in snake venom, many studies have been directed toward the attempted synthesis of ACE inhibitors, such as captopril, enalapril, alacepril, and lisinopril, which are currently used extensively in the treatment of essential hypertension and heart failure in humans (Ondetti, 1977; Patchett et al., 1980). However, these synthetic drugs are believed to have certain side effects, such as cough, taste disturbances and skin rashes (Atkinson & Robertson, 1979). Therefore, a search for ACE inhibitors from foods has become a major area of research. In recent years, many ACE inhibitory peptides have

* Corresponding author. Tel.: +82-51-620-6375; fax: +82-51-628-8147.
E-mail address: sknkim@mail.pknu.ac.kr (S.-K. Kim).

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.05.028

been isolated from various food proteins, such as cheese whey (Abubakar, Saito, Kitazawa, Kawai, & Itoch, 1998), casein (Kohmura et al., 1989; Maeno, Yamamoto, & Takano, 1996; Maruyama et al., 1989), zein (Miyoshi et al., 1991; Yano, Suzuki, & Funatsu, 1996), tuna muscle (Kohama et al., 1988), sardine (Ukeda et al., 1992), corn gluten (Suh & Whang, 1999) and bovine blood plasma (Hyun & Shin, 2000). In addition, some ACE inhibitors have also been reported in some fermented foods, such as soy sauce (Kinoshita, Yamakoshi, & Ikuchi, 1993), soybean (Okamoto, Hanagata, Kawamura, & Yanagida, 1995a, 1995b) and milk (Gobbetti, Ferranti, Smacchi, & Goffredi, 2000).

Fish and shellfish sauce are widely used in southeast and east Asian countries. In Korea, the production of oyster was estimated to be 182,229 ton in 2002, and only a few fish and shellfish sauces have survived in local areas in Korea. However, fish and shellfish sauces have recently been rediscovered because of increased consumer interest in their taste and flavour.

From this point of view, the present study intended to isolate an ACE inhibitor derived from fermented oyster sauce (FOS) and to characterize the purified inhibitor with respect to ACE inhibitory activity. Moreover, the antihypertensive action of the purified inhibitor, by oral administration in spontaneously hypertensive rats (SHRs), was also investigated.

2. Materials and methods

2.1. Materials

Oyster, *Crassostrea gigas*, was purchased from a local shellfish market (Samchunpo, Korea). ACE (from rabbit lung) and substrate peptide (hippuryl-histidyl-leucine) of ACE, SP-Sephadex C-25 and Sephadex G-50 were purchased from Sigma Chemical Co. (St. Louis, MO). Ohpak SB-800 HQ (2.5×250 mm), for gel permeation chromatography (GPC), was purchased from Showa Denko K.K. (Tokyo, Japan) and a Nucleosil 100-3 ODS C_{18} column (4.6×250 mm) was from Macherey-Nagel (Middleton Cheney OX17 2PA, UK). All other reagents used in this study were reagent grade chemicals.

2.2. Preparation of FOS

Oyster was washed with water to remove salt and other materials, and then fermented with 25% NaCl (w/w) at 20 °C for 6 months. Fermentation was terminated by boiling at 95 °C for 10 min. The FOS was desalted by an electrodialyzer (Micro Acilyzer Model G3, Asahi Chemical Industry Co., Tokyo, Japan) with a 100 Da MWCO (molecular weight cut off) membrane (Asahi Chemical Industry Co., Tokyo, Japan). The desalted FOS was lyophilized and stored at −20 °C until used.

2.3. Assay for ACE inhibitory activity

The ACE inhibitory activity was measured by the method of Cushman and Cheung (1971) with slight modifications. A sample solution (50 μl) with 50 μl of ACE solution (25 munits/ml) was pre-incubated at 37 °C for 10 min, and the mixture was incubated with 150 μl of substrate (8.3 mM Hip-His-Leu in 50 mM sodium borate buffer containing 0.5 M NaCl at pH 8.3) for 30 min at the same temperature. The reaction was terminated by the addition of 250 μl of 1 M HCl. The resulting hippuric acid was extracted with 0.5 ml of ethyl acetate. After centrifugation (800*g*, 15 min), 0.2 ml of the upper layer was transferred into a test tube, and evaporated at room temperature for 2 h in a vacuum. The hippuric acid was dissolved in 1.0 ml of distilled water, and the absorbance was measured at 228 nm using a UV-spectrophotometer (Cary 1C, Varian Inc., Australia). The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

2.4. Purification of ACE inhibitor form FOS

The lyophilized FOS (20 g) was loaded onto a SP-Sephadex C-25 ion-exchange column (4.0×40 cm) equilibrated with 20 mM sodium acetate buffer (pH 4.0) and eluted with a linear gradient of NaCl (0–2 M) in the buffer at a flow rate of 60 ml/h. Active fractions were collected, desalted using an electrodialyzer and lyophilized immediately. For further purification, a Sephadex G-50 gel filtration column (2.5×98 cm), equilibrated with distilled water, was employed. The column was eluted with distilled water and 5.0 ml fractions were collected at a flow rate of 60 ml/ h. The fraction with the highest ACE inhibitory activity was dissolved in distilled water and separated by high-performance liquid chromatography (HPLC) on a GPC column. The separation was performed with distilled water at a flow rate of 60 ml/h. Fractions showing ACE inhibitory activity were pooled and lyophilized. Further purification of ACE inhibitor was carried out by reversed-phase HPLC (RP-HPLC) using a Nucleosil 100-3 ODS C_{18} column. A linear gradient of acetonitrile from 0% to 11% containing 0.1% trifluoroacetic acid (TFA), was maintained at a flow rate of 1 ml/min. Elution peaks were monitored at 215 nm, and their ACE inhibitory activities were measured using the method previously described.

2.5. Determination of molecular weight

Molecular weight of the purified peptide was determined by a GPC column at a flow rate of 1 ml/min. Standard molecular weight makers used were as follows: cytochrome C (MW 12,900 Da), aprotinin (MW 6500 Da), angiotensin I (MW 1296.5 Da) and pentaphenylalanine (MW 753.9 Da).

2.6. Determination of the inhibition pattern on ACE

ACE inhibitor was added to each reaction mixture according to Bush, Herny, and Slusarchyk (1984) with some modifications. The enzyme activity was measured with different concentrations of the substrate. The kinetics of ACE in the presence of the inhibitor were determined by the Lineweaver–Burk plots.

2.7. Effects of ACE inhibitor on SHR

SHRs were obtained from the Korea Research Institute of Bioscience and Biotechnology (DaeJeon, Korea). SHRs were housed individually in steel cages in a room kept at 24 °C with a 12 h light-dark cycle, and fed a standard laboratory diet. Tap water was freely available. Peptide was dissolved in saline at a dose of 10 mg/kg body weight (BW) and injected orally using a metal gastric zoned in SHR. The systolic blood pressure (SBP) lowering efficacy of inhibitor was compared with that of captopril. Captopril was injected using the same method employed for peptide introduction. Control rats were administrated with the same volume of saline solution. Following oral administration of sample, SBP was measured by the tail cuff method using a programmed electro-sphygmomanometer (Model UR-5000, Ueda Co. Ltd., Tokyo, Japan) after warming up rats in a 40 °C chamber for 10 min.

2.8. Statistical analysis

All results were expressed as means ± standard errors of measurement (SEM). The significance of the differences between SBPs before and after administration was analyzed using the Student's *t* test.

3. Results and discussion

3.1. ACE inhibitory action of FOS

ACE inhibitors from fermented foods, such as temphe, miso, fish sauce and soy sauce (Okamoto et al., 1995a, 1995b) have already been recently isolated. Various products were studied during fermentation by action of bacteria or moulds. Okamoto et al. (1995a, 1995b) reported that the ACE inhibitory activity was changed by different cheese manufacturing processes, e.g., red cheddar, blue, camembert and cottage cheese. Blue, camembert and red cheddar cheeses are made by a process which includes a maturing step but that of cottage cheese does not involve a maturation. Production processes which included a maturing step showed a higher ACE inhibitory activity than those that excluded of maturing step. This result is caused by fermentation with bacteria or moulds, and it produces many kinds of fermentation products from α-, β-, γ-casein. It was further reported that the ratio between target material and the other raw material in the fermentation also affects the ACE inhibitory activity.

In this study, we fermented oyster with 25% (w/w) NaCl at 20 °C for 6 months. The mixture was boiled at 95 °C for 10 min, passed through a 40-mesh sieve, desalted using a Micro Acilyzer Model G3 electrodialyzer and then lyophilized. The ACE inhibitory activity test was performed as previously described. Results showed that FOS had an ACE inhibitory activity with an IC_{50} value of 2.45 mg/ml.

Captopril, an ACE inhibitor, has been adopted for oral hypertensive therapy. Hence, many studies have been launched to identify other food-derived ACE inhibitors that potentially prevent hypertension.

3.2. Purification of ACE inhibitor

For the purification of ACE inhibitor, FOS was loaded onto a SP-Sephadex C-25 ion-exchange column with a linear gradient of NaCl and fractionated into three portions (Fig. 1(a)). The pooled fractions were desalted and tested for ACE inhibitory activity. Fraction C was found to possess a strong activity with an IC_{50} of 1.54 mg/ml. The active fraction from the SP-Sephadex C-25 column was applied onto a Sephadex G-50 gel filtration column and separated into two fractions (Fig. 1(b)). Fractions were pooled and concentrated by rotary evaporation and then lyophilized. Further purification of active fraction CII from the Sephadex G-50 column was done by HPLC (on GPC) and it was separated into five fractions that were assayed for ACE inhibitory activity (Fig. 1(c)). The fraction with the highest ACE inhibitory activity was further purified by reversed-phase HPLC (Fig. 1(d)). The operation was performed with a linear gradient of acetonitrile, described previously. Finally, the purified ACE inhibitor from FOS had an IC_{50} value of 0.087 mg/ml. The molecular weight of the purified inhibitor from FOS was investigated using HPLC on a gel permeation column. The result showed that the molecular weight of the purified inhibitor was 592.9 Da (Fig. 2). Based on this result, the IC_{50} value of the purified inhibitor was 0.147 mM. Table 1 summarizes the results of purification of the ACE inhibitor from FOS. The ACE inhibitor was purified 28.2-fold from FOS using a four-step purification procedure, and the yield was 0.32%.

3.3. Determination of inhibition pattern of ACE inhibitor

The inhibition pattern of the purified ACE inhibitor from FOS was estimated using Lineweaver–Burk plots and it was found to be competitive (Fig. 3). Thus, ACE inhibitor from FOS binds competitively with the substrate at the active site of ACE. The ACE inhibitory

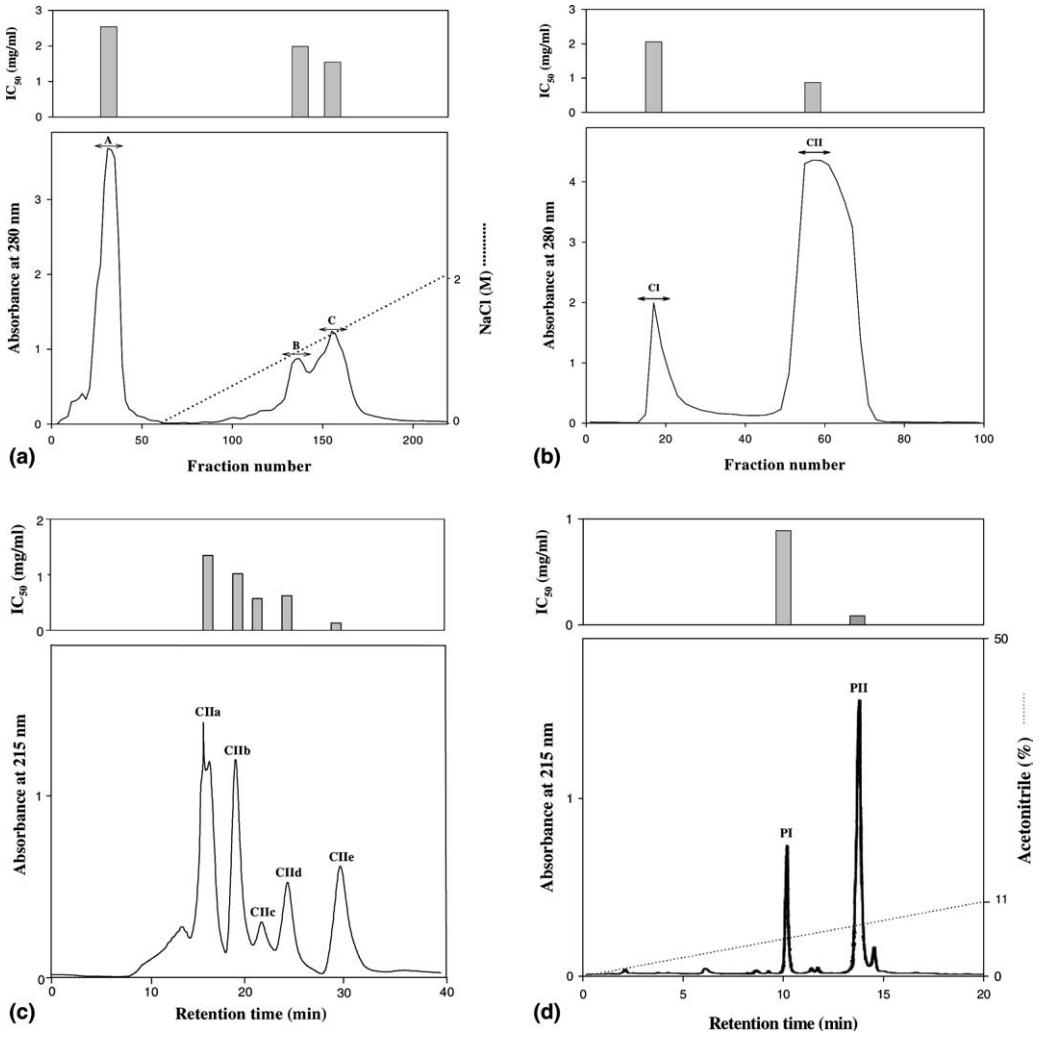

Fig. 1. Purification of ACE inhibitor from FOS. (a) SP-Sephadex C-25 chromatogram (lower panel) and ACE inhibitory activities of fractions (upper panel). (b) Sephadex G-50 chromatogram (lower panel) of active fraction from SP-Sephadex C-25 and ACE inhibitory activities (upper panel). (c) GPC profile (lower panel) of active fraction from Sephadex G-50 and ACE inhibitory activities (upper panel). (d) HPLC profile (lower panel) on a C_{18} column and ACE inhibitory activities (upper panel). All chromatography was performed as described in Section 2.

fraction from natto (Akiko, Hiroshi, & Eiko, 1994), a traditional soy fermented product, also showed a competitive inhibition similar to that observed in this study.

3.4. Effects of ACE inhibitor on SHR

Antihypertensive activity of the purified ACE inhibitor was evaluated by measuring the change of SBP at 1, 2, 3, 6, and 9 h after oral administration to rats of 10 mg/kg of body weight. There was no change in SBP in the control group during the investigation period. As shown in Fig. 4, a reduction of 12 mmHg of SBP, 3 h after administration of inhibitor was observed, and the activity was maintained for 6 h. Captopril lowered SBP significantly from 1 to 6 h after administration of the drug.

Recently, many ACE inhibitory peptides were isolated from food proteins and tested for their antihypertensive effects in SHR. Fujita and Yoshikawa (1999) reported that LKPNM is a pro-drug type of ACE inhibitory peptide because LKPNM was found to be hydrolyzed by ACE to produce LKP, which had an eightfold higher ACE inhibitory activity than LKPNM. After oral administration in SHR, the antihypertensive effect of LKPNM showed a maximal effect after 6 h, while LKP showed a maximal effect at 4 h. Some antihypertensive drugs are known to produce side effects, such as an abnormal elevation of the blood pressure after administration. However, we could not find any side effects after our sample administration. Oyster sauce is one of the favourite foods in southeast and east Asian countries. Thus, oyster sauce may be useful as functional food for the maintenance of blood pressure within the normal range.

The result of this study suggests that an ACE inhibitor derived from FOS could be utilized to develop

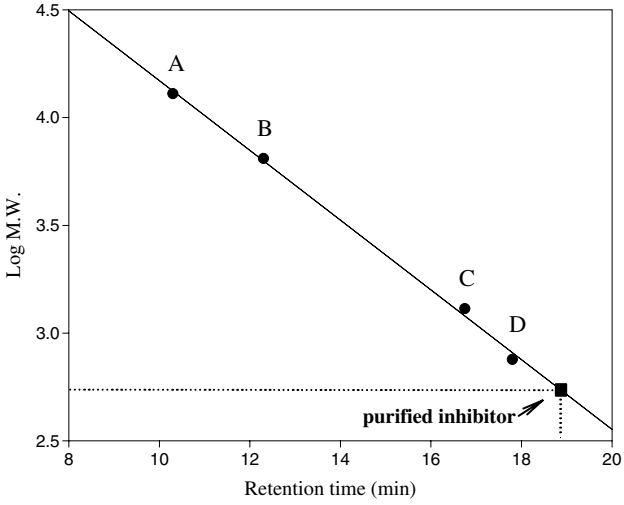

Fig. 2. Determination of molecular weight of the purified inhibitor on HPLC with GPC column. HPLC operation was carried out with deionized water as mobile phase at flow rate of 1.0 ml/min. The following molecular weight standards were used: A, cytochrome C (MW 12,900 Da); B, aprotinin (MW 6500 Da); C, angiotensin I (MW 1296.5 Da); and D, pentaphenylalanine (MW 753.9 Da).

Table 1
Purification of ACE inhibitor from FOS

Purification step	IC_{50}[a] (mg/ml)	Yield (%)	Purification fold
Freeze drying	2.45	100	1
SP-Sephadex C-25	1.54	15.8	1.59
Sephadex G-50	0.87	11.0	2.82
GPC	0.134	0.79	18.3
RP-HPLC	0.087	0.32	28.2

[a] IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

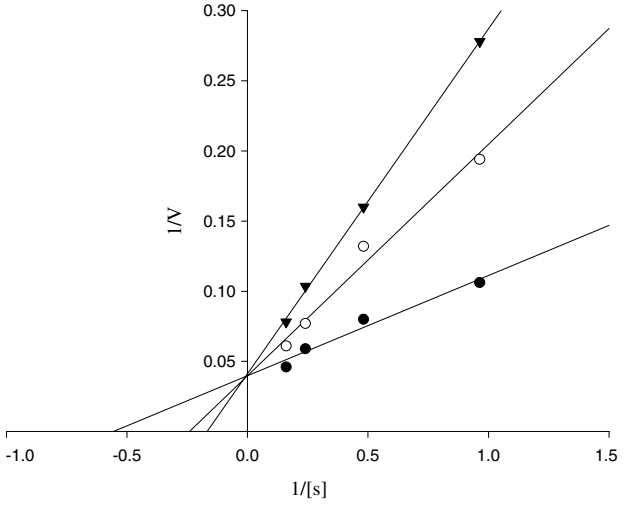

Fig. 3. Lineweaver–Burk plots of ACE inhibitory activity in the presence of the inhibitor. ●, control; ○, 25 μl inhibitor; ▼, 50 μl inhibitor.

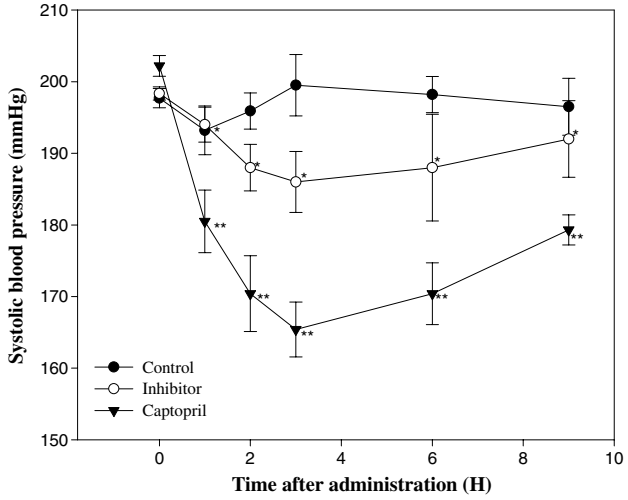

Fig. 4. Change in systolic blood pressure of SHR after administration of ACE inhibitor. Single oral administration was performed with the dose of 10 mg/kg BW, and SBP was measured 0, 1, 2, 3, 6 and 9 h after the administration. Significance of the difference from control at $^{*}P<0.05$, $^{**}P<0.01$.

physiologically functional foods. In addition, it is expected that this will contribute to developing interest in basic research and potential applications of bioactive peptides.

Acknowledgements

This research was funded by the MOMAF-SGRP, and this work was also supported by the Brain Korea 21 project in 2003.

References

Abubakar, A., Saito, T., Kitazawa, H., Kawai, Y., & Itoch, T. (1998). Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. *Journal of Dairy Science, 81*, 3131–3138.

Akiko, O., Hiroshi, H., & Eiko, M. (1994). Antihypertensive substances in viscous material of fermented soybean (NATTO). In . In K. Nishinar & E. Doi (Eds.), *Food hydrocolloids: Structures, properties, and functions* (pp. 497–502). New York: Plenum Press.

Atkinson, A. B., & Robertson, J. I. S. (1979). Captopril in the treatment of clinical hypertension and cardiac failure. *Lancet, 2*, 836–839.

Bush, K., Herny, P. R., & Slusarchyk, D. S. (1984). Muraceins-muramyl peptides produced by Norcardia orientalis as angiotensin converting enzyme inhibitors. *Journal of Antibiotics, 37*, 330–335.

Curtiss, C., Chon, J. N., Vrobel, T., & Francious, J. A. (1978). Role of the rennin-angiotensin system in the systemic vasoconstriction of chronic congestive heart failure. *Circulation, 58*, 763–770.

Cushman, D. W., & Cheung, H. S. (1971). Spectrophotometric assay and properties of the angiotensin I-converting enzyme of rabbit lung. *Biochemical Pharmacology, 20*, 1637–1648.

Fujita, H., & Yoshikawa, M. (1999). LKPNM: A prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology, 44*, 123–127.

Gobbetti, M., Ferranti, P., Smacchi, E., & Goffredi, F. (2000). Production of angiotensin I-converting enzyme inhibitory peptides in fermented milks started by *Lactobacillus delbruekii* subsp. *bulgaricus* SS1 and *Lactococcus lactis* subsp. *cremoris* FT5. *Applied and Environmental Microbiology, 66*, 3898–3904.

Hyun, C. K., & Shin, H. K. (2000). Utilization of bovine blood plasma proteins for the production of angiotensin I converting enzyme inhibitory peptides. *Process Biochemistry, 36*, 65–71.

Kinoshita, E., Yamakoshi, J., & Ikuchi, M. (1993). Purification and indentification of an antiotensin I-converting enzyme inhibitor from soy sauce. *Bioscience Biotechnology Biochemistry, 57*, 1107–1110.

Kohama, Y., Matsumoto, S., Oka, H., Teramoto, T., Okabe, M., & Mimura, T. (1988). Isolation of angiotensin-converting enzyme inhibitory from tuna muscle. *Biochemical and Biophysical Research Commutications, 155*, 332–337.

Kohmura, M., Nio, N., Kudo, K., Minoshima, Y., Munekata, E., & Ariyoshi, Y. (1989). Inhibition of angiotensin-converting enzyme by synthetic peptides of human β-casein. *Agricultural and Biological Chemistry, 53*, 2107–2114.

Maeno, M., Yamamoto, N., & Takano, T. (1996). Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790. *Journal of Dairy Science, 79*, 1316–1321.

Maruyama, S., Mitachi, H., Awaya, J., Kurono, M., Tomizuka, N., & Suzuki, H. (1989). Angiotensin I-converting enzyme inhibitory activity of the C-terminal hexapeptide of α_{s1}-casein. *Agricultural and Biological Chemistry, 53*, 2107–2114.

Miyoshi, S., Ishikawa, H., Kaneko, T., Fukui, F., Tanaka, H., & Maruyama, S. (1991). Structure and activity of angiotensin-converting enzyme inhibitors in an α-zein hydrolysate. *Agricultural and Biological Chemistry, 55*, 1313–1318.

Okamoto, A., Hanagata, H., Kawamura, Y., & Yanagida, F. (1995a). Anti-hypertensive substances in fermented soybean, natto. *Plant Foods for Human Nutrition, 47*, 39–47.

Okamoto, A., Hanagata, H., Matsumoto, E., Kawamura, Y., Koizumi, Y., & Yanagida, F. (1995b). Angiotensin I-converting enzyme inhibitory activities of various fermented foods. *Bioscience Biotechnology Biochemistry, 59*, 1147–1149.

Ondetti, M. A. (1977). Design of specific inhibitors of angiotensin-converting enzyme: New class of orally active antihypertensive agents. *Science, 196*, 441–444.

Patchett, A. A., Harris, E., Tristram, E. W., Wyvratt, M. J., Wu, M. T., Taub, D., Peterson, E. R., Ikeler, T. J., Broeke, J., Payne, L. G., Ondeyka, D. L., Thorsett, E. D., Greenlee, W. J., Lohr, N. S., Hoffsommer, R. D., Joshua, H., Ruyle, H. V., Rothrock, J. W., Aster, S. D., Maycock, A. L., Robinson, F. M., Hirschmann, R., Sweet, C. S., Ulm, E. H., Gross, D. M., Vassil, T. C., & Stone, C. A. (1980). A new class of angiotensin-converting enzyme inhibitors. *Nature, 298*, 280–283.

Skeggs, L. T., Kahn, J. E., Kahn, J. E., & Shumway, N. P. (1957). The preparation and function of the angiotensin-converting enzyme. *Journal of Experimental Medicine, 103*, 295–299.

Suh, H. J., & Whang, J. H. (1999). A peptide from corn gluten hydrolysate that is inhibitory toward angiotensin I converting enzyme. *Biotechnology Letters, 21*, 1055–1058.

Ukeda, H., Matsuda, H., Osajima, K., Matsufuji, H., Matsui, T., & Osajima, Y. (1992). Peptides from peptic hydrolyzate of heated sardine meat that inhibit angiotensin I-converting enzyme. *Nippon Nogeikagaku Kaishii, 66*, 25–29.

Yang, H. Y. T., Erdös, E. G., & Levin, Y. (1971). Characterization of a dipeptide hydrolase (Kininase II: Angiotensin I converting enzyme). *Journal of Pharmacology and Expermental Therapeutics, 177*, 291–300.

Yano, S., Suzuki, K., & Funatsu, G. (1996). Isolation from α-zein of thermolysin peptides with angiotensin I-converting enzyme inhibitory activity. *Bioscience Biotechnology Biochemistry, 60*, 661–663.