Biotechnology and Bioprocess Engineering **2004**, 9: 400-404

The Relationship between ACE Inhibitory Activity and Degradations of Sulfur Containing Materials in Dolsan Leaf Mustard Juice

Eun-Jeong Yoo, Myeong-Rak Choi, and Hyun-Soo Lim*

Department of Biotechnology, Yosu National University, Yeosu 550-749, Korea

Abstract This study was carried out to investigate the relationship ACE inhibitory activity and degradations of sulfur containing materials in Dolsan leaf mustard juice (DLMJ). The changes of sulfur containing materials which were treated with autolysis, myrosinase, ascorbate and papain were studied, as well as the changes of ACE inhibitory activity in DLMJ. At 37°C, sulfur containing materials by autolysis decreased most rapidly from 0.43% to 0.13% in the second day. Conversely, ACE inhibitory activity increased most from 66% to 87%, in the second day at 37°C. As myrosinase concentrations increased more, sulfur containing materials in DLMJ decreased more. The ACE inhibitory activities at 0, 0.5, 1, 2, and 4 Units of myrosinase for 240 min later were 70, 74, 75, 82, and 85%, respectively. At 1 mM ascorbate, concentrations of sulfur containing materials in DLMJ decreased more significantly on the second day than on the other days. At 1 mM ascorbate for 6 days, ACE inhibitory activity reached a maximum of about 92%. And, an increase of papain concentration was noted in accordance with a decreased sulfur containing materials. The maximum rate of ACE inhibitory activity at control, 3, 6, and 12 Units of papains treatments was shown as 70, 70, 75, and 78% at 60 min, respectively. These results suggested that the degradation of sulfur containing materials led to the increase of ACE inhibitory activity. Consequently, it was suggested that ACE inhibiting was significantly related to the degradatives of sulfur containing materials.

Keywords: leaf mustard, glucosinolates, myrosinase, angiotensin converting enzyme

INTRODUCTION

Recently, many of physiological effects of cruciferae have been reported. During the past decade, there has been continuous interest in studies of physiological effects of cruciferae (*Brassicaceae*), as these are suspected to play a role as anticancer agents, antimutagenics, antioxidants, and have antimicrobial properties. These plants are represented in the family cruciferae (*Brassicaceae*) which includes such vegetables as leaf mustard, broccoli, cabbage, cauliflower and radish. Leaf mustard (*Brassica juncea* Coss. var. *integrifolia*) is famous in Dolsan, Yeosu, Korea. Leaf mustard is the richest source of glucosinolates, ascorbic acid, β-carotene, chlorophyll, dietary fiber and flavonoides which are known to be physiological compounds [1,2]. Among these physiological compounds, glucosinolates, sinigrin and its major degradation products in cruciferae are known as major physiological compounds [3,4]. Glucosinolates are anionic β-D-*S*-glucosides that differ by their aglycon. Glucosinolates may contain at least 120 different aglycones that can be grouped into at least ten structural classes [5]. Glucosinolates are plant secondary products (β-thioglucoside N-hydroxysulfates) [6] which are responsible for its unique flavor and taste. Currently, many of plant secondary metabolites are isolated by solvent extraction from the naturally grown whole plants [7]. Glucosinolates are derived from many of the same amino acids as the cyanogens, although some amino acids serve a precursor for one group but not for the other. Glucosinolates that correspond to alanine, valine, leucine, isoleucine, phenylalnine, tyrosine, and tryptophane are known [8]. The degradation of glucosinolates, sulfur containing compounds in cruciferous plants, is catalyzed by plant and gut bacterial myrosinase [6]. The use of these enzymes is considerable importance for bioconversion to useful physiological compounds in biotechnology [9]. Myrosinase are released when the cells in plants are damaged. The myrosinase-mediated degradation of glucosinolates give rise to an unstable thiohydroximate o-sulfonates which, on release of sulphate, can result in the production of isothiocyanates, thiocyanates, nitriles and elementary sulfur, depending on the pH or other factors [10]. And, these hydrolysis are increased by the addition of ascorbic acid [11,12]. These hydrolysis products of glucosinolates are known to have mutagenic, antimicrobial agent [3], and

***Corresponding author**
Tel: +82-61-659-3306 Fax: +82-61-653-3319
e-mail: limbplab@yosu.ac.kr

anticarcinogenic properties [13]. In our further studies [14], antioxidative and ACE inhibitory activity in Dolsan leaf mustard Kimchi (DLMK) increased until the time of optimum ripening period. It might be that the production of naturally occurring isothiocyanates, on release of sulfate, which are known to physiological compounds by degradation of glucosinolates.

The cause of anticancer and antioxidative active compounds in leaf mustard is partly known. However, antihypertensive active compounds in leaf mustard remain unknown. Antihypertensive activities can be determined as angiotensin I-converting enzyme (ACE) inhibitory activity. ACE, a zinc containing enzyme, catalyzes the formation of the potent vasopressor angiotensin II from angiotensin I and inactivates bradykinin which has a vasodilating action [15,16]. ACE is also known as kininase II which is involved in the breakdown of kinins, potent vasodilators [17]. Recently, ACE inhibitors have been screened from natural sources. Because, synthetic drugs such as captopril and enalapril are thought to contribute to side effects, cough, taste disturbances, and skin rash [18]. ACE inhibitors from dried bonoto [19], sardine muscle [20], tuna [21], soy sauce, and miso were isolated. Some of ACE inhibitors were reported to have a bitter taste and fishy odor [22]. Hence, much attention has been focused on a new active compound found in edible plants and exhibiting potent ACE inhibitory activity.

This study was carried out investigate the relationship of ACE inhibitory activity and degradations of sulfur containing materials in DLMJ. And, the useful treating method among autolysis, ascorbate, papain and myrosinase were suggested to increase the antihypertensive activities in DLMJ.

MATERIALS AND METHODS

Material

The leaf mustard was obtained from Dolsan, Yeosu, Korea. The other ingredients such as garlic, ginger, red pepper powder and green onion were purchased from a local market in Yeosu, Korea. Myrosinase, papain, angiotensin converting enzyme (EC 3.4.15.1, 3.2 Unit/mg solid), HHL (Hippuryl-His-Leu), ascorbate and lead acetate were obtained from Sigma Chemical Co.

Preparation of Dolsan Leaf Mustard Juice (DLMJ)

5.00 kg of DLM were blended by a blender (Hanil, HMF-340, Seoul, Korea) and filtered with sterilized gauze. The filtrates were centrifuged at 1,500 rpm for 10 min. 3.96 kg of supernatants were filtered with a Whatman No. 2 paper and then stored at -20°C. And DLMJ were autolysis during 10 days at 4, 10, 20, and 37°C.

Enzyme Treatment

DLMJ were autoclaved and centrifuged at 1,500 rpm for 15 min. The supernants were adjusted with 0.1 N NaOH to pH 6.2 and 7.0. Papain (EC 3.4.22.2) was dissolved at 3, 6, and 12 Unit in 1 mL of DLMJ (pH, 6.2). Myrosinase (EC 3.2.3.1) was dissolved in 0.5, 1, 2, and 4 Unit in 1 mL of DLMJ (pH 7.0). The DLMJ-papain and DLMJ-myrosinase mixture were agitated and incubated at 25°C and 37°C during 240 min, respectively. The enzyme reaction was stopped in boiling water bath for 10 min.

Analysis of Organic Sulfur Concentration

The organic sulfur in DLMJ was autolysed and treated with ascorbate, protease, and myrosinase and analysed with elemental analyzer (EA-1120, CE Instrument, Rodanomilan, Italy). DLMJ was adjusted with 0.1 N NaOH or HCl to pH 6.0. And then 0.15 M lead acetate was added to DLMJ for removing of inorganic sulfur released from glucosinolates. And the mixture was agitated using a vortex mixer for 30 min. After precipitating, the sample was centrifuged at 12,000 rpm for 15 min and supernatants were filtered with 0.45 μm syringe. And the sample was dried at 105°C for 2 days. Analysis of sulfur with elemental analyzer was performed on GC columns and TCD (thermal conductive detector) with helium gas, at an oven temperature of 60°C and a flow rate of 120 mL/min.

Analysis of ACE Inhibitory Activity

The ACE (peptidyldipeptide hydrolase, EC 3.4.15.1) inhibitory activity was assayed by the method described by Cushman and Cheung with slight modification [13]. 100 μL of 25 mM Hip-His-Leu solution was mixed with 50 μL DLMJ and then preincubated at 37°C for 10 min. The reaction was initiated by adding 150 μL of ACE dissolved in a sodium borate buffer (pH 8.3), and the mixture was incubated at 37°C for 60 min. The reaction was stopped by adding 250 μL of 1 N HCl. The hippuric acid was extracted with 1.5 mL of ethyl acetate. The extracts were centrifuged at 2,500 rpm for 10 min. The supernatant was dried and dissolved in 3 mL of 1 M NaCl. The absorbance level at 228 nm was measured to evaluate the degree of inhibition in the ACE activity. The extent of inhibitory ratio was calculated as follows: inhibitory ratio (%) = $((C-S)/(C-S'))\times100$, where S is the absorbance of the sample, S' is the absorbance of a control sample and C is the absorbance without a sample.

RESULTS AND DISCUSSION

Autolysis of DLMJ

Changes of sulfur containing materials according to various temperatures were shown in Fig. 1. Sulfur containing materials at 37°C decreased most rapidly from 0.43% to 0.13% in the second day. Myrosinase are released when the cells in plants are damaged. Since breakdown of cells in DLM after sample (DLM)was treated by

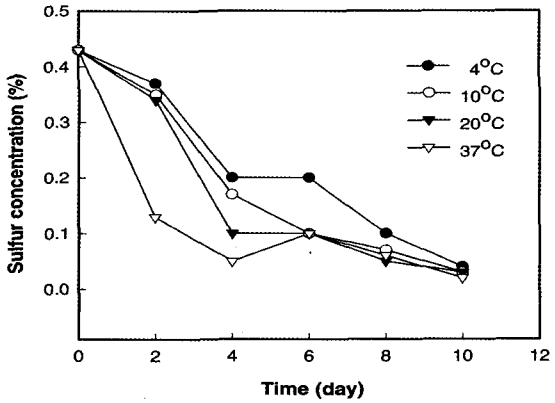

Fig. 1. Changes of sulfur containing materials in DLMJ according to autolysis.

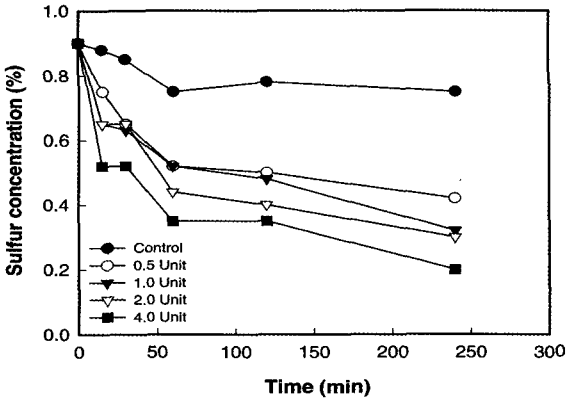

Fig. 3. Changes of sulfur containing materials in DLMJ according to myrosinase hydrolysis.

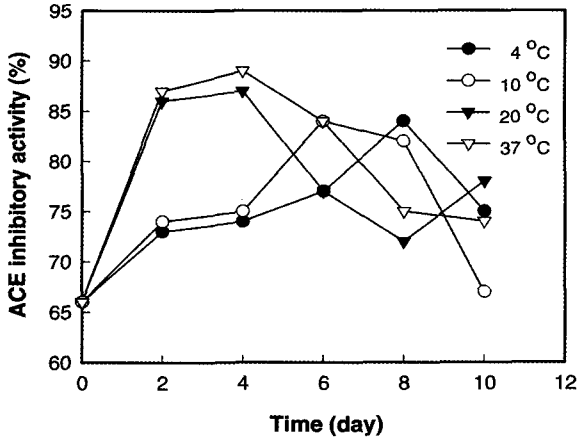

Fig. 2. Changes of ACE inhibitory activity in DLMJ according to autolysis.

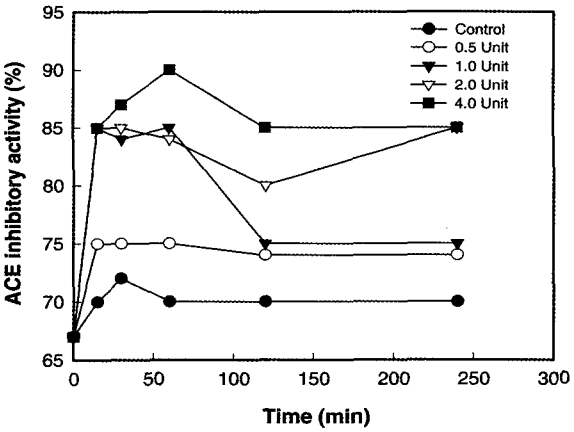

Fig. 4. Changes of ACE inhibitory activity in DLMJ according to myrosinase hydrolysis.

blender, autolysis could be occurred in DLMJ by myrosinase. And also, at optimal temperature of myrosinase, reported as 37°C [23], hence sulfur containing materials decreased most rapidly. Subsequently, there was 0.01~0.04% sulfur containing materials persisting until the 10th day.

Changes of ACE inhibitory activity increased most from 66% to 87% in the second day (Fig. 2). The maximum ACE inhibitory activities at 4, 10, 20, and 37°C were 82, 83, 87, and 89%, respectively. In addition, the higher temperature of autolysis in DLMJ was reached maximum ACE inhibitory activities in a short time. Thus, as sulfur containing materials decreased, ACE inhibitory activities increased. This tendency was the same at all temperatures. The degradation of sulfur containing compounds (glucosinolates) in DLMJ by autolysis, on release of sulfate, could be result in the production of isothiocyanates, thiocyanates and nitriles. These hydrolysis products of glucosinolates are known to have physiological activity [3,10]. It suggested that ACE inhibitor in DLMJ was closely related with the reduction of sulfur containing materials.

Effect of Myrosinase

The changes of sulfur containing materials in DLMJ according to myrosinase treatment are shown in Fig. 3. When DLMJ was treated with myrosinase at the early stage, concentration of sulfur containing materials were all about 0.9%. The concentration of sulfur containing materials at 0.5, 1, 2, and 4 Units for 240 min later were 0.39, 0.29, 0.25, and 0.19%, respectively. As myrosinase was treated to DLMJ, sulfur containing materials decreased rapidly than that of autolysis.

The changes of ACE inhibitory activity in DLMJ ac cording to myrosinase treatment are shown in Fig. 4. At 4 Units myrosinase treatment, ACE inhibitory activity was at its highest value, at 92% for 4 days. The ACE inhibitory activities at 0, 0.5, 1, 2, and 4 Units for 240 min later were 70, 74, 75, 82 and 85%, respectively.

Myrosianse catalyzed the hydrolysis of sulfur containing materials in DLMJ to give D-glucose and an aglycon, which then rearranges to give sulfate and isothiocyanates. And, an increase in myrosianse concentration led to the increase of ACE inhibitory activity. Consequently, it was

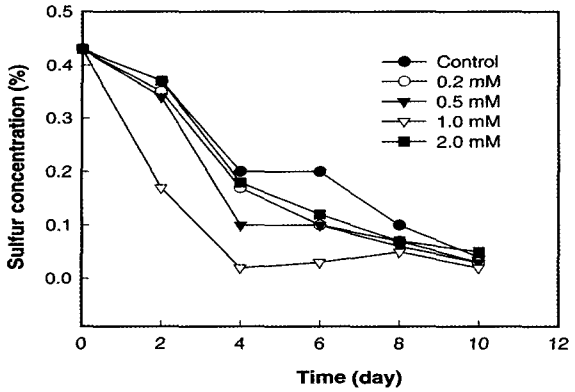

Fig. 5. Changes of sulfur containing materials in DLMJ according to ascorbate.

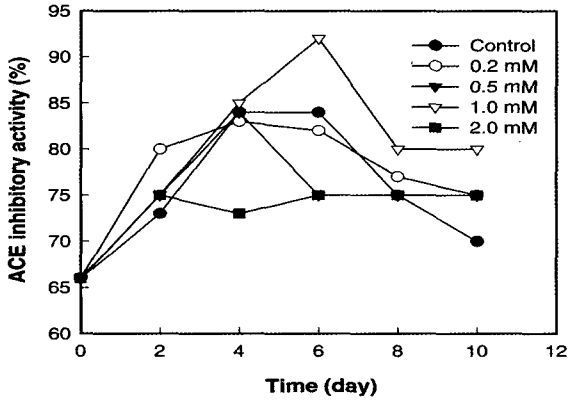

Fig. 6. Changes of ACE inhibitory activity in DLMJ according to ascorbate.

suggested that ACE inhibitor was significantly related to the degradation of sulfur containing materials. Although ACE inhibitors previously reported are classified in two groups, peptide and flavonoid inhibitors, mainly [24], this study suggested that isothiocyanates should also be ACE inhibitor.

Effect of Ascorbate

Myrosinase (*S*-glycosidase) hydrolyzes plant anionic 1-thio-β-D-glucosides (glucosinolate) and is considered part of the plant defense system. Ohtsuru and Hata [12] reported that myrosinase is activated by ascorbic acid. Hence, we observed the relationship between sulfur containing materials and ACE inhibitory activity in DLMJ according to ascorbate treatment.

The changes of sulfur containing materials in DLMJ according to ascorbate treatment are shown in Fig. 5. At 1 mM ascorbate, concentrations of sulfur containing materials in DLMJ decreased more significantly by the second day than on any other day. Ohtsuru *et al.* [12] reported that the rate of hydrolysis of sinigrin, catalyzed by the mustard enzyme, is increased more than 25-fold by the addition of 1 mM ascorbic acid. In our study, at a concentration of 1 mM ascorbate the effect was maximized. But, at 2 mM of ascorbate, although high concentration of ascorbate, the rate of hydrolysis was not increased over that of 1 mM ascorbate. These results suggested that high concentrations of ascorbates begin to competitively inhibit the myrosinase reaction [3,4]. The changes of ACE inhibitory activity in DLMJ according to ascorbate treatment are shown in Fig. 6. At a concentration of 1 mM ascorbate, the hydrolysis rate of sulfur containing materials was the most increased and ACE inhibitory activity was maximized at about 92% for 6 days.

These results suggest that the ACE inhibitor in DLMJ is related to sulfur containing materials and it could be myrosinase hydrolysis products from sulfur containing materials by specifically activated ascorbate.

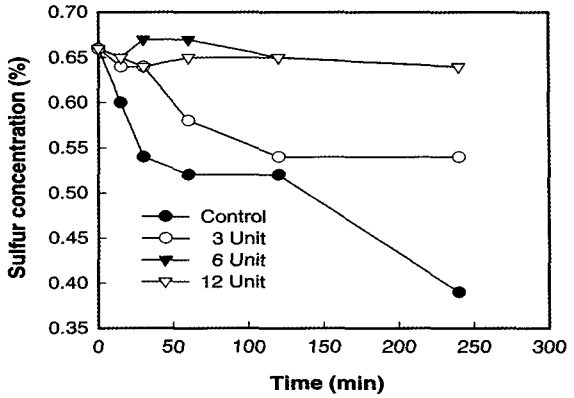

Fig. 7. Changes of sulfur containing materials in DLMJ according to papain hydrolysis.

Effect of Papain

The changes of sulfur containing materials in DLMJ according to papain treatment are shown in Fig. 7. Papains were administered to DLMJ at early stage (0 min), concentrations of sulfur containing materials of all samples were about 0.66%. And, control, 3, 6, and 12 Units of papains were treated to DLMJ 240 min later, concentration of sulfur containing materials were 0.64, 0.62, 0.54, and 0.39%, respectively. Thus, increase of papain concentrations were in accordance with a decreased in sulfur containing materials. It suggested that protease like papain could affect the reduction of sulfur containing materials in DLMJ. L-amino acids such as alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, and tryptophane are precursors for glucosinolates. And, also, sulfur containing materials (glucosinolates) may also occurred in association with proteins in plants [24]. Hence, sulfur containing materials which were adducted with protein in DLMJ could be extricated by papain treatments.

The ACE inhibitory activity in DLMJ corresponding to papain treatments are shown in Fig. 8. The maximum rate of ACE inhibitory activity at control (0), 3, 6, and 12 Units of papains treatments were shown as 70, 70, 75, and 78%, respectively 60 min later. Yuk *et al.* [24] reported that ACE inhibitory effect of the pronase treated

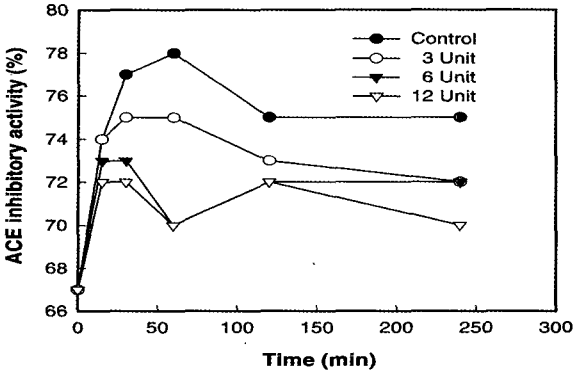

Fig. 8. Changes of ACE inhibitory activity in DLMJ according to papain hydrolysis.

sample from *Sinapis alba* L. extracts decreased compared with that of the untreated sample. In our research, the digetion of protein by papain treatment was affected to ACE inhibitory activity. These results suggest that sulfur containing materials in DLMJ was adducted with protein. These protein hydrolysis by papain resulted to the reduction of sulfate in DLMJ. And, the production of isothiocyanates gave rise to ACE inhibitory activity.

Acknowledgement This research was supported by the Korea Science Engineering Foundation(KOSEF) in 2003(2003-000-10415-0).

REFERENCES

[1] Cheigh, H. S. and K. Y. Park (1994) Biochemical, microbiological and nutritional aspects of Kimchi (Korea fermented vegetable products). *Crit. Rev. Food Sci. Nutr.* 34: 175-177.

[2] Cho, Y. S. and S. K. Park (1990) Studies on the chemical components of Korean *Brassica Juncea Cosson*. *Sunchon Natl. Univ. Bull.* 9: 169-172.

[3] Eilert. U., B. Wolters, and A. Nahrstedt (1981) The antibiotic principle of seeds of *Moringa oleifera and Moringa stenopetala*. *Planta Med.* 42: 55-61.

[4] Louda, S. and S. Mole (1991) Glucosinolates. pp.123-164. In: G. A. Rosenthal and M. R. Berenbaum (eds.). *Chemistry and Ecology in Herbivorous: Their Interactions with Secondary Plant Metabolites*. Kluwer Academic Publisher, London, UK.

[5] Cole, R. A. (1976) Isothiocyanates, nitriles, and thiocyanates as products of autolysis of glucosinolates in cruciferae. *Phytochemistry* 15: 759-762.

[6] Troyer, J. K., K. S. Ketherine, and W. F. Jed (2001) Analysis of glucosinolates from broccoli and other cruciferous vegetables by hydrophilic interaction liquid chromatography. *J. Chromatogr. A.* 31: 1-6.

[7] Saurabh C., S. Farkya, A. K. Srivastava, and V. S. Bisaria (2002) Bioprocess considerations for production of secondary metabolites by plant cell suspension cultures. *Biotechnol. Bioprocess Eng.* 7:138-149.

[8] Larsen, P. O. (1981) Glucosinolates in secondary plant products. pp. 35-37. In : P. K. Stumpf and E. E. Conn (eds.). *Biochemistry of Plants*. Vol. 7, Academic Press, New York, USA.

[9] Ramachandria, M., J. Bhat, and P. K. A. Muniswaran (2002) Prediction of continuous reactors performance based on batch reactor deactivation kinetics data of immobilized lipase. *Biotechnol Bioprocess Eng.* 7: 225-230.

[10] Atle, M. B. and T. R. John (1996) The myrosinase-glucosinolate system, its organisation and biochemistry. *Physiol. Plant* 97: 194-208.

[11] Tsuruo, I., M. Yosida, and T. Hata (1967) Studies on the myrosinase in mustard seed.: Part I. the chromatographic behaviors of the myrosinase and some of its characteristics *Agric. Biol. Chem.* 31: 27-32.

[12] Ohtsuru, M. and T. Hata (1979) The interaction of L-ascorbic acid with the active center of myrosinase. *Biochim Biophys. Acta.* 567: 384-391.

[13] Lim, H. S., E. J. Yoo, and M. R. Choi (2000) Changes of physiological activity of mustard leaf during its fermentation period. *J. Microbiol. Biotechnol.* 10: 43-47.

[14] Choi, Y. Y., E. J. Yoo, H. S. Lim, D. S. Kang, N. Nishizawa, and M. R. Choi (2001) The relationship between physiological activity and cell number in Dolsan leaf mustard Kimchi. *J. Food Sci. Nutr.*: 117-121.

[15] Curtiss, C., J. N. Chon, T. Vrobel, and J. A. Franciosa (1978) Role of the renin-angiotensin system in the systemic vasocontraction of chronic congestive heart failure. *Circulation* 58: 763-765.

[16] Erdoś, E. G. and R. A. Skidgel (1987) The angiotensin-I converting enzyme. *Lab Invest.* 56: 345-349.

[17] Erdos, E. G. (1990) Angiotensin-I converting enzyme and the changes in our concepts through the years. *Hypertension* 16: 363-365.

[18] Atkinson, A. B. and J. I. S. Robertson (1980) Benefits and risks in severe hypertension. *Lancet* 2: 105-107.

[19] Yokoyama, K., H. Chiba, and M. Yoshikawa (1992) Peptide inhibitors for angiotensin-I converting enzyme from thermolysin digest of dried bonoto. *Biosci. Biotechol. Biochem.* 56: 1541-1543.

[20] Matsui, T. H. Matsufuji, E. Seki, K. Osajima, M. Nakashima, and Y. Osajima (1993) Inhibition of angiotensin-I converting enzyme by *Bacillus licheniformis* alkaline protease hydrolysates derived from sardine muscle. *Biosci. Biotechnol. Biochem.* 57: 922-924.

[21] Kohama, Y., S. Matsumoto, H. Oka, T. Teramoto, M. Okabe, and T. Mimura. (1988) Isolation and ACE-inhibitor from tuna muscle. *Biochem. Biophys. Res. Commun.* 155: 332-334.

[22] Arai, S. (1996) Studies on functional foods in Japan-state of the art. *Biosci. Biotechnol. Biochem.* 60: 9-11.

[23] Shin, C. S., K. I. Seo, K. S. Kang, C. W. Ahn, Y. G. Kim, and K. H. Shim (1996) Purification and enzymatic of myrosinase in Korean mustard seed (*Brassica Juncea*). *J. Kor. Soc. Food Sci. Nutr.* 25: 687-694.

[24] Yuk, J. S., Y. H. Lim, and H. Y. Cho (2000) Purification and characterization of angiotensin I-converting enzyme inhibitors from *Sinapis alba* L. *J Food Sci. Nutr.* 5: 75-80.

[Received July 27, 2004; accepted September 28, 2004]