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RESEARCH NOTE

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Antioxidant and ACE Inhibitory Activities of Soybean Hydrolysates: Effect of Enzyme and Degree of Hydrolysis

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Abstract Native soy protein isolate (SPI) was hydrolyzed with 4 different proteolytic enzymes, including bromelain, papain, Neutrase, and Flavourzyme. SPI hydrolysates with the degree of hydrolysis (DH) in range of 6 to 15% were prepared by each enzyme. The angiotensin I converting enzyme (ACE) inhibitory and the antioxidant activities of the SPI hydrolysates, such as superoxide dismutase-like activity and inhibition of the linoleic acid autoxidation, were evaluated. Overall, as the DH increased, all evaluated bioactivities of the SPI hydrolysates significantly increased. The significantly highest ACE inhibitory and antioxidant activities were found in hydrolysates made with papain and bromelain, respectively. SPI hydrolysates by Flavourzyme showed the significantly lowest activity in all tested bioactivities. The results suggested that ACE inhibitory and antioxidant activities of SPI hydrolysates were determined by the DH and by the enzyme used.

Keywords: soy protein isolate, angiotensin I converting enzyme (ACE) inhibitory activity, antioxidant activity, degree of hydrolysis, proteolytic enzyme

Introduction

Soybean, one of the most cultivated plants in the world (1), is an important protein source and a potential source of various health effects (2). As the main components of soybean, soy proteins have been known to possess multiple health-promoting functions, including antioxidant (3), antitumor (4), hypocholesterolemic (5), and antihypertensive properties (6). In addition, the proteolytic hydrolyzed peptides from the soy proteins have been receiving more and more attention since the functional and biological improvement of soybean peptides has been reported (7-9).

In the past several decades, researchers have evaluated several biological activities of various enzymatic hydrolysates from the different food sources (2). Since Mellander (10) suggested in 1950 that casein-derived phosphorylated peptides enhanced vitamin D-independent bone calcification, numerous bioactive peptides have been isolated and characterized from different food protein sources, including milk (11-13), egg (14), fish (15), prawn (16), cereal (rice, wheat, buckwheat, barley, corn) (17), and soybean (7-9,18). Previous studies have shown that numerous food-derived bioactive peptides have higher biological activities, such as antihypertensive (12), anticancer (18), antiobesity (9), and antioxidant activities (7,8) than intact protein.

Dietary peptides are protein fragments or short amino acid chains (19) produced by the processing of food via acidic and enzymatic hydrolysis and fermentation or by enzymatic digestion in the gut (2,19). In a native protein molecule, hydrophobic groups, which have been reported to show some beneficial effect on various bioactivities, are buried inside the core of the folded structure. After partial hydrolysis, some of these groups are exposed, and various bioactivities as well as hydrophobicities of hydrolysates are also increased (20,21). Dietary bioactive peptides generally contain 2 to 9 amino acids; however, this range may be extended to 20 or more amino acid units (22). In addition, a number of proteolytic enzymes obtained from animal, plant, and microbial peptidases, such as pepsin, trypsin, papain, and bromelain, are used commonly for the proteolysis of food proteins (8,12,16-18,23-25). For these reasons, to obtain desirable dietary peptide having specific bioactive properties, the specifically suitable enzyme for its protein substrate should be selected through the screening step for the enzyme with regard to the target bioactivity. In addition, to control the degree of hydrolysis (DH) of hydrolysates is also very essential for the production of the targeted bioactive peptide (2). However, few studies have compared the suitability of enzymes and DH for soy protein to produce the bioactive peptide with various biological activities.

To understand the relation between various bioactivities and the principle hydrolysis condition, including enzyme used and DH, it is essential to know the bioactivities of hydrolysates with various DH using different proteolytic enzymes. Therefore, in this study, soy protein isolate (SPI) was hydrolyzed with different proteolytic enzymes, including bromelain, papain, Neutrase, and Flavourzyme. SPI hydrolysates with the DH in range of 6 to 15% were prepared by each enzyme and the effects of the enzyme used and DH on the angiotensin I converting enzyme (ACE) inhibitory and antioxidant activities were evaluated.

Materials and Methods

Materials SPI was obtained from Wonwoo Co., Ltd. (Chungnam, Korea). The rabbit lung acetone powder and hippuryl-L-histidyl-L-leucine (HHL) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Two plant-derived and 2 microbiological proteolytic enzymes

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were used to hydrolyze SPI. Neutrase (EC 3.4.24.28, from *Bacillus amyloliquefaciens*, 1.5 AU/g), Flavourzyme (EC 3.4.11.1, from *Aspergillus oryzae*), were purchased from Novo Nordisk (Bagsvaerd, Denmark). Papain (EC 3.4.22.2) and bromelain (EC 3.4.22.32) were purchased from Sigma-Aldrich. All the other reagents were of analytical grade.

Preparation of soybean hydrolysates SPI was dispersed in distilled water as a 10%(w/v) solution and incubated at 50°C for 30 min by stirring continuously. To be convenient for commercial use, SPI solution was not adjusted to pH and the pH of this solution was measured at 6.8 to 7.1. The reaction was initiated by the addition of the enzyme to give a final enzyme-to-substrate ratio of 0.5%(w/w). After the predetermined reaction time, the mixture was heated at 100°C for 10 min to inactivate the enzyme. In previous study (26), SPI was hydrolyzed in the same condition as mentioned above, hydrolysates were removed at various time intervals, and the DH of hydrolysates was measured by trinitrobenzensulfonic acid method according to Adler-Nissen (27). To prepare the SPI hydrolysates of the predetermined DH (6, 9, 12, and 15%), each reaction time was determined from the DH curve in previous study (26). After the preparation of hydrolysates, it was confirmed that their experimental DH were in a close agreement with the intended DH.

Assay of ACE inhibitory activity ACE inhibitory activity was measured by the method of Cushman and Cheung (28) with modification. The reaction mixture contained 50 μL of 20 mM HHL as a substrate, 50 μL of ACE powder (20 munit) in a 150 mM sodium borate buffer (pH 8.3), and 50 μL of the sample solution. The reaction was carried at 37°C for 30 min, and terminated by adding 100 μL of 1.75 N HCl and 1 mL of ethyl acetate. After centrifugation, the absorbance of the supernatants was measured at 228 nm.

Assay of antioxidant activity ***Superoxide dismutase (SOD)-like activity***: SOD-like activity was assayed by the method of Marklund and Marklund (29). The reaction mixture was prepared by 0.2 mL of the sample solution, 3.0 mL of the Tris-HCl buffer (50 mM Tris (hydroxymethyl) aminomethane+10 mM ethylene diamine tetraacetic acid, pH 8.5), 0.2 mL of 7.2 mM pyrogallol and stood at 25°C for 10 min. The oxidized pyrogallol was measured at 420 nm, using a spectrophotometer (DU 650; Beckman Coulter Inc., Fullerton, CA, USA), after stopping the reaction by adding 1.0 mL of 1.0 N HCl. The SOD-like activity was expressed as the reduction rate of the absorbance.

$$\text{SOD-like activity (\%)} = [1-(A_1/A_0)]\times 100 \quad (1)$$

where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample.

Inhibition of linoleic acid autoxidation: Antioxidant activity of SPI hydrolysates in a linoleic acid model system was measured according to the methods of Osawa *et al.* (30) with some modification. The sample (1.3 mg) was dissolved in 10 mL of 50 mM phosphate buffer (pH 7.0) and added to a mixed solution that consisted of 0.13 mL of linoleic acid and 10 mL of 99.5% ethanol. Then the total volume was adjusted to 25 mL with distilled water. The mixed solution was incubated at 40°C in a dark room for 5 days and the degree of oxidation was evaluated by measuring the thiobarbituric acid (TBA) value. The TBA value was measured using a modified version of the method of Ohkawa *et al.* (31). The inhibition of lipid peroxidation in percentage was calculated by following equation:

$$\text{\% Inhibition} = [(A_0 - A_1)/A_0]\times 100 \quad (2)$$

where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample.

Statistical analysis Statistical analysis was carried out using the Statistical Package for Social Science (SPSS) statistics program for Windows (SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) was used and mean comparison was performed by Duncan's multiple range test ($p<0.05$).

Results and Discussion

Effects of DH and enzyme on ACE inhibitory activities SPI was hydrolyzed by 4 different enzymes, bromelain, papain, Neutrase, and Flavourzyme; SPI hydrolysates with DH of 6, 9, 12, and 15% were produced. When all enzymes used are taken into account, it can be concluded that the ACE inhibitory activities of SPI hydrolysates significantly increased as the DH increased (Fig. 1). The most potent ACE inhibitory activity was 73.23%, which was observed in papain-treated hydrolysate with 15% DH and this value was about 22-fold more than that of native SPI. This result clearly showed that hydrolysis was required to release ACE inhibitory peptides from the unhydrolyzed intact SPI. Higher average ACE inhibitory activities were obtained in SPI hydrolysates with the highest DH, compared to those with the lower DH hydrolysates; however, no significant differences were observed for ACE inhibitory activities of SPI hydrolysates with DH of 12 and 15%, except Flavourzyme-treated hydrolysates. This result implies that the excess hydrolysis has little effect on the ACE inhibitory activity. This result was also consistent with the previous studies of ACE inhibitor from milk protein hydrolysates, in which the increase in ACE inhibitory activity due to hydrolysis was noted after 3 hr of hydrolysis and was still pertinent after 24 hr of hydrolysis (23). Chiang *et al.* (32) reported that when ACE inhibitory activities of the fractionated SPI hydrolysates (1, 10, and 30 kDa permeate) were evaluated, IC_{50} values of 1 and 10 kDa permeate were significantly lower than that of 30 kDa permeate; however, both IC_{50} values of 1 and 10 kDa permeate were not significantly different.

When considering all DH, there was a significant effect of enzyme used and SPI hydrolysates by papain treatment showed the significantly highest ACE inhibitory activities. This is probably related to the fact that papain is specific for many hydrophobic amino acid residues, resulting in a high number of small peptides with good inhibitory activity (20). Pripp *et al.* (33) reported that there was a positive correlation between the hydrophobicity of the C-terminal amino acid and the ACE inhibitory activity of proteolytic peptides.

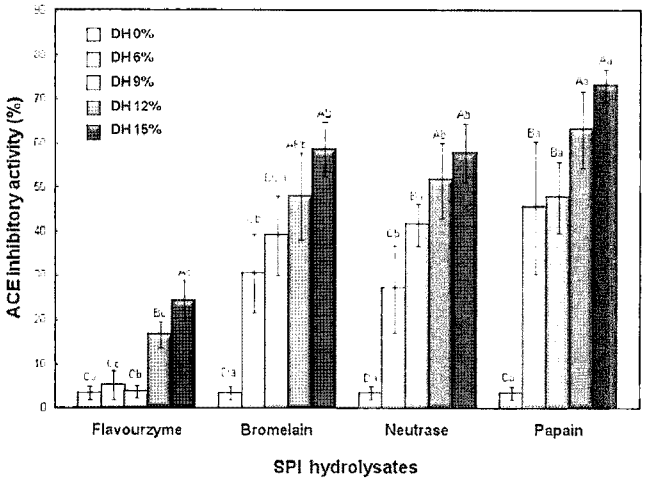

Fig. 1. ACE inhibitory activities of SPI hydrolysates. Different upper- and lower-case letters on the bars indicate a significant difference among the different DH of each SPI hydrolysate and among the different enzyme used of SPI hydrolysates with the same DH, respectively ($p<0.05$).

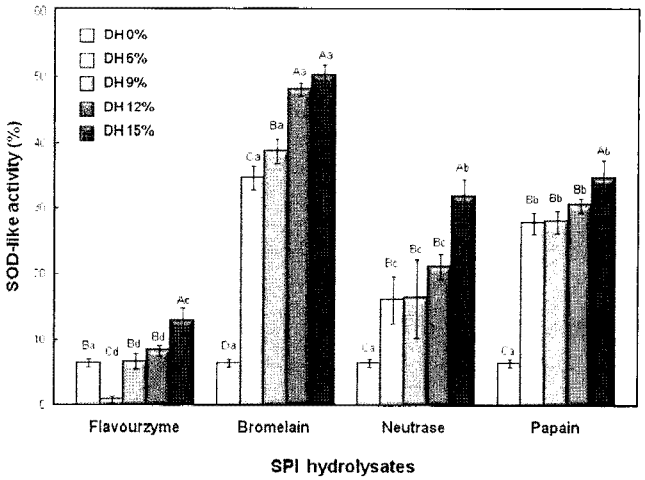

Fig. 2. SOD-like activities of SPI hydrolysates. Different upper- and lowercase letters on the bars indicate a significant difference among the different DH of each SPI hydrolysate and among the different enzyme used of SPI hydrolysates with the same DH, respectively ($p<0.05$).

SPI peptides by Flavourzyme showed the significantly lowest ACE inhibitory activities. This result could be due to the fact that Flavourzyme contains both endoprotease and exoprotease activities (32), and produces hydrolysates having higher DH values than other enzymes; however, compared with other enzyme-treated hydrolysates with the same DH, these hydrolysates have higher levels of high (>10 kDa) molecular mass material and lower levels of hydrophobic peptides (34). Chiang *et al.* (32) also suggested that the exoprotease activity may involve the inactivation of the active peptide sequence by cutting one or more amino acids from N-terminal or C-terminal positions.

Effects of DH and enzyme on antioxidant activities

SOD-like activity: The SOD-like activity, which is the important self-defense mechanism of body cell against oxidative damage, was evaluated as free radical scavenging activity of each SPI hydrolysate against superoxide anion radicals (Fig. 2). As DH increased, the SOD-like activities significantly increased. All proteolytic hydrolysates showed significantly higher SOD-like activity than the original SPI by 2- or 8-fold, except for Flavourzyme-treated SPI hydrolysates. This result implies that SOD-like activities could be caused by the smaller peptide with the higher DH. Among the 4 enzymes, bromelain showed the significantly highest SOD-like activities at any DH and SPI hydrolysates by Flavourzyme showed the significantly lowest SOD-like activities. However, little information is available on the SOD-like activities of soy protein and soy hydrolysates.

Inhibition of linoleic acid autoxidation: The inhibition of linoleic acid autoxidation by SPI hydrolysates was expressed as the relative antioxidant based on the days of the induction period according to the TBA method. Figure 3 shows the percentage of inhibition calculated from the absorbance after 5 day during treatment. Although proteolytic hydrolysates, except Flavourzyme-treated hydrolysates, showed significantly higher inhibition of linoleic acid autoxidation than the original SPI, the increasing effect due

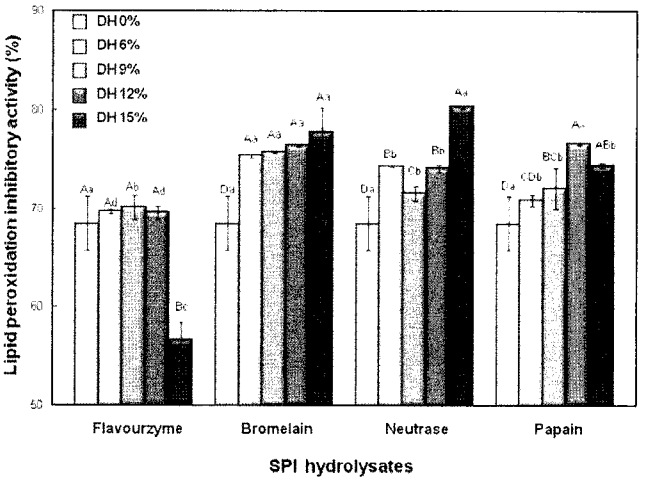

Fig. 3. Lipid peroxidation inhibitory activities of SPI hydrolysates. Different upper- and lowercase letters on the bars indicate a significant difference among the different DH of each SPI hydrolysate and among the different enzyme used of SPI hydrolysates with the same DH, respectively ($p<0.05$).

to hydrolysis was slight, compared with that of SOD-like activity. When the inhibition of linoleic acid autoxidation and the SOD-like activity of the SPI hydrolysates were compared with that of intact SPI, the maximum increasing effect of SPI hydrolysates due to hydrolysis increased by 1.2 and 8.0 times, respectively. This result could be due to the difference of the antioxidant properties measured to determine SOD-like activity and inhibition of linoleic acid autoxidation. To determine the overall antioxidant action, it was necessary to examine several methods, including the lipid peroxidation inhibitory, the reducing activity, the radical scavenging activity, and the peroxynitrite scavenging activity (2,7). These several methods seem to be necessary in understanding how the properties have their antioxidant activity. In addition, the native soy protein already possessed appreciable antioxidant activity of 68% inhibition of linoleic acid autoxidation.

When considering all DH, there was a significant effect of enzyme used; bromelain achieve higher inhibition of linoleic acid autoxidation than the other enzymes. This result could be due to the fact that bromelain is a plant cysteine-endoprotease, which has been generally known to have a catalytic dyad of cysteine and histidine residues (35), resulting in a high number of peptide with histidine residues exposed (36). Histidine-containing peptides have been known to be antioxidant (7), acting as metal-ion chelators, active-oxygen quenches, and hydroxyl-radical scavengers (37). However, Hirose and Miyashita (38) reported that SPI hydrolysates could function as a protective membrane around lipid droplets from oxidation initiators. To act as a membrane, proteins must possess a certain structural integrity; however, extensive degradation could reduce the peptide's ability to act as a physical barrier to prevent oxidants from reaching the lipid fraction in the linoleic emulsion solution. This might be the reason for the decreased inhibition of linoleic acid autoxidation observed in SPI hydrolysates with DH of 15% by Flavourzyme and papain.

In conclusion, our findings suggest that in the ACE inhibitory and the antioxidant activities, such as SOD-like activity and inhibition of the linoleic acid autoxidation, there were significant effects of enzyme used and DH. Overall, as the DH increased, all evaluated bioactivities of the SPI hydrolysates increased. The highest ACE inhibitory and antioxidant activities were found in hydrolysates made with papain and bromelain, respectively. In contrast, SPI hydrolysates by Flavourzyme showed the significantly lowest activity among all bioactivities. The results revealed that ACE inhibitory and antioxidant activities of SPI hydrolysates were specifically determined by the DH and by the enzyme used. In addition, SPI hydrolysates can be used as the more potent ACE inhibitory and antioxidant properties, as SPI was hydrolyzed with the proper DH and enzyme for target bioactivity.

Acknowledgments

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References

1. Gibbs BF, Zougman A, Masse R, Mulligan C. Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. Food Res. Int. 37: 123-131 (2004)
2. Wang W, de Mejia EG. A new frontier in soy bioactive peptides that may prevent age-related chronic diseases. Compr. Rev. Food Sci. F. 4: 63-78 (2005)
3. DiSilvestro RA, Mattern C, Wood N, Devor ST. Soy protein intake by active young adult men raises plasma antioxidant capacity without altering plasma testosterone. Nutr. Res. 26: 92-95 (2006)
4. Pollard MWW. Prevention of spontaneous prostate-related cancer in lobund-wistar rats by a soy protein isolate/isoflavone diet. Prostate 45: 101-105 (2000)
5. Potter SM. Overview of proposed mechanisms for the hypocholesterolemic effects of soy. J. Nutr. 125: 606S-611S (1995)
6. Burke V, Hodgson JM, Beilin LJ, Giangiulioi N, Rogers P, Puddey IB. Dietary protein and soluble fiber reduce ambulatory blood pressure in treated hypertensives. Hypertension 38: 821-826 (2001)
7. Chen HM, Muramoto K, Yamauchi F, Fujimoto K, Nokihara K. Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein. J. Agr. Food Chem. 46: 49-53 (1998)
8. Pena-Ramos EA, Xiong YL. Antioxidant activity of soy protein hydrolysates in a liposomal system. J. Food Sci. 67: 2952-2956 (2002)
9. Kim HJ, Bae IY, Ahn C-W, Lee S, Lee HG. Purification and identification of adipogenesis inhibitory peptide from black soybean protein hydrolysate. Peptides 28: 2098-2103 (2007)
10. Mellander O. The physiological importance of the casein phosphopeptide calcium salts. II. Peroral calcium dosage of infants. Acta Soc. Med. Ups. 55: 247-255 (1950)
11. Meisel H. Biochemical properties of regulatory peptides derived from milk proteins. Biopolymers 43: 119-128 (1997)
12. Tsai JS, Chen TJ, Pan BS, Gong SD, Chung MY. Antihypertensive effect of bioactive peptides produced by protease-facilitated lactic acid fermentation of milk. Food Chem. 106: 552-558 (2008)
13. Pihlanto A. Antioxidative peptides derived from milk proteins. Int. Dairy J. 16: 1306-1314 (2006)
14. Matoba N, Usui H, Fujita H, Yoshikawa M. A novel anti-hypertensive peptide derived from ovalbumin induces nitric oxide-mediated vasorelaxation in an isolated shr mesenteric artery. FEBS Lett. 452: 181-184 (1999)
15. Klompong V, Benjakul S, Kantachote D, Shahidi F. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (selaroides leptolepis) as influenced by the degree of hydrolysis and enzyme type. Food Chem. 102: 1317-1327 (2007)
16. Suetsuna K. Antioxidant peptides from the protease digest of prawn (*Penaeus japonicus*) muscle. Mar. Biotechnol. 2: 5-10 (2000)
17. Do J-R, Heo I-S, Back S-Y, Yoon H-S, Jo J-H, Kim Y-M, Kim K-J, Kim S-K. Antihypertensive, antimicrobial, and antifungal activities of buckwheat hydrolysate. Food Sci. Biotechnol. 38: 268-272 (2006)
18. Kim SE, Kim HH, Kim JY, Kang YI, Woo HJ, Lee HJ. Anticancer activity of hydrophobic peptides from soy proteins. Biofactors 12: 151-156 (2000)
19. De Mejia E, De Lumen BO. Soybean bioactive peptides: A new horizon in preventing chronic diseases. Sex. Reprod. Menopause 4: 91-95 (2006)
20. Wu WU, Hettiarachchy NS, Qi M. Hydrophobicity, solubility, and emulsifying properties of soy protein peptides prepared by papain modification and ultrafiltration. J. Am. Oil Chem. Soc. 75: 845-850 (1998)
21. Guan X, Yao H, Chen Z, Shan L, Zhang M. Some functional properties of oat bran protein concentrate modified by trypsin. Food Chem. 101: 163-170 (2007)
22. Kitts DD, Weiler K. Bioactive proteins and peptides from food sources. Applications of bioprocesses used in isolation and recovery. Curr. Pharm. Design 9: 1309-1323 (2003)
23. Otte J, Shalaby SM, Zakora M, Pripp AH, El-Shabrawy SA. Angiotensin-converting enzyme inhibitory activity of milk protein hydrolysates: Effect of substrate, enzyme, and time of hydrolysis. Int. Dairy J. 17: 488-503 (2007)
24. Je JY, Park PJ, Kim SK. Antioxidant activity of a peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate. Food Res. Int. 38: 45-50 (2005)
25. Saiga A, Tanabe S, Nishimura T. Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment. J. Agr. Food Chem. 51: 3661-3667 (2003)
26. Seo WH, Lee HG, Baek HH. Evaluation of bitterness in enzymatic hydrolysates of soy protein isolate by taste dilution analysis. J. Food Sci. 73: S41-S46 (2008)
27. Adler-Nissen J. Determination of the degree of hydrolysis of food proteins by hydrolysates by trinitrobenzenesulfonic acid. J. Agr. Food Chem. 27: 1256-1262 (1979)
28. Cushman DW, Cheung HS. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem. Pharmacol. 20: 1637-1648 (1971)
29. Marklund S, Marklund G. Involvement of superoxide anion radical in the oxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 47: 468-474 (1974)
30. Osawa T, Namiki M. A novel type of antioxidant isolated from leaf wax of eucalyptus leaves. Agr. Biol. Chem. Tokyo 45: 735-739

(1981)
31. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal. Biochem. 95: 351-358 (1979)
32. Chiang W-D, Tsou M-J, Tsai Z-Y, Tsai T-C. Angiotensin I-converting enzyme inhibitor derived from soy protein hydrolysate and produced by using membrane reactor. Food Chem. 98: 725-732 (2006)
33. Pripp AH, Sorensen R, Stepaniak L, Sorhaug T. Relationship between proteolysis and angiotensin-I-converting enzyme inhibition in different cheeses. Lebensm. -Wiss. Technol. 39: 677-683 (2006)
34. Smyth M, FitzGerald RJ. Relationship between some characteristics of wpc hydrolysates and the enzyme complement in commercially available proteinase preparations. Int. Dairy J. 8: 819-827 (1998)
35. Husain SS, Lowe G. Evidence for histidine in the active sites of ficin and stem-bromelain. Biochem. J. 110: 53-57 (1968)
36. Khalil AA, Mohamed SS, Taha FS, Karlsson EN. Production of functional protein hydrolysates from egyptian breeds of soybean and lupin seeds. Afr. J. Biotechnol. 5: 907-916 (2006)
37. Saito K, Jin DH, Ogawa T, Muramoto K, Hatakeyama E, Yasuhara T, Nokihara K. Antioxidative properties of tripeptide libraries prepared by the combinatorial chemistry. J. Agr. Food Chem. 51: 3668-3674 (2003)
38. Hirose A, Miyashita K. Inhibitory effect of proteins and their hydrolysates on the oxidation of triacylglycerols containing docosahexaenoic acids in emulsion. J. Jpn. Soc. Food Sci. 46: 799-805 (1999)