Prev. Nutr. Food Sci. 2015;20(3):183-189
http://dx.doi.org/10.3746/pnf.2015.20.3.183
pISSN 2287-1098 · eISSN 2287-8602

Preparation of Egg White Liquid Hydrolysate (ELH) and Its Radical-Scavenging Activity

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ABSTRACT: In the present study, an optimum protease was selected to hydrolyze the egg white liquid protein for the antioxidant peptides. Alcalase treatment yielded the highest amount of α-amino groups (15.27 mg/mL), while the control (no enzymatic hydrolysis) showed the lowest amount of α-amino groups (1.53 mg/mL). Alcalase also gave the highest degree of hydrolysis (DH) value (43.2%) and was more efficient for egg white liquid hydrolysis than the other enzymes. The Alcalase hydrolysate had the highest radical-scavenging activity (82.5%) at a concentration of 5.0 mg/mL. The conditions for enzymatic hydrolysis of egg white liquid with Alcalase were selected as substrate : water ratio of 2:1. Five percent Alacalse treatment did not show significant ($P>0.05$) increases of DH and α-amino nitrogen content after 24 h-hydrolysis. Thirty two hour-hydrolysis with 5% Alcalase is sufficient to make antioxidative egg white liquid hydrolysate from egg white liquid. DPPH and ABTS radical-scavenging activities were significantly ($P<0.05$) higher after enzymatic digestion. These results suggest that active peptides released from egg-white protein are effective radical-scavengers. Thus, this approach may be useful for the preparation of potent antioxidant products.

Keywords: egg-white liquid, hydrolysate, radical scavenger, Alcalase

INTRODUCTION

The importance of proteins in the diet has been increasingly acknowledged as a result of new scientific findings in the field of nutrition over the last two decades. The value of proteins as an essential source of amino acids is well documented, and, recently, it has been recognized that dietary proteins exert many other functions *in vivo* by means of biologically-active peptides (1).

Bioactive peptides have been defined as specific protein fragments that have a positive impact on body functions or conditions, and may ultimately influence one's health (1). Bioactive peptides have numerous potential physiological functions within the body; these include opioid, immunomodulatory, antibacterial, antithrombotic, and antihypertensive activity (2). Moreover, some of these peptides may exhibit multifunctional properties (3). Apart from these, amino acids, peptides, and proteins also contribute to the overall antioxidative capacity of cells and maintain the health of biological tissues.

Studies have been conducted to investigate the antioxidant properties of hydrolysates or bioactive peptides from plant or animal sources including peanut kernels (4), rice bran (5), sun flower protein (6), alfalfa leaf protein (7), corn gluten meal (8), and egg-yolk protein (9). In the previous study (10), the antioxidant properties of egg protein hydrolysate from egg powder prepared by Neutrase hydrolysis were investigated.

The use of eggs in food preparation depends primarily on the protein properties. Many attempts have been made to develop chemical or enzymatic modifications that alter the functional characteristics of egg white protein (11,12). Sakanaka et al. (13) found that the egg yolk protein hydrolysates, when compared with its original protein or amino acids mixture, showed stronger antioxidant activity in a linoleic acid oxidation system. In addition, a recent study reported the antioxidant activity of peptides produced from crude egg white by pepsin treatment (14). Nevertheless, information on the functional properties and antioxidant activity of peptides produced from enzyme hydrolysis is still limited and not well understood.

This study was conducted in order to prepare the antioxidant peptides from egg white using commercial proteases. In addition, the physicochemical properties of egg white peptides were also analyzed.

Received 2 June 2015; Accepted 21 July 2015; Published online 30 September 2015
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MATERIALS AND METHODS

Chemicals and enzymes

L-Leucine, 2,4,6-trinitrobenzenesulphonic acid (TNBS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Alcalase, Neutrase, Flavourzyme, and Protamex were purchased from Novozymes (Bagsvaerd, Denmark). Collupulin was purchased from DSM Corp. (Heerlen, Netherlands). Ficin was purchased from Sigma-Aldrich. The characteristics of each enzyme are summarized in Table 1. All chemicals used were of analytical grade.

Preparation of egg white liquid hydrolysate (ELH)

Egg white liquid (30 mL) was mixed with 100 mL deionized water. The suspension was incubated at 45°C for 20 min prior to enzymatic hydrolysis using various proteases. Hydrolysis was conducted at pH 6.0 for 12 h, and the hydrolysis conditions are reported in Table 1. After hydrolysis, the enzymes were inactivated by boiling for 15 min. The hydrolysates were centrifuged in a refrigerated centrifuge (Beckman J2-21, Beckman Coulter, Inc., Palo Alto, CA, USA) at 2,800 *g* for 20 min, and the supernatants were lyophilized (TFD, IlShin BioBase, Gyeonggi, Korea) and stored in a desiccator before further use.

Assay of α-amino nitrogen and degree of hydrolysis (DH)

The α-amino acid content was determined according to the method of Benjakul and Morrissey (15). To the diluted protein hydrolysate samples (125 μL), 2.0 mL of 0.2125 M phosphate buffer, pH 8.2, and 1.0 mL of 0.01% TNBS solution were added. The solution was mixed thoroughly and placed in a 50°C water bath (Model W350, Memmert Vertriebs-GmbH, Schwabach, Germany) for 30 min in the dark. The reaction was terminated by the addition of 2.0 mL of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was measured at 420 nm, and the α-amino acid content was expressed in terms of L-leucine. The DH was calculated as follows (15):

$$DH = \frac{L_t - L_0}{L_{max} - L_0} \times 100$$

where L_t is the amount of α-amino acid released at time t, and L_0 is that of α-amino acid in the original egg-white liquid. L_{max} is the total α-amino acid amount in the original egg white liquid obtained after acid hydrolysis with 6 M HCl at 100°C for 24 h.

Table 1. Enzyme characteristics

Enzyme	Source	Optimum condition		Type
		Temperature (°C)	pH	
Alcalase	*Bacillus* sp.	50~60	8.0~9.0	Endo
Neutrase	*B. amyloliquefaciens*	45	6.0~7.0	Endo
Protamex	*Bacillus* sp.	35~60	5.5~7.5	Complex
Flavourzyme	*Aspergillus* sp.	45~50	5.0~7.0	Complex
Collupulin	*Caruca papaya*	50~70	5.0~7.5	Endo
Ficin	*Ficus carica*	45~55	5.0~6.0	Endo

Free radical-scavenging activity

DPPH and ABTS were used to determine the free radical-scavenging activities of the ELH. The DPPH scavenging activity was measured using the method described by Cheung et al. (16) with slight modifications. ABTS radical-scavenging activity was determined as described by Re et al. (17) with slight modifications. The antioxidant activities of the test samples were expressed as IC_{50} (i.e., the amount of tested extract required for 50% decrease in the absorbance of DPPH and ABTS radicals).

In vitro digestion

In vitro digestion of ELH prepared with Alcalase was carried out by the modified method of Miguel et al. (18). Two grams of ELH were dissolved in 80 mL Milli-Q water and adjusted to pH 2.0 with 1 M HCl. Hydrolysis with pepsin (EC 3.4.23.1 type A, 10,000 U/mg from pork stomach obtained from Sigma-Aldrich; E/S 1/50, wt/wt) was carried out at 37°C for 2 h. Inactivation of pepsin was achieved by increasing the pH to 7.0 with 0.5 M $NaHCO_3$.

After neutralization, pancreatic digestion (from porcine gastric mucosa, Sigma-Aldrich) was conducted at an enzyme : substrate ratio of 1:50 w/w at pH 7.0~8.0 and 37°C for 150 min with continuous stirring. The reaction was stopped by heating at 95°C for 10 min in a water bath, followed by cooling to room temperature. The sample was used for measuring the radical-scavenging activity.

Statistical analysis

All experiments were carried out in triplicate. Data were analyzed using analysis of variance (ANOVA), and mean comparisons were performed using Duncan's multiple range test. Analysis was performed using SPSS software (SPSS 10.0 for windows, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Enzymatic hydrolysis of egg white liquid

The purpose of the present study was to maximize the hydrolysis of the substrate and to characterize the hydrolysate. Hydrolysis was monitored by measuring the α-amino nitrogen (A-N) amount of the supernatant after

centrifugation and measuring the DH.

Fig. 1 shows that Alcalase treatment yielded the highest α-amino groups (15.27 mg/mL), while the control (no enzymatic hydrolysis) showed the lowest level of α-amino group liberation (1.53 mg/mL). Treatment with Neutrase, Collupulin, and Ficin resulted in similar degrees of α-amino group liberation (1.72~7.86 mg/mL).

However, treatment with Neutrase showed the highest amino nitrogen amount in the hydrolysis of egg white powder in another study (10). During the production of egg white powder, the proteins are subjected to several processing steps of thermal, physical, interfacial, and chemical treatments that may damage the egg white functional properties (19). Lechevalier et al. (20) reported that heat treatment resulted in tertiary structural modifications, folding, or aggregation of the protein. Therefore, egg white powder and liquid show differences in the specificity of proteolytic cleavage.

DH is a measure of the extent of hydrolytic degradation of a protein, and it is also the most widely-used indicator for comparison among different proteolysis processes. The extent of egg white liquid degradation by proteolytic enzymes was also estimated by assessing the DH. DH values varied from 0% to 43.2% after 24 h of incubation, depending on the enzymes used. The treatment with Alcalase showed the highest DH value (43.2%) and was more efficient for egg white hydrolysis than the treatment with the other enzymes (Fig. 2). The high DH level obtained with Alcalase could be due to the endoproteinase content in the extracts resulting in a higher degree of hydrolysis of the egg white liquid.

The use of an endoproteinase is essential in the hydrolysis of food proteins. Occasionally, an endoproteinase is combined with an exopeptidase for complete degradation (21). It is known that Alcalase acts as an endoproteinase producing mainly small- and medium-sized oligopeptides or polypeptides.

Alcalase is an alkaline protease used not only for the production of protein hydrolysates with better functional and nutritional characteristics, but also for the generation of bioactive peptides with ACE-inhibitory activity (22). A higher level of DH indicates that a more hydrolyzed egg white protein could be obtained. Therefore, Alcalase may be ideal for use in commercial operations due to its low cost and high α-amino protein recovery.

Radical-scavenging activity of ELH prepared with various proteases

Bioactive peptides derived from food proteins with low molecular weight and useful bioactivities which are easily absorbed, have attracted attention, since they are safer and healthier than synthetic drugs (23). In recent years, bioactive peptides with antioxidant activity from food proteins have been under consideration.

As shown in Fig. 3, the control sample showed a radical-scavenging activity of 32.2% at a concentration of 5.0 mg/mL, but all of the hydrolysates showed higher radical-scavenging activities than the control. In particular, the Alcalase hydrolysate had the highest radical-scavenging activity (82.5%) at a concentration of 5.0 mg/mL. These results revealed that ELH contained substances that acted as electron donors and reacted with free radicals to terminate the radical chain reaction and convert them to more stable products. Based on our results, we selected Alcalase as the optimal enzyme to hydrolyze the egg white liquid to generate the antioxidant peptides.

A number of studies have shown that peptides and protein hydrolysates of plant and animal origins, such as the hydrolysates of soy protein (24), whey protein (24, 25), zein protein (26), gelatin (27), and egg albumin (14), possess significant antioxidant activities.

In our preliminary study, the ELH tested in the $ABTS^{+\cdot}$ system, though at a lower concentration (2.61 mg/mL), had an IC_{50} value of approximately 15 times higher than

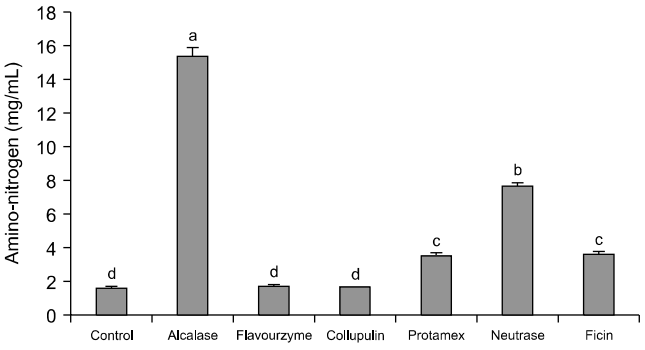

Fig. 1. α-amino nitrogen content of ELH prepared with various proteases. Fifty mL of egg white liquid was mixed with 100 mL of deionized water. The solution was incubated at 45°C for 20 min prior to enzymatic hydrolysis with various proteases. Hydrolysis was conducted at pH 6.0 for 12 h. Error bars represent the standard deviation from triplicate measurements. Different letters (a-d) indicate significant differences ($P<0.05$).

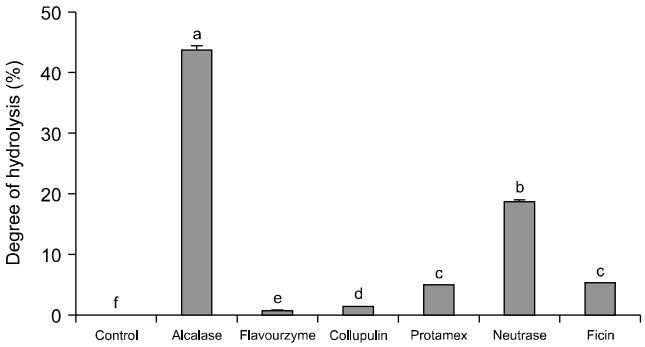

Fig. 2. Degree of hydrolysis of egg white liquid prepared by various proteases at 1:50 (w/w) enzyme/substrate and 1:2 (v/v) liquid egg/water. The hydrolysis was carried out at 50°C for 24 h. Error bars represent the standard deviation from triplicate measurements. Different letters (a-f) indicate significant differences ($P<0.05$).

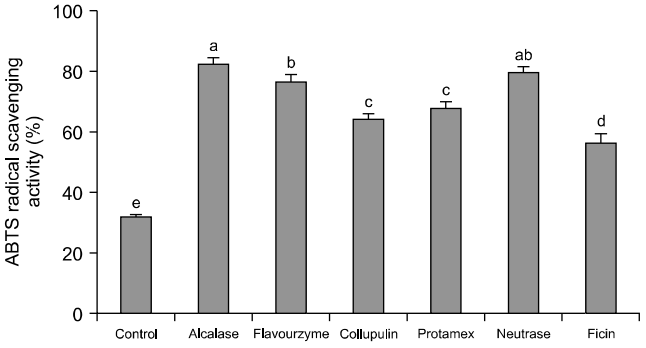

Fig. 3. Radical-scavenging activity of ELH prepared with various proteases. Error bars represent the standard deviation from triplicate measurements. Different letters (a-e) indicate significant differences ($P<0.05$).

that of the ELH sample (38.92 mg/mL) tested in the DPPH system.

The difference in efficacy can be attributed to the solubility and diffusivity of the radicals. A fat-soluble compound, DPPH, although dissolved in ethanol prior to being dispersed in the aqueous assay solution, may not readily diffuse to the target peptides; therefore, its reactivity could be limited. In contrast, as a water-soluble radical species, $ABTS^{+\cdot}$ would readily reach peptides in the aqueous assay solution and, hence, react effectively with peptides in the ELH. Data obtained from the present and preliminary studies suggest that the DPPH assay is not an appropriate method for the measurement of antioxidant activity of water-soluble proteins and peptides in an aqueous solution.

Antioxidant activities of protein hydrolysates depend on the proteases (28) and hydrolysis conditions employed (24,28). During hydrolysis, a wide variety of smaller peptides and free amino acids are generated, depending on enzyme specificity. Changes in size, level, and composition of free amino acids and small peptides affect the antioxidant activity (29). Jun et al. (28) reported that yellowfin sole hydrolysate, prepared using pepsin at lowest DH (22%), had a higher antioxidative activity than that of those produced using other enzymes, such as Alcalase, α-chymotrypsin, pepsin, Pronase E, Neutrase, and trypsin.

Changes of A-N and DH of ELH according to various ratios of egg white liquid to water during Alcalase hydrolysis

Substrate concentration greatly influences the molecular weight and amino acid sequence of the protein hydrolysates and, thus, their biological activities (30). In view of the economic interest on recovering the protein from egg white liquid, the most suitable substrate concentration were compared and selected. Fig. 4 shows the changes of amino-nitrogen and DH with various substrate concentrations. Substrate to water ratio of 1:1 showed the highest level of A-N compared with the other ratios of 1:2 and 2:1. With the increased addition of water to egg white liquid, the level of DH also increased; however, the cost required to produce the desired bioactive peptide is an important factor in the selection.

To minimize the cost of enzyme and energy for water removal to produce the dry hydrolysate as well as to reduce the reaction time, the conditions for enzymatic hydrolysis of egg white liquid with Alcalase were selected as the substrate:water ratio of 2:1.

Changes of A-N and DH during preparation of ELH with an egg white liquid/water ratio of 2:1 at 2% and 5% addition of Alcalase

The low ratio of egg white to water showed a high DH level after 24 h-hydrolysis (Fig. 4), leading to improve the production cost. Therefore, the appropriate ratio of enzyme to substrate could reduce the production cost with the improved DH of the hydrolysate. The amount of A-N and DH of ELH treated with Alcalase increased as the enzyme concentration increased (Fig. 5). Significant changes ($P<0.05$) of the amount of A-N and DH were observed between the enzyme treatments of 2% and 5% addition of Alcalase.

As expected, 2% and 5% addition of Alacalase resulted in a sharp increases in the amount of A-N and DH during the first 8 h of the reaction, but the rates slowed down thereafter (Fig. 5). Especially, 5% Alacalse treat-

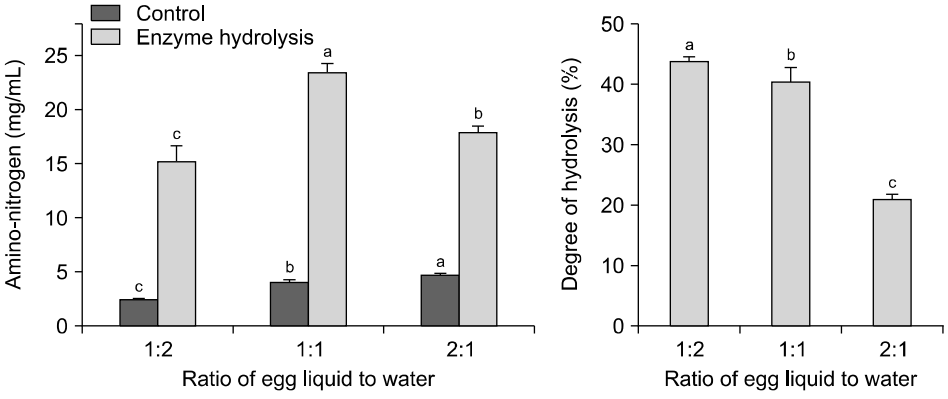

Fig. 4. Amino-nitrogen and degree of hydrolysis of ELH according to various ratio of egg liquid to water. The hydrolysis was carried out at 50°C for 24 h. Error bars represent the standard deviation from triplicate measurements. Different letters (a-c) indicate significant differences ($P<0.05$).

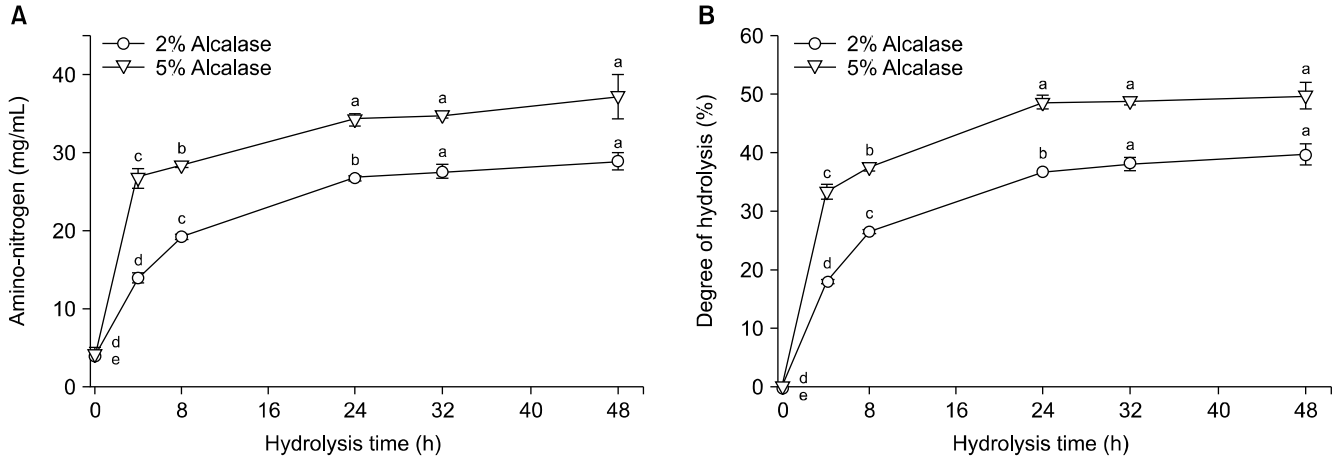

Fig. 5. Amino-nitrogen and degree of hydrolysis of ELH during hydrolysis with different amount of Alcalase of egg white liquid/water [2:1 (v/v)]. The hydrolysis was carried out at 50°C for 48 h. Error bars represent the standard deviation from triplicate measurements. Different letters (a-e) indicate significant differences with the same protease ($P<0.05$).

ment did not show a significant increase ($P>0.05$) in the amounts of DH and A-N after 24 h-hydrolysis (Fig. 5). This trend was consistent with the results obtained by other researchers using various proteases and substrates (31,32). The shape of the hydrolysis curve, particularly the decrease in the hydrolysis rate, is not fully understood. Alder-Nissen (21) emphasized that this behavior cannot be attributed to the substrate exhaustion. Constantinides and Aduamankwa (33) suggested several explanations including the depletion of the peptide bonds suitable for the enzyme, product inhibition, and/or enzyme inactivation.

The ratio of enzyme to substrate to obtain the complete digestion was between 2 and 10:100 (w/w) as the suppliers recommended (34), but, other research reported that in the range of 1.00~3.00%, considered as the economic usage range of the enzyme, the level of DH increased with the increase of E/S ratio up to about 2.40% and, after that, it slightly decreased (35).

Changes of ABTS radical-scavenging activity during preparation of ELH with an egg white liquid/water of 2:1 at 2% and 5% addition of Alcalase

Free radical-scavenging activities were analyzed using the ABTS radical assay (for water-soluble free radical). IC_{50} values of the hydrolysate were measured during the hydrolysis of egg white liquid (Fig. 6). Similar to the IC_{50} values during the hydrolysis with 2% and 5% Alcalase, the control (no treatment with Alcalase) had the lowest ABTS radical-scavenging ability. Both 2% and 5% addition of Alcalase resulted in sharp decreases in the IC_{50} value over the first 4 h of the reaction, and the rates slowed thereafter. For the hydrolysis with 2% Alcalase, little or no difference in ABTS radical-scavenging activity was observed after 4 h-hydrolysis. As for the 5% Alcalase treatment, 32 h-hydrolysis showed the lowest IC_{50} value (0.84 mg/mL), which confirmed that 32 h-hydrolysis with 5% Alcalase is sufficient to make antioxidative ELH from egg white liquid.

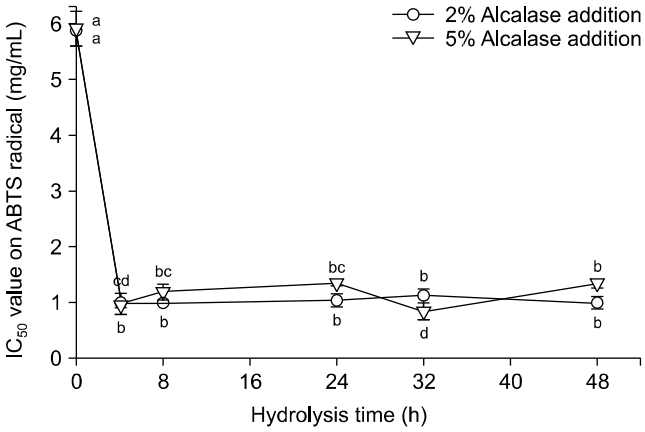

Fig. 6. Changes of IC_{50} value on ABTS radical during hydrolysis with different amount of Alcalase of egg white liquid/water [2:1 (v/v)]. The hydrolysis was carried out at 50°C for 48 h. Error bars represent the standard deviation from triplicate measurements. Different letters (a-d) indicate significant differences with the same protease ($P<0.05$).

Manso et al. (36) reported that the enzymatic hydrolysis of egg white with pepsin resulted in the production of peptides with free radical-scavenging capacity and lipid peroxidation inhibition activity (14). A number of studies have shown that peptides and protein hydrolysates of plant and animal origins possess significant antioxidant activities, such as the hydrolysates of soy protein (37), whey protein (24), zein protein (26), and egg albumin (14).

Radical-scavenging activity of ELH before and after digestive enzyme treatment

The antioxidant activity of hydrolyzed proteins and peptides in model food systems has been well-documented in the literature, but relatively few studies have been conducted to evaluate their activities and fate in the hu-

man upper digestive system. Because of the unique peptide bond specificity of digestive proteases, the products from pepsin and pancreatin (trypsin and chymotrypsin) digestion will depend on the characteristics of the ingested peptides and, in this study, the composition of Alcalase-hydrolyzed egg white protein. The results from the present study demonstrated that ELH, which possessed strong antioxidant activity (Fig. 7), had a decreased or increased activity during *in vitro* digestion, depending on the enzymes encountered and the duration of hydrolysis.

DPPH and ABTS radical-scavenging activities were significantly ($P<0.05$) higher after enzymatic digestion (Fig. 7). The IC_{50} values for DPPH and ABTS radicals, after the digestion, changed from 38.90 to 24.42 mg/mL and from 0.86 mg/mL to 0.21 mg/mL, respectively.

When the pepsin digest was further hydrolyzed with digestive enzyme, additional peptide bond cleavages would lead to the accumulation of shorter peptides (tri- and dipeptides) and amino acids, namely, the products became more hydrophilic. The digests with increased polarity (amino acids; small peptides) could readily react with water-soluble $ABTS^{+\cdot}$ but not with lipid-soluble $DPPH^{\cdot}$, and this would explain the complex scavenging behaviors of the protein digests in the two radical systems. Chen et al. (38) reported that peptides derived from the digests of soybean protein could not interact properly with hydrophobic peroxyl radicals due to the lack of hydrophobic patches in the peptides. Mendis et al. (27) indicated that the antioxidant reactivity of squid skin gelatin peptides was due to the hydrophilic-hydrophobic partitioning in the sequence.

Antioxidants might be able to prevent, or at least attenuate, the organic impairment caused by excessive oxidative stress. Since endogenous antioxidants may not be sufficient to prevent the damage, diet-derived or supplemented antioxidants could be important to maintain health. Some food proteins and peptides were found to have scavenging activity against free radicals (39) or to exert antioxidant activity against the peroxidation of lipids or fatty acids (40). However, neither the structure-activity relationship, nor the antioxidant mechanism of peptides is fully understood (41).

In addition to their nutritional value, hen eggs contain numerous substances with therapeutic effects. Several of these substances have already been produced on an industrial scale for food and medical applications. ELH may be a good source of desirable peptides and amino acids, and, furthermore, it may be used as an emulsifier and foaming agent with antioxidant activities. ELH prepared with Alcalase are preferred to those prepared using other enzymes, as it has the highest radical-scavenging activity. The approach developed in the present study provides a method for enzyme hydrolysis of the egg white powder and liquid treatment, which may be beneficial for time and energy costs.

A further requisite for the application of bioactive proteins and peptides is the large-scale availability of raw protein material and the cost of production of active components. Moreover, continued identification of the biological functions of egg components will define new methods for improving the utility of egg as a source of biologically-active compounds with specific health benefits and secure their role in the treatment and prevention of disease.

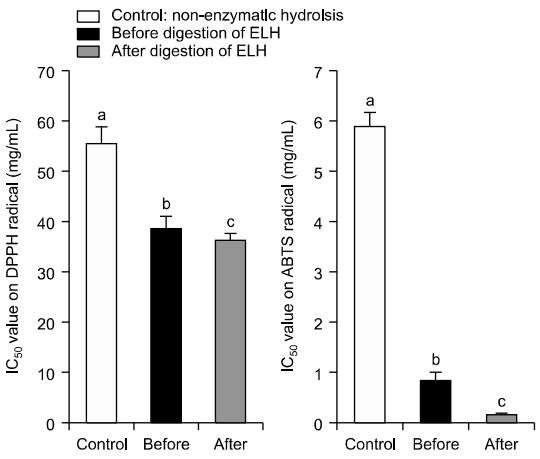

Fig. 7. Radical-scavenging activity of ELH before and after digestive enzyme treatment. Error bars represent the standard deviation from triplicate measurements. Different letters (a-c) indicate significant differences ($P<0.05$).

ACKNOWLEDGEMENTS

This work was supported by a Research Grant from Kaya University.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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