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Characteristics and Enhanced Antioxidant Activity of Egg White Protein Selenized by Dry-Heating in the Presence of Selenite

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ABSTRACT: This study reports a new method for the selenization of food proteins. Egg white protein (EWP) was selenized by dry-heating in the presence of selenite, and the physiochemical and functional properties of the selenized EWP were investigated. Selenization was accelerated with a decrease in pH from 7.0 to 3.0, an increase in heating time from 1 to 5 days, and an increase in incubation temperatures from 25 to 80 °C. The electrophoretic mobility of EWP increased with an increase in the level of selenization. Heat-induced polymerization of EWP was promoted by dry-heating in the presence of selenite. The selenite group was acid-stable and base-labile, which suggests that it was bound to the hydroxyl group of an amino acid and formed an $-O-SeHO_2$ linkage. ^{77}Se NMR spectral data also suggested that the selenite bond ($-O-SeHO_2$) was bound to EWP. The digestibility of EWP was improved by selenization. The antioxidant activities of EWP, including $ABTS^+$ free radical scavenging capacity, hydroxyl radical scavenging capacity, reducing power, and the Fe^{2+} chelating capacity, were remarkably enhanced by selenization. This finding is the first to describe that EWP can be selenized by dry-heating in the presence of selenite and that the antioxidant activities of EWP are markedly enhanced by selenization. This points to a potentially new method for generating antioxidant food protein and a new method for preparing organic Se.

KEYWORDS: *selenium, egg white protein, dry-heating, selenization, antioxidant activity*

INTRODUCTION

Free radicals have been shown to be responsible for the pathogenesis of certain human conditions, including cancer, aging, and chronic arterial disease.[1,2] The formation of free radicals, such as superoxide anion radicals and hydroxyl radicals, is an unavoidable consequence of aerobic respiration. These radicals are very unstable and react rapidly with other groups and substances in the body, which leads to cell and tissue injury.[3] Antioxidants can prevent oxidation chain reactions by interrupting the free radical chain of oxidation and hydrogen donation, thereby forming stable free radicals that do not initiate or propagate the oxidation of lipids or proteins. In this way, antioxidants may reduce the degree of injury that is caused by free radicals.[4,5]

Selenium (Se) is an essential trace element in human and animal nutrition. It has been shown to have several functions in a variety of systems and has received considerable attention over the past few decades. Many of the biochemical properties of Se are a result of its function in 25 selenoproteins, many of which are involved in redox control.[6] For instance, Se is an integral part of the catalytic sites of a large number of Se-containing enzymes, such as glutathione peroxidase and thioredoxin reductase. It is an essential component of several major metabolic pathways, including thyroid hormone metabolism, antioxidant defense systems, and immune function.[7,8] In recent years, some work on the antioxidant activity of Se-containing food has evaluated the relationship between enhanced antioxidant activity and Se in food components. For example, Se-containing phycocyanin and allophycocyanin from Se-enriched *Spirulina platensis* have been shown to exhibit more antioxidant activity than the control proteins without Se.[9,10] Some studies showed that biological enrichment with Se can significantly enhance the antioxidant capacity of aqueous extracts from meat, tea, and rice.[11−14] The selenized product of xyloglucan shows better antioxidant activity than xyloglucan, as recently reported by Cao and Ikeda.[15] These studies suggest that the enhanced antioxidant activity of food components is responsible for the conjugation of Se.

Egg white protein (EWP) is extensively used as a functional ingredient in the food industry because of its nutritional value and wide range of functional properties. Owing to the importance of its antioxidant properties, researchers have studied the enhanced antioxidant properties of EWP by using glycation.[16,17] However, because it is a mixture of protein, only limited information on the antioxidant properties of non-glycated forms of EWP is available.

To the best of our knowledge, no selenization of food proteins other than biotransformation has been reported to date. Moreover, it is also unclear whether the antioxidant activity of EWP is affected by conjugating it with Se. On the other hand, our previous work has demonstrated that EWP can be phosphorylated by dry-heating in the presence of phosphate.[18,19] We sought to determine whether food proteins can be selenized by dry-heating in the presence of selenite and

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whether selenization can induce or enhance its antioxidant activity. The purpose of the present study was to examine the occurrence of selenization and to elucidate the factors that affect the dry-heating-induced selenization of EWP in the presence of selenite. We also investigated the antioxidant activities of selenized EWP (Se-EWP).

MATERIALS AND METHODS

Materials. EWP was prepared as follows: Egg white, which was separated from infertile eggs, was homogenized, acidified to pH 5.5 with 1 N HCl, and then centrifuged. The resulting supernatant was diluted with an equal volume of water, dialyzed, and then lyophilized. Se-77 metal powder (99.2% isotopic enrichment) was purchased from Isoflex USA (San Francisco, CA, USA). 2,2′-Aminobis(2-ethylbenzothiazolinesulfonic acid) ammonium salt (ABTS), pepsin, and α-chymotrypsin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pronase E was purchased from Kaken Pharmaceutical Co., Ltd. (Tokyo, Japan). Alkaline protease was purchased from Nanning Doing-Higher Biotech Co. Ltd. (Nanning, China). All other reagents that we used were of analytical grade.

Synthesis of $H_2{}^{77}SeO_3$. $H_2{}^{77}SeO_3$ was synthesized by adding ^{77}Se metal powder to nitric acid according to the method of Suzuki et al.[20] with some modifications. Briefly, 30 mg of Se-77 metal powder was dissolved in 4 mL of 30% nitric acid and heated at 90 °C for 15 min in a 15 mL tube. The solution was then heated by using an alcohol burner until the water was completely evaporated. The white powder in the tube was dissolved in deionized water and used as an $H_2{}^{77}SeO_3$ solution.

Preparation of Se-EWP. Selenization of EWP was performed by using the same method as for the phosphorylation of food proteins[18] with the modification of replacing phosphate with selenite. Briefly, EWP was dissolved at a concentration of 2% in 2% selenite solution at various pH values from 3.0 to 7.0, which were adjusted with 1 N NaOH, and then lyophilized. Lyophilized samples were incubated at various temperatures (25−80 °C) and heating periods (1, 3, and 5 days). Dry-heated samples were dissolved and dialyzed for 3 days against deionized water to remove free selenite and then lyophilized. Dry-heated EWP (DH-EWP) was prepared as follows: EWP samples were dissolved at a concentration of 2% in deionized water, and the pH of the solution was adjusted to 3.0−7.0, after which the solutions were lyophilized, and residues were subjected to dry-heating under the same conditions as those for Se-EWP. ^{77}Se-EWP was prepared by dry-heating EWP at pH 3.0 and 60 °C for 3 days in the presence of $H_2{}^{77}SeO_3$.

Determination of Se Content of Se-EWP. The Se content of Se-EWP was measured by using inductively coupled plasma atomic emission spectroscopy (ICP-AES).[21] The Se-EWP samples were digested with a mixture of perchloric acid and concentrated nitric acid (2:1, v/v). The Se in the digest was regarded as being the total Se. For inorganic Se determination, 5 mL of 10% trichloroacetic acid was added to the same volume of 10 mg/mL Se-EWP solution, and the solution was centrifuged at 1000*g* for 20 min. The Se in the supernatant was regarded as being inorganic Se. The supernatant and the total Se were digested with mixed acids. The amount of Se that was bound to protein (organic Se) was estimated as being the difference between the total Se content and the inorganic content.

Measurement of Protein Solubility. Protein samples were dissolved at a concentration of 1 mg/mL in 50 mM Tris-HCl buffer (pH 7.0) and then centrifuged at 1000*g* for 20 min. The concentration of protein in the supernatant was determined by using the method of Lowry et al.[22]

Electrophoresis. Native polyacrylamide gel electrophoresis (native PAGE) was performed by using 10% gels in the absence of sodium dodecyl sulfate (SDS), and sodium dodecyl sulfate (SDS)-PAGE was performed by using 12.5% polyacrylamide gels under both reducing and nonreducing conditions in the presence or absence of 2-mercaptoethanol (2-ME), according to the method of Laemmli.[23] The gel sheets were stained with Coomassie Blue G-250 for 1 h.

High-Performance Liquid Chromatography (HPLC). Gel permeation HPLC was carried out at room temperature (25 °C) with an Agilent 1100 controller and a G1315B wavelength detector (Agilent Technologies, Palo Alto, CA, USA), using a TSK-GEL $G3000SW_{XL}$ column (7.8 mm × 30 cm, Toso Ltd., Tokyo, Japan) that was fitted to a TSK guard column (7.8 mm × 7.5 cm). The elution buffer was 0.1 M sodium phosphate buffer (pH 7.0) that contained 0.3 M NaCl. Individual 25 μL sample solutions (1 g of protein/L) were injected. The samples were then eluted with the same buffer solution at a flow rate of 0.5 mL/min, and the elution profile was monitored by examining the UV absorbance at 280 nm.

Acid and Base Stability of the Selenite Bonds in Se-EWP. Se-EWP (1 mg/mL) in deionized water was adjusted to pH 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, and 10.0 with 1 N NaOH or 1 N HCl or dissolved in 0.5 N HCl (pH 0.8−1.1) and 0.5 N NaOH (pH 12.2−13.2) and then incubated at 37 °C for 24 h. After incubation, total Se and inorganic Se were determined. The stability of the selenite group was evaluated as the degree of deselenization (the percentage of released Se) from Se-EWP at various pH values.

^{77}Se Nuclear Magnetic Resonance Spectroscopy. ^{77}Se nuclear magnetic resonance (NMR) spectroscopy was carried out with a Bruker Avance DRX spectrometer at 500 MHz and 20 °C. ^{77}Se-EWP, which was prepared by dry-heating at 60 °C and pH 3.0 for 3 days, was dissolved in a buffer that contained 50 mM 1, 4-piperazinebis-(ethanesulfonic acid), 5% SDS, and 5% urea (pH 7.0) at a concentration of 100 mg/mL. The chemical shift was externally referenced to $(CH_3)_2Se$. Also, the number of scans was 30000. The spectrum was obtained with a 30° tipping pulse and a 2 s repetition time.

Measurement of Digestibility. Protease digestion was performed as follows: 20 mL of 1 mg/mL EWP solution in a buffer of suitable pH was added to Pronase E, pepsin, alkaline protease, and α-chymotrypsin at enzyme/protein ratios of 1:100, 1:300, 1:10, and 1:10, respectively. Enzymatic reaction was carried out at 37 °C for 0−20 min. After protease digestion, to stop the enzymatic reaction and to remove native protein, 2 mL of protein solution removed from the reaction solution was added to 2 mL of 10% aqueous trichloroacetic acid and then a mixture of solution was centrifuged at 1000*g* for 20 min. The amount of peptides and amino acids in the supernatant was measured according to the method of Lowry et al.[22] The extent of digestion was expressed as the percentage of digestion of the total protein.

Measurement of $ABTS^+$Free Radical Scavenging Activity. The $ABTS^+$ free radical scavenging activity of EWP was measured according to the method reported by Zhang et al.[24] with some modifications. Briefly, 80 μL of the protein sample was mixed with 3920 μL of ABTS reagent with an absorbance of 0.60 at 734 nm. Then the absorbance at 734 nm was measured after the initiation of mixing for 10 min.

Measurement of Ferrous Ion Chelating Activity. Ferrous ion chelating activity was measured according to the method of Lee et al.[25] with a minor modification. Briefly, 0.1 mL of 2 mM $FeCl_2$ was added to 1.0 mL of protein (0−2 mg/mL), and the reaction was then initiated by adding 0.2 mL of 5 mM ferrozine at 25 °C for 60 min. The mixture was vortexed and left to stand at 25 °C for 10 min, after which the absorbance at 562 nm was measured. The ferrous ion chelating ability was calculated by using the equation

$$Fe^{2+}\ \text{chelating ability}\ (\%) = (1 - A_s/A_c)\times 100$$

where A_s and A_c represent the absorbances of the protein sample and the control (deionized water instead of protein), respectively.

Measurement of Hydroxyl Radical Scavenging Activity. The scavenging activity of hydroxyl radicals was measured with the *o*-phenanthroline Fenton system previously reported.[26] Reaction mixtures that contained different samples were incubated with *o*-phenanthroline (0.75 mM), H_2O_2 (2 mM), and $FeSO_4$ (0.75 mM) in sodium acetate−acetic acid buffer (0.1 M, pH 4.5) at 37 °C for 60 min. The absorbances of the mixtures were measured at 536 nm against a reagent blank. Hydroxyl radical scavenging activity was calculated by using the equation

hydroxyl radical scavenging activity (%)

$$= (A_s - A_0)/(A_c - A_0)\times 100$$

where A_c represents the absorbance of a sample, A_s represents the absorbance of a sample that has been reacted with H_2O_2, and A_0 represents the absorbance of the mixture without a sample.

Measurement of Reducing Power. The reducing power of the protein was determined according to the method of Zhang et al.[24] with some modifications. In brief, 1 mL of sample (1−10 mg/mL) was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The reaction mixtures were incubated in a water bath at 50 °C for 20 min followed by the addition of 2.5 mL of 10% trichloroacetic acid after the mixtures had cooled to room temperature. The mixtures were then centrifuged at 3000*g* for 10 min at 25 °C. One milliliter of the supernatant was mixed with 2.0 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The absorbance of the reaction mixture was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

RESULTS AND DISCUSSION

Selenization. Table 1 shows the Se contents of various Se-EWPs. The Se content of Se-EWP increased with decreasing pH from 7.0 to 3.0 and increasing temperatures from 25 to 80 °C, which was similar to the results for the dry-heat-induced phosphorylation of EWP.[18,19] The Se contents of EWP that was incubated at 60 °C and pH 3.0 for 3 and 5 days were 0.69 and 0.95%, respectively. This Se level of EWP was much higher than that of some Se-enriched foods as reported previously.[9,21,24,27−29] However, the solubility of Se-EWP that was subjected to dry-heating at pH 3.0 and 60 °C for 5 days markedly decreased. Thus, taken together, Se-EWP can be prepared by heating at pH 3.0 and 60 °C for <5 days. To our knowledge, this study is the first to report that food protein (i.e., EWP) can be selenized by dry-heating in the presence of selenite, which may potentially be a new method for preparing organic Se. Furthermore, this method may have some advantages as follows, when it is used in food industrial. First, the procedure to prepare Se-EWP by dry-heating is much shorter than that for biotransformation, which may provide a new and simple method for preparing organic Se. Second, dry-heating selenization seems to be more economical than biotransformation, because the reagents that are used are all normal and may be low cost. Third, the selenization level of EWP by dry-heating is higher than that of the biotransformational method.

Table 1. Se Content of EWP by Dry-Heating in the Presence of Selenite at Various pH Values and Temperatures for 1, 3, and 5 Days

pH	incubation time (days)	incubation temp (°C)	Se content[a] (%)	solubility[b] (%)
7.0	1	60	0.14	99.5 ± 2.5
6.0	1	60	0.18	98.1 ± 1.7
5.0	1	60	0.22	98.4 ± 0.9
4.0	1	60	0.35	97.5 ± 1.5
3.0	1	60	0.55	96.2 ± 1.3
3.0	3	60	0.69	92.4 ± 0.8
3.0	5	60	0.95	81.8 ± 3.4
3.0	1	25	0.15	98.3 ± 1.0
3.0	1	37	0.22	98.6 ± 2.1
3.0	1	50	0.45	99.1 ± 1.6
3.0	1	60	0.55	96.2 ± 1.3
3.0	1	70	0.72	92.5 ± 1.1
3.0	1	80	1.01	72.3 ± 2.9

[a]Data shown are the mean value of the two determinations, with a deviation of <1%. [b]The solubility of Se-EWP was measured at pH 7.0. Each value is the mean with its SD (n = 3).

The selenization process in the dry state may be explained in simple terms by the following reaction:

$$\blacklozenge - OH + H_2SeO_3 \rightarrow \blacklozenge - OHSeO_2 + H_2O$$

Here, ◆ represents a protein. Its formation is promoted with an increase in dry-heat temperature from 25 to 80 °C. The removal of water from this system promotes the forward reaction, because of Le Chatelier's principle, and hence drying promotes the formation of esters. A small amount of residual water, however, appears to facilitate the reaction. This is similar to the formation of Schiff bases in dry mixtures of proteins and reducing sugars, for which an optimum moisture content has been reported for the maximum reaction rate.[30,31] The role of water in this reaction could be physical, thereby providing a degree of mobility within the solid matrix that may facilitate any possible reactions. Water may also, in the present case, be involved in the reaction itself. Selenization was also enhanced by a decrease in pH from 7.0 to 3.0. Selenite has two ionizable hydrogen atoms with the following dissociation constants: pk_1 = 2.62 and pk_2 = 8.32. At pH 8.32, the two main anionic selenite species ($HSeO_3^-$ and SeO_3^{2-}) are equal in concentration. If the pH decreases from 8.3 to 2.6, the proportion of $HSeO_3^-$ increases, whereas that of SeO_3^{2-} decreases, and thus selenoester formation between hydroxyl groups and $HSeO_3^-$ may be favored. Therefore, greater selenization may be expected at a low pH values. On the other hand, $HSeO_3^-$ may be one of the components of the mixture at an equilibrium mixture and not the reactive species. The solution chemistry of selenite is complex: A variety of selenite oxyacid species are known to coexist in equilibrium. Thus, it is possible that a common component of the equilibrium is the agent that is responsible for selenization. In this study, at pH 7.0 and lower, $HSeO_3^-$ is the main anionic selenite species. By increasing the amount of this species, it is reasonable to assume that selenoester formation between hydroxyl and $HSeO_3^-$ is more favorable than that between hydroxyl and SeO_3^{2-}. Thus, a decrease in pH from 7.0 to 3.0 could enhance selenization.

Characteristics of Selenized EWP. Given the observation that the selenization of EWP was high at pH 3.0 (Table 1), we prepared Se-EWP by dry-heating EWP at pH 3.0 and various temperatures for 1 day in the presence of selenite. Figure 1A shows the native PAGE profiles of Se-EWP that was reacted at various temperatures. Native ovalbumin (Ova) was dissociated into three bands (A_1, A_2, and A_3) depending on the number of phosphoserine residues in it.[32] In the absence of selenite, there were almost no changes in the mobility of the EWP components (Figure 1B). On the other hand, the mobility of Se-EWP was greater than that of EWP that was dry-heated under the same conditions in the absence of selenite, and its mobility was consistent with the Se content of Se-EWP, as shown in Table 1. These results suggest that the negative charges of the selenite groups were bound to the protein and that the higher number of introduced selenite groups caused greater mobility of the EWP components.

Protein polymerization during dry-heating was analyzed by PAGE in the absence of SDS. In Figure 1B, almost no polymeric proteins were observed in DH-EWP, which was dry-

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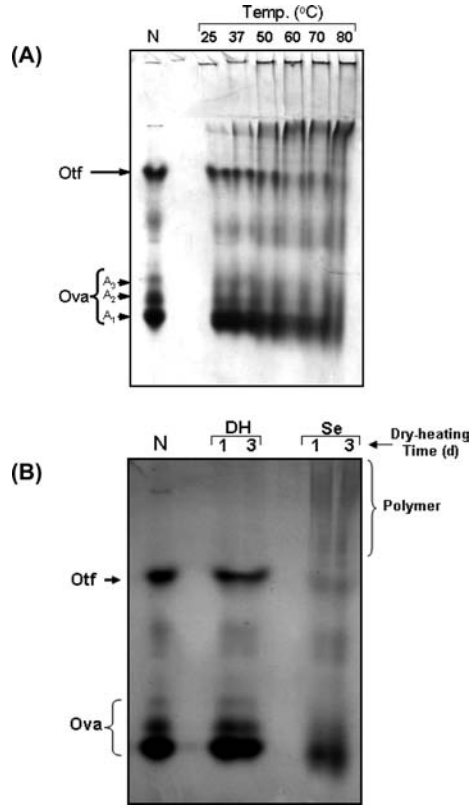

Figure 1. Native PAGE patterns of N-EWP and Se-EWP, which were prepared by dry-heating at pH 3.0 and 25−80 °C for 1 (A) and 3 days (B) in the presence of selenite. PAGE (10% polyacrylamide gel without SDS) was performed at a constant current of 8 mA, and 15 μL of 1 mg/mL sample solution was applied to each lane. N, native EWP; Ova, ovalbumin; Otf, ovotransferrin; DH, EWP dry-heated in the absence of selenite; Se, EWP dry-heated in the presence of selenite.

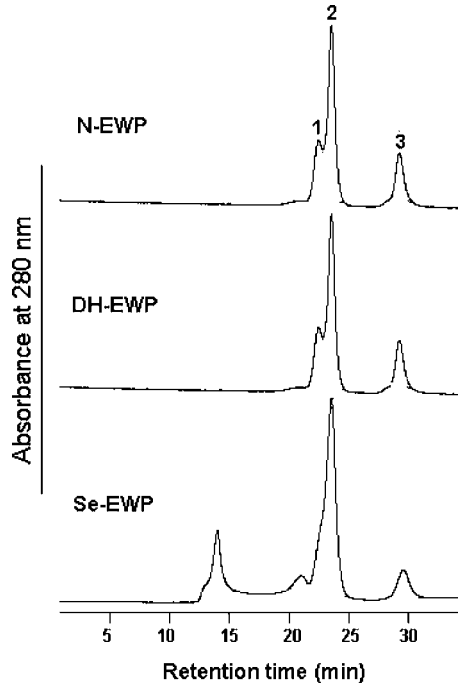

Figure 2. HPLC patterns of native EWP, DH-EWP, and Se-EWP. DH-EWP and Se-EWP were incubated at pH 3.0 and 60 °C for 3 days. Column, TSK-GEL G3000SW$_{XL}$ column (7.8 mm × 30 cm); elution buffer, 0.1 M phosphate buffer (pH 7.0) containing 0.3 M NaCl, for which the flow rate was 0.5 mL/min. Peaks: 1, ovotransferrin; 2, ovalbumin; 3, lysozyme.

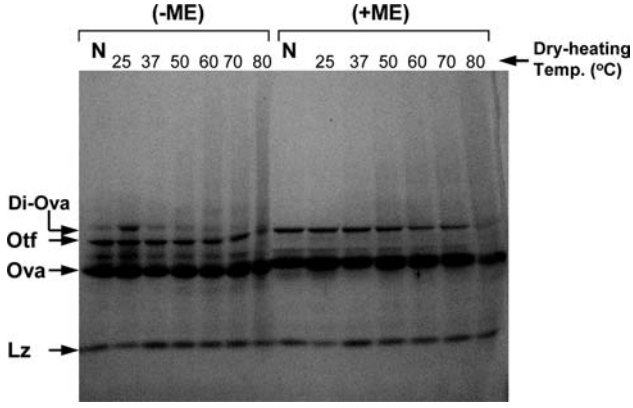

Figure 3. Sodium dodecyl sulfate−polyacrylamide electrophoresis (SDS-PAGE) patterns of native egg white protein (EWP) and selenized egg white protein that was prepared by dry-heating at pH 3.0 and 25−80 °C for 1 day in the presence of selenite. PAGE (12.5% polyacrylamide gel) was performed at a constant current of 8 mA, and 15 μL of 1 mg/mL sample solution was applied to each lane. N, native EWP; Ova, ovalbumin; Otf, ovotransferrin; Lz, lysozyme; Di-Ova, Ova dimer.

heated at pH 3.0 and 60 °C for 1 and 3 days in the absence of selenite. In contrast, polymers were detected in the Se-EWP that was dry-heated in the presence of selenite under the same conditions. Protein polymerization was further investigated by gel permeation HPLC in the absence of SDS. Figure 2 shows the typical elution profiles of EWP that was dry-heated at 60 °C and pH 3.0 in the absence or presence of selenite for 3 days. Peaks 1, 2, and 3 are identified as ovotransferrin (Otf), Ova, and lysozyme, respectively. The fraction eluted before peak 1 represents polymerized protein. The elution pattern of N-EWP was almost identical to that of DH-EWP, which was dry-heated at pH 3.0 and 60 °C for 3 days. However, the fraction that was eluted before peak 1 clearly increased after dry-heating in the presence of selenite, which suggested that polymerized proteins were formed during dry-heating in the presence of selenite.

This result was greatly different from that of EWP phosphorylation,[18] where polymerization of EWP was almost unaffected by dry-heating in the presence of phosphate. To carefully assess the binding type of aggregates of Se-EWP, SDS-PAGE was performed with or without 2-ME. As seen in Figure 3, dry-heating in the presence of selenite induced partial aggregation of the protein, and the aggregation increased with increasing temperature from 25 to 80 °C in the absence or presence of 2-ME. In the presence of SDS and 2-ME, the Ova dimer was considerably reduced to monomers. However, most high-molecular-weight aggregates remained undissociated, which indicated that not only disulfide bonds but also other types of bonds were formed by dry-heating. More importantly, the major components of EWP (Ova and Otf) were not degraded by dry-heating at temperatures <70 °C at pH 3.0 in the presence of selenite. The result further supported the result that the greater mobility of the Se-EWP (Figure 1) was caused by the negative charge of the introduced selenite group and not by protein degradation.

It has been reported that the polymerization of EWP is not affected by dry-heating in the presence or absence of phosphate.[18] Remarkably, though, the polymerization of EWP

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occurred more easily during dry-heating in the presence of selenite than in its absence, which may have been due to the strong oxidizing property of selenite under acidic conditions. Lawrence reported that sodium selenite catalyzes disulfide interchange reactions in strongly acidic media.[33] In the present study, EWP was selenized by heating under acidic and dry conditions in the presence of selenite, which appeared to form an acidic medium and may have catalyzed disulfide interchange reactions. Thus, the presence of selenite may also cause greater polymerization of EWP via disulfide linkages that are formed by dry-heating in acidic media. Covalent bonds other than disulfide bonds form in proteins during dry-heating,[34] but their structures have not yet been elucidated. It has been reported that cross-linking by amidation between carbonyl and ε-amino groups or by transamidation between such groups with the elimination of ammonia occurs when proteins are severely heated.[35]

Protease digestion of EWPs was performed by incubating EWPs with Pronase E, pepsin, alkaline protease, and α-chymotrypsin, respectively (Figure 4). The digestion velocities

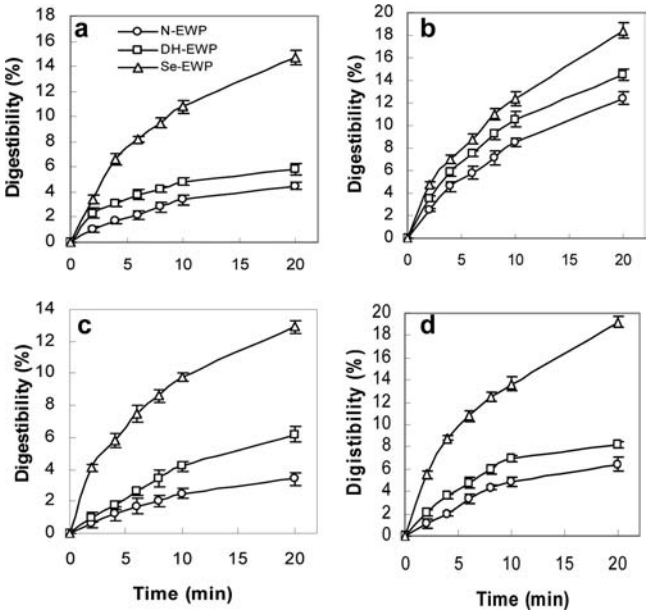

Figure 4. Time course of proteolytic the digestion of N-, DH-, and Se-EWP with Pronase E (a), pepsin (b), alkaline protease (c), and α-chymotrypsin (d). The enzyme/protein ratios for Pronase E (pH 8.0), pepsin (pH 3.0), alkaline protease (pH 9.0), and α-chymotrypsin (pH 8.0) were 1:100, 1:300, 1:10, and 1:10, respectively, at the optimal pH of the corresponding enzyme. DH- and Se-EWP were prepared as described in Figure 2. Each value is the mean with its SD (n = 3).

of Se-EWP with the different proteases were much higher than those of native EWP (N-EWP) and DH-EWP. The digestion velocities of Se-EWP, which were estimated at 20 min, were 14.7, 18.4, 12.9, and 19.2%, respectively. These values were 3.3-, 1.5-, 3.8-, and 3.0-fold higher than the values for N-EWP, respectively. In the present study, interestingly, although more polymers were formed in Se-EWP, the digestibility of Se-EWP was far superior to that of N- or DH-EWP under the same reaction conditions (Figure 4). This result implies that Se-EWP may be more easily digested and then absorbed after being eaten. Moreover, the higher digestibility of Se-EWP by proteases may be advantageous for manufacturing peptides.

Characteristics of Selenite Bonds. The stability of the selenite bonds in Se-EWP at various pH levels was examined by incubating EWP at 37 °C for 24 h (Figure 5). The

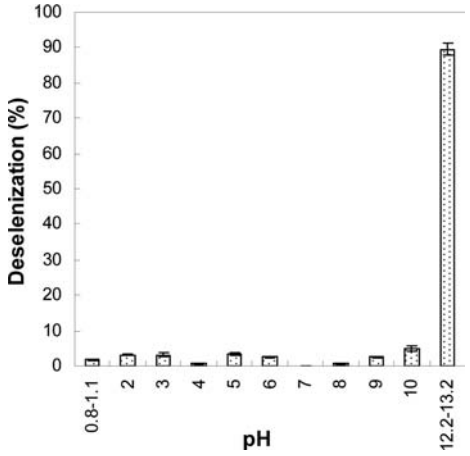

Figure 5. Stability of selenite bonds in Se-EWP under various pH treatments. Se-EWP was prepared by dry-heating at pH 3.0 and 60 °C for 1 day in the presence of selenite. Each value is the mean with its SD (n = 3).

deselenization of Se-EWP was <5% after incubation at 37 °C and over the pH range 2−10 for 24 h, which suggested that the selenite bonds of Se-EWP were stable under these conditions. In 0.5 N HCl, the deselenization level of Se-EWP was only 1.76%, which indicated that the selenite bonds of Se-EWP were acid-stable. On the other hand, in 0.5 N NaOH, the deselenization levels of Se-EWP was approximately 90%, indicating that the selenite bonds were base-labile. The phosphate groups of riboflavin-binding protein (containing eight phosphate groups) exist as oxygen-bound phosphate group (−$OHPO_3$) and are identified as acid-stable and base-labile.[18] Similarly, the selenite bonds in Se-EWP were determined to bind to the hydroxyl group of an amino acid or sugar and form oxygen-bound selenite bonds (−$OHSeO_2$). ^{77}Se NMR spectroscopy is a powerful tool for characterizing the chemical structures of Se-containing compounds.[36] Because of the low natural abundance of ^{77}Se (~7.6%), Se-EWP was prepared in an isotopically enriched form by dry-heating it in the presence of $H_2{}^{77}SeO_3$ (^{77}Se, 99.2%) in our study. As shown in Figure 6, the ^{77}Se NMR spectrum of ^{77}Se-EWP exhibited resonance at 1314 ppm, which was close to the values reported for H_2SeO_3 and $NaHSeO_3$ previously (1300 and 1308 ppm, respectively).[36,37] This indicated that the selenite group was bound to the hydroxyl group of the protein and formed a −$OHSeO_2$ linkage. On the other hand, a signal for a −$NHSeO_2$ linkage[36] (1057 or 1076 ppm) was not detected in Se-EWP. This result was consistent with that for the pH stability of the selenite group (Figure 5).

ABTS$^+$ Free Radical Scavenging Activity. The total antioxidant activity of Se-EWP was evaluated by using an ABTS$^+$ free radical scavenging assay. ABTS$^+$ is a relatively long-lived free radical. It is generated by the direct oxidation of ABTS with manganese dioxide and is decolorized when it reacts with hydrogen-donating antioxidants. The ABTS$^+$ assay includes a direct comparison of the antioxidant activities of tested samples as presented in terms of percentage inhibition. The ABTS$^+$ free radical scavenging activities of N-EWP, DH-EWP, and Se-EWP increased as the concentration of the

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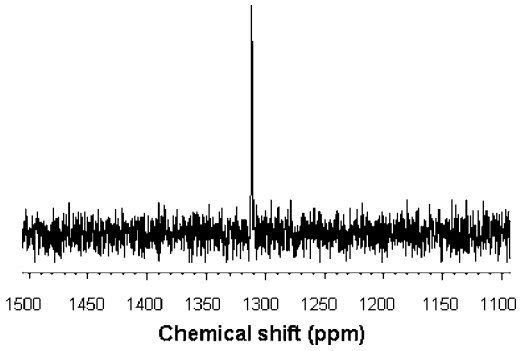

Figure 6. ^{77}Se NMR spectrum of ^{77}Se-EWP at pH 7.0 and 20 °C. ^{77}Se-EWP was dry-heated at pH 3.0 and 60 °C for 3 days in the presence of selenite and dissolved in a buffer that contained 50 mM PIPES (pH 7.0) at a concentration of 120 mg/mL. $(CH_3)_2Se$ was the external standard. Scan totals were 30000.

sample increased from 0.5 to 5.0 mg/mL (Figure 7). However, the scavenging activity of Se-EWP was much higher than that of

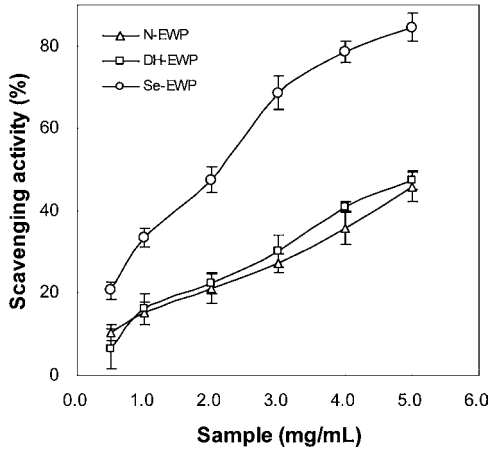

Figure 7. Scavenging effects of N-EWP, DH-EWP, and Se-EWP on ABTS$^+$ free radical at protein concentrations of 0.5−5.0 mg/mL. DH- and Se-EWP were prepared as described in Figure 2. Each value is the mean with its SD (n = 3).

N-EWP or DH-EWP at the same sample concentration. Se-EWP, N-EWP, and DH-EWP inhibited ABTS oxidation by 84.62, 45.80, and 47.54%, respectively, at a protein concentration of 5.0 mg/mL, which suggests that the antioxidant capacity of EWP was markedly enhanced by selenization. This was consistent with results reported by Shen et al.,[38] who showed that the Se-containing protein from the Se-enriched *Bifidobacterium animalis* 01 had a more pronounced antioxidant activity than the same protein without Se. Similar results were observed in previous studies.[9,24,28]

Ferrous Ion Chelating Activity. Redox active transition metals such as iron and copper are capable of catalyzing the reduction of hydroperoxides into reactive radical species and are important prooxidants. Some protein chelators can inhibit oxidative reactions by changing the physical location of transition metals, forming insoluble metal complexes, reducing the chemical reactivity of transition metals, and/or sterically hindering the interaction of metals and dispersed lipids.[39] As indicated in Figure 8, all of the EWP samples showed increased Fe^{2+}-chelating activity as the concentration increased from 0.1 to 2.0 mg/mL. However, DH-EWP exhibited less chelating

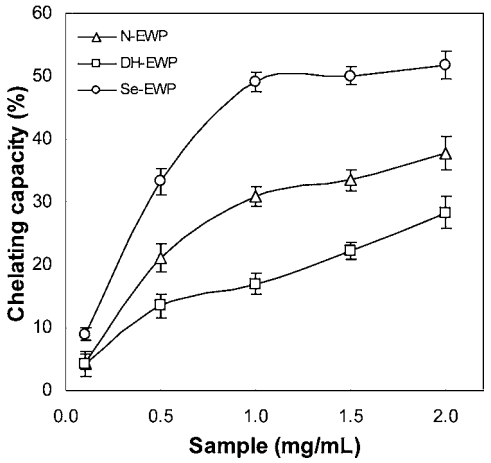

Figure 8. Chelating activities of N-EWP, DH-EWP, and Se-EWP on Fe^{2+} at protein concentrations of 0.1−2.0 mg/mL. DH- and Se-EWP were prepared as described in Figure 2. Each value is the mean with its SD (n = 3).

capacity than N-EWP. This indicated that the Fe^{2+}-chelating activity of EWP could be decreased by dry-heating, which may have been due to the partial loss of iron-binding capacity of Otf that was caused by dry-heating unfolding.[40] However, Se-EWP exhibited more Fe^{2+}-chelating activity than N-EWP or DH-EWP. This was attributed to the electrostatic interactions of the negatively charged selenite groups that were introduced into Se-EWP.

Hydroxyl Radical Scavenging Activity. The hydroxyl radical scavenging activity of Se-EWP was evaluated by using the *o*-phenanthroline Fenton system. The hydroxyl radical was found to be the most reactive free radical. It can be formed from superoxide anion and hydrogen peroxide in the presence of metal ions such as copper and iron, and it can react with nonselective compounds such as protein, DNA, unsaturated fatty acids, and biological membranes. As shown in Figure 9, N-EWP and DH-EWP exhibited dose-dependent hydroxyl radical scavenging activity. However, the hydroxyl radical scavenging activity of Se-EWP was significantly more pronounced than that of N-EWP and DH-EWP. For example, Se-EWP scavenged

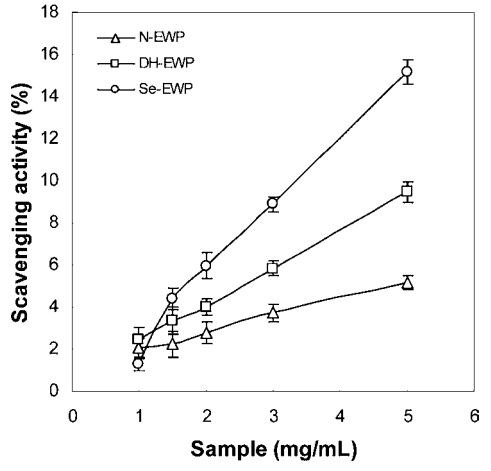

Figure 9. Scavenging effects of N-EWP, DH-EWP, and Se-EWP on hydroxyl radical at protein concentrations of 1−5 mg/mL. DH- and Se-EWP were prepared as described in Figure 2. Each value is the mean with its SD (n = 3).

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15.16% of the hydroxyl radicals at a concentration of 5 mg/mL, and N-EWP and DH-EWP scavenged 5.18 and 9.46%, respectively, under the same conditions. This result suggested that selenization could enhance the hydroxyl radical scavenging activity of EWP. The enhanced hydroxyl radical scavenging activity of Se-EWP could be partially attributed to its strong metal chelating ability via electrostatic interaction between the introduced selenite groups. However, the hydroxyl radical scavenging capacity as assessed by using this assay was still lower than that observed by using the ABTS assay.

Reducing Power. We used a ferric reducing antioxidant assay to determine the reducing power of Se-EWP. This assay was based on the ability of the antioxidant to reduce Fe^{3+} to Fe^{2+} in a redox-linked colorimetric reaction. As seen in Figure 10, N-EWP, DH-EWP, and Se-EWP exhibited more

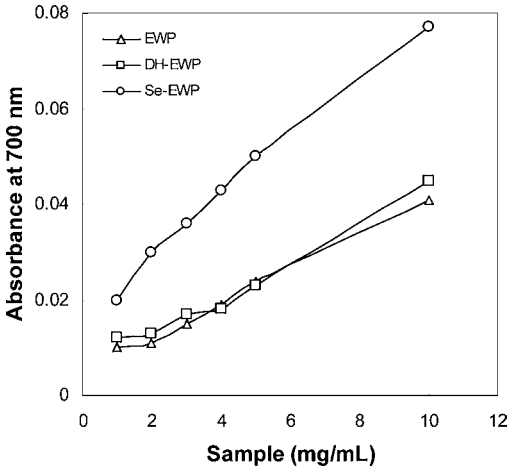

Figure 10. Reducing power of N-EWP, DH-EWP, and Se-EWP at protein concentrations of 1−10 mg/mL. DH- and Se-EWP were prepared as described in Figure 2. Data shown are the mean values of two determinations.

pronounced reducing powers as the concentration of the samples increased from 1 to 12 mg/mL. The reducing power of Se-EWP was superior to that of N-EWP and DH-EWP at the same sample concentration, which indicated that the reducing power of EWP was enhanced by selenization.

To determine the mechanism underlying the enhanced antioxidant activity of Se-containing proteins, several factors were considered. First, selenium compounds exercise free radical scavenging activity through the central free radical of selenium[41] and then increase the antioxidant activity of the protein. Second, the increased chelation of prooxidative transition metals (Fe^{2+}) by selenized EWP is responsible for its increased hydroxyl radical scavenging activity. Third, it has been reported that the antioxidant activity of proteins is dependent on the complex interactions between their ability to inactivate reactive oxygen species, scavenge free radicals, and reduce hydroperoxides. For example, some amino acids, such as tyrosine, phenylalanine, tryptophan, and cysteine, can donate protons to free radicals,[42,43] but basic amino acids, such as lysine and arginine, and acidic amino acids, such as aspartate acid and glutamate acid, exercise antioxidant activity by chelating metal ions.[44,45] It has been reported that the basic amino acid histidine may behave both as a radical scavenger and as a metal chelator due to its imidazole ring.[43,44] Moreover, phosphorylated serine and threonine can bind metal ions.[46] If these amino acids are exposed on the surfaces of proteins, they can chelate metals and may enhance the protein's antioxidant capacity. The antioxidant activity of these amino acids residues is limited by the tertiary structure of the polypeptide, because many amino acids with antioxidant potential can be buried within the protein core, where they are inaccessible to prooxidants.[46] We have shown in previous work that the tertiary structure of EWP is affected by dry-heating and is further affected by phosphorylation.[19] In this study, we speculated that the changes in the structure of the EWP may occur during dry-heating and selenization and that the exposed amino acids may change. In this way, the enhanced antioxidant capacity of Se-EWP may also be related to the conformational change (especially the tertiary structure of the polypeptide) that is caused by dry-heating. Further studies are needed to determine the real relationship between enhanced antioxidant capacity and the structural changes that take place during selenization. This work is currently in progress in our laboratory.

In summary, EWP was successfully selenized by dry-heating in the presence of selenite. The antioxidant activity, including $ABTS^{+}$ free radical scavenging activity, hydroxyl radical scavenging activity, reducing power, and Fe^{2+}-chelating activity of EWP were markedly enhanced by selenization. These results indicate that dry-heating selenization could be a useful means for generating antioxidant food proteins. Other potential physiological functions besides the antioxidant activity of Se-EWP may be expected. However, it is well-known that overdosing on Se may cause dangerous side effects. These include brittle nails and hair, bad breath, nausea, fever, exhaustion, weight loss, hair loss, heart attack, cardiac arrest, hypothyroidism, infertility in males, skin cancer, allergies, and death. Therefore, the safety of Se-EWP should be evaluated carefully before it is used in foods.

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Author Contributions

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Notes

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ABBREVIATIONS USED

ABTS, 2,2′-aminobis(2-ethylbenzothiazolinesulfonic acid) ammonium salt; EWP, egg white protein; DH-EWP, egg white protein dry-heated in the absence of selenite; N-EWP, native EWP; PAGE, polyacrylamide gel electrophoresis; Se-EWP, selenized EWP; SDS, sodium dodecyl sulfate; 2-ME, 2-mercaptoethanol; HPLC, high-performance liquid chromatography; Ova, ovalbumin; Otf, ovotransferrin; NMR, nuclear magnetic resonance

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