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Investigation of the Protein−Protein Aggregation of Egg White Proteins under Pulsed Electric Fields

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Egg whites were exposed to pulsed electric fields (PEFs) to investigate the protein−protein aggregation. No insoluble protein aggregate was found when egg whites were exposed to PEFs at 25, 30, and 35 kV/cm for 400 μs. However, atomic force microscopy showed that the sizes of the protein particles increased. Native polyacrylamide gel electrophoresis (PAGE) demonstrated the existence of aggregates under PEFs at 35 kV/cm for 400 μs. Sodium dodecyl sulfate (SDS)-PAGE in the presence and absence of 2-mercaptoethanol further indicated that sulfhydryl-disulfide interchange reactions occurred under PEFs. Differential scanning calorimetry scans showed 400 μs of PEF treatment at 35 kV/cm denatured 16.5% proteins. Insoluble egg white protein aggregates were induced by PEF (35 kV/cm, 800 μs) and heat (60 °C, 3.5 min) treatments. Disulfide bonds were the dominant binding forces in the formation of protein aggregates. However, the weakly noncovalent bonds play a much more important role in the protein aggregation forming in heat treatment (60 °C, 3.5 min) than that in PEF treatment (35 kV/cm, 800 μs).

KEYWORDS: Pulsed electric fields (PEF); egg white; protein; aggregation

INTRODUCTION

A pulsed electric field (PEF) is a promising nonthermal food preservation technique that uses short pulses of high-intensity electric fields to inactivate most microorganisms and some enzymes at room or moderate temperatures (*1*, *2*). As compared to thermal treatment, PEFs can offer freshlike "minimally"-processed foods, mainly low acid juices with little loss of color, flavor, and nutrients (*3*−*5*). They are now receiving considerable attention by scientists, governments, and the food industry and have undergone substantial developments nearing commercial application (*6*). However, the mechanisms involved in inactivation of enzymes by PEFs and the actions of PEFs on proteins are not now fully understood. Researchers found some proteins with enzymatic activity could be inactivated under some PEF processing conditions due to the structural changes. Castro et al. (*7*) reported that disruptions of side chain interactions by an electric field of 22 kV/cm with pulses of 700−800 μs altered the tertiary structure and denatured bovine alkaline phosphatase. Concomitantly, many hydrophobic groups originally in the interior of the molecule were exposed to a more polar solvent to form aggregates by hydrophobic interactions. Budi et al. (*8*) found that the intrinsic flexibility of insulin was constrained by PEFs, thus potentially restricting access to the protein's active state. Zhao et al. (*9*) applied a 35 kV/cm PEF treatment on lysozyme in phosphate buffer and found that the unfolding of lysozyme structure was induced by PEF, accompanied by the cleavage of disulfide bonds. Published studies reported that the inactivation of PEF-treated papain (*10*), peroxidase (*11*), and polyphenol oxidase (*11*) was correlated to the alternation of secondary structures.

PEF processing has been applied to a variety of protein-based foods such as milk (*12*, *13*), soymilk, liquid whole egg (*14*), egg white (*15*−*17*), and soybean protein isolates (*18*). Several researchers also studied the structural alterations of food protein components. Jeantet et al. (*16*) investigated the effects of exponential decay pulses, up to 35 kV/cm for 2−8 pulses (10 μs pulse width, 100−900 Hz pulse frequency), on dia-ultrafiltered egg white, suggesting that PEF treatment did not modify the surface hydrophobicity of egg white proteins. Fernández-Diaz et al. (*17*) found no changes in the stucture of dialyzed egg white proteins after 50−400 exponential decay pulses with an electric field strength of 27−33 kV/cm. Barsotti et al. (*19*) studied the effects of PEF on β-lactoglobulin at low and high protein concentrations. The results showed that PEF processing at 30−32 kV/cm with a pulse width of 1.3 μs for 200 exponential decay pulses at 1 Hz did not cause marked unfolding of the β-lactoglobulin molecule at low protein concentration (2%, w/v) and protein aggregation at higher protein concentration (16.7%, w/v). However, Perez and Pilosof (*15*) observed that the structures of egg white protein and β-lactoglobulin were partially modified when subjected to long pulse width and high-strength PEF by measuring thermal denaturation temperatures and the gelation rate. Li et al. (*18*) reported that the solubility of soybean protein isolates decreased due to denaturation when the PEF strength and treatment time were above 30 kV/cm and 288 μs. From these results, the conclusions about the effects of PEF on proteins elucidated by different research groups are inconsistent.

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Effects of PEF on enzymes or other proteins appear to depend on the protein, PEF system, and parameters. This is an area that has not been fully elucidated; more research work is still required, and more detailed information about the action of PEF on protein is necessary.

As is known, electric fields can profoundly affect the physical properties and reactivity of dissolved protein molecules. An external high-intensity electric field could affect the local electrostatic fields in proteins and then disrupt the electric interactions of peptide chains (*20*). The exposure of hydrophobic residues, the cleavage of disulfide bonds, and the changes in tertiary and secondary structures of protein molecules under PEFs were demonstrated in previous studies (*7*−*11*), which represent the initial steps of subsequent macroscopic changes such as exposure of regions suitable for different kinds of interactions with other proteins; however, little is known about the protein−protein interactions and the nature of the chemical bonds involved. From the point of view of theory, understanding the protein−protein interactions under PEFs is necessary for the further development and commercialization of PEFs. Egg white, a natural multiprotein system, is a good model to investigate the protein−protein aggregation under PEFs. From the point of view of PEF application, it is almost impossible to ignore the significance of eggs in the world of food. Egg whites, as a potential application target of PEFs, represent an extensively used and multipurpose food ingredient, meeting the requirements of a variety of food formulations.

MATERIALS AND METHODS

Materials. Fresh eggs were obtained from a local chicken farm. The egg whites were separated from the egg yolks and chalazae. The separated egg whites were then manually homogenized by gentle stirring and then centrifuged at 10000*g* for 20 min at 4 °C to remove small amounts of insoluble materials. The egg whites were stored at −40 °C until use. According to the studies of Van der Plancken et al. (*21*), there was no conversion of N-ovalbumin to S-ovalbumin at this storage temperature. The pH of the supernatant used in this study was 7.2 as determined with a pH meter (Delta 320 pH acidometer, Shanghai Mettler-Toledo Apparatus Co. Ltd., China). The protein content was determined to be 92.4 mg/mL by Kjeldahl method (AOAC) (*22*). Hen egg white lysozyme was purchased from Amresco Inc. (Solon, OH). Hen egg white lysozyme powder was dissolved in sodium phosphate buffer (10 mM, pH 7.2) with an electrical conductivity of 0.5 S/m at 25 °C determined with a conductivity meter (Hydac, Cambridge Scientific Instruments, Cambridge, MD).

PEF Devices and Treatments. A bench scale continuous system (OSU-4 L, The Ohio State University, Columbus, OH) with square-wave pulses was used in this work. The PEF apparatus has been described in our previous study (*9*). After going through each pair of chambers, the sample was cooled by passing through a coiled tube with a 2.3 mm inner diameter, which was submerged in a heat exchange bath (Fisher Scientific Inc., Pittsburgh, PA) with cold water (5 °C). The pre- and post-PEF exposure temperatures at the inlet and outlet of the treatment chamber were measured using a K type thermocouple (OMEGA, Stamford, CT). The highest temperature achieved in all of the tests was lower than 42 °C.

The egg whites were exposed to PEFs with 25, 30, and 35 kV/cm of electric field intensity for 400 μs, respectively. In this study, the pulse repetition rate and pulse width were set to 200 Hz and 2 μs, respectively. To get insoluble egg white protein aggregates under electric fields, the egg whites were subjected to PEF treatment at 35 kV/cm for 800 μs. Lysozyme solutions (20 mg/mL) were exposed to PEF at 35 kV/cm for 1200 μs.

Thermal Treatments. According to current regulations in the United States, the pasteurization of liquid egg in the food industry requires at least 60 °C for a minimum of 3.5 min. In this investigation, an experimental setup was constructed to simulate the heat treatment of liquid egg using a plate heat exchanger in the food industry. The egg whites were preheated to 45 °C, held at 60 °C, and then cooled to 5 °C by passing through stainless steel coils with a 2.3 mm inner diameter, which were submerged in several heat exchange bathes (Fisher Scientific Inc.) with different temperatures. When egg whites were running in the coils, the time needed for the temperature to rise from 45 to 60 °C was very short and could be ignored due to the very small inner diameter and wall thickness of the stainless steel coil. The time that it took the egg whites to pass the temperature holding (60 °C) container was 3.5 min. Lysozyme solutions (20 mg/mL) were preheated to 60 °C, held at 100 °C for 3.5 min, and then cooled to 5 °C.

Atomic Force Microscopy (AFM) Imaging. AFM imaging was performed with a Multimode Nanoscope IIIa atomic force microscope from Digital Instruments (Santa Barbara, United States). The experiments were conducted at room temperature (20 °C) and at atmospheric pressure (760 mmHg) operating in tapping mode, in which the space between the tip and the sample was from 10 to 100 Å and the total force was very low. This low force was advantageous to study soft and deformable samples. Triangular silicon tips were used for this analysis. To visualize the protein aggregates, the noncontact mode was more appropriate because the vesicles were only a little susceptible to the load forces applied. Just before the analysis, the samples were diluted in water (1: 100) to obtain a less sticky fluid for analyses. A 3−4 μL droplet of protein dispersion was placed onto a small mica disk with a diameter of 1 cm and dried in ambient air. After 2 min, the sample chamber was mounted onto the AFM scanner, and the measurements were performed and completed within a few minutes to avoid deformation. Images were treated with the Digital Nanoscope Software (Version 5.30r3, Digital Instruments) for three-dimensional (3D) representation.

Turbidity Measurements. Turbidity was measured using a UV−visible spectrophotometer (UV1201, Beijing Ruili Instrument Co., Beijing, China) at 650 nm according to the method of Van der Plancken et al. (*23*). The percent transmittance (T%) of the (un)treated egg white was recorded and 100 T% was used as an indicator of turbidity. A turbidity of 0% corresponded to a totally clear solution.

Gel Electrophoresis. Native polyacrylamide gel electrophoresis (native-PAGE) and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) were carried out at a constant current (20 mA) in a Mini-Protean Tetra systerm with PowerPac Basic Power Supply (Bio-Rad Laboratories, Inc., Richmond, CA). Because of the low current, the produced heat was not significant. Native-PAGE was performed using 4% stacking gel and 7.5% separating gel. The stacking gel was 4% acrylamide and 0.32% *N*,*N*′-methylene-bis-acrylamide in 0.125 M Tris−HCl buffer, pH 6.8, and the separating gel was 12.5% acrylamide and 0.27% *N*,*N*′-methylene-bis-acrylamide in 0.43 M Tris−HCl buffer, pH 8.9. The electrode buffer was 0.05 M Tris−0.384 M glycine buffer, pH 8.3. The sample buffer was 0.01 M Tris−HCl, 10% glycerol, and 0.02% bromophenol blue, pH 8.0. The samples were not heated before application. SDS-PAGE was performed using 4% stacking gel and 12.5% separating gel under both reducing and nonreducing conditions in the presence and absence of β-mercaptoethanol (ME). Both gels and electrode buffer contained 0.1% SDS. The reductive sample buffer was 2% SDS, 5% ME, 10% glycerol, 0.02% bromophenol blue, and 0.01 M Tris−HCl buffer, pH 8.0, while the ME was omitted in nonreducing sample buffer. The sample solution was boiled for 3 min and centrifuged at 1500*g* for 10 min to remove undissolved debris. After separation, the proteins were stained with Coomassie Brilliant Blue R-250 (0.02 g/100 mL) in methanol (50 mL/100 mL) and acetic acid (7.5 mL/100 mL) and destained with methanol (50 mL/100 mL) and acetic acid

(7.5 mL/100 mL), followed by methanol (5 mL/100 mL) and acetic acid (7.5 mL/100 mL). The images of gels were scanned with GEL-DOC 2000 gel documentation system (Bio-Rad).

Differential Scanning Calorimetry (DSC). Thermal properties of egg white were determined using DSC (Perkin−Elmer Co., Norwalk, CT). Twenty-five microliters of egg white before and after PEF treatments were placed into aluminum DSC pans. The pans were hermetically sealed, and the samples were scanned from 15 to 100 °C at a heating rate of 5 °C/min. The enthalpy of denaturation (ΔH) and the denaturation temperature (T_d) were determined.

Preparation of Insoluble Protein Aggregates and the Binding Forces Measurement. PEFs and heat-treated egg whites were centrifuged at 10000*g* for 20 min at 4 °C to obtain the insoluble protein aggregates. These aggregates were washed three times with distilled water and then centrifuged as above. The solubilization of the insoluble egg white protein aggregates induced by PEFs and heat was conducted as described by Matsudomi et al. (*24*) using various agents, which differ from each other according to their ability to cleave intermolecular bonds: electrostatic and hydrogen bonds (0.6 M NaCl), hydrogen bonds (1.5 M urea), hydrogen and hydrophobic bonds (8 M urea), and disulfide bonds [10 mM dithiothreitol (DTT)]. The insoluble protein aggregates were treated independently with 0.6 M NaCl (solution A), 0.6 M NaCl plus 1.5 M urea (solution B), 0.6 M NaCl plus 8 M urea (solution C), and 0.6 M NaCl and 8 M urea plus 10 mM DTT (solution D). The aggregates containing each solution were fully stirred and then centrifuged. The supernatant fractions were analyzed for protein solubility. The protein solubility was calculated as a percentage of protein content of the supernatant as compared with the total protein content. The protein content of protein aggregates was determined by the Kjeldahl method (AOAC). For solution D, all of the proteins were solubilized completely, and the protein solubility was 100%. For solutions A−C, the protein concentration was determined using a Non-Interfering Protein Assay Kit as Calbiochem Procedure No. 488250 (Calbiochem, United States) with bovine serum albumin as the standard, which uses an universal protein precipitating agent to precipitate and immobilize the protein in the tube, while the interfering reagents are removed and the protein concentration is based on the specific binding of copper to the peptide backbone.The assay can overcome interference of agents in protein solutions including 8 M urea.

Statistical Analyses. Analysis of variance (ANOVA) was performed by Statistical Analysis System (SAS, version 8.0, 2000, Cary, NC). Experiments were triplicated, and the means of the three data sets are presented.

RESULTS AND DISCUSSION

Monitoring Soluble Protein Aggregation Induced by PEF. As is known, proteins under several physical stresses such as heat and pressure (*25*) can form aggregates ranging from dimers and small oligomers to gel-like structures and insoluble macroscale particles and fibers. An electric field is a very different physical stress from heat and pressure, which had an effect on the proteins (*8*−*10*). In this work, egg white was exposed to PEFs at several electric field strength levels from 25 to 35 kV/cm for 400 μs. There was no significant change ($p > 0.05$) in the turbidity (approximately 15%) of egg white induced by PEFs, and no insoluble aggregate was found after centrifugation at 10000*g* for 20 min at 4 °C. The surface topography of highly diluted egg white proteins on mica was measured by AFM. As illustrated in **Figure 1**, the AFM 3D images indicate a different topography for the control sample (untreated) and the samples exposed to PEF at 25, 30, and 35 kV/cm for 400 μs. The resonant frequencies of this cantilever were found to be about 300 kHz. Higher resonant frequencies of the cantilever are necessary to minimize the sensitivity to vibrational noise. It is clear from **Figure 1a** that AFM 3D representation displayed quite homogeneous protein particles of the untreated sample. However, the size of protein particles increased with the increase of electric field strength from 25 to 35 kV/cm (**Figure 1b**−**d**). It is especially obvious that protein particles were roughly agglomerated under 35 kV/cm of electric field, suggesting that the protein aggregation occurred, probably due to stronger protein−protein interactions under the stress of electric field. Previous studies have demonstrated that the protein response to electric field stress includes cleavage of disulfide bonds, exposure of hydrophobic amino acid residues, constraint of intrinsic flexibility of protein molecule, and unfolding of secondary and tertiary structures (*7*−*10*). These responses represent the initial steps of subsequent macroscopic changes such as self-aggregation and kinds of protein interactions in complex systems, because aggregation can be based on noncovalent, electrostatic, or hydrophobic interactions or on covalent binding, usually through the formation of disulfide bonds between cysteinyl residues (*26*). The use of AFM in this work puts forward the evidence for the protein aggregation formation induced by PEFs.

The native-PAGE patterns of native egg white proteins and egg whites exposed to PEF at several electric field strength levels from 25 to 35 kV/cm for 400 μs are presented in **Figure 2A**. As compared with lane a (native egg white proteins) in the native-PAGE, more protein bands appeared in the stacking gel and the beginning of the separating gel in lane d (egg white proteins exposed to PEFs at 35 kV/cm for 400 μs). It indicates the formation of soluble aggregates. These soluble aggregates could not migrate into the stacking gel or remained at the beginning of the separating gel. The results suggest that polymerization between or among egg white proteins occurred under electric field stress. The SDS-PAGE in the presence and absence of 2-mercaptoethanol was also performed to assess the binding type of PEF-induced soluble protein aggregates of egg white proteins. From the SDS-PAGE patterns in the presence of 2-mercaptoethanol (**Figure 2B**), there were no differences between the bands of native egg white proteins and those of PEF-treated egg white proteins, indicating that the soluble aggregates were almost dissociated by SDS and 2-mercaptoethanol and covalent bonds other than disulfide bonds were not involved in the protein polymerization under PEF. However, when SDS-PAGE was performed in the absence of 2-mercaptoethanol (**Figure 2C**), the aggregation phenomenon was illustrated by the higher molecular weight protein bands that appeared in the stacking and separating gels under 35 kV/cm, indicating that most of the aggregates remained undissociated without 2-mercaptoethanol. In light of these results, the main type of binding of soluble aggregates is likely to be disulfide bonds between egg white proteins. These results suggest that sulfhydryl−disulfide interchange reactions occurred under PEFs.

DSC scans for native egg white proteins and egg white proteins exposed to PEF at several electric field strength levels from 25 to 35 kV/cm for 400 μs are in **Figure 3**. Unfolding of globular proteins during thermal denaturation involves absorption of heat to break intramolecular bonds (noncovalent and, in some cases, disulfide) and is therefore endothermic (*27*). DSC scans in **Figure 3** exhibited two main endothermic peaks (T_{P1} and T_{P2}). DSC scans of egg white proteins exposed to PEFs mainly showed a decrease in enthalpy (ΔH). From the values of ΔH of egg white proteins

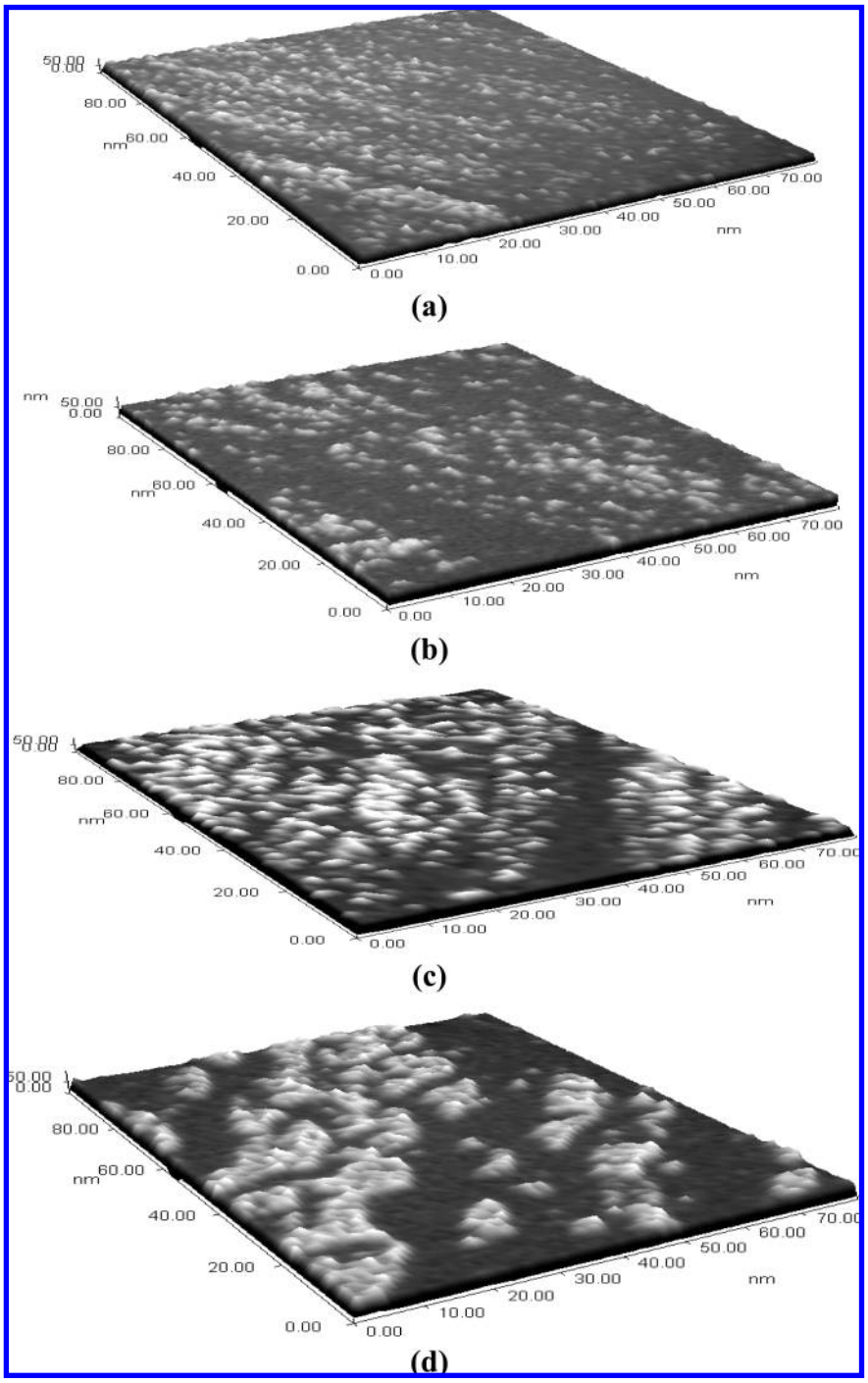

Figure 1. Three-dimensional plots obtained from AFM of (**a**) native egg white protein solution and egg white protein solution exposed to PEF at (**b**) 25, (**c**) 30, and (**d**) 35 kV/cm for 400 μs.

(**Table 1**), the degree of denaturation was about 16.5% when 400 μs of PEF at 35 kV/cm was applied. As shown in **Table 1**, T_{onset} and T_{P2} were not significantly ($p > 0.05$) modified by an electric field, whereas T_{P1} increased by approximately 2 °C when egg white proteins were exposed to 35 kV/cm of PEFs for 400 μs. It indicates that PEFs would partially enhance the thermoresistance of egg white proteins, which is in agreement with the previous report (*15*).

Insoluble Protein Aggregation Induced by PEFs. To get insoluble egg white protein aggregates induced by PEFs, the egg whites were subjected to a higher intensity of PEFs. The exposure time of egg white proteins to PEFs has been prolonged to 800 μs. The heat-induced aggregation among heterogeneous egg white proteins was formed at 60 °C for 3.5 min. The turbidity values of egg whites under these PEFs and heat conditions were approximately equal (approximately 60%), indicating that these two treatments might induce a similar degree of protein aggregation.

The SDS-PAGE in the presence and absence of 2-mercaptoethanol of egg white protein aggregates induced by PEFs (35 kV/cm, 800 μs) and heat (60 °C, 3.5 min) are illustrated in **Figure 4**. From the SDS-PAGE patterns in the presence of 2-mercaptoethanol (**Figure 4A**), the PAGE pattern of egg white protein aggregates induced by PEF and heat was the same as that of native egg white proteins. It indicates that the insoluble protein aggregates were almost dissociated by SDS and 2-mercaptoethanol; covalent bonds other than disulfide bonds were not involved in the protein polymerization

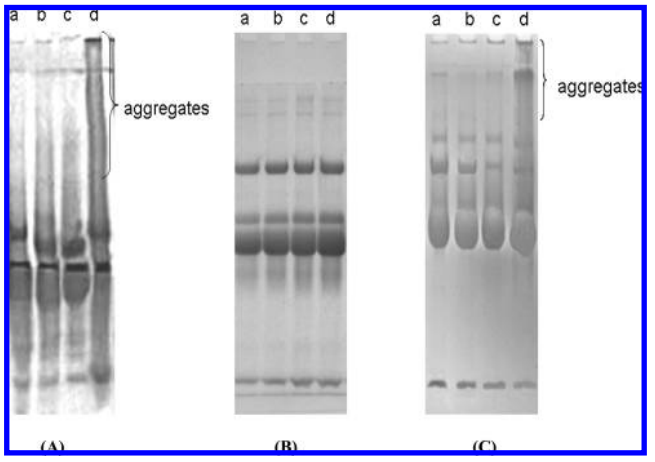

Figure 2. Native-PAGE (**A**) and SDS-PAGE in the presence (**B**) and absence (**C**) of 2-mercaptoethanol of (a) native egg white proteins and egg white proteins exposed to PEF at (b) 25, (c) 30, and (d) 35 kV/cm for 400 μs. Approximately 500 μg of protein was applied on each lane.

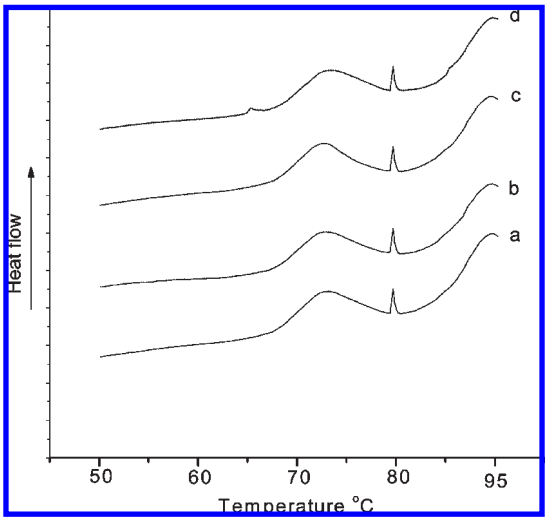

Figure 3. DSC of (a) native egg white proteins and egg white proteins exposed to PEF at (b) 25, (c) 30, and (d) 35 kV/cm for 400 μs.

Table 1. Differential Scanning Calorimetric Data in the Native Egg White Proteins (a) and Egg White Proteins Exposed to PEF at (b) 25, (c) 30, and (d) 35 kV/cm for 400 μs[a]

sample	T_{onset} (°C)	T_{P1} (°C)	T_{P2} (°C)	ΔH (J/g)
a	68.2 ± 0.2	72.7 ± 0.2	79.7 ± 0.1	29.8 ± 0.6
b	68.0 ± 0.3	72.7 ± 0.1	79.7 ± 0.2	29.3 ± 1.0
c	68.5 ± 0.2	73.0 ± 0.2	79.7 ± 0.3	27.8 ± 1.2
d	68.0 ± 0.4	74.6 ± 0.1	79.7 ± 0.2	24.9 ± 0.7

[a] Data are the means ± standard deviations. Significance level, $p = 0.05$.

induced by PEF or heat, and all protein components in the heterogeneous egg white proteins were involved in the protein interactions under PEF and heat to form protein aggregates. However, the protein aggregates were demonstrated by the existing of higher molecular weight protein bands in the stacking gel and the beginning of the separating gel of SDS-PAGE in the absence of 2-mercaptoethanol (**Figure 4B**) in both PEF and heat-induced samples, suggesting that egg white proteins had been incorporated covalently into insoluble protein aggregates through disulfide cross-links with the other proteins under PEF and heat treatments. It is interesting to note that there were some differences between the SDS-PAGE profile in the absence of 2-mercaptoethanol

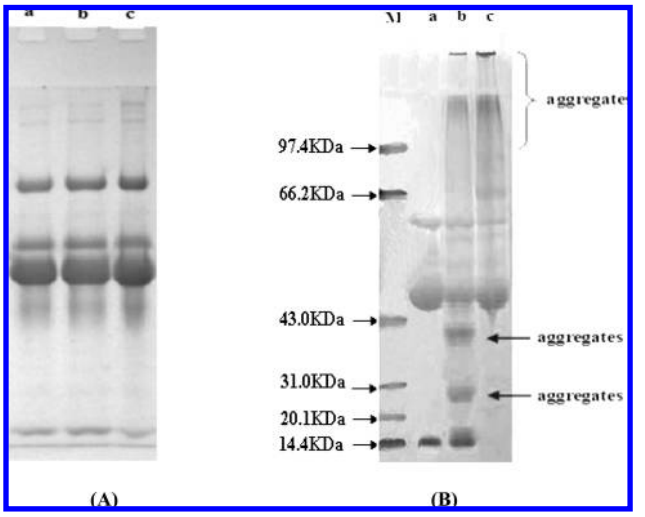

Figure 4. SDS-PAGE in the presence (**A**) and absence (**B**) of 2-mercaptoethanol of (a) native egg white proteins and egg white protein aggregates induced by (b) PEF (35 kV/m, 800 μs) and (c) heat (60 °C, 3.5 min). Approximately 500 μg of protein was applied on each lane. Lane M, the molecular weight markers with an arrow in the left: hen egg white lysozyme (14.4 kDa), trypsin inhibitor (20.1 kDa), bovine carbonic anhydrase (31 kDa), rabbit actin (43 kDa), bovine serum albumin (66.2 kDa), and rabbit phosphorylase b (97.4 kDa), respectively.

(**Figure 4B**) of PEF-induced protein aggregates and that of heat-induced protein aggregates. For the SDS-PAGE profile in the absence of 2-mercaptoethanol of heat-induced egg white protein aggregates (**Figure 4B**, lane c), high molecular weight soluble protein aggregates could not migrate into the stacking gel and appeared as dark stains at the top of stacking gel. Some large molecular weight egg white protein aggregates also appeared at the top of separating gel. However, the band of lysozyme (14.4 kDa) is almost hard to be observed. The similar phenomenon could be observed from the study of Mine et al. (*28*) when the egg white proteins were heated at a temperature ranged from 60 to 100 °C. Egg white comprises hetereogenerous proteins such as ovalbumin, conalbumin, ovomucoid, and lysozyme. The heat aggregation of egg white could be facilitated by interactions among such heterogeneous proteins (*28*). However, for the SDS-PAGE profile of the PEF-induced egg white protein aggregates in the absence of 2-mercaptoethanol (**Figure 4B**, lane b), two protein aggregate bands corresponding to relatively low molecular weight occurred (as indicated by the arrows) besides the protein aggregate bands corresponding to high molecular weight at the top of stacking gel and the beginning of the separating gel. It may be concluded that lysozyme dimers and trimers were formed under an electric field based on the comparison with the molecular weight markers.

In the present work, the aggregation behaviors of lysozyme under an electric field and heat were further investigated. The insoluble lysozyme aggregates induced by PEFs (35 kV/cm, 1200 μs) and heat (100 °C, 3.5 min) began to appear. The SDS-PAGE profiles in the absence of 2-mercaptoethanol of native lysozyme, insoluble lysozyme aggregates induced by PEF, and heat are shown in **Figure 5**. On the basis of the comparison with the molecular weight markers, lysozyme dimers and trimers (in **Figure 5A**, lane b) were clearly found when lysozyme solution was exposed to PEFs at 35 kV/cm for 1200 μs. The results confirm the deduction that the formation of lysozyme dimers and trimers

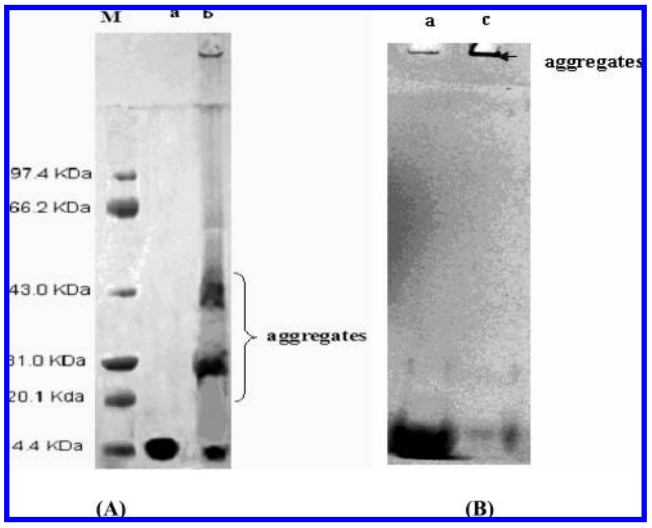

Figure 5. SDS-PAGE in the absence of 2-mercaptoethanol of insoluble lysozyme aggregates induced by (**A**) PEF (35 kV/cm, 1200 μs) and (**B**) heat (100 °C, 3.5 min). Approximately 100 μg of protein was applied on each lane. Lane M, the molecular weight markers with arrow in the left: hen egg white lysozyme (14.4 kDa), trypsin inhibitor (20.1 kDa), bovine carbonic anhydrase (31 kDa), rabbit actin (43 kDa), bovine serum albumin (66.2 kDa), and rabbit phosphorylase b (97.4 kDa), respectively; lane a, native lysozyme; and lanes b and c, insoluble lysozyme aggregates induced by PEF and heat, respectively.

occurred when egg white proteins were under electric field. Elmnasser et al. (*29*) found that after a pulsed-light treatment of β-lactoglobulin with 5, 7, and 10 pulses, the formation of dimers was observed. Liu et al. (*30*) reported the presence of dimers of β-lactoglobulin after high hydrostatic pressure treatment. These results may indicate the presence of small protein aggregates such as dimers and trimers induced by nonthermal tretments such as high hydrostatic pressure, pulsed light, and PEF treatments. However, different protein aggregation behaviors occurred under thermal treatment, which is characterized mainly by high molecular weight protein aggregates. These aggregates could not migrate into the stacking gel and appeared as dark stains at the top of stacking gel.

Binding Forces Measurement of Protein Aggregates Induced by PEFs. To elucidate the major forces involved in the formation of protein−protein aggregates induced by PEFs and to obtain more information of the action of PEF on the proteins, egg white protein aggregates induced by PEFs and heat were solubilized by various protein-denaturing reagents. As illustrated in **Figure 6**, only a small portion (less than 10%) of PEF-induced egg white protein aggregates was soluble in solutions A (0.6 M NaCl), B (0.6 M NaCl plus 1.5 M urea), and C (0.6 M NaCl plus 8 M urea). However, heat-induced egg white protein aggregates treated with solution A gave protein solubility values of around 20% of protein aggregates, and when the heat-induced egg white protein aggregates were treated with solution C, more than 40% of protein aggregates was solubilized. The fractions soluble in solutions A−C would be related to proteins bound weakly through noncovalent bondings such as electrostatic interactions, hydrogen bonds, or hydrophobic interactions. The heat-induced egg white protein aggregates exhibited more solubilization than did the PEF-induced, suggesting that the weakly noncovalent bonds such as electrostatic interactions, hydrogen bonds, and hydrophobic interactions play a much more important role in the protein aggregates

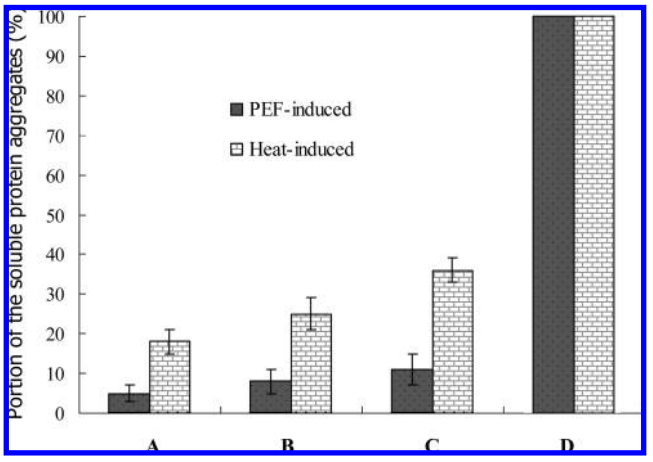

Figure 6. Binding forces measurement of egg white protein aggregates induced by PEF and heat. The solubilization of egg white protein aggregates induced by PEF and heat by various protein-denaturing reagents: 0.6 M NaCl (solution A), 0.6 M NaCl plus 1.5 M urea (solution B), 0.6 M NaCl plus 8 M urea (solution C), and 0.6 M NaCl and 8 M urea plus 10 mM DTT (solution D).

formed in the heat treatment than that in PEF treatment. With solution D (0.6 M NaCl and 8 M urea plus 10 mM DTT), proteins were solubilized completely in both types of protein aggregates, indicating that disulfide bonds are the uppermost binding forces in the formation of protein aggregates no matter whether the protein aggregates were induced by PEFs or heat. Another conclusion is also confirmed that covalent bonds other than disulfide bonds were not involved in the protein polymerization under stress of PEFs or heat.

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