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Toward Better Understanding of Salt-Induced Hen Egg White Protein Aggregation Using Field-Flow Fractionation

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Field-flow fractionation techniques including sedimentation field-flow fractionation (SdFFF) and flow field-flow fractionation (FlFFF) were applied to investigate hen egg white protein aggregation. The thermally induced aggregation of hen egg white protein was observed at temperatures of 60 °C and higher. Particle size and size distribution of hen egg white protein aggregates were characterized by SdFFF to investigate parameters affecting $ZnCl_2$-induced aggregation of hen egg white protein. At a fixed concentration of 1.0 M $ZnCl_2$ and an incubation time of 15 min, the mean particle diameters of the aggregates were determined to be 0.43, 0.67, and 0.80 μm for hen egg white protein contents of 5, 6.25, and 7.5% (w/v), respectively. With the incubation time of 15 min, increasing the concentration of $ZnCl_2$ from 0.5 to 1.0 and to 1.5 M caused the mean particle diameter of the aggregates to grow from 0.37 to 0.42 and to 0.68 μm, respectively at 5% (w/v) hen egg white protein. Upon prolonged contact time, larger aggregates were formed. Furthermore, FlFFF was employed as a novel approach to determine the efficiency of protein utilization for aggregation. The pH values as well as $ZnCl_2$ and protein concentrations influenced the efficiency of protein utilization for aggregation. With the optimum condition, that is, a protein concentration higher than 2% (w/v) and a pH greater than 5, the efficiency of protein utilization was approximately 65%.

KEYWORDS: Hen egg white; field-flow fractionation; particle size; aggregate; efficiency of protein utilization

INTRODUCTION

Hen egg white has been widely used in many types of food products as it has several excellent functional properties such as gelling, foaming, water binding, and emulsifying capacity (*1*). Various types of proteins are found in hen egg white, including 54.0% ovalbumin (44.5 kDa), 12.0% ovotransferrin (77.7 kDa), 11.0% ovomucoid (28.0 kDa), 3.5% ovomucin (5500−8300 kDa), 3.4% lysozyme (14.3 kDa), and a few other proteins (*1*). One of the important functional properties of hen egg white is due to its ability to undergo aggregation upon heat treatment (*2*−*4*) or addition of salt (*5*−*7*), which plays an important role in the textural properties of final food products. Therefore, it is crucial to understand how to control the aggregation behavior of hen egg white protein. Aggregation and subsequent gel formation, which are complicated processes, depend on several factors such as protein concentration, ionic strength, pH, and interaction with other components (*2, 8*).

The aggregation of hen egg white protein has been extensively described by many researchers (*1, 9*). Heat treatment causes changes in surface hydrophobicity and flexibility, which have an impact on viscosity and aggregation (*10*−*12*). The pH and the ionic strength of the protein environment were reported to alter the charge distribution of amino acid side chains, which could either decrease or increase the protein−protein interaction (*8*). The effect of type of salts (NaCl and $CaCl_2$) on the aggregate formation of ovalbumin, a dominant protein in hen egg white, was examined (*13*). The results showed that $CaCl_2$ lowered the denaturation temperature of ovalbumin and also influenced the microstructure and rheological properties of thermally denatured ovalbumin, whereas NaCl exhibited no effect. According to Barbut and Foegeding (*14*), despite its simplicity and rapidity, thermally induced aggregation was not always desirable, as the efficiency of protein utilization was not near 100%. Therefore, cold gelation or salt-induced aggregation is highlighted in this study. Our preliminary results suggested that at the same concentration, $ZnCl_2$ could promote aggregation of hen egg white protein more readily than the other types of salts, that is, $CaCl_2$ and $FeCl_3$. Consequently, this study was aimed toward gaining an insight into $ZnCl_2$-induced aggregation of hen egg white protein.

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Many analytical techniques have been applied to investigate the structure and particle size of hen egg white protein aggregates such as transmission electron microscopy (*15*), static and dynamic light scattering (*7*), atomic force microscopy (*3, 16*), and field-flow fractionation (FFF)−liquid chromatography (*17*). FFF was employed in this study because of its distinct advantages offering particle size information as well as size separation. As compared to other size separation techniques, FFF is considered gentle as the separation of particles or macromolecules is achieved solely through the interaction of sample with an external perpendicular physical field, rather than by the interaction with a stationary phase in chromatographic systems. The purpose of the perpendicular field is to drive different kinds of particles and macromolecules to different localized regions or positions between the channel walls, which are then intercepted with different regions of the parabolic flow profile. These particles are then carried toward the channel exit at different speeds. The perpendicular field can be of many kinds, by which a cross-flow of liquid and centrifugation field is most commonly used under the name "flow FFF (FlFFF)" and "sedimentation FFF (SdFFF)", respectively. In FlFFF, fractionation is based on diffusion characteristics of the separated particles, whereas the boyuancy of the particles also plays an important role in the retention mechanism of SdFFF. The characterization of food protein aggregates by FFF has been reported by many investigators (*17*−*19*).

This study was undertaken to investigate parameters affecting hen egg white protein aggregation including pH and hen egg white protein and $ZnCl_2$ concentrations using FFF techniques. SdFFF was employed to provide evidence of heat- and salt-induced hen egg white protein aggregation and particle size information of the resulting aggregates. FlFFF was proposed as a new method to measure the efficiency of protein utilization for aggregation. This information indicates how much percentage of protein underwent aggregation. The unique information obtained from SdFFF and FlFFF experiments can be used as guidelines how to control the aggregate size of hen egg white protein and how to efficiently utilize hen egg white protein for aggregation.

MATERIALS AND METHODS

Chemicals. $ZnCl_2$, HCl, and NaOH were purchased from Merck (Darmstadt, Germany). Tris(hydroxymethyl aminomethane), which was used as a carrier liquid for FlFFF experiment, was purchased from Fisher Scientific (Pittsburgh, PA). FL-70 detergent and NaN_3 for the preparation of SdFFF carrier liquid were from Fisher Scientific and Merck, respectively. Coomassie blue-G-250, 95% ethanol, and phosphoric acid (H_3PO_4) were purchased from USB Corp. (OH), Merck, and J. T. Baker (Phillipsburg, NJ), respectively.

Preparation of Hen Egg White Protein Powder. Fresh hen egg was purchased from a local market. Hen egg white protein powder was prepared using the method reported by Croguennec et al. (*6*). After separation of the egg white from the yolk, the egg white was diluted with 2 volumes of deionized water, and the mixture was adjusted to pH 6.0 with 3 M HCl. The solution was gently stirred and kept at 4 °C for 12 h, enabling ovomucin precipitation. Insoluble materials including ovomucin and impurities were removed by centrifugation at 2300 rpm for 4 min. The supernatant, adjusted to pH 7.5 with 0.1 M NaOH, was then lyophilized. The resulting hen egg white protein powder was stored at 4 °C until use.

Observation of Hen Egg White Protein Aggregation. Hen egg white protein of 10% (w/v) was prepared in deionized water at pH 7. To prepare thermally denatured hen egg white protein, the 10% (w/v) hen egg white protein solution was heated at 40 °C for 120 min and cooled to room temperature (27 °C). The hen egg white protein suspension was filtered through a 0.45 μm cellulose acetate membrane filter to remove any undesirable particles and to obtain a clear solution. To induce hen egg white protein aggregation, the thermally denatured hen egg white protein and salt solution ($ZnCl_2$) were mixed and diluted to a specified concentration in deionized water with the resulting pH of approximately 6, and the mixture was left for incubation at room temperature. To investigate parameters affecting the size distribution of hen egg white protein aggregation, the egg white protein aggregates were directly introduced into the SdFFF channel after various contact times with $ZnCl_2$ for 15−1440 min. To evaluate the degree of aggregate formation or the efficiency of protein utilization, hen egg white protein aggregate was centrifuged at 2300 rpm for 4 min to separate the nonaggregated part from the particles containing protein aggregates. The supernatant part was introduced into the FlFFF channel.

Hen Egg White Protein Determination Using Bradford Protein Assay. The Coomassie brilliant blue protein assay, known as the Bradford assay (*20*), was employed to measure the remaining nonaggregated protein content after $ZnCl_2$-induced aggregation of hen egg white protein. Bradford reagent was prepared by mixing Coomassie blue-G-250 with 95% ethanol and phosphoric acid (H_3PO_4). After separation of the nonaggregated protein from the particles containing protein aggregates by centrifugation at 2300 rpm for 4 min, the supernatant was mixed with Bradford reagent to obtain a blue solution, which was monitored for its absorbance value at 595 nm using a UV−vis spectrometer (V530, Jasco UV/vis spectrophotometer, Tokyo, Japan).

FlFFF. An FlFFF system (model PN-1021-FO, Postnova Analytik, Landsberg, Germany) equipped with a 1 kDa molecular mass cutoff and a regenerated cellulose acetate membrane (Postnova Analytik) was used. The FlFFF channel was 27.7 cm long, 2.0 cm wide, and 254 μm thick. Samples were injected into an injector valve (Rheodyne) with a fixed loop (20 μL) attached to the FlFFF channel front end. A 30 mM TRIS buffer (pH 8) was used as a carrier liquid throughout this study. A high-pressure liquid chromatography (HPLC) pump (model PN 2101, Postnova Analytik) delivered the channel flow at 1 mL/min. Another HPLC pump of the same model was employed to regulate the cross-flow rate at 2 mL/min. A relaxation time of 1.1 min was allowed for sample particles and macromolecules situated at the top wall to move to the accumulation wall. The UV detector (model S3210 UV/vis detector, Postnova Analytik) was set at 254 nm to monitor light attenuation of the flowing stream.

SdFFF. The SdFFF system used in this study was the model S-101 Particle/Colloid Fractionator purchased from Postnova Analytik. The SdFFF channel was 89.5 cm long, 2.0 cm wide, and 0.0254 cm thick, with a rotor radius of 15.1 cm. The channel volume was calculated to be 4.45 mL. The carrier solution was introduced into the SdFFF channel by an HPLC pump (model PN1122, Postnava Analytik). Light attenuation by the eluted particles was monitored by a UV detector operating at the fixed wavelength of 254 nm (model UV2075, Jasco). Samples of 50 μL were injected into a Rheodyne model 7725i loop injector. A carrier liquid was deionized water containing 0.02% (v/v) FL-70 detergent (Fisher Scientific) and 0.02% (w/v) NaN_3 (Merck) to prevent bacterial growth, with the final pH of 8.0. Fractionations of hen egg white protein aggregate samples were performed in SdFFF normal mode of retention, by which the smaller particles elute earlier than the larger ones.

Scanning Electron Microscope (SEM). A Hitachi scanning electron microscope (S-2500, Tokyo, Japan) was operated at an accelerating voltage of 15 kV to observe particle size of hen egg white aggregates. The samples were dropped onto a slide, left until dry, and coated with platinum/palladium before SEM analysis.

Data Treatment. Raw fractograms were translated into size distribution profiles using an Excel (Microsoft Excel 2002, Redmond, WA) spreadsheet. Peak evaluation, baseline adjustment, and cumulative area plotting were performed by PeakFit (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

Observation of Heat-Induced Denaturation of Hen Egg White Protein Using SdFFF. The effect of temperature on the aggregate formation of hen egg white protein was examined by heating 5% (w/v) hen egg white protein at 40, 60, and 80

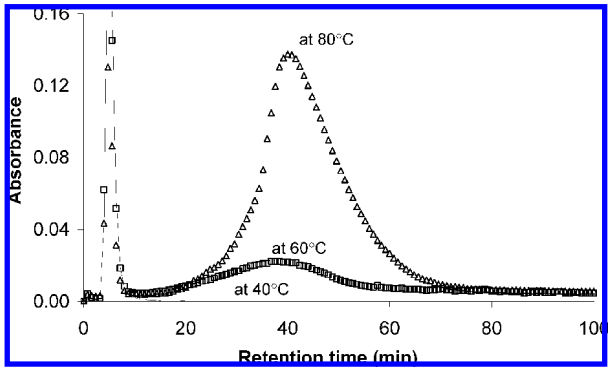

Figure 1. SdFFF fractograms of 5% (w/v) hen egg white protein at different heating temperatures, where —, □, and Δ represent 5% hen egg white protein at 40, 60, and 80 °C heating temperatures, respectively.

°C for 40 min. The resulting protein suspensions were introduced to SdFFF for size fractionation, and the raw fractograms are illustrated in **Figure 1**. At 40 °C, only one peak was observed at approximately 5 min of elution time (void fraction), suggesting the absence of protein aggregates, similar to what observed for the unheated protein as a control (the results are not shown). At higher temperature, nonetheless, two peaks were observed, one at the void fraction and the other at 38.6 and 40.7 min for the protein treated at 60 and 80 °C, respectively, indicating the occurrence of aggregates formation. These results imply that hen egg white protein was thermally denatured and began to form aggregates when heated to 60 °C and that the extent of aggregate formation increased at higher temperature as evidenced by a larger peak obtained for the protein treated at 80 °C similar to that obtained at 60 °C. These observations are in agreement with those reported by Mine et al. (*1*). As the objective of this work was to gain an insight into salt-induced protein aggregation, the temperature of 40 °C was selected to partially unfold the protein to expose its inner hydrophobic part without causing heat-induced aggregate formation.

Evidence of $ZnCl_2$-Induced Aggregation of Hen Egg White Protein. To observe if $ZnCl_2$ can induce aggregation of hen egg white protein, 5% (w/v) hen egg white protein with and without addition of 1 M $ZnCl_2$ at an incubation time of 15 min was subjected to size characterization by SEM and SdFFF, as illustrated in **Figure 2**. From the SEM photograph (**Figure 2a**), the particle diameter of hen egg white protein aggregates was determined to be approximately 0.42 μm. The fractogram of 5% (w/v) hen egg white protein (**Figure 2b**) in the absence of $ZnCl_2$ showed a single peak at approximately 5 min as a void fraction, suggesting that hen egg white protein remained in its nonaggregated state. In the presence of 1 M $ZnCl_2$, an additional peak at 34 min was observed, indicating the occurrence of protein in the aggregated form.

To obtain particle size information from the SdFFF experiment, it is necessary to know the exact density of the sample particle to get the value of the density difference between sample particle and carrier liquid (ρ), which is further used for converting a retention time (t_r) or retention volume to a diameter size information using eq 1 (*21*)

$$|\Delta\rho| = \frac{36kTt_r}{\pi G w t_0 d^3} \quad (1)$$

where k is Boltzmann's constant, T is an absolute temperature, G is a centrifugal acceleration, which has the unit of gravities ($G = \omega^2 r$, where ω is angular velocity around radius r), w is a channel thickness, and t_0 is a void time. Initially, the value of

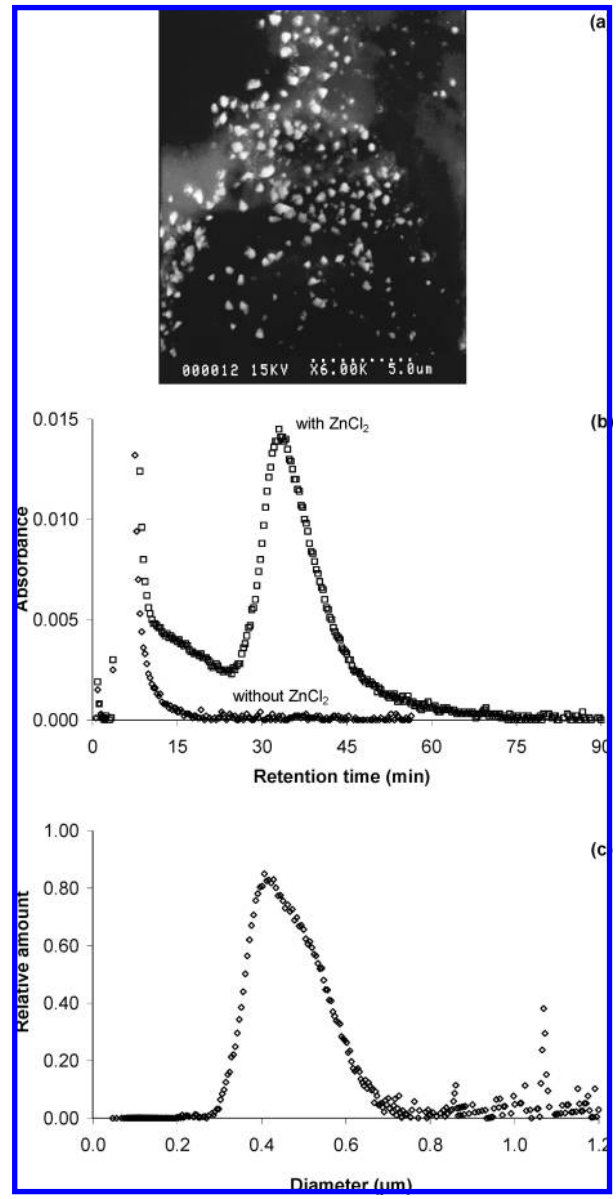

Figure 2. (**a**) SEM photograph of 5% (w/v) hen egg white protein mixed with 1 M $ZnCl_2$ at 15 min of incubation time, (**b**) SdFFF fractograms of 5% (w/v) hen egg white protein in the absence (◇) and presence (□) of 1.0 M $ZnCl_2$ at 15 min of incubation time, and (**c**) particle size distribution of 5% (w/v) hen egg white protein mixed with 1.0 M $ZnCl_2$ at 15 min of incubation time.

density difference is not known. To estimate the density of the aggregate particle, the particle diameter value of 0.42 μm obtained from the SEM experiment was therefore used for calculation of the density difference between the hen egg white protein aggregates and the carrier liquid. This calculation yielded the number of 0.12 g cm^{-3}, and this value was used throughout this study for the estimation of particle diameter obtained from SdFFF fractogram. With this density information, the raw fractogram in **Figure 2b** was translated into a particle size distribution profile of hen egg white protein aggregates as shown in **Figure 2c**. It should be noted, however, that the particle size information of the aggregates obtained from SdFFF would only be accurate when the density value is correct. Therefore, the particle size information obtained from our experiment is only an approximate value as SEM is not an ideal method for size measurement of hydrated protein aggregates because of the possible occurrence of dehydration and changes in the particle

size. Nonetheless, as the objective of this study was to observe relative changes in the particle size at various experimental conditions, the use of approximate values of particle size is forgivable.

Parameters Affecting $ZnCl_2$-Induced Aggregation of Hen Egg White Protein. The aggregation process and the subsequent textural properties of the final gel product depend on several factors including protein concentration and pH (*22*). Manipulation of the above factors can alter hen egg protein functionality and affect its rheological behavior (*23*). As the objective of this study was to examine salt-induced aggregation, the pH value of the mixture was kept constant at around 6. In this study, parameters affecting the size distribution of hen egg white protein aggregates, including hen egg white protein and $ZnCl_2$ concentrations as well as incubation time, were investigated using SdFFF.

At a fixed incubation time of 15 min and 1 M $ZnCl_2$, the effect of protein concentration on the salt-induced aggregate formation was examined. Particle size distributions of hen egg white protein aggregates at various hen egg white protein concentrations are presented in **Figure 3a**. Larger aggregates were observed as the particle diameter at peak (d_p value) increased from 0.42 to 0.80 μm when hen egg white protein concentrations increased from 5 to 7.5% (w/v).

Similarly, the concentration of $ZnCl_2$ plays a significant role in the extent of aggregate formation, as illustrated in **Figure 3b**. At a fixed incubation time of 15 min and a hen egg white protein concentration of 5% (w/v), the effect of zinc concentration on the aggregate formation was examined. At higher $ZnCl_2$ concentration, the particle size of hen egg white protein aggregate was larger by which the d_p value increased from 0.37 to 0.68 μm in the presence of 0.5 and 1.5 M $ZnCl_2$, respectively. This might be due to the increase in the amount of Zn^{2+} as a positive charge to shield hen egg white protein negative charges (*24, 25*), leading to larger particle size of hen egg white protein aggregate. This finding corresponded with the Derjaguin−Landau−Verwey−Overbeek (DLVO) theory (*26*), which states that the addition of salt suppresses the repulsive potential among the negatively charged particles, causing particles to aggregate. At higher Zn^{2+} concentrations, the energy barrier drops faster, resulting in faster aggregate formation as evidenced by the increase in the d_p value.

To examine the effect of incubation time on the extent of aggregate formation, 5% (w/v) hen egg white protein was mixed with 1.0 M $ZnCl_2$, and the size distributions of aggregates were observed from 15 to 1440 min contact time as shown in **Figure 3c**. With the enhancement of time for the association between hen egg white protein and Zn^{2+}, the particle diameter of hen egg white protein aggregates was found to be larger from 0.42 (at 15 min) to 0.84 μm (at 1440 min).

The particle size information including d_p and d_{mean} values as the particle diameter at peak maximum and the mean particle diameter values obtained from various experimental conditions are summarized in **Table 1**. As the two values are almost equal, the particle size distributions of hen egg white protein aggregates are shown to be close to the normal distribution pattern.

Efficiency of Hen Egg White Protein Utilization for Aggregation−New Information from FlFFF. Aggregation of protein requires an optimum range of protein level, pH, and heating conditions as reported by many other investigators (*5, 12, 23*). As illustrated earlier by SdFFF, the concentrations of hen egg white protein as well as $ZnCl_2$ showed significant impact on the growth of hen egg white protein aggregates. At higher $ZnCl_2$ and protein concentrations, the aggregates grew larger. Nonetheless, the question remains as to how much protein undergoes aggregation or how efficient the aggregation process is in term of protein utilization. The higher efficiency of protein utilization suggests that a larger proportion of protein can form aggregates with only a smaller amount of the remaining nonaggregated protein. In this study, FlFFF was proposed as a novel method to estimate the remaining protein content, which was still in the nonaggregated form by considering the peak area of the FlFFF fractogram. With FlFFF, a raw fractogram of 1% (w/v) hen egg white protein without addition of $ZnCl_2$ showed a peak maximum at 4.1 min as illustrated in **Figure 4**. This corresponds to the molecular mass of approximately 40 kDa or a particle diameter of 5 nm. Upon addition of $ZnCl_2$, large aggregates were formed. To observe the remaining nonaggregated protein, the aggregates suspension was therefore centrifugally separated at 2300 rpm before introduction of the supernatant part into

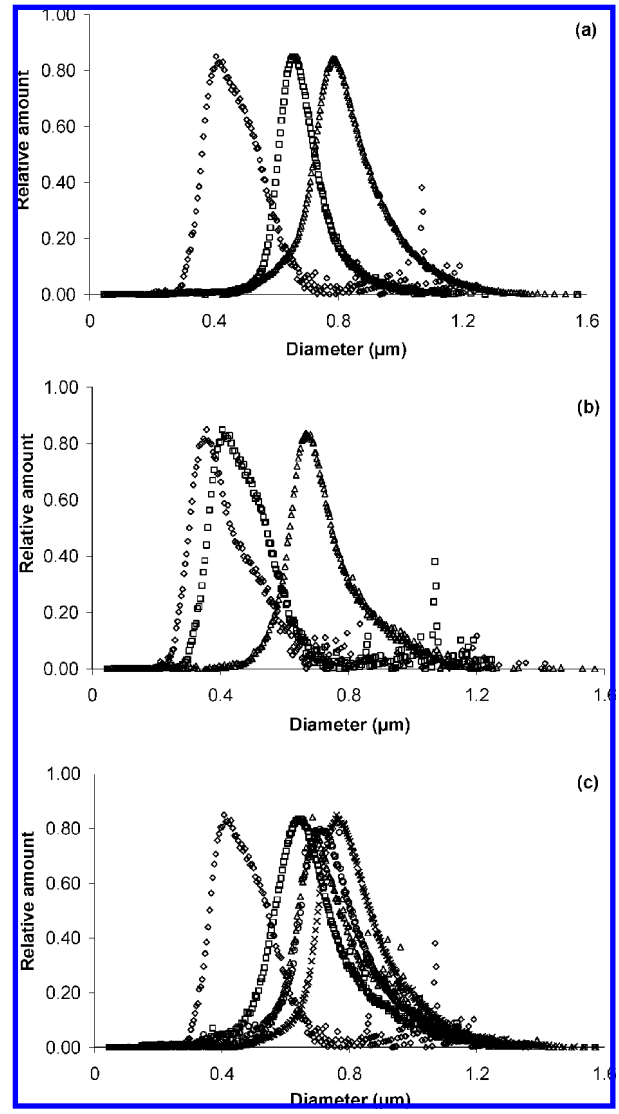

Figure 3. Particle size distributions of hen egg white protein aggregates obtained from SdFFF: (**a**) effect of hen egg white protein concentration (w/v) at 1.0 M $ZnCl_2$ and 15 min of incubation time, where ◇, □, and Δ represent 5, 6.25, and 7.5% (w/v) hen egg white protein, respectively; (b) effect of $ZnCl_2$ content (M) at 5% (w/v) hen egg white protein and 15 min of contact time, where ◇, □, and Δ represent 0.5, 1.0, and 1.5 M $ZnCl_2$, respectively; and (**c**) effect of incubation time for 5% (w/v) hen egg white protein mixed with 1.0 M $ZnCl_2$, where ◇, □, Δ, ○, and × represent 15, 180, 360, 540, and 1440 min of incubation time, respectively.

Table 1. Particle Size Information of Hen Egg White Protein Aggregates Obtained from Various Experimental Conditions

effect of heating temperature [at 5% (w/v) hen egg white protein]		
heating temperature (°C)	d_p (μm)	d_{mean}[a] (μm)
40	0.00	0.00
60	0.58	0.56
80	0.58	0.59

effect of hen egg white protein content (at 1 M $ZnCl_2$ and 15 min of incubation time)		
hen egg white protein content (%, w/v)	d_p (μm)	d_{mean}[a] (μm)
5	0.42	0.46
6.25	0.67	0.67
7.5	0.80	0.81

effect of $ZnCl_2$ concentration [at 5% (w/v) hen egg white protein and 15 min of incubation time]		
$ZnCl_2$ concentration (M)	d_p (μm)	d_{mean}[a] (μm)
0.5	0.37	0.39
1	0.42	0.46
1.5	0.68	0.70

effect of incubation time [at 5% (w/v) hen egg white protein and 1 M $ZnCl_2$]		
incubation time (min)	d_p (μm)	d_{mean}[a] (μm)
15	0.42	0.46
180	0.66	0.67
360	0.70	0.74
540	0.73	0.74
1440	0.84	0.80

[a] d_{mean} is defined as the particle size at which 50% of the total accumulative area is detected.

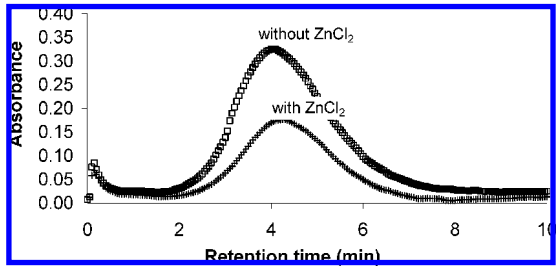

Figure 4. FlFFF fractograms of 1% (w/v) hen egg white protein in the absence (□) and presence (+) of 1.5 M $ZnCl_2$.

the FlFFF channel. In the presence of 1.5 M $ZnCl_2$, the peak magnitude of hen egg white protein at 4.1 min (the nonaggregated protein) decreased to about half of the original. This suggests the possibility of using FlFFF to measure the efficiency of protein utilization or the degree of aggregation using eq 2.

$$\text{efficiency of protein utilization (\%)} = \frac{(A-B)}{A} \times 100 \quad (2)$$

where A is the peak area at 4.1 min from the fractogram of hen egg white protein without the addition of $ZnCl_2$, and B is that with the addition of $ZnCl_2$. One may argue that the information on efficiency of protein utilization can be easily obtained from the Bradford assay. Nonetheless, FlFFF provides additional

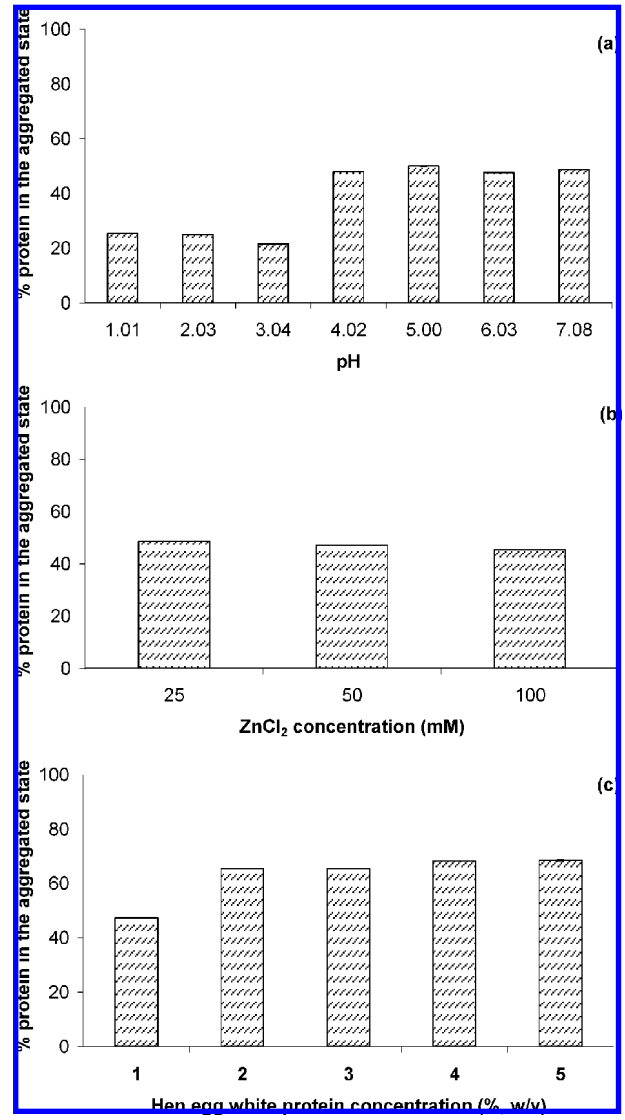

Figure 5. Parameters affecting efficiency of hen egg white protein usage for aggregation: (**a**) effect of pH for 1% (w/v) hen egg white protein mixed with 50 mM $ZnCl_2$, (**b**) effect of $ZnCl_2$ concentration (mM) for 1% (w/v) hen egg white protein at pH 6.0, and (**c**) effect of hen egg white protein content (%, w/v) at 50 mM $ZnCl_2$ and pH 6.0.

benefit to observe whether shifts in molecular weight distribution occur. In our experiment, a shift in molecular weight distribution was not observed, suggesting that all types of proteins in the mixture of proteins in egg white underwent aggregation to the same extent. A shift in molecular weight distribution could imply that only some types or certain types of proteins underwent aggregation. With FlFFF, additional information could be gained.

In this work, the effect of pH values as well as the concentrations of $ZnCl_2$ and hen egg white protein on the efficiency of protein utilization were examined using FlFFF and the Bradford assay. With FlFFF, the effect of the pH values (from 1.01 to 7.08) on the degree of aggregate formation of 1% (w/v) hen egg white protein mixed with 50 mM $ZnCl_2$ is illustrated in **Figure 5a**. At a pH of 1.01−3.04, percentages of protein in the aggregated form were lower than those at pH of 4.02−7.08, which corresponded with the observation by Bradford assay showing that percentages of protein in the aggregated form were found to range around 52−56 at a pH higher than 4. This can be explained by considering the pI values of various proteins in egg white, which range around 4.1−6.1, except that

it was 10.7 for lysozyme (~3.4% in egg white protein mixture) (*1*). At a pH higher than pI values, hen egg white protein exhibits a negative charge, which could be neutralized with Zn^{2+}, leading to aggregation as a result of reduction in electrostatic repulsion between protein molecules. Furthermore, three different concentrations of $ZnCl_2$ (25, 50, and 100 mM) were investigated for their effect on the efficiency of protein utilization of 1% (w/v) hen egg white protein at pH value around 6 using FlFFF, as demonstrated in **Figure 5b**. The degree of aggregate formation was found to be approximately half of the original protein content for all concentrations of $ZnCl_2$ studied, suggesting that $ZnCl_2$ of only 25 mM was already adequate for charge neutralization. The Bradford assay provided the same trend that the efficiency of protein utilization of 1% (w/v) hen egg white protein at pH value around 6 was experimentally observed to range around 45−49%. Furthermore, the effect of hen egg white protein concentration on the degree of aggregate formation was also evaluated by FlFFF as shown in **Figure 5c**. Increasing hen egg white protein content from 1 to 2% (w/v) in the presence of 50 mM $ZnCl_2$ resulted in a higher degree of aggregate formation from 47 to 65%, while the degree of aggregate formation remained almost constant when increasing hen egg white protein content from 2 to 5% (w/v), implying that to maximize the aggregate formation, the hen egg white protein concentration of at least 2% is needed. The percentages of protein content in the nonaggregated and aggregated forms obtained by FlFFF experiment were in good agreement with those determined by the Bradford assay, which showed that the efficiency of protein utilization ranged between 48 and 64%.

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