รายงานการวิจัยฉบับสมบูรณ์

โครงการ การวิเคราะห์ขนาดอนุภาคของสารที่มีฤทธิ์ทางชีวภาพในอนุภาคเภสัชภัณฑ์ขนาดนาโนเมตรโดยใช้เทคนิคการแยกแบบไหลภายใต้สนาม

โดย ผศ.ดร. อทิตยา ศิริภิญญานนท์ และคณะ

สิงหาคม 2551

สัญญาเลขที่ TRG4980002

รายงานการวิจัยฉบับสมบูรณ์

การวิเคราะห์ขนาดอนุภาคของสารที่มีฤทธิ์ทางชีวภาพในอนุภาคเภสัชภัณฑ์ขนาดนาโนเมตร โดยใช้เทคนิคการแยกแบบไหลภายใต้สนาม

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

ขอขอบคุณสำนักงานกองทุนสนับสนุนการวิจัยที่ได้สนับสนุนทุนการวิจัย “ทุนส่งเสริมนักวิจัยรุ่นใหม่” เพื่อใช้ในการดำเนินงานวิจัยในโครงการ “การวิเคราะห์ขนาดอนุภาคของสารที่มีฤทธิ์ทางชีวภาพในอนุภาคเภสัชภัณฑ์ขนาดนาโนเมตรโดยใช้เทคนิคการแยกแบบไหลภายใต้สนาม” โดยมีระยะเวลาของโครงการ 2 ปี ตั้งแต่ สิงหาคม 2549 ถึง สิงหาคม 2551 และขอขอบคุณนักวิจัยที่ปรึกษา “ศาสตราจารย์ ดร. ยุวดี เชี่ยววัฒนา” สำหรับคำแนะนำและข้อคิดเห็นที่เป็นประโยชน์ต่องานวิจัย รวมถึงขอขอบคุณศูนย์ความเป็นเลิศด้านนวัตกรรมทางเคมี โครงการพัฒนาบัณฑิตศึกษาและการวิจัยทางเคมี สำหรับทุนการศึกษาของนักศึกษา วัสดุวิจัยและครุภัณฑ์บางส่วนในโครงการวิจัย และภาควิชาเคมี คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล สำหรับสถานที่ เครื่องมือและอุปกรณ์ต่างๆ

คณะผู้วิจัย

บทคัดย่อ

รหัสโครงการ: **TRG4980002**

ชื่อโครงการ: **การวิเคราะห์ขนาดอนุภาคของสารที่มีฤทธิ์ทางชีวภาพในอนุภาคเภสัชภัณฑ์ขนาดนาโนเมตรโดยใช้เทคนิคการแยกแบบไหลภายใต้สนาม**

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การวิจัยนี้ได้ประยุกต์ใช้เทคนิคการแยกแบบไหลภายใต้สนามประเภทอาศัยแรงหนีศูนย์กลางและแรงของการไหล เพื่อใช้ในการวิเคราะห์ขนาดอนุภาคและติดตามการเปลี่ยนแปลงของขนาดและการกระจายตัวของขนาดอนุภาคโปรตีนในอาหารเมื่อมีการเติมเกลือลงในโปรตีน ซึ่งเกลือทำให้โปรตีนเกิดการจับรวมตัวกัน ได้ทำการศึกษาโปรตีนสองชนิด คือ โปรตีนแลคโทโกลบูลินและโปรตีนไข่ขาว โดยได้ศึกษาถึงปัจจัยต่างๆ ที่ส่งผลต่อขนาดอนุภาคของโปรตีนที่เกิดการจับรวมตัวกัน ได้แก่ ประเภทของเกลือ ความเข้มข้นของเกลือและโปรตีน ระยะเวลาในการทำปฏิกิริยา พบว่าทุกปัจจัยมีอิทธิพลต่อขนาดอนุภาคของโปรตีนที่เกิดการจับรวมตัวกัน ซึ่งขนาดอนุภาคใหญ่ขึ้นเมื่อความเข้มข้นของเกลือและโปรตีนสูงขึ้น และระยะเวลาในการทำปฏิกิริยานานขึ้น นอกจากนี้ได้ศึกษาถึงความสามารถของอนุภาคของโปรตีนแลคโทโกลบูลินที่เกิดการจับรวมตัวกันในการเป็นอนุภาคกักเก็บสารเสริมสุขภาพ คือ ไวตามินอี พบว่าความเข้มข้นของโปรตีนแลคโทโกลบูลินและไวตามินอี มีผลต่อประสิทธิภาพในการกักเก็บ

คำหลัก: การวิเคราะห์ขนาดอนุภาค เทคนิคการแยกแบบไหลภายใต้สนาม แลคโทโกลบูลิน โปรตีนไข่ขาว ไวตามินอี การกักเก็บ

ABSTRACT

Project Code: **TRG4980002**

Project Title: **Size Characterization of Bioactive Ingredients in Pharmaceutical Nanoparticles Using Field-Flow Fractionation**

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Project Period: August 30, 2006 – August 29, 2008

Field-flow fractionation (FFF) techniques including sedimentation field-flow fractionation (SdFFF) and flow field-flow fractionation (FlFFF) were applied to investigate food protein aggregation induced by addition of salt. Two types of food proteins, i.e., β-lactoglobulin and hen egg white protein, were examined. Parameters affecting the extent of aggregate formation were investigated. These include the types of salts, concentrations of salt and protein, as well as contact time. All factors exhibited a combined effect on the size of aggregates, whereby larger aggregates were obtained at increased concentrations of salt and protein. Upon prolonged contact time, larger aggregates were formed. Further, with the finding that salt could induce aggregation of β-lactoglobulin, the possibility of using β-lactoglobulin aggregates to encapsulate α-tocopherol, a sensitive nutraceutical compound, was examined. Concentrations of α-TOC and β-lactoglobulin were found to have an influence on encapsulation efficiency.

Keywords: size characterization, field-flow fractionation, β-lactoglobulin, hen egg white protein**,** α-tocopherol, encapsulation

Executive Summary

Project Title: Size Characterization of Bioactive Ingredients in Pharmaceutical Nanoparticles Using Field-Flow Fractionation

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Project Period: August 30, 2006 – August 29, 2008

Understanding the aggregation of food proteins has been an issue of interest for food scientists, owing to its important role to control the functional properties of food products. In order to get accurate information on particle size and size distribution of food protein aggregates, a reliable and gentle size separation technique is needed. Field-flow fractionation (FFF) was used as an alternative technique for size characterization of food protein aggregates. Furthermore, encapsulation technologies have gained increased interest to the food industry as they are used to increase stability of the bioactive compounds during processing and storage and also to prevent undesirable interactions with the food matrix. A real benefit of encapsulation is due to the ability to control the release behavior of the incorporated ingredients and deliver them to the specific target at a suitable time. Therefore, the use of food protein aggregates to encapsulate a sensitive nutraceutical compound was investigated.

Two papers have been published in the international journals as follows:

1. Saeseaw, S., Shiowatana, J. & Siripinyanond, A. 2006, "Observation of Salt-induced β-Lactoglobulin Aggregation Using Sedimentation Field-Flow Fractionation", *Anal. Bioanal. Chem.,* vol. 386, pp. 1681–1688. (impact factor = 2.591)
2. Samontha, A., Nipattamanon, C., Shiowatana, J. & Siripinyanond, A. 2008, "Toward Better Understanding of Salt-Induced Hen Egg White Protein Aggregation Using Field-Flow Fractionation", *J. Agric. Food Chem.,* vol. 56, pp. 8809–8814. (impact factor = 2.322)

Four papers have been presented at the international conferences during the past two years as follows:

1. Sudarat Saeseaw, Juwadee Shiowatana and Atitaya Siripinyanond, Understanding Aggregation of β-Lactoglobulin by Sedimentation Field-Flow Fractionation, **6th International Colloquium on Process Related Analytical Chemistry**, 21-23 March 2007, Dortmund, Germany.
2. Atitaya Siripinyanond, Sumattana Worapanyanond, Sudarat Saeseaw, Pranee Phukphatthanachai and Juwadee Shiowatana, Field-Flow Fractionation: An Alternative Tool for Size Characterization of Nanometer and Micrometer Particles, **Pure and Applied Chemistry International Conference (PACCON 2008)**, 30 January - 1 February 2008, Bangkok, Thailand.
3. Atitaya Samontha, Chiraya Nipattamanon, Juwadee Shiowatana and Atitaya Siripinyanond, Investigation of Egg White Protein Aggregation Using Field-Flow Fractionation, **Pure and Applied Chemistry International Conference (PACCON 2008)**, 30 January - 1 February 2008, Bangkok, Thailand.
4. Atitaya Siripinyanond, Sudarat Saeseaw, Atitaya Samontha and Juwadee Shiowatana, Field-Flow Fractionation: Size Characterization of Food Protein, **Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy (PITTCON 2008),** 2-6 March 2007, New Orleans, Louisiana, U.S.A..

Chapter 1

Size Characterization of Salt-Induced β-lactoglobulin Aggregates Using Field-Flow Fractionation

1.1 Introduction

1.2 Experimental

1.2.1 Instrumentation

1.2.2 Chemicals

1.2.3 Salt-induced β-lactoglobulin aggregates

1.2.4 Data transformation

1.3 Results and discussion

1.3.1 SdFFF and evidence for β-lactoglobulin aggregation

1.3.2 Factors affecting zinc-induced β-lactoglobulin aggregation

1.3.3 Comparison between Ca^{2+} and Zn^{2+} in terms of extent of aggregation induced

1.4 Summary

1.5 References

1.1 Introduction

The aggregation of food proteins is a topic that has generated much research in the field of food science, due to its important influence over the functional properties of food products [*1*]. Whey proteins are important functional ingredients that are used as emulsifiers, texture modifiers, thickeners, and gelling agents [*2*]. The functionality of whey proteins is primarily based on their ability to undergo aggregation and eventually gelation [*2*]. The major protein in whey is β-lactoglobulin, which accounts for approximately 50% of the total whey protein [*3*]. Therefore, β-lactoglobulin is often used as a model protein to study aggregation behavior [*2*, *4*]. β-lactoglobulin is a globular protein that has a molecular mass of 18.3 kDa, a radius of approximately 2 nm, one thiol group and two disulfide bonds [*3*, *5*, *6*]. Upon heat treatment above the denaturation temperature (~78 °C), the conformational structure of β-lactoglobulin changes, causing the nonpolar and the thiol groups to become exposed [*3*]. Consequently, aggregate formation occurs through intermolecular associations involving disulfide bridging, electrostatic interactions, hydrogen bonding, and hydrophobic interactions [*7–10*]. The conformation of the protein and its physicochemical properties depend on environmental factors (e.g., pH and ionic strength) [*11*], protein concentrations [*12*], as well as time and temperature [*13*, *14*].

Zinc is an important essential trace element for human nutrition, and deficiency of zinc is a worldwide nutritional issue. To overcome this problem, zinc sulfate is often prepared as a zinc supplement, owing to its adequate bioavailability [*15*]. Nonetheless, zinc sulfate interacts with the food matrix, modifying the sensorial characteristics of the food, and sometimes making the flavor unacceptable [*15*]. In order to incorporate minerals into food matrix, iron sulfate has been added to β-lactoglobulin; this is reported to have resulted in a network of β-lactoglobulin aggregates with the iron encapsulated inside [*16*]. As a consequence, the iron could be protected and transported for further absorption by the human gastrointestinal tract [*16*]. Therefore, we decided to investigate the effect of zinc ion on structural changes of β-lactoglobulin, for two reasons. The main reason was to examine whether the particle size of β-lactoglobulin aggregates, and hence food sensorial characteristics, could be controlled by the addition of zinc ion. A second reason was that we wished to investigate whether a new source of zinc supplement could be prepared by encapsulating zinc in a network β-lactoglobulin aggregates. However, in this work, only the first objective is addressed.

The molecular mass of β-lactoglobulin has been extensively characterized using ultracentrifugation [*17*], gel electrophoresis [*18*, *19*], and size exclusion chromatography (SEC) [*20*, *21*]. Among the size separation techniques used, SEC is the most common approach. However, the applicable range of the SEC columns available is limited to masses of below a few million, and therefore this technique is not suited to the separation of very large protein aggregates [*22–26*]. In addition, potential interactions of protein

aggregates with the stationary phase restrict the use of SEC. To avoid these problems, field-flow fractionation (FFF) has been used as an alternative technique for separating very large protein aggregates. The characterization of protein aggregates by FFF—either flow FFF (FlFFF) [*22–24*] or sedimentation FFF (SdFFF) [*25*, *26*]—has been reported by many investigators. Only recently, the application of ceramic hollow fiber FlFFF in order to characterize the particle sizes of β-lactoglobulin aggregates was demonstrated by Zhu et al. [*27*]. In their study, heat (65 °C) was applied to induce the aggregation of β-lactoglobulin, with particle sizes reportedly ranging from 30 to 200 nm. Although FlFFF appears to be a valuable technique for the size separation of protein aggregates, interactions between the samples and the membrane generally used in the FlFFF channel may occur at high ionic strengths.

Since it did not require a membrane inside the channel, and therefore potential interactions between β-lactoglobulin aggregates and the channel wall are minimized, SdFFF was therefore chosen to characterize the particle sizes of β-lactoglobulin aggregates. The aims of this investigation were to examine the salt-induced aggregation of β-lactoglobulin and to characterize the particle size distributions of β-lactoglobulin aggregates induced by Ca^{2+} and Zn^{2+}. The effects of β-lactoglobulin and salt concentrations, as well as the contact time, were examined.

1.2 Experimental

1.2.1 Instrumentation

An SdFFF system (Model S-101 Particle/Colloid Fractionator, Postnova Analytik, Landsberg, Germany) was used to characterize the particle size distributions of β-lactoglobulin aggregates. The SdFFF channel wall was made of a polished Hastelloy C alloy, which is principally Ni (56%) with 15% Cr, 17% Mo, 5% Fe, 4% W, and traces of Mn and Si. The SdFFF channel was 89.5 cm long (tip to tip), 2.0 cm wide, and 0.0254 cm thick, with a rotor radius of 15.1 cm. The channel volume was calculated to be 4.5 mL. The carrier solution was introduced into the SdFFF channel by an HPLC pump (Model PN1122, Postnova Analytik). The elution of particles was monitored by a UV absorption detector operating at the fixed wavelength of 280 nm (UV-2000 Spectra System, Thermo Electron Corporation, Waltham, MA, USA). Samples were introduced into a Rheodyne (Rohnert Park, CA, USA) injector at a fixed loop of 50 μL. The operating conditions of SdFFF are summarized in Table 1.1.

Table 1.1 SdFFF operating conditions

Conditions	Measurements
SdFFF channel dimensions (cm^3)	89.5 × 2.0 × 0.0254
SdFFF rotor radius (cm)	15.1
Carrier liquid	0.02% (v/v) FL-70 containing 0.02% (w/v) sodium azide
Channel flow rate (mL min^{-1})	1.0
Equilibration time (min)	15
Power field programming	Initial field strength, 1500 rpm for 5 min; field decay parameter, −40; final field strength, 150 rpm

1.2.2 Chemicals

One hundred millimoles of $CaCl_2$ and 100 mM $ZnSO_4$, both of analytical reagent grade from Merck (Darmstadt, Germany), were prepared in doubly distilled deionized water and used as stock solutions for further dilution. The carrier liquid was 0.02% (v/v) FL-70 detergent (Fisher Scientific, Pittsburgh, PA, USA) containing 0.02% (w/v) NaN_3 (Merck).

Four percent 4% (w/v) β-lactoglobulin (Sigma, St. Louis, MO, USA) solution was prepared in deionized water at pH 7. To prepare denatured β-lactoglobulin, the 4% (w/v) β-lactoglobulin solution was heated at 80 °C for 30 min and cooled to room temperature (27 °C) [*28*].

1.2.3 Salt-induced β-lactoglobulin aggregates

To induce β-lactoglobulin aggregation, the thermally denatured β-lactoglobulin and salt solutions (i.e., $CaCl_2$ or $ZnSO_4$) were mixed and diluted to a specified concentration in deionized water. After various contact times of between 0 to 2880 min, the β-lactoglobulin aggregates were introduced into the SdFFF channel.

1.2.4 Data transformation

Raw fractograms were translated into size distribution profiles using Microsoft Excel spreadsheet software. Peak evaluation, baseline adjustment, and cumulative area distribution plotting were performed using PeakFit (SPSS, Chicago, IL, USA).

1.3 Results and discussion

1.3.1 SdFFF and evidence for β-lactoglobulin aggregation

Size characterization by SdFFF is quite straightforward provided that the density of the sample particle is known. If the exact geometry of the SdFFF channel, the field, flow rate, and the density difference between the particle and carrier liquid are all known, the diameter of the separated particle can be calculated directly from the experimental retention time [*29*]. Once the size distribution profile has been plotted, the particle size at peak maximum (d_p), and the mean particle size (d_{mean}) can be measured. The particle size at peak maximum (d_p) is then used to identify the dominant particle size of the investigated β-lactoglobulin aggregates. To obtain the mean particle size (d_{mean}) of the β-lactoglobulin aggregates, the diameter distribution profiles are converted into cumulative area plots. The d_{mean} is defined as the particle size at which 50% of the total accumulative area is detected.

In this study, particle size information obtained from SdFFF is used to obtain evidence of β-lactoglobulin aggregation. The aggregation of 1% (w/v) β-lactoglobulin induced by 20 mM Ca^{2+} was investigated by comparing the fractograms of calcium-induced β-lactoglobulin aggregates at 60 and 1440 min contact times with that of the 1% (w/v) β-lactoglobulin without the addition of Ca^{2+} (Fig. 1.1a). The original 1% (w/v) β-lactoglobulin displayed a peak maximum at approximately 5.3 min, which coeluted with the void fraction (4.5 min), suggesting that the β-lactoglobulin was not retained or was only slightly retained in the SdFFF channel. With the addition of 20 mM Ca^{2+}, however, two peak maxima were observed. The first peak was detected at the void fraction and the second peak was observed at 22 and 32 min after contact times of 60 and 1440 min, respectively, with the corresponding size distributions shown in Fig. 1.1b. Although β-lactoglobulin aggregates have a fractal structure where the density decreases with the size of the aggregates, in this experiment the density of the β-lactoglobulin aggregates was assumed to be 1.05 g cm^{-3}. Usually, for a fixed particle size, the retention time decreases with particle density. Therefore, the increase in the retention time actually indicates that the aggregates grew in size. The particle sizes (d_p) increased from <0.01 to 0.26 and 0.38 μm after contact times of 60 and 1440 min, respectively, with broader size distributions. The cumulative area distribution plots (Fig. 1.1c) indicate that the d_{mean} of β-lactoglobulin aggregates shifted from 0.015 to 0.26 and 0.38 μm after being in contact with 20 mM Ca^{2+}

for 60 and 1440 min, respectively. The increase in particle size at longer contact times confirms that aggregation of β-lactoglobulin took place. The delayed elution was not caused by interactions between the SdFFF channel wall and the β-lactoglobulin particles. This investigation shows that SdFFF is a suitable tool for obtaining evidence of β-lactoglobulin aggregation. Moreover, information could be obtained on the particle size distributions of the aggregates.

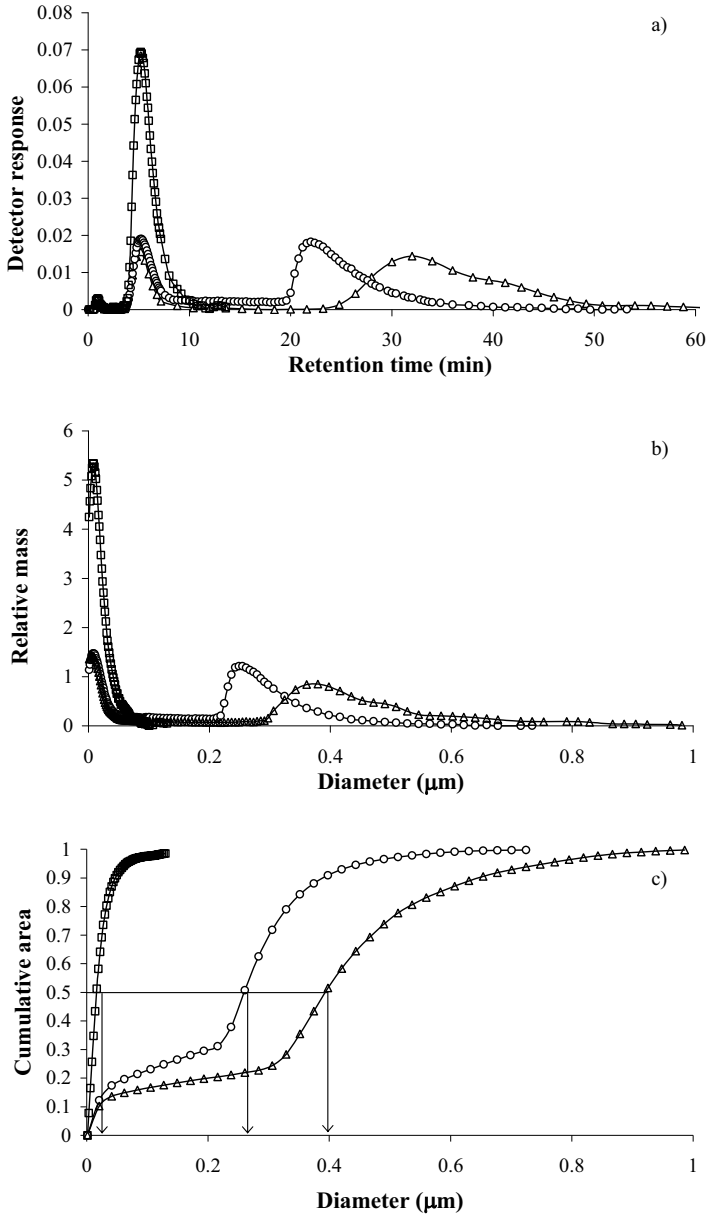

Figure 1.1a Raw fractograms of 1% (w/v) β-lactoglobulin (*squares*) and 1% (w/v) β-lactoglobulin in 20 mM Ca^{2+} at contact times of 60 (*circles*) and 1440 (*triangles*) min; **b** the corresponding size distributions; **c** the corresponding cumulative area plots. The *arrow lines* indicate the mean diameters (d_{mean})

1.3.2 Factors affecting zinc-induced β-lactoglobulin aggregation

Since the factors that affect calcium-induced β-lactoglobulin have been reported by many other investigators [*12*, *28*, *30*, *31*], only factors affecting zinc-induced aggregation were examined in this study. The effects of the concentrations of Zn^{2+} and β-lactoglobulin, as well as the contact times, on the extent of aggregate formation were investigated. At a fixed contact time of 15 min, three different concentrations of Zn^{2+} (5, 10, and 15 mM) were examined for their effect on the aggregate formation of 2% (w/v) β-lactoglobulin. Particle size distributions of β-lactoglobulin aggregates at various Zn^{2+} concentrations are illustrated in Fig. 1.2a. At higher Zn^{2+} concentrations, larger aggregates were formed, as the d p value increased from 0.11 to 0.16 μm when the Zn^{2+} concentration increased from 5 to 15 mM Zn^{2+}. Adding Zn^{2+} resulted in the negatively charged surface of β-lactoglobulin becoming shielded, leading to intermolecular associations or aggregation. Furthermore, increasing the concentration of Zn^{2+} resulted in an increase in the number of bridges binding the denatured β-lactoglobulin, leading to larger β-lactoglobulin aggregates [*30*]. According to the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory [*30*], which describes the force attributed to van der Waals attraction and the repulsion due to counterions between surfaces interacting through a liquid medium, the addition of salt suppresses the repulsive potential, the energy barrier between the negatively charged particles, causing particles to aggregate. At higher Zn^{2+} concentrations, the energy barrier drops faster, resulting in faster aggregate formation, and therefore larger aggregates are formed.

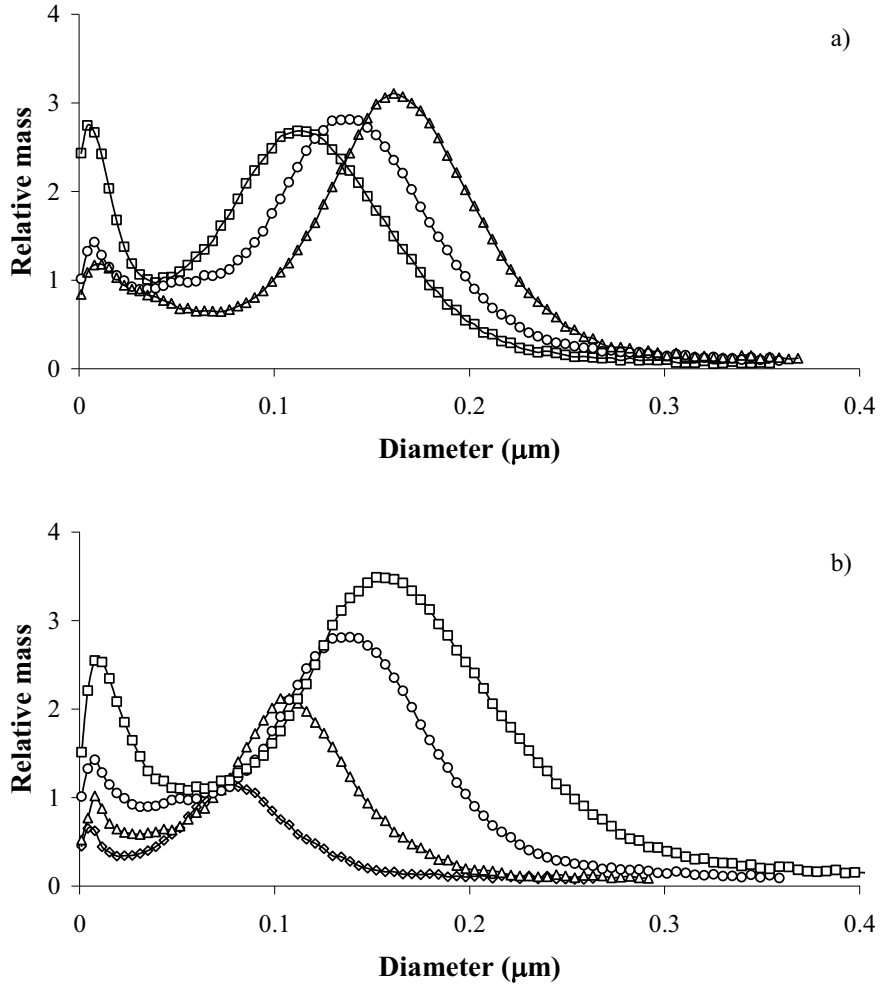

Figure 1.2 a Raw fractograms of 1% (w/v) β-lactoglobulin (*squares*) and 1% (w/v) β-lactoglobulin in 20 mM Ca^{2+} at contact times of 60 (*circles*) and 1440 (*triangles*) min; **b** the corresponding size distributions; **c** the corresponding cumulative area plots. The *arrow lines* indicate the mean diameters (d_{mean})

Similarly, the concentration of β-lactoglobulin plays a significant role in the extent of aggregate formation, as illustrated in Fig. 1.2b. At higher β-lactoglobulin concentrations, larger aggregates were formed, as the d_p value increased from 0.08 to 0.16 μm when the β-lactoglobulin concentration increased from 0.5% to 3.0% (w/v). Naturally, both intermolecular hydrophobic interactions and disulfide bond formation increased as the β-lactoglobulin concentration was increased [*31*].

In order to investigate the aggregation dynamics of β-lactoglobulin, various contact times of 15, 120, 360, 1440, and 2880 min were examined. Figure 1.3 demonstrates the

particle size distributions and cumulative area distributions of 2% (w/v) β-lactoglobulin in 10 mM Zn^{2+} at various contact times. The d_p value increased from 0.19 (at 15 min) to 0.38 μm (at 2880 min). At 120 min contact time, the particle size distribution of the β-lactoglobulin aggregates started to show a deviation from monomodal characteristics, implying that more than one group of β-lactoglobulin aggregates existed.

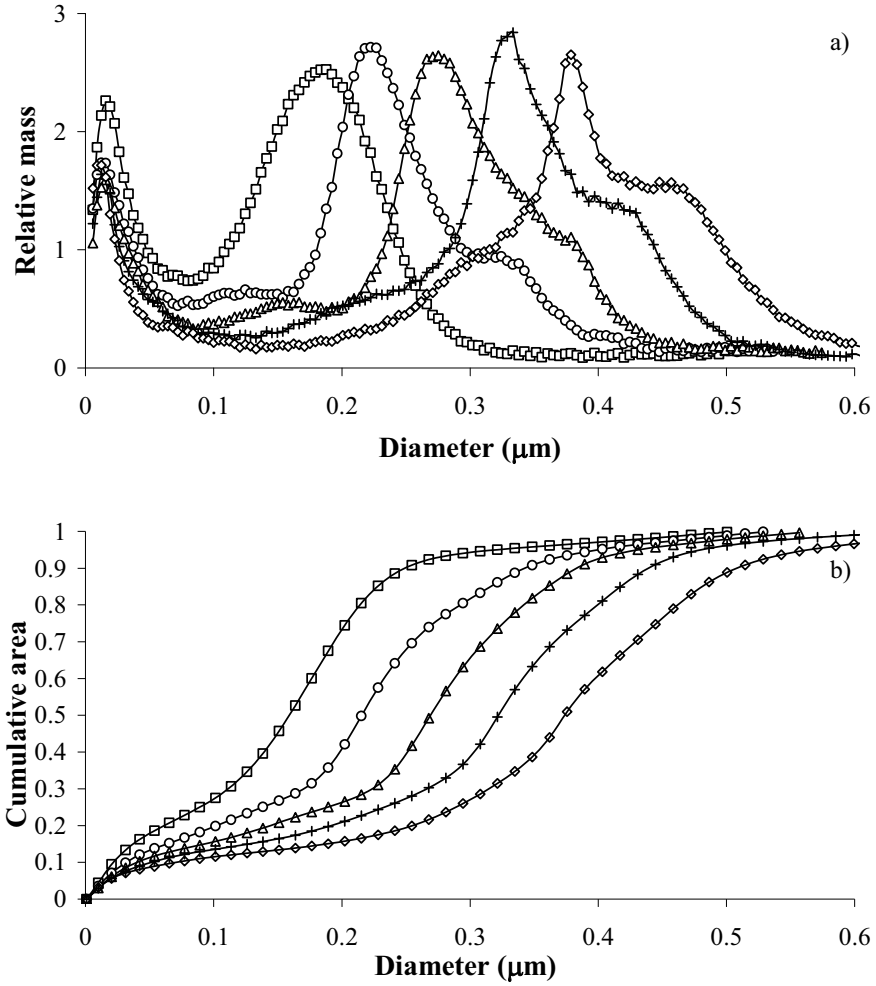

Figure 1.3a Raw fractograms of 1% (w/v) β-lactoglobulin (*squares*) and 1% (w/v) β-lactoglobulin in 20 mM Ca^{2+} at contact times of 60 (*circles*) and 1440 (*triangles*) min; **b** the corresponding size distributions; **c** the corresponding cumulative area plots. The *arrow lines* indicate the mean diameters (d_{mean})

Zinc and β-lactoglobulin concentrations, as well as contact times, exhibited a combined effect on the size of the aggregates. Figure 1.4a contains three-dimensional plots

showing the effects of β-lactoglobulin and zinc concentrations on the mean particle size (d_{mean}) of the aggregates after being in contact with Zn^{2+} for 15 min. As discussed previously, the concentrations of both play important roles in aggregation. Three-dimensional plots showing the effects of contact time and β-lactoglobulin concentration on the mean particle size (d_{mean}) of the aggregates are illustrated in Fig. 1.4b. Both factors contribute significantly to the aggregate formation.

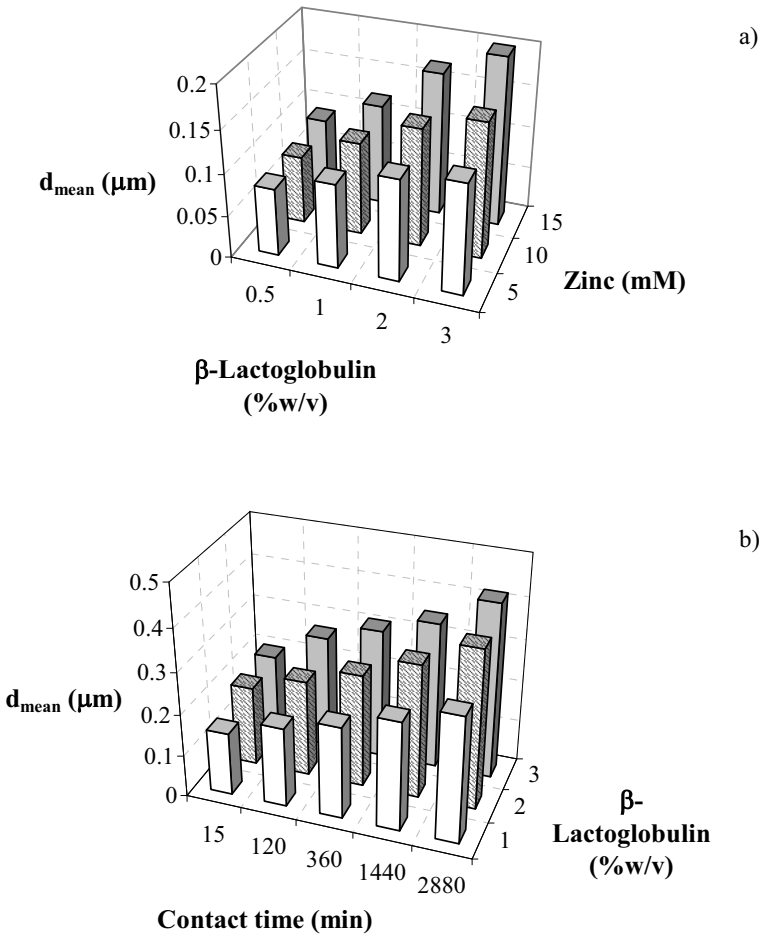

Figure 1.4a Relationship between β-lactoglobulin and Zn^{2+} concentrations and the particle size of the aggregates; **b** relationship between β-lactoglobulin concentration, the contact time and the particle size of the aggregates

1.3.3 Comparison between Ca^{2+} and Zn^{2+} in terms of extent of aggregation induced

To compare the extent of aggregate formation induced by Ca^{2+} or Zn^{2+}, 1% (w/v) β-lactoglobulin was mixed with 10 mM Ca^{2+} or Zn^{2+}, and the size distributions of the aggregates were observed at 15 and 1440 min contact times (Fig. 1.5). Figure 1.5 illustrates that 1% (w/v) β-lactoglobulin in Ca^{2+} coeluted with the void fraction (<0.01 μm), whereas that in Zn^{2+} displayed a peak maximum at 0.14 μm, indicating that at 15 min contact time Zn^{2+} could induce larger aggregates than Ca^{2+}. These results suggest that the kinetics of aggregate formation induced by Zn^{2+} are faster than those associated with Ca^{2+}. Similarly, at a prolonged contact time of 1440 min, β-lactoglobulin aggregates induced by Zn^{2+} (~0.30 μm) were larger than those induced by Ca^{2+} (~0.25 μm). Furthermore, at a contact time of 1440 min, Zn^{2+}-induced β-lactoglobulin aggregates started to show bimodal characteristics, exhibiting peak maxima at both 0.30 and 0.32 μm, suggesting the presence of more than one group of aggregates.

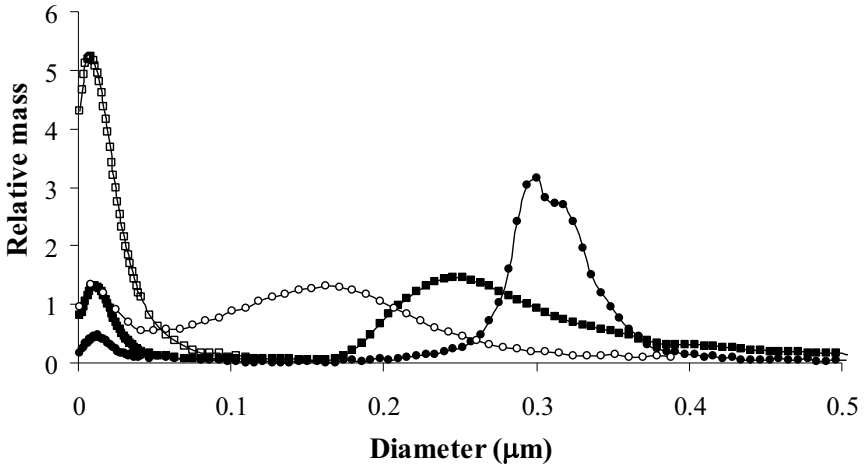

Figure 1.5 Size distributions of salt-induced β-lactoglobulin aggregates, where *open squares* and *circles* represent 1% (w/v) β- lactoglobulin in 10 mM Ca^{2+} and Zn^{2+}, respectively, at 15 min contact time, and *filled squares* and *circles* represent 1% (w/v) β-lactoglobulin in 10 mM Ca^{2+} and Zn^{2+}, respectively, at 1440 min contact time

Additionally, changes in the mean particle sizes of the salt-induced β-lactoglobulin aggregates were examined at various contact times (Fig. 1.6). The mean particle size

increased with contact time in all cases (Fig. 1.6). For calcium-induced aggregation, the effect of contact time on the aggregate formation of 1% and 2% (w/v) β-lactoglobulin in 20 mM Ca^{2+} was investigated. The aggregation of 2% (w/v) β-lactoglobulin proceeded faster than that of 1% (w/v) β-lactoglobulin. At contact times longer than 720 min, the size of the aggregates remained constant. The maximum attainable aggregate sizes for 1% and 2% (w/v) β-lactoglobulin in 20 mM Ca^{2+} were 0.39 and 0.46 μm, respectively. For zinc-induced aggregation, the effect of contact time on the aggregate formation of 1%, 2%, and 3% (w/v) in 10 mM Zn^{2+} was investigated. From our observations made at 60 min contact time, the concentration of Ca^{2+} required to trigger β-lactoglobulin aggregation was higher than that of Zn^{2+}. Therefore, the concentration of Zn^{2+} (10 mM) used in this experiment was lower than the concentration of Ca^{2+} (20 mM). Similar to that observed with calcium-induced aggregation, the mean particle size of Zn^{2+}-induced β-lactoglobulin aggregates increased with contact time. The aggregation during the first 360 min proceeded faster than at longer contact times, and the size of the aggregates appeared to continue to grow even after a contact time of 2880 min. The trends in the growth of mean particle size were similar for all three β-lactoglobulin concentrations. As the β-lactoglobulin concentration was increased, the particle sizes of aggregates increased. The d_{mean} values of 3% (w/v) β-lactoglobulin in 10 mM Zn^{2+} were larger than those of 2% and 1% (w/v) β-lactoglobulin, respectively. Differences in the growth behavior of β-lactoglobulin aggregates induced by Ca^{2+} and Zn^{2+} might be due to differences in the molecular mechanisms involved in aggregate formation.

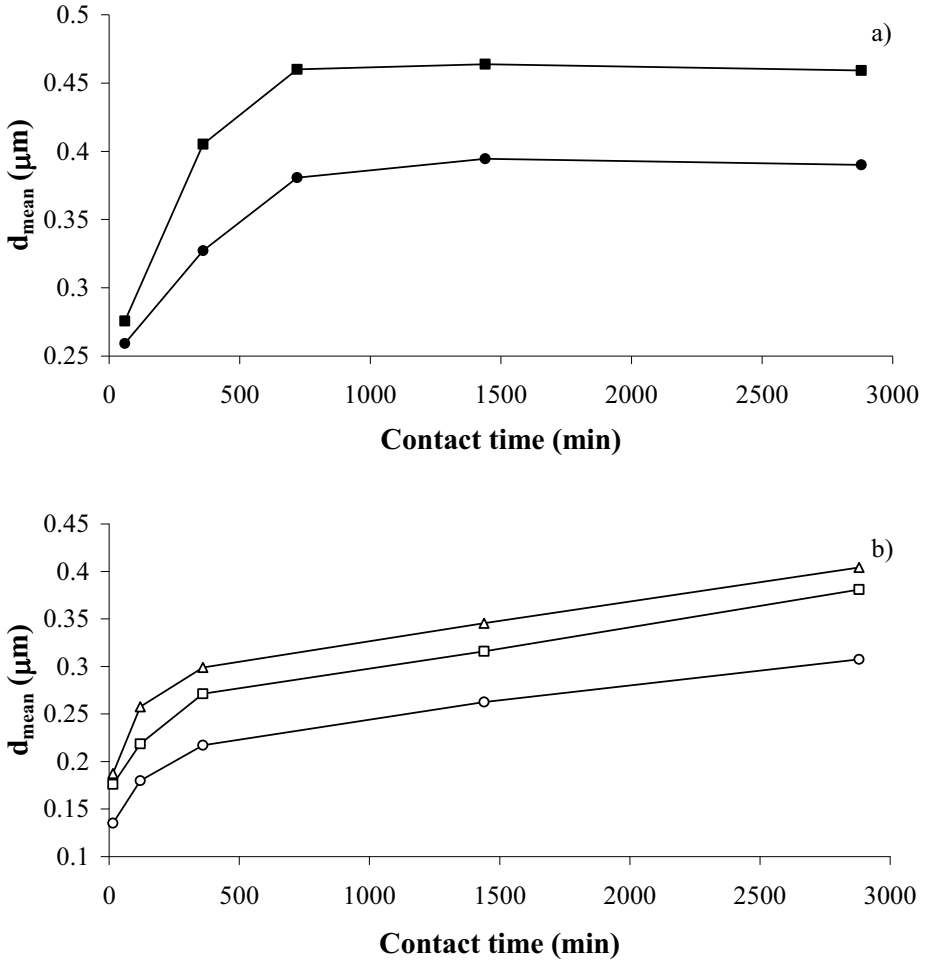

Figure 1.6 Relationship between particle sizes of β-lactoglobulin aggregate and contact time for: **a** Ca^{2+}-induced β- lactoglobulin aggregate, where *filled circles* and *squares* represent 1% and 2% (w/v) β-lactoglobulin, respectively, in 20 mM Ca^{2+}; **b** Zn^{2+}-induced β-lactoglobulin aggregate, where *open circles*, *squares*, and *triangles* represent 1%, 2%, and 3% (w/v) β-lactoglobulin, respectively, in 10 mM Zn^{2+}

1.4 Summary

This study demonstrates a novel application of SdFFF to study the process of β-lactoglobulin aggregation after thermal denaturation. The concentrations of both the β-lactoglobulin and the added metal ion, as well as the type of metal ion and the contact time, exhibited a combined effect on the extent of aggregate formation. The results from this study could be used to guide food manufacturers in how to manipulate the aggregate size of β-lactoglobulin, which is used as an ingredient in food.

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Chapter 2

Size Characterization of Hen Egg White Protein Aggregates Using Field-Flow Fractionation

2.1 Introduction

2.2 Experimental

2.2.1 Chemicals

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2.2.6 Data treatment

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2.3.3 Parameters affecting $ZnCl_2$ induced aggregation of hen egg white protein

2.3.4 Efficiency of hen egg white protein utilization for aggregation – new information from FlFFF

2.4 Summary

2.5 References

2.1 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.

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.

2.2 Experimental

2.2.1 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.

2.2.2 Instrumentation

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.

2.2.3 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.

2.2.4 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.

2.2.5 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).

2.2.6 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).

2.3 Results and Discussion

2.3.1 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 °C for 40 min. The resulting protein suspensions were introduced to SdFFF for size fractionation, and the raw fractograms are illustrated in Figure 2.1. 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.

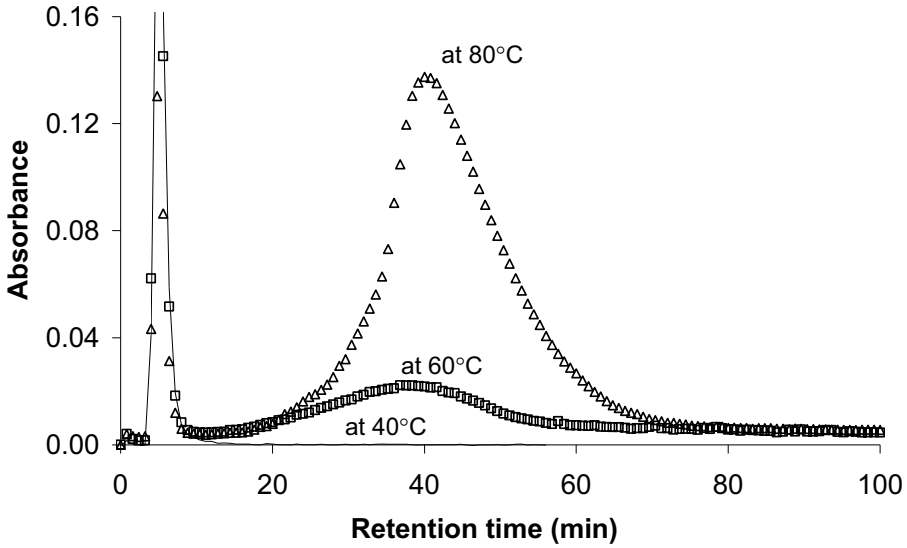

Figure 2.1 SdFFF fractograms of 5% (w/v) hen egg white protein at different heating temperatures, where —, □, and Δ represent 5% hen egg white protein at 40, 60, and 80 °C heating temperatures, respectively.

2.3.2 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 2.2. From the SEM photograph (Figure 2.2**a**), 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 2.2**b**) 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.

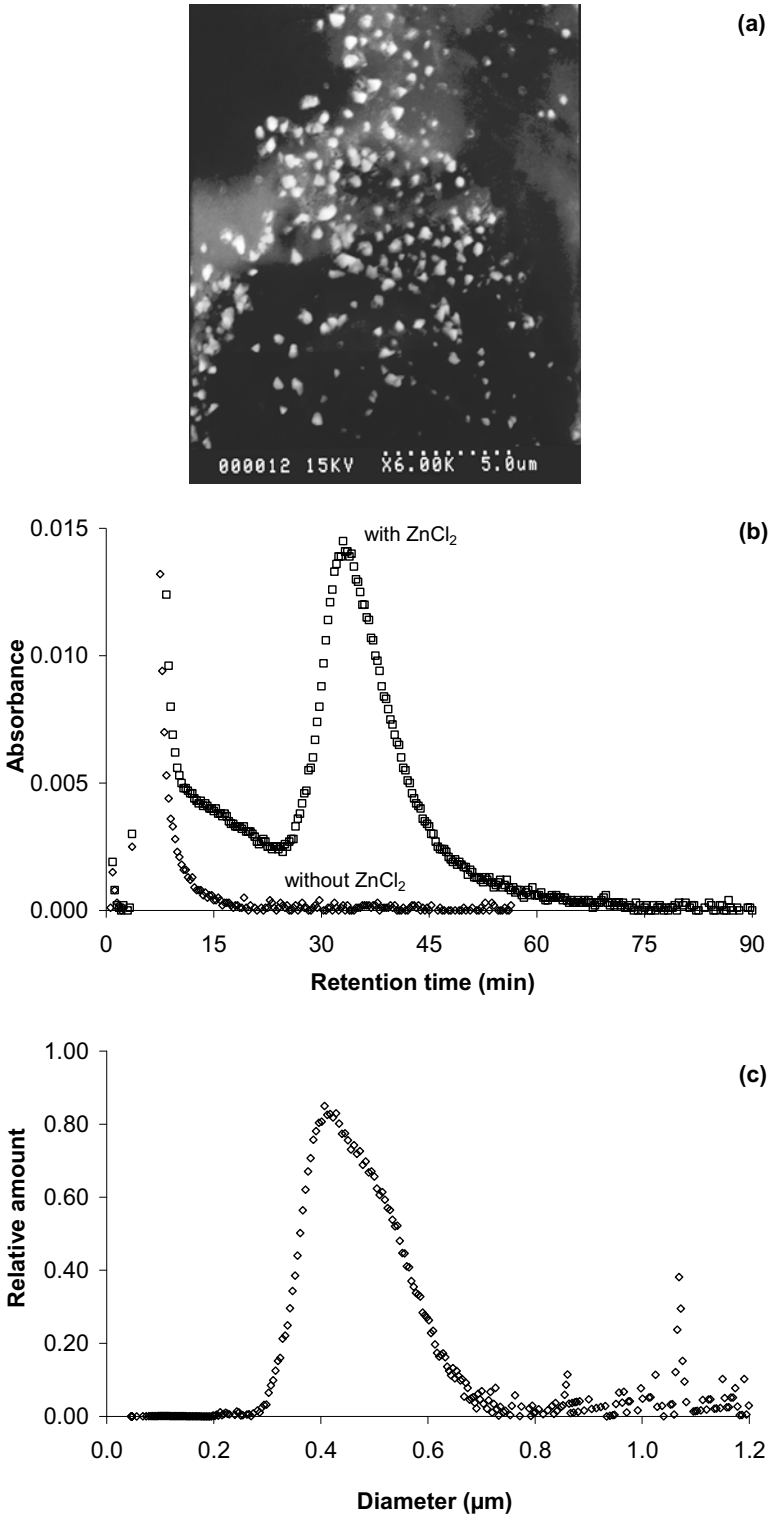

Figure 2.2 (**a**) SEM photograph of 5% (w/v) hen egg white protein mixed with 1 M $ZnCl_2$ at 15 min of incubation time, (**b**) SdFFF fractograms of 5% (w/v) hen egg white protein in the absence (◊) and presence (□) of 1.0 M $ZnCl_2$ at 15 min of incubation time, and (**c**) particle size distribution of 5% (w/v) hen egg white protein mixed with 1.0 M $ZnCl_2$ at 15 min of incubation time.

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*)

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

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 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 2.2**b** was translated into a particle size distribution profile of hen egg white protein aggregates as shown in Figure 2.2**c**. 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.

2.3.3 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 2.3**a**. 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 2.3**b**. 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.

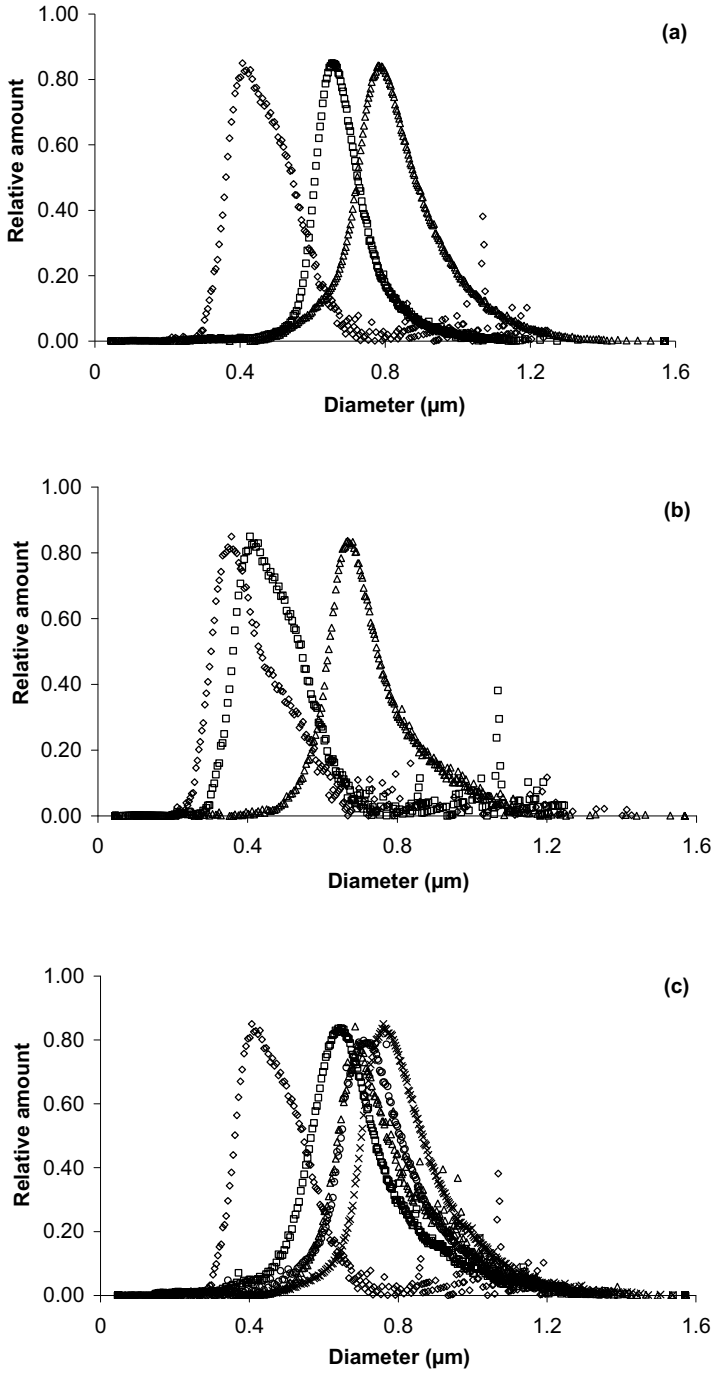

Figure 2.3 Particle size distributions of hen egg white protein aggregates obtained from SdFFF: (**a**) effect of hen egg white protein concentration (w/v) at 1.0 M $ZnCl_2$ and 15 min of incubation time, where ◊, □, and Δ represent 5, 6.25, and 7.5% (w/v) hen egg white protein, respectively; (b) effect 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.

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 2.3**c**. 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 2.1. 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.

Table 2.1 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}* (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 incubation time)

Hen egg white protein content (%, w/v)	d_p (μm)	d_{mean}* (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 incubation time)

$ZnCl_2$ concentration (M)	d_p (μm)	d_{mean}* (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}* (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

* d_{mean} is defined as the particle size at which 50% of the total accumulative area is detected

2.3.4 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 2.4. 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 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**.

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

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 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.

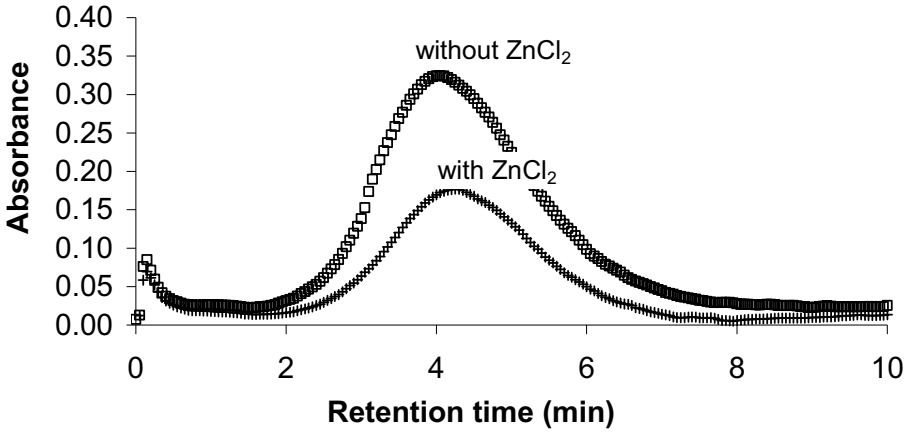

Figure 2.4 FlFFF fractograms of 1% (w/v) hen egg white protein in the absence (□) and presence (+) of 1.5 M $ZnCl_2$.

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 2.5**a**. 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 2.5**b**. 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 2.5**c**. 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%.

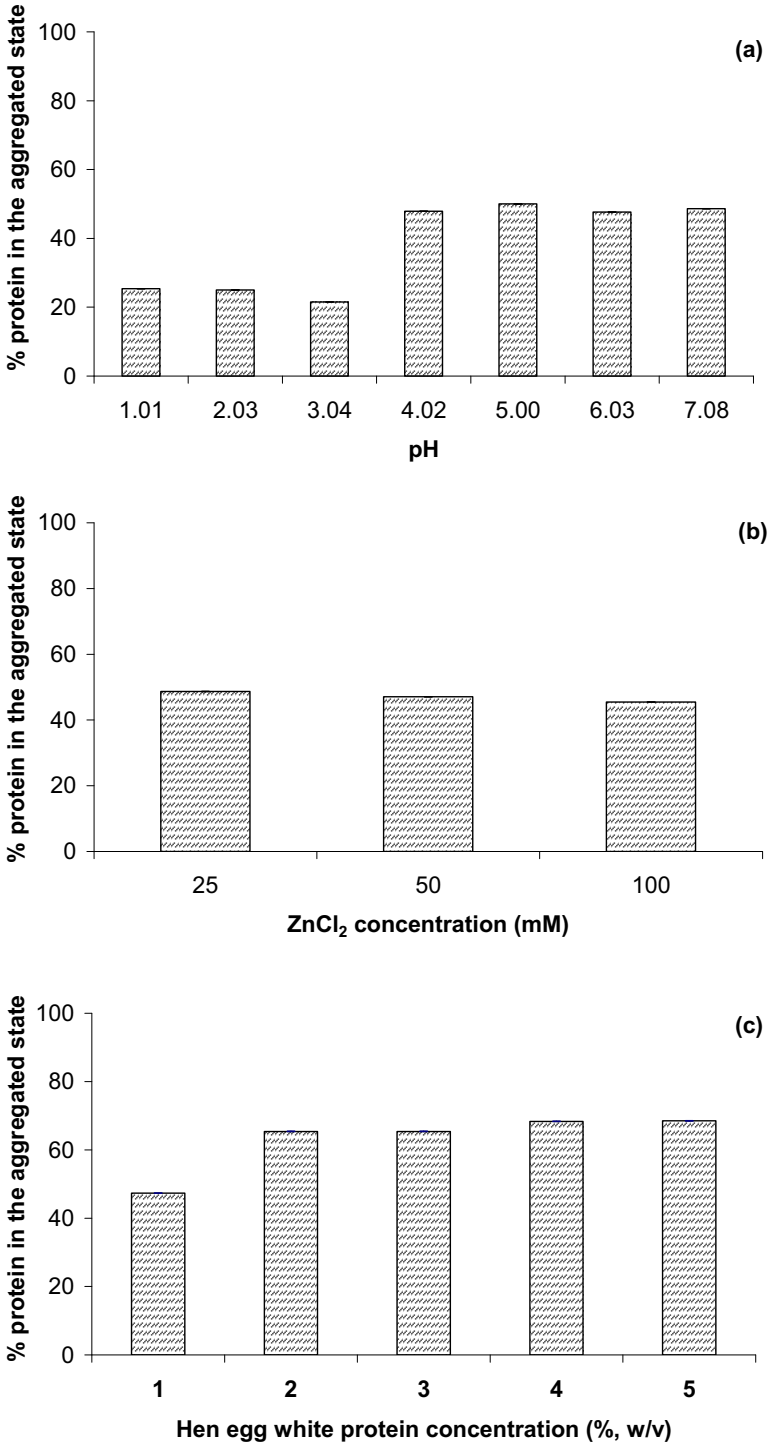

Figure 2.5 Parameters affecting efficiency of hen egg white protein usage for aggregation: (**a**) effect of pH for 1% (w/v) hen egg white protein mixed with 50 mM $ZnCl_2$, (**b**) effect of $ZnCl_2$ concentration (mM) for 1% (w/v) hen egg white protein at pH 6.0, and (**c**) effect of hen egg white protein content (%, w/v) at 50 mM $ZnCl_2$ and pH 6.0.

2.4 Summary

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%.

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Chapter 3

Characterization of β-lactoglobulin Aggregates Encapsulated α-Tocopherol

3.1 Introduction

3.2 Experimental

3.2.1 Chemicals

3.2.2 Instrumentation

3.2.3 Preparation of β-lactoglobulin solutions

3.2.4 Preparation of BLG-encapsulated α-TOC

3.2.5 Determination of α-TOC loading

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3.3.1 Selection of divalent cation for the preparation of BLG-encapsulated α-TOC

3.3.2 Parameters affecting encapsulation efficiency of BLG-encapsulated α-TOC

3.3.3 Size characterization of BLG-encapsulated α-TOC

3.4 Summary

3.5 References

3.1 Introduction

Vitamin E is an important antioxidant that can help reduce risk of diseases such as cardiovascular diseases (*1-5*). Although vitamin E deficiency is rare in humans, absorption of vitamin E is reduced in the patients with some diseases including pancreatic diseases, biliary obstruction, coeliac disease, and abetalipoproteinaemia. Therefore, supplementation of vitamin E is needed in some cases. Vitamin E exists in eight different forms including four tocopherols (α, β, γ, δ) and four tocotrienols (α, β, γ, δ), but vitamin E supplements are usually available in forms of relatively stable all-rac α-tocopherol acetate and all-rac α-tocopherol succinate. Nonetheless, α-tocopherol was believed to be most absorbable in human intestine. Although it was believed that tocopherol esters were hydrolyzed to free tocopherol on the duodenum (*6-8*), the need of hydrolytic enzyme to de-esterify the supplement cannot guarantee the efficient bioavailability. Consequently, it would be beneficial if vitamin E supplements can be prepared in the free phenol form of α-tocopherol (α-TOC). As it is relatively sensitive and unstable to temperature, oxygen, and light, encapsulation is needed for protection of α-tocopherol during shelf storage. Protein based particles has been used for encapsulation of bioactive ingredients as protein contains high nutritional value and is generally recognized as safe. This study was carried out to evaluate the ability of β-lactoglobulin aggregates (BLG) as a particle for encapsulation of α-TOC. Size characterization of BLG-encapsulated α-TOC was also carried out using sedimentation field-flow fractionation (SdFFF).

3.2 Experimental

3.2.1 Chemicals

(±)-α-tocopherol, sodium phosphate monohydrate and sodium phosphate dibasic dodecahydrate were purchased from Fluka Chemie GmbH, Buchs, Germany. β-lactoglobulin (from bovine milk approx. 90 % PAGE chromatographically purified and lyophilized), pepsin 1:10,000 (from porcine stomach mucosa, crystallized and lyophilized), 2,2'-bipyridyl and $ZnCl_2$ were purchased from Sigma, St. Louis, MO, USA. $FeCl_3$ and $CaCl_2$ were purchased from Riedel-de Haën, Seelze, Germany. α-Tocopherol stock solutions of the appropriate concentrations (mM) were diluted in ethanol. Reagents used were of analytical grade (MERCK KGaA, Darmstadt, Germany, Labscan Asia Co., Ltd, Bangkok, Thailand).

3.2.2 Instrumentation

An SdFFF system (Model S-101 Particle/Colloid Fractionator, Postnova Analytik, Landsberg, Germany) was used to characterize the particle size distributions of BLG-encapsulated α-TOC. The elution of particles was monitored by a UV absorption detector operating at the fixed wavelength of 280 nm (UV-2000 Spectra System, Thermo Electron Corporation, Waltham, MA, USA). Samples were introduced into a Rheodyne (Rohnert Park, CA, USA) injector at a fixed loop of 50 μL.

3.2.3 Preparation of β-lactoglobulin solutions

BLG powder was dissolved in deionized water to obtain a stock solution of 6% (w/v). To prepare denatured BLG, the 6% (w/v) BLG solution was heated at 80 °C for 30 min and cooled to room temperature (27 °C) (*9*). The BLG protein solution was filtered through 0.45 μm cellulose acetate syringe filter before use.

3.2.4 Preparation of BLG-encapsulated α-TOC

Salt induced gelation technique was used for preparation of BLG-encapsulated α-TOC. α-TOC was mixed with appropriate concentration of BLG solution and subsequently $CaCl_2$ was added to induce aggregation of BLG. The molar ratio of protein and α-TOC and salts was 2:1:1.

3.2.5 Determination of α-TOC loading

The BLG-encapsulated α-TOC was centrifuged at 12,000 rpm for 30 min (Beckman Allegra X-22R Centrifuge, Palo Alto, USA) to separate the unencapsulated α-TOC from the mixture. The concentration of unencapsulated α-TOC was determined by colorimetric method exploiting the ability of α-TOC to reduce Fe^{3+} into Fe^{2+} which could subsequently form red-orange color with 2,2-bipyridyl. The absorbance of the resulting solution was measured at 521 nm. Subsequently, the precipitate part containing protein aggregates was dispersed in 30 ml of simulated gastric fluid (SGF) (HCl solution, pH 1.2) with 0.1% pepsin (w/v) at 37 °C under vigorous agitation for 2 h (*16*). The resulting mixture was extracted by hexane solution and α-TOC concentration in the aggregate was determined by measuring the absorbance at 297 nm with a UV–visible spectrophotometer (JASCO V530 UV-Visible Spectrophotometer, JASCO, Inc., Tokyo, Japan). The

encapsulated α-TOC was calculated by subtraction of unencapsulated α-TOC from the total amount of added α-TOC. The encapsulation efficiency (EE) was calculated from the ratio of the encapsulated α-TOC and the added α-TOC. All experiments were performed at least in five replicates ($n = 5$).

3.3 Results and Discussion

3.3.1 Selection of divalent cation for the preparation of BLG-encapsulated α-TOC

Cold gelation of protein can be achieved by adding salts including monovalent or divalent cations, e.g., Na^{+}; Ca^{2+}; or Zn^{2+} to the solution containing charged protein molecules (9, *11*). To perform salt induced gelation of proteins, $CaCl_2$ and $ZnCl_2$ were used as they are listed as generally recognized as safe (GRAS) by the United States of America Food and Drug Administration when used in accordance with good manufacturing practice (*12*). Our investigation indicated that salt induced gelation of BLG was best achieved by $CaCl_2$ or $ZnCl_2$, respectively. One might argue that aggregate formation could already be obtained by mixing BLG with α-TOC. Without addition of $CaCl_2$, however, the added α-TOC was mostly found in the supernatant part suggesting that they were not encapsulated in BLG aggregates.

3.3.2 Parameters affecting encapsulation efficiency of BLG-encapsulated α-TOC

The concentrations of BLG and α-TOC showed combined influence on encapsulation efficiency of BLG-encapsulated α-TOC as illustrated in Figure 3.1. At fixed concentration of BLG, the encapsulation efficiency decreased with increasing concentration of α-TOC for each BLG concentration (Figures 3.1a-c). Nonetheless, the mole of α-TOC in the aggregate increased with increasing concentration of α-TOC added and reached a plateau at a certain concentration of α-TOC because of the limited amount of BLG.

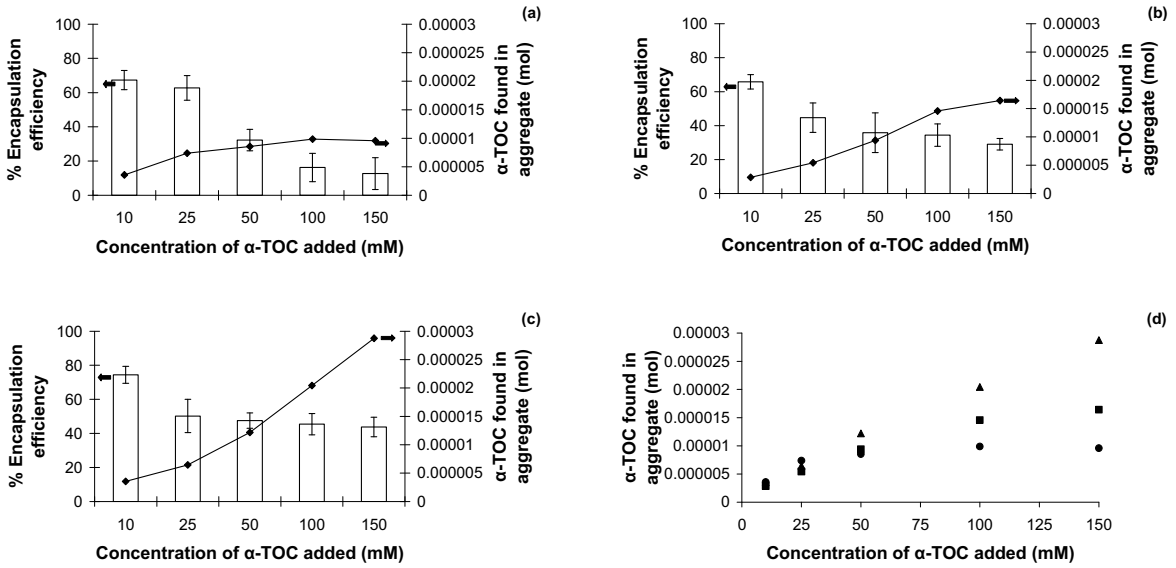

Figure 3.1 Effect of α-TOC concentration on encapsulation efficiency at various concentrations of β-lactoglobulin: a) 0.5% (w/v); b) 1.0% (w/v); c) 2.0% (w/v), in 100 mM $CaCl_2$. d) relationship between concentration of α-TOC added and α-TOC encapsulated.

Figure 3.1d shows the relationship between the added α-TOC concentration and the mole of α-TOC encapsulated at various BLG concentrations. In the excess of BLG at 2% (w/v), a straight line relationship was obtained. Deviation from the straight line was observed when BLG was not adequate for encapsulation of α-TOC. At the plateau, however, the mole ratio of α-TOC and BLG was calculated to be 140 and 104 for BLG of 0.5% (w/v) and 1% (w/v), respectively, suggesting that the average mole ratio of the two compounds might be at approximately 120. In general, higher protein concentrations can hold higher amount of α-TOC at the fixed concentrations of α-TOC and $CaCl_2$. This statement was confirmed by determining the encapsulation efficiency and the absolute mole of α-TOC in the aggregate at various concentrations of BLG at fixed concentrations of α-TOC and $CaCl_2$. The amount of α-TOC encapsulated increased with increasing in BLG concentration (Figure 3.2). BLG was reported to form continuous and homogeneous membranes around the α-TOC oil droplets through intermolecular β-sheets interaction because of its flexibility and amphiphilic nature (*13, 14*). At fixed concentrations of BLG and α-TOC, the change in encapsulation efficiency was not so significant with the change in $CaCl_2$ concentrations (from 10 to 100 mM), as

illustrated in Figure 3.3. Therefore, only small amount of Ca^{2+} was needed for neutralization of the negatively charged BLG and lowering electrostatic repulsion with the subsequent aggregates formation through intermolecular bridges (*15-17*). Concentration of $CaCl_2$ higher than 100 nM was not tested, as too high concentration of salt weakened electrostatic interactions, which could suppress aggregate formation because of charge screening effects (*17*).

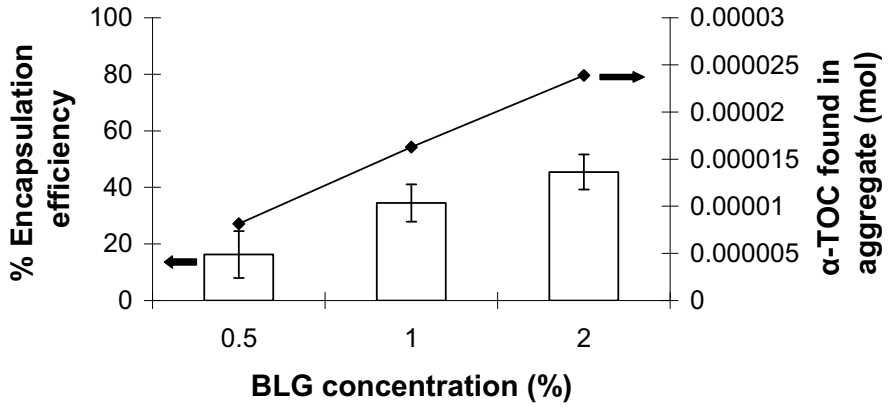

Figure 3.2 Effect of β-lactoglobulin concentration on encapsulation efficiency of 100 mM α-TOC in 100 mM $CaCl_2$

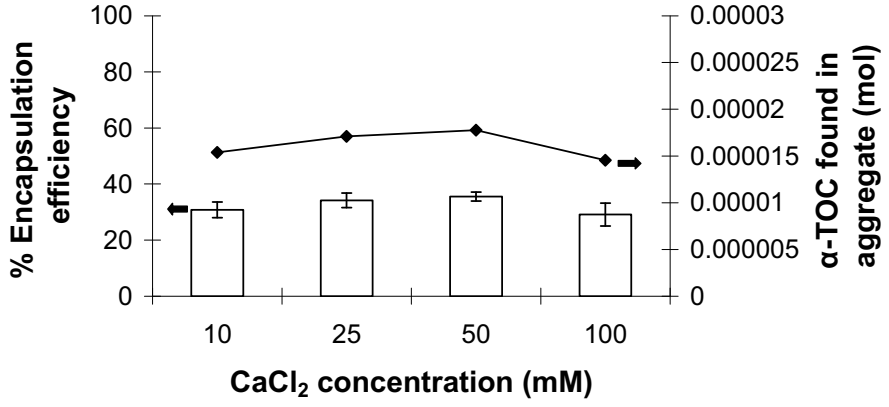

Figure 3.3 Effect of $CaCl_2$ concentration on encapsulation efficiency of 100 mM α-TOC with 0.5% (w/v) β-lactoglobulin

3.3.3 Size characterization of BLG-encapsulated α-TOC

In this study, particle size distribution of BLG-encapsualted α-TOC was examined using SdFFF. Figure 3.4 shows particle size distributions of 1% (w/v) β-lg in 20 mM $CaCl_2$ with 25 mM α-tocopherol at various contact times of 15, 300, 600, 1440 and 2880 min. At prolonged contact time, particle size of BLG-encapsualted α-TOC grew larger than that at short contact time.

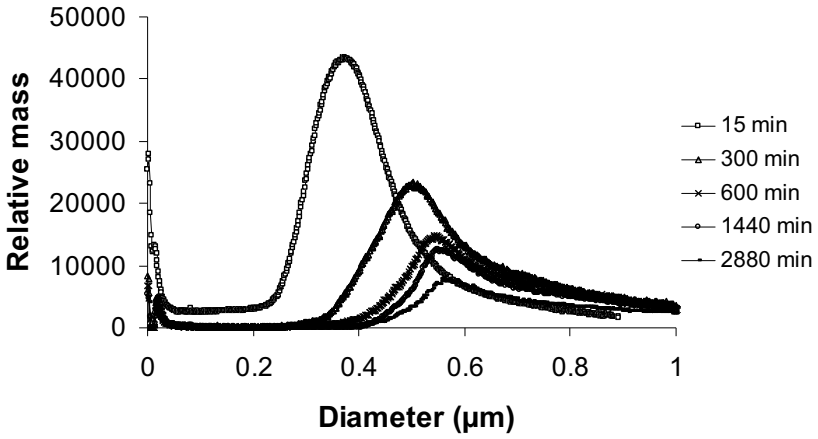

Figure 3.4 Particle size distributions of BLG-encapsulated α-TOC at various incubation times. Conditions: 1% (w/v) β-lactoglobulin in 20 mM $CaCl_2$ with 25 mM α-tocopherol at contact time 15, 300, 600, 1440 and 2880 min

3.4 Summary

BLG can be used for encapsulation of α-TOC. Concentrations of α-TOC and β-lactoglobulin were found to exhibit effect on encapsulation efficiency. Sedimentation FFF can be employed for size characterization of BLG-encapsulated α-TOC particles.

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โครงการ การวิเคราะห์ขนาดอนุภาคของสารที่มีฤทธิ์ทางชีวภาพในอนุภาคเภสัชภัณฑ์ขนาดนาโนเมตรโดยใช้เทคนิคการแยกแบบไหลภายใต้สนาม

ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ 2 เรื่อง

1. Saeseaw, S., Shiowatana, J. & Siripinyanond, A. 2006, "Observation of Salt-induced β-Lactoglobulin Aggregation Using Sedimentation Field-Flow Fractionation", *Anal. Bioanal. Chem.,* vol. 386, pp. 1681–1688. (impact factor = 2.591)
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ผลงานเสนอในการประชุมวิชาการนานาชาติ 4 เรื่อง

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ORIGINAL PAPER

Observation of salt-induced β-lactoglobulin aggregation using sedimentation field-flow fractionation

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Abstract Sedimentation field-flow fractionation (SdFFF) was applied in order to characterize particle sizes of β-lactoglobulin aggregates induced by Ca^{2+} or Zn^{2+}. Aggregation induced by Zn^{2+} was faster than that induced by Ca^{2+}. Effects of Zn^{2+} and β-lactoglobulin concentrations, as well as contact time, on the aggregation of β-lactoglobulin were examined. All factors exhibited a combined effect on the size of aggregates, whereby larger aggregates were obtained at increased concentrations of Zn^{2+} and β-lactoglobulin. At fixed concentrations of 2% (w/v) β-lactoglobulin and 10 mM Zn^{2+}, the particle size of the aggregates increased from 0.19 μm (at 15 min) to 0.38 μm (at 2880 min). Further, a hyphenated technique of SdFFF and inductively coupled plasma–optical emission spectrometry (ICP–OES) was used to examine whether intermolecular ionic bridges take part in salt-induced β-lactoglobulin aggregation. With SdFFF–ICP–OES, protein–cation–protein cross-linkages were observed for β-lactoglobulin aggregation induced by Zn^{2+}, but not for that induced by Ca^{2+}.

Keywords Lactoglobulin · Aggregation · Field-flow fractionation · Inductively coupled plasma–optical emission spectrometry · Calcium and zinc ions

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Introduction

The aggregation of food proteins is a topic that has generated much research in the field of food science, due to its important influence over the functional properties of food products [1]. Whey proteins are important functional ingredients that are used as emulsifiers, texture modifiers, thickeners, and gelling agents [2]. The functionality of whey proteins is primarily based on their ability to undergo aggregation and eventually gelation [2]. The major protein in whey is β-lactoglobulin, which accounts for approximately 50% of the total whey protein [3]. Therefore, β-lactoglobulin is often used as a model protein to study aggregation behavior [2, 4]. β-lactoglobulin is a globular protein that has a molecular mass of 18.3 kDa, a radius of approximately 2 nm, one thiol group and two disulfide bonds [3, 5, 6]. Upon heat treatment above the denaturation temperature (~78 °C), the conformational structure of β-lactoglobulin changes, causing the nonpolar and the thiol groups to become exposed [3]. Consequently, aggregate formation occurs through intermolecular associations involving disulfide bridging, electrostatic interactions, hydrogen bonding, and hydrophobic interactions [7–10]. The conformation of the protein and its physicochemical properties depend on environmental factors (e.g., pH and ionic strength) [11], protein concentrations [12], as well as time and temperature [13, 14].

Zinc is an important essential trace element for human nutrition, and deficiency of zinc is a worldwide nutritional issue. To overcome this problem, zinc sulfate is often prepared as a zinc supplement, owing to its adequate bioavailability [15]. Nonetheless, zinc sulfate interacts with the food matrix, modifying the sensorial characteristics of the food, and sometimes making the flavor unacceptable

[15]. In order to incorporate minerals into food matrix, iron sulfate has been added to β-lactoglobulin; this is reported to have resulted in a network of β-lactoglobulin aggregates with the iron encapsulated inside [16]. As a consequence, the iron could be protected and transported for further absorption by the human gastrointestinal tract [16]. Therefore, we decided to investigate the effect of zinc ion on structural changes of β-lactoglobulin, for two reasons. The main reason was to examine whether the particle size of β-lactoglobulin aggregates, and hence food sensorial characteristics, could be controlled by the addition of zinc ion. A second reason was that we wished to investigate whether a new source of zinc supplement could be prepared by encapsulating zinc in a network β-lactoglobulin aggregates. However, in this manuscript, only the first objective is addressed.

The molecular mass of β-lactoglobulin has been extensively characterized using ultracentrifugation [17], gel electrophoresis [18, 19], and size exclusion chromatography (SEC) [20, 21]. Among the size separation techniques used, SEC is the most common approach. However, the applicable range of the SEC columns available is limited to masses of below a few million, and therefore this technique is not suited to the separation of very large protein aggregates [22–26]. In addition, potential interactions of protein aggregates with the stationary phase restrict the use of SEC. To avoid these problems, field-flow fractionation (FFF) has been used as an alternative technique for separating very large protein aggregates. The characterization of protein aggregates by FFF—either flow FFF (FlFFF) [22–24] or sedimentation FFF (SdFFF) [25, 26]—has been reported by many investigators. Only recently, the application of ceramic hollow fiber FlFFF in order to characterize the particle sizes of β-lactoglobulin aggregates was demonstrated by Zhu et al. [27]. In their study, heat (65 °C) was applied to induce the aggregation of β-lactoglobulin, with particle sizes reportedly ranging from 30 to 200 nm. Although FlFFF appears to be a valuable technique for the size separation of protein aggregates, interactions between the samples and the membrane generally used in the FlFFF channel may occur at high ionic strengths.

Since it did not require a membrane inside the channel, and therefore potential interactions between β-lactoglobulin aggregates and the channel wall are minimized, SdFFF was therefore chosen to characterize the particle sizes of β-lactoglobulin aggregates. The aims of this investigation were to examine the salt-induced aggregation of β-lactoglobulin and to characterize the particle size distributions of β-lactoglobulin aggregates induced by Ca^{2+} and Zn^{2+}. The effects of β-lactoglobulin and salt concentrations, as well as the contact time, were examined. Further, inductively coupled plasma–optical emission spectrometry (ICP–OES) was used for elemental detection after size separation by SdFFF in order to perform the elemental fractionation of β-lactoglobulin, providing information on the molecular mechanisms involved in aggregate formation.

Experimental

Instrumentation

An SdFFF system (Model S-101 Particle/Colloid Fractionator, Postnova Analytik, Landsberg, Germany) was used to characterize the particle size distributions of β-lactoglobulin aggregates. The SdFFF channel wall was made of a polished Hastelloy C alloy, which is principally Ni (56%) with 15% Cr, 17% Mo, 5% Fe, 4% W, and traces of Mn and Si. The SdFFF channel was 89.5 cm long (tip to tip), 2.0 cm wide, and 0.0254 cm thick, with a rotor radius of 15.1 cm. The channel volume was calculated to be 4.5 mL. The carrier solution was introduced into the SdFFF channel by an HPLC pump (Model PN1122, Postnova Analytik). The elution of particles was monitored by a UV absorption detector operating at the fixed wavelength of 280 nm (UV-2000 Spectra System, Thermo Electron Corporation, Waltham, MA, USA). Samples were introduced into a Rheodyne (Rohnert Park, CA, USA) injector at a fixed loop of 50 μL.

A Spectro Ciros (Spectro Analytical Instruments, Kleve, Germany) end-on-view inductively coupled plasma–optical emission spectrometer (ICP–OES) was used to detect elements in the fractionated β-lactoglobulin aggregates. Owing to the similarity of the SdFFF channel flow and ICP–OES sampling flow rates, the ICP–OES nebulizer was connected directly to the UV detector outlet with a 50-cm length of poly(tetrafluoroethylene) tubing (PTFE, 0.58 mm id). The operating conditions of SdFFF and ICP–OES measurements are summarized in Table 1.

Chemicals

One hundred millimoles of $CaCl_2$ and 100 mM $ZnSO_4$, both of analytical reagent grade from Merck (Darmstadt, Germany), were prepared in doubly distilled deionized water and used as stock solutions for further dilution. The carrier liquid was 0.02% (v/v) FL-70 detergent (Fisher Scientific, Pittsburgh, PA, USA) containing 0.02% (w/v) NaN_3 (Merck).

Four percent 4% (w/v) β-lactoglobulin (Sigma, St. Louis, MO, USA) solution was prepared in deionized water at pH 7. To prepare denatured β-lactoglobulin, the 4% (w/v) β-lactoglobulin solution was heated at 80 °C for 30 min and cooled to room temperature (27 °C) [28].

Table 1 SdFFF–ICP–OES operating conditions

Conditions	Measurements
SdFFF operating conditions	
SdFFF channel dimensions (cm^3)	89.5 × 2.0 × 0.0254
SdFFF rotor radius (cm)	15.1
Carrier liquid	0.02% (v/v) FL-70 containing 0.02% (w/v) sodium azide
Channel flow rate (mL min^{-1})	1.0
Equilibration time (min)	15
Power field programming	Initial field strength, 1500 rpm for 5 min; field decay parameter, −40; final field strength, 150 rpm
ICP–OES operating conditions	
Rf generator frequency (MHz)	27.2
Rf power (W)	1350
Nebulizer gas flow rate (L min^{-1})	0.8
Coolant gas flow rate (L min^{-1})	12.0
Auxiliary gas flow rate (L min^{-1})	1.0
Lines monitored (nm)	Ca(II) 393.366, S(I) 180.731, and Zn(I) 334.502

Salt-induced β-lactoglobulin aggregates

To induce β-lactoglobulin aggregation, the thermally denatured β-lactoglobulin and salt solutions (i.e., $CaCl_2$ or $ZnSO_4$) were mixed and diluted to a specified concentration in deionized water. After various contact times of between 0 to 2880 min, the β-lactoglobulin aggregates were introduced into the SdFFF channel.

Data transformation

Raw fractograms were translated into size distribution profiles using Microsoft Excel spreadsheet software. Peak evaluation, baseline adjustment, and cumulative area distribution plotting were performed using PeakFit (SPSS, Chicago, IL, USA).

Results and discussion

SdFFF and evidence for β-lactoglobulin aggregation

Size characterization by SdFFF is quite straightforward provided that the density of the sample particle is known. If the exact geometry of the SdFFF channel, the field, flow rate, and the density difference between the particle and carrier liquid are all known, the diameter of the separated particle can be calculated directly from the experimental retention time [29]. Once the size distribution profile has been plotted, the particle size at peak maximum (d_p), and the mean particle size (d_{mean}) can be measured. The particle size at peak maximum (d_p) is then used to identify the dominant particle size of the investigated β-lactoglobulin aggregates. To obtain the mean particle size (d_{mean}) of the β-lactoglobulin aggregates, the diameter distribution profiles are converted into cumulative area plots. The d_{mean} is defined as the particle size at which 50% of the total accumulative area is detected.

In this study, particle size information obtained from SdFFF is used to obtain evidence of β-lactoglobulin aggregation. The aggregation of 1% (w/v) β-lactoglobulin induced by 20 mM Ca^{2+} was investigated by comparing the fractograms of calcium-induced β-lactoglobulin aggregates at 60 and 1440 min contact times with that of the 1% (w/v) β-lactoglobulin without the addition of Ca^{2+} (Fig. 1a). The original 1% (w/v) β-lactoglobulin displayed a peak maximum at approximately 5.3 min, which coeluted with the void fraction (4.5 min), suggesting that the β-lactoglobulin was not retained or was only slightly retained in the SdFFF channel. With the addition of 20 mM Ca^{2+}, however, two peak maxima were observed. The first peak was detected at the void fraction and the second peak was observed at 22 and 32 min after contact times of 60 and 1440 min, respectively, with the corresponding size distributions shown in Fig. 1b. Although β-lactoglobulin aggregates have a fractal structure where the density decreases with the size of the aggregates, in this experiment the density of the β-lactoglobulin aggregates was assumed to be 1.05 g cm^{-3}. Usually, for a fixed particle size, the retention time decreases with particle density. Therefore, the increase in the retention time actually indicates that the aggregates grew in size. The particle sizes (d_p) increased from <0.01 to 0.26 and 0.38 μm after contact times of 60 and 1440 min, respectively, with broader size distributions. The cumulative area distribution plots (Fig. 1c) indicate that the d_{mean} of β-lactoglobulin aggregates shifted from 0.015 to 0.26 and 0.38 μm after being in contact with 20 mM Ca^{2+} for 60 and 1440 min, respectively. The increase in particle size at longer contact times confirms that aggregation of β-lactoglobulin took place. The delayed elution was not caused by interactions between the SdFFF channel wall and the β-lactoglobulin particles. This investigation shows that

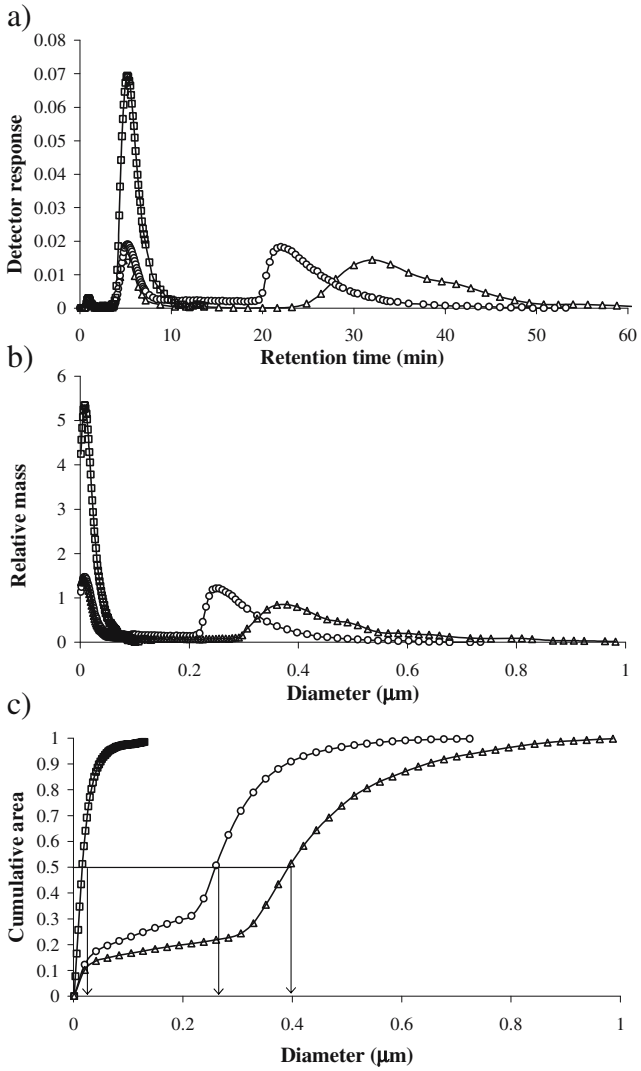

Fig. 1a–c **a** Raw fractograms of 1% (w/v) β-lactoglobulin (*squares*) and 1% (w/v) β-lactoglobulin in 20 mM Ca^{2+} at contact times of 60 (*circles*) and 1440 (*triangles*) min; **b** the corresponding size distributions; **c** the corresponding cumulative area plots. The *arrow lines* indicate the mean diameters (d_{mean})

SdFFF is a suitable tool for obtaining evidence of β-lactoglobulin aggregation. Moreover, information could be obtained on the particle size distributions of the aggregates.

Factors affecting zinc-induced β-lactoglobulin aggregation

Since the factors that affect calcium-induced β-lactoglobulin have been reported by many other investigators [12, 28, 30, 31], only factors affecting zinc-induced aggregation were examined in this study. The effects of the concentrations of Zn^{2+} and β-lactoglobulin, as well as the contact times, on the extent of aggregate formation were investigated. At a fixed contact time of 15 min, three different concentrations of Zn^{2+} (5, 10, and 15 mM) were examined for their effect on the aggregate formation of 2% (w/v) β-lactoglobulin. Particle size distributions of β-lactoglobulin aggregates at various Zn^{2+} concentrations are illustrated in Fig. 2a. At higher Zn^{2+} concentrations, larger aggregates were formed, as the d_p value increased from 0.11 to 0.16 μm when the Zn^{2+} concentration increased from 5 to 15 mM Zn^{2+}. Adding Zn^{2+} resulted in the negatively charged surface of β-lactoglobulin becoming shielded, leading to intermolecular associations or aggregation. Furthermore, increasing the concentration of Zn^{2+} resulted in an increase in the number of bridges binding the denatured β-lactoglobulin, leading to larger β-lactoglobulin aggregates [30]. According to the Derjaguin–Landau–Verwey–Overbeek (DLVO) theory [30], which describes the force attributed to van der Waals attraction and the repulsion due to counterions between surfaces interacting through a liquid medium, the addition of salt suppresses the repulsive potential, the energy barrier between the negatively charged particles, causing particles to aggregate. At higher Zn^{2+} concentrations, the energy barrier drops faster, resulting in faster aggregate formation, and therefore larger aggregates are formed.

Similarly, the concentration of β-lactoglobulin plays a significant role in the extent of aggregate formation, as

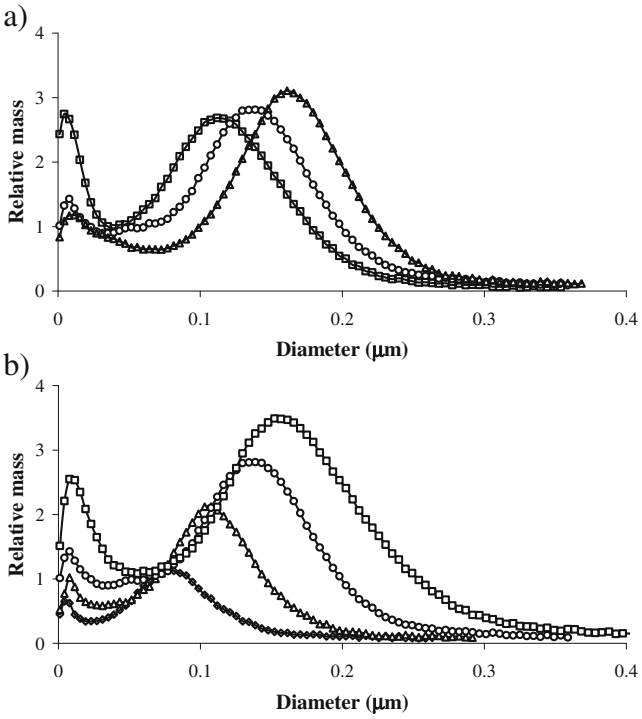

Fig. 2a–b Size distributions of Zn^{2+}-induced β-lactoglobulin aggregates at 15 min contact time: **a** effect of Zn^{2+} concentration on the aggregation of 2% (w/v) β-lactoglobulin; where *squares*, *circles*, and *triangles* represent 5, 10, and 15 mM Zn^{2+}, respectively; **b** the effect of β-lactoglobulin concentration at 10 mM Zn^{2+}; where *diamonds*, *triangles*, *circles*, and *squares* represent 0.5%, 1%, 2%, and 3% (w/v) β-lactoglobulin, respectively

illustrated in Fig. 2b. At higher β-lactoglobulin concentrations, larger aggregates were formed, as the d_p value increased from 0.08 to 0.16 μm when the β-lactoglobulin concentration increased from 0.5% to 3.0% (w/v). Naturally, both intermolecular hydrophobic interactions and disulfide bond formation increased as the β-lactoglobulin concentration was increased [31].

In order to investigate the aggregation dynamics of β-lactoglobulin, various contact times of 15, 120, 360, 1440, and 2880 min were examined. Figure 3 demonstrates the particle size distributions and cumulative area distributions of 2% (w/v) β-lactoglobulin in 10 mM Zn^{2+} at various contact times. The d_p value increased from 0.19 (at 15 min) to 0.38 μm (at 2880 min). At 120 min contact time, the particle size distribution of the β-lactoglobulin aggregates started to show a deviation from monomodal characteristics, implying that more than one group of β-lactoglobulin aggregates existed.

Zinc and β-lactoglobulin concentrations, as well as contact times, exhibited a combined effect on the size of the aggregates. Figure 4a contains three-dimensional plots showing the effects of β-lactoglobulin and zinc concentrations on the mean particle size (d_{mean}) of the aggregates after being in contact with Zn^{2+} for 15 min. As discussed previously, the concentrations of both play important roles

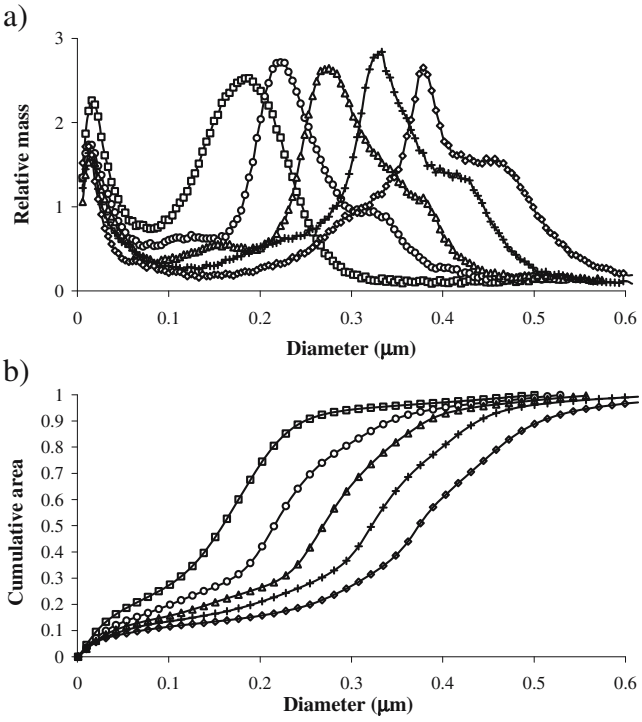

Fig. 3a–b Effect of contact time on the 2% (w/v) β-lactoglobulin aggregate formation induced by 10 mM Zn^{2+}: **a** size distributions; where *squares*, *circles*, *triangles*, *crosses*, and *diamonds* represent 15, 120, 360, 1440, and 2880 min, respectively; **b** the corresponding cumulative area plots

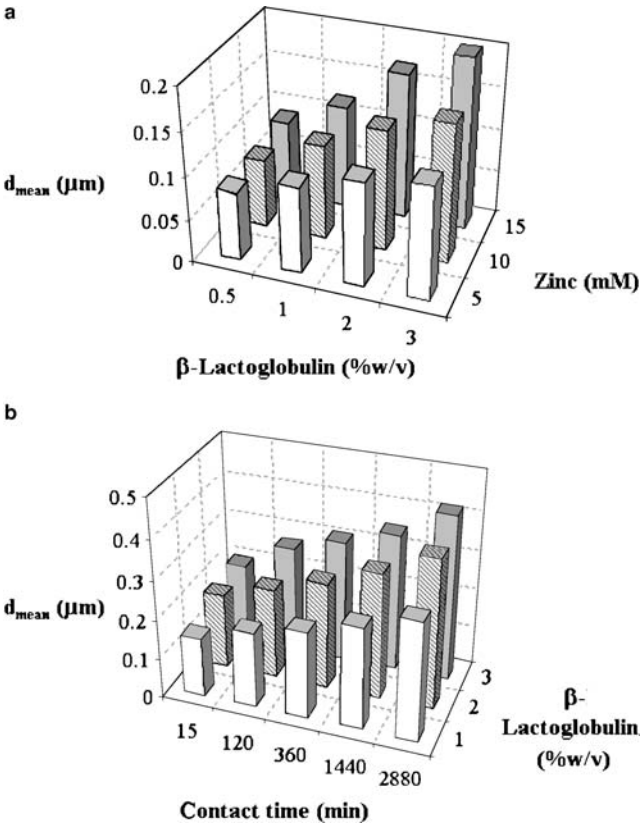

Fig. 4a–b **a** Relationship between β-lactoglobulin and Zn^{2+} concentrations and the particle size of the aggregates; **b** relationship between β-lactoglobulin concentration, the contact time and the particle size of the aggregates

in aggregation. Three-dimensional plots showing the effects of contact time and β-lactoglobulin concentration on the mean particle size (d_{mean}) of the aggregates are illustrated in Fig. 4b. Both factors contribute significantly to the aggregate formation.

Comparison between Ca^{2+} and Zn^{2+} in terms of extent of aggregation induced

To compare the extent of aggregate formation induced by Ca^{2+} or Zn^{2+}, 1% (w/v) β-lactoglobulin was mixed with 10 mM Ca^{2+} or Zn^{2+}, and the size distributions of the aggregates were observed at 15 and 1440 min contact times (Fig. 5). Figure 5 illustrates that 1% (w/v) β-lactoglobulin in Ca^{2+} coeluted with the void fraction (<0.01 μm), whereas that in Zn^{2+} displayed a peak maximum at 0.14 μm, indicating that at 15 min contact time Zn^{2+} could induce larger aggregates than Ca^{2+}. These results suggest that the kinetics of aggregate formation induced by Zn^{2+} are faster than those associated with Ca^{2+}. Similarly, at a prolonged contact time of 1440 min, β-lactoglobulin aggregates induced by Zn^{2+} (~0.30 μm) were larger than those induced by Ca^{2+} (~0.25 μm).

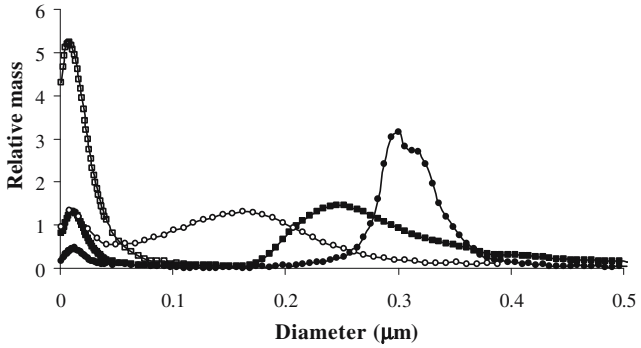

Fig. 5 Size distributions of salt-induced β-lactoglobulin aggregates, where *open squares* and *circles* represent 1% (w/v) β- lactoglobulin in 10 mM Ca^{2+} and Zn^{2+}, respectively, at 15 min contact time, and *filled squares* and *circles* represent 1% (w/v) β-lactoglobulin in 10 mM Ca^{2+} and Zn^{2+}, respectively, at 1440 min contact time

Furthermore, at a contact time of 1440 min, Zn^{2+}-induced β-lactoglobulin aggregates started to show bimodal characteristics, exhibiting peak maxima at both 0.30 and 0.32 μm, suggesting the presence of more than one group of aggregates.

Additionally, changes in the mean particle sizes of the salt-induced β-lactoglobulin aggregates were examined at various contact times (Fig. 6). The mean particle size increased with contact time in all cases (Fig. 6). For calcium-induced aggregation, the effect of contact time on the aggregate formation of 1% and 2% (w/v) β-lactoglobulin in 20 mM Ca^{2+} was investigated. The aggregation of 2% (w/v) β-lactoglobulin proceeded faster than that of 1% (w/v) β-lactoglobulin. At contact times longer than 720 min, the size of the aggregates remained constant. The maximum attainable aggregate sizes for 1% and 2% (w/v) β-lactoglobulin in 20 mM Ca^{2+} were 0.39 and 0.46 μm, respectively. For zinc-induced aggregation, the effect of contact time on the aggregate formation of 1%, 2%, and 3% (w/v) in 10 mM Zn^{2+} was investigated. From our observations made at 60 min contact time, the concentration of Ca^{2+} required to trigger β-lactoglobulin aggregation was higher than that of Zn^{2+}. Therefore, the concentration of Zn^{2+} (10 mM) used in this experiment was lower than the concentration of Ca^{2+} (20 mM). Similar to that observed with calcium-induced aggregation, the mean particle size of Zn^{2+}-induced β-lactoglobulin aggregates increased with contact time. The aggregation during the first 360 min proceeded faster than at longer contact times, and the size of the aggregates appeared to continue to grow even after a contact time of 2880 min. The trends in the growth of mean particle size were similar for all three β-lactoglobulin concentrations. As the β-lactoglobulin concentration was increased, the particle sizes of aggregates increased. The d_{mean} values of 3% (w/v) β-lactoglobulin in 10 mM Zn^{2+} were larger than those of 2% and 1% (w/v) β-lactoglobulin, respectively. Differences in the growth behavior of β-lactoglobulin aggregates induced by Ca^{2+} and Zn^{2+} might be due to differences in the molecular mechanisms involved in aggregate formation.

Association of Ca^{2+} or Zn^{2+} with β-lactoglobulin aggregates: the role of SdFFF–ICP–OES

Since the molecular mechanisms involved in the salt-induced aggregation are still debatable, it would be beneficial to develop an efficient method for studying the nature of protein aggregation. As stated by other investigators, the aggregation of proteins involves protein–protein interactions, via either the formation of disulfide linkages, sulfhydryl–disulfide interchange or protein–cation–protein cross-linkages [1, 7–10]. In this study, the use of a hyphenated technique of SdFFF with an element-specific detector (i.e., ICP–OES) has been proposed in order to investigate whether ionic bridges (i.e., protein–cation–protein cross-linkages) contribute to intermolecular association. Using SdFFF–ICP–OES, elemental fractograms of 1% (w/v) β-lactoglobulin incubated with 50 mM Ca^{2+} for 120 min were obtained, as shown in Fig. 7a and b. Also, elemental fractograms of 1% (w/v) β-lactoglobulin

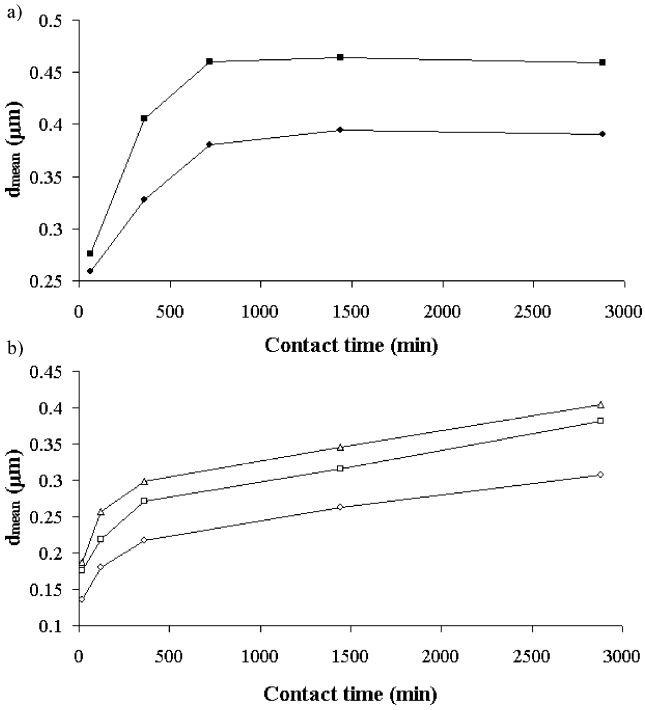

Fig. 6a–b Relationship between particle sizes of β-lactoglobulin aggregate and contact time for: **a** Ca^{2+}-induced β- lactoglobulin aggregate, where *filled circles* and *squares* represent 1% and 2% (w/v) β-lactoglobulin, respectively, in 20 mM Ca^{2+}; **b** Zn^{2+}-induced β-lactoglobulin aggregate, where *open circles*, *squares*, and *triangles* represent 1%, 2%, and 3% (w/v) β-lactoglobulin, respectively, in 10 mM Zn^{2+}

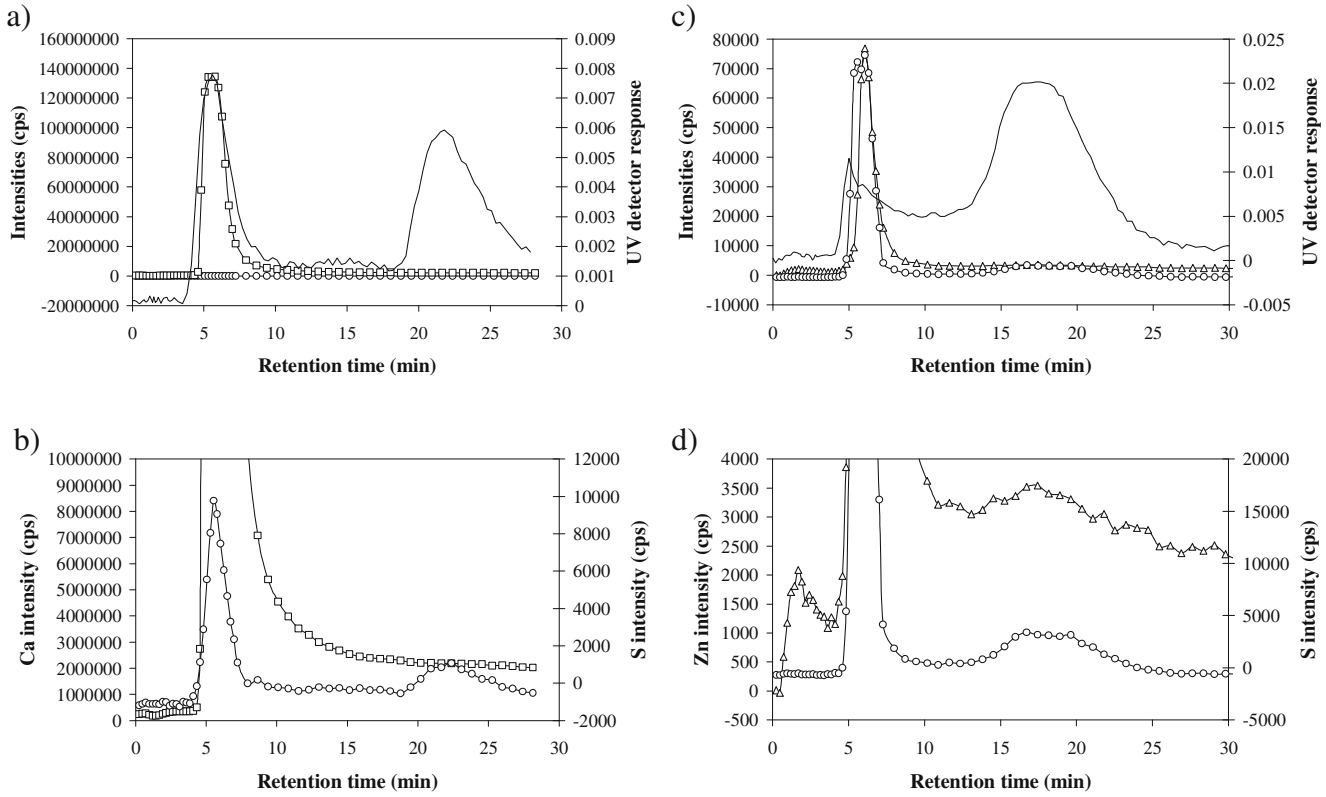

Fig. 7a–d **a** Fractograms of Ca^{2+}-induced β-lactoglobulin aggregates after 120 min contact time for 1% (w/v) β-lactoglobulin in 50 mM Ca^{2+}, where the *solid line* represents a UV fractogram, and *squares* and *circles* represent the fractograms of Ca and S, respectively; **b** an exploded view of **a**; **c** fractograms of Zn^{2+}-induced β-lactoglobulin aggregates after 120 min contact time for 1% (w/v) β-lactoglobulin in 5 mM Zn^{2+}, where the *solid line* represents a UV fractogram, and *circles* and *triangles* represent the fractograms of S and Zn, respectively; **d** an exploded view of **c**

incubated with 5 mM Zn^{2+} for 120 min were obtained, as shown in Fig. 7c and d. The hypothesis is that if the proteins were linked together by ionic bridges, both sulfur and the metal added should be detected in the large aggregate fraction. In contrast, if the metal added was not detected in the large aggregate fraction, linkages via ionic bridges were unlikely. By monitoring the Ca, Zn, and S in the fractionated β-lactoglobulin, S was found in both the small fraction close to the void fraction, as well as in the large aggregate fraction (Fig. 7b and d), confirming the presence of β-lactoglobulin aggregates. Similary, Zn was detected in both the void and the large aggregate fractions (Fig. 7c and d), suggesting that Zn^{2+} ionic bridges may also contribute to β-lactoglobulin aggregation. In contrast, Ca was detected only in the void fraction and was not detectable in the large aggregate fraction (Fig. 7a and b), indicating that intermolecular ion bridges between charged or carboxylic groups of β-lactoglobulin and Ca^{2+} were not observed in this study. This finding was in agreement with that reported earlier by Simons et al. [12] using aggregation studies of a chemically modified β-lactoglobulin. These studies suggest that the protein aggregation induced by different metal ions may occur via different mechanisms. The addition of Ca^{2+} might result in a local unfolding of the β-lactoglobulin, subsequently exposing the –SH, which might participate in disulfide linkages and hence cause aggregation [31].

In conclusion, the results from SdFFF–ICP–OES indicated that intermolecular ion bridges were unlikely for Ca^{2+}- but may occur for Zn^{2+}-induced β-lactoglobulin aggregation. Nonetheless, other techniques should also be used to confirm these conclusions. However, the SdFFF–ICP–OES definitely shows that their is an association between Zn^{2+} and β-lactoglobulin, but not between Ca^{2+} and β-lactoglobulin, which might be due to different binding constants of Zn^{2+} and Ca^{2+} with β-lactoglobulin.

Conclusion

This study demonstrates a novel application of SdFFF to study the process of β-lactoglobulin aggregation after thermal denaturation. The concentrations of both the β-lactoglobulin and the added metal ion, as well as the type of metal ion and the contact time, exhibited a combined effect on the extent of aggregate formation. The results

from this study could be used to guide food manufacturers in how to manipulate the aggregate size of β-lactoglobulin, which is used as an ingredient in food. Furthermore, SdFFF–ICP–OES was introduced as an invaluable and convenient tool for studying the mechanism of aggregation that avoids the need to use tedious chemical modification methods (e.g., succinylation and methylation of proteins [12]).

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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

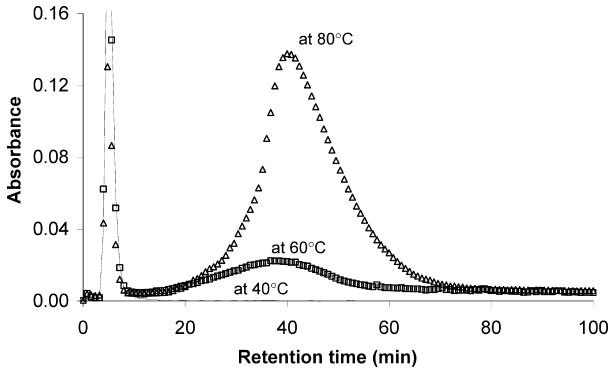

Figure 1. SdFFF fractograms of 5% (w/v) hen egg white protein at different heating temperatures, where —, □, and Δ represent 5% hen egg white protein at 40, 60, and 80 °C heating temperatures, respectively.

°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

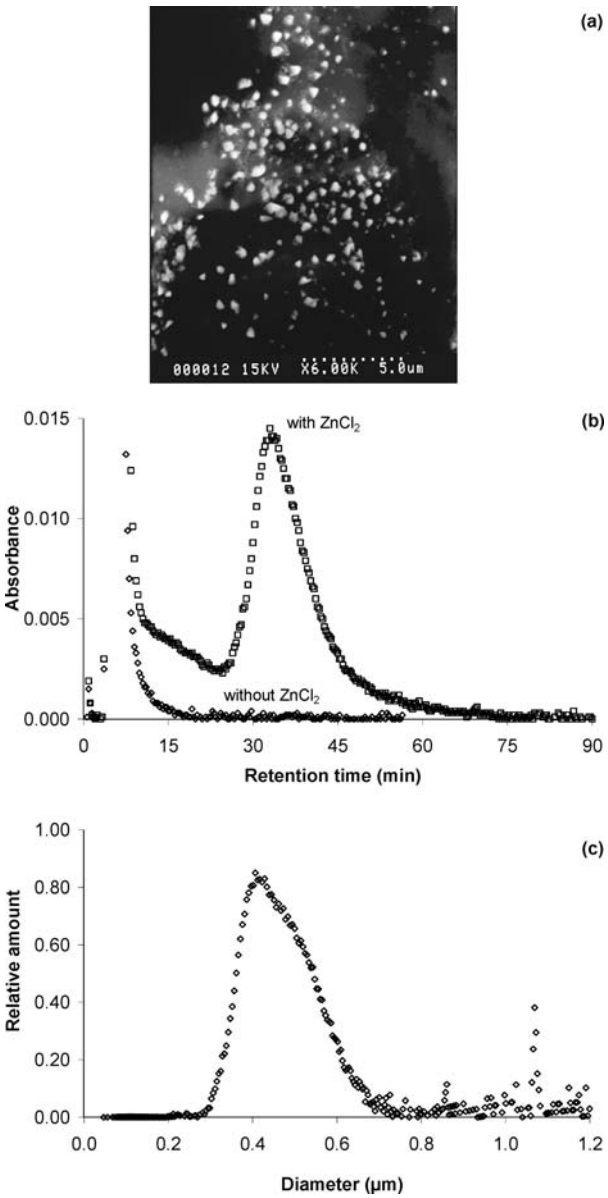

Figure 2. (**a**) SEM photograph of 5% (w/v) hen egg white protein mixed with 1 M $ZnCl_2$ at 15 min of incubation time, (**b**) SdFFF fractograms of 5% (w/v) hen egg white protein in the absence (◇) and presence (□) of 1.0 M $ZnCl_2$ at 15 min of incubation time, and (**c**) particle size distribution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

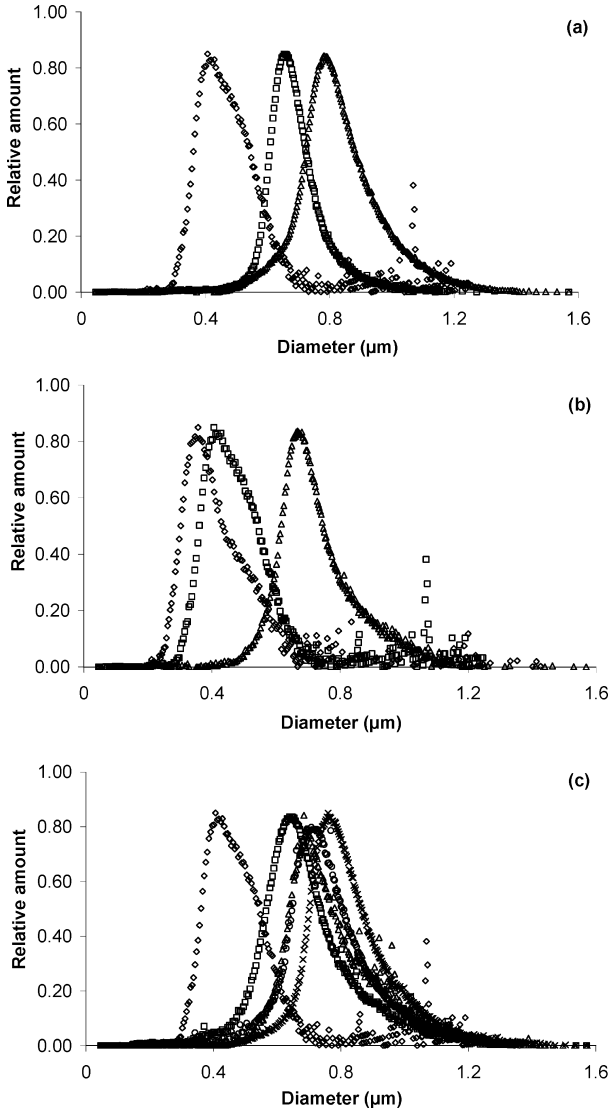

Figure 3. Particle size distributions of hen egg white protein aggregates obtained from SdFFF: (**a**) effect of hen egg white protein concentration (w/v) at 1.0 M $ZnCl_2$ and 15 min of incubation time, where ◇, □, and Δ represent 5, 6.25, and 7.5% (w/v) hen egg white protein, respectively; (b) effect 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.

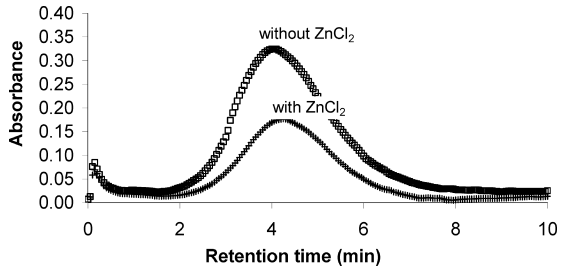

Figure 4. FlFFF fractograms of 1% (w/v) hen egg white protein in the absence (□) and presence (+) of 1.5 M $ZnCl_2$.

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

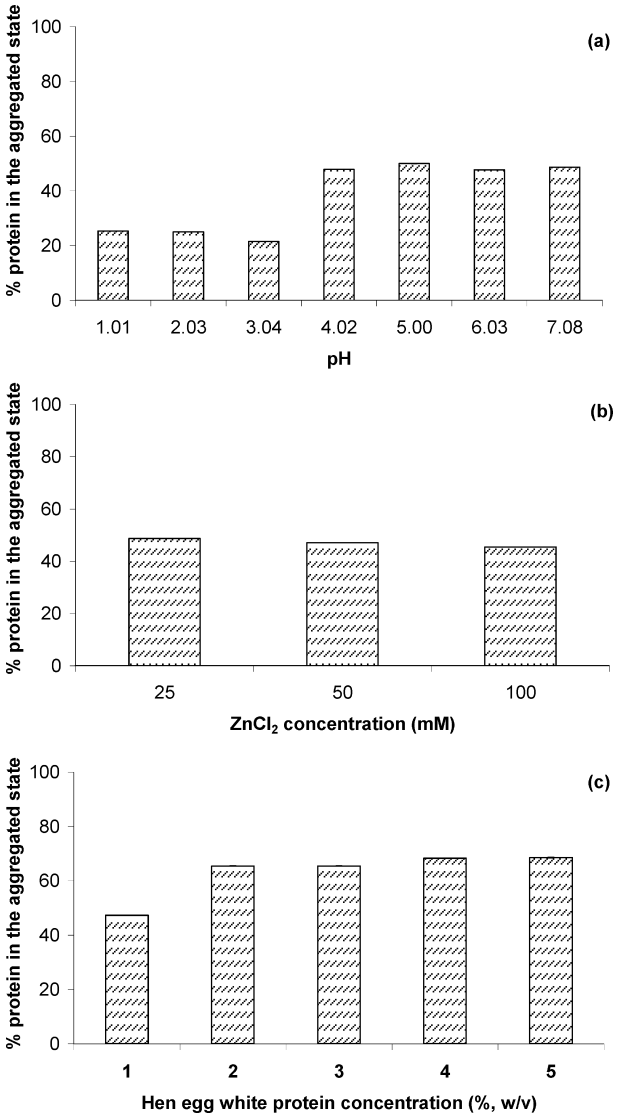

Figure 5. Parameters affecting efficiency of hen egg white protein usage for aggregation: (**a**) effect of pH for 1% (w/v) hen egg white protein mixed with 50 mM $ZnCl_2$, (**b**) effect of $ZnCl_2$ concentration (mM) for 1% (w/v) hen egg white protein at pH 6.0, and (**c**) effect of hen egg white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