JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Effects of Caseins on Thermal Stability of Bovine β-Lactoglobulin

YIE HUI YONG AND E. ALLEN FOEGEDING*

Department of Food, Bioprocessing and Nutrition Sciences and the Southeast Dairy Foods Research Center, Box 7624, North Carolina State University, Raleigh, North Carolina 27695-7624

Casein fractions have been shown to act as molecular chaperones and inhibit aggregation of whey proteins in dilute solutions (≤1% w/v). We evaluated if this approach would stabilize protein solutions at higher concentration and thermal processing temperatures desired for beverage applications. Mixtures of β-lactoglobulin (BLG) (6% w/v) with either β-casein (BCN) (0.01−2% w/v) or α_s-casein (ACN) (2% w/v) were adjusted to pH 6.0 and heated (70−90 °C) for 20 min, cooled, and then analyzed to determine the degree of aggregation. Aggregation was determined by solution turbidity as optical density (OD) at 400 or 600 nm. The addition of 0.05% (w/v) BCN or greater caused a drop in turbidity for solutions heated at 70−90 °C. In contrast, inhibition was observed in BLG−ACN mixtures at 70 °C but not at ≥75 °C. Moreover, prolonged heating (90 min) of BLG with 2% (w/v) BCN (pH 6.0) at 90 °C produced a clear solution while BLG−ACN solutions formed translucent gels after heating for 15 min. The weight-averaged molar mass and root-mean-square (rms) radius of soluble aggregates were determined by size exclusion chromatography in conjunction with multiangle laser light scattering (SEC-MALS). SEC-MALS confirmed the turbidity results by showing that the BLG−BCN mixture (8% w/v protein) produced aggregates with lower molar mass and smaller rms radius (majority 20−40 nm). These results showed that BCN is a feasible component to stabilize higher concentrations of whey proteins in beverages.

KEYWORDS: β-Lactoglobulin; β-casein; α-casein; thermal stability; aggregation

INTRODUCTION

Whey protein offers numerous functional benefits to food formulators in diverse applications including beverages (*1–3*). However, to make the labeling claim of a "high protein beverage", the FDA requires a protein concentration of ≥4.2% (w/v) (*4*). Most beverages above pH 4.6 require thermal processing before sale (*5*). The Code of Federal Regulations requires pasteurization for all milk and milk products in final package form intended for direct human consumption. The specified temperature and time are either 63 °C for 30 min, 72 °C for 15 s, or 89 °C for 1 s for milk products with fat contents less than 10% (*6*). However, heat treatment of milk above 70 °C during commercial processing operations results in a number of physicochemical changes, including denaturation and aggregation of whey proteins (*7*). Etzel (*5*) showed that beverages containing whey protein isolate were clear at pH 2−7 before heating, but only beverages at pH ≤ 3 remained clear after thermal processing at 88 °C for 120 s. Therefore, heat stability in the context of a beverage application refers to the ability of the solution to remain stable after thermal processing.

Bovine β-lactoglobulin (BLG), the most abundant whey protein, is a lipocalin that has been studied extensively in the past six decades (*8*). Although this globular protein has a well-defined structure (*9–12*) and has been shown to bind a variety of ligands at more than one binding site with different modes (*13–16*), its true function is still an enigma.

Thermal denaturation and aggregation of BLG are relevant to functional applications, such as gelation and stability in beverages, as well as being related to fouling in heat exchangers (*17*). Therefore, it is not surprising that a vast number of heat-induced aggregation studies were conducted from the aspect of pH effects (*18–23*), heating temperatures (*24–26*) and times (*24, 26*), protein concentrations (*27–29*), genetic variation (*30–32*), and ionic strength (*20, 33*). Kinetic models have been proposed for heat-induced aggregation of BLG (*27, 34*).

Attempts have been made to improve the thermal stability of BLG by adding different compounds. One way to monitor this improvement is through increases in denaturation temperature. Conjugating BLG with carboxymethyl cyclodextrin increases the denaturation temperature by 4 °C (*35*). The addition of 10 mM sodium dodecyl sulfate increases the denaturation temperature of BLG A and B by 3.4 and 4.4 °C, respectively (*36*). Also, Zhang et al. (*37*) showed that in the presence of dextran sulfate, the denaturation temperature of BLG was about 4.6 °C higher.

Another approach is to determine how various compounds alter the aggregation process. Toward that end, mixtures of BLG

* To whom correspondence should be addressed. Tel: +1-919-513-2244. Fax: +1-919-513-8023. E-mail: allen_foegeding@ncsu.edu.

10.1021/jf801658u CCC: $40.75
Published on Web 10/02/2008

and caseins have the potential to alter aggregation. Caseins are unusual as they are neither globular nor fibrous proteins in nature (*38*). They do not have well-defined secondary and tertiary structures but exist in nature as micellar aggregates (*39*). The four caseins differ markedly in many respects, including charge, hydrophobicity, and calcium sensitivity (*40*). Molecular chaperone-like properties have been reported with β-casein (BCN) and α_s-casein (ACN) (*39, 41, 42*). Molecular chaperones are able to stabilize proteins from unfolding, aggregating, and precipitating under stressed conditions, such as elevated temperature (*42*).

Studies have shown that caseins (ACN and/or BCN) decreased turbidity of whey proteins (single or multiple proteins) when heated at 70−85 °C (pH 6.0−7.1) (*42, 43*). Also, gel filtration showed that in the presence of ACN, soluble aggregates formed by BLG and α-lactalbumin were smaller than those formed in the absence of ACN (*39*). However, these investigations only involved total protein concentrations of ≤10 mg/mL, which are too low for industrial beverage applications. Thus, it is still not clear if this effect will be observed at higher concentrations. Moreover, the mechanism responsible for improved heat stability has not been established. Therefore, the objective of this investigation was to see if caseins would inhibit aggregation of BLG at a concentration more representative of what would be found in beverages. Additionally, the size and shape of the aggregates were analyzed as a means to help understand the mechanism of heat stability of BLG with and without caseins.

MATERIALS AND METHODS

Materials. Three different batches of purification of BLG (named as lots 1, 2, and 3) were prepared from BioPURE BLG (Davisco Foods International, Le Sueur, MN) using the method of Mailliart and Ribadeau-Dumas (*44*) and isolating conditions of pH 3.0 with 7% w/v NaCl. The protein solution was increased to pH 6.7 before dialysis at 4 °C and lyophilization. Two lots of BCN (lots 015K7410 and 30K7442, minimum of 90% BCN, named as BCN1 and BCN2, respectively) and one lot of ACN (lot 075K7425 with minimum of 70% ACN) were purchased from Sigma Chemical Co. (St. Louis, MO). One crude preparation of BCN (East BCN) was supplied by Eastern Regional Research Center (Wyndmoor, PA). Other chemicals were purchased from Fisher Scientific (Suwanee, GA) and Sigma Chemical Co. as reagent grade.

Electrophoresis. A NuPAGE Bis-Tris Electrophoresis System was used to identify the protein composition in each protein preparation. All gels and reagents were purchased from Invitrogen Corp. (Carlsbad, CA). Proteins were separated on precast NuPAGE 12% Bis-Tris gels using the procedure in the NuPAGE electrophoresis system instruction manual. The proteins were dissolved in NuPAGE LDS Sample Buffer with NuPAGE Sample Reducing Agent [containing 2,3-butanediol,1,4-dimercapto-(θ,θ)-(±)-], and 7 μg of protein was loaded in each well. NuPAGE MOPS SDS Running Buffer was used to separate the bands, and electrophoresis was done at a constant 200 V with the unit in an ice water bath. Gels were stained with Colloidal Blue Stain Kit and dried using Gel-Dry Drying Solution.

Determination of pH and Temperature Effects on Turbidity of BLG Solutions. BLG solutions (6% w/v protein) were prepared by hydrating in deionized water and adjusted to pH 5.8−6.8 with 1 N HCl prior to adjusting to final volume. The BLG solutions in 12 mm × 75 mm borosilicate glass tubes (1.7 mL) were heated in a water bath at 65−85 °C for 20 min. Heated samples were cooled immediately in a cold water bath. Overall aggregation was determined by solution turbidity as optical density (OD) at 400 nm using a Shimadzu UV160U spectrophotometer (Shimadzu, Columbia, MD). On the basis of these results, a constant pH of 6.0 was used for all subsequent experiments.

Evaluation of Effect of Caseins on Turbidity of BLG Solutions. All solutions were at pH 6.0 and heated in a water bath at 70−90 °C for 20 min before they were cooled immediately in an ice water bath. The overall aggregation was determined by solution turbidity (OD) at 400 and 600 nm.

Concentration-Dependent Effect of BCN1 on Turbidity of BLG Solutions. BCN1 (0−0.2 g) and 0.6 g of BLG were dissolved in 8 mL of deionized water. Once dissolved, the pH was adjusted to 6.0 using 1 N HCl before the volume was adjusted to 10 mL.

Effect of Different Caseins on Turbidity of BLG Solutions. BLG (6% w/v) was mixed with BCN2, East BCN, or ACN individually (2% w/v) using the aforementioned preparation.

Time-Dependent Development of Turbidity of BLG−Casein Solutions. BLG (6% w/v) was dissolved by mixing with or without BCN1 or ACN (2% w/v) in deionized water, and the pH was adjusted to 6.0 with 1 N HCl. The solutions were heated in a water bath for 120 min at 90 °C before they were cooled immediately in an ice water bath. The turbidity (OD 400 and 600 nm) of cooled solutions was measured.

Determining Molar Mass and Root-Mean-Square (rms) Radius of Using Size Exclusion Chromatography and a Multiangle Laser Light Scattering Detector (SEC-MALS). The average soluble molecular mass and rms radius of unheated and heated (75 °C for 20 min and 90 °C for 10 min) 6% (w/v) BLG solutions with or without 2% (w/v) BCN1 or ACN were determined using SEC-MALS. Unheated and heated samples were diluted with 20 mM imidazole buffer (pH 6.0) containing 0.05% NaN_3 to respective concentrations of 5 and 1 mg/mL BLG (stock solutions) and filtered through a 0.45 mm PVDF filter membranes (Whatman Inc., Florham Park, NJ). The protein concentration in stock solutions after filtration was measured using Pierce BCA Protein Assay Kit according to the instruction manual (Pierce, Rockford, IL). Protein separation of filtered stock solutions was carried out using a Shodex PROTEIN KW-804 column (Shoko America, Inc., La Jolla, CA) attached to a Waters HPLC system (Waters Corp., Milford, MA). A solution containing 20 mM imidazole and 0.05% NaN_3 at pH 6.0 was used as an eluent buffer. The sample injection volume was 170 μL for unheated samples and 70 μL for heated samples, the flow rate was 0.6 mL/min, and separation was performed at 30 °C column temperature. The concentration of each eluting slice was detected using a photodiode array (PDA) detector model 2996 (Waters Corp.) operating at 280 nm. A DAWN-EOS MALS photometer (Wyatt Technology, Santa Barbara, CA) fitted with a helium−neon laser (λ = 690 nm) and a K5-flow cell, combined with a differential refractive index (RI) detector model 2414 (Waters Corp.), was used for calculation of weight-averaged molar mass and rms radius of protein aggregates. The RI detector was used as the concentration detector. The weight-averaged molar mass and the rms radius of aggregates were calculated with a first-order Debye fit for unheated solutions and Zimm fit for heated solutions, using the refractive index increment (dn/dc) of 0.185 (*45*) for all samples. Data from the A_{280nm} measurement were processed by Waters Empower software. The amount of native protein was calculated as % area relative to unheated protein area (A_{280nm}). Light scattering data were processed with ASTRA software version 4.90.08 (Wyatt Technology). For heated samples, the formation of large aggregates resulted in the saturation of the MALS detectors (>10 V). The sensitivity of the photodiodes at selected angles was reduced 100-fold by putting jumpers on the detectors. The reduced sensitivity was compensated for in the ASTRA software by an adjustment in the normalization coefficient for those angles. The sensitivity of the 90° detector was not reduced to avoid any changes in the Wyatt DAWN EOS MALS detector calibration; therefore, the 90° detector was not included in data analysis.

Data Analysis. Each experiment was replicated three times, and mean values plus standard deviations are reported.

RESULTS AND DISCUSSION

Protein Composition. Electrophoretic patterns of BLGs and caseins under reducing conditions are shown in **Figure 1**. The three lots of purified BLG (lanes C−E in **Figure 1**) showed good repeatability, and their band patterns were almost identical

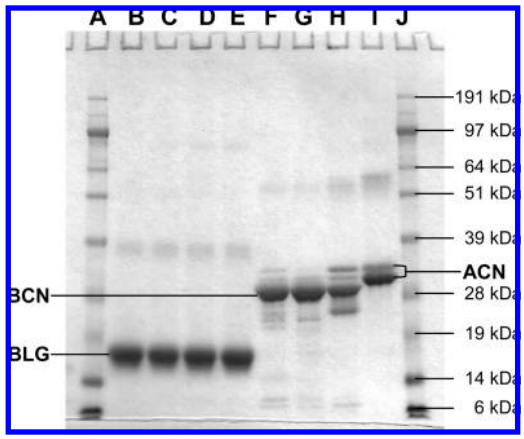

Figure 1. NuPAGE patterns of BLGs and caseins with 10% (v/v) NuPAGE sample reducing agent. Lanes: A, molecular weight markers; B, Sigma BLG (approximately 90% pure); C, purified BLG lot 1; D, purified BLG lot 2; E, purified BLG lot 3; F, BCN1; G, BCN2; H, East BCN; I, ACN; and J, molecular weight markers.

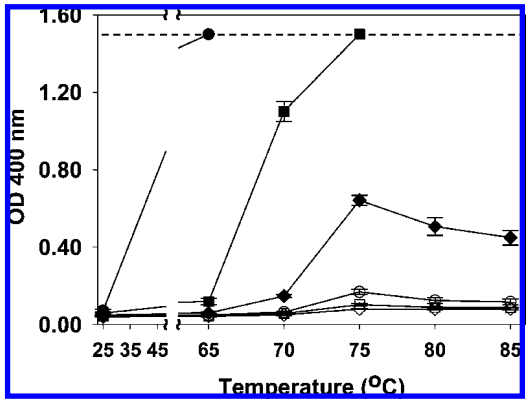

Figure 2. Temperature-dependent changes of turbidity of BLG at different pH values. A 6% (w/v) solution of BLG was dissolved in deionized water. pH treatments: pH 5.8 (●), pH 6.0 (■), pH 6.2 (◆), pH 6.4 (○), pH 6.6 (□), and pH 6.8 (◇). All points on the dashed line exceeded the maximum linear region for turbidity (standard deviations were not shown). Error bars represent one standard deviation.

to high purity Sigma BLG (lane B). The figure also illustrated well-resolved band patterns of BCNs (lanes F−H) and ACN (lane I) using the same electrophoretic system.

Determination of pH and Temperature Effects on Turbidity of BLG Solutions. The turbidity of 6% (w/v) BLG solutions (pH 5.8−6.8), heated at 65−85 °C, is shown in **Figure 2**. The profiles identified pH 6.0 as the suitable condition for further study as BLG solution remained as a homogeneous milk-white fluid when heated at 70 °C and above. For pH 6.4 and above, the turbidity of the heated solutions was insignificant, while at pH 5.8, large aggregates started to be apparent. The turbidity result was in agreement with turbidity development of 0.5% (w/w) whey protein isolate heated at 85 °C for 10 min (*43*). In that study, turbidity was evident on heating at pH 6.3, reached a maximum at pH 6.0, and formed precipitates when the pH was less than 6.0. There was minimum turbidity at pH 6.0 when solutions were heated at 65 °C. This coincided with a previous observation that a 5% (w/v) BLG solution (pH 6.0) heated at 65 °C for 24 h had a very slow decrease in concentration of native BLG (*22*). Therefore, we decided to assay in the heating range 70−90 °C to create a more stressed heating condition for subsequent experiments.

Concentration-Dependent Effect of BCN on Turbidity of BLG Solutions. The effect of 0.01−2% BCN on the aggregation

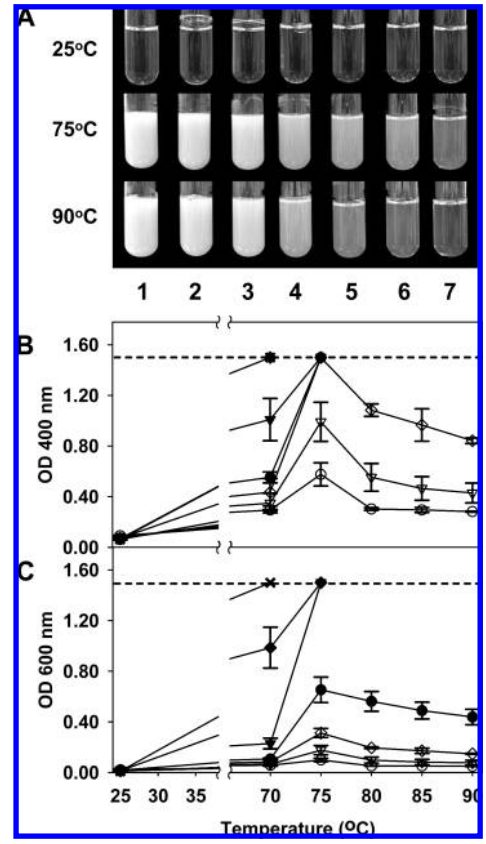

Figure 3. (**A**) Appearance of BLG−BCN1 solutions at 25, 75, and 90 °C. Amount of added BCN1 (w/v): 1, 0.00%; 2, 0.01%; 3, 0.05%; 4, 0.20%; 5, 0.50%; 6, 1.00%; and 7, 2.00%. Temperature-dependent changes of turbidity of BLG−BCN1 solutions at pH 6.0. OD: (**B**) 400 and (**C**) 600 nm. Amount of added BCN1 (w/v): 0.00% (×), 0.01% (◆), 0.05% (▼), 0.20% (●), 0.50% (◇), 1.00% (▽), and 2.00% (○). All points on dashed lines exceeded maximum linear region for turbidity (standard deviations were not shown). Error bars represent one standard deviation.

of BLG is seen in **Figure 3**. Turbidity was determined at 400 and 600 nm to cover a range of aggregates (*39, 43*). The appearance showed that unheated solutions were clear and that BCN decreased the turbidity of heated solutions (**Figure 3A**). Interestingly, heating at 90 °C produced clearer solutions than at 75 °C (lanes 5−7). The solution appearance coincided with turbidity measurements (**Figure 3B**,**C**). The maximum linear region for turbidity (OD = 1.5) was exceeded when BCN was below 0.2 (600 nm) or 0.5% (400 nm). The addition of 2% (w/v) BCN reduced turbidity (600 nm) to only 0.049 ± 0.002 after heating at 90 °C (**Figure 3C**). Even a small amount of BCN1 (0.05% w/v) decreased the turbidity of the BLG solution at 70 °C. On the basis of **Figure 3C**, a suppression of heat-induced aggregation of BLG could be achieved at a molar ratio 1:0.13 BLG to BCN or greater, similar to inhibition of heat-induced aggregation of whey protein isolate by α_{s1}/BCN at a mole ratio of 1:0.15 (*43*).

One interesting observation was the maximum turbidity at 75 °C, regardless of pH value (**Figure 2**) or BCN concentrations (**Figure 3B**). This could be related to differences in surface hydrophobicity brought about by changes in temperature.

The result in **Figure 3** showed a great potential of BCN to alter heat-induced aggregation of BLG solutions at concentrations higher than previously reported (*39, 42, 43*). The effects of BCN were concentration-dependent, which was also previously shown with ACN (0.5−5 mg/mL) incrementally decreasing turbidity of BLG (5 mg/mL) at pH 7.1 when heated at

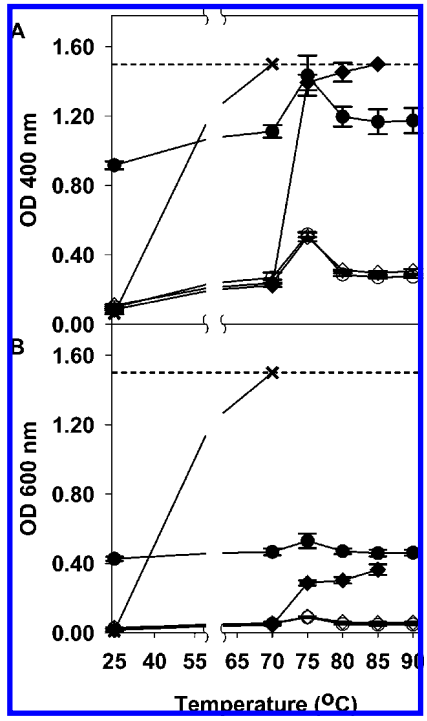

Figure 4. Temperature-dependent changes of turbidity of BLG−casein solutions (6% w/v BLG and 2% w/v casein) at pH 6.0. OD: (**A**) 400 and (**B**) 600 nm. Type of caseins: no casein added (×), BCN1 (○), BCN2 (◇), East BCN (●), and ACN (◆). All points on dashed lines exceeded the maximum linear region for turbidity (standard deviations were not shown). The BLG−ACN solution formed a gel after it was heated at 90 °C. Error bars represent one standard deviation.

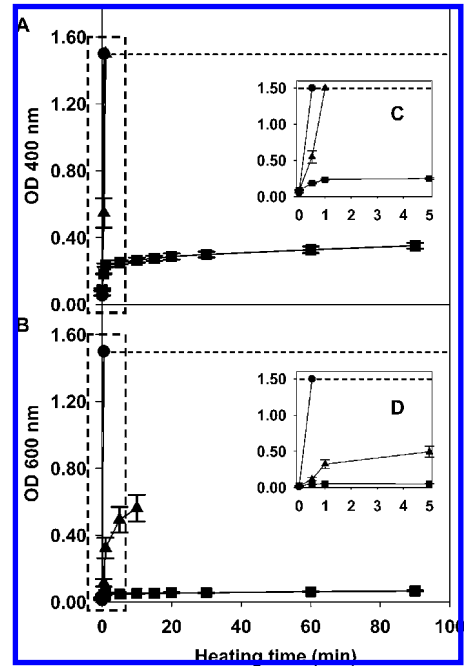

Figure 5. Time-dependent changes of turbidity of BLG−casein solutions (6% w/v BLG and 2% w/v casein) at pH 6.0. The heating was carried out at 90 °C for 120 min. OD: (**A**) 400, (**B**) 600, (C) 400 (enlarged 0−5 min), and (D) 600 nm (enlarged 0−5 min). Type of caseins: no casein added (●), BCN1 (■), and ACN (▲). All points on the dashed lines exceeded the maximum linear region for turbidity (standard deviations were not shown). BLG−BCN1 and BLG−ACN solutions formed a gel after they were heated for 120 and 15 min, respectively. Error bars represent one standard deviation.

70 °C (*42*). Of all the caseins, BCN is the most hydrophobic and devoid of cysteine residue (*40*). Its highly nonuniform distribution of hydrophilic and hydrophobic residues produces a distinct amphipathic character (*46*). As a result, BCN can form spherical micelles, depending on concentration and temperature (*47*). BCN has a critical micelle concentration of 0.05% (w/v) at 40 °C (*48*). In this investigation, in the presence of 6% BLG (w/v), BCN was soluble even at concentrations as high as 2% (w/v).

Effect of Different Caseins on Turbidity of BLG Solutions. Previous investigations have shown inhibition of whey protein aggregation by ACN (*39, 42*) and a mixture of α_{s1}/BCN (*43*) in dilute solutions. We tested ACN, a second isolation lot of BCN (BCN2) and a crude BCN (East BCN), to determine if similar trends were observed. Electrophoretic results (**Figure 1**) confirmed each main casein band pattern. There were slight differences of minor bands between BCN1 and BCN2 (lanes F and G), and the crude BCN (East BCN) contained ACN in addition to a faster migrating band below the major BCN band (lane H).

The turbidity profiles for each casein (2% w/v) mixed with BLG (6% w/v) are shown in **Figure 4**. The two lots of Sigma BCN produced identical turbidity profiles despite their differences in minor non-BCN components. Even though the BLG solution containing East BCN showed a higher turbidity before heating (0.92 ± 0.023), it was able to inhibit turbidity as was observed for the more pure Sigma BCN. In fact, normalized data (not shown) of East BCN gave a matching turbidity profile as Sigma BCN. This proved that BCN indeed suppressed turbidity development of BLG solutions regardless of the degree of impurities. On the other hand, BLG−ACN solutions showed different turbidity profiles than the BCNs. The solutions remained clear after they were heated at 70 °C, showing that ACN was able to inhibit turbidity formation at this lower heating temperature. However, when the heating temperature was increased to 75 °C, the turbidity of the BLG−ACN solutions increased drastically (1.4 ± 0.045). Heating at 80−85 °C further increased the turbidity, and the solutions formed gels at 90 °C (**Figure 4B**). It was obvious that BCN was a more effective inhibitor of turbidity development than ACN, especially for temperature ≥75 °C. Previous research has shown that ACN had a better chaperone activity (suppression of OD at 360 nm) than BCN at 25 and 37 °C (*42*) and can inhibit aggregation of BLG (*42*) and whey protein isolate (*39*) at 70 °C. It appears that ACN loses its chaperone-like activity after heating to 75 °C (**Figure 4**). In contrast, BCN maintains its ability to inhibit aggregation at temperatures up to 90 °C.

Time-Dependent Development of Turbidity of Casein-Added BLG Solutions. Previous experiments were all conducted using a 20 min heating period. It was possible that the caseins were just slowing down the reaction rather than altering the mode of aggregation. Data in **Figure 5** clearly show that for BCN1, the aggregation process was altered such that soluble aggregates were formed and terminated for up to 120 min when heating at 90 °C. The turbidity profile of BLG−BCN1 (**Figure 5A**,**C**) reached a plateau after heating for 1 min. Even though the turbidity did not change significantly, the viscosity of the solution increased through prolong heating and formed soft transparent gel after 120 min (data not shown). On the other hand, BLG−ACN solutions had lower turbidity than control BLG at OD 600 nm, but the solutions formed gels after heating for ≥15 min (**Figure 5B**). BLG alone remained a turbid solution after it reached its maximum turbidity in <1 min (**Figure 5C**,**D**)

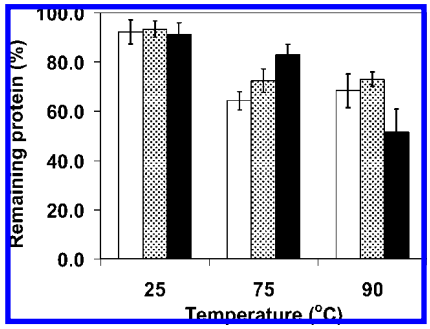

Figure 6. Relative remaining protein (%) of BLG−casein solutions (6% w/v BLG and 2% w/v casein) at pH 6.0 after filtration with 0.45 mm PVDF filter membranes as determined by the BCA protein assay. Type of caseins: no casein added (white bars), BCN1 (dotted bars), and ACN (black bars). Error bars represent one standard deviation.

without obvious visual increase in viscosity. These results suggested that all three solutions had different aggregation modes when heated at 90 °C.

Characterization of Soluble Aggregates. Turbidity provides a general measurement of aggregation. In this part, soluble heated aggregates were further characterized using SEC-MALS. Three temperatures (25, 75, and 90 °C representing nonheated, the highest turbidity, and the highest heat-treated nongelled samples, respectively) were used to compare the SEC-MALS profiles of the BLG control and BCN1- and ACN-added BLG solutions (based on results in **Figures 4** and **5**). After each treatment, the solutions were diluted and filtered before injection. The percentage of protein remaining after filtration was determined, and all samples at 25 °C had >90% of the protein passed through the filter (**Figure 6**). After heating at 75 °C (20 min), the protein passing through the filter dropped to 64, 72, and 83% for BLG control, BCN1, and ACN, respectively. At 90 °C (10 min), there was little change in the BLG control (68%) and BLG−BCN1 mixture (73%) whereby only 50% of the BLG−ACN aggregates were able to pass through the filter (**Figure 6**).

All profiles of samples at 25 °C showed the expected weight-averaged molar mass for BLG dimers (data not shown). The weight-averaged molar mass profiles for heated solutions (**Figure 7A**) showed trends that were similar to turbidity profiles (**Figures 4** and **5**). When heated at 75 °C, BLG−BCN1 aggregates had the lowest molar mass distribution (majority 4.5 $\times$ 10^6−2.1 $\times$ 10^7 g/mol) among all heated samples. This was followed by BLG−ACN aggregates (majority 5.6 $\times$ 10^6−8.2 $\times$ 10^7 g/mol). BLG without any casein showed the highest molar mass range among the three. After it was heated at 90 °C, the molar mass range for control BLG sample remained similar as the one at 75 °C. However, BLG solutions containing either BCN or ACN at 90 °C increased in molar mass. Changes in rms radius are shown in **Figure 7B**. When heated at 75 °C, the rms radius for BLG−BCN1 aggregates was only 20−40 nm, while the range for BLG−ACN aggregates was higher (majority 30−63 nm). These BLG−casein mixtures had lower rms radius (particularly BCN1) as compared to heated BLG without any casein (majority 55−98 nm). After heating at 90 °C for 10 min, the rms radius of BLG control (48−88 nm) was slightly lower than the one heated at 75 °C (**Figure 7B**), even though they had a similar molar mass range (**Figure 7A**). On the other hand, the rms radius of the filtered BLG−BCN1 aggregates at 90 °C was increased to 37−100 nm, although their molar mass was 10 times smaller than aggregates of BLG control. This suggested that BLG−BCN1 aggregates were more linear, which matched with the increasing viscosity of heated BLG−BCN1 samples

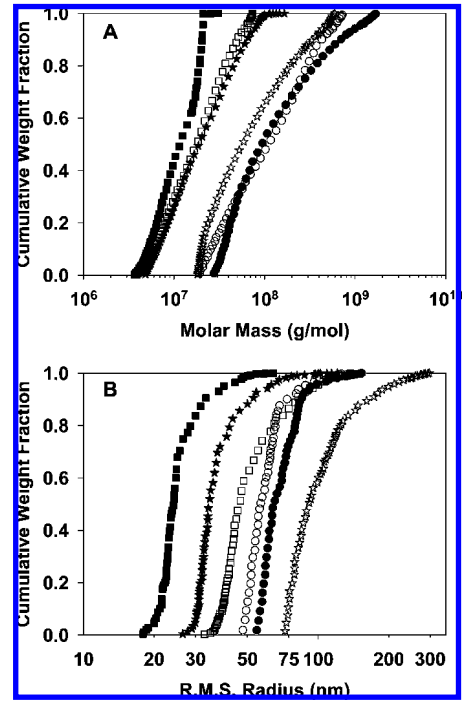

Figure 7. Typical (**A**) weight-averaged soluble molar mass and (**B**) rms radius distribution of BLG−casein solutions (6% w/v BLG and 2% w/v casein) at pH 6.0 as determined by SEC-MALS. Type of caseins and temperature treatments: no casein added, 75 °C (20 min) (●); no casein added, 90 °C (10 min) (○); BCN1, 75 °C (20 min) (■); BCN1, 90 °C (10 min) (□); ACN, 75 °C (20 min) (★); and ACN, 90 °C (10 min) (☆).

as previously mentioned in the time-dependent development of turbidity section. Among the three BLG systems, BLG−ACN aggregates showed the highest range of rms radius (74−210 nm) at 90 °C, and this did not account for 50% of the total protein that was removed by filtration. Clearly, much larger aggregates were formed in BLG−ACN mixtures. Both BLG−BCN1 and BLG−ACN solutions showed a major increase in rms radius at the higher heating temperature (**Figure 7**) and gelled at longer heating times (**Figure 5**), suggesting that the elongation of the aggregates was continuing until gel networks were formed.

On the basis of the results from this study and a model proposed by Lefevre and Subirade (*49*), we suggest the possible mechanism. When heated at 70 °C and above, the dimers of 6% (w/v) control BLG solutions were partially unfolded and formed opaque particulate aggregates. Because of concentration limits, these particulate aggregates were not crowded enough to form particulate gels. In the presence of BCN or ACN at 70 °C, the usual path to form the particulate aggregates was blocked. Instead, dimer or monomer intermediates interacted with BCN or ACN to form fine-stranded aggregates. However, when the temperature was increased to 75 °C and above, the BLG monomer was extensively unfolded and the previous binding site was changed. Instead, a new binding site was exposed that had weaker binding site for ACN than BCN. The BLG−BCN system formed clear fine-stranded aggregates, while the BLG−ACN system proceeded to form larger, translucent aggregates. Through prolong heating time or at higher heating temperature, these aggregates continued to elongate and associate to form a three-dimensional gel network.

In summary, our results revealed that both BCN and ACN showed chaperone effects by altering aggregation of BLG at pH 6 at high protein concentration (8% w/v total protein). While the addition of BCN was shown to be effective at decreasing

turbidity of BLG over a range of temperatures (70−90 °C), ACN lost its chaperone ability when the temperature was ≥75 °C. It is also apparent that gelling behavior of BLG under the influence of BCN and ACN will be an interesting topic for future study.

ACKNOWLEDGMENT

This is Paper FSR-08-13 of the Journal Series of the Department of Food, Bioprocessing and Nutrition Science, North Carolina State University (Raleigh, NC). The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service of the products named nor criticism of similar ones not mentioned. We are very grateful for the proteins donated by Davisco Foods International and the Eastern Regional Research Center and to Dr. Bongkosh Vardhanabhuti for technical assistance on SEC-MALS analysis.

LITERATURE CITED

(1) Walzem, R. L.; Dillard, C. J.; German, J. B. Whey components: millennia of evolution create functionalities for mammalian nutrition: What we know and what we may be overlooking. *Crit. Rev. Food Sci. Nutr.* **2002**, *42*, 353–375.

(2) U.S. Dairy Export Council. Reference Manual for U.S. Whey and Lactose Products; Arlington, VA, 2004; www.usdec.org/files/PDFs/2008ReferenceManuals/Whey% 5FLactose%5FReference%5FManual%5FComplete2%5FOptimized.pdf.

(3) Johnson, M. E.; Lucey, J. A. Major technological advances and trends in cheese. *J. Dairy Sci.* **2006**, *89*, 1174–1178.

(4) Code of Federal Regulations. Nutrient content claims for "good source", "high", "more" and "high potency". *Code of Federal Regulations*; Office of the Federal Register: College Park, MD, 2008; Title 21, Part 101.54.

(5) Etzel, M. R. Manufacture and use of dairy protein fractions. *J. Nutr.* **2004**, *134*, 996–1002.

(6) Code of Federal Regulations. Mandatory pasteurization for all milk and milk products in final package form intended for direct human consumption. *Code of Federal Regulations*; Office of the Federal Register: College Park, MD, 2008; Title 21, Part 1240.61.

(7) Patel, H. A.; Singh, H.; Anema, S. G.; Creamer, L. K. Effects of heat and high hydrostatic pressure treatments on disulfide bonding interchanges among the proteins in skim milk. *J. Agric. Food Chem.* **2006**, *54*, 3409–3420.

(8) Sawyer, L.; Kontopidis, G. The core lipocalin, bovine β-lactoglobulin. *Biochim. Biophys. Acta* **2000**, *1482*, 136–148.

(9) McKenzie, H. A.; Ralston, G. B.; Shaw, D. C. Location of sulfhydryl and disulfide groups in bovine β-lactoglobulins and effects of urea. *Biochemistry* **1972**, *11*, 4539–4547.

(10) Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadarao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **1986**, *324*, 383–385.

(11) Brownlow, S.; Morais Cabral, J. H.; Cooper, R.; Flower, D. R.; Yewdall, S. J.; Polikarpov, I.; North, A. C. T.; Sawyer, L. Bovine β-lactoglobulin at 1.8 Å resolution—Still an enigmatic lipocalin. *Structure* **1997**, *5*, 481–495.

(12) Kontopidis, G.; Holt, C.; Sawyer, L. Invited review: β-lactoglobulin: Binding properties, structure, and function. *J. Dairy Sci.* **2004**, *87*, 785–796.

(13) Sawyer, L.; Brownlow, S.; Polikarpov, I.; Wu, S. Y. β-Lactoglobulin: Structural studies, biological clues. *Int. Dairy J.* **1998**, *8*, 65–72.

(14) Wu, S. Y.; Dolores Pérez, M.; Puyol, P.; Sawyer, L. β-Lactoglobulin binds palmitate within its central cavity. *J. Biol. Chem.* **1999**, *274*, 170–174.

(15) Kontopidis, G.; Holt, C.; Sawyer, L. The ligand-binding site of bovine β-lactoglobulin: Evidence for a function. *J. Mol. Biol.* **2002**, *318*, 1043–1055.

(16) Konuma, T.; Sakurai, K.; Goto, Y. Promiscuous binding of ligands by β-lactoglobulin involves hydrophobic interactions and plasticity. *J. Mol. Biol.* **2007**, *368*, 209–218.

(17) Bromley, E. H. C.; Krebs, M. R. H.; Donald, A. M. Aggregation across the length-scales in β-lactoglobulin. *Faraday Discuss.* **2005**, *128*, 13–27.

(18) Harwalkar, V. R. Measurement of thermal denaturation of β-lactoglobulin at pH 2.5. *J. Dairy Sci.* **1980**, *63*, 1043–1051.

(19) Casal, H. L.; Köhler, U.; Mantsch, H. H. Structural and conformational changes of β-lactoglobulin B: An infrared spectroscopic study of the effect of pH and temperature. *Biochim. Biophys. Acta* **1988**, *957*, 11–20.

(20) Renard, D.; Lefebvre, J.; Griffin, M. C. A.; Griffin, W. G. Effect of pH and salt environment on the association of β-lactoglobulin revealed by intrinsic fluorescence studies. *Int. J. Biol. Macromol* **1998**, *22*, 41–49.

(21) Verheul, M.; Roefs, S. P.; de Kruif, K. G. Kinetics of heat-induced aggregation of β-lactoglobulin. *J. Agric. Food Chem.* **1998**, *46*, 896–903.

(22) Hoffmann, M. A.; van Mil, P. J. Heat-induced aggregation of β-lactoglobulin as a function of pH. *J. Agric. Food Chem.* **1999**, *47*, 1898–1905.

(23) Wada, R.; Fujita, Y.; Kitabatake, N. Effects of heating at neutral and acid pH on the structure of β-lactoglobulin A revealed by differential scanning calorimetry and circular dichroism spectroscopy. *Biochim. Biophys. Acta* **2006**, *1760*, 841–847.

(24) Prabakaran, S.; Damodaran, S. Thermal unfolding of β-lactoglobulin: Characterization of initial unfolding events responsible for heat-induced aggregation. *J. Agric. Food Chem.* **1997**, *45*, 4303–4308.

(25) Panick, G.; Malessa, R.; Winter, R. Differences between the pressure- and temperature-induced denaturation and aggregation of β-lactoglobulin A, B and AB monitored by FT-IR spectroscopy and small-angle X-ray scattering. *Biochemistry* **1999**, *38*, 6512–6519.

(26) Sava, N.; Van der Plancken, I.; Claeys, W.; Hendrickx, M. The kinetics of heat-induced structural changes of β-lactoglobulin. *J. Dairy Sci.* **2005**, *88*, 1646–1653.

(27) Roefs, S. P.; de Kruif, K. G. A model for the denaturation and aggregation of β-lactoglobulin. *Eur. J. Biochem.* **1994**, *226*, 883–889.

(28) Iametti, S.; Cairoli, S.; De Gregori, B.; Bonomi, F. Modifications of high-order structures upon heating of β-lactoglobulin: Dependence on the protein concentration. *J. Agric. Food Chem.* **1995**, *43*, 53–58.

(29) Qi, X. L.; Brownlow, S.; Holt, C.; Sellers, P. Thermal denaturation of β-lactoglobulin: Effect of protein concentration at pH 6.75 and 8.05. *Biochim. Biophys. Acta* **1995**, *1248*, 43–49.

(30) Nielsen, B. T.; Singh, H.; Latham, J. M. Aggregation of bovine β-lactoglobulins A and B on heating at 75°C. *Int. Dairy J.* **1996**, *6*, 519–527.

(31) Manderson, G. A.; Hardman, M. J.; Creamer, L. K. Effect of heat treatment on the conformation and aggregation of β-lactoglobulin A, B and C. *J. Agric. Food Chem.* **1998**, *46*, 5052–5061.

(32) Le Bon, C.; Durand, D.; Nicolai, T. Influence of genetic variation on the aggregation of heat-denatured β-lactoglobulin. *Int. Dairy J.* **2002**, *12*, 671–678.

(33) Baussay, K.; Le Bon, C.; Nicolai, T.; Durand, D.; Busnel, J. P. Influence of the ionic strength on the heat-induced aggregation of the globular protein β-lactoglobulin at pH 7. *Int. J. Biol. Macromol* **2004**, *34*, 21–28.

(34) Arnaudov, L. N.; de Vries, R. Theoretical modeling of the kinetics of fibrilar aggregation of bovine β-lactoglobulin at pH 2. *J. Chem. Phys.* **2007**, *126*, 145106.

(35) Hattori, M.; Okada, Y.; Takahashi, K. Functional changes in β-lactoglobulin upon conjugation with carboxymethyl cyclodextrin. *J. Agric. Food Chem.* **2000**, *48*, 3789–3794.

(36) Boye, J. I.; Ma, C. Y.; Ismail, A. Thermal stability of β-lactoglobulins A and B: Effect of SDS, urea, cysteine and N-ethylmaleimide. *J. Dairy Res.* **2004**, *71*, 207–215.

(37) Zhang, G.; Foegeding, E. A.; Hardin, C. C. Effect of sulfated polysaccharides on heat-induced structural changes in β-lactoglobulin. *J. Agric. Food Chem.* **2004**, *52*, 3975–3981.
(38) Alaimo, M. H.; Farrell, H. M.; Germann, M. W. Conformational analysis of the hydrophobic peptide α_{s1}-casein (136−196). *Biochim. Biophys. Acta* **1999**, *1431*, 410–420.
(39) Bhattacharyya, J.; Das, K. P. Molecular chaperone-like properties of an unfolded protein, α_s-casein. *J. Biol. Chem.* **1999**, *274*, 15505–15509.
(40) Swaisgood, H. E. Chemistry of the caseins. In *Advanced Dairy Chemistry: Proteins*, 3rd ed.; Fox, P. F., McSweeney, P. L. H., Eds.; Kluwer Academic/Plenum Publishers: New York, NY, 2003; Vol. 1, pp 139−202.
(41) Zhang, X.; Fu, X.; Zhang, H.; Liu, C.; Jiao, W.; Chang, Z. Chaperone-like activity of β-casein. *Int. J. Biochem. Cell Biol.* **2005**, *37*, 1232–1240.
(42) Morgan, P. E.; Treweek, T. M.; Lindner, R. A.; Price, W. E.; Carver, J. A. Casein proteins as molecular chaperones. *J. Agric. Food Chem.* **2005**, *53*, 2670–2683.
(43) O'Kennedy, B. T.; Mounsey, J. S. Control of heat-induced aggregation of whey proteins using casein. *J. Agric. Food Chem.* **2006**, *54*, 5637–5642.
(44) Mailliart, P.; Ribadeau-Dumas, B. Preparation of β-lactoglobulin and β-lactoglobulin-free proteins from whey retentate by NaCl salting out at low pH. *J. Food Sci.* **1988**, *53*, 743–745, 752.
(45) Huglin, M. B. Specific refractive index increments. In *Light Scattering from Polymer Solutions*; Huglin, M. B., Ed.; Academic Press: New York, NY, 1972; pp 165−331.
(46) Dickinson, E. Interfacial, emulsifying and foaming properties of milk proteins. In *Advanced Dairy Chemistry: Proteins*, 3rd ed.; Fox, P. F., McSweeney, P. L. H., Eds.; Kluwer Academic/Plenum Publishers: New York, NY, 2003; Vol. 1, pp 1229−1260.
(47) Leclerc, E.; Calmettes, P. Interactions in micellar solutions of β-casein. *Phys. Rev. Lett.* **1998**, *78*, 150–153.
(48) O'Connell, J. E.; Grinberg, V. Y.; de Kruif, C. G. Association behavior of β-casein. *J. Colloid Interface Sci.* **2003**, *258*, 33–39.
(49) Lefevre, T.; Subirade, M. Molecular differences in the formation and structure of fine-stranded and particulate β-lactoglobulin gels. *Biopolymers* **2000**, *54*, 578–586.

Received for review May 29, 2008. Revised manuscript received August 25, 2008. Accepted August 29, 2008. Support from the North Carolina Agricultural Research Service, Dairy Management Inc., and The Southeast Dairy Foods Research Center is gratefully acknowledged.

JF801658U