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β-Lactoglobulin Self-Assembly: Structural Changes in Early Stages and Disulfide Bonding in Fibrils

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Supporting Information

ABSTRACT: Bovine β-lactoglobulin (β-Lg) self-assembles into long amyloid-like fibrils when heated at 80 °C, pH 2, and low ionic strength (<0.015 mM). Heating β-Lg under fibril-forming conditions shows a lag phase before fibrils start forming. We have investigated the structural characteristics of β-Lg during the lag phase and the composition of β-Lg fibrils after their separation using ultracentrifugation. During the lag phase, the circular dichroism spectra of heated β-Lg showed rapid unfolding, and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of samples showed increasing hydrolysis of β-Lg. The SDS-PAGE profiles of fibrils separated by ultra centrifugation showed that after six hours, the fibrils consisted of a few preferentially accumulated peptides. Two-dimensional SDS-PAGE under reducing and nonreducing conditions showed the presence of disulfide-bonded fragments in the fibrils. The sequences in these peptide bands were characterized by in-gel digestion electrospray ionization (ESI)-MS/MS. The composition of solubilized fibrils was also characterized by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) MS/MS. Both MS analyses showed that peptides in fibrils were primarily from the N-terminal region, although there was some evidence of peptides from the C-terminal part of the molecule present in the higher molecular weight gel bands. We suggest that although the N-terminal region of β-Lg is almost certainly involved in the formation of the fibrils, other peptide fragments linked through disulfide bonds may also be present in the fibrils during self-assembly.

KEYWORDS: *β-lactoglobulin, self-assembly, hydrolysis, disulfide bond, lag phase, SDS-PAGE, mass spectrometry, circular dichroism spectroscopy, amyloid-like nanofibrils*

INTRODUCTION

Bovine β-lactoglobulin (β-Lg) is a water-soluble globular protein present in milk at concentrations of approximately 0.18 to 0.50% (w/v)[1] and accounts for approximately 50% of the total whey proteins. It has a molecular weight of approximately 18.4 kDa and mainly exists in polymorphic forms A and B that differ, respectively, by the substitutions Asp for Gly at position 64 (on an exposed loop) and Val for Ala at position 118 (at a partly internal site).[1] In its native state, β-Lg has two disulfide bonds between residues Cys66−Cys160, linking the CD loop with the C terminal region and Cys106−Cys119 linking loops G and H, and a free thiol group at Cys121.[2] At pH 2 and low ionic strength β-Lg exists as a monomer[3] and shows high heat stability.[4] It has a well-defined secondary structure consisting of 50% β-sheet, 10% α-helix, and 35% random coil,[5] the latter mostly found on the structure-stabilizing flexible loops connecting the different β-strands in the structure.[6] β-Lg self-assembles into amyloid-like fibrils upon incubation with solvents[7] or concentrated urea[8] and on heating at low ionic strength and low pH.[7,9−11] Protein-based fibrils have received intense attention due their potential applications in biotechnology[12] and as food ingredients to improve functionality.[13]

Heating at low pH and low ionic strength has been the most common method used for investigating β-Lg fibril formation, due to relatively rapid kinetics of self-assembly under these conditions. β-Lg self-assembly shows typical characteristics of amyloid fibril formation[14−16] with fibrils showing β-sheets running perpendicular to the fibril axis.[14] Fibrils formed during heating at pH 2 appear long and semiflexible, and they have a diameter of ∼4 nm and lengths of up to 10 μm.[10] The factors affecting the kinetics of self-assembly at low pH include the temperature of heating,[17−19] mode of heating,[20] pH,[15] seeding[13,21] and shear conditions during self-assembly.[21,22] In unstirred solutions, β-Lg fibril formation shows an initial lag phase, followed by a rapid increase in the rate of self-assembly, i.e. a growth phase, and finally a stationary phase, during which the rate of self-assembly declines.[14,15] It is reported that during the lag phase the monomers undergo activation that leads to the formation of nuclei that then promote self-assembly.[9,14,21] Most studies characterizing β-Lg self-assembly[16,18,23] use shear during heating. Shear force during self-assembly affects the kinetics of fibril formation by inducing changes in protein structure[24] and by enhancing nucleation[22] in the lag phase. Enhanced nucleation can come about through shear-induced fragmentation of protofibrils, whose fragments act as nuclei and promote self-

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assembly, i.e. secondary or 'heterogeneous' nucleation.[21,25] Thus, information about the behavior of β-Lg before formation of nuclei in the lag phase and the nature of the nuclei still remains elusive.

A recent study has suggested a relationship between an increase in the rate of self-assembly and the rate of hydrolysis of β-Lg at high temperatures.[18] Akkermans et al.[23] reported that peptides generated during heating at low pH are the building blocks of fibrils, but this analysis was carried out on fibrils formed after heating β-Lg for 20 h at 85 °C under constant shear conditions. Separately, fibrils from hen egg-white lysozyme have been reported to contain intact monomers as well as peptides generated upon hydrolysis.[26] Thus, it is not yet known whether hydrolysis is a prerequisite for β-Lg self-assembly. Nor is the composition of fibrils or protofibrils formed from β-Lg at short heating times in the growth phase known with certainty.

In the current study, we explore the structural changes in the β-Lg monomer during the lag phase and investigate the role of hydrolysis in the formation of β-Lg fibrils, to gain insight into the structural transitions and chemical changes in β-Lg preceding self-assembly. Accordingly the composition of fibrils formed after different heating times during self-assembly was studied by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after their separation using ultracentrifugation. 1D and 2D nonreducing and reducing SDS-PAGE were used to determine disulfide bonding in fibrils, while mass spectrometry was used to characterize the makeup of β-Lg fibrils in the growth phase. The results of this study may inform the design of protocols for control of β-Lg self-assembly into fibrils.

MATERIALS AND METHODS

Unless mentioned otherwise, all chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louis, MO) and all samples and reagents were made in Milli Q water.

Isolation of β-Lactoglobulin. β-Lactoglobulin (β-Lg) was isolated from whey protein isolate (WPI) 8855 (Fonterra Cooperative Ltd., Auckland, New Zealand) using a modification of the salt precipitation method described previously.[27] The composition of WPI was total protein content 93.5% w/w (as is basis), moisture 4.7%w/w, ash 0.3%w/w, with the rest being carbohydrate and fat. The final precipitates obtained from the salt precipitation were dissolved in water at pH 2 and dialyzed for 48 h at 4 °C followed by freeze-drying. The freeze-dried β-Lg was used for experiments without further purification. The total protein content of β-Lg was analyzed to be 97% by Leco, total combustion method (AOAC 968.06); nondenaturing PAGE on the sample showed a mixture of variants A and B.

Preparation of Fibrils. β-Lg powder was dissolved in water at pH 2 and stirred at 4 °C for at least 8 h to allow complete hydration of the protein. The hydrated protein solution was centrifuged at 44000*g* for 30 min at 20 °C, and the supernatant was filtered (pore size 0.2 μm syringe filter (Minisart CE, Sartorius Stedim Biotech GmbH, Goettingen, Germany) to remove any insoluble material. Water was added to achieve a final β-Lg concentration of 1% (w/v), and then pH was readjusted to 2.00 ± 0.02 using 6 M HCl. Samples were transferred to 1.5 mL polypropylene tubes (Eppendorf, Hamburg, Germany, sample volume 1 mL) or Kimax glass tubes (Schott, Elmsford, NY, volume 10 mL) and then heated in a temperature-controlled water bath (Jeio Tech, Seoul, South Korea) maintained at 80 ± 0.2 °C in triplicates. Since the objective of this study was to investigate changes during the lag phase all experiments were conducted without shear. Concentrations of β-Lg were determined by measuring the absorbance at 278 nm (Ultraspec 2000, Pharmacia Biotech, Cambridge, U.K.) and applying an extinction coefficient of 0.94 cm^2/mg.[28] Samples were taken at different holding times and quenched by rapid cooling in an ice bath. The cooled samples were analyzed by the Thioflavin T assay at 20 °C with a maximum delay of 1 h after their removal. Samples intended for SDS-PAGE were frozen and stored at −18 °C until analysis.

Thioflavin T Assay for Detection of Fibrils. The thioflavin T (ThT) assay reported previously[15] was used for studying the rate of self-assembly. ThT fluorescence intensities in samples were measured at 486 nm on a spectrofluorometer (FP-6200, Jasco, Tokyo, Japan). The intensities were recorded as average values of triplicate measurements after subtracting the intensity of the ThT solution (blank). The intensity of an unheated control was subtracted from all samples to get net fluorescence intensities, which were then plotted against heating time.

The ThT fluorescence intensities were fitted using the same eq 1 used to characterize β-Lg self-assembly[15] and originally proposed by Morris et al.[29] in which f_t denotes fluorescence at time t (h), while α, β, and γ are constants.

$$f_t = \alpha - \frac{\frac{\beta}{\gamma} + \alpha}{1 + \frac{\beta}{\alpha\gamma}\exp[t(\beta + \alpha\gamma)]} \quad (1)$$

The time for the lag phase t_{lag}, and the time required for attaining half of the maximum fluorescence value $t_{1/2\ max}$ were calculated using eqs 2 and 3 respectively.

$$t_{lag} = \frac{1}{\beta + \alpha\gamma}\left(\ln\left(\frac{\alpha\gamma}{\beta}\right) - 4\frac{\alpha\gamma}{\beta + \alpha\gamma} + 2\right) \quad (2)$$

$$t_{1/2\ max} = \frac{\ln\left(2 + \frac{\alpha\gamma}{\beta}\right)}{(\beta + \alpha\gamma)} \quad (3)$$

Separation of Fibrils Using Ultracentrifugation. Heated and quenched β-Lg solutions (9 mL) were ultracentrifuged (Kendro Laboratory Products, U.S.A.) using a Sorvall T-890 fixed angle rotor at different centrifugation speeds ranging from $1.1 \times 10^5 g$ to $2.9 \times 10^5 g$ for either 30 or 60 min. The extent of separation of fibrils from the heated solution into the pellet was determined by measuring the residual ThT intensity of the supernatant solutions. Complete separation of fibrils was achieved when samples were centrifuged at $2.3 \times 10^5 g$ for 60 min (see Supporting Information, Figure S1). Under these conditions the unheated β-Lg did not form a pellet. Transmission electron microscopy on the supernatant obtained after ultracentrifugation of β-Lg heated for 12 h, using the method described by Loveday et al.,[15] did not show the presence of any fibrils (results not shown), indicating complete separation. After centrifugation, the supernatant was collected, while the pellet was washed twice with 2 mL of water (pH 2), without dispersion, to remove any residual supernatant. This pellet will be referred to here as the 'surface-washed pellet'. The supernatant and surface-washed pellets from heated solutions were used for further analysis.

SDS-PAGE. Tricine SDS-PAGE methodology, based on the method described previously,[30] was used to analyze the samples. Heated solutions were diluted 1:10 using reducing PAGE sample buffer containing 2% SDS and 100 mM dithiothreitol (DTT). For centrifuged samples, 100 μL aliquots of supernatant solutions were diluted as above while the pellets were suspended in the PAGE sample buffer, with or without DTT, without further dilution. Samples meant for analysis under reducing conditions were heated to 56 °C for 15 min to reduce disulfide bonds. About 5−10 μL samples were loaded onto an SDS polyacrylamide gel with a resolving gel concentration of 16% (49.5%T, 3%C) and a gel thickness of 0.75 mm (prepared in-house). The loaded gel was then subjected to a voltage of 100 V until the dye front approached the bottom of the gel (MiniProtean III Bio-Rad Laboratories, Hercules, CA). The gels were then stained using 0.3% Coomassie brilliant blue R 250 for 1 h at 20 °C and destained (10% v/v acetic acid containing 10% v/v 2-propanol) for 24−48 h at 20 °C. Gels were scanned using a molecular imager Gel Doc XR system (Bio-Rad Laboratories, CA) and images analyzed using QuantityOne software. The residual intensities of the β-Lg band at different times (I) were measured and normalized against the intensity of the unheated sample in the same gel (I_0). The data from three independent experiments were

fitted using a first-order rate equation (eq 4) in which t is time in h, while k_h is the rate constant.

$$I = I_0\, e^{-k_h t} \tag{4}$$

Two-Dimensional Nonreducing Reducing (NR-R) SDS-PAGE. β-Lg (1% w/v) that had been heated for 12 h was subjected to ultracentrifugation at $2.3 \times 10^5 g$ for 60 min, and the pellets formed were washed as described above. The surface-washed pellet was diluted with 9 mL of pH 2 water and then mixed by vortexing to disperse the pellet and remove any residual traces of the supernatant. The above procedure of centrifugation and vortexing was repeated twice to produce the sample for analysis. The pellet washed using the method described in this section is referred to as the 'complete-washed pellet'. This pellet was dispersed in nonreducing SDS-PAGE sample buffer containing 2× SDS (4%) and dissolved with constant agitation at 20 °C over 7 days. At the end of this time, the sample was divided in two and 1 M DTT solution was added to one-half (final concentration 100 mM), while a volume of water equivalent to that of DTT was mixed with the other half. The sample containing DTT was heated at 56 °C for 15 min to ensure complete reduction of all disulfide bonds in the sample. Ten microliters of the nonreduced sample was subjected to SDS PAGE at 100 V as described above. For 2D NR-R PAGE analysis, methodology described previously[31] was followed. Lanes with protein bands were excised from the native gel using a clean scalpel and reduced in 200 mM DTT solution at 56 °C for 15 min. The reduced gel strips were then washed with excess water for 10 min before being placed horizontally between the gel casting plates in the stacking gel area on top of the resolving gel. The resolving gel was poured from the sides to a height that left a gap of 10 mm between the lower part of the gel strip and the surface of resolving gel. Stacking gel was poured around and over the reduced gel strip to cement it in place and remove air from around it. The reduced sample was loaded into a single well on the side as a control. The resolving gel concentration of 20% (49.5%T, 3%C) was used for both dimensions. The gels were run and scanned using the method as described above.

Circular Dichroism (CD) Spectroscopy. β-Lg spectra were measured on a Chirascan CD spectrometer (Applied Photophysics Ltd., U.K.) using a cuvette of path length 10 mm. The concentration of β-Lg for scans in the near ultraviolet (NUV) region was 1 mg/mL and 0.01 mg/mL for the far ultraviolet (FUV) region. A stock solution of β-Lg was made in HPLC grade water at pH 2 (adjusted using 6 M HCl), and the sample was allowed to hydrate overnight at 4 °C. The samples were then diluted to the desired concentration, filtered (syringe filter, pore size 0.2 μm, Minisart CE, Sartorius Stedim Biotech GmbH, Goettingen, Germany), and pH readjusted to pH 2.00 ± 0.02. Protein concentrations were determined as described above.

Scans of native β-Lg were obtained at 20 °C. The temperature of the sample was raised from 20 to 80 °C in the instrument at a rate of 12 °C/min. As soon as the temperature reached 80 °C, continuous scans were taken for one hour at a scan rate of 1 nm/s. For NUV region, scans were taken between wavelengths 260 and 320 nm in a total scan time (including the interval between two successive scans) of 40 s. Data from three scans were averaged over 2 min time intervals. FUV scans between wavelengths 180 and 250 nm took 1 min (including interval between two successive scans), and data from two scans were averaged over 2 min time intervals. The baseline scans for both NUV and FUV regions were recorded and averaged as above using water at pH 2 instead of β-Lg. Averaged scans of baselines were subtracted from β-Lg scans, and molar ellipticity was calculated according to the following equation[32]

$$[\theta]^{\lambda}_{M} = \frac{100\theta_{\lambda}}{LC} \tag{5}$$

where θ_λ is the measured ellipticity (deg) at wavelength λ, L is path length of the cuvette used (cm), and C is the protein concentration (dmol/L).

The subtracted scans were smoothed using SigmaPlot 12.0 software. Smoothing used a fixed bandwidth of 5 points and a second-order polynomial function with negative exponential weighting. To compare the kinetics of structural changes in β-Lg upon heating, ellipticities at 293 nm in the NUV region and 208 nm in the FUV region were used for calculating relative loss of ellipticities using the following equations

$$\left(\frac{\Delta[\theta]_t}{\Delta[\theta]_0}\right)_{293} = \frac{[\theta]_t - [\theta]_{60}}{[\theta]_0 - [\theta]_{60}} \tag{6}$$

$$\left(\frac{\Delta[\theta]_t}{\Delta[\theta]_{60}}\right)_{208} = \frac{[\theta]_t - [\theta]_0}{[\theta]_{60} - [\theta]_0} \tag{7}$$

where $[\theta]_t$ and $[\theta]_{60}$ represent the molar ellipticities at wavelengths 293 or 208 nm at a given time t min and that after 60 min at 80 °C respectively, while $[\theta]_0$ represents the molar ellipticity at these wavelengths in the unheated sample. The plot of $(\Delta[\theta]_t/\Delta[\theta]_0)_{293}$ against time at 80 °C was fitted with the first-order exponential model represented in eq 4 to calculate the rate constant (k_{NUV}). Similarly the plot of $(\Delta[\theta]_t/\Delta[\theta]_{60})_{208}$ was fitted with the first-order exponential model represented by eq 8 below

$$\left(\frac{\Delta[\theta]_t}{\Delta[\theta]_{60}}\right) = 1 - e^{k_{FUV}t} \tag{8}$$

where k_{FUV} is the rate constant.

Mass Spectrometry. *1. Sample Preparation.* For matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), the complete-washed pellets from β-Lg (1% w/v) that had been heated for for 12 h were suspended in 5 mL of solubilization buffer (8 M guanidinium chloride, 0.15 M Tris and 0.1 M DTT, pH of 8), then held at room temperature until they were dissolved. After 14 days the sample appeared transparent and did not show the presence of any insoluble material. A 1 mL aliquot of this solution was diluted to a final guanidinium chloride concentration of 5 M then centrifuged at 14500 rpm for 10 min and filtered (0.2 μm filter).

A 100 μL aliquot of the filtered sample was subjected to RP-HPLC (C_{18}, Jupiter, Torrance, CA) at a flow rate of 1 mL/min. After loading, the sample was washed in buffer A (10% acetonitrile (MeCN) and 0.1% trifluoroacetic acid (TFA)) for 1 min, then eluted using the following program: 0 to 20% buffer B (90% MeCN and 0.08% TFA) over 10 min; 20% to 100% B over 1 min 100% B for 5 min; 100 to 0% B in 1 min; 0% B for 5 min. The elution was monitored by absorbance at 214 and 280 nm and peaks due to buffer sample components identified by running each of the buffer components separately. Peaks eluting at an acetonitrile concentration of 25% and 90% collected from five runs were pooled and stored at 4 °C until analysis. The samples from two runs were collected, pooled, and concentrated by centrifugation (Savant SpeedVac SC100, Holbrook, NY) under vacuum (Varian DS 102, Varian Vacuum Technologies, Torino, Italy) prior to analysis.

Peptides present in fibrils were also characterized by in-gel digestion followed by electrospray ionization (ESI) MS-MS as described previously.[33] For in-gel digestion ESI-MS, the final surface-washed pellet was suspended in 10% SDS and allowed to stir for three days. After three days, the samples appeared clear and 250 μL of this solution was diluted 1:1 in reducing PAGE loading buffer (2× strength) then analyzed by tricine SDS-PAGE as described above. A control sample suspended in PAGE loading buffer (4% SDS) was run on the same gel. The concentration of the resolving gel was 20% and the stacking gel 4%. The gels were stained using colloidal Coomassie blue to identify peptide bands.[34] Five major bands identified in the pellet were cut from the gels and destained using warm water. For the control sample the single band corresponding to the β-Lg monomer band was excised. Samples were reduced using 50 mM tris(2-carboxyethyl) phosphine hydrochloride and alkylated with 360 mM acrylamide to prevent reformation of disulfide bridges during subsequent steps. The gel pieces were digested using trypsin Gold (VSS280, Promega Corporation, U.S.A.) at 37 °C for 20 h. Peptides obtained after digestion were extracted from the gel pieces by washing with acetonitrile containing 1% (v/v) formic acid and concentrated to a minimum volume of 25 μL in a vacuum concentrator. The samples were stored at −80 °C until analysis by ESI-mass spectrometry.

2. MALDI-TOF MS/MS for Peptides in Fibrils. The analysis of samples was carried out by the Centre for Protein Research, University of Otago, Dunedin, New Zealand. A 1 μL of sample was premixed with 1 μL of matrix (10 mg/mL, cyano-4-hydroxycinnamic acid, dissolved in 65% (v/v) acetonitrile containing 0.1% (v/v) TFA). A sample (0.8 μL) was spotted onto a MALDI sample plate (opti-TOF 384-well plate, Applied Biosystems, MA) and air-dried. Samples were analyzed on a 4800 MALDI-TOF analyzer (MALDI TOF/TOF, Applied Biosystems, MA). Calibration was done for the mass range 1000 to 25000 m/z on a 5 peptide/protein calibration mix, and the mass range was between 20 000 and 100 000 m/z on the BSA 1+ and 2+ ions (66000 and 33000 m/z). All MS spectra were acquired in linear, positive-ion mode with 1200 laser pulses per sample spot. The 15 to 20 strongest precursor ions of each sample spot were selected for MS/MS collision-induced dissociation (CID) analysis. CID spectra were acquired with 2000–4000 laser pulses per selected precursor using the 2 kV mode and air as the collision gas at a pressure of 1×10^{-6} Torr.

For protein identification, MS/MS data were searched against the SWISS-PROT sequence database using the Mascot search engine.[35] The search was set up for no enzyme cleavage specificity and with deamidation of asparagine (N) and glutamine (Q) and oxidation of methionine (M) included as variable modifications. The precursor mass tolerance threshold was 15 ppm, and the maximum fragment mass error was 0.4 Da. A probability cutoff of $p < 0.05$ was used, which gave a threshold cutoff ion score of 55. As the sample contained only a single protein (β-Lg), the traditional decoy approach for database search and false positive calculations was not followed. Since a traditional false positive rate was not calculated, the integrity of the results was based on the quality of the data. A typical MS/MS spectrum for a peptide above the cutoff ion threshold is shown in Figure S2 (see Supporting Information). The spectrum shows all major ion peaks assigned with masses. Table S1 (see Supporting Information) shows the masses of most of the ions shown in Figure S2.

3. LC-ESI-MS/MS on In-Gel Digest Extracts. Aliquots (5 μL) of samples were injected into an HPLC-Chip Cube Interface (G4240A) with ProtID-Chip-43 (II) chip on an Agilent 1260 infinity LC system and analyzed using a Q-TOF mass spectrometer (6520, Agilent Technologies Ltd.). The capillary voltage was 1850 V, the drying gas temperature was 300 °C at a flow-rate of 4 μL/min, the fragmenter voltage was 175 V, and the skimmer voltage was 65 V. Precursor ions were scanned from 100 to 1700 m/z at 6 spectra/s. For MS-MS analysis, ions were scanned from 50 to 1700 m/z at 4 scans/s. The data were analyzed using the Mascot search engine allowing the variable modifications described above with trypsin cleavage, a precursor mass tolerance threshold of 15 ppm, and the maximum fragment mass error of 0.4 Da. For the control β-Lg band, since the sample was not heated, deamidation of asparagine and glutamine (N or Q) and oxidation of methionine (M) were not included as variable modifications. The probability cutoff $p < 0.05$ was used for all searches for the peptide bands which gave a threshold ion score cutoff of 46, while the threshold cut off for the control β-Lg band was 42.

RESULTS AND DISCUSSION

1. Self-Assembly from β-Lg. Self-assembly of β-Lg was studied using the ThT assay (Figure 1). ThT binds only to the β-sheets present in the β-Lg fibrils.[36] A net increase in fluorescence can therefore be used to indicate an increase in the concentration of fibrils. The samples showed low fluorescence intensity for approximately 4 h suggesting a distinct lag phase. It is widely believed that during the lag phase β-Lg forms nuclei capable of forming mature fibrils upon prolonged incubation. All samples with heating times of more than 4 h showed an increase in fluorescence intensity indicating the presence of fibrils in the heated samples. The fluorescence showed a rapid increase in intensity between 6 and 12 h, indicating the occurrence of a growth phase. The fluorescence intensity data were fitted to the model represented by eq 1 with $R^2 = 0.9932$. The value for t_{lag} was 5.7 h, which agreed with the experimental data that showed an increase in fluorescence after only 6 h. The value of $t_{1/2\ max}$ was calculated to be 9.1 h.

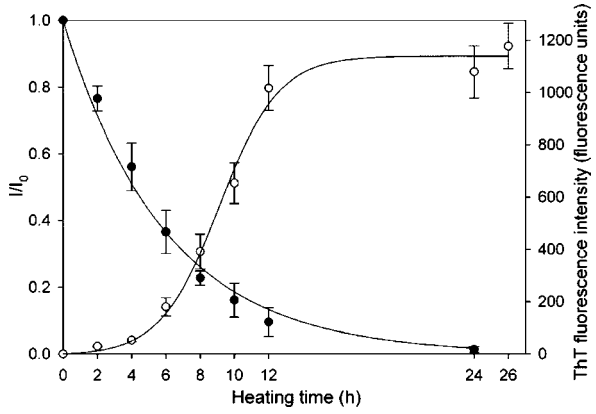

Figure 1. (○) Thioflavin T (ThT) fluorescence intensity at 486 nm for 1% β-Lg at pH 2 heated at 80 °C. Error bars show standard deviations for triplicate measurements for three separate samples, and the solid lines show fit of eq 1. (●) The normalized intensity of the SDS-PAGE band corresponding to intact monomer. Error bars show standard deviations from three separate samples while the solid lines shows fit of eq 4.

2. Hydrolysis of β-Lg in Heated Samples. β-Lg samples after heating for the times shown in Figure 1 were analyzed by tricine SDS-PAGE under reducing conditions (Figure 2). The unheated sample did not show evidence of hydrolysis in contrast to all the heated samples, which showed differing degrees of hydrolysis of the β-Lg monomer into peptides with molecular masses ranging from 15 kDa to less than 3.5 kDa. As the time of heating increased, the intensity of the band corresponding to the β-Lg monomer (β-Lg band) decreased with a corresponding increase in the intensities of the peptide bands. This β-Lg band was quantified using a densitometer and normalized relative to the intensity of unheated β-Lg and plotted against heating time (Figure 1).

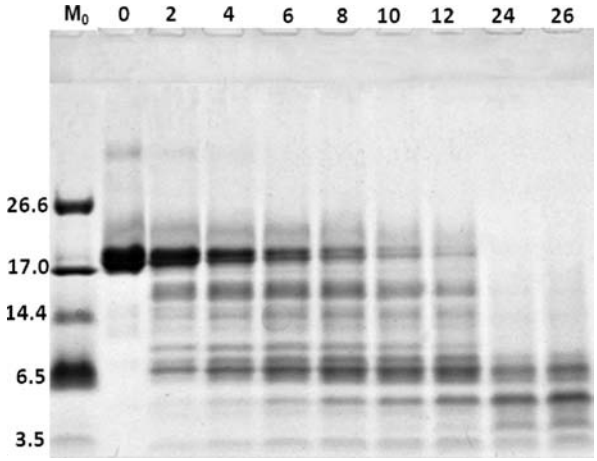

Figure 2. Reduced SDS-PAGE of β-Lg heated at 80 °C and pH 2 for different times: M_0, molecular mass marker in kDa; 0, unheated sample; numbers above the lanes indicate heating time in hours.

The rate of hydrolysis was fitted using a first-order model (eq 4), giving a value for the rate constant (k_h) of 2.8×10^{-3} min^{-1} ($R^2 = 0.9902$, standard deviation of 0.5×10^{-3}). This value is lower than that reported by Kroes-Nijboer et al.[18] for β-Lg hydrolysis at 80 °C during self-assembly (3.9×10^{-3} min^{-1}) ($R^2 = 0.88$). The latter study involved heating with continuous

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shearing, whereas we heated without shearing in order to minimize secondary nucleation, which would have interfered with a close analysis of events during the lag phase. The percentage difference in k_h values is typical of interlaboratory variation in statistically derived parameters, so the effect of shear on k_h was probably negligible.

The time required to hydrolyze 50% of the β-Lg was 4.1 h. The intensity of the β-Lg band at the beginning of the growth phase, after ~6 h incubation, was calculated to be approximately 36% of the initial intensity, indicating that a major proportion of the monomer had undergone hydrolysis before significant formation of any fibrils.

Hydrolysis of β-Lg continued in the growth phase, with increasing losses of higher molecular weight peptides at >8 h. No intact β-Lg remained after 24 h and the samples contained only peptides with molecular weights below 10 kDa, suggesting that larger peptides formed first and then these underwent further hydrolysis as heating continued, resulting in the formation of smaller peptides. The stacking gels in all samples did not show any evidence of high molecular weight aggregates. The SDS-PAGE buffer containing the sample that had been heated for 12 h did not show any intact fibrils when analyzed by transmission electron microscopy (results not shown), confirming complete dissociation of fibrils in the PAGE sample buffer.

3. Structural Transitions in the Lag Phase. *(i). Hydrolysis of β-Lg.* Changes to β-Lg during the lag phase were explored by analyzing samples heated at 80 °C for times less than 4 h (i.e., in the lag phase) using SDS-PAGE under reducing conditions. This showed that peptides with approximate molecular weights from 15 kDa to less than 3.5 kDa appeared within 30 min of heating at 80 °C (Figure 3). The molecular weights of the bands observed

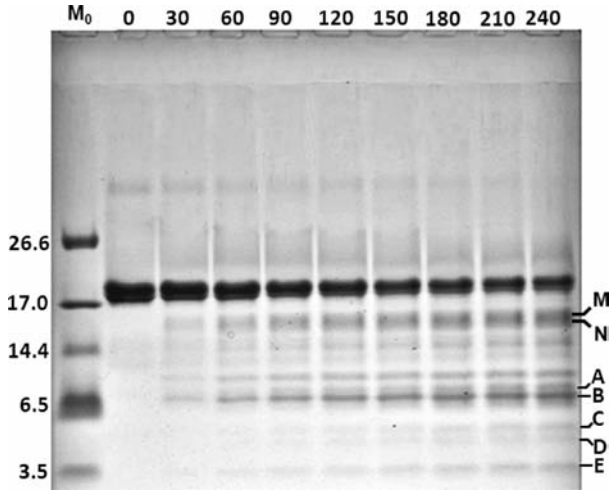

Figure 3. Reduced SDS-PAGE of β-Lg (1% w/v) heated at 80 °C and pH 2 during the lag phase: M_0, molecular mass marker (kDa); 0, unheated sample; numbers above the lanes indicate heating time in minutes. For description of bands A–E, M, and N, see text.

in these samples were similar to those observed in the growth phase (Figure 2), and their intensities steadily increased throughout the lag phase. This suggests that heating β-Lg monomer at pH 2 initiates hydrolysis almost immediately. Moreover, hydrolysis of the monomer proceeds more rapidly than the subsequent hydrolysis of the higher molecular weight peptides (8–15 kDa) during this period as revealed in Figures 1 and 2. Indeed, only after longer heating times (>8 h), when the concentration of β-Lg drops to below 20% of its initial concentration, does the concentration of the higher molecular weight fragments (8–15 kDa) start to fall.

The results from SDS-PAGE analysis of samples in the lag phase suggest that hydrolysis may have an important role in the formation of the nuclei that initiate β-Lg self-assembly. Moreover, once initiated, hydrolysis of the β-Lg monomer and larger peptides continues during heating irrespective of fibril formation. At pH 2, the free cysteine residue in β-Lg remains protonated and hence the likelihood of aggregation by intermolecular thiol-disulfide exchange reactions is greatly reduced.[37,38] Peptide bonds with either aspartic or glutamic acid on the C-terminal side have been shown to be preferentially cleaved during heating in acidic conditions.[39]

There have been several previous reports of β-Lg hydrolysis during fibril formation at low pH, high temperature, and protein concentrations ≤2%.[11,16,38,40,41] However the shortest heating times were in most cases ≥3 h,[11,16,38,40] and where shorter heating times were used, fibrils were not separated from nonfibril material.[41] An increase in the rate of fibril formation at temperatures above 80 °C was recently shown to be related to an increase in the rate of hydrolysis of the monomer at these temperatures.[18] In that study, samples were stirred during heating in order to eliminate the lag phase by promoting secondary nucleation, thereby simplifying kinetic modeling. Thus, previous studies have not presented a comprehensive view of events during the lag phase, particularly the role of hydrolysis.

(ii). Unfolding of β-Lg during Heating. Structural changes in the β-Lg monomer in the early stages of the lag phase, before significant hydrolysis had occurred, were investigated using CD spectroscopy. Since the unfolding of the monomer is rapid, the samples were heated to 80 °C in a temperature-controlled cell holder in the instrument and scans were recorded continuously once the temperature reached 80 °C. The duration of heating was restricted to 1 h since the samples with heating times less than 1 h showed that the major proportion of β-Lg still remained intact (approximately 80%, from Figure 1). Continued heating of β-Lg beyond 1 h resulted in significant hydrolysis of the protein monomer (Figure 3), so that the resulting spectra would be a mixture of those arising from β-Lg monomer and peptides. The NUV CD spectrum of native β-Lg provides information about the tertiary structure of the protein and is characterized by two sharp troughs at about 286 and 293 nm that are due to Trp at position 19.[42] Small troughs observed in the region between 262 and 269 nm are attributed to Phe residues.[43] Heating of β-Lg from 20 to 80 °C resulted in a slight loss of intensities of these troughs, indicating a change in the environment around Trp.[19] A similar change in β-Lg tertiary structure has been reported to occur on heating from 37 and 75 °C.[44] Continued heating of β-Lg caused a rapid decrease in intensity of these bands (Figure 4A). Complete loss of CD signal for Trp was observed within the first 10 min of heating, after which the spectrum remained unchanged for the rest of the heating time. These results suggest that heating of β-Lg leads to complete destruction of its native tertiary structure within the first 10 min after reaching 80 °C. The plot of $(\Delta[\theta]_t/\Delta[\theta]_0)_{293}$ against time is shown in Figure S3A (see Supporting Information). The k_{NUV} for unfolding calculated with fitting experimental data by eq 5, was determined to be 0.20 min^{-1} (R^2 = 0.9665, standard deviation 0.06 min^{-1}).

The far UV (FUV) CD spectrum (180 to 230 nm) gives information on the secondary structure of a protein, as it measures the absorption of polarized light by peptide bonds of the polypeptide chain.[32,45] Scans for unheated β-Lg show a characteristic trough with a minimum at 217 nm suggesting the

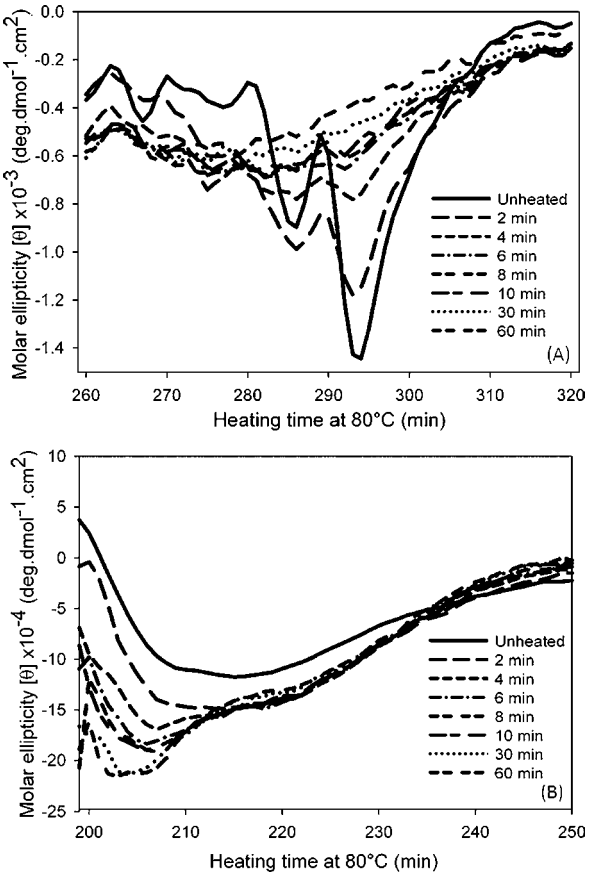

Figure 4. CD spectra of β-Lg at pH 2 and 80 °C. (A) Near-UV scans, (10 mg/mL). (B) Far-UV spectra (0.01 mg/mL). Conditions for data collection were the following: path length, 10 mm; temperature, 80 °C; scans were averaged at 2 min intervals each.

presence of β-sheets, consistent with the known structure.[3,5,44] Averaged scans for samples recorded during continued heating showed that almost immediately after commencement of heating the trough deepened with ellipticity between 220 and 230 nm becoming increasingly more negative. The CD spectrum then remained approximately constant with continued heating (Figure 4B). Simultaneously there was a shift in the trough at 217 nm, characteristic of β-sheets in the unheated protein, toward shorter wavelengths (<210 nm). The bulk of this shift occurred during the first 10 min of heating and continued heating caused only a further small deepening of the trough. The relative change in ellipticity at 208 nm represented by $(\Delta[\theta]_t/\Delta[\theta]_{60})_{208}$ was plotted against heating time and fitted with a first-order exponential model in eq 8 (Figure S3B, see Supporting Information). The rate constant for the change in secondary structure upon heating was 0.25 min^{-1} (R^2 = 0.9864, standard deviation 0.03 min^{-1}). The rate constants for loss of tertiary and loss of secondary structure are the same within experimental error.

The data below 200 nm were characterized by a high detector high-tension (HT) value (>1000 V), which represents high absorbance by the sample making the spectra difficult to interpret. (Figure S4, see Supporting Information). Chloride ions strongly absorb polarized light below 200 nm.[32] Since HCl was used for adjusting the sample pH, chloride ions contributed to the high absorbance at wavelengths in this region. The increase in the ellipticity at 222 nm and the shift of the trough toward lower wavelengths suggest that the heating of β-Lg led to a decrease in the β-sheet content with a concomitant increase in α-helix structure. Such a shift in the trough for the FUV region has previously been reported upon heating β-Lg at pH 2[44] and during β-Lg self-assembly under shear after 12 h at 80 °C[22] and has been attributed to the formation of a non-natively folded α-helix.[46] A similar shift in the trough at 222 nm was seen in a diluted sample containing β-Lg fibrils.[16,20] However, within the time scales used in those studies, it is likely that a significant proportion of β-Lg monomer has undergone hydrolysis so that the CD signals represent a composite signal of residual unhydrolyzed monomer, peptides, and fibrils. In contrast, the time scale used for data collection in this study allows structural measurements to be made before significant hydrolysis occurred. The increase in non-native α-helix upon protein unfolding is thought to be the same intermediate characterized during the refolding of β-Lg.[47,48] This intermediate is thought to involve residues 12−24, which form part of the β-A strand of β-Lg.[48] Our study shows that this change is one of the first structural changes

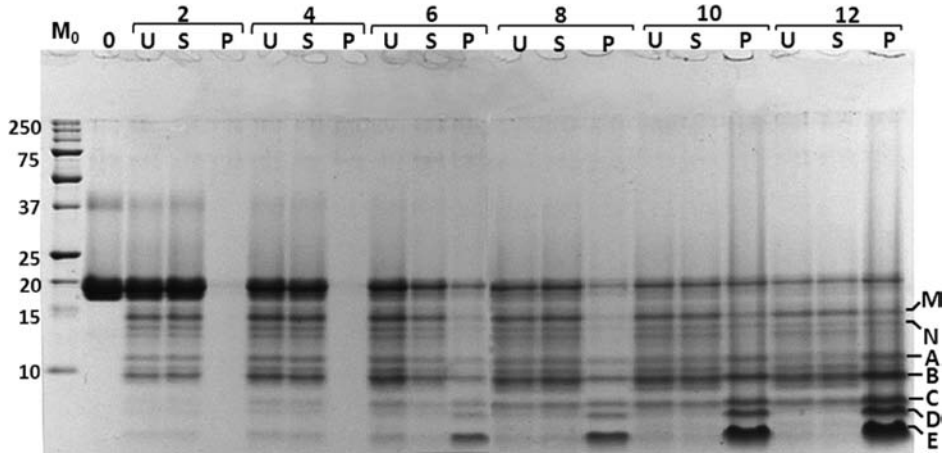

Figure 5. Comparison of reduced SDS-PAGE profiles of centrifuged samples at different heating times: M_0, molecular mass marker, weights in kDa; 0, unheated sample; U, heated and uncentrifuged; S, supernatant; P, pellet. Numbers above the lanes indicate heating times (h). Uncentrifuged sample and supernatants were diluted 1:10 by means of the PAGE sample buffer. Surface-washed pellets were suspended in loading buffer without dilution. For description of bands A−E, M, and N, see text.

to occur during the lag phase of β-Lg self-assembly and precedes hydrolysis.

4. Characterization of Fibril Composition. *(i). Composition of Fibrils after Different Heating Times.* Akkermans et al.[23] reported that peptides are the building blocks of fibrils formed at pH 2, 85 °C, and 3% protein, after 20 h under constant shear. The makeup of fibrils at earlier stages of self-assembly, when a fraction of β-Lg remains unhydrolyzed, is not yet known. We used ultracentrifugation to sediment fibrils while leaving the unassembled material in the supernatant. The supernatants analyzed by ThT assay showed low fluorescence, indicating complete separation of fibrils from the heated solutions (Figure S5, see Supporting Information). All samples heated for more than 4 h gave a transparent pellet upon ultracentrifugation, whereas those heated for 2 or 4 h produced no pellet. The heated solutions and their corresponding separated components were examined by SDS-PAGE under reducing conditions to determine their composition (Figure 5). At all heating times, the peptide compositions of the supernatants were similar to those of the parent uncentrifuged solutions. Most of the β-Lg monomers in the heated samples at all heating times remained in the supernatant, while very little intact monomer was observed in the pellets. This suggests that the fibrils formed under these conditions (pH 2, 80 °C, 1% protein) consist primarily, and probably exclusively, of peptide fragments. It is interesting to note that not all peptides generated upon hydrolysis were observed in the fibrils (pellets). Five distinct peptides (Figure 5, bands A–E) preferentially accumulated in fibrils, with peptides labeled D and E with approximate masses 4.1 kDa and 3.2 kDa (Table S3 see Supporting Information) being the most intense bands. The intensities of all bands, except full-length β-Lg, increased with the length of heating. Bands M and N with an approximate molecular weight of 14 kDa showed slightly reduced intensity after subsequent washing and recentrifugation steps to obtain the complete-washed pellet (Figure 6, lane 4). It is likely that one or more peptides in these bands before washing represented contaminant unassembled peptides from the supernatant that were entrapped within the fibril pellet and were dislodged during washing.

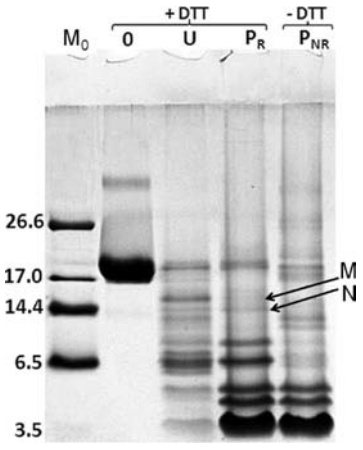

Figure 6. SDS-PAGE comparison of peptide bands in fibrils under reducing and nonreducing conditions. Complete-washed pellets obtained after ultracentrifugation for 12 h: M_0, molecular mass marker (weights in kDa); 0, unheated sample; U, heated and uncentrifuged sample; P_R, complete-washed pellet suspended in reducing PAGE buffer; P_{NR}, complete-washed pellet suspended in nonreducing PAGE buffer. For description of bands M and N, see text.

The profiles of fibrils separated at 10 and 12 h showed a light smear in addition to the peptide bands (Figure 5), which may result from incomplete dispersion of the protein in the compact pellet obtained at these heating times. Ultracentrifugation of the sample heated for 24 h gave a very compact pellet that was difficult to disperse in PAGE buffer and hence rendered SDS-PAGE analysis difficult. Since continued heating of β-Lg for 24 h resulted in complete hydrolysis of the β-Lg monomer (Figure 2), it was expected that the fibrils after 24 h would be made up of only peptide fragments of β-Lg. To confirm this, the complete-washed pellet from a sample heated for 24 h was dispersed in reducing PAGE sample buffer with 2× SDS (4%) and allowed to dissolve for 7 days at 20 °C. The PAGE profiles of the sample were compared with those from pellets from samples that had been heated for both 6 and 12 h (suspended in sample buffer containing 4% SDS) under reducing conditions. A comparison of peptide bands from these samples showed the presence of bands almost identical to those in material subjected to less intense heat treatment (see Supporting Information, Figure S6). This suggests that fibrils are made up of similar peptides regardless of how long they are heated. Comparing the PAGE profiles of these fibrils with those of heated samples from the lag phase (Figures 3 and 5), it is clear that peptides A–E from the fibrils were first observed after just 30 min of heating. At heating times of less than 30 min, these peptides were not visible by SDS-PAGE, although it is possible that they could be present in solution at very low concentrations. While the intensity of these bands increased throughout the lag phase, there was no accompanying increase in ThT fluorescence (Figure 1), suggesting that self-assembly of these peptides into fibrils proceeds only once a certain minimum or critical concentration of peptides is reached.

(ii). Disulfide Bonding in Fibrils. To determine the presence of disulfide bonds in fibrils, a sample heated for 12 h was divided into two and ultracentrifuged. Complete-washed pellets (obtained after vortexing and recentrifugation) from one of the samples were suspended in nonreducing PAGE loading buffer while the other was suspended in loading buffer with reducing agent DTT. Figure 6 shows the comparison of fibril peptides under reducing and nonreducing conditions. The nonreducing gel (lane 5, Figure 6) showed three distinct peptides corresponding to bands C–E in Figure 5, suggesting that these three peptides do not contain disulfide bonds. Additionally, the sample showed several faint bands with molecular weights higher than that of the monomer. Bands corresponding to peptides A and B appeared faint under nonreducing conditions, but gain considerably in intensity under reducing conditions, as is especially evident in the 1D SDS-PAGE of Figure 6. This indicates that some peptides bands eluting with bands A and B may be disulfide linked under nonreducing conditions.

The disulfide linkage in fibrils was further investigated by subjecting complete-washed pellets obtained after 12 h heating to 2D NR-R PAGE.[31] Using this technique, peptides without disulfide bonds will lie on the diagonal, while those with disulfide bonds will separate into multiple spots off the diagonal, due to different electrophoretic mobilities, after reduction. As expected bands C–E eluted as distinct spots (Figure 7) along with spots A and B indicating that the bands A–E did not contain any disulfide linkages. Two additional spots marked P and Q appeared on the electophoretic paths of spots M and N indicating that peptides labeled M and N were involved in disulfide linkages with peptides labeled P and Q, respectively. The relative mobilities of the peptides represented by spots P and Q were coincident with

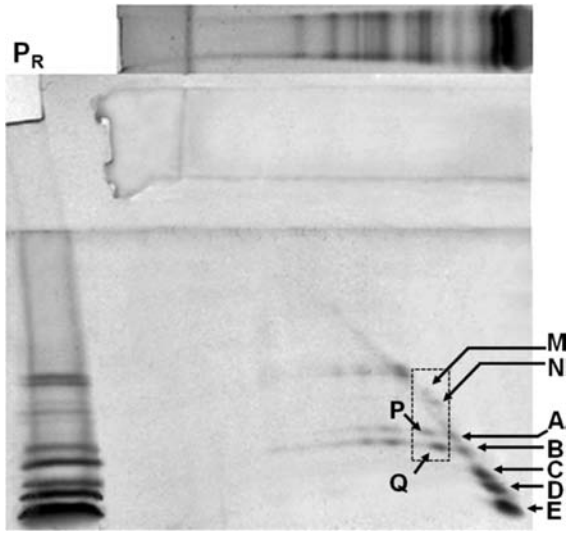

Figure 7. Two-dimensional SDS-PAGE of the pellet from a sample heated for 12 h. Complete-washed pellet suspended in buffer containing 4% SDS and run under nonreducing conditions in 1D. The target gel lane was excised, reduced, and run in the second dimension. P_R: reduced sample obtained from the same sample. For description of bands A–E, M, N, P, and Q, see text.

those of peptides in A and B respectively, indicationg they could be the same peptides. In 2D NR-R PAGE several additional peptide spots appeared adjacent to P and Q indicating that they are most likely to be disulfide linked to P and Q. Further characterization work is necessary to determine the identity the peptides represented by these additional spots. Nonetheless, these results, together with the 1D SDS-PAGE results, indicate that the fibrils contain disulfide-linked peptides.

(ii). Characterization of Sequences of Fibril Peptides. Peptides present in the pellet (fibrils) were identified using MALDI-TOF MS/MS. Table 1 shows the peptides observed in the fibrils with molecular weights of 1.2 to 3.4 kDa are derived from sequences 1–53 and very probably 152–162. All peptides showed cleavage at peptide bonds involving aspartic acid (D) residues. A similar composition of peptides in β-Lg fibrils has been reported previously.[20,23] The fact that fewer peptides were observed in our study can be explained on the basis of the difference in method of fibril separation and sample preparation. Whereas the previous studies employed ultrafiltration to separate the fibrils, we used ultracentrifugation. We also carefully washed fibrils by multiple cycles of vortexing and recentrifugation to ensure that there was minimal contamination from nonfibril material.

To determine the exact sequences of fibril peptides marked A–E in Figure 5, we followed in-gel digestion of these bands using trypsin. The digested peptides were extracted from the gel matrix using acetonitrile and analyzed by nano LC-ESI-MS/MS. As the bands are digested using trypsin, the digest will contain small peptides cleaved at peptide bonds involving lysine or arginine residues and the sequences obtained from MS/MS represent a part of the original peptide sequence, not the whole sequence. Table 2 summarizes the sequences present in fibril peptides. All bands contained a few peptides with cleavage of peptide bonds at hydrophobic amino acids in addition to those involving lysine and arginine. An explanation for this is that the trypsin used in the study must have contained chymotrypsin despite being treated with N-tosyl-L-phenylalanine chloromethyl ketone (TCPK) which inhibits chymotrypsin. Chymotrypsin is known to cleave peptide bonds involving hydrophobic amino acids tyrosine (Y), methionine (M), or leucine (L) and alanine (A) upon prolonged incubation. ESI MS/MS of fragments from the tryptic digest of unheated β-Lg also showed cleavage at hydrophobic residues, supporting the notion that chymotrypsin was present (Table S2, see Supporting Information). A schematic comparison of the sequences found in different peptide bands is shown in Figure 8. All the five bands (A to E, Figure 5) showed peptides from the region containing residues 25–53 suggesting this region is important in fibril formation as it is present in all peptides. In addition, although at reduced significance, band A showed sequences 92–101, 125–135, and 149–162, while band B showed 92–101. The sequences 92–100/101 and 125–135 have signs of tryptic hydrolysis (cleavage involving a Lys (K) or an Arg (R) residue) at both ends and do not have any sites of acid hydrolysis between them. In addition, cysteine residues at positions 106, 119, and 121 are in the vicinity of these peptide fragments. A possible explanation is that this arises from the presence of a large peptide involving residues 92 to 135 and probably up to 162 accounting for the sequence 149–162 in band A. It is likely that the chymotrypsin further cleaved the tryptic-digest peptides into smaller sequences which were not able to be detected by MS. A comparison of SDS-PAGE data for fibril peptides in 1D and 2D NR-R (Figures 6 and 7) showed that peptides in spots P and Q eluted in bands A and B under reducing conditions. The fragments from this region have also been found in fibrils formed by conventional[23] and microwave heating.[20] Hamada et al.[49] showed that sequences 102–109 (β-strand G) and 118–123 (β-strand H) and β-strand I (residues 146–152) in the C terminal region were capable of forming fibrils, but did not

Table 1. Peptides from β-Lg Hydrolysis Present in Fibrils Analyzed by MALDI-TOF MS/MS

amino acid sequence of peptides in fibrils[a]	seq	obsvd molar mass (Da)	Δ mass (ppm)[d]	expect values[e]	ion score[f]
LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL(D)[b]	1–32	3437.7673	−21.80	7.0×10^{-9}	130
(D)IQKVAGTWYSLAMAAS(D)	12–27	1695.8839	10.9	1.2×10^{-2}	63
(D)IQKVAGTWYSLAMAASD(I)	12–28	1810.9064	7.70	1.3×10^{-6}	104
(D)ISLLDAQSAPLRVYVEELKPTPEG(D)	29–52	2624.4136	2.87	5.3×10^{-4}	78
(D)IQKVAGTWYSLAMAASDISLLD(A)	12–33	2352.2144	4.64	1.9×10^{-3}	73
(D)ISLLDAQSAPLRVYVEELKPTPEGD(L)	29–53	2739.4434	3.79	2.0×10^{-3}	73
(N)PTQLEEQCHI(-)[c]	153–162	1197.5506	0.89	1.5×10^{-2}	61

[a]Letters in the parentheses indicate the amino acids adjacent to the peptides. [b]Peptide from N-terminal region. [c]Peptide from the C-terminal region. [d]Delta mass indicates the difference (error) between experimental and calculated mass. [e]Expect value (expectation value) represents the number of times the peptide score is equal or higher, purely by chance. The smaller the expectation value, the better is the significance of the match. [f]The ion score represents the probability of the match being a random event.[52]

Table 2. Peptide Sequences of Peptide Bands A–E (Figure 5) in the Pellet (12 h) Analyzed by ESI-MS/MS after Extraction Following In-Gel Digestion with Trypsin

band	amino acid sequence of peptides in bands[a]	seq	obsvd molar mass (Da)	Δ mass (ppm)[e]	expect value[f]	ion score[g]
A	(K)VAGTWYSLAMAASDISLLDAQSAPLR(V)[b,c,d]	15–40	2706.3690	0.12	1.1×10^{-8}	100
	(Y)SLAMAASDISLLDAQSAPLR(V)[b,c,d]	21–40	2029.0547	1.63	3.1×10^{-8}	115
	(M)AASDISLLDAQSAPLR(V)[c,d]	25–40	1626.8595	1.11	1.4×10^{-7}	89
	(R)VYVEELKPTPEG(D)	41–52	1359.6932	0.76	1.2×10^{-3}	57
	(R)VYVEELKPTPEGD(L)	41–53	1474.7236	3.04	2.7×10^{-4}	59
	(R)VYVEELKPTPEGDLEILLQK(W)	41–60	2312.2600	3.69	8.7×10^{-5}	57
	(K)VLVLDTDYK(K)	92–100	1064.5803	4.62	4.0×10^{-5}	71
	(R)TPEVDDEALEK(F)	125–135	1244.5794	1.79	1.5×10^{-3}	53
	(R)LSFNPTQLEEQCHI	149–162	1728.8148	0.40	6.1×10^{-4}	47
B	LIVTQTMK(G)[c,d]	1–8	932.5406	4.44	8.0×10^{-4}	60
	(K)VAGTWYSLAMAASDISLLDAQSAPLR(V)[b,c,d]	15–40	2706.3766	2.93	8.0×10^{-7}	81
	(Y)SLAMAASDISLLDAQSAPLR(V)[b,c,d]	21–40	2029.0612	4.88	5.0×10^{-7}	88
	(L)AMAASDISLLDAQSAPLR(V)	23–40	1828.9366	0.75	6.0×10^{-6}	78
	(M)AASDISLLDAQSAPLR(V)[c,d]	25–40	1626.8592	0.96	7.0×10^{-9}	107
	(R).VYVEELKPTPEGD(L)	41–53	1474.7280	6.02	4.0×10^{-3}	54
	(R)VYVEELKPTPEGDLEILLQK(W)	41–60	2312.2615	4.33	6.0×10^{-6}	65
	(K)VLVLDTDYK(K)	92–100	1064.5747	−0.65	8.0×10^{-5}	57
	(K)VLVLDTDYKK(Y)	92–101	1192.6718	1.27	1.0×10^{-4}	52
C	(K)VAGTWYSLAMAASDISLLDAQSAPLR(V)[b]	15–40	2706.3777	3.34	1.4×10^{-6}	77
	(Y)SLAMAASDISLLDAQSAPLR(V)[b,c,d]	21–40	2029.0525	0.55	6.3×10^{-7}	100
	(L)AMAASDISLLDAQSAPLR(V)	23–40	1828.9375	1.21	4.1×10^{-4}	59
	(M)AASDISLLDAQSAPLR(V)[c,d]	25–40	1626.8582	0.36	2.6×10^{-8}	101
	(R)VYVEELKPTPEGD(L)	41–53	1474.7225	2.29	6.2×10^{-5}	76
	(R)VYVEELKPTPEGDLEILLQK(W)	41–60	2312.2609	4.09	2.2×10^{-5}	58
D	LIVTQTMK(G)	1–8	932.5411	4.90	4.3×10^{-3}	53
	(K)VAGTWYSLAMAASDISLLDAQSAPLR(V)[b,c,d]	15–40	2706.3814	4.69	1.7×10^{-6}	72
	(Y)SLAMAASDISLLDAQSAPLR(V)[b]	21–40	2029.0600	4.25	1.1×10^{-4}	62
	(Y)SLAMAASDISLLDAQSAPLR(V)[b,c,d]	22–40	2029.0519	0.25	8.5×10^{-10}	115
	(L)AMAASDISLLDAQSAPLR(V)	23–40	1828.9310	−2.33	9.5×10^{-8}	102
	(A)MAASDISLLDAQSAPLR(V)	24–40	1757.8996	0.84	3.7×10^{-8}	99
	(M)AASDISLLDAQSAPLR(V)[c,d]	25–40	1626.8573	−0.24	2.0×10^{-6}	86
	(R)VYVEELKPTPE(G)	41–51	1302.6732	1.90	1.1×10^{-3}	56
	(R)VYVEELKPTPEG(D)	41–52	1359.6947	1.83	6.0×10^{-3}	54
	(R)VYVEELKPTPEGD(L)	41–53	1474.7336	9.82	2.3×10^{-5}	76
	(R)VYVEELKPTPEGDLEILLQK(W)	41–60	2312.2591	3.30	5.4×10^{-5}	56
E	(A)LIVTQTMK(G)[b,c]	1–8	933.5230	2.73	3.9×10^{-3}	54
	(R)VYVEELKPTPEG(D)	41–52	1359.6957	2.55	9.0×10^{-4}	56
	(R)VYVEELKPTPEGD(L)	41–53	1474.7352	10.90	5.9×10^{-2}	51
	(M)AASDISLLDAQSAPLR(V)	25–40	1626.8591	0.89	1.9×10^{-5}	70

[a]Letters in parentheses indicate the amino acids adjacent to the peptides. [b]Deamidation of glutamine (Q). [c]Deamidation of asparagines (N). [d]Oxidation of methionine (M). [e]Delta mass indicates the difference (error) between experimental and calculated mass. [f]Expect value (expectation value) represents the number of times the peptide score is equal or higher, purely by chance. The smaller the expectation value, the better is the significance of the match. [g]The ion score represents the probability of the match being a random event.

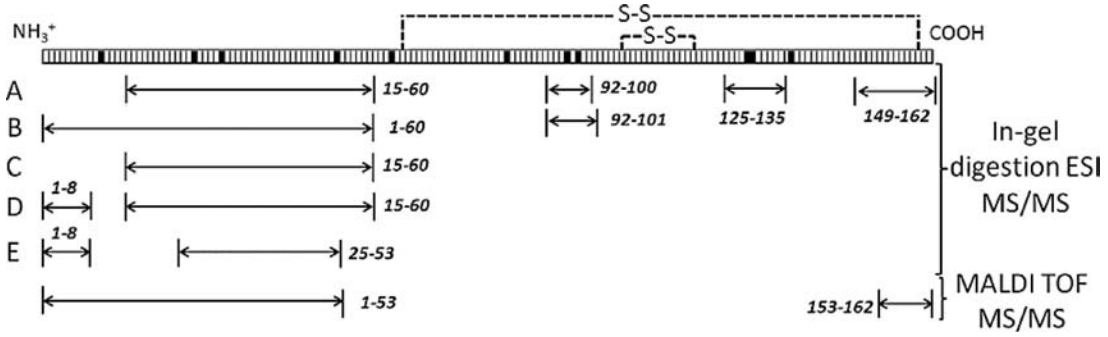

Figure 8. Schematic representation of regions found in peptides from in-gel digestion ESI-MS/MS and MALDI-TOF MS/MS. The dark blocks in the sequence show location of aspartic acid residues indicating potential sites of acid hydrolysis of β-Lg. Sequence of β-Lg variant A is shown. Variant B has glycine instead of aspartic acid at residue 64 and alanine instead of valine at residue 118.

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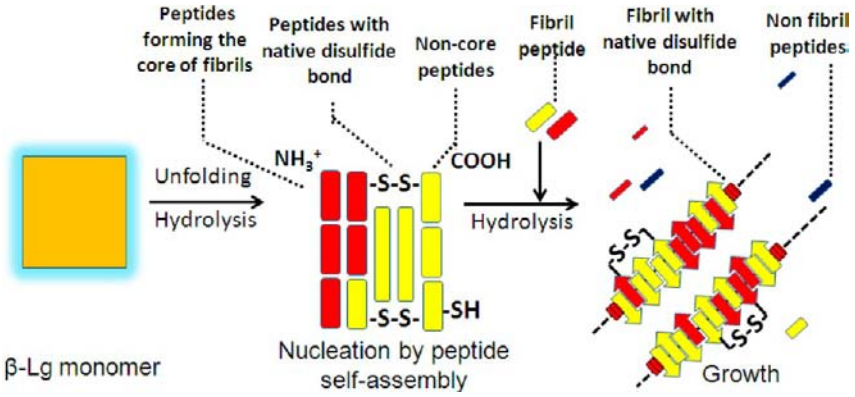

Figure 9. Schematic illustration of how disulfide-bonded peptides come to be present in fibrils.

promote self-assembly from full length β-Lg upon seeding in the presence of urea. This suggests that the peptides from these regions may primarily contribute to growth of nuclei during self-assembly at pH 2.

It is interesting to note that band A contains the sequences 12−60 and 149−162, which are located at opposite ends of the β-Lg polypeptide chain. The peptides from these regions were also found in the MALDI-TOF MS analyses from this study (Table 1) and consistently appear in previous reports of β-Lg fibril composition.[20,23] In the absence of conditions promoting unrestrained disulfide exchange reactions at pH 2, it is possible that the disulfide bond between Cys160 and Cys66 is conserved in fibrils and may be responsible for disulfide-bonded peptides found in 2D NR-R SDS-PAGE (spots M and P or N and Q, Figure 7). This disulfide linkage is one of the two disulfide bonds present in the native β-Lg molecule.[2] Since the sequence between cysteine residues 106 and 119 does not have any aspartate residues, the most common site of acid hydrolysis, as observed in the ESI MS/MS results, it is likely that these residues were present on the same peptide after reduction in 2D SDS-PAGE. We note that although the region with Cys66 was not found in the in-gel digestion of band A, this could be explained due to a number of closely located lysine residues (at residues 60, 69, 70, and 75) which act as sites of tryptic cleavage. Since small peptides are often not detected by MS analysis, it is not surprising that peptides from this region were not detected after in-gel digestion. To test this hypothesis, in-gel digestion of unheated β-Lg was carried out and ESI MS/MS analysis of this sample also failed to detect any peptides covering 60−83 which contains the residue Cys66 (Table S2, see Supporting Information). Since all previous studies to characterize composition of fibrils involved dissolving fibrils under reducing conditions, the presence of disulfide bonds was not considered, even though the relevant cysteines were shown to be present in fibrils from both A and B variants of β-Lg.[20,23] This suggests that fibrils can contain peptides from disparate regions if they are linked by the disulfide bridge. This hypothesis is illustrated schematically in Figure 9.

The in-gel digestion ESI-MS found that the peptide bands A−E contained peptides from the region 15−53, which includes most of the β-A strand (residues 16−21). This region is involved in a non-native α-helix formation during refolding[47,48] and is highly amyloidogenic in concentrated urea.[49] It is the predominant component of heat-induced fibrils, and we believe that once tertiary structure is disrupted (which is very rapid), this region is prevented from assembling by adjacent nonamyloidogenic regions, which hinder the mutual alignment of assembling regions into a stable β-sheet structure of fibrils. That constraint can be overcome by removing the nonassembling regions with acid hydrolysis, as shown in this study, enzymatic hydrolysis of β-Lg,[50] or by microwave excitation of the protein.[20] Nonthermal effects of microwaves apparently speed up the alignment of assembling regions into correct positions and/or speed up the movement of noncore regions into sterically favorable positions.[20]

(iv). Proposed Composition of Fibril Components A−E. From the SDS-PAGE under reducing conditions and the results of the mass spectrometric analyses, likely compositions of peptides comprising bands A−E can be predicted. The molecular weights of reduced SDS-PAGE bands of Figure 6 were calculated and corrected to the known mass of bovine β-Lg, giving values about 5% less than those derived from molecular weight markers (Table S3, Supporting Information). Band A (estimated mass 8.2 kDa and containing tryptic fragments spanning residues 12−162) may be identified with peptides comprising residues 12−85 (calculated mass 8.2 kDa and pI 4.9) and with 'lower probability' residues 86−162 and/or 96−162 (calculated mass 8.8 kDa and pI 4.7, and 7.8 kDa and 4.8). The latter bands are most likely to be associated with band P in the 2D NR-R SDS-PAGE. Band B (estimated mass 6.7 kDa and containing tryptic fragments spanning residues 1−101) may be associated with peptides 1−62 (hydrolysis at exposed Glu62 on loop CD, calculated mass 6.8 kDa) and 54−114 (hydrolysis at exposed Glu114, calculated mass 7.1 kDa). Band C (estimated mass 4.7 kDa and containing tryptic fragments spanning residues 12−60) may be associated with peptides 1−53 (calculated mass 4.6 kDa) and 28−64 (calculated mass 4.1 kDa). Similarly, band D (estimated mass 4.1 kDa and containing tryptic fragments spanning residues 1−60) may be associated with peptides 34−64 (calculated mass 3.7 kDa) and 12−51 (hydrolysis at exposed Glu51 on loop BC, calculated mass 4.4 kDa). Band E (estimated mass 3.2 kDa and containing tryptic fragments spanning residues 1−53) may be associated with peptides 1−28 (calculated mass 3.0 kDa), 1−34 (calculated mass 3.4 kDa), and 34−62 (hydrolysis at exposed Glu62 on loop CD, calculated mass 3.3 kDa). Generally, the tryptic/chymotryptic digests with fragments beyond Asp53 have lower ion scores and expect values. This may be due to intrinsically lower abundance due to hydrolysis at an exposed glutamate residue (especially the exposed Glu62 on loop CD), instead of the more hydrolytically susceptible aspartate residues.

5. Mechanistic Considerations. The prevailing mechanistic models for β-Lg nanofibril self-assembly assume nucleation-dependent polymerization, with activation of β-Lg monomers and nucleation followed by growth of nuclei into mature fibrils.[9,14,21] For self-assembly to proceed, the nuclei must

grow to a critical size, below which their self-assembly is reversible.[51] Light-scattering studies have shown that nucleation/aggregation during the early stages of self-assembly is reversible upon cooling.[9,51] The fibril-forming peptides predominantly from the N-terminal region, probably self-assemble into weakly associated nuclei, which disintegrate upon cooling. The end of the lag phase therefore represents the point at which the concentration of fibrillogenic peptides is sufficiently large that the rate of assembly and growth of nuclei exceeds the rate of disassembly. The peptides from other regions of β-Lg molecule then self-assembled onto the nuclei resulting in their growth into fibrils. Assembly is rapid beyond this critical point, as indicated by the sharp increase in ThT fluorescence.

Under the conditions used here (pH 2, 80 °C, 1% protein), hydrolysis occurs at a constant rate and constant temperature, irrespective of fibril formation. The rate of fibril formation in the growth phase will depend on environmental conditions (e.g., temperature, pH, salts, shear) as well as the supply of fibril-forming peptides, which are formed by monomer hydrolysis and potentially destroyed by overhydrolysis. Self-assembly is ultimately curtailed by the depletion of these peptides as the stationary phase is reached. Hydrolysis and self-assembly occurred concurrently toward the end of the lag phase, but these two reactions have quite different underlying mechanisms. Hydrolysis is effectively a unimolecular reaction at pH 2 in aqueous solution, whereas self-assembly is a diffusion-limited multimolecular reaction. We are currently investigating ways to decouple the two processes by manipulating the composition of the solution.

■ ASSOCIATED CONTENT

Supporting Information

Figure S1, showing residual ThT intensities in supernatants obtained by ultracentrifugation of β-Lg (1% w/v) heated for 12 h at different speed/time combinations; Figure S2, showing a typical MS/MS spectrum of peptide using the Mascot search to identify major ion peaks. Figure S3, showing relative change in ellipticities at 293 and 208 nm; Figure S4, showing the circular dichroism detector HT values for FUV scans shown in Figure 4B; Figure S5, showing the residual ThT intensities in supernatants used for SDS-PAGE analysis in Figure 5; Figure S6, showing PAGE profiles of pellets containing fibrils after 6, 12, and 24 h; Figure S7, showing the coverage of β-Lg sequence found in peptides characterized by in-gel digestion ESI MS-MS of unheated β-Lg band; Figure S8 showing the standard curve used for estimation of molecular weights of peptide bands in fibrils. Table S1, showing assigned masses to most of the major ion peaks in the spectrum shown in Figure S2; Table S2 showing peptides characterized by in-gel digestion of the unheated β-Lg band; and Table S3 listing possible sequences in SDS-PAGE peptide bands of fibrils. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Sawyer, L. β-Lactoglobulin. In *Advanced Dairy Chemistry - 1 Proteins*, 3rd ed.; Fox, P. F., Mcsweeney, P. L. H. , Eds.; Kluwer Academic/Plenum Publishers: New York, 2003; pp 319−386.

(2) 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* (4), 481−495.

(3) Uhrínová, S.; Smith, M. H.; Jameson, G. B.; Uhrín, D.; Sawyer, L.; Barlow, P. N. Structural changes accompanying pH-induced dissociation of the β-lactoglobulin dimer. *Biochemistry* **2000**, *39*, 3565−3574.

(4) Kella, N. K.; Kinsella, J. E. Enhanced thermodynamic stability of β-lactoglobulin at low pH. A possible mechanism. *Biochem. J.* **1988**, *255*, 113−118.

(5) Qi, X. L.; Holt, C.; McNulty, D.; Clarke, D. T.; Brownlow, S.; Jones, G. R. Effect of temperature on the secondary structure of β-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: a test of the molten globule hypothesis. *Biochem. J.* **1997**, *324*, 341−346.

(6) Jameson, G. B.; Adams, J. J.; Creamer, L. K. Flexibility, functionality and hydrophobicity of bovine β-lactoglobulin. *Int. Dairy J.* **2002**, *12*, 319−329.

(7) Gosal, W. S.; Clark, A. H.; Pudney, P. D. A.; Ross-Murphy, S. B. Novel amyloid fibrillar networks derived from a globular protein: β-lactoglobulin. *Langmuir* **2002**, *18*, 7174−7181.

(8) Hamada, D.; Dobson, C. M. A kinetic study of β-lactoglobulin amyloid fibril formation promoted by urea. *Protein Sci.* **2002**, *11*, 2417−2426.

(9) Arnaudov, L. N.; de vries, R.; Ippel, H.; van Mierlo, C. P. M. Multiple steps during the formation of β-lactoglobulin fibrils. *Biomacromolecules* **2003**, *4*, 1614−1622.

(10) Ikeda, S.; Morris, V. J. Fine-stranded and particulate aggregates of heat-denatured whey proteins visualized by atomic force microscopy. *Biomacromolecules* **2002**, *3*, 382−389.

(11) Bolder, S. G.; Vasbinder, A. J.; Sagis, L. M. C.; van der Linden, E. Heat-Induced whey protein isolate fibrils: Conversion, hydrolysis, and disulphide bond formation. *Int. Dairy J.* **2007**, *17*, 846−853.

(12) Raynes, J. K.; Pearce, F. G.; Meade, S. J.; Gerrard, J. A. Immobilization of organophosphate hydrolase on an amyloid fibril nanoscaffold: Towards bioremediation and chemical detoxification. *Biotechnol. Prog.* **2011**, *27*, 360−367.

(13) Loveday, S. M.; Su, J.; Rao, M. A.; Anema, S. G.; Singh, H. Whey protein nanofibrils: The environment-morphology-functionality relationship in lyophilization, rehydration, and seeding. *J. Agric. Food Chem.* **2012**, *60*, 5229−5236.

(14) 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.

(15) Loveday, S. M.; Wang, X. L.; Rao, M. A.; Anema, S. G.; Creamer, L. K.; Singh, H. Tuning the properties of β-lactoglobulin nanofibrils with pH, NaCl and $CaCl_2$. *Int. Dairy J.* **2010**, *20*, 571−579.

(16) Lara, C.; Adamcik, J.; Jordens, S.; Mezzenga, R. General self-assembly mechanism converting hydrolyzed globular proteins into giant multistranded amyloid ribbons. *Biomacromolecules* **2011**, *12*, 1868−1875.

(17) Loveday, S. M.; Wang, X. L.; Rao, M. A.; Anema, S. G.; Singh, H. β-Lactoglobulin nanofibrils: Effect of temperature on fibril formation kinetics, fibril morphology and the rheological properties of fibril dispersions. *Food Hydrocolloids* **2012**, *27*, 242−249.

(18) Kroes-Nijboer, A.; Venema, P.; Bouman, J.; van der Linden, E. Influence of protein hydrolysis on the growth kinetics of β-Ig fibrils. *Langmuir* **2011**, *27*, 5753−5761.
(19) Loveday, S. M.; Wang, X. L.; Rao, M. A.; Anema, S. G.; Singh, H. Effect of pH, NaCl, $CaCl_2$ and temperature on self-assembly of β-lactoglobulin into nanofibrils: A central composite design study. *J. Agric. Food Chem.* **2011**, *59* (15), 8467−8474.
(20) Hettiarachchi, C. A.; Melton, L. D.; Gerrard, J. A.; Loveday, S. M. Formation of β-lactoglobulin nanofibrils by microwave heating gives a peptide composition different from conventional heating. *Biomacromolecules* **2012**, *13*, 2868−2880.
(21) Bolder, S. G.; Sagis, L. M. C.; Venema, P.; van der Linden, E. Effect of stirring and seeding on whey protein fibril formation. *J. Agric. Food Chem.* **2007**, *55*, 5661−5669.
(22) Dunstan, D. E.; Hamilton-Brown, P.; Asimakis, P.; Ducker, W.; Bertolini, J. Shear-induced structure and mechanics of β-lactoglobulin amyloid fibrils. *Soft Matter* **2009**, *5*, 5020−5028.
(23) Akkermans, C.; Venema, P.; van der Goot, A. J.; Gruppen, H.; Bakx, E. J.; Boom, R. M.; van der Linden, E. Peptides are building blocks of heat-induced fibrillar protein aggregates of β-lactoglobulin formed at pH 2. *Biomacromolecules* **2008**, *9*, 1474−1479.
(24) Morinaga, A.; Hasegawa, K.; Nomura, R.; Ookoshi, T.; Ozawa, D.; Goto, Y.; Yamada, M.; Naiki, H. Critical role of interfaces and agitation on the nucleation of Aβ amyloid fibrils at low concentrations of Aβ monomers. *Biochim. Biophys. Acta − Proteins Proteomics* **2010**, *1804*, 986−995.
(25) Hill, E. K.; Krebs, B.; Goodall, D. G.; Howlett, G. J.; Dunstan, D. E. Shear flow induces amyloid fibril formation. *Biomacromolecules* **2006**, *7* (1), 10−13.
(26) Mishra, R.; Sorgjerd, K.; Nystrom, S.; Nordigarden, A.; Yu, Y.-C.; Hammarstrom, P. Lysozyme amyloidogenesis is accelerated by specific nicking and fragmentation but decelerated by intact protein binding and conversion. *J. Mol. Biol.* **2007**, *366*, 1029−1044.
(27) 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.
(28) Swaisgood, H. E. Chemistry of milk protein. In *Developments in Dairy Chemistry-1*; Fox, P. F., Ed.; Applied Science Publishers: London, U.K., 1982; Vol. *1*, p 1−59.
(29) Morris, A. M.; Watzky, M. A.; Agar, J. N.; Finke, R. G. Fitting neurological protein aggregation kinetic data via a 2-step, minimal/"Ockham's razor" model: The Finke-Watzky mechanism of nucleation followed by autocatalytic surface growth. *Biochemistry* **2008**, *47*, 2413−2427.
(30) Laemmli, U. K. Cleavage of structural proteins during assembly of head of bactariophage-T4. *Nature* **1970**, *227*, 680−685.
(31) Havea, P.; Singh, H.; Creamer, L. K.; Campanella, O. H. Electrophoretic characterization of the protein products formed during heat treatment of whey protein concentrate solutions. *J. Dairy Res.* **1998**, *65*, 79−91.
(32) Woody, R. W. Circular-dichroism. *Methods Enzymol.* **1995**, *246*, 34−71.
(33) Soskic, V.; Godovac-Zimmermann, J. Improvement of an in-gel tryptic digestion method for matrix-assisted laser desorption/ionization-time of flight mass spectrometry peptide mapping by use of volatile solubilizing agents. *Proteomics* **2001**, *1*, 1364−1367.
(34) Candiano, G.; Bruschi, M.; Musante, L.; Santucci, L.; Ghiggeri, G. M.; Carnemolla, B.; Orecchia, P.; Zardi, L.; Righetti, P. G. Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. *Electrophoresis* **2004**, *25*, 1327−1333.
(35) Perkins, D. N.; Pappin, D. J. C.; Creasy, D. M.; Cottrell, J. S. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **1999**, *20*, 3551−3567.
(36) Krebs, M. R. H.; Bromley, E. H. C.; Donald, A. M. The binding of thioflavin-T to amyloid fibrils: localisation and implications. *J. Struct. Biol.* **2005**, *149*, 30−37.
(37) Otte, J.; Zakora, M.; Qvist, K. B. Involvement of disulfide bands in bovine β-lactoglobulin B gels set thermally at various pH. *J. Food Sci.* **2000**, *65*, 384−389.
(38) Mudgal, P.; Daubert, C. R.; Clare, D. A.; Foegeding, E. A. Effect of disulfide interactions and hydrolysis on the thermal aggregation of β-lactoglobulin. *J. Agric. Food Chem.* **2010**, *59* (5), 1491−1497.
(39) Harris, J. I.; Cole, R. D.; Pon, N. G. Kinetics of acid hydrolysis of dipeptides. *Biochem. J.* **1956**, *62*, 154−159.
(40) Bateman, L.; Ye, A. Q.; Singh, H. In vitro digestion of β-lactoglobulin fibrils formed by heat treatment at low pH. *J. Agric. Food Chem.* **2010**, *58*, 9800−9808.
(41) Oboroceanu, D.; Wang, L. Z.; Brodkorb, A.; Magner, E.; Auty, M. A. E. Characterization of β-lactoglobulin fibrillar assembly using atomic force microscopy, polyacrylamide gel electrophoresis, and *in situ* Fourier transform infrared spectroscopy. *J. Agric. Food Chem.* **2010**, *58*, 3667−3673.
(42) Manderson, G. A.; Creamer, L. K.; Hardman, M. J. Effect of heat treatment on the circular dichroism spectra of bovine β-lactoglobulin A, B, and C. *J. Agric. Food Chem.* **1999**, *47* (11), 4557−4567.
(43) Strickland, E. H. Aromatic contributions to circular dichroism spectra of proteins. *Crit. Rev. Biochem.* **1974**, *2*, 113−175.
(44) Molinari, H.; Ragona, L.; Varani, L.; Musco, G.; Consonni, R.; Zetta, L.; Monaco, H. L. Partially folded structure of monomeric bovine β-lactoglobulin. *FEBS Lett.* **1996**, *381*, 237−243.
(45) Johnson, W. C. Protein secondary structure and circular-dichroism − a practical guide. *Proteins: Struct., Funct., Genet.* **1990**, *7*, 205−214.
(46) Aouzelleg, A.; Bull, L. A.; Price, N. C.; Kelly, S. M. Molecular studies of pressure/temperature-induced structural changes in bovine β-lactoglobulin. *J. Sci. Food Agri.* **2004**, *84*, 398−404.
(47) Hamada, D.; Segawa, S.; Goto, Y. Non-native α-helical intermediate in the refolding of β-lactoglobulin, a predominantly β-sheet protein. *Nat. Struct. Biol.* **1996**, *3*, 868−873.
(48) Kuwata, K.; Shastry, R.; Cheng, H.; Hoshino, M.; Batt, C. A.; Goto, Y.; Roder, H. Structural and kinetic characterization of early folding events in β-lactoglobulin. *Nat. Struct. Biol.* **2001**, *8*, 151−155.
(49) Hamada, D.; Tanaka, T.; Tartaglia, G. G.; Pawar, A.; Vendruscolo, M.; Kawamura, M.; Tamura, A.; Tanaka, N.; Dobson, C. M. Competition between folding, native-state dimerisation and amyloid aggregation in β-lactoglobulin. *J. Mol. Biol.* **2009**, *386*, 878−890.
(50) Akkermans, C.; Venema, P.; van der Goot, A. J.; Boom, R. M.; van der Linden, E. Enzyme-induced formation of β-lactoglobulin fibrils by AspN endoproteinase. *Food Biophys.* **2008**, *3* (4), 390−394.
(51) Aymard, P.; Nicolai, T.; Durand, D.; Clark, A. Static and dynamic scattering of β-lactoglobulin aggregates formed after heat-induced denaturation at pH 2. *Macromolecules* **1999**, *32*, 2542−2552.
(52) Matrix science help: summary reports for MS/MS. http://www.matrixscience.com/help/msms_summaries_help.html (25.01.2013)

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