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Re-formation of Fibrils from Hydrolysates of β-Lactoglobulin Fibrils during in Vitro Gastric Digestion

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ABSTRACT: In this study, in vitro digestion of β-lactoglobulin (β-Lg) fibrils and the re-formation of fibril-like structures after prolonged enzymatic hydrolysis (up to 48 h) were investigated using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS), thioflavin T fluorescence photometry, and transmission electron microscopy (TEM). Pure β-Lg fibrils that had been formed by heat treatment at pH 2.0 were rapidly hydrolyzed by pepsin in the simulated gastric fluid (pH 1.2), and some new peptides that were suitable for further fibril formation were produced. TEM showed that the new fibrils were long and straight but thinner than the original fibrils, and both TEM and MALDI-MS indicated that the peptides in the new fibrils were shorter/smaller than the peptides in the original fibrils. The formation of new fibrils was found to be affected more by pH than by enzyme activity or temperature.

KEYWORDS: β-Lactoglobulin, fibrils, in vitro pepsin digestion, re-formation

INTRODUCTION

β-Lactoglobulin (β-Lg) is widely used as a functional ingredient in foods. However, β-Lg is little hydrolyzed by pepsin under gastric conditions because of its stable, globular tertiary structure at low pH (pH < 3.0). The highly hydrophobic β-barrel buries most hydrophobic sequences, making it very difficult for enzymes to gain access.[1−3] It has been found that modification of the tertiary and secondary structures by heat or high-pressure treatment allows β-Lg to be more easily accessed by enzymes.[4−6] At neutral pH after heat treatment at 55−65 °C, bovine β-Lg has been found to undergo increased proteolysis with trypsin and chymotrypsin;[7] high-pressure treatment can lead to rapid in vitro digestion of β-Lg by pepsin;[6] microwave treatment has also been shown to accelerate the digestion of β-Lg by Pronase, α-chymotrypsin, and pepsin.[8]

It has been found that heat treatment results in long, straight, flexible amyloid-like fibrils at a pH lower than the isoelectric point (e.g., pH 2−3), whereas only short, curly aggregates are formed at high pH (higher than the isoelectric point, e.g., pH > 7).[9−11] During the past decade, many studies to understand the mechanisms of fibril formation and to identify the building blocks of fibrils have been carried out.[12−17] Using atomic force microscopy, Radford and co-workers[18] attempted to achieve detailed understanding of the formation of amyloid fibrils from β_2-microglobulin at different protein concentrations, pH values, and ionic strengths. This group found two distinct competitive pathways for fibril formation as well as heterogeneity of the amyloidal assembly.[18] In contrast, Akkermans et al.[19] claimed that rather than denatured intact proteins, the building blocks of β-Lg fibrils are peptides that are derived through acid hydrolysis on heat treatment at low pH. This group suggested that only specific peptides with characteristics of high hydrophobicity, the ability to form β-sheets, low charge, and proper charge distribution along the peptide can form fibrils, which results in a relatively low yield (20% on average) of fibrils.[19]

In addition to the possibility of using β-Lg fibrils to improve food texture and nutritional value,[20,21] they have also been used in drugs and supplements.[22−24] Kim et al.[25] studied the antigenicity of whey proteins after hydrolysis by pepsin and trypsin and found that heat treatment of whey protein concentrate before enzymatic hydrolysis resulted in lower antigenicity. β-Lg fibrils may also be used as substitutes for β-Lg to reduce the allergenicity that is caused during digestion in the gastrointestinal system, while providing similar nutrition. After evaluating various proteins, Astwood et al.[26] proposed that the more stable a protein is toward enzymes in the gastric fluid, the greater is the possibility that the protein will reach the intestinal mucosa to cause a food allergy. Our previous study[27] on the in vitro digestion of β-Lg fibrils in a heat-treated mixture has provided evidence that the amyloid-like fibrils can be digested by pepsin under gastric conditions in as little as 2 min, which may thus greatly reduce the allergenicity that is caused by milk products containing β-Lg when they are added into foods to improve their nutritional value.

Except for a few studies that found that after enzymatic hydrolysis, the resultant peptides were able to form fibrils under suitable conditions,[28−30] there has to date been little work on β-Lg fibril digestion in the gastrointestinal system. In our previous study,[27] we found that β-Lg fibrils in a heat-treated mixture could be digested in vitro by pepsin within 2 min and that the peptides in the fibrils were degraded to smaller peptides. To better understand fibril digestion, in the current study, pure β-Lg fibrils were digested by pepsin in a designated in vitro system for a prolonged period of time (up to 2 days). The formation of new fibrils from hydrolysates after rapid enzymatic digestion was studied in detail; factors that may affect digestion and new fibril formation, such as pH, temperature, and enzyme activity, were investigated. The whole process was monitored by transmission electron microscopy (TEM), matrix-assisted laser desorption/ionization

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mass spectrometry (MALDI-MS), and thioflavin T (ThT) fluorescence.

MATERIALS AND METHODS

Materials. Bovine milk β-Lg (approximately 90% pure; containing β-Lg A and β-Lg B) and pepsin were obtained from Sigma-Aldrich (St. Louis, MO). Unless otherwise stated, all of the chemicals used were of analytical grade and were obtained from Sigma.

Preparation of Protein Samples. Bovine β-Lg was dissolved in pH 2.0 HCl solution and Milli-Q water to give solutions with a 10 mg/mL concentration at pH 2.0 (adjusted using 1 M HCl). The β-Lg solutions were shaken in a REAX Control shaker (John Morris Scientific Pty Ltd., Chatswood, Australia) for 5 min before being stirred at room temperature for 2 h. They were then centrifuged at 27000*g* using a Sorvall Evolution RC Superspeed Centrifuge (Thermo Scientific, Asheville, NC) and filtered using 0.2 μm membrane filters. The protein concentrations were measured using a Genesys 10 Series UV/vis spectrophotometer (Thermo Electron Corp., Waltham, MA) at an absorption wavelength of 278 nm.

Preparation of Pure β-Lg Fibrils. The protein samples were shaken at a speed of 50 rpm in a shaking water bath (BS-06/31, Jeio Tech Co., Ltd., Korea) at 80 °C for 20 h. They were cooled at room temperature before being stored in a refrigerator at 4 °C.

Pure fibrils were obtained by filtering the preheated samples using 4 mL Amicon Ultra Centrifugal Filter Devices (regenerated cellulose, 10 kDa molecular weight cutoff). A Heraeus Multifuge 3SR+ centrifuge (Thermo Scientific) operating at 3000 rpm was used. The samples with reduced volume were then washed twice using pH 2.0 HCl solution, followed by centrifugation each time to wash off the filtrate. The final volumes were made to one-fifth of the original volumes using the same HCl solution (thus, the final concentration of the fibrils was about 1% based on a 20% yield of fibrils). The retentate of each sample was then kept in the refrigerator at 4 °C. The filtrates from each sample were combined and dried using a Savant SC250EXP SpeedVac Concentrator (Thermo Scientific). The procedure for preparing pure aggregates (short, wormlike fibrils) at neutral pH was exactly the same as above except that the original protein solutions were prepared using Milli-Q water instead of HCl solution.

Enzymatic Hydrolysis. The fibril samples obtained were diluted with simulated gastric fluid (SGF, containing 0.034 M NaCl and using concentrated HCl to adjust the pH to 1.2, without pepsin) at a volume ratio of 1:2. Thus, the final β-Lg fibril concentration was 3.3 mg/mL. All samples were shaken at a speed of 50 rpm in a 37 °C water bath. Freshly made pepsin solution (10 mg/mL in pH 2.0 HCl solution and shaken in a REAX Control shaker for 5 min before filtering using 0.2 μm membrane filters) was added to these solutions, to a final pepsin concentration of 2 mg/mL. Aliquots were withdrawn into Eppendorf vials containing 0.2 M Na_2CO_3 (sample:Na_2CO_3 = 1:0.7, v/v) after 0, 0.5, 2, 5, 10, 30 min, etc. For some samples, the enzyme was inactivated by heating in a boiling water bath (~95 °C) for 30 s.

TEM. A Philips CM10 transmission electron microscope (Eindhoven, The Netherlands) was used together with a Modara digital camera system (Olympus Soft Imaging Solutions) to observe the images. The images were optimized and recorded using ITEM software and were enlarged up to 92000 times by the microscope.

All samples were examined after dilution with Milli-Q water (1:3, v/v) and mixing using a REAX Control shaker for 30 s. The samples were deposited on to carbon-coated copper grids (Agar 200 mesh copper grids with a Formvar support film stabilized by evaporated carbon) by putting a grid on to a drop of sample with the coated side contacting the sample. After 3 min, excess sample was removed using a piece of filter paper. The grid was then put on to a drop of 2% uranyl acetate solution for another 3 min for staining, excess liquid was removed using a piece of filter paper, and the grid was then air-dried at room temperature and transferred on to a carrier in the TEM system for examination.

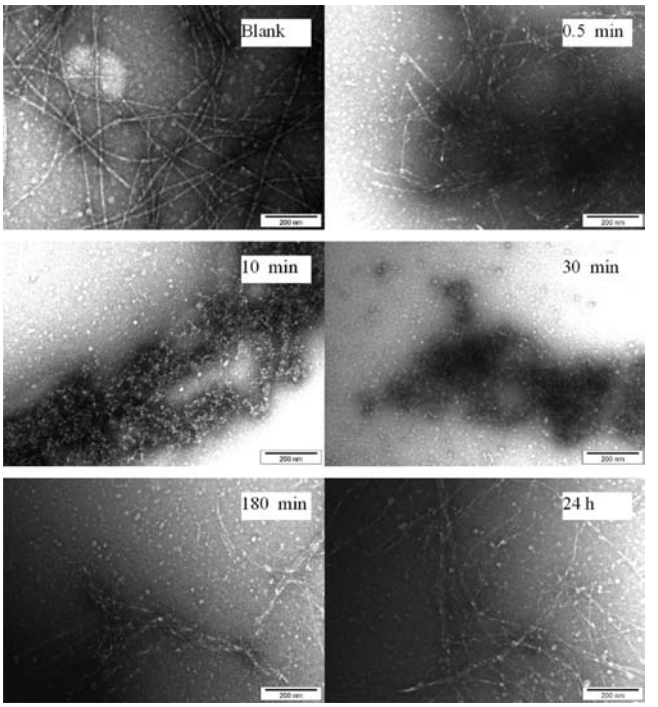

Figure 1. TEM images of samples that were enzymatically hydrolyzed for up to 24 h. (A−F) Images for the control and after incubation for 0.5, 10, 30, 180 min, and 24 h, respectively. All images were obtained using the negative stain method. All samples were diluted three times using Milli-Q water and were enlarged up to 92000 times on the microscope. All scale bars are 200 nm.

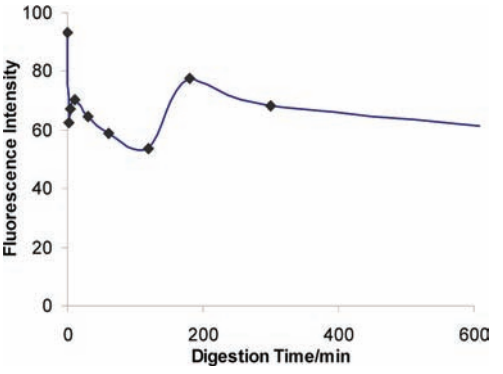

Figure 2. ThT fluorescence intensities of samples that were enzymatically hydrolyzed at 37 °C for 24 h. The net intensities were obtained by subtracting the intensity of a ThT solution from the intensity of the samples. The excitation and emission wavelengths were 442 and 485 nm, respectively.

ThT Fluorescence Measurement. An enzymatically hydrolyzed sample (100 μL) was added into a four-sided quartz cuvette containing 2.9 mL of 0.02% ThT solution (pH 7.0, 0.1 M phosphate buffer) and mixed well. An FP-6200 spectrofluorometer (Jasco Corp., Tokyo, Japan) was used in fixed-wavelength mode, with an excitation wavelength of 442 nm (with a bandwidth of 5 nm) and an emission wavelength of 485 nm (with a bandwidth of 10 nm). The intensities were recorded as average values of triplicate measurements. The net

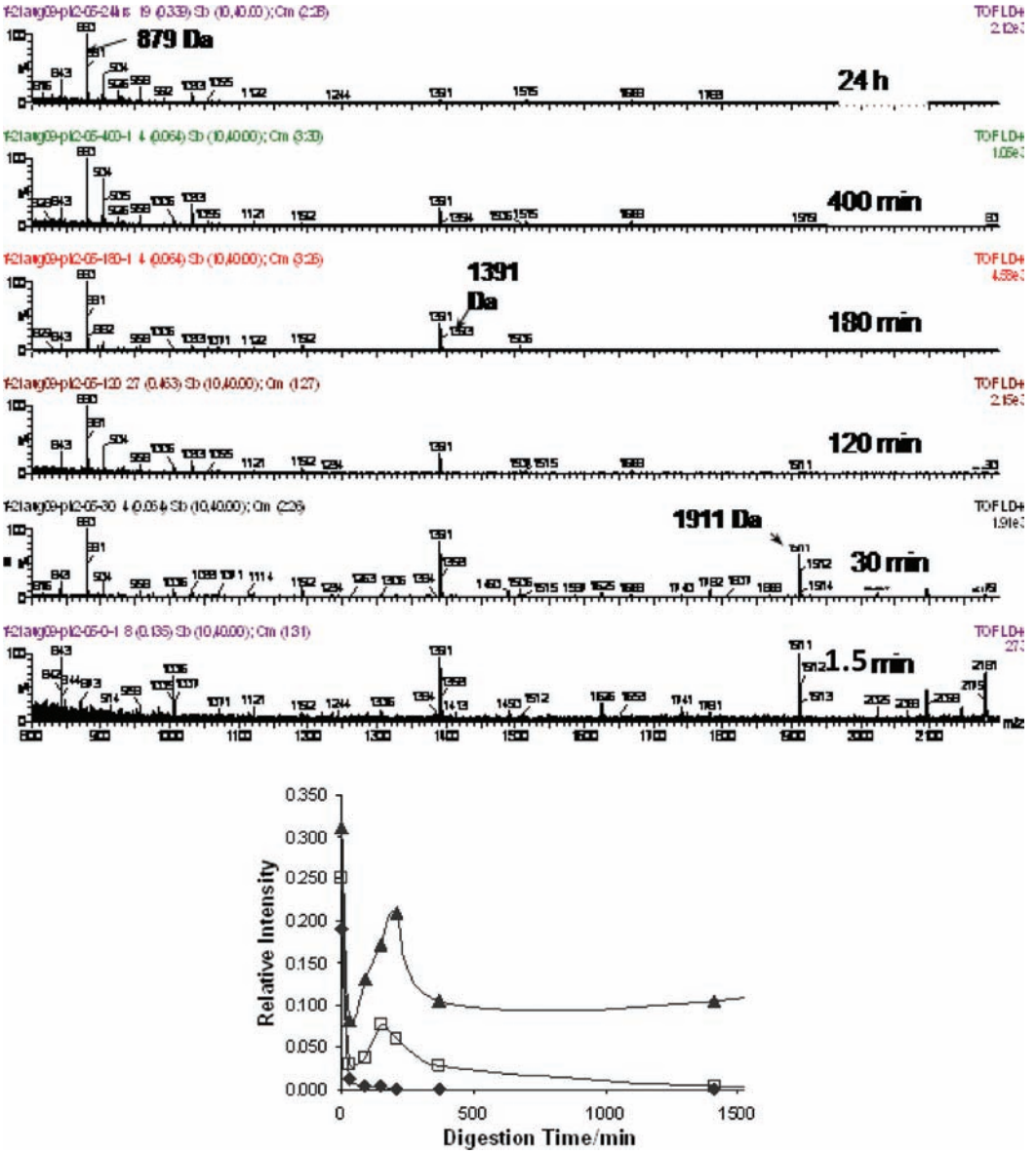

Figure 3. Upper: MALDI-MS spectra of samples standing at room temperature after 30 min of enzymatic hydrolysis. From bottom to top, the standing times were 0, 30, 120, 180, and 400 min and 24 h with base addition to inactivate the enzyme. The spectra were obtained using reflectron mode. Three peaks at 879, 1391, and 1911 Da, with obvious changes during the enzymatic hydrolysis process, are marked in the spectra. Lower: relative intensities of three major peaks [879 Da (▲), 1391 Da (□), and 1911 Da (◆), respectively] as compared with the peak at 442 Da from MALDI mass spectra as a function of digestion time for the above samples.

intensities were obtained by subtracting the intensity of the ThT solution (blank) when the data were analyzed.

MALDI-MS. Measurements were performed on a Waters-LMW 1974 MALDI mass spectrometer (Waters Corp., Milford, MA) equipped with a pulsed nitrogen laser (337 nm wavelength, 3 ns pulse width, and 3 Hz frequency) and a delayed extraction ion source. Ions generated by laser desorption of digestion samples of β-Lg were analyzed in positive ion reflectron mode (for small peptides) or linear mode (for large peptides or proteins).

Samples were mixed with matrix agents at a volume ratio of 1:1. For linear mode, sinapinic acid (10 mg/mL, in 40% acetonitrile and 60% water containing 1% trifluoroacetic acid) was used; for reflectron mode, α-cyano-4-hydroxycinnamic acid (20 mg/mL, in methanol:acetonitrile = 1:1, v/v) was used. The maximum masses (monoisotopic ion) detected in the reflectron and linear modes were 4000 and 50000 Da, respectively. The mass spectra were analyzed using MassLynx software.

Na^+ charged peptides may have been detected in addition to H^+ charged peptides when using both matrix agents, such that the detected molecular mass would be 22 Da higher than the calculated value. Other situations, such as oxidation of methionine (16 or 32 Da higher), were also considered. The error in molecular mass was considered to be less than 1 Da for reflectron mode and 1−2 Da for linear mode.

The above data were confirmed by ABI 4800 MALDI-TOF/TOF-MS (Applied Biosystems, Mulgrave, Auatralia), and the built-in software, Mascot, was used for peptide mapping, in which only peptides with a matching ion score above 40 were considered to be well-matched results. The software, MS-Seq-Prospector, was also used for manual sequence matching.

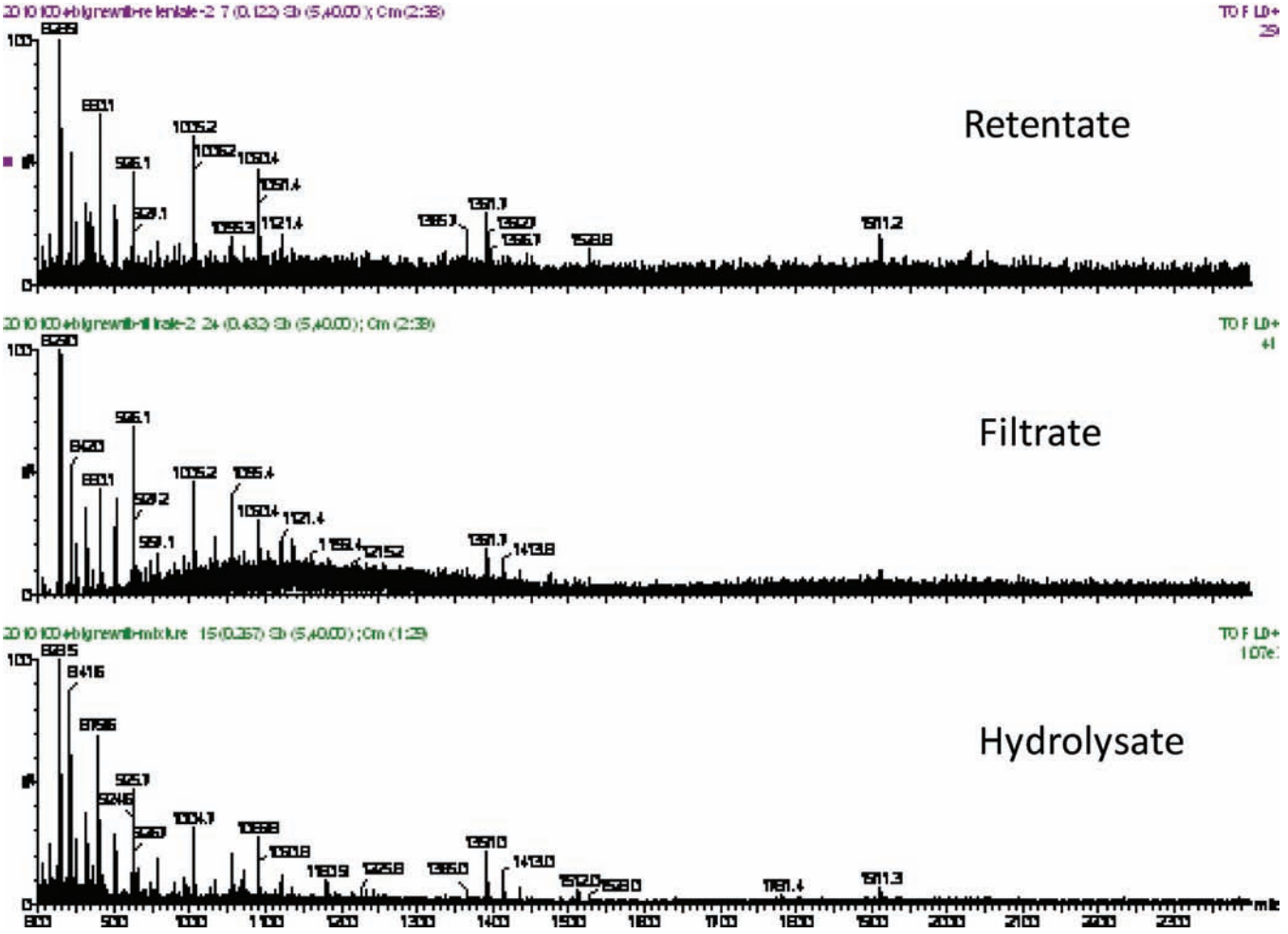

Figure 4. MALDI-MS spectra of samples standing at room temperature for 180 min after 30 min of enzymatic hydrolysis. The retentate was obtained by centrifugation of the hydrolysates in a 10000 Da membrane filter and washing twice with pH 2.0 HCl solution. Top, retentate; middle, filtrate; and bottom, hydrolysate mixture.

RESULTS

Pepsin Hydrolysis and Re-formation of β-Lg Fibrils. The fibrils obtained from centrifugation were added to SGF containing pepsin in a 37 °C water bath. Aliquots were withdrawn at different times up to 24 h and were examined using TEM, ThT fluorescence, and MALDI-MS.

The TEM images showed long, straight fibrils in the control sample (no pepsin addition) (Figure 1A). During incubation from 0.5 to 30 min (Figures 1B−D), the fibrils gradually became shorter and had almost disappeared at 30 min. However, surprisingly, fibril-like structures appeared again when the incubation time was extended to 180 min; long, straight fibrils were apparent after incubation for 24 h (Figure 1E,F). These new fibrils appeared to be smaller in diameter and had a somewhat different appearance as compared with those in the control sample (Figure 1A). Using the ITEM software on the TEM equipment, the diameters of the original fibrils and of the new fibrils in different samples were estimated. The average diameters of the original fibrils in two samples were 8.28 and 8.22 nm; those of the newly formed fibrils in two samples were 6.65 and 6.52 nm.

ThT fluorescence analysis (Figure 2) showed that the fluorescence intensity decreased up to incubation for 120 min, which indicated a decrease in ThT binding on to β-sheets/fibrils, suggesting that the fibrils had been hydrolyzed by pepsin. However, at 180 min, the fluorescence intensity increased to another maximum, which implied an increase in ThT binding to β-sheets. A slow decrease in fluorescence intensity was observed when the incubation time was extended to 24 h.

MALDI spectra using reflectron mode were obtained for β-Lg fibrils incubated in SGF (with pepsin) for different times. As shown in Figure 3 (upper), about two dozen peaks were detected for the original fibrils, that is, the control sample (no pepsin addition). The samples incubated in SGF with pepsin showed fewer peaks; after 30 min of incubation, the peptides with molecular masses of 879, 1391, and 1911 Da were dominant in the spectra, along with other peptides. The peptide with molecular mass 1911 Da was hydrolyzed to smaller peptides at and above an incubation time of 120 min. The peptide with molecular mass 1391 Da was not detected after 24 h of incubation. The relative peak intensity of the peptide with molecular mass 1911 Da decreased during digestion, and no signal was detected after 120 min, whereas the relative peak intensities of the peptides with molecular masses 1391 and 879 Da increased during digestion and reached their maximum intensities at 120 and 180 min, respectively (Figure 3, lower).

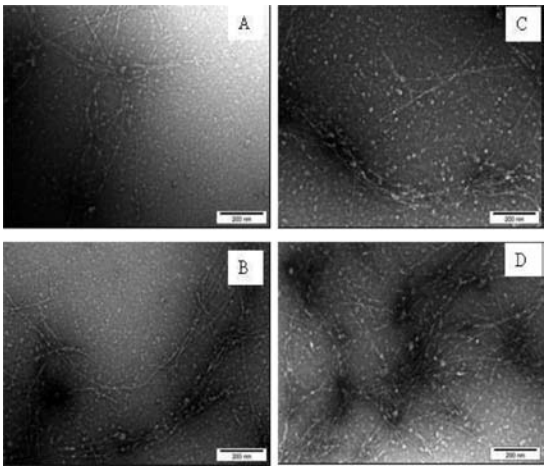

Figure 5. TEM images of samples after (A) 0, (B) 20, (C) 80, and (D) 140 min of enzymatic hydrolysis and then enzyme inactivation by heating at 95 °C for 30 s. All images were obtained using the negative stain method. All samples were diluted three times using Milli-Q water and were enlarged up to 92000 times on the microscope. All scale bars are 200 nm.

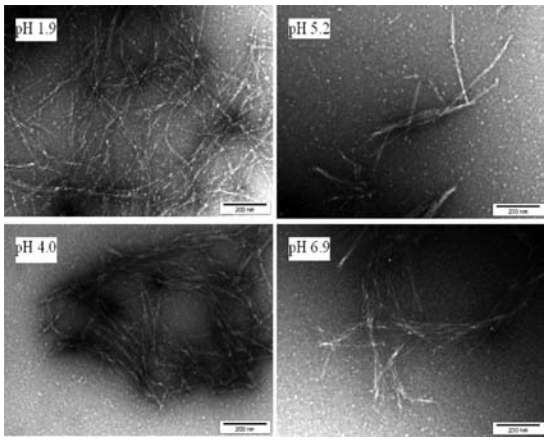

Figure 6. TEM images of hydrolysate samples of different pH values. All samples were obtained by heat inactivation of pepsin after 30 min of enzymatic hydrolysis. The hydrolysates were adjusted to different pH values and were held at 20 °C for 180 min. All images were obtained using the negative stain method. All samples were diluted three times using Milli-Q water and were enlarged up to 92000 times on the microscope. All scale bars are 200 nm.

The decreasing trend in molecular mass of the peptides from the original fibrils during pepsin digestion suggested that the larger peptides in the original fibrils were hydrolyzed to smaller peptides during the digestion process, in agreement with our earlier work.[27]

To examine the peptides that may be involved in forming the new fibrils, the sample that had been hydrolyzed for 30 min was passed through a 10 KDa membrane. Both the retentate and the filtrate were analyzed by MALDI. It was noted that the MALDI spectrum obtained for the retentate, rather than the filtrate, was more consistent with that obtained for the hydrolysate. The major peptides, with molecular masses of 1911, 1391, and 879 Da, were observed in the retentate, whereas the filtrate contained mainly small peptides (Figure 4).

Fibril Re-formation in the Absence of Enzyme Activity. After enzymatic hydrolysis for different times (e.g., 0, 20, 80, and 140 min), the enzyme was inactivated by heating at 90 °C for 30 s. The samples were then left at room temperature overnight (~18 h) before analysis by TEM. As shown in Figure 5, long, straight, thin fibrils were observed in all samples, and it appeared that more fibrils were formed in the samples with longer hydrolysis times. These results suggest that, in the absence of pepsin, fibrils will reform once there are sufficient peptides in the sample.

Effect of pH on Fibril Re-formation. After incubation for 30 min, the pepsin-hydrolyzed samples (the pepsin was inactivated by heat treatment) were adjusted to pH values of 1.9, 4.0, 5.2, and 6.9 and were then left at room temperature for about 3 h. As shown in Figure 6, a large amount of long, straight, thin fibrils was observed in the sample at pH 1.9, somewhat shorter fibrils were observed in the sample at pH 4.0, and very few short fibrils were detected in the samples at pH 5.2 (close to the isoelectric point) and at neutral pH (pH 6.9). These results suggest that fibril formation from pepsin-derived peptides is facilitated on the acidic side of the isoelectric point of β-Lg.

DISCUSSION

The results clearly showed that peptides derived from the pepsin-induced hydrolysis of β-Lg can self-assemble into fibril-like structures after prolonged storage. Long, straight fibrils were observed after 180 min of pepsin hydrolysis of β-Lg under simulated gastric conditions (Figure 1E). ThT fluorescence gave supporting evidence for β-sheet/fibril formation after 180 min of hydrolysis (Figure 2).

The greatest amount of new fibrils was formed in a highly positively charged environment (pH 1.9) (Figure 6); more new fibrils were formed from hydrolysates when the pH was higher than the isoelectric point (e.g., pH 6.9) than when the pH was close to the isoelectric point (e.g., pH 5.2). In contrast, the observation was that there was no fibril re-formation for hydrolysates of aggregates obtained through heat treatment at pH 7.4. These results may suggest that the peptides available for fibril reformation should inherit the characteristics of the parent peptides for fibril formation. Alternatively, the newly produced peptides from the breaking down of the peptides in the original fibrils should retain most of the characteristics that are necessary for fibril formation.

Akkermans et al.[30] reported that after β-Lg had been enzymatically hydrolyzed by AspN endoproteinase, fibrils formed from the hydrolysates when the pH was adjusted to pH 2.0. A few other studies on the formation of fibrils from enzymatic hydrolysates of proteins under different conditions have been carried out as follows: Otte et al.[31] used a Glu- and Asp-specific microbial protease to hydrolyze β-Lg at neutral pH and low temperature, proposing that peptide 135−158 from the C terminus was responsible for the initiation of aggregation;[31] Creusot et al.[32] argued that the dominating peptide for aggregate formation was the N terminus peptide, 1−45. Although these studies were carried out in different systems, they led to a similar conclusion, that is, certain peptides of β-Lg (with the ability to form fibrils) that result from enzymatic hydrolysis may form fibrils under suitable conditions.

In our previous study, peptide mapping assigned the peptide with molecular mass 1911 Da at sequence 37−53 or 134−149, the peptide with molecular mass 1391 Da at sequence 1−12 or 138−149, and the peptide with molecular mass 879 Da at sequence 46−53, 23−32, or 143−149. All of the matches fell

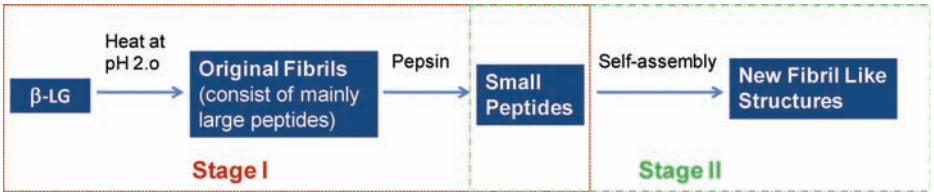

Figure 7. Illustration of the two-stage in vitro digestion of β-Lg by pepsin and new fibril formation during incubation for up to 48 h.

at either the N terminus or the C terminus of β-Lg, where the peptides were expected to be involved in fibril formation on heat treatment at low pH (the original fibrils before enzymatic hydrolysis).[19]

The changes in the peak intensities of the peptides with molecular masses 1911, 1391, and 879 Da during pepsin hydrolysis could be clearly observed (Figure 3): The release and consumption of the peptide with molecular mass 1911 Da occurred very rapidly during the first 30 min; the maximum release of the peptide with molecular mass 1391 Da was at 2 h, whereas the maximum release of the peptide with molecular mass 879 Da at 3 h coincided with fibril formation, observed by both TEM and ThT fluorescence (Figures 1 and 2). This indicates the possible involvement of the peptides with molecular masses 1391 and 879 Da in the formation of new fibrils; the increase in the peptide with molecular mass 879 Da at 24 h also suggested that it was a final product peptide.

The thickness varied from one fibril to another, as well as from one part to another part in the same fibril because of the heterogeneity and the partially twisted morphology of the fibrils, both of which led to reasonably high standard deviations in the measurement of the fibril diameters. However, a large number of measurements on many samples overcame the shortcomings, making the measurement of the overall average fibril diameters fairly reasonable. On the basis of the suggestions of Loveday et al.[33] and Gosal et al.,[18] that is, that the peptides constitute a fibril in a perpendicular direction to the fibril chain, the diameter of the fibril should be a good indication of the length/size of the building peptides. Therefore, the smaller diameters of the new fibrils also suggested that the peptides in the new fibrils were shorter/smaller than the peptides in the original fibrils, which coincided with the observations from MALDI-MS (Figure 3).

After hydrolysis of the peptides in the fibrils by pepsin, equilibrium could develop between the self-assembly of peptides into the new fibrils and the dissociation/degradation of the peptides from the original fibrils (as illustrated in Figure 7). According to Kroes-Nijboer et al.,[34] β-Lg fibril formation is an entropy-driven process, that is, the initial fibril formation (above the critical aggregate concentration) is dominated by the amount of proteins or peptides in the sample that are available for fibril formation. Thus, the dissociation and reassociation of peptides from or with the fibrils should be related to the amount of peptides available for fibril formation as well as the charge interactions between the peptides. Therefore, during enzymatic hydrolysis, if many of the peptides produced are suitable for fibril formation, the high entropy would drive the equilibrium in the direction of new fibril formation. However, if the peptides produced are not in favor of fibril formation (i.e., fewer fibril formation peptides are available), the equilibrium might be driven to the direction of the dissociation of peptides from the fibrils and, consequently, might lead to complete fibril hydrolysis during prolonged incubation (up to 2 weeks). In our case, it seemed that the hydrophobic-favoring pepsin did produce sufficient suitable peptides from the original β-Lg fibrils to achieve new fibril development.

Overall, this study on the formation of β-Lg fibrils by pepsin under gastric conditions during prolonged incubation revealed rapid hydrolysis of the original fibrils followed by the formation of new fibrils from the hydrolysates. The new fibrils were found by TEM to be long and straight but thinner and containing more shorter/smaller peptides, as compared with the original fibrils. Maintaining a low pH was found to be critical for new fibril formation; temperature and enzyme activity had less effect than pH. For the first time, this work has provided insight into β-Lg fibril digestion and subsequent new fibril formation under various conditions.

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REFERENCES

(1) Fox, P. F.; McSweeney, P. L. H. *Advanced Dairy Chemistry*, 3rd ed.; Kluwer Academic/Plenum Publishers: New York, 2003; Vol. 1, p 741.

(2) Kontopidis, G.; Holt, C.; Sawyer, L. Invited Review: β-Lactoglobulin: Binding Properties, Structure, and Function. *J. Dairy Sci.* **2004**, *87*, 785–796.

(3) Sakurai, K.; Oobatake, M.; Goto, Y. Salt-dependent monomer-dimer equilibrium of bovine beta-lactoglobulin at pH 3. *Protein Sci.* **2001**, *10*, 2325–2335.

(4) Qi, X. L.; Brownlow, S.; Holt, C.; Sellers, P. Thermal denaturation of [beta]-lactoglobulin: Effect of protein concentration at pH 6.75 and 8.05. *Biochim. Biophys. Acta, Protein Struct. Mol. Enzymol.* **1995**, *1248*, 43–49.

(5) Dufour, E.; Herve, G.; Haertle, T. Hydrolysis of Beta-Lactoglobulin by Thermolysin and Pepsin under High Hydrostatic-Pressure. *Biopolymers* **1995**, *35*, 475–483.

(6) Zeece, M.; Huppertz, T.; Kelly, A. Effect of high-pressure treatment on in-vitro digestibility of beta-lactoglobulin. *Innovative Food Sci. Emerging Technol.* **2008**, *9*, 62–69.

(7) Iametti, S.; Rasmussen, P.; Frokiaer, H.; Ferranti, P.; Addeo, F.; Bonomi, F. Proteolysis of bovine β-lactoglobulin during thermal treatment in subdenaturing conditions highlights some structural features of the temperature-modified protein and yields fragments with low immunoreactivity. *Eur. J. Biochem.* **2002**, *269*, 1362–1372.

(8) Izquierdo, F. J.; Alli, I.; Yaylayan, V.; Gomez, R. Microwave-assisted digestion of beta-lactoglobulin by Pronase, alpha-chymotrypsin and pepsin. *Int. Dairy J.* **2007**, *17*, 465–470.

(9) Loveday, S. M.; Rao, M. A.; Creamer, L. K.; Singh, H. Factors affecting rheological characteristics of fibril gels: The case of beta-lactoglobulin and alpha-lactalbumin. *J. Food Sci.* **2009**, *74*, R47–R55.

(10) Krebs, M. R. H.; Devlin, G. L.; Donald, A. M. Amyloid fibril-like structure underlies the aggregate structure across the pH range for β-lactoglobulin. *Biophys. J.* **2009**, *96*, 5013–5019.

(11) Mahmoudi, N, M. S.; Nicolai, T; Durand, D; Riaublanc, A. Light-scattering study of the structure of aggregates and gels formed by heat-denatured whey protein isolate and beta-lactoglobulin at neutral pH. *J. Agric. Food Chem.* **2007**, *55*, 3104–3111.

(12) Ferrone, F. Analysis of protein aggregation kinetics. In *Amyloid, Prions, and Other Protein Aggregates*; Academic Press Inc.: San Diego, 1999; Vol. 309, pp 256−274.

(13) Yan, H.; Frielinghaus, H.; Nykanen, A.; Ruokolainen, J.; Saiani, A.; Miller, A. F. Thermoreversible lysozyme hydrogels: properties and an insight into the gelation pathway. *Soft Matter* **2008**, *4*, 1313–1325.

(14) Veerman, C.; Ruis, H.; Sagis, L. M. C.; van der Linden, E. Effect of electrostatic interactions on the percolation concentration of fibrillar beta-lactoglobulin gels. *Biomacromolecules* **2002**, *3*, 869–873.

(15) Veerman, C.; Sagis, L. M. C.; Venema, P.; van der Linden, E. Shear-induced aggregation and break up of fibril clusters close to the percolation concentration. *Rheol. Acta* **2005**, *44*, 244–249.

(16) Donato, L.; Schmitt, C.; Bovetto, L.; Rouvet, M. Mechanism of formation of stable heat-induced [beta]-lactoglobulin microgels. *Int. Dairy J.* **2009**, *19*, 295–306.

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

(18) Gosal, W. S.; Morten, I. J.; Hewitt, E. W.; Smith, D. A.; Thomson, N. H.; Radford, S. E. Competing Pathways Determine Fibril Morphology in the Self-assembly of [beta]2-Microglobulin into Amyloid. *J. Mol. Biol.* **2005**, *351*, 850–864.

(19) 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 B-lactoglobulin formed at pH 2. *Biomacromolecules* **2008**, *9*, 1474–1479.

(20) Graveland-Bikker, J. F.; de Kruif, C. G. Unique milk protein based nanotubes: Food and nanotechnology meet. *Trends Food Sci. Technol.* **2006**, *17*, 196–203.

(21) Foegeding, E. A. Food biophysics of protein gels: A challenge of nano and macroscopic proportions. *Food Biophys.* **2006**, *1*, 41–50.

(22) Hartmann, R. M., H. Food-derived peptides with biological activity: From research to food applications. *Curr. Opin. Colloid Interface Sci.* **2007**, *18*, 163–169.

(23) Korhonen, H.; Pihlanto, A. Technological options for the production of health-promoting proteins and peptides derived from milk and colostrum. *Curr. Pharm. Des.* **2007**, *13*, 829–843.

(24) Hernandez-Ledesma, B. R. I.; Amigo, L. β-Lactoglobulin as source of bioactive peptides. *Amino Acids* **2008**, *35*, 257–265.

(25) Kim, S. B.; Ki, K. S.; Khan, M. A.; Lee, W. S.; Lee, H. J.; Ahn, B. S.; Kim, H. S. Peptic and tryptic hydrolysis of native and heated whey protein to reduce its antigenicity. *J. Dairy Sci.* **2007**, *90*, 4043–4050.

(26) Astwood, J. D.; Leach, J. N.; Fuchs, R. L. Stability of food allergens to digestion in vitro. *Nat. Biotechnol.* **1996**, *14*, 1269–1273.

(27) Bateman, L.; Ye, A.; Singh, H. In Vitro Digestion of β-Lactoglobulin Fibrils Formed by Heat Treatment at Low pH. *J. Agric. Food Chem.* **2010**, *58*, 9800–9808.

(28) Groleau, P. E.; Morin, P.; Gauthier, S. F.; Pouliot, Y. Effect of physicochemical conditions on peptide-peptide interactions in a tryptic hydrolysate of β-lactoglobulin and identification of aggregating peptides. *J. Agric. Food Chem.* **2003**, *51*, 4370–4375.

(29) Otte, J.; Lomholt, S. B.; Ipsen, R.; Qvist, K. B. Effect of partial hydrolysis with an immobilized proteinase on thermal gelation properties of beta-lactoglobulin B. *J. Dairy Res.* **2000**, *67*, 597–608.

(30) Akkermans, C.; Venema, P.; Van Der Goot, A. J.; Boom, R. M.; Van Der Linden, E. Enzyme-induced formation of beta-lactoglobulin fibrils by AspN endoproteinase. *Food Biophys.* **2008**, *3*, 390–394.

(31) Otte, J.; Lomholt, S. B.; Halkier, T.; Qvist, K. B. Identification of peptides in aggregates formed during hydrolysis of β-lactoglobulin B with a Glu and Asp specific microbial protease. *J. Agric. Food Chem.* **2000**, *48*, 2443–2447.

(32) Creusot, N.; Gruppen, H. Enzyme-induced aggregation and gelation of proteins. *Biotechnol. Adv.* **2007**, *25*, 597–601.

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

(34) Kroes-Nijboer, A.; Venema, P.; Bouman, J.; van der Linden, E. The Critical Aggregation Concentration of beta-Lactoglobulin-Based Fibril Formation. *Food Biophys.* **2009**, 1–5.