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ARTICLE

In Vitro Digestion of β-Lactoglobulin Fibrils Formed by Heat Treatment at Low pH

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Extensive studies have been done on β-lactoglobulin (β-Lg) fibrils in the past decade due to their potential as functional food ingredients, gelling agents, and encapsulation devices etc. (van der Goot, A. J.; Peighambardoust, S. H.; Akkermans, C.; van Oosten-Manski, J. M. Creating novel structures in food materials: The role of well-defined shear flow. *Food Biophys.* **2008**, *3* (2), 120−125 and Loveday, S. M.; Rao, M. A.; Creamer, L. K.; Singh, H. Factors affecting rheological characteristics of fibril gels: The case of β-lactoglobulin and α-lactalbumin. *J. Food Sci.* **2009**, *74* (3), R47−R55). However, most of the studies focus on the formation and mechanism of the fibrils. Little is known about fibril digestibility to date. In this work, in vitro pepsin digestion of bovine β-lactoglobulin (β-Lg) fibrils in simulated gastric fluid was investigated using thioflavin T fluorescence photometry, sodium dodecyl sulfate−polyacrylamide gel electrophoresis, size-exclusion chromatography, matrix-assisted laser desorption/ionization mass spectrometry, and transmission electron microscopy (TEM). The fibrils were formed by heating β-Lg solutions at 80 °C and pH 2.0 for 20 h. The fibrils were found to be digested completely by pepsin within 2 min, when long, straight fibrils were no longer observed by TEM. The peptides in the fibrils (2000−8000 Da) could be digested to smaller peptides (mostly <2000 Da) by pepsin. The peptides in the fibrils were believed to be more susceptible for pepsin to access and attack because of their hydrophobic nature. For comparison purposes, solutions of β-Lg heated at neutral pH (pH 7.4) were also studied under the same conditions.

KEYWORDS: β-Lactoglobulin; fibrils; in vitro pepsin digestion; heat treatment

INTRODUCTION

Bovine β-lactoglobulin (β-Lg) is a globular protein that contains 162 amino acids and has a molecular weight of 18.3 kDa. It is composed of nine β-strands and one α-helix, in which the hydrophobic sequences are mostly buried. At room temperature and neutral pH, it exists in the form of a dimer, which dissociates into monomers at acidic pH (<pH 3) (*2*).

It is known that β-Lg is scarcely hydrolyzed by enzymes such as pepsin under gastric conditions because of its stable, globular tertiary structure at low pH (<pH 3). The highly hydrophobic β-barrel makes it very difficult for enzymes to access the target peptide bonds (*2*−*4*). However, it has been found that under certain circumstances such as heating, high pressure, high ionic strength, or in organic solvents, denatured or hydrolyzed β-Lg can be digested by enzymes more easily than the native protein (*5*, *6*).

Under denaturing conditions, β-Lg is able to form gels and aggregates or precipitates, depending on the protein concentration and various conditions, such as pH, temperature, and heating time and speed (*2*, *7*). β-Lg gels formed through heating treatment are widely used in the food industry (*8*). This whey protein contributes not only to the consistency and texture of foods but also to their nutritional value. Applications for β-Lg gels in both drug and food supplements have also been reported (*9*−*12*).

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Recently, it was found that β-Lg can form fibrils on heating treatment at low pH, on heat treatment under high pressure, or in the presence of organic solvents (*1*, *13*−*16*). Many studies on the mechanism of fibril formation and on the building blocks of fibrils have been carried out (*8*, *17*−*19*). Akkermans et al. (*20*) claimed that rather than denatured intact proteins, the building blocks of β-Lg fibrils are peptides obtained through acid hydrolysis of β-Lg molecules on heat treatment. They found that after heat treatment at 85 °C and pH 2.0 for 20 h, β-Lg molecules were hydrolyzed into peptides that subsequently formed fibrils. They argued that only specific peptides with characteristics of high hydrophobicity, the ability to form β-sheets, low charge, and proper charge distribution along the peptide are able to form fibrils. This may explain the low yield (about 20% on average) of β-Lg fibril formation on heat treatment at low pH.

As well as extensive studies on the mechanism of β-Lg fibril formation, some work on the use of β-Lg fibrils to improve both the texture and the nutritional value of foods has been carried out (*7*, *21*). However, to our knowledge, there has been little work on the digestion of β-Lg fibrils in the gastrointestinal system. This work attempts to address this issue in a designated in vitro system, in which β-Lg fibrils formed by heat treatment at pH 2.0 and 80 °C for 20 h were subjected to digestion by pepsin under simulated gastric conditions. The digestion processes were monitored using transmission electron microscopy (TEM), matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS),

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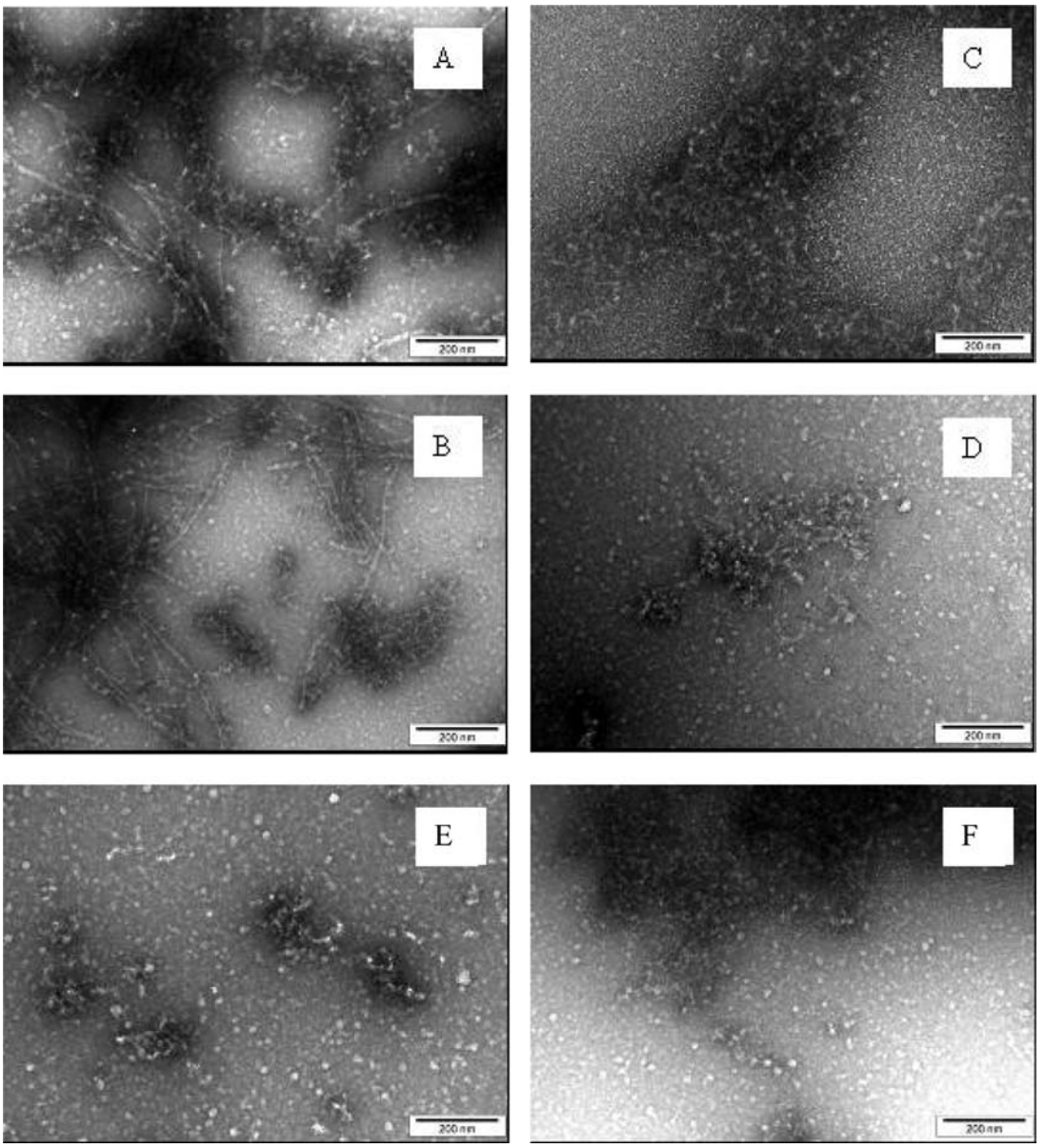

Figure 1. Negative stain TEM images of β-Lg digestion samples preheated at pH 2.0 (**A**−**D**) and 7.4 (**E** and **F**) and 80 °C for 20 h. Control sample without digestion (**A**, pH 2.0; **E**, pH 7.4) and samples with pepsin added at 0 (**B**), 2 (**C**), and 30 min (**D**, pH 2.0; **F**, pH 7.4), respectively.

size-exclusion chromatography high-performance liquid chromatography (SEC-HPLC), thioflavin T (ThT) fluorescence photometry, and sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). The results for digestion samples preheated at pH 2.0 were compared with those for digestion samples preheated at neutral pH (pH 7.4) under the same conditions.

MATERIALS AND METHODS

Bovine milk β-Lg (approximately 90% PAGE; contains variant A and variant 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.

Protein Sample Preparation. Bovine β-Lg was dissolved in pH 2.0 HCl solution to give protein solutions of 10 mg/mL concentration at pH 2.0 (adjusted using 1 M HCl). β-Lg was also dissolved in Milli-Q at the same protein concentration; the pH of this solution was 7.4. 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 15000 rpm 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.

Heat Treatment of β-Lg Solutions. The samples of both pH values (pH 2.0 and pH 7.4) were put into a shaking water bath (BS-06/31, Jeio Tech Co., Ltd., Korea) at 80 °C with a shaking speed of 50 rpm for 20 h. The samples were cooled at room temperature before being stored in a refrigerator at 4 °C.

Enzymatic Hydrolysis. The preheated protein samples were diluted with simulated gastric fluid (SGF, containing 0.034 M NaCl and using HCl to adjust the pH to 1.2, without pepsin) at a ratio of 1 to 2 (v/v). Thus, the final β-Lg concentration was 3.3 mg/mL. All samples, with or without preheating treatment, were shaken in a 37 °C water bath at a speed of 50 rpm. A freshly made pepsin solution (10 mg/mL in pH 2.0 HCl solution and shaken in a REAX Control shaker for 5 min before it was filtered 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) to inactivate the enzyme after 0, 0.5, 2, 5, 10, and 30 min.

ThT Fluorescence Measurement. A protein sample (250 μL) was added into a four-sided quartz cuvette containing 2.75 mL of 0.02% ThT solution 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 intensities were obtained by subtracting the intensity of the ThT solution (blank) when the data were analyzed.

SDS-PAGE. The method and the sample treatment are described in detail elsewhere (*22*). Protein samples were diluted using sample buffer (containing 5% 2-mercaptoethanol as a reducing agent) at a ratio of 1 to 3 (v/v) and then reduced by heating in a boiling water bath for 4 min. The final protein concentration was 0.25%. Gels (15% acrylamide gel) were run in a Protean II slab electrophoresis cell (Bio-Rad Laboratories, Hercules, CA) using an electrode stock buffer at a voltage of 210 V and an electric current of 70 mA. The gels obtained were scanned using a Molecular Imager Gel Doc XR system (Bio-Rad Laboratories) on an Anthos Fluido plate using Trans-White light. Quantity One software was used for scanning and analysis of the gels.

SEC-HPLC. Experiments were carried out on an Agilent Technology 1200 Series system (Agilent Technologies, Forest Hill, Australia) using a PolySep-GFC-P 2000 column (Phenomenex, 7.8 mm × 300 mm, molecular mass range 2000−9000 Da). A UV detector at wavelengths of both 280 and 214 nm was used. The samples were eluted in an isocratic mode using an eluting solution of 0.08% NaCl with 0.02% NaN_3 at a flow rate of 0.5 mL/min for 60 min.

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.

All samples were examined after dilution with Milli-Q water (1 to 3, v/v) and mixing using a REAX Control shaker for 30 s. 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 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.

MALDI-MS. Measurements were performed on a Waters-LMW 1974 MALDI mass spectrometer (Waters Corp., Milford, MA) equipped with a pulsed nitrogen laser (λ = 337 nm, 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 (small peptides) or linear mode (large peptides or proteins).

Samples were mixed with matrix agents at a ratio of 1 to 1 (v/v). 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 mode were 4000 and 50000 Da, respectively. Mass spectra were analyzed using MassLynx software.

Besides H^+ charged peptides, Na^+ charged peptides might be detected using both matrix agents, in which 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), should be considered, too. For reflectron mode, the error in molecular mass was considered to be less than 1 Da; for linear mode, it was considered 1−2 Da.

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 those with matching ions score above 40 were considered as well-matched results. The software, MS-Seq-Prospector, was also used for the purpose of sequence matching manually.

RESULTS

After heat treatment at 80 °C for 20 h, the pH 2.0 samples became translucent, viscous solutions, whereas the pH 7.4 samples were similar in appearance to the β-Lg solutions before heating treatment.

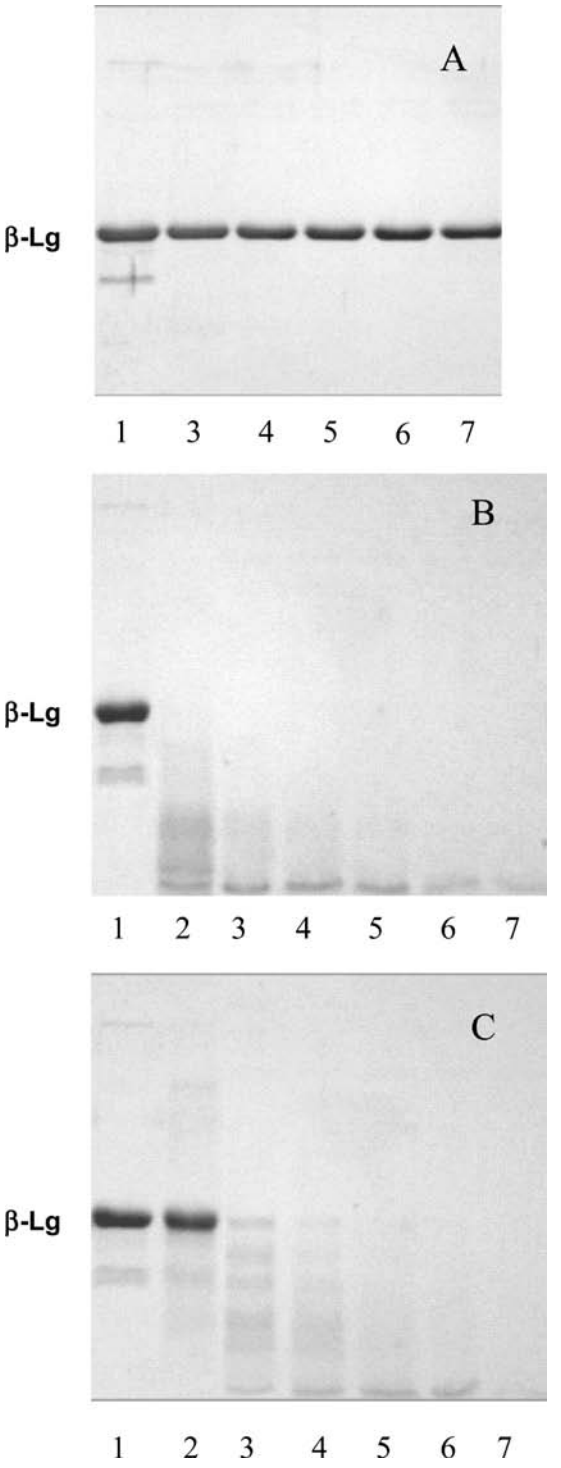

Figure 2. SDS-PAGE gels (15% acrylamide gel) for β-Lg samples in SGF without heating treatment (**A**) or with heating treatment at pH 2.0 (**B**) and pH 7.4 (**C**) at different digestion times. Lane 1, β-Lg solution; lane 2, preheated samples; and lanes 3−7, samples digested by pepsin for 0, 0.5, 2, 5, and 10 min, respectively.

TEM. TEM images of the samples were taken during the enzymatic hydrolysis (digestion). As shown in **Figure 1A**, long, straight fibrils formed after heat treatment at pH 2.0 and 80 °C for 20 h, in agreement with previous studies (*20*, *23*−*25*). The long, straight fibrils were still observable immediately after the enzyme had been added (**Figure 1B**). At digestion times from 2 to 30 min, only short, wormlike aggregates were observed (**Figure 1C**,**D**).

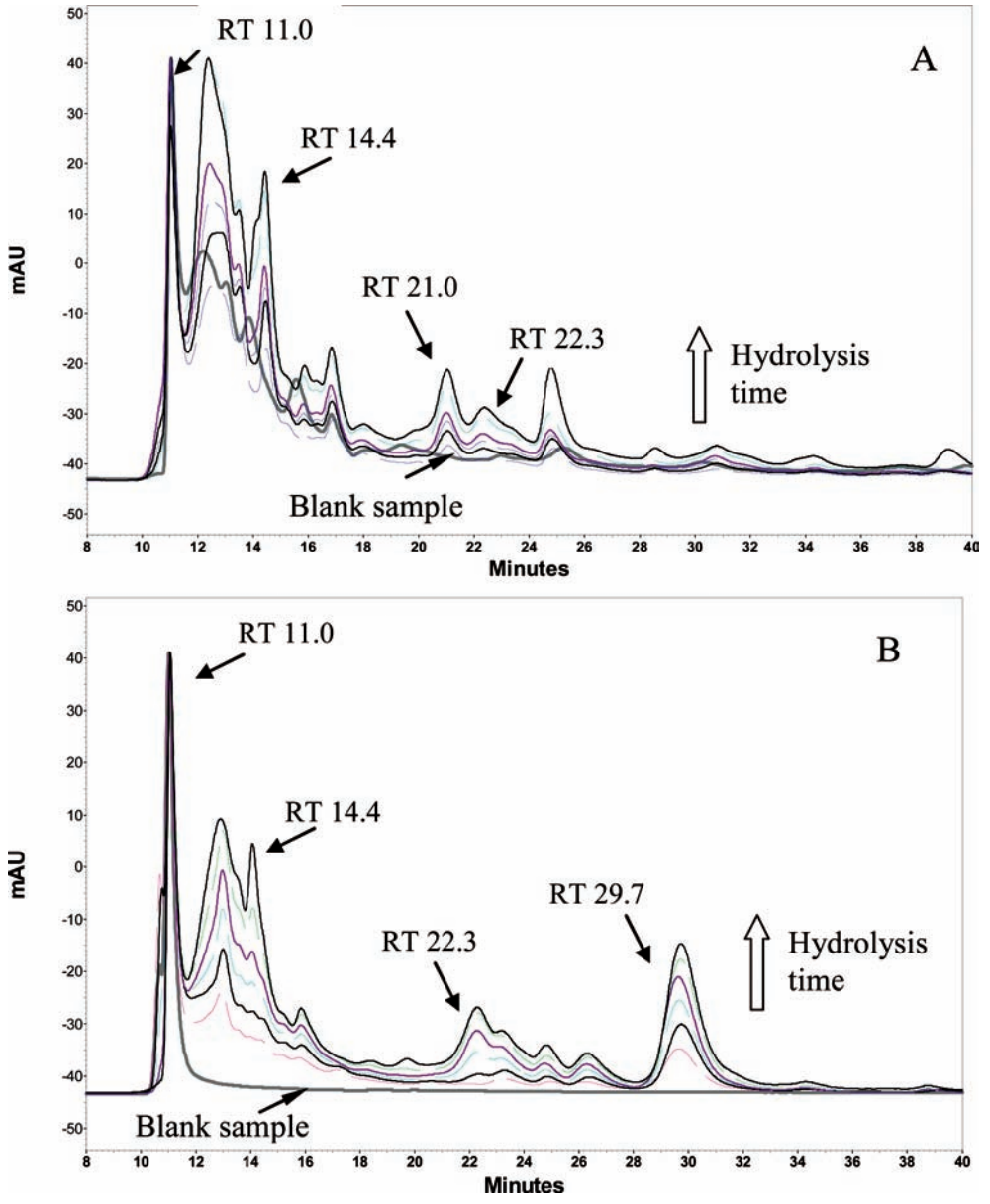

Figure 3. SEC-HPLC spectra for digestion samples preheated at pH 2.0 (**A**) and pH 7.4 (**B**) at digestion times of 0, 0.5, 2, 5, 10, and 30 min, respectively (indicated as block arrows). The profiles of samples preheated at pH 2.0 and pH 7.4 without pepsin added (indicated as blank sample) were overlapped with those of digestion samples. In addition to the peak RT 11.0, four other peaks with obvious changes during the process, RT 14.4, RT 21.0, RT 22.3, and RT 29.7, are marked in the spectra.

For samples preheated at pH 7.4 and 80 °C for 20 h, only short, wormlike aggregates were observed before enzyme addition (**Figure 1E**). After pepsin had been added, the aggregates became smaller as the digestion time was increased from 0.5 to 30 min (**Figure 1F**).

SDS-PAGE. In vitro digestion patterns of native β-Lg and preheated β-Lg at pH 2.0 and pH 7.4 were examined using SDS-PAGE (**Figure 3A**−**C**). For the unheated β-Lg samples, there was almost no change in the β-Lg bands when the digestion time was increased from 0.5 to 30 min (**Figure 2A**, lanes 3−8).

Almost no intact β-Lg band was observed on the gel for samples preheated at pH 2.0 before pepsin was added (**Figure 2B**, lane 2). Instead, some bands with a molecular mass lower than 10 kDa were observed, which indicated acid hydrolysis of the intact protein molecules during the heating treatment at pH 2.0. This result is in agreement with Akkermans et al. (*20*), who reported that the molecular mass range of peptides obtained from acid hydrolysis of β-Lg on heat treatment at 85 °C and pH 2.0 for 20 h was between 2000 and 8000 Da. In the presence of pepsin, peptide bands with lower molecular masses were observed (**Figure 2B**, lanes 3 and 4). As the digestion time was increased to 2 min and then up to 30 min, almost all peptide bands became undetectable (**Figure 2B**, lanes 5−8). The results indicated further cleavages of the original peptides from the acid hydrolysis by pepsin during the enzymatic digestion.

For the samples preheated at pH 7.4, the β-Lg band was observed before the enzyme was added (**Figure 2C**, lane 2) but disappeared within 2 min of digestion; some bands with lower molecular masses were also observed (**Figure 2C**, lanes 3−5). These bands disappeared completely from samples digested for longer than 5 min (**Figure 2C**, lane 6). The results are consistent with previous reports (*26*).

SEC-HPLC. To monitor the changes to the fibrils and the peptides in the samples after different digestion times, SEC-HPLC experiments using a column with a molecular mass range of 2000−9000 Da were carried out. It was expected that large aggregates

Table 1. Some Peptides Detected in Blank Samples (Heat-Treated Only)[a]

molecular mass (Da)			
measured	calculated	location	sequence
1218.8	1217.6	1−11	LIVTQTMKGLD
1391.1	1390.9	138−149	KALKALPMHIRL
1625.3	1625.0	138−151	KALKALPMHIRLSF
2027.4	2026.1	34−51	AQSAPLRVYVEELKPTPE
2084.5	2083.1	34−51	AQSAPLRVYVEELKPTPEG
2199.5	2198.1	34−53	(D)AQSAPLRVYVEELKPTPEG(D)
3554.7	3553.1	1−33	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLL(D)
5736.0	5735.5	1−53	LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEG(D)

[a] The peptides listed here are well-matched ones from Mascot search (ion score >40) with a sequence coverage of 26% of β-Lg A and B.

and fibrils would elute from the column earlier than smaller peptides. Pepsin eluted at a void volume of 12.4 min, and all molecules that were larger than pepsin (34000 Da), including fibrils, eluted within 12 min (**Figure 3**). The fractions that eluted later (after 16 min) were assumed to be peptides with lower molecular masses, which was confirmed by MALDI-MS results for different fraction collections (data not shown).

The sample at pH 2.0, in the absence of enzyme (marked as blank sample in **Figure 3A**), had a different elution profile from the enzymatically digested samples. The fractions that eluted after 16 min suggested that an acid hydrolysis had occurred during the heat treatment at pH 2.0, which was clearly seen on the SDS-PAGE gels (**Figure 2B**, lane 2). When the digestion time was increased, the peaks with retention times (RTs) of 14.4, 21.0, and 22.3 min increased in size (data not shown). MALDI-MS also detected peptides with a molecular mass of less than 3000 Da for the three fractions (data not shown). The peak at 25.0 min was from the enzyme, so it would be excluded from further analysis.

The only peak that decreased as the digestion time was increased had a RT of 11.0 min, which implied a decrease with time during digestion. The initial relative area of peak RT 11.0 in the samples preheated at pH 2.0 (**Figure 3A**) essentially matched the average yield of fibrils of about 20% from heating treatment at 85 °C and pH 2.0 for 20 h (*20*), suggesting that the decease in this fraction coincided with the decrease in the fibrils formed on heat treatment.

Samples preheated at pH 7.4 were also subjected to SEC-HPLC. **Figure 3B** shows combined elution profiles of the samples at different digestion times. In contrast to the samples preheated at pH 2.0, only one peak, at an RT of 11.0 min, was observed before enzyme was added (**Figure 3B**, marked as blank sample). This indicated that no smaller peptides were formed during the heat treatment at pH 7.4. Therefore, the easier digestion of the samples preheated at neutral pH (than native proteins) could be because the denatured protein molecules are more prone to hydrolysis than the native proteins, which is in agreement with previous studies (*2*, *27*, *28*). When the digestion time was increased, the relative areas of peaks RT 14.4, RT 22.3, and RT 29.7 increased (**Figure 3B**), implying that the digestion progressed over time, which was similar to samples preheated at pH 2.0.

In addition to the three common peaks RT 11.0, RT 14.4, and RT 22.3 for samples preheated at both pH 2.0 and pH 7.4, two other peaks were observed. Peak RT 21.0 was obvious for the pH 2.0 samples, whereas peak RT 29.7 dominated for the pH 7.4 samples, indicating that different peptides formed during the digestion process for samples preheated at different pH. The dominant peptides for the acidic pH samples were larger than those for the neutral pH samples (RT 21.0 vs RT 29.7).

MALDI-MS. *MALDI Spectra for Samples Preheated at pH 2.0.* Both linear mode (1000−10000 Da) and reflectron mode (400−4000 Da) were applied in MALDI-MS examination. Approximately 50 peptides were detected for the β-Lg sample heated at 80 °C and pH 2.0 for 20 h before digestion. The two peaks of 3554 (peptide 1−33) and 5736 Da (peptide 1−53) that were claimed by Akkermans et al. (*20*) as the major fibril formation peptides were detected along with other peptides possibly involving in fibril formation. Some of the peptides that were well matched with theoretical values using Mascot search software are listed in **Table 1** (above). No intact protein molecules (or dimers) were detected using MALDI-MS in β-Lg samples preheated at pH 2.0 when the molecular mass maximum was extended to 30000 or 50000 Da. The peptides detected in this sample were mostly less than 8000 Da, which was also in agreement with the previous studies (*20*).

Table 2. Some Peptides Detected in Samples Digested for 2 min[a]

molecular mass (Da)			
measured	calculated	location	sequence
841.8	840.4	34−41	AQSAPLRV
879.5	878.5	143−149	LPMHIRL
	878.6	23−32	(L)AMAASDISL(L)
	878.4	46−53 (Na^+)	LKPTPEGD
950.8	949.5	142−149	ALPMHIRL
1004.8	1003.5	34−42	AQSAPLRVY
1069.7	1068.5	17−26	GTWYSLAMAA
	1068.6	32−41	LDAQSAPLRV
1218.8	1217.6	1−11	LIVTQTMKGLD
1390.8	1389.8	138−149	KALKALPMHIRL
	1389.4	1−12 (acetyl oxidation)	(-)LIVTQTMKGLDI(Q)
1782.2	1781.1	135−149	KFDKALKALPMHIRL
1911.3	1910.1	134−149	(L)EKFDKALKALPMHIR(L)
	1910.4	37−53	APLRVYVEELKPTPEGD
2224.3	2223.3	131−149	EALEKFDKALKALPMHIRL
2339.4	2338.3	130−149	DEALEKFDKALKALPMHIRL

[a] The peptides listed here are well-matched ones from Mascot search (ion score >40) with a sequence coverage of 42% of β-Lg A and B.

MALDI Spectra for Digestion Samples. The digestion samples were examined directly using a MALDI-MS in both reflectron mode and linear mode with molecular mass ranges of 400−4000 and 1000−30000 Da, respectively. For samples preheated at pH 2.0, approximately two dozens of peaks were detected in the region of 800−3000 Da. Peaks with molecular masses of 879, 1391, and 1911 Da were dominant in the spectra along with other peptides from the same region (**Figure 4**). They were among the best matching peptides in Mascot search results using MALDI TOF/TOF MS. Peptides detected in digestion samples that were well matched with theoretical values (matching score >40) are listed in **Table 2**. Only about 5−6 peaks were detected in the region of 800−3000 Da for samples preheated at neutral pH. The peak with molecular mass 879 Da was dominant in the spectra of samples with different digestion time. The signal of the peak of molecular mass 1911 Da was much weaker as compared to that of pH 2.0 samples (**Figure 4**).

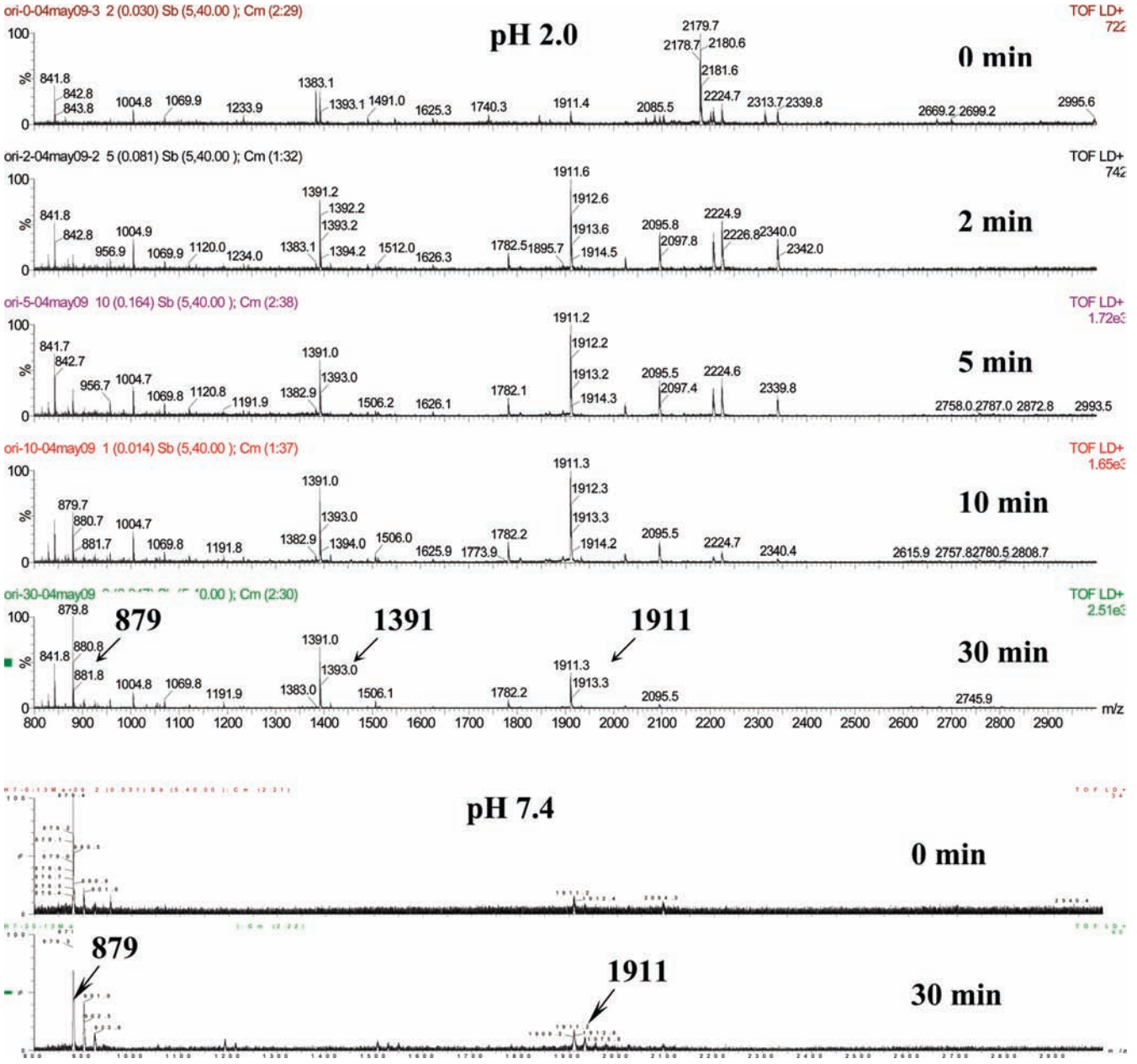

Figure 4. MALDI spectra obtained using reflectron mode for digestion samples preheated at pH 2.0 at digestion times of 0, 2, 5, 10, and 30 min (above) and pH 7.4 at digestion times of 0 and 30 min (below), respectively. All peaks in one cluster were considered to be one peptide. Three peaks at 879, 1391, and 1911 Da with obvious changes during the digestion were marked in the spectra.

The relative intensities of peaks 879, 1391, and 1911 Da against the peak 443 Da (matrix peak, consistent during the digestion process) were calculated and plotted against the digestion time. **Figure 5A** shows the normalized data for digestion samples with preheating treatment at pH 2.0. The peptides with molecular masses of 879, 1391, and 1911 Da increased during the digestion, with the maximum values being reached at a digestion time of about 2 min (**Figure 5A**, hollow diamonds, solid circles, and triangles, respectively). The peptides with molecular masses of 1911 and 879 Da were not observed in samples digested for less than 2 min (**Figure 4**).

For samples preheated at pH 7.4, the amount of the peptide with a molecular mass of 879 Da increased with an increase in the digestion time, indicating an increasing release of this peptide during the digestion process (**Figure 5B**, hollow diamonds). The peptide with a molecular mass of 1391 Da was not observed in the digestion samples preheated at neutral pH. The peptide with a molecular mass of 1911 Da was also not as intense as that in the samples preheated at pH 2.0 (**Figure 4**).

ThT Fluorescence. As ThT is known for its specificity in binding on β-sheet structure in proteins, it is used widely as an indicator of fibrils and β-sheets in solution (*29*, *30*). No changes in fluorescence intensity were observed during the digestion of unheated samples, which implied no binding of ThT on either β-sheets or fibrils (**Figure 6**, hollow circles).

The initial high fluorescence intensities for β-Lg samples preheated at both pH 7.4 and pH 2.0 indicated that fibrils or aggregates/β-sheets were formed during heating treatment (**Figure 6**). For samples preheated at pH 2.0 (**Figure 6**, solid circles), the intensities decreased rapidly and reached a minimum within a digestion time of 2 min. For samples preheated at pH 7.4 (**Figure 6**, squares), as the digestion time was increased, the intensities reduced rapidly and started to level off after 10 min. As the digestion time was increased to 30 min, the intensities decreased to nearly zero.

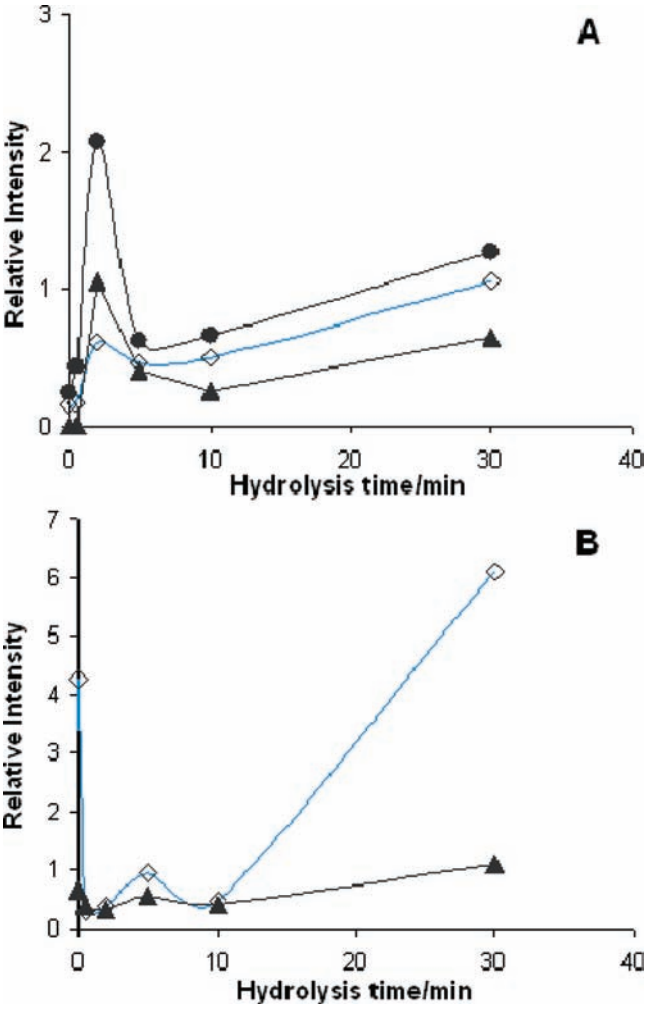

Figure 5. Normalized intensities of the peaks at 879 (◇), 1391 (●), and 1911 (▲) Da over the intensity of the peak at 443 Da from MALDI-MS as a function of the digestion time for the digestion samples preheated at pH 2.0 (**A**) and pH 7.4 (**B**).

DISCUSSION

The study on samples preheated at pH 2.0 suggests that fibrils of β-Lg can be digested by pepsin in vitro in 2 min with only short, curly aggregates observed in the TEM images (**Figure 1C**). Both samples containing base (0.2 M Na_2CO_3) to inactivate the enzyme, either without any enzyme addition or with the enzyme just applied (**Figure 1A**,**B**), had no observation in morphological changes to the long, straight fibrils. This indicated that the curly aggregates observed in the digestion samples did not result from direct morphological transformation of the fibrils as a result of the change in the pH from acidic to basic. Therefore, it is reasonable to suggest, given the disappearance of the long, straight fibrils, an enzymatic digestion of the β-Lg fibrils when pepsin was applied. The ThT fluorescence results also support this argument with the fluorescence intensities decreased rapidly when the enzyme was added and reached a minimum at a digestion time of 2 min (**Figure 6**, solid circles). MALDI-MS also revealed the emergence and an increase in the release of the three major peptides during the enzymatic digestion, which coincidently reached a maximum after a digestion time of 2 min (**Figure 5A**).

This study also discovered that during the enzymatic digestion, the peptides in the fibrils became smaller in size. Both the disappearance of the β-Lg band and the simultaneous appearance of bands of lower molecular mass on SDS-PAGE gels for the

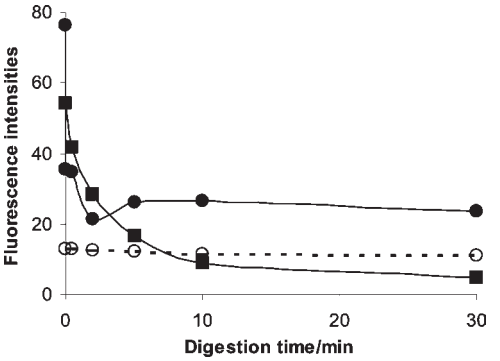

Figure 6. ThT fluorescence intensities of digestion samples preheated at pH 2.0 (circles) and pH 7.4 (squares) as a function of the digestion time. The net intensities were obtained by subtraction of the intensity of ThT solution. The excitation and emission wavelengths were 442 and 485 nm, respectively. The measurements for digestion of unheated samples at pH 2.0 (hollow circles) were included for comparison.

samples preheated at pH 2.0 (**Figure 2B**, lane 2) suggested acid hydrolysis of the intact proteins to peptides during the heat treatment, which was in line with the findings of Akkermans et al. (*20*); over the time of enzymatic hydrolysis, the peptide bands moved to smaller molecular weight until no longer observable in the gel after 2 min (**Figure 2B**, lanes 5−8). SEC-HPLC also gave evidence of decreases in larger peptides in preheated samples before digestion (eluted in smaller void volumes, **Figure 3A**, blank sample) and simultaneous increases in smaller peptides in the digestion samples (eluted in bigger void volumes, **Figure 3A**). Furthermore, the peptides detected by MALDI-MS became less than 2000 Da from 2000 to 8000 Da before the digestion (**Table 1**, below, and **Figure 4**).

On heat treatment at low pH, the aspartic acid sites (D-X or X-D) of the β-Lg peptide bonds are preferentially hydrolyzed, resulting in relatively bigger peptides (*31*, *32*) [mostly with molecular masses between 2000 and 8000 Da (*20*)]. According to Akkermans et al. (*20*), only certain peptides, fulfilling the requirements of hydrophobicity, low charge, proper charge distribution along the peptide chain, and the ability to form β-sheets, are involved in fibril formation. In contrast to acid hydrolysis, it has been found that pepsin tends to attack highly hydrophobic amino acid and aromatic amino acid residues (*33*) (e.g., leucine, isoleucine, phenylalanine, valine, and tryptophan), and it has a preferential tendency toward the cleavage of peptide bonds adjacent to leucine residues, that is, L-X (or X-L) bonds (*34*). The peptides detected by MALDI-MS were mostly related to L-X (or X-L) or I-X (or X-I) bond cleavage by pepsin (**Table 1**, below, and **Figure 7**). Therefore, it was reasonable that these highly hydrophobic, fibril-forming peptides were more accessible and susceptible to attack by pepsin than the peptides that were not in the fibrils.

Using MALDI-MS and two peptide mapping software, the major product peptides from the enzymatic digestion were found from both termini of the β-Lg chain. The Mascot searching basically assigned the three major peptides at the C terminus region of bovine β-Lg (879 Da, 143−149; 1391 Da, 138−149; and 1911 Da, 134−149), while the MS-Seq-Prospector program allocated them well at the N terminus of bovine β-Lg (879 Da, 23−32 or 46−53; 1391 Da, 1−12; and 1911 Da, 37−53) (**Table 2**, and **Figure 7**). In either case, they coincided with the results of Akkermans et al. that fibril formation peptides were mainly from both termini of the protein chain (*20*). This provided positive evidence to the above discussion based on TEM and ThT fluorescence data, which the peptides in the fibrils are more likely to be digested by pepsin in vitro. Although the coverage of peptide obtained by

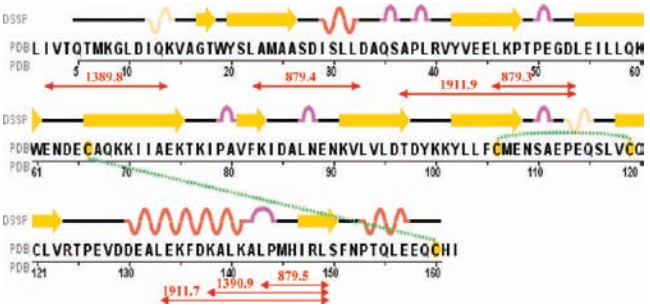

Figure 7. Sequence of bovine β-Lg A (the B variant has G64 and A118 instead) adapted from PCSB Protein Data Bank with PDB ID of 2AKQ. Some of the well-matched sequences using Mascot and MS-Seq-Prospector for three major peptides, 1911, 1391, and 879 Da, are marked in arrows.

MALDI-MS (46%) is not high enough to explain why few peptides detected are from the middle region of the protein chain, the consistency of peptides detected from both termini in fibrils and enzymatic digested samples clearly suggested a digestion of fibrils (and peptides in fibrils) when pepsin was applied to samples containing β-Lg fibrils in vitro. Parallel studies on fibrils (retentate) obtained by filtering preheated β-Lg samples at pH 2.0 confirmed the results for the mixture samples (data not shown).

After all, the β-Lg fibrils formed on heating treatment at 80 °C and pH 2.0 for 20 h were composed of peptides with molecular masses between 2000 and 8000 Da. These fibrils were digested by pepsin in SGF to release small peptides, mostly with molecular masses of less than 2000 Da. The fibril digestion process was rapid; the long, straight fibrils formed through heating treatment became undetectable by TEM, ThT fluorescence, SDS-PAGE, and SEC-HPLC within 2 min of addition of the enzyme. MALDI-MS and peptide mapping revealed that three of the major peptides released during the enzymatic digestion probably resulted from the peptides in the original fibrils formed on heating treatment. Because of the increasing interest in the application of milk protein fibrils in functional foods, studies on digestion of milk protein fibrils in gastrointestinal system are important. For the first time, this work has provided valuable information on the digestibility of a whey protein, β-Lg, and fibrils under a simulated gastric condition.

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