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In vitro gastric digestion of heat-induced aggregates of β-lg

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Interpretive summary

The 3-D structure of native proteins is altered by food processing treatments such as heating and high pressure, and structural changes may be either desirable or undesirable. Heating produces an array of denatured proteins and aggregates of various sizes. Heated β β-lactoglobulin has higher gastric digestibility than the native protein, which is particularly resistant because of its acid-stable structure. This study examined the heat-induced structural modifications to β-lactoglobulin, such as the denaturation and aggregation, and measured the *in vitro* digestibility of denatured and aggregated species. Such information will be useful in fabricating food products with improved nutritional properties.

ABSTRACT

An *in vitro* gastric digestion of heat-induced aggregates of β-lactoglobulin (β-lg) in simulated gastric fluid was investigated using sodium dodecyl sulfate-PAGE under nonreducing and reducing conditions, native-PAGE, 2-dimensional electrophoresis, and size exclusion chromatography. Heating at 90ºC significantly increased the digestibility of β-lg, with a high initial digestion rate followed by a relatively constant rate of digestion at a high enzyme:substrate (E:S) ratio of 3:1. At a low E:S ratio (1:6), the rate of digestion of β-lg was slower, and intermediate and low molecular weight species could be seen. The high molecular weight nonnative aggregates (pentamers, tetramers, trimers, etc.) were digested relatively rapidly, whereas some of the nonnative dimers were resistant to digestion and others were digested rapidly. The intermediate molecular weight species (21 to 23 kDa) were digested slowly. These results indicated that the digestibility of nonnative β-lg aggregates varied significantly depending on the E:S ratio and the types of aggregate. Further investigation is necessary to identify and characterize slowly digested dimers and intermediate molecular weight species.

Key words: β-lactoglobulin, heat-induced aggregate, *in vitro* gastric digestibility, pepsin

INTRODUCTION

The majority of food proteins are subjected to technological processes such as heating, cooling, drying, exposure to high pressure, extrusion, irradiation, fermentation, homogenization, and membrane processes. The native structure of food proteins is readily altered as a result of these processing treatments (Li-Chan, 2004). The most common structural changes that food proteins undergo during processing include unfolding of the native tertiary structure and aggregation of the unfolded molecules. Such changes may have both desirable and undesirable effects on protein digestibility (Mills et al., 2009). For example, mild heating improves the digestibility by partial denaturation of the proteins, but severe heat treatment may cause a reduction in protein digestibility by cross-linking, racemization, and the Maillard reaction (Damodaran, 2007). A fundamental understanding of the structural modifications of proteins during food processing and relating these changes to digestibility could be useful in fabricating food products with improved nutritional properties.

β-Lg is a globular protein, comprising almost 50% of the total whey protein in bovine milk. At ambient temperature and physiological pH, β-lg exists as a dimer, which dissociates into two identical monomers below pH 3 (Fox and McSweeney, 2003). Monomeric β-lg consists of 162 amino acid residues and has a molecular weight of 18.3 kDa. It has two disulfide bonds at Cys 66−Cys 160 and Cys 106−Cys 119 and one free sulfhydryl group at Cys 121. The secondary structure of β-lg contains 9 β-strands: 8 are arranged to form a β-barrel and 1 forms a short α-helix on the surface (Papiz et al., 1986). The center of the barrel is highly hydrophobic and is capable of binding to vitamin A and fatty acids.

It is well known that native β-lg is resistant to some proteases, particularly to pepsin, because of its unique structural stability at low pH (Miranda and Pelissier, 1983; Reddy et al., 1988). Most of the hydrophobic amino acids, which are potential cleavage sites for pepsin, are buried inside the hydrophobic core and are not readily accessible. Heating, high pressure treatment, addition of alcohols, and esterification have been reported to increase the susceptibility of β-lg to hydrolysis by pepsin (Chobert et al., 1995; Dalgalarrondo et al., 1995; Guo et al., 1995; Zeece et al., 2008). These treatments induce conformational changes in β-lg, resulting in increased exposure of peptic cleavage sites and thus increased susceptibility to pepsin action.

The heat-induced aggregation of β-lg has been extensively studied under a wide range of experimental conditions, such as pH, ionic strength, protein concentration, and heating temperature (Xiong et al., 1993; Iametti et al., 1995; Manderson et al., 1998; Hoffmann and Van Mil, 1999). Heating above 70°C results in the dissociation of the dimer into monomers and the exposure of hydrophobic amino acids and free thiol/sulfhydryl groups (McKenzie, 1971; Iametti et al., 1995, 1996). This leads to the formation of a reactive monomer that undergoes sulfhydryl−disulfide exchange reactions to form nonnative dimers. These nonnative dimers react further to form intermediate oligomers and larger aggregates (Schokker et al., 1999).

It has been reported previously that heating at 80 to 90°C induces conformational changes in the β-lg molecule that increase the exposure of peptic cleavage sites, thus increasing the hydrolysis of β-lg by pepsin (Reddy et al., 1988; Guo et al., 1995). As several different kinds of intermediates and aggregates are formed during heat treatment, it is not known how different aggregates and modified monomers behave under gastrointestinal conditions, particularly in the presence of pepsin. Therefore, the objective of the present study was to examine the *in vitro* gastric digestibility of β-lg aggregates formed by heat treatment.

MATERIALS AND METHODS

Materials

Bovine β-lg (> 90% pure; genetic variants A and B) and pepsin (porcine gastric mucosa; 800 to 2500 units/mg protein) were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals used were of analytical grade and were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise specified.

Heat Treatment of β-lg

A stock solution of β-lg (0.5%) was prepared by dissolving β-lg in Milli-Q water. The pH of the solution was 7.4. Solutions of β-lg (0.5%) were transferred into glass tubes and were heated in a shaking water bath (BS-11, Jeio Tech Co., Ltd., Korea) at 90 ± 0.1°C for different time periods. After heating, the samples were immediately cooled in ice water for 5 min and were kept at room temperature.

In vitro Gastric Digestion

The method described by Thomas et al. (2004) was used for the determination of the digestibility of β-lg and its aggregates, with minor modifications. Simulated gastric fluid (SGF) was prepared according to the United States Pharmacopoeia and consisted of 3.2 mg pepsin/mL in 0.035 M NaCl and 0.084 N HCl at pH 1.2. Aliquots of SGF were placed in glass vials and were incubated in a shaking water bath at 37°C for 5 min prior to the addition of β-lg samples. The preheated β-lg solution was added to the SGF to give enzyme:substrate (E:S) ratios of 3:1 and 1:6 (w/w), and the mixture was incubated at 37°C for the appropriate digestion time. Four samples (200 μL for alkaline native-PAGE, 200 μL for reduced SDS-PAGE, 200 μL for nonreduced SDS-PAGE, and 1 mL for size exclusion chromatography (SEC)−HPLC) were withdrawn after 0, 0.5, 1, 2, 5, 10, 30, 60, 90, and 120 min. Control samples contained β-lg in SGF without pepsin. Samples of pepsin in SGF without β-lg were also prepared and treated, to assess the auto-digestion of pepsin.

The samples for alkaline native-PAGE were transferred to Eppendorf tubes containing 70 μL of 0.2 M Na_2CO_3 and 70 μL of native-PAGE sample buffer (40% glycerol, 0.062 M Tris, and 0.01% bromophenol blue). The samples for reduced SDS-PAGE were transferred to Eppendorf tubes containing 70 μL of 0.2 M Na_2CO_3 and 70 μL of 5X Laemmli sample buffer (40% glycerol, 10% SDS, 0.33 M Tris, 0.05% bromophenol blue, and 5% β-mercaptoethanol) and were heated at 90°C for 5 min. The samples for nonreduced SDS-PAGE were transferred to Eppendorf tubes containing 70 μL of 0.2 M Na_2CO_3 and 70 μL of 5X Laemmli sample buffer (40% glycerol, 10% SDS, 0.33 M Tris, and 0.05% bromophenol blue). The samples for SEC−HPLC were transferred to Eppendorf tubes containing 350 μL of 0.2 M Na_2CO_3. All samples were stored at −20°C until further analysis.

Polyacrylamide Gel Electrophoresis

Electrophoresis experiments were carried out using a Mini Protean® 3-cell system (Bio-Rad Laboratories, Hercules, CA). Alkaline native-PAGE and SDS-PAGE (reduced and nonreduced) were performed as described by Manderson et al. (1998) using a 16% resolving gel and a 4% stacking gel. Samples from each time point and controls were loaded into each well and were subjected to electrophoretic separation at a constant voltage of 200 V for 1 h. Following separation, the gels were stained with Coomassie brilliant blue staining solution (Coomassie brilliant blue R-250, 20% isopropanol, and 10% acetic acid) for 30 min and were destained with isopropanol/acetic acid solution (10% each). The gels were scanned and quantified using the Molecular Imager Gel Doc XR system (Bio-Rad Laboratories) and Quantity One 1-D

analysis software. Two-dimensional (2D) SDS-PAGE (first dimension nonreducing, second dimension reducing) was performed as described by Havea et al. (1998).

Size Exclusion Chromatography

Experiments were carried out using a Superdex 75 10/300 GL column (GE Healthcare, Piscataway, NJ; molecular mass range 3,000 to 70,000 Da) attached to an AKTA FPLC system (GE Healthcare). The samples were filtered through a 0.22 µm membrane filter (Millipore) and 200 µL was applied to the column equilibrated with 0.02 M imidazole in 0.05 M NaCl, pH 7.0 at a flow rate of 0.3 mL/min. The UV absorption was measured at 280 nm.

RESULTS AND DISCUSSION

Two procedures were used in these experiments to investigate the *in vitro* gastric digestion of native and heated β-lg samples. The first procedure, described by Zeece et al. (2008), used a pepsin:β-lg ratio of 3:1 (w/w). The second procedure used a pepsin:β-lg ratio of 1:6 (w/w). Both protein heating and *in vitro* digestion steps were replicated and found to be highly reproducible.

In vitro gastric digestion of native β-lg and β-lg heated at 90°C for 5, 60, and 120 min was examined by reduced SDS-PAGE (Figures 1A to 1D). For native β-lg, there was little change in the intensity of the β-lg bands when the digestion time was increased from 0.5 to 120 min (Figure 1A), indicating that native β-lg was resistant to digestion by pepsin. However, the intensity of the β-lg band decreased during 120 min of digestion for β-lg heated at 90°C for 5 min (Figure 1B). An increase in the heating time at 90°C caused further decreases in the intensity of the β-lg band during digestion (Figures 1C and 1D). In all heated samples, the decrease in the intensity of the β-lg band was rapid during the initial 0.5 min of digestion and was followed by a much slower decrease over 120 min of digestion.

The reduced SDS-PAGE results were quantified using densitometry, measuring the quantity of β-lg remaining undigested after each time point of digestion (Figure 2). The quantity of β-lg remaining undigested after 120 min of digestion was 65% for native β-lg whereas it was 33, 15, and 7% when β-lg was heated at 90°C for 5, 60, and 120 min, respectively. These results suggested that the digestibility of β-lg increased as the heating time was increased from 5 to 120 min. In the case of heated β-lg, there was a rapid loss of β-lg in the initial 0.5 min of digestion, and heating for longer increased the amount of β-lg that was lost ininitially. Thereafter, the amount of β-lg decreased slowly during 120 min of digestion. In agreement with other studies (Reddy et al., 1988; Dalgalarrondo et al., 1995), these results confirm that native β-lg is largely resistant to digestion by pepsin. It is considered that most of the hydrophobic amino acids are buried inside the β-barrel and are not easily accessible to pepsin, but that heating causes the unfolding of protein molecules, thereby increasing the accessibility of exposed hydrophobic amino acids to pepsin (Reddy et al., 1988; Schmidt and Van Markwijk, 1993). In addition, the present study showed that the rate of digestion of heated β-lg was very high in the initial few minutes, and subsequent digestion was much slower, possibly indicating different susceptibilities to pepsin of the intermediate species created during the heat-induced unfolding and aggregation process. In order to explore this further, we examined the relative digestibilities of different aggregates and intermediates produced during the heat treatment of β-lg, using an *in vitro* gastric digestion model.

Figure 1. Reduced SDS-PAGE profiles of *in vitro* gastric digestion of (A) native β-lg, (B) β-lg heated at 90°C for 5 min, (C) β-lg heated at 90°C for 60 min, and (D) β-lg heated at 90°C for 120 min. Pepsin:β-lg ratio 3:1. Lane M corresponds to the molecular weight markers. C_0 and C_{60} represent preheated β-lg without pepsin in SGF at t = 0 and 60 min. The number at the bottom of each lane represents the time of digestion (min). P_0 and P_{60} represent pepsin controls at t = 0 and 60 min.

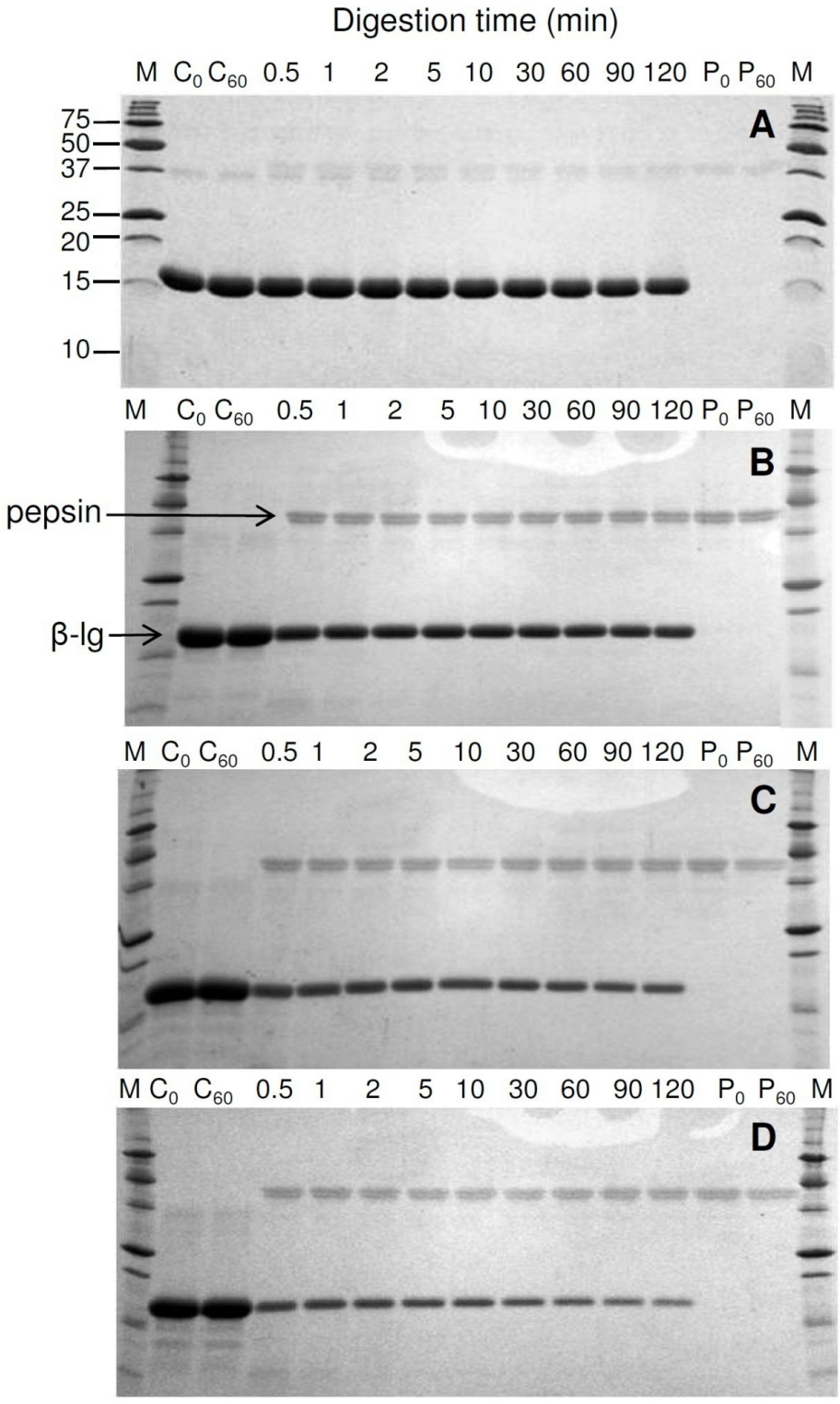
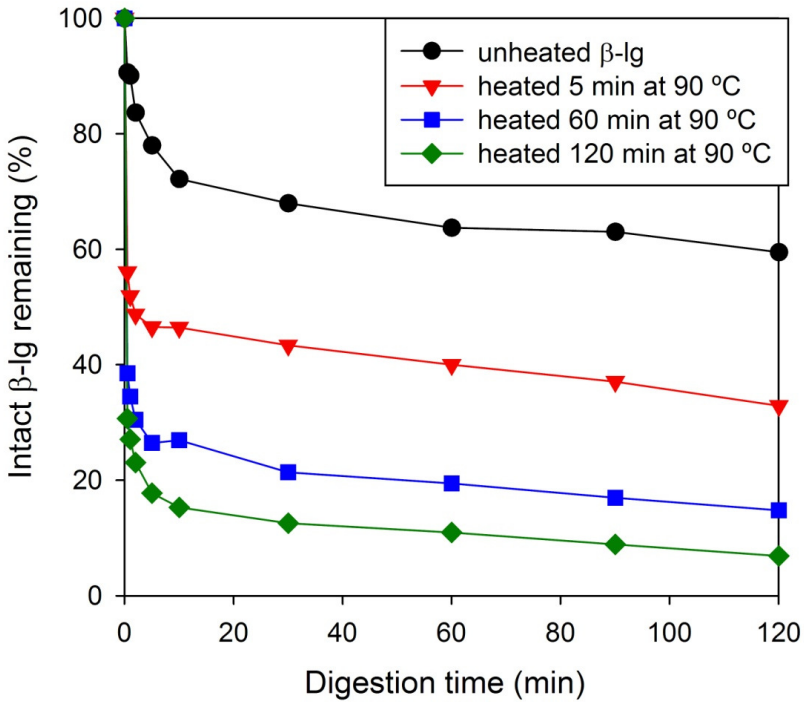


Figure 2. Quantification of intact β-lg remaining after gastric digestion of native and heated β-lg (90°C for 5, 60, and 120 min).



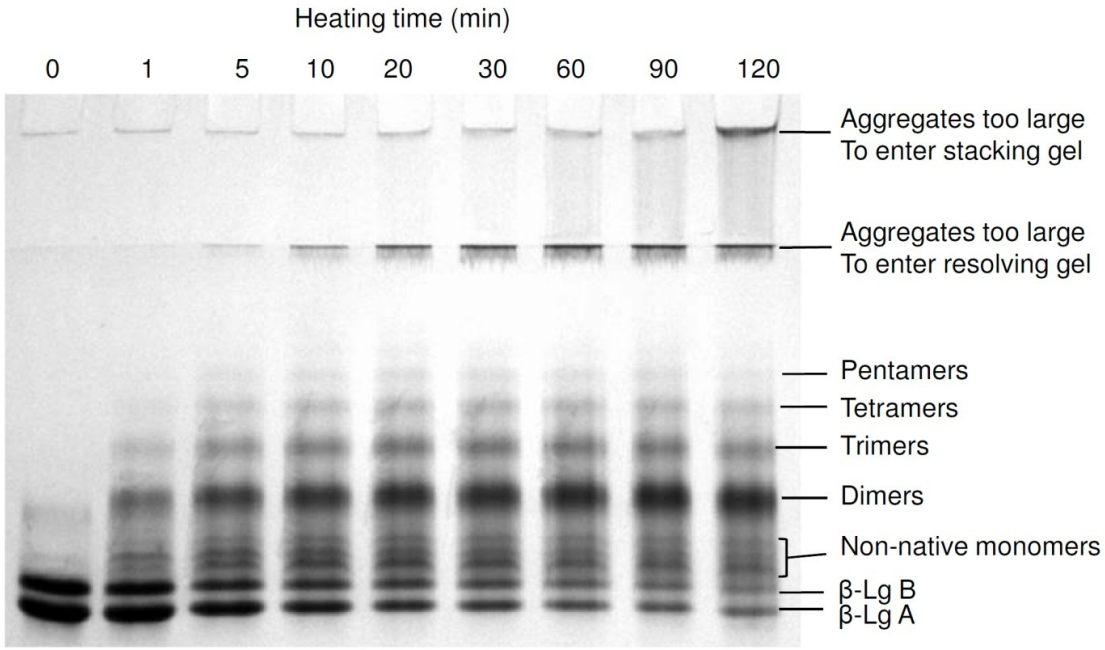
The alkaline native-PAGE patterns of β-lg heated at 90°C for 0, 1, 5, 10, 20, 30, 60, 90, and 120 min in Milli-Q water are shown in Figure 3. As expected, heating resulted in a decrease in the intensity of the native β-lg band and a corresponding increase in the intensity of protein bands with electrophoretic mobility lower than that of native β-lg. These bands were identified as nonnative monomers, dimers, trimers, tetramers, pentamers, etc. by comparison with previous results (Manderson et al., 1998; Schokker et al., 1999). Nonnative monomers may have undergone irreversible conformational alterations (Harwalkar, 1980), such as those induced by intramolecular disulphide interchange (Creamer *et al.*, 2004). In addition, some large aggregates that did not enter the resolving gel and the stacking gel were also observed. A small quantity of β-lg remained in the native state after heating at 90°C for 120 min.

In order to understand the digestion behavior of aggregates formed during the heat treatment of β-lg, the *in vitro* gastric digestion profile of β-lg heated at 90°C for 60 min was analyzed by alkaline native-PAGE (Figure 4A) and nonreduced SDS-PAGE (Figure 4B). Alkaline native-PAGE is used to study aggregates that are linked through both disulfide and noncovalent bonds, whereas nonreduced SDS-PAGE is used for only disulfide-linked aggregates because it dissociates the aggregates linked through noncovalent bonds. The alkaline native-PAGE analysis showed that heating β-lg at 90°C for 60 min resulted in the formation of nonnative aggregates of different sizes, i.e., dimers, trimers, tetramers, pentamers, and aggregates, present at the interface between the resolving gel and the stacking gel. The bands related to trimers, tetramers, and pentamers (oligomers) were not well separated in nonreduced SDS-PAGE. Most of the nonnative aggregates were completely digested by pepsin within 0.5 min. Aggregates present at the interface between the resolving gel and the stacking gel were digested by pepsin within 5 min. There was little or no change in the intensity of the native β-lg band after 120 min of digestion with pepsin. From these results, it is clear that the rapid decrease in the intensity of the β-lg band during the initial 0.5

min of digestion in reduced SDS-PAGE gels (Figures 1B to 1D) was due to the complete digestion of disulfide-linked aggregates, which appear as monomeric β-lg bands in reduced SDS-PAGE gels; the β-mercaptoethanol present in the reduced SDS-PAGE sample buffer disrupts all disulfide-linked aggregates.

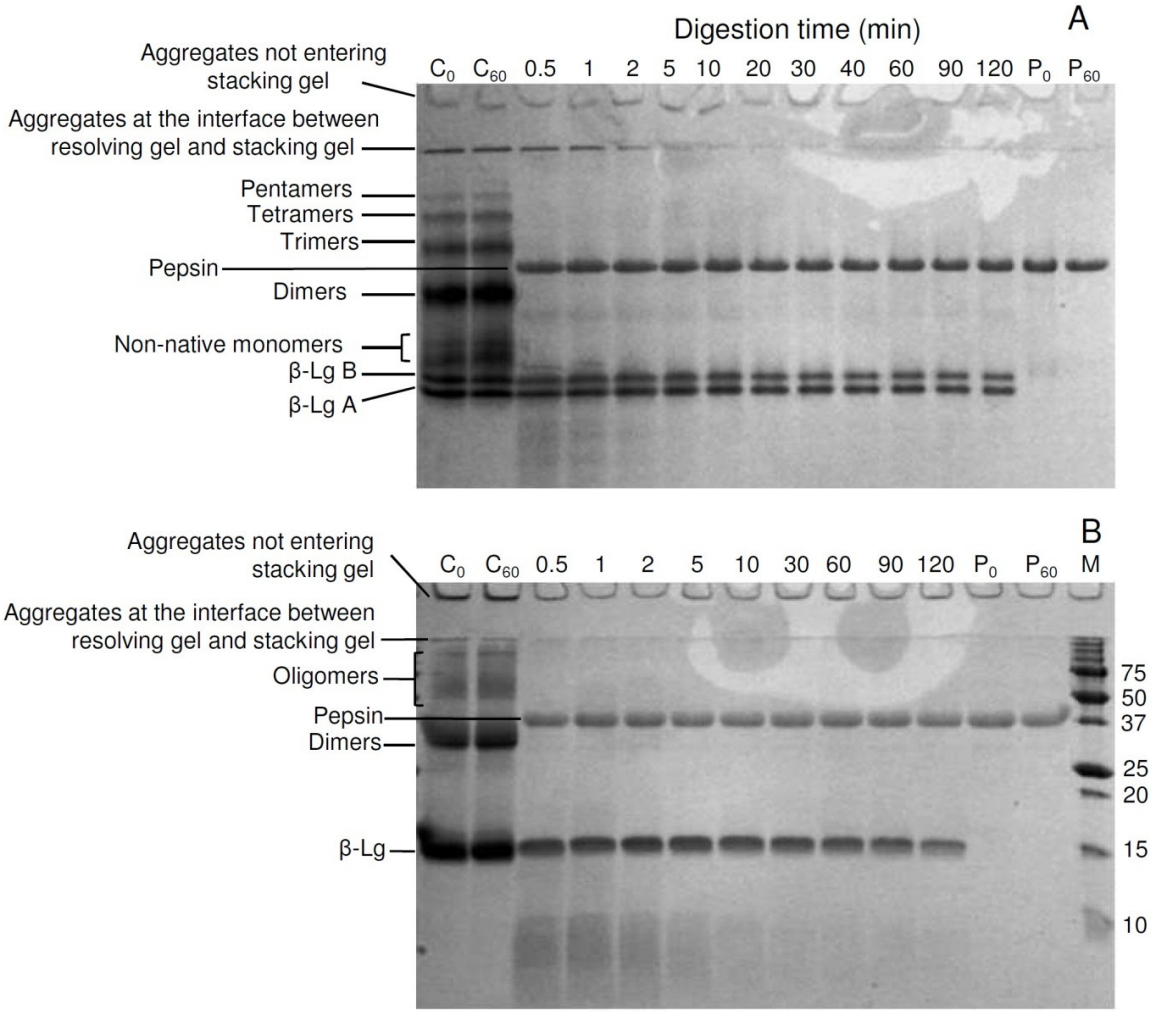
The rate of protein digestion is greatly influenced by the E:S ratio used in the study. The exact ratio of pepsin to food protein present during the human digestive process is not well known and varies with the nature of the food consumed. We used a lower E:S ratio (1:6) to further understand the digestion behavior of heat-induced aggregates of β-lg, which were digested very rapidly at the higher E:S ratio (3:1). Supplementary material contains a side-by-side comparison showing how the enzyme to substrate ratio affected digestion of β-lg heated at 90°C for 5, 60 or 120 min.

Figure 3. Alkaline native-PAGE of β-lg heated at 90°C for 0, 1, 5, 10, 20, 30, 60, 90, and 120 min.



In vitro gastric digestion of β-lg heated at 90°C for 5 and 120 min was performed using SGF at pH 1.2 and a pepsin:β-lg ratio of 1:6. Alkaline native-PAGE (Figures 5A and 5B) analysis showed that heating β-lg at 90°C for 5 min resulted in the formation of nonnative aggregates of different sizes, i.e., dimers, trimers, and tetramers (Figure 5A). The trimers and tetramers were completely digested within 0.5 min, but the intensity of the dimer band decreased during the first 60 min of digestion and remained almost constant thereafter. A small decrease in the intensity of the β-lg A and B bands was observed after 0.5 min of digestion, compared with the control samples. Heating β-lg at 90°C for 120 min **(**Figure 5B**)** resulted in the formation of more aggregates, i.e., dimers, trimers, tetramers, pentamers, and large aggregates (aggregates at the interface of the stacking and resolving gels and aggregates that were too large to enter the stacking gel) (Figure 5B). The dimers, trimers, tetramers, and pentamers were completely digested by pepsin within the first 0.5 min. Large aggregates were digested by pepsin within 10 min. A slight decrease in the intensity of the β-lg band was observed after 0.5 min of digestion.

Figure 4. Gastric digestion of β-lg heated at 90°C for 60 min and analyzed by (A) native-PAGE and (B) nonreduced SDS-PAGE. Pepsin:β-lg ratio 3:1. Lane M corresponds to the molecular weight markers. C_0 and C_{60} represent preheated β-lg without pepsin in SGF at t = 0 and 60 min. The number at the bottom of each lane represents the time of digestion (min). P_0 and P_{60} represent pepsin controls at t = 0 and 60 min.



Nonreduced SDS-PAGE (Figures 6A and 6B) analysis showed similar trends. Heating at 90°C for 5 min resulted in the formation of aggregates of different sizes, i.e., dimers and oligomers (Figure 6A). All oligomers were digested completely within 0.5 min but some dimers were quite resistant to digestion. The dimer band decreased in intensity in the first 0.5 min and then remained constant until 120 min of digestion. There were some bands just above the β-lg band, which we have termed "intermediate molecular weight" (IMW) species. The IMW species were digested slowly. Some peptide bands with a molecular mass lower than 10 kDa were observed and were fully digested within 60 min. In addition to dimers and oligomers, large aggregates were also formed when β-lg was heated at 90°C for 120 min (Figure 6B). The dimers and oligomers were completely digested by pepsin in 0.5 min whereas the large aggregates were digested by pepsin within 10 min. The IMW species formed after 120 min of heating followed almost the same digestion pattern as those formed after 5 min of heating. Digestion for 0.5 min produced several peptides with molecular masses that were lower than 10 kDa; some were digested completely in 2 min and some were digested slowly and resisted 120 min of digestion.

Figure 5. Alkaline native-PAGE analysis of *in vitro* gastric digestion of β-lg heated at 90°C for (A) 5 min and (B) 120 min. Pepsin:β-lg ratio 1:6. Lane M corresponds to the molecular weight markers. C_0 and C_{60} represent preheated β-lg without pepsin in SGF at t = 0 and 60 min. The number at the bottom of each lane represents the time of digestion (min). P_0 and P_{60} represent pepsin controls at t = 0 and 60 min.

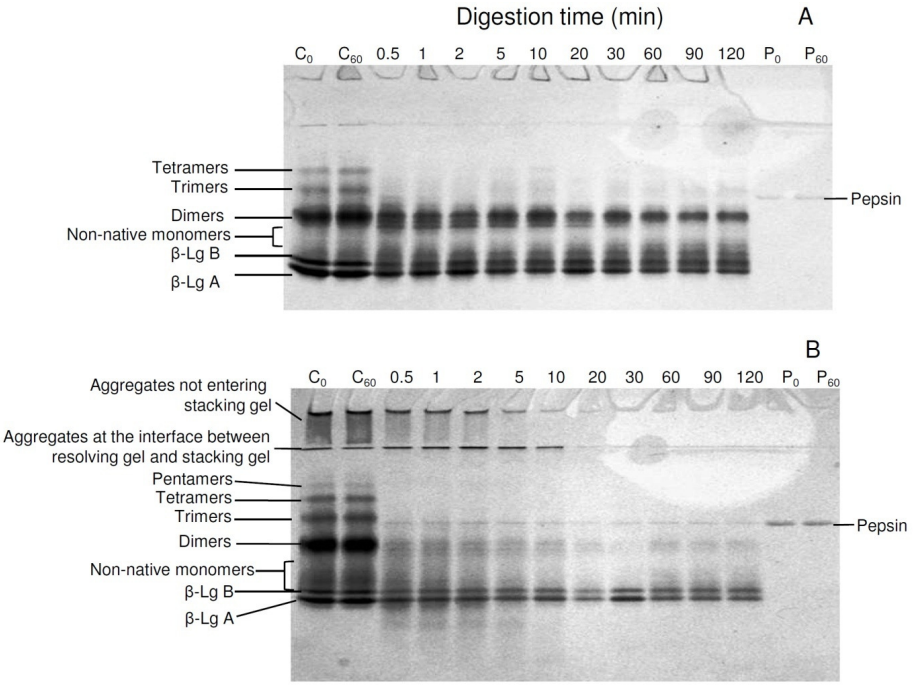
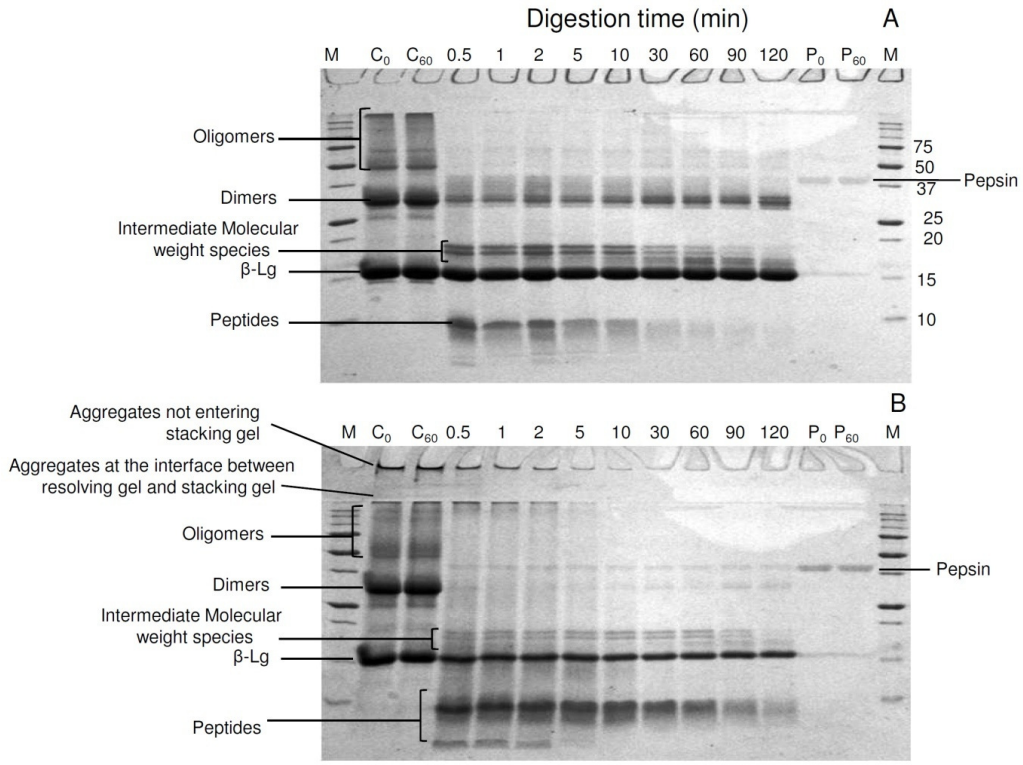


Figure 6. Nonreduced SDS-PAGE analysis of *in vitro* gastric digestion of β-lg heated at 90°C for (A) 5 min and (B) 120 min. Pepsin:β-lg ratio 1:6. Lane M corresponds to the molecular weight markers. C_0 and C_{60} represent preheated β-lg without pepsin in SGF at t = 0 and 60 min. The number at the bottom of each lane represents the time of digestion (min). P_0 and P_{60} represent pepsin controls at t = 0 and 60 min.



These results clearly showed that the stable high molecular weight aggregates (pentamers, tetramers, trimers, etc.) generated during the heat-induced aggregation of β-lg were digested relatively rapidly; in contrast, some of the nonnative dimers were relatively resistant to digestion whereas others were digested readily. It is interesting to note that the dimers formed during the early stages of heating (e.g., 5 min of heating) were quite resistant to digestion whereas the dimers formed after 120 min of heating were easily digested by pepsin. The dimers formed during the early stages of unfolding probably retained some of their secondary and tertiary structural features, and the pepsin cleavage sites remained buried and were not accessible to pepsin. Heating for longer resulted in significant changes in the secondary and tertiary structures of the dimers and consequently promoted their digestibility with pepsin. Further work is required to understand the structural changes in these dimers with an increase in the heating time. The IMW species are also interesting. They were probably formed from interactions of the nonnative monomeric β-lg with the exposed thiol group and peptides resulting from the digestion of nonnative aggregates.

Reduced SDS-PAGE analysis of the *in vitro* gastric digestion of β-lg heated at 90°C for 5 and 120 min using a pepsin:β-lg ratio of 1:6 is shown in Figures 7A and 7B. At an E:S ratio of 1:6, the rate of digestion of β-lg was slower (compared with that observed at an E:S ratio of 3:1) and a number of low molecular weight peptides (less than 10 kDa) were observed after 0.5 min of digestion (Figure 7B). Most of these peptides were further hydrolyzed during 120 min of digestion. The number of peptides observed after pepsin digestion was greater in the reduced SDS-PAGE gels (Figure 7B) than in the nonreduced SDS-PAGE gels (Figure 6B), as indicated by the larger number of bands and wider spreading of bands below the β-lg band. This may have been because some of the peptides were cross-linked through disulfide bonds to form IMW species; these species were broken down by the β-mercaptoethanol present in the sample buffer of the reduced SDS-PAGE. To explore this further, the IMW species observed in the nonreduced SDS-PAGE were characterized using 2D electrophoresis (SDS-PAGE nonreduced and then SDS-PAGE reduced). β-Lg heated at 90°C for 5 min and digested with pepsin for 10 min was analyzed (Figure 8**)**. The first dimension nonreduced SDS-PAGE pattern of the heated and digested β-lg sample showed bands corresponding to native monomeric β-lg, IMW species, and nonnative dimers, and very faint bands corresponding to nonnative oligomers. In the second dimension reduced SDS-PAGE, all the oligomers and dimers appeared as monomeric β-lg, whereas the IMW species appeared as 2 spots, 1 at the monomeric β-lg position and another faint band just below the monomeric β-lg position. These results indicate that the IMW species consisted of nonnative monomeric β-lg and some peptides linked through disulfide bonds.

The *in vitro* gastric digestion of β-lg heated at 90°C for 5 min was analyzed by SEC−HPLC to observe the changes in the digestion pattern of the peptides (Figure 9). Heated β-lg showed 2 peaks corresponding to nonnative dimers and native-like β-lg and eluting at retention times of 33 and 38 min respectively. Pepsin was eluted as a single peak with a retention time of 30 min. After 1 min of digestion, the peaks of nonnative dimers and native-like β-lg decreased and 6 different peaks (P1 to P6) corresponding to peptides were generated. There was no significant change in the peak height for nonnative aggregates and native-like β-lg beyond 60 min. The peptides indicated by P1 and P4 were completely digested, whereas the peptides corresponding to P5 and P6 increased during the first 60 min of digestion and there was no significant change in the peak height thereafter. P3 increased throughout 120 min of digestion, whereas P2 appeared after the first minute of digestion and neither increased nor decreased with longer digestion. The number of peptides observed after β-lg digestion with pepsin was greater for SEC−HPLC than for SDS-PAGE (Figure 7A), because of the inability of SDS-PAGE gels to detect lower molecular weight peptides.

Figure 7. Reduced SDS-PAGE analysis of *in vitro* gastric digestion of β-lg heated at 90°C for (A) 5 min and (B) 120 min. Pepsin:β-lg ratio 1:6. Lane M corresponds to the molecular weight markers. C_0 and C_{60} represent preheated β-lg without pepsin in SGF at t = 0 and 60 min. The number at the bottom of each lane represents the time of digestion (min). P_0 and P_{60} represent pepsin controls at t = 0 and 60 min.

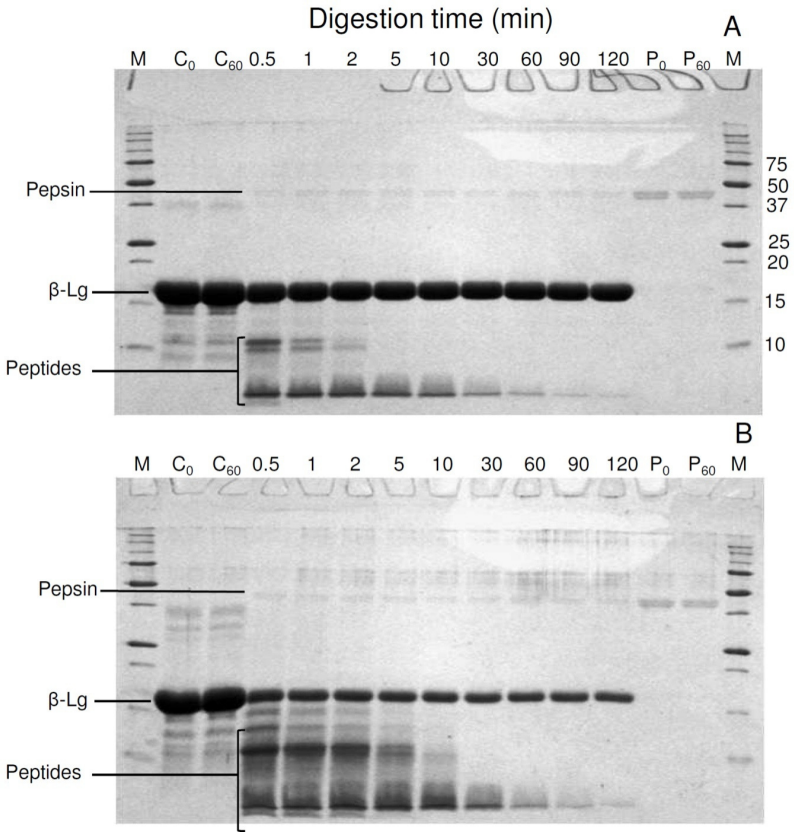


Figure 8. 2D electrophoresis (first dimension nonreducing SDS-PAGE, second dimension reducing SDS-PAGE) of β-lg heated at 90°C for 5 min and digested with pepsin for 10 min.

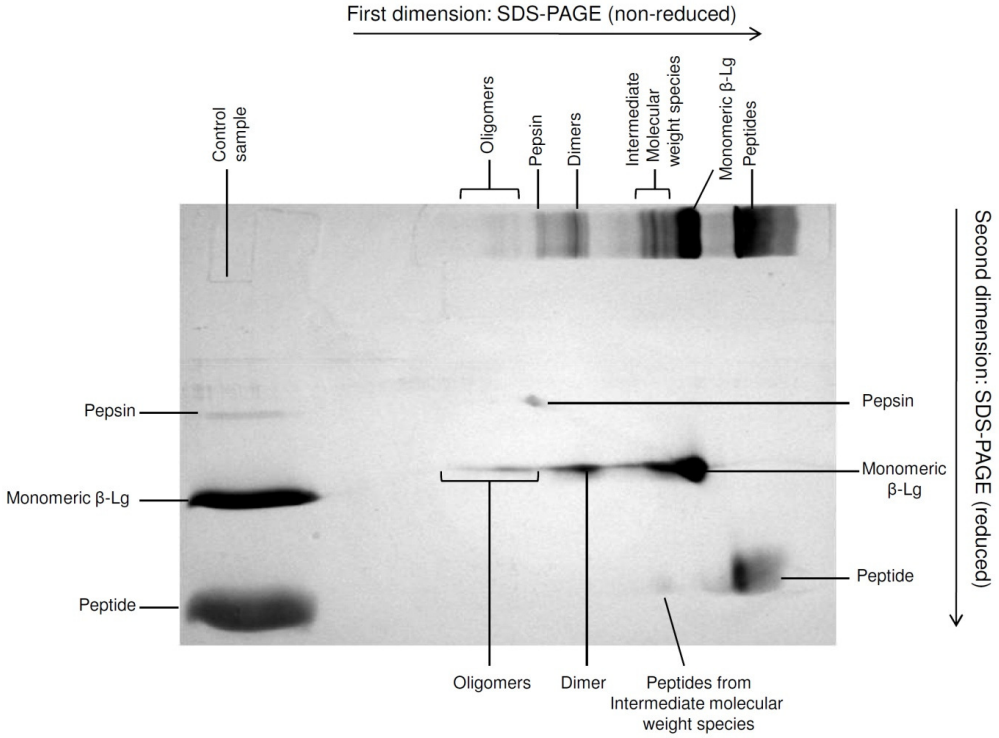
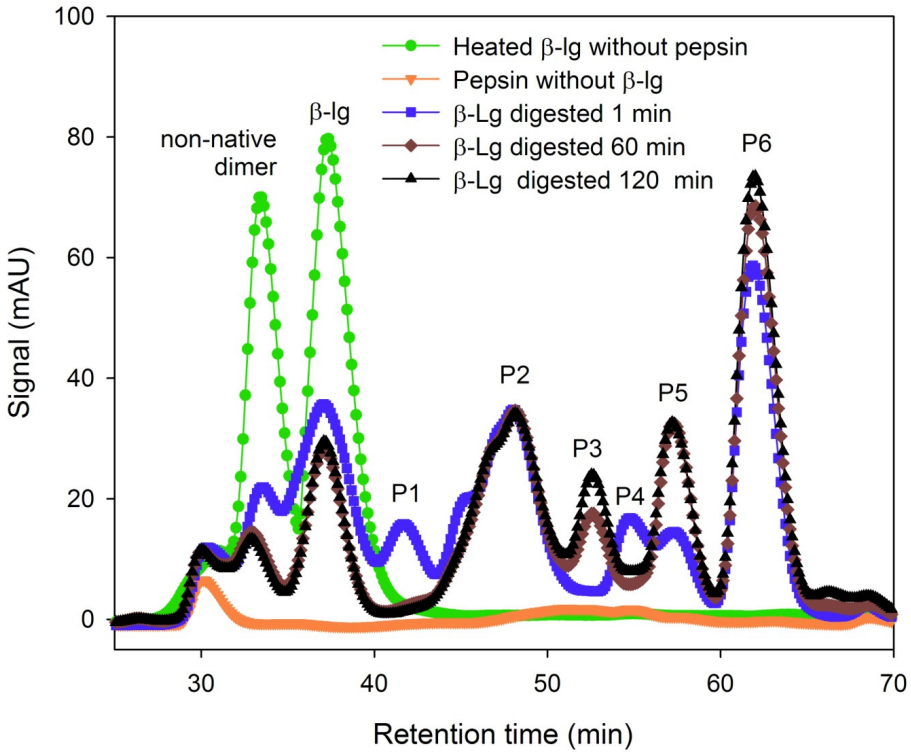
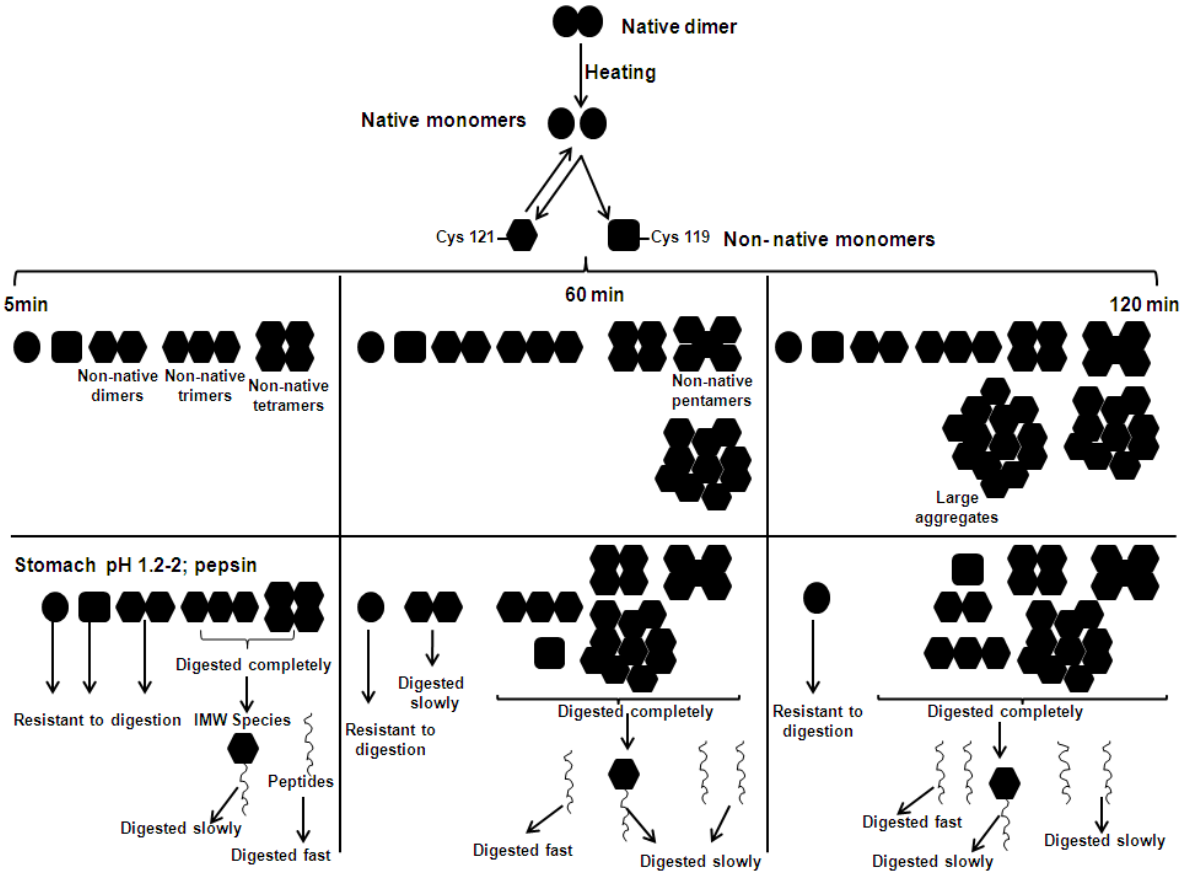


Figure 9. SEC−HPLC profile of gastric digestion of β-lg heated at 90°C for 5 min.



A schematic diagram showing the possible sequence of events during the formation of heat-induced aggregates of β-lg and their digestion in the gastric environment is given in Figure 10. At room temperature and neutral pH, β-lg exists as a noncovalently linked dimer. Upon heating to 90°C, this dimer dissociates into monomers, which partially unfold, leading to the formation of nonnative monomers and the exposure of inner hydrophobic amino acids and the free thiol group. There are 2 types of nonnative monomers, 1 with the free thiol group at Cys 121 and the other with the free thiol group at Cys 119 (Creamer et al., 2004). The nonnative monomer with the free Cys 119 is formed from the intramolecular sulfhydryl−disulfide exchange reaction between Cys 121 and Cys 106−Cys 119. The nonnative monomer with the free Cys 121 undergoes intermolecular disulfide exchange reactions, leading to the formation of dimers and large aggregates (Creamer et al., 2004) . On cooling, the nonnative monomer with the free Cys 121 switches back to a native monomer whereas the nonnative monomer with the free Cys 119 remains as a nonnative monomer. A heated and cooled β-lg solution contains native monomers with free Cys 121, nonnative monomers, and other disulfide-linked aggregates. The digestibility of heat-induced aggregates of β-lg varies significantly. All aggregates and intermediate species, except some dimers, are readily digested, suggesting that aggregation does not hinder the access of pepsin to the cleavage sites. New IMW fragments that are linked through disulfide bonds are generated from the hydrolysis of the aggregates. Further studies are necessary to understand the structural aspects of slowly and rapidly digested dimers of β-lg, and the peptides generated during the digestion need to be identified.

Figure 10. Scheme of events taking place during the pepsin digestion of heat-induced aggregates of β-lg.



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SUPPLEMENTARY MATERIAL

Effect of enzyme to substrate ratio on digestion of β-lactoglobulin heated at 90ºC

Ratio: **1:3** **1:6**