

Characterization of the molecular properties and allergenicity (IgE-binding capacity) of β -lactoglobulin by heat treatment using asymmetric-flow field-flow fractionation and ultra-performance liquid chromatography quadrupole time of flight mass chromatography

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ABSTRACT

In this study, the heat product (90 °C, 10 min) of β -lactoglobulin (β -LG) was analyzed by asymmetric-flow field-flow fractionation (AF4) to observe the effect of heat treatment. The changes in molar mass (M) and molar size induced by heat treatment were characterized by AF4, and changes in molar shape were observed by transmission electron microscopy (TEM). The results showed that β -LG dissociated and aggregated into four fractions with different M values, sizes, and shapes after heat treatment. The vast aggregations with the highest allergenicity (IgE-binding capacity) might enhance the allergenicity of β -LG. However, the number of characterized epitope peptides was decreased due to heat treatment. The above results provide some references for related studies of β -LG and its allergenicity. Further separation and characterization of the high-allergenicity fractions and peptides will help to eliminate allergens in dairy products and reduce the occurrence of allergic reactions.

1. Introduction

Food allergies are one of the most important issues of public health and have become a focus in the field of food safety. Generally, food allergies can be divided into non-immunoglobulin E (IgE)-mediated allergic reactions and IgE-mediated allergic reactions. IgE-mediated food allergies account for the majority of food allergic reactions (van Wijk & Knippels, 2007). More than 10% of 1-year-old infants suffer from this type of allergy (Osborne et al., 2011). Bovine milk allergies are one of the most common food allergies, especially in early childhood. Among all the identified allergenic proteins in milk, β -lactoglobulin (β -LG) is considered an important allergen (Bu et al., 2009) since as much as approximately 82% of humans who have allergic reactions to bovine milk are allergic to β -LG (He et al., 2019). Moreover, β -LG has been

extensively used as an ingredient in food formulations because of its functional and nutritional properties, but its industrial application is still limited by its high allergenicity (Corzo-Martínez et al., 2010).

Heat treatment is an important process in the manufacturing of food. Health and food authorities strongly require raw milk to be heated before consumption, and most commercially available milk is processed through heat treatment and several other processing steps, such as centrifugation and filter (Brick et al., 2020). However, heat treatment will induce changes in the structure, function and even further allergenicity of proteins (Hussain et al., 2012). Studies have been carried out on the properties of allergen proteins under heat treatment in many aspects, such as conformational stability, allergenicity, and digestion resistance (Apostolovic et al., 2016; Chihi et al., 2016; Teodorowicz et al., 2017). Among these properties, allergenicity is a prominent research topic.

Abbreviations: β -LG, β -lactoglobulin; AF4, asymmetric-flow field-flow fractionation; TEM, transmission electron microscopy; MALS, multiangle light scattering; dRI, differential refractive index; UPLC-Q-TOF-MS, ultra-performance liquid chromatography quadrupole time of flight mass chromatography; M, molar mass; SEC, size exclusion chromatography; IgE, immunoglobulin E; ELISA, enzyme-linked immunosorbent assay; ACN, acetonitrile; DTT, dithiothreitol; TFA, trifluoroacetic acid; BSA, bovine serum albumin; IAA, iodoacetamide; FA, formic acid; PBS, phosphate buffer saline; DLS, dynamic light scatter; RT, retention time; TMB, tetramethylbenzidine.

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Through heat treatment, most of the original tertiary structure of the protein is lost, and many of the antibody recognition sites on the native molecule are destroyed, leading to changes and reductions in allergenicity. However, some new antigenic sites can be revealed, leading to an increase in allergenicity (Davis & Williams, 1998). It has been reported that heating will result in a significant decrease in the intensity of native β -LG and a corresponding increase in the intensity of nonnative proteins, such as some large aggregates caused by irreversible conformational alterations (Peram et al., 2013). In addition, some epitopes of β -LG are lost during heat treatment, while binding with IgE is significantly increased after pasteurization, which will promote the allergic sensitization of proteins by enhancing uptake through Peyer's patches, and the aggregates formed due to heat treatment play an important role (Pranger et al., 2021; Roth-Walter et al., 2008). Existing studies have mainly focused on the property change of whole β -LG proteins, but few studies have focused on the fractions of β -LG brought by heat treatment. The existence of these fractions may directly contribute to the property change, especially for its allergenicity enhancement. Therefore, it is important to investigate the effect of heat treatment on the molecular properties of β -LG and its contribution to allergenicity changes.

To achieve this aim, it is essential to develop convenient methods for direct, rapid, and cost-effective evaluation of the allergenicity of the fractions in the β -LG heat product. Size exclusion chromatography (SEC) and agarose gel electrophoresis are the two traditional methods in the study of aggregates, but they are not suitable for some large-particle samples due to the lack of suitable markers and an inability to maintain the complete biological activity of molecules among others (Loiseleux et al., 2018). Alternatively, transmission electron microscopy (TEM) can be used to image heated β -LG and its fractions, but it cannot achieve quantitative measurements. Asymmetric-flow field-flow fractionation (AF4) is a versatile method and has been widely used to separate and characterize biomacromolecules from different origins (Fuentes et al., 2019; Williams et al., 2011; Zhang & Lyden, 2019). Moreover, AF4 is a nondestructive method that does not change the properties of analytes during fractionation and is amenable to subsequent analyses (Loiseleux et al., 2018).

Therefore, the purpose of this study was to adopt AF4 connected to multiangle light scattering (MALS) and differential refractive index (dRI) detectors to directly and effectively investigate the change in protein fractions and molecular properties, especially for the change in allergenicity (IgE-binding capacity) by heat treatment, taking the β -LG heat product (90 °C, 10 min) as an example. First, the IgE-binding capacity of β -LG heat products was characterized by indirect competitive enzyme-linked immunosorbent assay (ELISA) to select a heat product (90 °C, 10 min) with high IgE-binding capacity. The molecular properties and the binding behavior between the heat product and IgE were then characterized by AF4. In addition, estimation of IgE-binding capacity was performed among different AF4 collected fractions of the selected β -LG heat product that were collected during AF4 separation. Moreover, an AF4 combined with ultra-performance liquid chromatography quadrupole time of flight mass chromatography (UPLC-Q-TOF-MS) strategy was used to characterize the epitopes of β -LG and its heat product to investigate the effect of epitope change on allergenicity brought by heat treatment.

2. Materials and methods

2.1. Materials

Ammonium bicarbonate (NH_4HCO_3), acetonitrile (ACN), dithiothreitol (DTT), trifluoroacetic acid (TFA), trypsin from porcine pancreas (Proteomics-grade), β -LG (>90%), and bovine serum albumin (BSA, >98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Iodoacetamide (IAA) and formic acid (FA) were purchased from Acros Organics (New Jersey, USA). Native human IgE (azide free) was purchased from Abcam (Cambridge, UK). Phosphate-buffered saline (PBS)

was purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). The Quick Start™ Bradford Protein Assay Kit including dye reagent and bovine serum albumin (BSA) standard were purchased from Bio-Rad (Hercules, USA). Mouse anti- β -LG serum was purchased from Abiocenter Biotechnology Co., Ltd. (Beijing, China). The working buffer solutions for ELISA part were obtained as an ELISA supplemental solution set (Cat: SEKCR02) developed by Sino Biological (Beijing, China). Deionized water (18.2 M Ω /cm) was obtained from a Milli-Q Advantage A10 Ultra-Pure Water system (Millipore, MA, USA). All chemicals were of analytical reagent grade and used without further purification.

2.2. Heat treatment of β -LG

The β -LG solution was prepared in PBS and mixed thoroughly to a final concentration of 10 mg/mL. Solutions in glass test tubes sealed with rubber stoppers were put in a water bath at 78.3 °C and 90 °C for different times. Room-temperature β -LG solution without heat treatment was used as a control. All samples were stored at -20 °C for further analysis.

A thermal stability test using dynamic light scattering (DLS) was performed in a DynaPro Plate Reader III (Wyatt Technology, Santa Barbara, CA, USA) equipped with an 830 nm wavelength laser. Continuous temperature ramp mode was adopted. DLS acquisition times and the number of DLS acquisitions were set as 5 s and 5, respectively. The starting temperature, ending temperature and delta temperature per scan were 25 °C, 80 °C and 1 °C, respectively. The increase rate was 0.75 °C/min.

2.3. Evaluation of β -LG heat product by AF4-MALS-dRI-UV

A 1100 pump (Agilent Technologies, Waldbronn, Germany) and an Eclipse system (Wyatt Technology, Dernbach, Germany) were used for analysis. The Eclipse system was equipped with a DAWN MALS detector (Wyatt Technology, Santa Barbara, CA), an Optilab dRI detector (Wyatt Technology, Santa Barbara, CA) and an Agilent UV detector (Agilent Technologies, Waldbronn, Germany). A 0.1 μm membrane filter (Millipore, MA, USA) was placed between the pump and the AF4 channel to ensure that the carrier liquid entered the AF4 channel free of particles. A Wyatt short channel assembled with a 350 μm -thick Mylar spacer and a regenerated ultrafiltration cellulose membrane with a nominal cut-off of 1.0×10^4 Da were used. The carrier liquid for AF4 was PBS filtered through a 0.22 μm regenerated cellulose membrane filter before use. During elution, the detector flow rate was constant at 0.8 mL/min. The sample was injected with a flow rate of 0.2 mL/min for 2.0 min. After injection, a 3 min focusing/relaxation step was performed. The cross-flow rate was constant at 3.2 mL/min until the end of elution to study the binding between β -LG and its heat product with IgE. The detection wavelength of the UV detector was set at 215 nm to increase the sensitivity to the targets (Huang et al., 2007; Ni & Song, 2002). For the analysis of β -LG and its heat product, a UV detection wavelength of 280 nm was used, and the cross-flow rate was started at 3.2 mL/min with a duration of 10 min, decreased exponentially to 0 mL/min in 15 min and held for 5 min to flush out the remaining sample.

Through analysis of the β -LG heat product, four fractions were collected according to the retention time (RT) as follows: fraction 1 (RT: 12.5–15 min), fraction 2 (RT: 15–17 min), fraction 3 (RT: 22–25.5 min) and fraction 4 (RT: 25.5–29 min). All collected fractions were stored at -20 °C for further analysis.

2.4. Characterization and validation of the fractions separated from AF4 by TEM

Before TEM analysis, fractions collected from AF4 as described in Section 2.3 were diluted with deionized water to appropriate concentrations. Then, the diluted solutions were placed on formvar- and carbon-coated copper grids. After 1 min, the solution was removed.

Fifteen milliliters of a 2% (w/v) uranyl acetate solution was placed on the grid and then removed after 30 s. The above step was repeated once. The grids were studied under a JEM-1400 Plus electron microscope (JEOL, Peabody, MA, USA) after drying with an accelerating voltage of 100 kV.

2.5. Evaluation of IgE-binding capacity by indirect competitive ELISA

To evaluate the IgE-binding capacity of the heat products of β -LG, the four fractions (Section 2.3) were collected, and the IgE-binding capacity of these four fractions *in vitro* was analyzed by indirect competitive ELISA (Wróblewska & Jedrychowski, 2002; Wroblewska et al., 2004). The brief procedure was as follows. First, the collected fractions were freeze-dried using a Christ ALPHA 1–4 LSC vacuum freeze drier (Marin Christ, Osterode, Germany) at -90°C overnight. Then, the dried fractions were dissolved using 1 mL of PBS. The concentrations were quantified using a Quick Start™ Bradford Protein Assay Kit and Multiskan MK3 microplate reader (Thermo Fisher, Franklin, MA, USA). The quantification procedure was performed according to the standard protocol (<https://www.bio-rad.com/sites/default/files/webroot/web/pdf/lsr/literature/4110065A.pdf>). The appropriate amount of PBS was added to each fraction to dilute the fractions at a final concentration of 10.0 $\mu\text{g/mL}$ for subsequent analysis.

Microtiter plates with 96 wells were coated with 100 μL /well antigen (β -LG: 0.1 $\mu\text{g/mL}$) diluted in PBS and incubated overnight at 4°C . Then, the liquid in the wells was dropped off, and the wells were filled with 300 μL of 2% BSA to block residual free binding sites and incubated for 1 h at room temperature. The plates were washed twice with 300 μL of washing buffer. Solutions of PBS (negative control) or analytes (above diluted fractions) with an equivalent volume of mouse anti- β -LG serum diluted in PBS (1:50000 for anti- β -LG serum) were incubated overnight at 4°C in the test tube. The preparation method of mouse anti- β -LG serum is described in the supplementary materials according to a previously reported method (Bu et al., 2009). Then, 100 μL /well reactive mixtures were added and incubated for 2 h at room temperature. After washing the plates, 100 μL of 0.09 $\mu\text{g/mL}$ Peroxidase AffiniPure Rabbit Anti-Mouse IgG, F(ab')₂ fragment specific (Jackson ImmunoResearch Laboratories Inc., Pennsylvania, USA), was added and further incubated at room temperature for 1 h. The plates were washed again, and then 200 μL of a 1:1 mixture of chromogenic solution A (stabilized hydrogen peroxide) and B (stabilized tetramethylbenzidine, TMB) was added to each well. The plates were incubated for 20 min at room temperature in the dark. Finally, 50 μL /well stop solution was added to stop the reaction. Absorptions were read spectrophotometrically at 450 nm on a Multiskan MK3 ELISA plate reader (Thermo Fisher, Franklin, MA, USA). In this study, the IgE-binding capacity was calculated by the average absorption of analyses ($\text{OD}_{450\text{analyse}}$) and absorption of the negative control ($\text{OD}_{450\text{PBS}}$) using Formula (1) as follows:

$$\text{IgE-binding capacity} = 1 - \frac{\text{OD}_{450\text{analyse}}}{\text{OD}_{450\text{PBS}}} \quad (1)$$

2.6. Characterization of β -LG epitope peptides

AF4 combined with UPLC-Q-TOF-MS was used to characterize the epitopes of shrimp tropomyosin, as reported by our previous research (Li et al., 2019). The digestion process of β -LG and its heat product was used according to the reported method with slight modification for the characterization of epitope peptides and is shown in the Supplementary Material. The UPLC-Q-TOF-MS (Waters Vion IMS-Q-TOF) method was used in the current study with slight modifications to achieve a better separation of those peptides. The sample solutions were loaded onto a Waters ACQUITY UPLC® Peptides BEH™ C18 column (130 Å, 1.7 μm , 2.1 mm $\times$ 100 mm) with a column temperature set at 60°C . The mobile phase consisted of water with 0.1% FA (Solvent A) and ACN with 0.1% FA (Solvent B). A binary solvent was run at 0.3 mL/min. The binary

solvent gradient was 2% B held for 2.0 min, then linearly increased from 2% to 40% B in 13.0 min, linearly increased from 40% to 95% B for 1.0 min, held for 1.0 min, decreased to 2% B for 1.0 min and held at 2% for 2.0 min. Positive electrospray ionization (ESI +) was utilized. The MS data were acquired from 100 Da to 2000 Da using MS^E mode. The scan time was set as 1.0 s. The capillary voltage was 3.0 kV. The low collision energy was 6.0 eV, and the high collision energy was set as 20–45 eV with a gradient. The cone gas and desolvation gas were 50 L/h and 500 L/h, respectively. The source temperature and desolvation temperature were set as 100°C and 300°C , respectively.

2.7. Statistical analysis

All tests were repeated at least three times, and the results are expressed as the mean $\pm$ standard deviation. Statistical analysis was performed by one-way analysis of variance and T test using Prism (version 8.0, GraphPad). A value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Heat treatment of β -LG

For some thermally unstable proteins, changing the temperature will lead to denaturalization and aggregation so that an increase in the radius can be observed (Borzova et al., 2017; Parthasarathy & Murthy, 2000). It was reported that the antigenicity of β -LG after heat treatment will reach its maximum when heated at 90°C for 10 min (Bu et al., 2009). Validation studies were performed in advance to check the thermal stability of β -LG. First, the thermal stability of β -LG was investigated by DLS. As a result, 78.3°C was the critical temperature for the thermal stability of β -LG, as shown in Fig. S1. Second, IgE-binding capacity *in vitro* estimation was performed among β -LG (without treatment) and its different heat products to represent allergenicity, and the concentration of β -LG (without treatment) among all those samples was kept at the same level at approximately 100 $\mu\text{g/mL}$. According to the results in Fig. 1, the IgE-binding capacity of all the β -LG heat products (78.3°C and 90°C for different durations) was significantly increased ($P < 0.05$) when compared with that of β -LG (without treatment). In addition, a

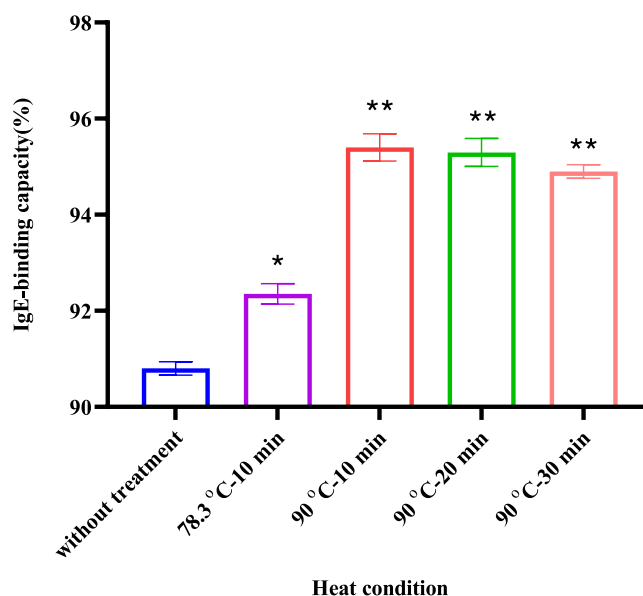


Fig. 1. IgE-binding capacity of β -LG (without treatment) and its different heat products (78.3°C , 10 min; 90°C , 10 min; 90°C , 20 min; 90°C , 30 min) estimated by indirect competitive ELISA. * $P < 0.05$, ** $P < 0.01$, versus the without treatment group.

significant difference among the IgE-binding capacity of those five groups was analyzed by ordinary one-way ANOVA ($P < 0.05$). The β -LG heat product (90 °C, 10 min) had the highest IgE-binding capacity, which is highly consistent with reported results. However, the IgE-binding capacity of β -LG at 90 °C under different heat times (10 min, 20 min, 30 min) was very close. Therefore, the heating condition of β -LG was set at 90 °C for 10 min for further investigation.

3.2. Effect of heat treatment on the molecular properties of β -LG

Initially, the β -LG control solution (without heat treatment) was characterized by AF4 coupled with MALS and UV detector. As shown in Fig. 2a, β -LG was detected in a single peak at an elution time of approximately 15 min by both UV and MALS detectors, and the molar mass (M) was 1.965×10^4 Da, as measured by the MALS detector. The measured M was higher than the theoretical M (1.8×10^4 Da). The mechanism of separation by AF4 is based on the shape and size of the molecules, which would affect the measured M by AF4. For example, if two molecules have the same theoretical M, the measured M of one molecule with a larger size would be higher than that of a molecule with a smaller size. Therefore, it would be reasonable that the M measured by AF4 was slightly different from the theoretical M.

The effect of heat treatment on β -LG at 90 °C for 10 min is shown in Fig. 2b. Through heat treatment, β -LG suffered significant changes. The only peak at approximately 15 min in the control β -LG turned to four peaks (Fraction 1 to Fraction 4; F1-F4) observed by the UV detector, although F1 and F2 had poor separation. Only two aggregate peaks (F3 at 24 min with M from 1.0×10^6 Da to 1.0×10^7 Da, F4 at 27 min with M from 1.0×10^7 Da to 5.0×10^8 Da) were observed by the MALS detector. F1 and F2 almost disappeared because of the low M of F1 at 14 min (1.0×10^4 Da) and F2 at 16 min (1.0×10^3 Da to 1.0×10^4 Da). The M of F2 slightly decreased compared with F1 but was eluted later, which did not agree with the principle of AF4 detection that particles with a relatively low M are prone to be eluted relatively early. A possible explanation of F1 and F2 could be a result of dissociation brought by high temperature. These dissociated fragments may differ in M and other features, such as viscosity and shape. Thus, F2 may be prone to attach to the regenerated cellulose membrane, resulting in delayed elution and poor separation. In addition, it is speculated that there may be some smaller fragments with an M far lower than the cut-off of M existing in the solution but had been filtered into waste during separation by AF4 and were therefore not detected. Therefore, not only a remarkable aggregation process but also a dissociation process were observed in the β -LG heat product, and both processes led to changes in the molecular properties of β -LG.

3.3. Effect of heat treatment on β -LG binding with IgE

The mechanism of IgE-mediated allergic reaction involves antigen presentation, IgE production, mast cell activation, mediator release, and symptom exhibition (Chen et al., 2008). The binding between IgE and allergic proteins is the beginning of food allergies and an important indicator reflecting allergenicity. β -LG is one of the main allergen in milk and can bind specifically to IgE. To investigate the effect of heat treatment on its binding behavior with IgE and its allergenicity change, the binding of β -LG and its heat product (90 °C, 10 min) with IgE was characterized *in vitro* by AF4. As shown in Fig. 2c, there were two main peaks of IgE detected by both UV and MALS detectors. One peak was seen at an elution time of approximately 21.5 min, corresponding to IgE monomers plotting M at approximately 2.0×10^5 Da, which was slightly higher than the reported 1.9×10^5 Da (Ishizaka & Ishizaka, 2016), and another peak was observed at approximately 25 min, corresponding to IgE polymers plotting M at approximately 3.0×10^5 Da to 4.0×10^5 Da. When binding with β -LG (without treatment) and its heat product, both the peak of the IgE- β -LG complex and IgE- β -LG heat product complex show right movement compared to IgE by UV and MALS detectors. No obvious change in M was seen between IgE and the IgE- β -LG complex.

There was a clear increase in the M of the IgE- β -LG heat product complex compared with that of IgE, especially for its polymers (the peak at approximately 26 min), with its M at approximately 3.4×10^5 Da to 4.4×10^5 Da. Heat treatment did not damage the binding activity between IgE and β -LG instead of obviously forming a binding complex, which could also verify the result of the IgE-binding capacity change described in Section 3.1.

3.4. Characterization and validation of the fractions separated from AF4 by TEM

During AF4 separation, four fractions were collected and then analyzed by TEM to further characterize the fractions and validate the AF4 results. Fig. 3 shows TEM micrographs of the four fractions with the same dilution factor. As displayed in Fig. 3a, F1 was denser than the other fractions and contained regular spots with particle sizes below 10 nm. The F2 particles (Fig. 3b) were slightly smaller than F1 particles at the same scale of 50 nm, consistent with the results described in Section 3.3. In addition, the shape of Fraction 2 particles was not as regular as that of F1 particles. More oval or linear particles could be seen in F2 than in F1, which may be due to the dissociation occurring at high temperatures. The particle numbers of both F3 (Fig. 3c) and F4 (Fig. 3d) were obviously decreased compared to those of F1 and F2 and were therefore characterized under a larger TEM scale (100 nm). F3 showed a regular globular shape with a size of approximately 100–150 nm. F4 showed an irregular shape with a particle size over 300 nm, and some holes could be seen in the aggregates. Overall, the TEM results demonstrated good coherence with the above AF4 results, which validated the separate mechanism of AF4 and confirmed the existence of four fractions with different molecular properties. The difference in sizes, shapes, and particle numbers of those fractions may contribute to their properties change, such as IgE-binding capacity, which was evaluated and discussed in the following section.

3.5. IgE-binding capacity estimation of fractions separated from AF4

For allergic reactions mediated by IgE, the IgE-binding capacity of allergens *in vivo* and *in vitro* reflected the allergenicity of compounds. In this study, the IgE-binding capacities *in vitro* of the fractions separated and collected from AF4 were estimated to represent allergenicity. Before the test of IgE-binding capacity *in vitro*, the four fractions were freeze-dried and redissolved in PBS according to the procedure detailed in Section 2.5. As shown in Fig. 4, the IgE-binding capacities of the four fractions *in vitro* exceeded 75%. It was suggested that all heat products of β -LG still maintained high allergic activity after heat treatment. However, the IgE-binding capacities of F1, F2 and F3 were significantly lower than those of F4 ($P < 0.05$). In all four fractions, the M and particle size of F4 was the largest according to the above results. This result was similar to the reported aggregates caused by pasteurization, which could enhance uptake through Peyer's patches and result in promoting the allergic sensitization of β -LG (Roth-Walter et al., 2008). However, the IgE-binding capacity of F3 was slightly lower than F1 and F2, although the M and particle size of F3 was higher than F1 and F2. Therefore, the increase in M and particle size might not be critical factors for the increase in IgE-binding capacity. On the other hand, according to Fig. 3, the molecular shape of F4 was changed except for the increase in M and particle size. The molecular shape of F4 changed to an irregular clump shape from a regular globular shape. It was supposed that the holes of the irregular clump may contain more binding sites, which could increase the binding area of F4 with IgE. Therefore, the increase in the binding site and the irregular clump shape due to the aggregate behavior of molecules might be a potential key factor for the increase in IgE-binding capacity.

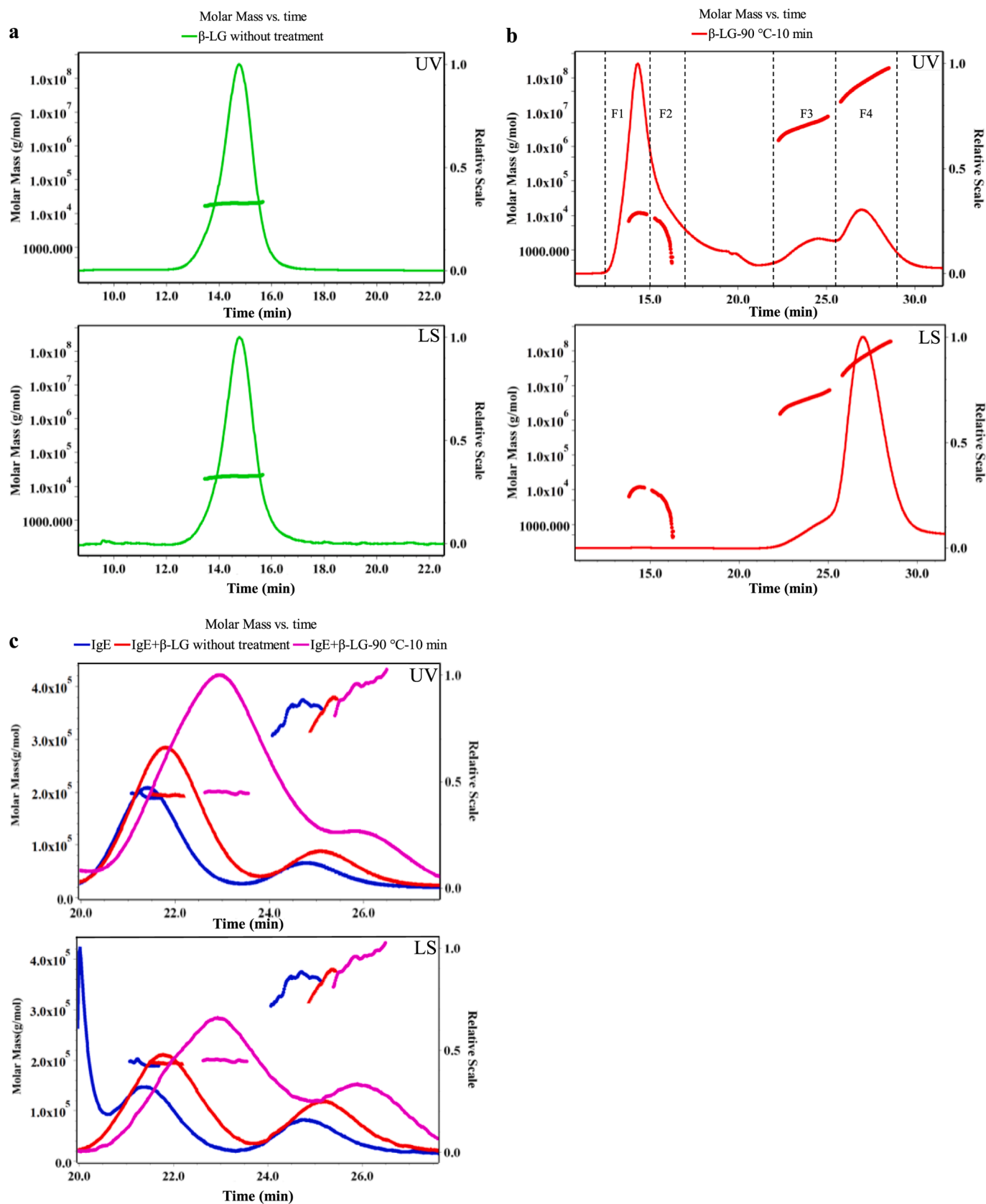


Fig. 2. AF4-UV and MALS fractograms with the calculated molar mass (M) of β -LG without treatment (a), β -LG heat product (b: the heat condition was 90 °C lasting 10 min, F1-F4 are fractions that were collected during AF4 separation), IgE, IgE binding with β -LG without treatment and IgE binding with β -LG heat product (c).

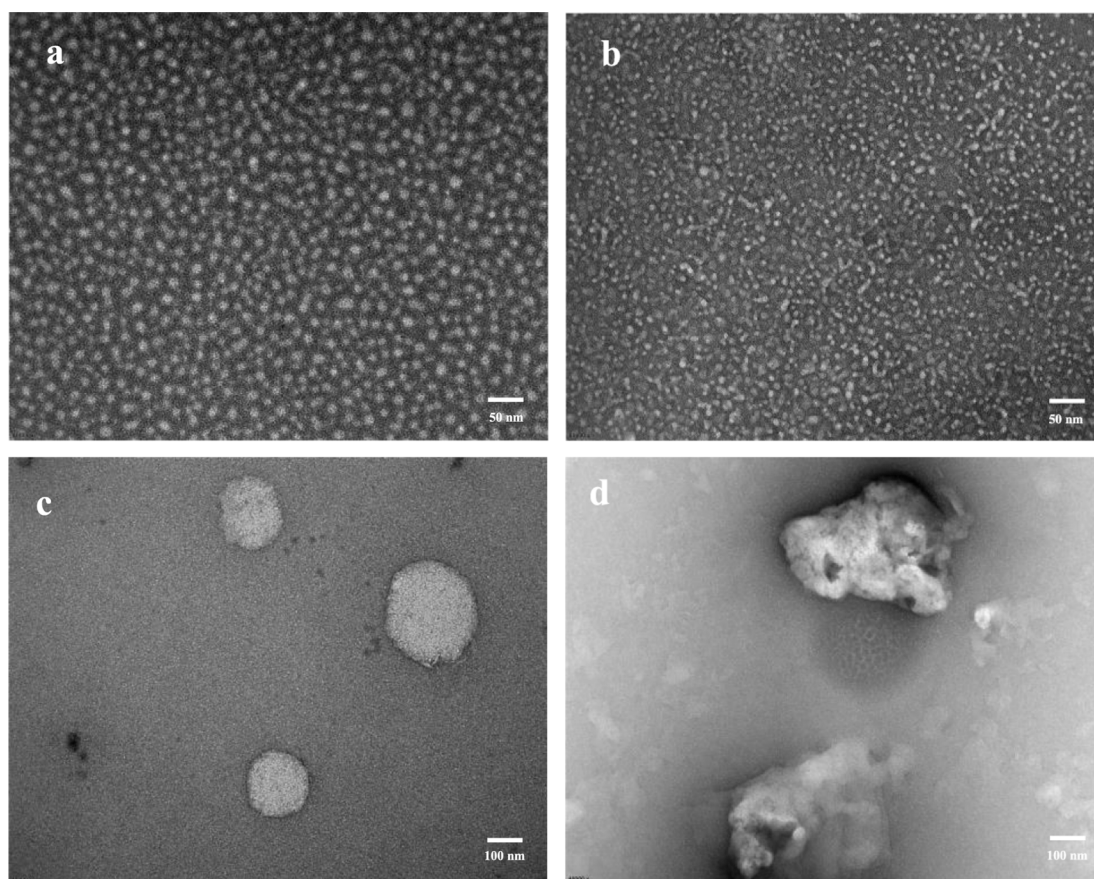


Fig. 3. Transmission electron micrographs of Fraction 1 (a), Fraction 2 (b), Fraction (3) and Fraction 4 (d) of the β -LG heat product. The reference scale of a and b was 50 nm, and the reference scale of c and d was 100 nm.

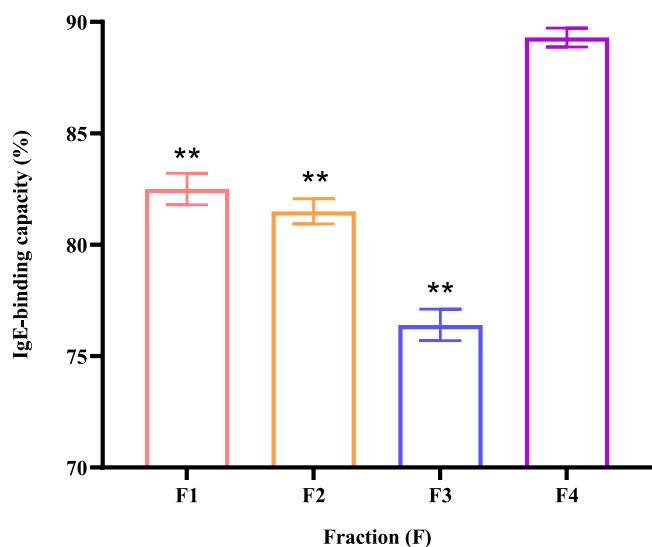


Fig. 4. The IgE-binding capacity of different AF4-collected fractions of the β -LG heat product (90 °C, 10 min) was estimated by indirect competitive ELISA. *P < 0.05, **P < 0.01, versus F4.

3.6. Effect of heat treatment on epitope peptides of β -LG

Through heat treatment, β -LG experienced dramatic changes reflected by changes in molecular properties and allergenicity (IgE-binding capacity) according to our above results. However, the reason for the change in allergenicity is still unclear. Epitope peptides are the binding

sites of allergens and are associated with allergenicity. Therefore, the effect of heat treatment on epitopes of β -LG was investigated in this study to explain the effect of heat treatment on allergenicity. The linear epitope peptides of β -LG were characterized by a previously described strategy (Li et al., 2019). First, digested peptides of β -LG were incubated with IgE before injection into AF4. In the incubation procedure, the peptide segments with specific antigen epitopes will bind with IgE *in vitro*. After separation by AF4, the combined peptides flowed from the outlet along with IgE, while unbound peptides were filtered and were eluted as waste due to their lower M values. The components of the outlet were collected, desalted and then analyzed by UPLC-Q-TOF-MS. Finally, five peptides as potential epitopes were characterized through this strategy. The chromatograms of characterized epitope peptides of β -LG (without heat treatment) are shown in Fig. 5a, and detailed information about those peptides is listed in Table 1.

The same characterization strategy was used to study the epitopes of the β -LG heat product. As a result, only two of five identified epitopes of β -LG were characterized as epitopes of the β -LG heat product. As shown in Fig. 5b, peptide 1 and peptide 5 were detected in correspondence to β -LG. Peptide 2 to peptide 4 were not detected. However, a new peptide at positions 1 to 8 (LIVTQTMK) was detected as a potential epitope of the β -LG heat product. This peptide (LIVTQTMK) has also been reported as an epitope peptide of β -LG (Järvinen et al., 2001; Sélo et al., 1999). Except for those three detected peptides, some new peptides with unclear amino acid sequences were present as potential epitopes of the β -LG heat product. Lee's study suggested that the loss of tertiary structure, S—S bond cleavage or interaction, and aggregation would be present under heat treatment (Lee, 1992). Therefore, thermal processing induces the loss of epitopes and the formation of new epitopes (Davis & Williams, 1998). This viewpoint about the change in the epitope

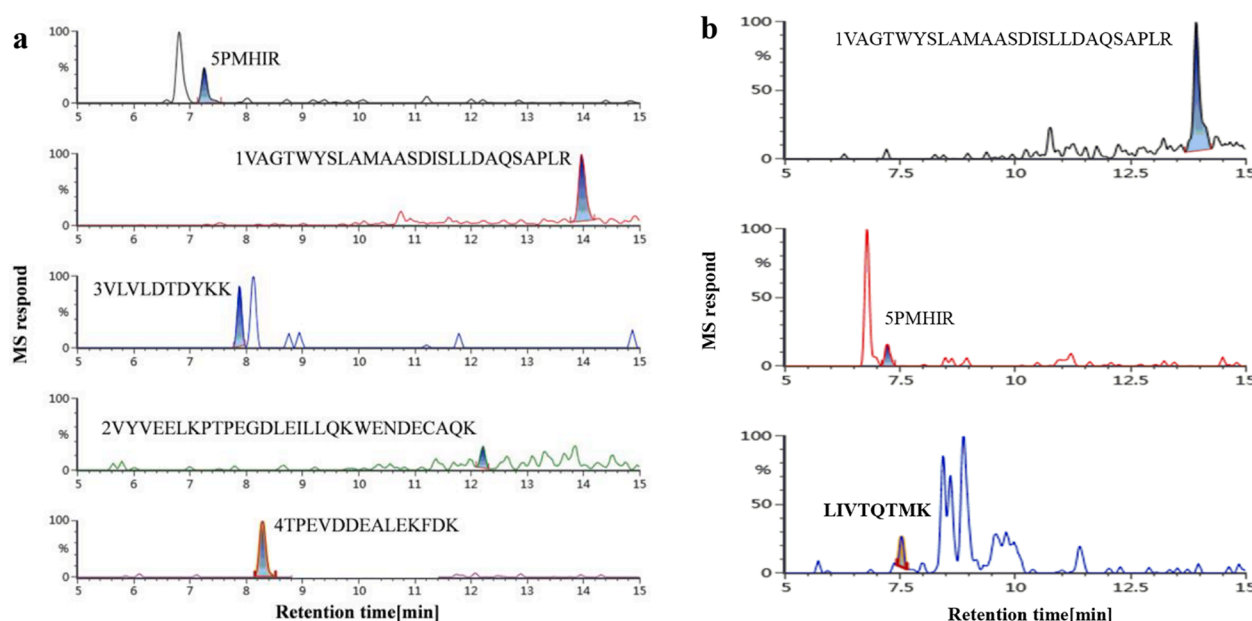


Fig. 5. Chromatograms of epitope peptides characterizing β -LG (a: without heat treatment) and its heat product (b: 90 °C, 10 min).

Table 1

β -LG peptide segments detected in combination with IgE by AF4-UPLC-Q-TOF-MS.

Number	Peptide position	Peptide sequence	Peptide length
1	15–40	VAGTWYSLAMAASDISLLDAQSAPLR	26
2	41–69	VYVEELKPTPEGDLLEILLQKWEDECAQK	29
3	92–101	VLVLDTDYKK	10
4	125–138	TPEVDDEALEKFDK	14
5	144–148	PMHIR	5

Note: Underlined parts in the peptides are identical to the reported epitopes.

induced by heat treatment was consistent with our results.

According to Selo's study, peptide 2 was a major epitope of native β -LG, which accounted for 10–15% of the total allergenicity of whole β -LG. While peptide 1, peptide 3 and peptide LIVTQTMK accounted for approximately 4% of the total allergenicity of whole native β -LG, peptide 4 showed very weak allergenicity (Séto et al., 1999). Generally, the loss of peptide 4 had little effect on the allergenicity of the β -LG heat product. However, the loss of peptide 2 and peptide 3 would seriously influence the allergenicity of the β -LG heat product. However, it was suggested that the *in vitro* IgE-binding capacity of the β -LG heat product was increased compared with that of β -LG according to our above result. It was speculated that the reason for the increase in the IgE-binding capacity of the β -LG heat product might be related to the new potential epitopes. According to the estimation by Unify software, the ratio of unknown peptides could reach 30% (Fig. S2). Amino acid sequences of those peptides are mainly focused on the part marked with purple underline in Fig. S2, which have a high degree of overlap with peptides 2–4 that cannot be characterized in the β -LG heat product. Regions of these amino acids were fragmented after heat treatment, and unknown peptides were produced. These newly produced peptides may have a different or even stronger antigenicity than the other peptides and will be further studied in our future research.

4. Conclusion

After heat treatment, the molecular properties and IgE-binding capacity of β -LG *in vitro* changed greatly. As shown from our results, β -LG without heat treatment exhibited a single peak, which would become

four fractions that exhibited different properties in terms of M, size, and shape through heat treatment (90 °C, 10 min). The product maintains its binding ability with IgE *in vitro* and was even higher than β -LG without heat treatment. All these fractions showed various degrees of IgE-binding capacity *in vitro*. The large aggregates (F4) with a high M, a large molecular size and an irregular shape had the highest IgE-binding capacity *in vitro*. It was suggested that the irregular shape due to aggregation and an increase in the binding site were potential causes of the enhancement of IgE-binding capacity. The characterized epitope peptide number of β -LG was decreased due to heat treatment. Some epitopes were damaged, while peptide LIVTQTMK and some new (unknown) peptides were observed, which may be attributed to the change in allergenicity and will be further studied in our future research. According to our results, a new possibility is proposed that fractions with high allergenicity could be properly separated and removed in manufacturing to reduce the occurrence of allergic reactions. In addition, fractions with relatively low allergenicity could be used in allergy vaccine research since they maintain the antigenicity of β -LG but may not cause a strong allergic effect in the future.

CRediT authorship contribution statement

Yang Li: Conceptualization, Experiment preparation, Data curation, Writing – original draft preparation. **Yunjia Yang:** Data curation, Methodology, Language polishing, Writing - review & editing. **Yue Zou:** Language polishing. **Lin Shu:** Language polishing. **Nan-yin Han:** Supervision, Guiding the experiment. **Yi Yang:** Supervision, Guiding the experiment, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2021.131748>.

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Supplementary Material

Characterization of the molecular properties and allergenicity (IgE-binding capacity) of β -lactoglobulin by heat treatment using asymmetric-flow field-flow fractionation and ultra-performance liquid chromatography quadrupole time of flight mass chromatography

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1. The method for the preparation of mouse-anti- β -LG-serum

Balb/c mice at 6-8 weeks old with the weight of 21 ± 1 g were obtained from the Jiangnan Experimental Animal Base of Abiocenter Biotechnology (Wuxi, China). Before dosing, mice were housed for 7 days in a barrier facility for laboratory animals under the environmental room (temperature: 25 ± 2 °C, humidity: 60 ± 5 %, 12/12 h light/dark cycle). Mice were allowed free access to food (soy-free custom diet) and water. All experiments were performed according to procedures approved by the animal care and using committee of Abiocenter Biotechnology Co., Ltd.

Mice were sensitized with β -LG (Sigma, L3908) by parenteral administration for antigen (β -LG) immunization. For the first immunization, 50 μ g antigen per mouse with the Freud complete adjuvant 1:1 mixed emulsification was given through multiple subcutaneous injection. On the fourth and thirty-two day, 50 μ g antigen per mouse with the Freud incomplete adjuvant 1:1 mixed emulsification was given through multiple subcutaneous injection to perform the second and third immunization, respectively. Three weeks after the third immunization, the titer of antibody was measured by the indirect ELISA method and the titer of antibody was 243000. Another week later, 50 μ g antigen per mouse with PBS 1:1 mixed was given through intraperitoneal injection to perform the stimulation before collecting of blood. Blood samples then were incubated for 1 h at room temperature and left overnight at 4 °C, then centrifuged for 20 min at 3000 g to obtain serum. The serum was stored at -20 °C until the following analyses.

2. The process of β -LG digestion

The digest produce was as following: 50 μ L β -LG solution (1 mg/mL in PBS) was mixed into 50 μ L ammonium bicarbonate solution (0.05 mol/L), 0.5 μ L DTT (1 mol/L) then was added and incubating last 1 h at 37 °C. 1.5 μ L IAA (1 mol/L) was added after the incubated solution was cooled down to room temperature and the solution was put at room temperature for 45 min avoiding of light. 1 μ L trypsin (1 mg/mL) then was added to perform digest through incubating overnight at 37 °C with gentle agitation. After incubation, the digested solution was cool down at 37 °C for 30 min to stop the digestion. After that, it was centrifugated at 14 000 r/min for 10 min, the upper solution was taken for the following step. The digest process of β -LG heat product was same as above process expect a heat treatment of β -LG solution described in section 2.2 was performed before the mixing with ammonium bicarbonate solution.

Supplementary Figures

Figure Captions

Fig. S1. Thermal stability of β -LG by dynamic light scattering

Fig. S2. Comparison chart of detected peptide amino acid sequences between β -LG and its heat product (Reference refer to β -LG digest solution; Unknown refer to those amino acids existed only in the digest solution of β -LG heat product; Common refer to the identical parts of digest solution between β -LG heat product with β -LG)

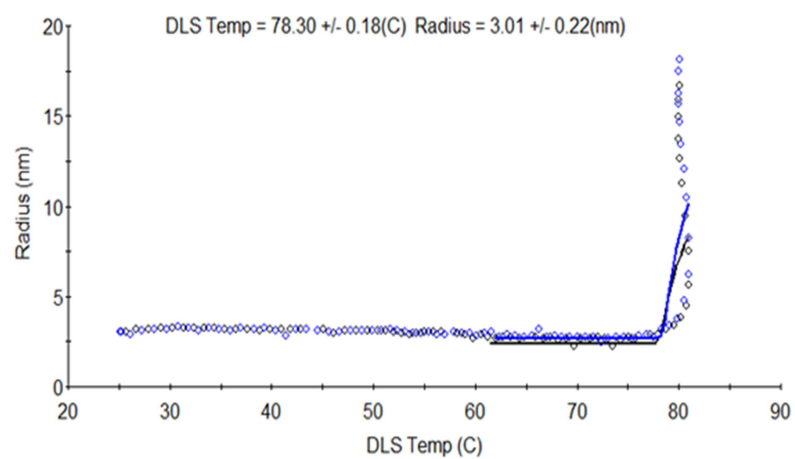


Fig. S1. Thermal stability of β -LG by dynamic light scattering

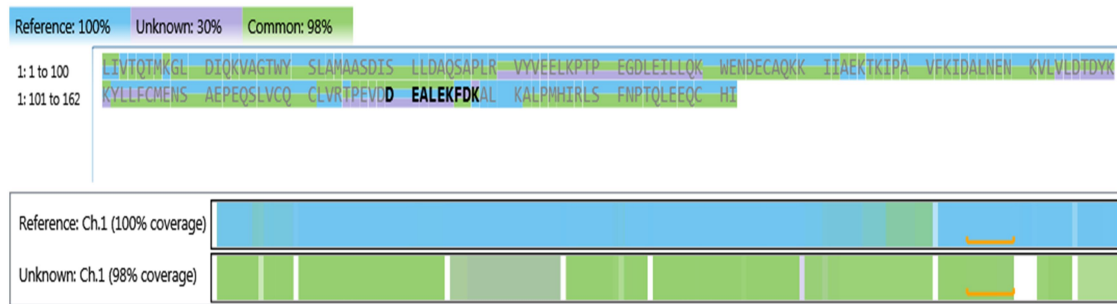


Fig. S2. Comparison chart of detected peptide amino acid sequences between β -LG and its heat product (Reference refer to β -LG digest solution; Unknown refer to those amino acids existed only in the digest solution of β -LG heat product; Common refer to the identical parts of digest solution between β -LG heat product with β -LG)