



Studying the effects of high pressure–temperature treatment on the structure and immunoreactivity of β -lactoglobulin using experimental and computational methods

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ABSTRACT

The effects of high hydrostatic pressure (HHP) on the conformation and immunoreactivity of bovine β -lactoglobulin (BLG) were studied. BLG was treated under 100–600 MPa at the temperature of 20–60 °C. The immunoglobulin E (IgE) binding ability of BLG decreased when the pressure increased from 0.1 to 200 MPa. However, the IgE binding increased with the increase in pressure from 200 to 400 MPa, followed by a gradual decrease until a pressure of 600 MPa. The IgE binding ability continuously decreased with an increase in pressure at 60 °C. The conformation of HHP-treated BLG was evaluated using fluorescence spectroscopy, circular dichroism spectroscopy and molecular dynamics (MD) simulation. Increasing the temperature and pressure promoted the unfolding of BLG, causing the disappearance of some α -helices and some β -sheets. Based on ELISA analysis, it was revealed that HHP-temperature treatment altered the immunoreactivity of BLG by altering the structures and conformational states of BLG.

1. Introduction

Bovine milk, which contains abundant proteins, is one of the commonly consumed foods, providing nutrition and energy for the body health (Kleber et al., 2007; Zhou et al., 2016). It is generally used to produce infant milk powder, which serves as a breast milk substitute or supplement. However, bovine milk allergy represents a common allergic disease in infants and young children (Chicon, López-Expósito, Belloque, Alonso, & López-Fandiño, 2008; Johnson et al., 2010; Leeb et al., 2011; Zhang et al., 2017). Milk has several types of proteins, e.g. α -lactalbumin, β -lactoglobulin (BLG) and casein, which result in the potential allergens (Bu, Luo, & Zheng, 2009; Costa et al., 2021; Euston, 2013; Johnson et al., 2010; Leeb et al., 2011). BLG, as the main fraction in whey protein, is one of the major allergen responsible for the bovine milk allergy.

The allergenic epitopes of proteins can be divided into conformational epitopes and linear epitopes (Kleber, Maier, & Hinrichs, 2007; Zhou, Wang, Ye, Chen, Tao, & Cao, 2016). The reactivity of the former one depends on the molecular conformation of the protein, while that of the latter mainly depends on the primary structure of the protein.

Currently, a number of physicochemical methods (heating, steam flash-explosion, high pressure processing) have been attempted to eliminate the allergenicity of BLG during food processing (Johnson et al., 2010; Elena, Ulrich, & Seronei, 2011; Zhang, Yang, Zhang, Hu, & Zhao, 2017). Generally, thermal treatment is regarded as a powerful tool to modify proteins and to prevent antigen epitopes from being recognized by immunoglobulins (Bu et al., 2009; Euston, 2013). However, this strategy often results in a loss of food quality. It has been reported that glycation can give rise to different impacts on allergens, increasing the IgE-binding capacity or causing effective desensitization (Costa et al., 2021; Yuan et al., 2018). However, chemical modifications have been reported to result in byproducts, such as advanced glycation end products, which contribute to lesion formations in the body (Rabbani & Thornalley, 2018).

Enzymatic hydrolysis of proteins reduces the allergenicity of BLG. However, the unique dimer structure of BLG is resistant to both acid and enzymatic hydrolysis (Peñas et al., 2006). The applications of high hydrostatic pressure (HHP) treatment, an emerging nonthermal processing technology, could alter the immunoreactivity of proteins by affecting the intermolecular forces and by changing the structure of these

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molecules (Bamdad, Bark, Kwon, Suh, & Sunwoo, 2018; Costa et al., 2021; Faisala, Buckowb, Vasiljevica, & Donkor, 2019; Kato, Katayama, Matsubara, Omi, & Matsuda, 2000; Somkuti & Smeller, 2013).

It has been reported that the structural changes of BLG caused by HHP result in potential allergic response (López-Expósito, Chicón, Bel-loque, López-Fandino, & Berin, 2012; Meng, Bai, Gao, Li, & Chen, 2017; Russo et al., 2013). The structural transformation of allergenic epitopes is the apparent primary cause of a change in the allergenicity of BLG (Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001). Thus, the conformational state of the BLG molecule is critical for understanding the allergic response induced by BLG. Recently, published literature has highlighted the impact of temperature in emerging technologies aiming to decrease immunoreactive properties of BLG, e.g. ohmic heating, electric fields and HHP (Chizoba Ekezie, Cheng, & Sun, 2018; Pereira et al., 2021). Generally, heat stability (upon extreme heat conditions) contributes to the increase in the allergenicity of tropomyosins and preserve the allergenicity of parvalbumins, caseins and serum albumins, but decrease in the allergenicity for proteins of the ovomucoid family, e.g. Gal d 1 (Costa et al., 2021). According to the report by Iametti et al., the allergenic epitopes of BLG were exposed after thermal treatment at 55–65 °C, which increased the allergenicity of BLG (Iametti et al., 1997). Structural changes (unfolding, destruction of conformational epitopes, and formation of aggregates) enables to reduce the IgE-binding capacity of arginine kinases, transferrins, lipocalins and serpins (Costa et al., 2021). However, the combinational effects of HHP and heat treatments on BLG have not still been completely understood because of the structural changes of the proteins after depressurization.

To date, the causal relationships between physicochemical properties and the basis of BLG allergenicity has not been explained at molecular level. Therefore, this work aims to study the effects of HHP-heating treatment on the immunoreactivity and structure of BLG, as well as the possible mechanism. The indirect competitive enzyme-linked immunosorbent assay was used to evaluate the immunoreactivity of BLG. Molecular dynamics simulation (MD) is a powerful tool to study the state of molecules, for example, the conformational states of BLG during unfolding. In this study, the combination of simulation and experimental methods such as circular dichroism (CD) spectroscopy and fluorescence spectroscopy, was utilized for understanding the change in the immunoreactivity of BLG. The result of this study may provide important information on the change in the allergenicity of BLG.

2. Materials and methods

2.1. Material

The pure BLG and 8-anilino-1-naphthalene sulfonate (ANS) were purchased from Sigma-Aldrich (Shanghai, China). The ELISA kit was from Sinobestbio Co., Ltd. (Shanghai, China). The protein marker in denaturing polyacrylamide gel electrophoresis was from Solarbio Science and Technology Co., Ltd. (Beijing, China). The native protein marker was from Thermo Fisher Scientific Inc. (MA, USA). All other chemicals and reagents were of analytical grade and purchased from Sinopharm (Shanghai, China).

2.2. High hydrostatic pressure (HHP) treatment

The HHP treatment was conducted with a pressurization rate of 10 MPa/s using high pressure device (MICRO FOODLAB FPG5740, Standard Fluid Power Ltd., Harlow, UK) with a temperature control system (0–100 °C). Before the treatment the high pressure chamber was pre-heated to the target temperature. The BLG was diluted properly using 25 mmol/L phosphate potassium buffer (pH 7.5). The protein solution (1 mg/mL) was filled in a 5-mL polypropylene tube, loaded into the chamber and treated under various pressures (0.1–600 MPa) for 25 min. After treatment, samples were rapidly cooled by liquid nitrogen and stored at –20 °C for analysis.

2.3. Circular dichroism (CD) spectrum determination

The secondary structure of BLG was investigated using CD spectrometry (Bio-Logic MOS-450, France) (Chen et al., 2018). All the CD measurements were carried out at 25 °C. Before measurement, the protein solution was diluted to 0.1 mg/L. For the far-UV CD spectra determination, each spectrum was the average of three independent replicates.

2.4. Intrinsic fluorescence spectra of BLG

The intrinsic fluorescence spectrum was determined using a spectrofluorimeter (Cary Eclipse1260, Agilent Technologies, Inc. Chandler, AZ, USA) at 25 °C with a 1 cm path length cell. The protein fluorescence was excited at 295 nm and the emission spectra were recorded from 300 to 450 nm. The emission and excitation slit were fixed at 5 nm, respectively. The intrinsic fluorescence plots of BLG were scanned at least three times. The protein concentration was 0.1 mg/mL in the measurement. All measurements were carried out immediately after HHP treatment.

2.5. Extrinsic fluorescence spectra of BLG

The extrinsic fluorescence spectra were measured using the extrinsic fluorescence probe (ANS). A stock solution of ANS was prepared with a concentration of 4 mmol/L. The ratio of protein solution to ANS was 50:1 (v/v). The excitation wavelength was fixed at 380 nm and the emission spectra were gathered in the range of 400–600 nm. The extrinsic fluorescence was scanned at least three independent times. The protein concentration was 0.1 mg/mL in the measurements.

2.6. Molecular dynamics simulation

The crystal structure of BLG was obtained from PDB bank (Protein Data Bank ID: 3NPO). The GROMACS software (version 5.0) was used to run molecular dynamics simulation with G43a1 force field and the protein was solvated by SPC water (Berendsen, van der Spoel, & van Drunen, 1995; Chen et al., 2019). The system was neutralized by adding counter ions in the form of 8Na⁺ ions. Energy minimization was done by steepest descents method (Mukund et al., 2019). The system was equilibrated for 400 ps under NVT and NPT conditions before an all-atom molecular dynamics simulation for 400 ns. All bonds in the system were constrained by LINCS algorithm. The system was weakly coupled to an external bath by Bredsen's methods. The pressure coupling was respectively performed for desired pressures (0.1, 200, and 600 MPa) through isotropic Parrinello-Rahman protocol. The unfolding process of protein could be accelerated by high temperature. In order to study the effects of HHP on BLG structure at different temperatures, periodic boundary conditions were applied to all simulations at 20, 60 and 100 °C, respectively.

2.7. Gel electrophoresis of the protein

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and native-polyacrylamide gel electrophoresis (Native-PAGE) were carried out through a Mini-Protean Tetra system (Bio-Rad Laboratories, Inc, Richmond, CA, USA). SDS-PAGE was applied to evaluate protein composition according to the method described by Laemmli (1970), which confirmed that the BLG is pure. In the analysis, a 4% stacking gel and 12% separating gel under both reducing and non-reducing conditions were prepared in the presence and absence of β-mercaptoethanol, respectively. Both the gels and electrode buffer contained 0.1% SDS for SDS-PAGE, which differs from their compositions in native-PAGE. Protein samples were mixed with a reducing sample buffer [0.1 M Tris–HCl, pH 6.8; 20% (v/v) glycerol, 2% (v/v) SDS 5%, (v/v) β-mercaptoethanol and 0.01% bromophenol blue] at a

volume ratio of 2:1, and β -mercaptoethanol was not induced in the nonreducing sample buffer. Native-PAGE was performed according to the method described by Lin, Sun, Cao, Cao, & Jiang (2010) with some modifications. Native gel electrophoresis was performed with a 12% polyacrylamide running gel and a 4% polyacrylamide stacking gel. The protein samples for electrophoresis were prepared before and after treatment. Electrophoresis for native -PAGE was carried out at 120 V with $1 \times$ Tris-glycine electrophoresis buffer (50 mmol/L, pH 8.0) without SDS. While electrophoresis for SDS-PAGE was carried out at 120 V with $1 \times$ Tris-Glycine electrophoresis buffer (25 mmol/L, pH 8.0) containing SDS (0.1%, w/w). After electrophoresis, the gels were stained with Coomassie brilliant blue R-250 and treated with a de-staining solution.

2.8. Sandwich enzyme-linked immunosorbent assay (ELISA)

An indirect ELISA was applied to estimate the immunoreactivity of the samples according to the methods described by Kleber, Maier, & Hinrichs (2007) with some modifications. To evaluate the content of BLG-IgE in the sample, polystyrene microtiter plates were used as solid supports. Single wells were coated with 50 μ L of BLG-IgE in 0.02 mol/L phosphate buffer (pH 7.5). The plates were washed 3 times with PBS containing 0.05% Tween 20. Each sample was added in triplicate to microplate wells that had been precoated with BLG-specific IgE. 50 μ L of each diluted samples (0.1 mg/mL) was added to a sample well. Each well was blocked with 100 μ L of horseradish peroxidase-conjugated rabbit BLG-specific IgE (diluted 1:1000 in PBS containing 0.05% Tween 20) and covered with an adhesive strip and then the plates were incubated for 1 h at 37 °C. After the reaction, each well was immediately aspirated and washed three times. The commercial chromogen solution containing 0.15% of tetramethylbenzidine (Sinobestbio Co., Ltd., Shanghai, China) of 100 μ L was then added to each well, followed by a gentle mixing and incubation at 37 °C for 15 min before termination by the addition of 50 μ L of stop solution (H_2SO_4 , 2 mol/L) to each well. After the termination of chromogenic reaction, the content of BLG-IgE in samples was estimated by the absorbance at 450 nm using an ELISA plate reader (Thermo Fisher Scientific, MA, USA).

2.9. Statistical analysis

All the experiments were replicated at least three times and the values were presented by the mean value $\pm$ standard error. The plots were prepared through Excel 2013. The statistical significance analysis in this work was conducted using SPSS program (version 13.0, SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1. Conformational change of BLG

3.1.1. Secondary structure of BLG determined by far-UV CD

The effect of HHP on the secondary structure of BLG at different temperatures was evaluated through far-UV CD spectra. Fig. 1 displays the effects of HHP on BLG at 40 and 60 °C. It shows that the secondary structure was not altered under the pressure of 100 MPa at 40 °C, which is suggested by the overlapping lines representing the spectra 0.1 and 100 MPa in CD plot. According to the report of Russo et al. (2013), BLG likely does not undergo significant unfolding under high pressures below 150 MPa. However, the secondary structure begins to dissolve as the pressure is increased to 200 MPa (Fig. 2a). For example, the intensity of the negative peak at 208 nm became weaker, which suggests the loss of the secondary structure (Loupjac, Bonetti, Pin, & Calmettes, 2006). Similarly, Huppertz and coauthors observed that 5% of BLG was denatured at a pressure of 200 MPa for 30 min, while more than 45% of BLG was denatured under a pressure of 400 MPa (Huppertz, Fox, & Kelly, 2004).

The intensity of negative peaks (at 208 and 222 nm) of the CD plot appeared to be marginal under a pressure of 600 MPa at 40 °C, although the characteristic negative peak at 208 corresponding to the α -helical structure was retained in the CD spectrum (Chen et al., 2018). Similarly, the peak at 216 nm characterizing β -sheets, is retained in the CD curves with the increase in pressure. The results indicate that there are residual α -helices and sheets in the BLG structure after HHP treatment (Huppertz, Fox, & Kelly, 2004; Meng, Bai, Gao, Li, & Chen, 2017). It was documented that 61% of the BLG was denatured under 600 MPa for 10 min (Kleber, Maier, & Hinrichs). The change in the secondary structure of BLG with increasing pressure at 20 °C shows a pattern similar to that at 40 °C (cf. supporting information). It was reported that more than 60% of BLG was denatured by the application of a pressure of 600 MPa at 20 °C (Huppertz, Fox, & Kelly, 2004). However, the effect of temperature on the BLG structure was weak under the same pressures. It was reported that the 5% BLG solution (w/w) was denatured at 80 °C other than 37 °C (Elena et al., 2011).

The pressure levels in the CD plots at 60 °C shown in Fig. 1b are different from those at 40 °C. As shown in Fig. 1b, the peaks at 218, 216, and 206 nm became unremarkable after the treatments, which implies the unfolding of the secondary structure at 0.1 MPa and 60 °C. It should be attributed to that high temperature enables to disrupt the ordered structure of proteins (Costa et al., 2021; Pereira et al., 2021). In addition, the unfolding of BLG was not promoted by treatment with low pressures (Considine, Patel, Anema, Singh, & Creamer, 2007). In contrast to this observation, disruption of the secondary structure of BLG was promoted by the combination of the heat treatment with HHP, indicating the synergistic effect of HHP and temperature on the protein structure. However, the secondary structure of BLG, i.e., a partial helical structure and sheet structure, was preserved under 600 MPa at 60 °C, as suggested

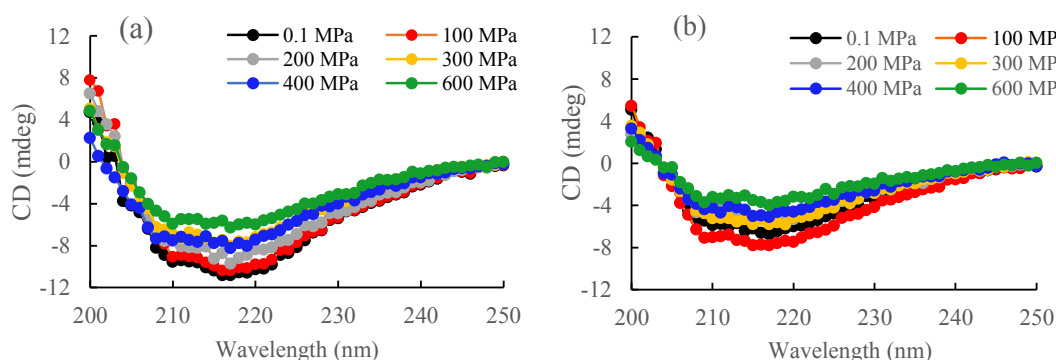


Fig. 1. Changes in CD spectra of BLG under various pressures at 40 (a) and 60 °C (b), respectively.

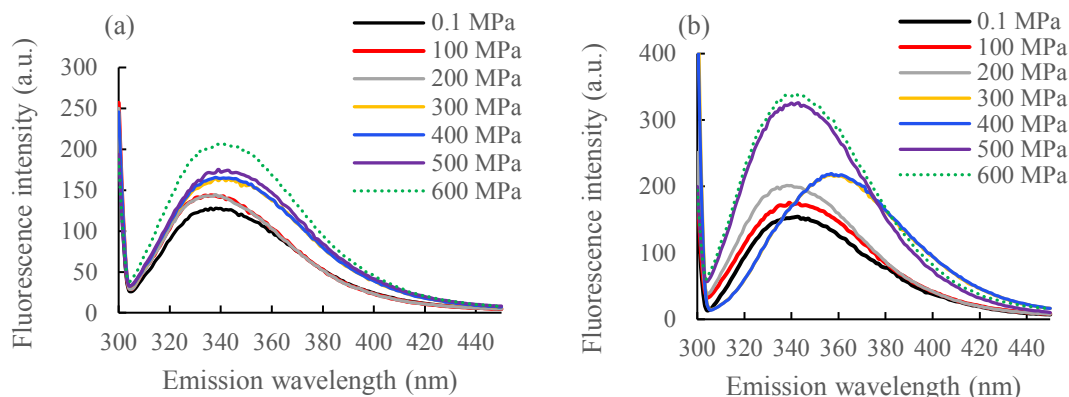


Fig. 2. Intrinsic fluorescence evolution of BLG before and after HHP treatments at 40 (a) and 60 °C (b), respectively.

by the negative peaks at 206 and 216 nm. The result is similar to the observations at 20 and 40 °C under 600 MPa, indicating a residual secondary structure of BLG after treatments. The unfolding of BLG upon heating or high-pressure treatment may make β -lactoglobulin more susceptible to proteases, by exposing the hydrolysis site of the protein (Elena et al., 2011; Zeece, Huppertz, & Kelly, 2008). It has been documented that thermal processing causes a change in conformational epitopes (Peyron, Mouécoucou, Frémont, Sanchez, & Gontard, 2006). While, it has been reported that some linear epitopes could not be changed by HHP treatment (Peñas et al., 2006). Therefore, the conformation caused by HHP and heating treatment may alter the immunoreactivity of BLG (Costa et al., 2021).

3.1.2. Intrinsic fluorescence of BLG

To study the BLG conformation, the tryptophan (Trp) fluorescence of BLG was measured, and the results are shown in Fig. 2. The intrinsic fluorescence intensity of BLG increases with the elevation of pressure, indicating that the conformation of the local region around Trp residues was altered by the HHP treatment. Additionally, the maximal emission wavelength shifted to a longer wavelength. For example, the wavelength showed a shift from 337 nm to 343 nm, when the pressure increases from 0.1 to 500 MPa. However, the intensity increases from 171 to 204 a.u. at 600 MPa, a finding consistent with the results reported by other researchers (Meng, Bai, Gao, Li, & Chen, 2017). The result indicates that the surroundings of Trp residues becomes more polar. This conformational change indicated by intrinsic fluorescence is consistent with the change in secondary structure of BLG.

The pressure effects on the intrinsic fluorescence of BLG at 60 °C are shown in Fig. 2b, which clearly displays that the fluorescence intensity at 60 °C is higher than that under the same pressure at 40 °C. BLG began unfolding under a thermal treatment at 60 °C. Together with thermal treatment, high pressure synergistically caused the unfolding of the BLG molecules, and the similar effect may be observed for other proteins (Chen et al., 2019; Kobayashi, Otake, Kobayashi, & Sakurai, 2016). The maximal emission wavelength shifted from 337 nm to 358 nm as the pressure increased to 300 and 400 MPa. However, a shift of wavelength from 358 to 340 nm was observed when the pressure increased from 500 to 600 MPa. These results suggest that the local region around Trp residues became less polar at pressures over 400 MPa. It has been confirmed that HHP treatment can cause intermolecular cross-bonding or aggregation through the transformation of the thiol groups (Considine, Patel, Anema, Singh, & Creamer, 2007; Russo et al., 2013). The shift of the maximal emission wavelength is likely attributed to the dramatic conformational change and aggregation of BLG after HHP treatment.

3.1.3. Extrinsic fluorescence analysis

ANS is a common chemical that specifically binds to the hydrophobic

regions of proteins producing extrinsic fluorescence. The increase in extrinsic fluorescence generally indicates the exposure of previously buried hydrophobic regions to the environments or the loosening of protein folding (Chen et al., 2019). Thus, the fluorescence property of proteins is a unique property that allows us to probe the conformational changes of proteins caused by physical and chemical treatments. In this study, ANS was used to evaluate the effects of treatments on BLG.

As shown in Fig. 3, the fluorescence intensity of the BLG treated by the pressure of 0.1 MPa treatment at 40 °C showed little change compared to that of the native BLG. However, the fluorescence intensity increased remarkably with increasing pressure, which implies that some hydrophobic regions were exposed to the surroundings. The thermal treatment also enhanced the fluorescence intensity. For example, the fluorescence intensity of BLG under 0.1 MPa at 60 °C was higher than that at 40 °C. The same pattern was observed after treatment within a pressure range of 200–600 MPa, at 60 °C. However, the fluorescence intensity was at the same levels after treatment with pressures of 500 and 600 MPa at both temperatures. This outcome may be attributed to the fact that the increase in the pressure did not cause additional unfolding of the structure of BLG (Chen et al., 2019). A similar observation was also reported in other research works (Considine, Patel, Anema, Singh, & Creamer, 2007; Meng, Bai, Gao, Li, & Chen, 2017). The increasing fluorescence intensity suggests structural changes of BLG, which is in agreement with the CD results and the intrinsic fluorescence results.

3.2. Structural changes of BLG revealed by MD simulation

To evaluate the changes to local regions of the BLG subunit, which has 162 amino acid residues, we performed MD simulations at temperatures as high as 100 °C (Fig. S2). BLG has eight strands (A, B, C, D, E, F, G and H). Strands A, B, C, E, G and H, are related to the IgE binding activity of BLG (Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001). In addition, there is one three-turn helix (Asp129-Ala142) along strands F, G and H. BLG has two disulfide bonds and one free Cys (Cys121), which is covered by the α -helix (Fig. 4).

To clarify using conformational difference of the BLG structure, the BLG structure generated using MD simulation after different treatments were overlapped with the initial structure (Fig. 4). The structure of BLG changed slightly at 20 °C under 600 MPa when the MD simulation was run for 200 ns (cf. supporting information). However, the secondary structure was well preserved with the partial disappearance (Leu140-Ala142) and displacement of α -helix (Asp129-Ala142) 400 ns (Fig. 5). Therefore, the average structure generated in the final 10 ns of the simulation was used to estimate the conformational changes of BLG. As shown in Fig. 4, the ordered helical structure gradually disappeared with the increase in temperature from 20 °C to 100 °C at 0.1 MPa. In addition to the loss of the helical structure, the β -sheet was also partially

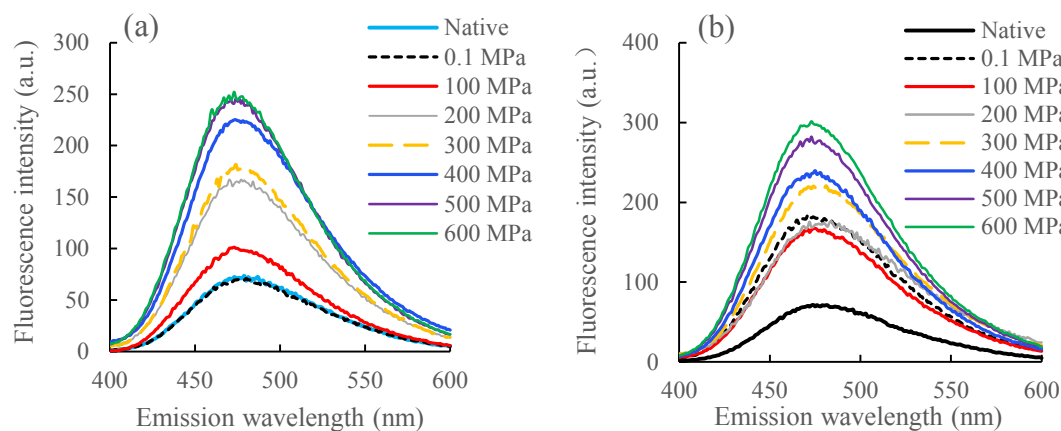


Fig. 3. Extrinsic fluorescence Changes of BLG before and after HHP treatments at 40 (a) and 60 °C (b), respectively.

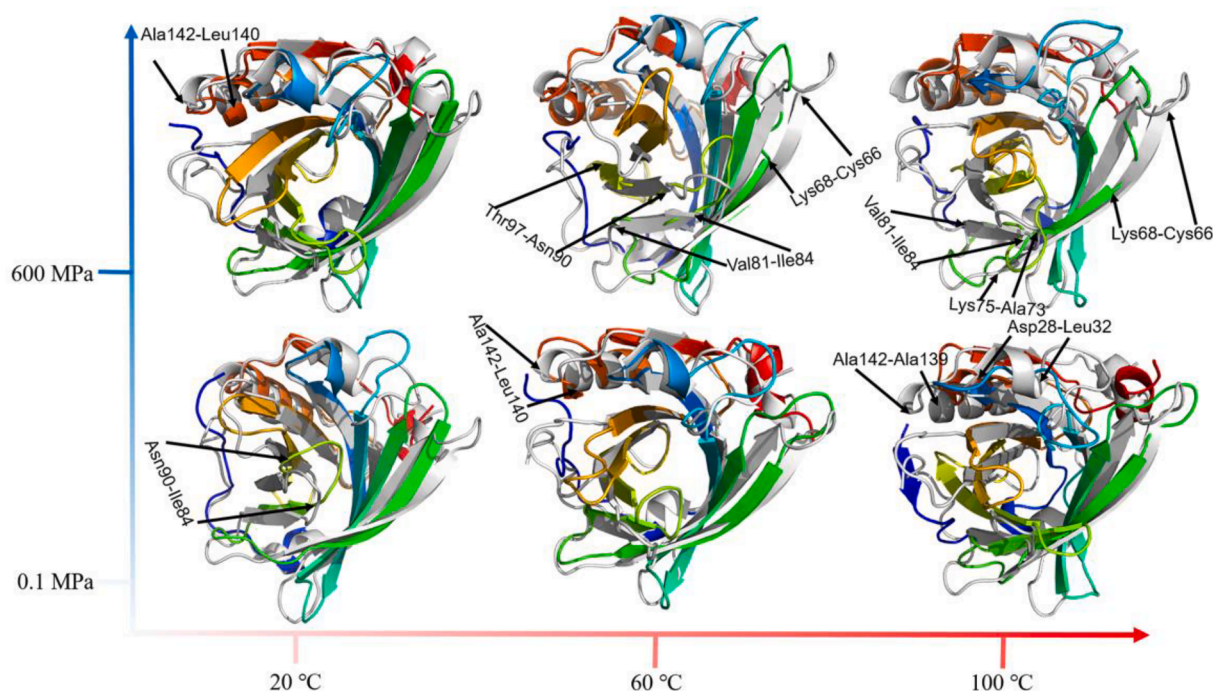


Fig. 4. The conformational change of BLG under different pressures revealed through MD simulation. The structure colored by gray is the initial structure of BLG while the structure colored by rainbow is the structures under different conditions.

disrupted. For example, together with the transition of the helix of Asp28-Leu32 to a random coil, the β -sheets Leu54-Trp61 and Cys66-Lys75 colored by green, became shorter at 100 °C. The structural disruption of the protein induced by thermal treatment is consistent with the result documented by Euston (2013), who found only small changes to the tertiary fold and secondary structure in a MD simulation at 77 °C for 110 ns.

Similarly, the loss of ordered structure was also observed under 600 MPa, which became more significant at 60 °C and 100 °C. Together with the above mentioned partial disappearance of the β -sheet (Leu54-Trp61, Cys66-Lys75) in the protein molecule, the β -sheets (Asn90-Thr97 and Cys66-Lys75) became much shorter at 60 °C under 600 MPa compared to the initial structure. In addition to these observations, the β -sheet of Val81-Ile84 completely disappeared, transforming from a β -sheet into a random coil at 60 °C and 100 °C under 600 MPa. As the temperature increased to 100 °C, more than one-half of the sheet (Cys66-Lys75) disappeared, including peptides Ala73-Lys75 and Cys66-Lys68. The results indicate that the combination of high pressure and high

temperature acted on BLG and disrupted the core of this protein. It is assumed that intermolecular exchanges occur between free thiol groups of Cys residues and disulfide bonds in BLG molecules (Belloque, López-Fandiño, & Smith, 2000; Creamer et al., 2004). Therefore, the conformational changes caused by these treatments may induce the dissociation and association of BLG molecules through Cys residues. The helix (residues153-156) and the helical turn (residues159-161) were easily disrupted by the pressure and temperature treatments (cf. supporting information). It is inferred that Cys66-Cys160 bond may be broken and reformed with the thiol group of other Cys residues, thus contributing to intermolecular association.

There are seven IgE-binding epitopes on BLG, and they include the residues 1–16, 31–48, 49–60, 67–78, 75–86 and 129–144 (Järvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001). The binding of IgE to these epitopes (residues 67–78, 75–86 and 129–144) was influenced by the treatments at 60 °C and 100 °C. These peptide bonds were broken by the treatments, which disrupted the interaction between IgE and the epitopes (Zhong et al., 2011). In addition, the conformation, particularly

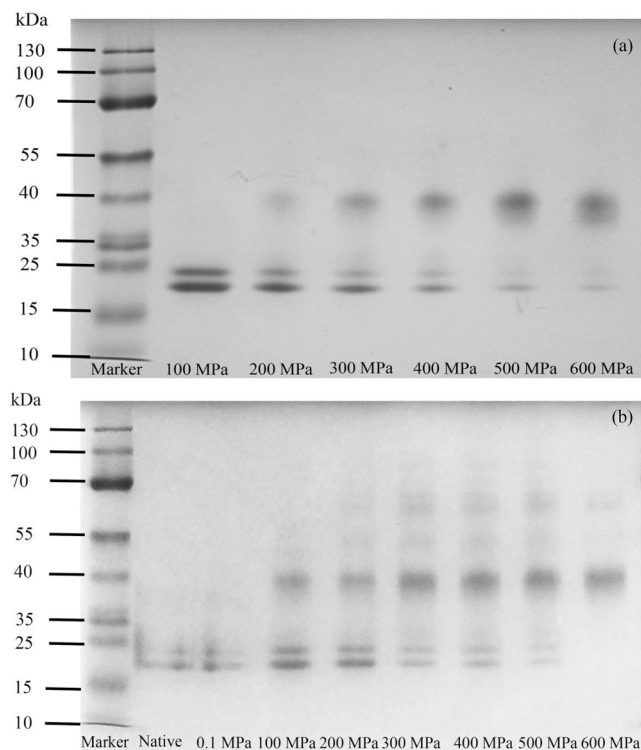


Fig. 5. Native-PAGE of BLG before and after HHP treatments at 40 °C (a) and 60 °C (b), respectively. The untreated BLG protein (Native) was regarded as the control.

the “barrel” shape of BLG, was changed when the linker (residues Ile84-Asn90) moved toward the interior. Thus, the conformational states of the epitopes in this region were thus modified with the changes in tertiary fold and secondary structure of BLG. In addition, some residues in the epitopes displayed a significant conformational displacement (cf. [supporting information](#)). For example, the side chain of Lys75 and Phe82 moved away from their initial positions. The conformational change of proteins contributes to the allergenic responses. Thus, it is likely that the HHP treatments at high temperature altered the allergenicity of BLG (Kleber, Krause, Illgner, & Hinrichs, 2004).

3.3. Gel electrophoresis analysis

To estimate the influence of HHP on the dissociation/aggregation of BLG, SDS-PAGE and native-PAGE were performed. As indicated by the SDS-PAGE (cf. [supporting information](#)), BLG subjected to various conditions (0.1–600 MPa) displayed a single band between 14.4 and 22 kDa, which is consistent with the molecular weight of the BLG subunit (18 kDa). This result indicates that the BLG was pure. Untreated BLG was used as a control and the native electropherogram of BLG before and after treatment is shown in [Fig. 5](#).

The monomer and dimer of BLG are known to be in equilibrium (Bello, Fragoso-Vázquez, & Basurto, 2016). The bands of the control sample indicate that native BLG exists in two forms in solution (pH 7.5), a monomer and dimer. In addition, the dimer constitute a very small part of these forms. The native-PAGE patterns showed that the proportion of the dimer was not enhanced by pressures applied within the range of 0–100 MPa at 40 °C. This result is supported by the secondary and tertiary structure of the protein under the same conditions. The protein structure was difficult to be perturbed by relatively low pressures at moderate temperatures (Meng, Bai, Gao, Li, & Chen, 2017). However, the proportion of the dimer significantly increased with increasing pressure. For example, the intensity of the band corresponding to the low-molecular-weight form (18 kDa) grew gradually weaker with the

increase in pressure, whereas that corresponding to the high-molecular-weight form (36 kDa) became more visible. In particular, the band corresponding to the low molecular weight was almost invisible after the treatment under 600 MPa. This observation indicates that the pressure treatment changed the conformational form of the protein in solution. In contrast to the molecular weight distribution at 40 °C, the number of bands increased significantly under pressure of 100–600 MPa at 60 °C suggesting the formation of multimers of this protein. This result implies that the heating treatment promotes the unfolding and refolding of BLG. Additionally, together with pressure treatment, thermal treatment induced the dissociation and association of the BLG monomer/dimer after treatment. However, the multimers were mainly composed of dimers under 600 MPa at 60 °C, which was attributed to the exposure of specific charged groups, such as free thiol groups that result in specific association between monomers (Considine, Patel, Anema, Singh, & Creamer, 2007). The formation of multimers or aggregates after treatment will influence the allergenicity of BLG by hiding the epitopes on the protein molecule surface.

3.4. Effects of HHP on the immunoreactivity of BLG

In order to evaluate the effect of pressure at different temperatures on the immunoreactivity of BLG, an indirect ELISA assay was performed. As shown in [Fig. 6](#), the OD value of HHP-treated BLG fluctuated with HHP at 20 °C revealing a profile similar to that obtained at 40 °C. The OD value at 450 nm was lower for the samples treated with pressures of 100–200 MPa compared to the untreated BLG, which suggests the lower IgE binding affinity after treatments. The OD value decreased from 0.39 to 0.32 and 0.30 as the pressure increased from 0.1 MPa to 200 MPa at 20 °C and 40 °C, respectively. The reduced IgE binding ability was attributed to the loss of secondary of BLG by pressure treatment, which disrupted the conformational epitope (Costa et al., 2021). However, the OD value indicated that the binding of IgE to the protein gradually increased when the pressure increased from 200 MPa to 400 MPa. The increase in IgE binding of BLG under 400 MPa has also been observed in other studies (Meng, Bai, Gao, Li, & Chen, 2017). It may be attributed to that an incomplete unfolding and conformational rearrangement of BLG caused by mild treatments result in a transient increased IgE-binding capacity (Costa et al., 2021). Interestingly, the IgE binding capacity was reduced after treatment with pressures higher than 500 MPa. The displayed fluctuation of OD may be primarily attributed to the marked unfolding and disruption of the BLG structure, which caused the exposure of some hidden IgE-binding epitopes. This observation supports the view that the degree of denaturation does not correlate with the allergenicity of BLG (Kleber, Maier, & Hinrichs, 2007).

As the temperature increased to 60 °C, the OD value changed slightly at 0.1 MPa. In contrast to the changes observed at temperatures below 60 °C, the OD value decreased with increasing pressure from 100 MPa to

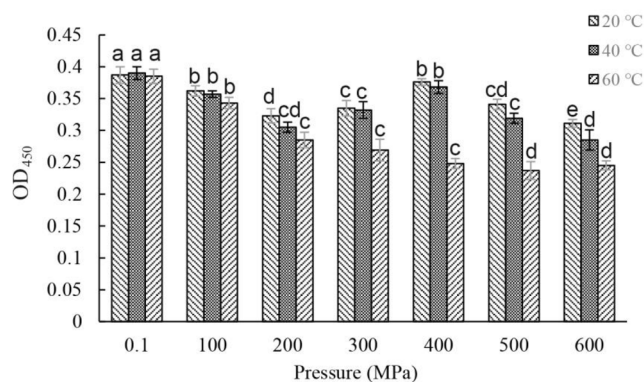


Fig. 6. The OD value at 450 nm of IgE-BLG complex after HHP treatment (* $P < 0.05$).

600 MPa. The decrease in the amount of IgE-binding to BLG was mainly caused by the formation of BLG multimers and the aggregation of BLG molecules caused by the HHP treatment at 60 °C. According to other reports, the potential allergenicity of BLG can be remarkably reduced after HHP treatment under 600 MPa at high temperature, such as at 75–90 °C for 6 min (Orcajo, Mara  n, & Lavilla, 2015; Sathe, Teuber, & Roux, 2005; Xu, Shi, Yao, Jiang, & Luo, 2016). The decrease in immunoreactivity of BLG should be also attributed to the consequence of heat treatments, e.g. the loss of tertiary/secondary structures and destruction of conformational epitopes, or the formation of protein aggregates (Costa et al., 2021). However, one study described the opposite observation that the allergenicity of BLG in a mixture was enhanced by thermal treatment for 10 and 30 min at 60 °C. This different outcome may be due to that the pure BLG was obtained using different preparation methods.

Most IgE epitopes have been identified to be specific for the surface features of a protein (Kleber, Maier, & Hinrichs, 2007). Further, MD simulations have also showed that some IgE epitopes can be modified by HHP treatment. In addition, it is notable that some epitopes are near Cys residues (Cys66 and Cys121) that play critical roles in the associating the subunits of the BLG protein. The observed results may be attributed to the aggregation of the protein, which buries the surface peptides (Kleber, Maier, & Hinrichs, 2007; Xu, Shi, Yao, Jiang, & Luo, 2016). This observation is supported by the increasing intensity of the bands representing the higher-molecular-weight protein in the native-PAGE results at 60 °C. These results indicate that changes in the conformation of protein enable to alter the immunoreactivity of the protein, either by enhancing or reducing it (Costa et al., 2021).

4. Conclusions

This work describes the conformational states and immunoreactivity of BLG observed under HHP treatment. The secondary structures of BLG changed slightly at relatively low pressures and temperature. However, increasing the temperature and pressure promoted the significant unfolding of the protein, which changed the state of IgE binding epitopes within the protein. Compared to the case for untreated BLG, the IgE binding ability of BLG fluctuated with the pressure at temperature below 40 °C. However, the IgE binding ability of BLG decreased notably at 60 °C. The results of spectroscopic analysis and MD simulation revealed that HHP treatment influenced the tertiary structure and potential binding sites of BLG. Specifically, the aggregation of BLG causes the exposed epitopes in these molecules to be hidden, thereby reducing their immunoreactivity. However, these results were only obtained from *in vitro* experiments, and the further *in vivo* investigations are required to be performed to confirm whether HHP-treated BLG elicits notably reduced the allergic responses.

CRedit authorship contribution statement

Gang Chen;Chenyu Wu;Xiaojie Chen;Zhennai Yang;Huqing Yang:.

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.2021.131226>.

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Supporting Information

Fig. S1 Secondary structural changes of BLG subjected to HHP treatments at 20 °C.

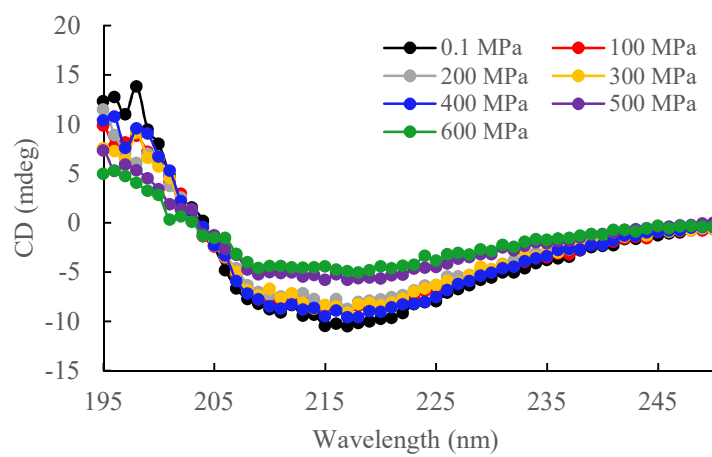


Fig. S2 The structure of BLG in monomeric state. (a) represents the structure shown with code of secondary structural elements and (b) represents the structure indicated by amino acid.

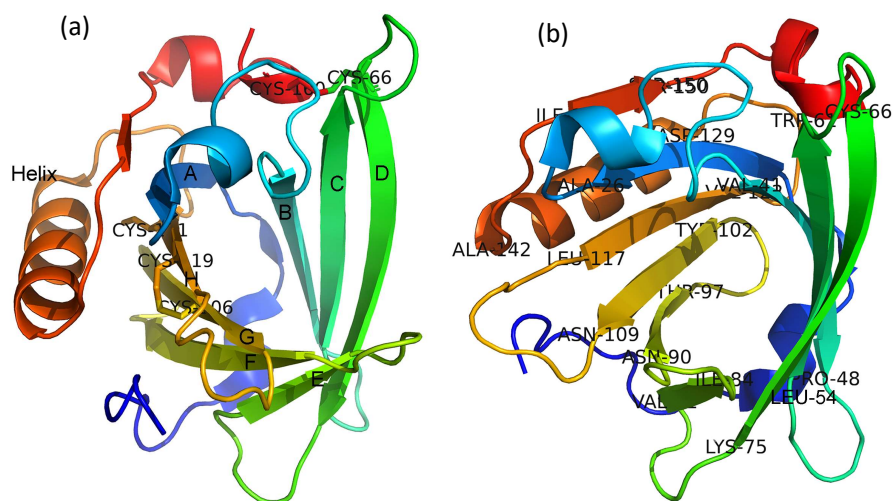


Fig. S3 Snaps of BLG under 600 MPa at 20 °C in the MD simulation for 200 ns (a) and 400 ns (b). The white and the colored represents the structures of BLG before and after the HHP treatment, respectively.

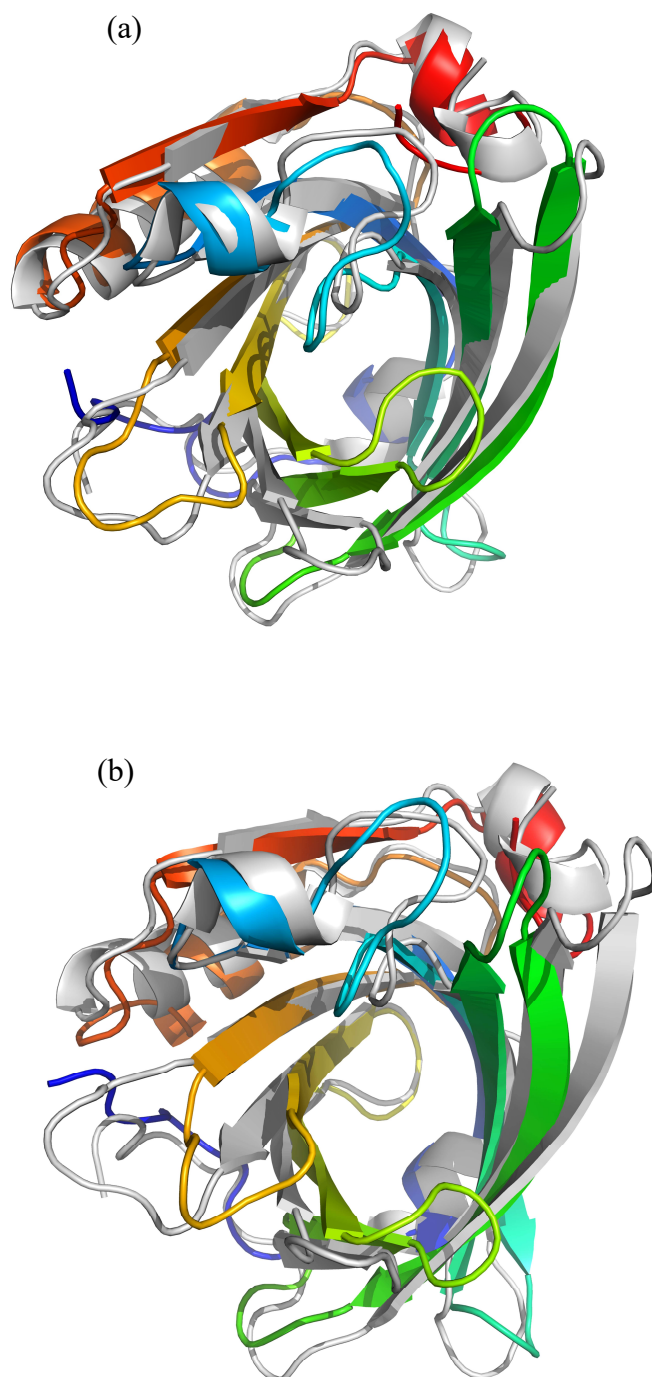


Fig. S4 Change of the disulfide bond between Cys66 and Cys160 in the C-terminus (residues153-161) of BGL at different temperatures, under 600 MPa. The gray represents the initial structure, while the blue, green and magenta represent the structures at 20, 60 and 100 °C, respectively.

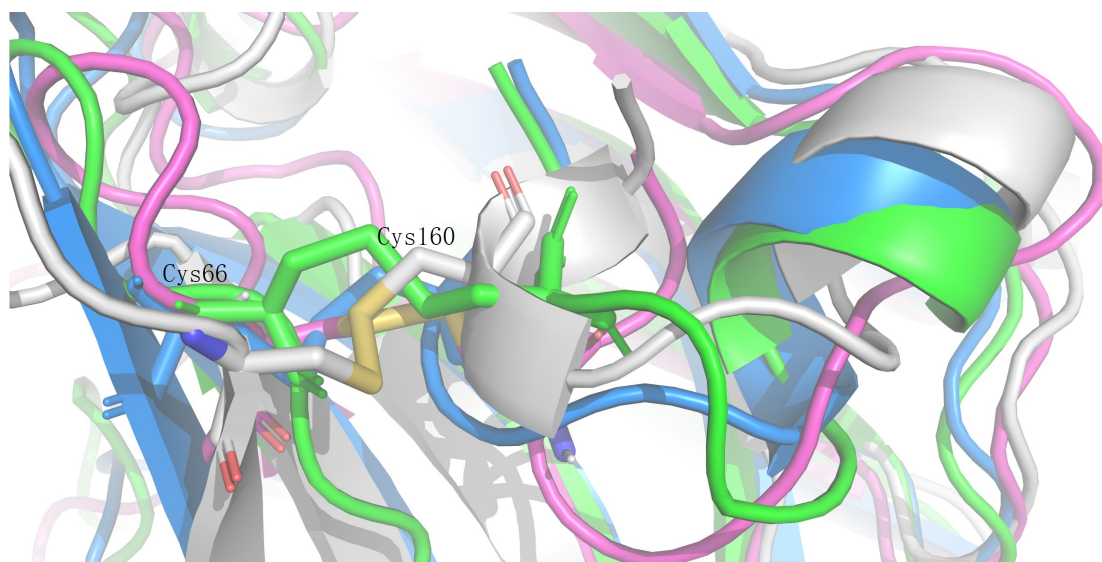


Fig. S5 Conformational displacement the local region of BGL under different conditions. The gray represents the initial structure and the cyan represents the structure at 20 °C under 0.1 MPa. While the blue, green and magenta represent the structures under 600 MPa at 20, 60 and 100 °C, respectively.

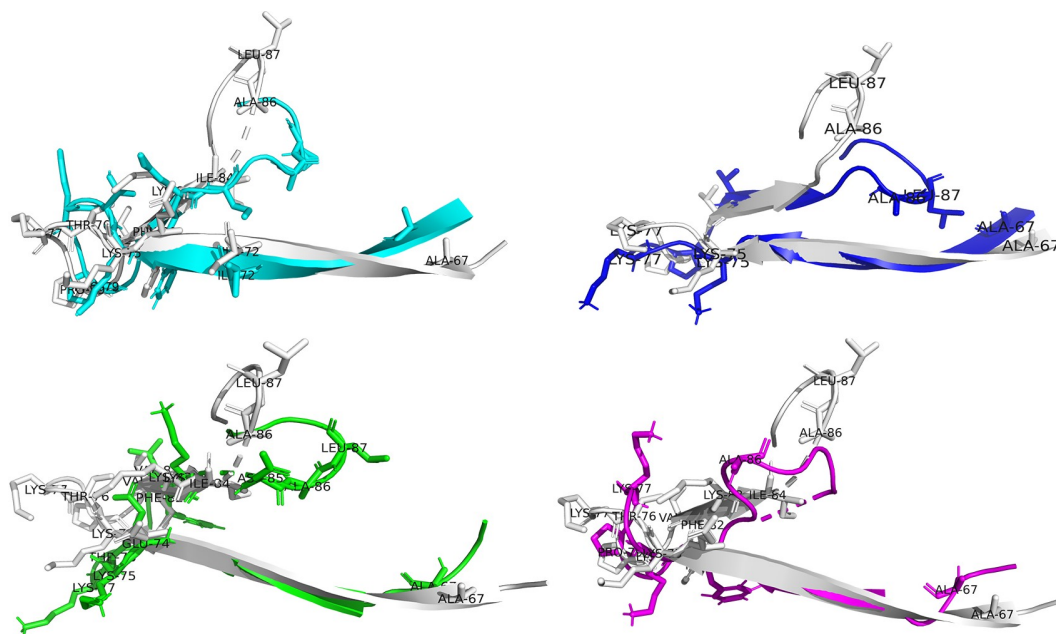


Fig. S6 SDS-PAGE of BGL under different pressures at 40 °C and 60 °C, respectively.

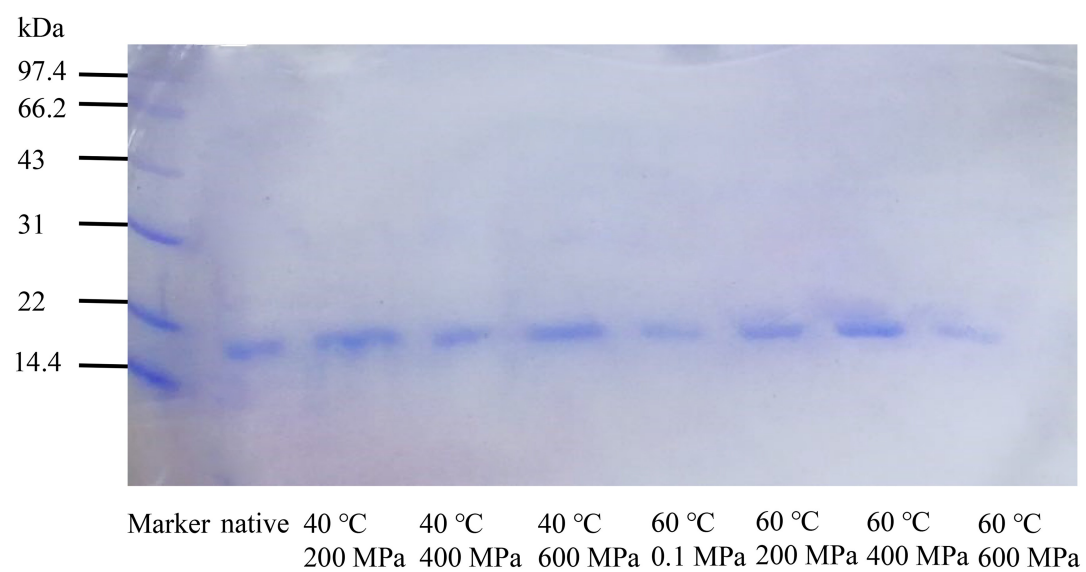


Fig. S7 The correlation between the OD_{450 nm} and the concentration of IgE-BLG complex.

