



Effects of high hydrostatic pressure on the structure and potential allergenicity of the major allergen bovine β -lactoglobulin



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ABSTRACT

Bovine β -lactoglobulin (β -Lg) is recognized as a significant milk allergen in several countries. In this study, β -Lg was isolated and treated with high hydrostatic pressure (HHP) at 100, 200, 300, 400, and 500 MPa. The allergenic properties of the HHP-treated β -Lg were characterized by indirect competitive enzyme-linked immunosorbent assay with anti- β -Lg rabbit antibody and the sera of patients allergic to cows' milk. The conformation of the HHP-treated β -Lg was examined with ultraviolet absorption spectroscopy, endogenous fluorescence spectroscopy, exogenous fluorescence spectroscopy, and circular dichroism spectroscopy analyses. The results indicated that IgG binding increased with treatment pressure, and IgE binding was lowest at 200 MPa and highest at 400 MPa. The tertiary structure of β -Lg changed significantly after HHP, whereas the primary and secondary structures remained stable. Overall, this study suggests that the conformational changes in HHP-treated β -Lg contribute to its altered allergenicity.

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1. Introduction

Cows' milk commonly causes allergic reactions in children. Approximately 2–3% of infants and young children are allergic to cows' milk proteins during the first year of life (Host & Halken, 2014; Høst, 2002). To date, the only way to avoid milk allergy is to refrain from consuming milk components. However, doing so is difficult because many processed foods contain cows' milk (Bu, Luo, Chen, Liu, & Zhu, 2013; Nowak-Węgrzyn et al., 2008). Therefore, reducing the allergenic potential of milk would be beneficial in reducing the allergenic risk. Several studies have demonstrated that conventional thermal treatment can be effective in decreasing allergenicity by altering the structures of food proteins (Achouri & Boye, 2013; Mondoulet et al., 2005; Tong et al., 2012). However, this treatment causes numerous changes in the sensory qualities and nutritional value of milk, which affect customer acceptance and demand for high-quality products (Contador, Delgado, García-Parra, Garrido, & Ramírez, 2015; Shriver & Yang, 2011; Singh, 2004). To overcome these problems, other novel non-

thermal methods have been explored with special interest in the field of hypoallergenic products (Shriver & Yang, 2011).

High hydrostatic pressure (HHP), a non-thermal food processing method, is used to inactivate microorganisms and enzymes at room temperature, which retains the freshness and nutritional value of foods (Huang, Hsu, Yang, & Wang, 2014; Sarker, Akhtar, Torres, & Paredes-Sabja, 2015; Somkuti & Smeller, 2013). Numerous reviews have stated that HHP alters the allergen reactivity of several proteins by causing a reversible or irreversible structural modification, leading to aggregation, fragmentation, denaturation, or gelatinization (Demazeau & Rivalain, 2011; Rahaman, Vasiljevic, & Ramchandran, 2016). For example, Kato, Katayama, Matsubara, Omi, and Matsuda (2000) explained that the content of major allergens in rice decreased and almost completely disappeared after treatment with a proteolytic enzyme at 300 MPa for 30 min (Kato et al., 2000). Hildebrandt et al. (2010) discovered that the immunoreactivity of egg white allergens decreased with increasing pressure after HHP treatment (400–700 MPa, 20 min), and changes in the protein structure were positively correlated with pressure (Hildebrandt et al., 2010). Li, Zhu, Zhou, and Peng (2012) reported that the allergenicity of soy protein isolate (SPI) decreased by >50% after HHP treatment (300–500 MPa, 20 min) and that HHP-treated proteins showed higher hydrophobicity than native SPI (Li et al., 2012). HHP treatment effectively reduces the allergenicity of

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certain food allergens. Therefore, the effects of HHP on milk proteins should be further investigated.

β -Lactoglobulin (β -Lg) is a major milk allergen that significantly influences the overall allergenicity of bovine milk (Caffarelli et al., 2010). Previous research demonstrated that HHP is useful in changing the antigenicity of β -Lg. For instance, data from Kleber, Maier, and Hinrichs (2007) suggested that the antigenicity of β -Lg increased with increasing pressure (200–600 MPa) (Kleber et al., 2007). Chicón, Lopez-Fandino, Alonso, and Belloque (2008) found that the combination of HHP and proteolysis could be used to produce hypoallergenic β -Lg (Chicón et al., 2008). However, all of these findings are only based on the antigenicity of HHP-treated β -Lg. The relationship between changes in potential allergenicity and conformational modification of β -Lg has not been explained to data.

This study aims to investigate the effects of HHP on the potential allergenicity and structure of β -Lg, as well as the possible mechanism underlying immunoreactivity changes after HHP-induced structural modification. Secondary and tertiary structures were analyzed through circular dichroism (CD) spectroscopy, ultraviolet (UV) absorption spectroscopy, and fluorescence spectroscopy. Immunoglobulin G (IgG) and immunoglobulin E (IgE) binding to β -Lg were assessed by indirect competitive enzyme-linked immunosorbent assay (ELISA) with anti- β -Lg rabbit antibodies and sera of patients allergic to cows' milk, respectively.

2. Materials and methods

2.1. Reagents and chemicals

1-Anilinonaphthalene-8-sulfonate (ANS), Freund's complete adjuvant, Freund's incomplete adjuvant, and *o*-phenylenediamine (OPD) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). All other reagents were analytical grade.

2.2. Preparation of β -Lg

Isolated β -Lg was prepared by combining Sephadex G-75 gel chromatography column and anion-exchange chromatography on a DEAE-Sepharose Fast Flow column in tandem operation (Li, Luo, Chen, & Cao, 2008). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and mass spectrometry confirmed that the purity of the isolated β -Lg reached 99%. This value was significantly higher than that of commercial β -Lg (Sigma, St. Louis, MO, USA; purity > 90%).

2.3. Preparation of specific polyclonal antibodies

Anti- β -Lg rabbit antibodies were produced from two male New Zealand rabbits by injecting isolated β -Lg. The selected feed for the rabbits did not contain milk proteins. Two rabbits were fed and treated according to the principles of the Experimental Animal Center, Nanchang University (Nanchang, China; permit number: "SCXK [Gan] 2014-0005"). Prior to immunization, 1 mL of blood from an untreated rabbit was collected using the ear-bleeding method, as a negative serum sample. Two rabbits were pre-immunized with 1 mg of β -Lg solution emulsified with an equal volume of Freund's complete adjuvant. Subsequent injections of β -Lg in Freund's incomplete adjuvant were repeated six times at two-week intervals. Sera titers were determined by indirect ELISA. When the titer reached a stable level, the rabbits were finally bled, and antiserum was separated by centrifugation at 4000×g and 4 °C for 10 min. All sera were stored at −80 °C until further use.

2.4. HHP treatment

The experiment was conducted with HHP equipment in a laboratory at the Guangdong Academy of Agricultural Sciences, China. The isolated β -Lg samples (0.4 mg/mL in phosphate buffer, pH 6.8) were packed in polyethylene bags. The bags were sealed under vacuum and placed in the prototype pressurization apparatus with a maximum pressure of 600 MPa (RLGY-600). The pressure-transmitting medium was water, which maintained a temperature of 25 °C during pressure processing. The samples were subjected to five pressure treatments at 100, 200, 300, 400, and 500 MPa for 30 min at 25 °C. The pressurized samples were collected in sterile bottles and stored at −20 °C for subsequent assays.

2.5. Human sera

Human sera were pooled from 11 patients allergic to cows' milk (Table 1). Specific IgE and total IgE levels were determined (specific IgE level > 1 IU/mL; total IgE level > 100 IU/mL). Sera from non-allergic individuals ($n = 3$) were also pooled and used as negative serum. All serum samples were generously provided by the First Affiliated Hospital of Guangxi Medical University (Guangxi, China). The availability of the samples was approved by the internal ethical committee of the hospital. The serum samples were stored at −80 °C until further use.

2.6. IgG and IgE binding by indirect competitive ELISA

The IgG and IgE binding of HHP-treated β -Lg were identified by indirect competitive ELISA with rabbit polyclonal antibodies and specific sera from patients allergic to cows' milk.

Briefly, 2.5 μ g/mL untreated β -Lg in 0.05 M carbonate buffer (pH 9.6) was used to coat each well (100 μ L/well) of a 96-well microtiter plate at 4 °C overnight. The plates were washed three times with PBS containing 0.05% Tween 20. Each well was blocked for 1 h at 37 °C with 3% gelatin in PBS. Equal volumes of antiserum samples (diluted 1:50,000 for the pooled rabbit sera to test IgG binding to β -Lg; diluted 1:20 for the human sera to test IgE binding to β -Lg) and HHP-treated β -Lg (inhibitors) were mixed and incubated for 1 h at 37 °C. The blocked plates were washed three times. Subsequently, 100 μ L of each inhibited antiserum sample was added to each well and incubated for 1 h at 37 °C. The plate was washed, and 100 μ L of the diluted 1:5000 antisera (HRP-labeled anti-rabbit IgG or biotin-labeled anti-human IgE) was added to the wells and incubated at 37 °C for 1 h. For the IgE binding experiments only, avidin was added in an additional step before the chromogenic substrate in order to increase chromogenic signal. After washing again, 100 μ L of the substrate solution (0.4 mg/mL OPD in a citrate buffer with 0.04% H₂O₂) was added to the wells. The chromogenic reaction was terminated by adding 50 μ L of 2 M sulfuric acid per well. Absorbance was determined at 490 nm with an ELISA plate reader (Model 1860; Bio-Rad, USA). The inhibition rates of antigenicity and allergenicity for the HHP-treated β -Lg were calculated with the following equation:

$$\text{Inhibition (\%)} = \frac{\text{total reactivity} - \text{remaining reactivity after inhibition}}{\text{total reactivity}} \times 100\%$$

2.7. Protein identification by SDS-PAGE

Proteins were analyzed by SDS-PAGE according to the method of Laemmli (1970) (Laemmli, 1970). First, a 4% stacking gel and a 12% separating gel were used with a constant current of 6 to

Table 1

Clinical data of 11 patients allergic to cows' milk (No. 1–11) and 3 non-allergic individuals (No. 12–14).

No.	Sex	Age	Clinical symptoms	Total IgE Level (IU/mL)	Specific IgE Level (IU/mL)
1	Female	ND*	ND*	>100	2.7
2	Male	2	ND*	>100	3.7
3	Male	4	Urticaria	>200	5.9
4	Male	2	Acute Upper Respiratory Infection	>200	2.4
5	Female	3	ND*	>200	1.1
6	Male	20	Urticaria	>200	1.9
7	Male	1	Bronchial Asthma	>200	2.0
8	Female	4	ND*	100–200	9.7
9	Male	3	Chronic Rhinitis	>200	2.4
10	Male	2.5	Asthma, Bronchitis	>200	1.8
11	Male	2	Bronchial Asthma	>200	1.3
12	Female	3	No symptoms	<100	–
13	Female	2.5	No symptoms	<100	–
14	Male	4	No symptoms	<100	–

* Not Done; –: Negative.

12 mA per gel. Samples (10 μ L) were added to 10 μ L loading buffer (2% [w/v] SDS, 50 mM Tris-HCl, 0.02% [w/v] bromophenol blue, 25% [v/v] glycerol, and 3% [v/v] β -mercaptoethanol), boiled at 100 $^{\circ}$ C for 5 min, and centrifuged. The supernatants were loaded onto the gel. After running the gel, the proteins were stained with Coomassie Brilliant Blue R250 for 15 min. Destaining was performed by incubation with 7.5% acetic acid and 5% methanol for at least 30 min or overnight.

2.8. Protein structure analysis

Conformational changes in the HHP-treated β -Lg were identified by CD spectroscopy, UV absorption spectroscopy, endogenous fluorescence spectroscopy, and exogenous fluorescence spectroscopy analyses.

2.8.1. CD analysis

The secondary structure of the HHP-treated and untreated β -Lg was characterized with a JASCO-715 spectropolarimeter (Jasco, Tokyo, Japan). Briefly, all samples were homogeneous mixed at 0.2 mg/mL. Under blowing nitrogen gas, CD spectra were obtained by far-UV CD spectroscopy from 240 nm to 190 nm. Quartz cells were used at a scan rate of 60 nm/min, with a bandwidth of 1.0 nm, and measurements were made at room temperature (25 $^{\circ}$ C). This process was performed at least twice. The contents of different secondary structures were calculated with JASCO secondary structure software.

2.8.2. UV absorption spectrum analysis

UV absorption spectra were obtained and analyzed with a UV spectrophotometer (TU-1901; Purkinje General, Beijing). All samples were homogeneous mixed at 0.1 mg/mL and scanned from 280 nm to 350 nm. The optical path was 1 cm with a spectrum path of 2.0 nm, and measurements were made at room temperature. The response time was 0.2 s. Each sample was analyzed at least in duplicate.

2.8.3. Intrinsic fluorescence spectrum analysis

Unfolding of HHP-treated and untreated β -Lg was determined with a fluorospectrophotometer (F-4500, Hitachi, Japan) through endogenous fluorescence spectroscopy. All samples were homogeneous mixed at 0.1 mg/mL. The UV absorption spectra were obtained from 280 nm to 350 nm, with 1.0 nm intervals. The optical path was 1 cm, with a spectrum path of 2.0 nm and a response time of 0.2 s. The result for each sample was presented as the average of two consecutive scans.

2.8.4. Exogenous fluorescence spectrum analysis

The surface hydrophobicity of HHP-treated and untreated β -Lg was determined using ANS fluorescence probes. Approximately 20 μ L of 0.05 M ANS solution (0.015 g ANS in 0.05 M PBS, pH 6.8) was added to 4 mL of the sample (0.1 mg/mL) solutions. Fluorescence spectra were obtained with a fluorospectrophotometer (F-4500, Hitachi, Japan) from 400 nm to 650 nm, with an excitation wavelength of 375 nm. Scanning was conducted at 1200 nm/min with a 0.5 s response time. The optical path was 1 cm with a slit width of 5.0 nm, and measurements were made at room temperature. Each sample was measured in duplicate.

2.9. Statistical analysis

All data were analyzed for statistical significance by ANOVA (SPSS for Windows 2003, version 13.0, Microsoft Corp., Chicago, IL, USA) and Fisher's least significant difference (LSD, $p < 0.05$) test.

3. Results

3.1. SDS-PAGE

SDS-PAGE patterns for HHP-treated β -Lg are shown in Fig. 1. Electrophoresis produced bands with an apparent molecular mass of 18 kDa. The HHP-treated samples did not obviously change

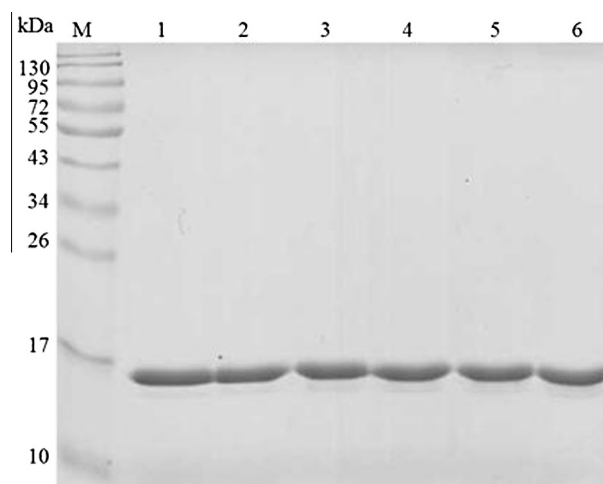


Fig. 1. SDS-PAGE of β -Lg treated with HHP. Lane M: pre-stained marker; lane 1: untreated β -Lg; lanes 2–6: β -Lg treated with 100, 200, 300, 400, and 500 MPa, respectively. The loading amount of each sample was 4 μ g.

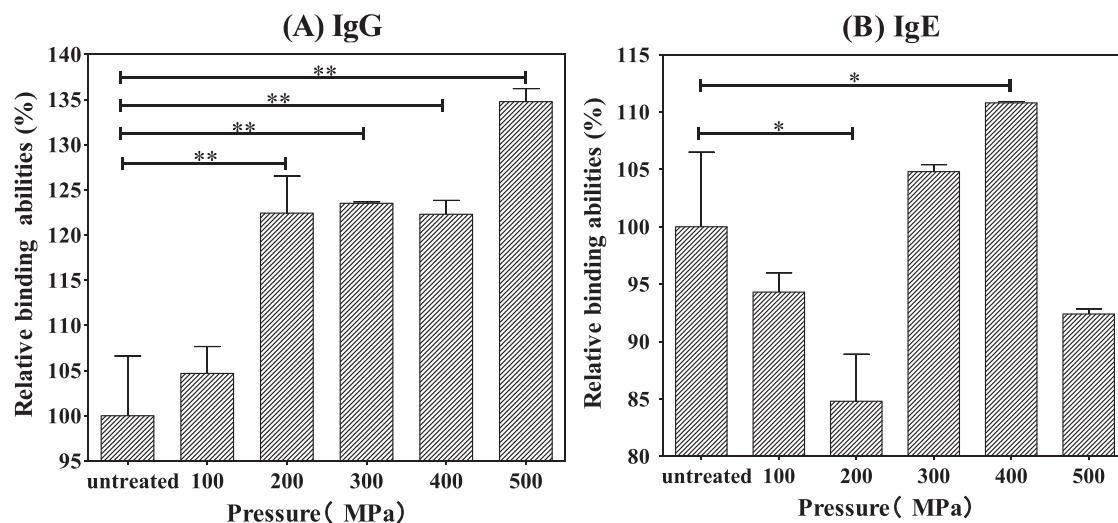


Fig. 2. Effect of HHP on relative binding abilities to β -Lg as determined by indirect competitive ELISA. Effect of different pressures on the (A) IgG binding to β -Lg and (B) IgE binding to β -Lg. Data are presented as the mean $\pm$ standard deviation ($X \pm SD$). Student's *t*-test was used to determine statistical significance. * $P < 0.01$, * $P < 0.05$.

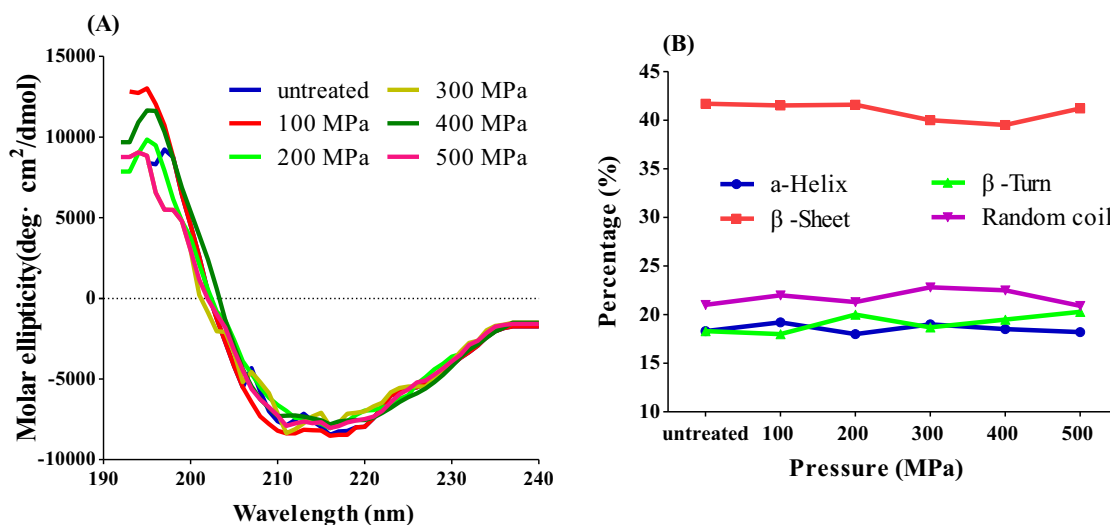


Fig. 3. Effect of HHP on the secondary structure of β -Lg at different pressures determined by far-UV CD spectroscopy. (A) Far-UV CD spectra of untreated and HHP-treated β -Lg. (B) Content of each secondary structure in the untreated and HHP-treated β -Lg.

compared with the untreated β -Lg. These results indicate that the primary structure of β -Lg may remain stable after HHP treatment from 100 MPa to 500 MPa.

3.2. Effects of HHP on the antigenicity and the potential allergenicity of β -Lg

Indirect competitive ELISA was used to determine the antigenicity and potential allergenicity of HHP-treated β -Lg with anti- β -Lg rabbit antibodies and the sera of patients allergic to cows' milk, respectively. As shown in Fig. 2(A), IgG binding increased with increasing pressure, indicating that HHP treatment induces an increase in the antigenicity of β -Lg. In contrast to untreated β -Lg, the antigenicity rapidly increased in samples treated with 100 MPa and 200 MPa, with the greatest increase observed was 23% ($p < 0.01$) at 200 MPa. Antigenicity remained the same among samples treated with 200–400 MPa, and sharply increased again by approximately 35% ($p < 0.01$) at 500 MPa.

The potential allergenicity of HHP-treated β -Lg fluctuated with pressure (Fig. 2(B)). IgE binding was lower when samples were

treated with 100 MPa or 200 MPa compared with the untreated β -Lg. The greatest decrease in IgE binding (15.2%) was at 200 MPa ($P < 0.05$). However, binding of serum IgE to the HHP-treated β -Lg gradually increased from 200 MPa to 400 MPa, and reached a maximum at 400 MPa with an increase of 10.8% ($P < 0.05$) compared with the untreated β -Lg. Interestingly, the serum IgE binding reduced at 500 MPa and was similar to that obtained when the pressure was 200 MPa.

3.3. Effects of HHP treatment on β -Lg structure

HHP-induced alterations of β -Lg structure were analyzed. The secondary and tertiary structures of β -Lg were defined using CD spectroscopy, UV absorption spectroscopy, and fluorescence spectroscopy analyses. The secondary structure of the treated β -Lg at various pressures (100–500 MPa) was detected using far-UV CD spectra at 190–240 nm. Fig. 3 shows the spectrum of treated β -Lg did not change significantly from untreated β -Lg at different pressures. This result indicates that HHP treatment exerted no obvious effects on the secondary structure. At 190 nm to 200 nm, the

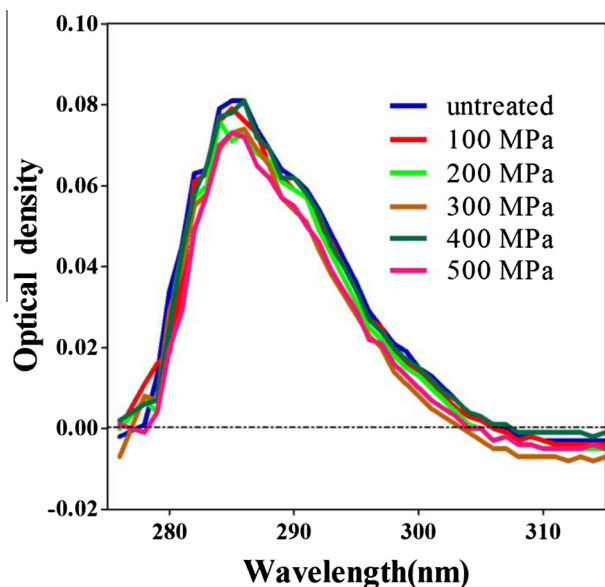


Fig. 4. Effect of HHP on β -Lg structure at different pressures for 30 min as determined by UV spectroscopy.

positive molar ellipticity peaks of the samples significantly increased to the maximum, especially for sample at 100 MPa.

The alteration in the tertiary structure of HHP-treated β -Lg can be characterized by the UV absorption and fluorescence spectra. The UV absorbance of HHP-treated β -Lg was observed at 280–350 nm (Fig. 4). The untreated β -Lg exhibited a maximum absorbance at 285 nm. Compared with the untreated sample, the HHP-treated samples showed a slight decrease in UV absorbance and the maximum absorption peak was similarly located.

The tertiary structure of β -Lg was sensitive to pressure as revealed by fluorescence spectra. Fig. 5A shows the extent of β -Lg unfolding after HHP treatment. The fluorescence intensity of HHP-treated β -Lg increased with pressure treatment from 100 MPa to 500 MPa. This result indicates that the fluorescence quenching effect of β -Lg was weakened under different pressures (100–500 MPa). Changes in the tertiary structure of HHP-treated β -Lg were also probed by surface hydrophobicity. Fig. 5B shows

the ANS-induced fluorescence spectra of β -Lg treated at various pressures. When the sample was treated at 100 MPa, the ANS-fluorescence intensity showed a profile similar to the untreated sample. In contrast, the ANS-fluorescence intensity of the HHP-treated samples increased by 1.39-, 1.88-, 2.55-, and 2.87-fold at 200, 300, 400, and 500 MPa, respectively. In addition, a slight blue shift (3–18 nm) of the location of the ANS-fluorescence peak was observed between the HHP-treated and untreated samples.

4. Discussion

Bovine β -Lg is an antigen characterized by antigenicity and allergenicity. Antigenicity refers to the ability of an antigen to stimulate specific antibody synthesis and react with pre-synthesized antibodies (Owen, Punt, Stranford, & Jones, 2013; Rosendal & Barkholt, 2000). Allergenicity refers to the ability of an antigen to induce an abnormal immune response, which disrupts physiological functions or damages tissues (i.e., a capacity to induce an IgE response) (Verhoeckx et al., 2015; Zhang & Tao, 2015). The study of antigenicity in humans is difficult because they are exposed to several allergens in an uncontrolled and unpredictable manner. Moreover, experimental tests of antigenicity on human are unethical. Accordingly, we chose rabbits to produce specific anti- β -Lg polyclonal antibodies (IgG antibodies), which were used to detect the antigenicity (IgG-binding ability to the prepared anti- β -Lg antibodies) of HHP-treated β -Lg. Clinical studies have confirmed that most abnormal immune responses triggered by milk proteins are caused by IgE (Restani, Ballabio, Di Lorenzo, Tripodi, & Fiocchi, 2009). Accordingly, investigations of allergenicity have primarily focused on food allergies mediated by IgE. In the current study, the potential allergenicity of HHP-treated β -Lg, measured as the ability to bind IgE from the sera of patients allergic to cows' milk, was characterized by indirect competitive ELISA *in vitro*.

This study demonstrated that HHP treatment increased the antigenicity of β -Lg with increasing pressure. Similarly, HHP treatment (200–600 MPa) enhanced the antigenicity of β -Lg in whey protein isolate solution, sweet whey, and skim milk (Kleber et al., 2007). Another study showed that β -Lg treated at 200–400 MPa increased binding to β -Lg-specific rabbit IgG (Chicón et al., 2008). According to Somkuti and Smeller (2013), the increase in antigenicity of the HHP-treated β -Lg is caused by protein unfolding

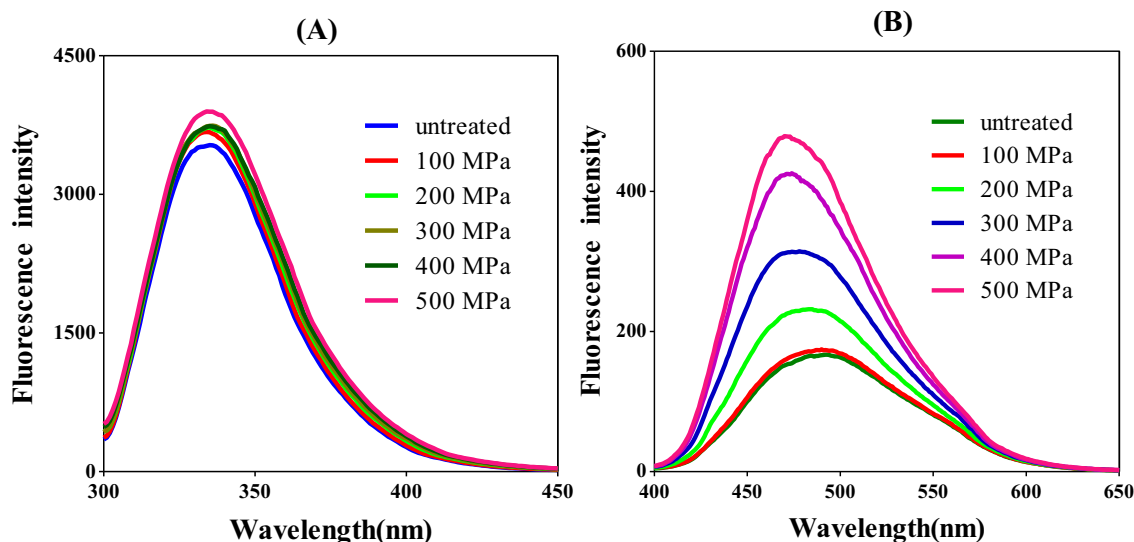


Fig. 5. Effect of HHP on β -Lg at different pressures for 30 min as determined by fluorescence spectroscopy. (A) Extent of β -Lg unfolding. (B) Surface hydrophobicity of β -Lg.

triggered by HHP; such unfolded states may expose some hidden IgG binding epitopes, which are usually buried within the protein structure but become accessible to antibodies in the unfolded state (Somkuti & Smeller, 2013). Previous studies also showed that the linear IgG binding epitopes contribute to the total antigenicity (Dhakal et al., 2014; Knudsen, Otte, Olsen, & Skibsted, 2002); thus, we inferred that the increase in antigenicity after HHP treatment is mainly caused by the exposed linear epitopes.

IgE binding was tested by ELISA to assess the potential allergenicity of HHP-treated β -Lg. Our results indicated that the IgE binding did not show a regular increase or decrease but fluctuated with the pressure applied. However, several contradictions were noted between our results and other reports. Peñas, Préstamo, Luisa Baeza, Martínez-Molero, and Gomez (2006) observed that hydrolysates obtained from the enzymatic treatment of β -Lg under high pressure exhibited reduced IgE binding (Peñas et al., 2006). Other studies reported that the application of high pressure during enzymatic hydrolysis can reduce the serum IgE binding to β -Lg hydrolysates (Beran et al., 2009; Chicón et al., 2008). This discrepancy can be ascribed to enhancement of β -Lg hydrolysis under high pressure, which in turn reduces the allergenicity of the hydrolysates. In the present study, β -Lg is an intact protein subjected to HHP treatment and its IgE binding differs from the hydrolyzed fragments produced by enzymatic hydrolysis in previous studies. Therefore, to further elucidate the effects of HHP on allergenicity, structural modification induced by HHP must be investigated.

The primary structure of β -Lg is composed of an amino acid sequence connected by covalent bonds. However, HHP treatment only affects non-covalent bonds, such as hydrogen, ionic, and hydrophobic bonds (Huang et al., 2014; Rahaman et al., 2016). Accordingly, the primary structure of a protein is maintained during HHP (Somkuti & Smeller, 2013). This view was confirmed by the present results, which showed the SDS-PAGE patterns for HHP-treated β -Lg did not change compared with the untreated β -Lg. Moreover, the secondary structure of HHP-treated β -Lg was not destroyed, indicating that hydrogen bonds remained intact because the secondary structure elements (α -helices or β -sheets/strands) are created and maintained through intramolecular or intermolecular hydrogen bonds. Previous studies indicated that secondary structures change at very high pressure levels (>500 MPa), probably because of the cleavage of hydrogen bonds (Huppertz, Fox, & Kelly, 2004; Mazri, Sánchez, Ramos, Calvo, & Pérez, 2012; Yaldagard, Mortazavi, & Tabatabaie, 2008). However, the maximum pressure was set at 500 MPa for this study because of the limitations of the facility. Consequently, the hydrogen bonds in the secondary structure were undamaged.

The HHP-induced alterations in the β -Lg tertiary structure were more significant than the primary and secondary structures. The increased intensity of fluorescence indicated that the protein unfolded, and numerous hydrophobic regions were exposed after HHP treatment. Thus, the hydrophobicity of the HHP-treated β -Lg increased. This finding demonstrates that the tertiary structure of β -Lg was strikingly modified by pressure. This phenomenon is not unique because similar results have been observed in other proteins, such as SPI and α -casein (Hu, Zheng, Liu, Deng, & Zhao, 2016; Li et al., 2012). The tertiary structure is a 3D configuration caused by non-covalent interactions among amino acid side chains. HHP substantially affects the β -Lg tertiary structures, which are mainly maintained by non-covalent bonds.

The changes in the antigenicity and potential allergenicity of the HHP-treated β -Lg are correlated with tertiary structure modification. The increased antigenicity of HHP-treated β -Lg was due to protein unfolding, which can expose hydrophobic groups and antigenic epitopes (IgG linear epitopes) inside the native molecule to the surface. In contrast to IgG binding epitopes, both linear and

conformation epitopes contributed to IgE binding (Zhou et al., 2016). Therefore, HHP did not completely destroy the recognition of β -Lg by serum IgE from patients allergic to cows' milk. These findings suggest that linear epitopes recognized by IgE probably persist after HHP treatment, which destroyed predominantly the conformational-dependent epitopes on β -Lg. Several researchers also described similar results in crushed peanut, squid tropomyosin (Tod p1 allergen), and soy seed (Huang, Yang, & Wang, 2014; Jin et al., 2015; Peñas, Gomez, Frias, Baeza, & Vidal-Valverde, 2011).

5. Conclusions

This study helps to characterize the relationship between the protein structure and allergenic properties of HHP-treated β -Lg. The primary and secondary structures of β -Lg did not change after HHP treatment; however, the tertiary structure changed significantly. Compared to untreated β -Lg, IgG binding increased with increasing pressure, but IgE binding fluctuated during the HHP treatment. Spectroscopic analysis revealed that HHP treatment influenced the antigenicity and potential allergenicity of β -Lg by changing its tertiary structure. However, these results were only obtained from *in vitro* experiments. Further investigations involving animal or clinical studies must be performed to confirm whether HHP-treated β -Lg can significantly reduce the allergic symptoms compared to consumption of untreated β -Lg. Upon confirmation, HHP processing could be used as a food processing technology to reduce the allergenic properties of milk allergens.

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