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## Glycation of $\beta$ -lactoglobulin under dynamic high pressure microfluidization treatment: Effects on IgE-binding capacity and conformation

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### ABSTRACT

The effects of dynamic high-pressure microfluidization (DHPM) (80, 120, and 160 MPa) treatment and glycation with galactose on the IgE-binding capacity and conformation of  $\beta$ -lactoglobulin ( $\beta$ -Lg) were investigated. The binding capacity of immunoglobulin E (IgE) from patients' sera with cow's milk allergy on  $\beta$ -Lg glycated with galactose decreased after DHPM treatment.  $\beta$ -Lg treated after different DHPM methods and pressures yielded a significant discrepancy in IgE-binding capacity. When  $\beta$ -Lg was pretreated by DHPM, the IgE-binding capacity of  $\beta$ -Lg–galactose conjugates decreased with increasing pressure; however, the conjugates showed higher IgE-binding capacity at 120 MPa than that at 80 and 160 MPa when the  $\beta$ -Lg–galactose mixture was treated by DHPM. Results of thermal properties, intrinsic fluorescence spectroscopy, surface hydrophobicity, and circular dichroism (CD) spectra indicated the occurrence of protein unfolding, as well as the tertiary and secondary structural changes of  $\beta$ -Lg. The results suggested pretreatment by DHPM and glycation with galactose was a promising approach for eliminating the IgE-binding capacity of  $\beta$ -Lg.

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### 1. Introduction

$\beta$ -Lactoglobulin ( $\beta$ -Lg) is the main constituent of whey protein in milk and is composed of a single polypeptide chain that contains 162 amino acids.  $\beta$ -Lg molecular weight is about 18.3 kDa (Papiz et al., 1986).  $\beta$ -Lg is widely used as a functional additive in industrial application with numerous nutritional properties (Baez, Busti, Verdini, & Delorenzi, 2013; Chen, Chen, Guo, & Zhou, 2015; Croguennec, Renault, Bouhallab, & Pezennec, 2006).  $\beta$ -Lg possesses the best proportion of amino acid, but the high allergenicity of  $\beta$ -Lg has greatly limited its application in the food industry (Rahaman, Vasiljevic, & Ramchandran, 2016).

Numerous methods have been investigated to improve the functional properties and reduce allergenicity of  $\beta$ -Lg through physical treatments, such as heat treatments (Rahaman, Vasiljevic, & Ramchandran, 2015) and high hydrostatic pressure (Lopez-Exposito, Chicon, Belloque, Lopez-Fandino, & Berin, 2012); and chemical treatments, such as glycation and phosphorylation (Enomoto et al., 2009; Taheri-Kafrani et al., 2009; Wong, Li, Wong, Jiang, & Shaw, 2012). The Maillard reaction is a chemical reaction between amino acid and reducing sugars, which forms non-enzymatic brown complex and included three stages. Glycation which is based on the Amadori rearrangement steps of Maillard reaction generally occurs between proteins and reducing sugars in safe conditions and without extraneous chemical reagents. This reaction is a promising chemical modification for food protein. It can improve food proteins' functional properties and endows them with novel functionality. This reaction is complicated, which is affected by intrinsic (Oliver, Melton, & Stanley, 2006; Thomsen et al., 2012) and extrinsic factors (Guan et al., 2010; Shi, Sun, Yu, & Zhao, 2010), including molecular structure of reagents, temperature, pH, and water activity. High hydrostatic pressure processing (HPP) influences the Maillard reaction. High pressure could accelerate the formation of Amadori rearrangement products and increase the levels of intermediate and advanced reaction products (Moreno, Molina, Olano, & Lopez-Fandino, 2003). A previous study revealed the impact of HPP on  $\beta$ -Lg and found

**Abbreviations:**  $\beta$ -Lg,  $\beta$ -lactoglobulin; DHPM, dynamic high-pressure microfluidization; IgE, immunoglobulin E; HPP, high hydrostatic pressure processing; CMA, cow's milk allergy; CD, circular dichroism; SDS, sodium dodecyl sulphate; ANS, 1-anilinonaphthalene-8-sulfonate; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate buffer solution; PBST, PBS/Tween solution; CBS, carbonate buffer solution;  $H_0$ , surface hydrophobicity; SDS-PAGE, Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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that HPP (500 MPa) gave rise to complete unfolding of protein with the formation of intermolecular aggregates (Considine, Patel, Singh, & Creamer, 2005).

Dynamic high pressure microfluidization (DHPM) is an emerging high pressure technology that uses a collective force of high-velocity impact, high-frequency vibration, instantaneous pressure drop, intense shear, cavitation, and ultrahigh pressures up to 200 MPa. DHPM is an effective method for modifying food biomacromolecules, including proteins (Shen & Tang, 2012), enzymes (Liu et al., 2009), and polysaccharides (Chen et al., 2013). DHPM combined with glycation treatment could change protein structure and decline the antigenicity of  $\beta$ -Lg (Zhong et al., 2014). In our previous work, DHPM pretreatment on the glycation of bovine serum albumin can elevate glycation level and increase the glycation sites, and DHPM pretreatment can significantly change bovine serum albumin's conformation and promote the Maillard reaction (Huang et al., 2013). However, studies about glycation under different DHPM treatments on the IgE-binding capacity and conformational changes of  $\beta$ -Lg are lacking.

In this work, we initially prepared  $\beta$ -Lg and  $\beta$ -Lg-galactose mixture under different DHPM treatments. The effects of glycation of  $\beta$ -Lg in dry-state on the binding capacity of immunoglobulin E (IgE) from cow's milk allergy (CMA) patients' sera were investigated in relation to the conformational changes of  $\beta$ -Lg. These changes were characterized by SDS-PAGE, thermal properties, intrinsic fluorescence spectra, surface hydrophobicity ( $H_0$ ), and circular dichroism (CD) spectra.

## 2. Materials and methods

### 2.1. Materials

$\beta$ -Lg from bovine milk (L3908; purity, 90%; containing  $\beta$ -Lg A and  $\beta$ -Lg B; lyophilized powder) was purchased from Sigma Chemical Co. (St. Louis, MO). D-(+)-galactose was obtained from Shanghai Lanji Technology Development Company (Shanghai, China). Goat anti-human IgE-HRP (GtxHu-002-DHRPX) conjugate was from Immunology Consultants Laboratory, Inc. (Oregon, Portland). All other reagents used were of analytical grade.

Human sera: sera of patients allergic to milk were purchased from PlasmaLab International (Everett, W.A., USA) and stored at  $-80^\circ\text{C}$  until used. The four patients were numbered P1, P2, P3, P4. Their specific IgE levels in milk allergic human sera are 13.8, 14.2, 96.1 and 96.6 KUA/L (measured by ImmunoCAP). According to the documented clinical history, all patients had the milk-related clinical symptoms (such as hives) begin childhood. Sera from people with no allergy to milk were used as the negative control. The same volume of mixed serum pool (four patients' sera) was used for the inhibition ELISA.

### 2.2. Preparation of $\beta$ -Lg-galactose Maillard reaction products

The solution containing  $\beta$ -Lg (1 mg/mL) and galactose (1 mg/mL) was prepared in 50 mM phosphate buffer at pH 8.0 and then split into four aliquots. The  $\beta$ -Lg solution was poured into the product inlet reservoir of microfluidizer (M-110EH; Microfluidics Co., Newton, MA) at ambient temperature and atmospheric pressure. One aliquot was circulated in the high pressure microfluidizer without any pressure. The other three aliquots were treated thrice under pressures of 80, 120, and 160 MPa. The DHPM treated samples were referred to as 1 (0 MPa), 5 (80 MPa), 6 (120 MPa), and 7 (160 MPa).  $\beta$ -Lg solution without galactose was pretreated by DHPM under the same conditions. After the  $\beta$ -Lg solution was pretreated by DHPM, the protein content was measured using the Bradford method. Galactose (mass ratio (galactose:  $\beta$ -Lg) = 1:1) was then added to the  $\beta$ -Lg solution pretreated by DHPM. The DHPM-pretreated samples were referred to as 2 (80 MPa), 3 (120 MPa), and 4 (160 MPa). The samples were lyophilized and stored at  $4^\circ\text{C}$  until used.

The lyophilized samples were incubated  $55^\circ\text{C}$  and 65% relative humidity for 6 h. The reaction was stopped by placing the sample tubes into an ice bath. Each sample in the tube was then dissolved in 100  $\mu\text{L}$  of distilled water and filtered by a Centricon centrifugal filter unit (3000 Da cutoff; Slariob, Beijing, China) to remove salts and free galactose. The concentration of protein was adjusted to 2 mg/mL and stored at  $4^\circ\text{C}$  until used.

$\beta$ -Lg heated at conjugate conditions without sugar (heat control) was referred to as 0 ( $\beta$ -Lg heated).

### 2.3. SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed for  $\beta$ -Lg according to reference (Schagger, Borchart, Machleidt, Link, & Vonjagow, 1987) with modifications. A 12% acrylamide separating gel and a 5% acryl amide stacking gel were used in a Bio-Rad Mini protean Tetra MP4 electrophoresis (Bio-Rad Mini-Protein system). The samples were prepared by mixing  $\beta$ -Lg at a 3:1 (vol/vol) ratio with loading buffer ( $4 \times$  loading buffer with  $\beta$ -mercaptoethanol, Solarbio Chemical Co). The solutions were then heated in boiling water for 5 to 10 min before electrophoresis. Low molecular weight markers (14.4–97.4 kDa) were run as reference. Electrophoresis was performed in electrophoresis buffer (containing 0.025 M Tris-HCl, 0.192 M glycine and 0.1% SDS) at a current of 15–35 mA. Gels were stained with Coomassie Brilliant Blue R250 and destained in a 5% ethanol/7.5% acetic acid mixture.

### 2.4. Thermal properties

Thermal behavior measurements were performed on a differential scanning calorimetry (DSC 200 F3; Netzsch, Germany). Approximately 10 mg of the protein sample was weighed into the aluminum pans. The pans were hermetically sealed, and then a temperature ramp from  $20^\circ\text{C}$  to  $120^\circ\text{C}$  was applied; at a heating rate of  $10^\circ\text{C}/\text{min}$ .

### 2.5. Intrinsic fluorescence emission spectroscopy

The intrinsic emission fluorescence spectra of  $\beta$ -Lg samples were obtained in using a fluorophotometer (F-4500; Hitachi, Tokyo, Japan) in accordance with the technique described by Tang, Sun, and Foegeding (2011) with modifications. Protein concentration of 0.2 mg/mL was prepared in phosphate buffer (20 mM, pH 7.4). The excitation wavelength was 280 nm. The emission spectrum was recorded from 300 nm to 500 nm, with excitation and emission slits of 2.5 and 5.0 nm, respectively, and a scan speed of 240 nm/min.

### 2.6. Measurement of $\beta$ -Lg surface hydrophobicity

Surface hydrophobicity ( $H_0$ ) of samples was obtained in accordance with the method of Haskard and Li-Chan (1998). 1-anilinonaphthalene-8-sulfonate (ANS; Sigma Chemical, Co) was used as the fluorescent dye. The protein solutions were prepared in phosphate buffer (20 mM, pH 7.4) ranging from 0.02 mg/mL to 1.0 mg/mL. The assay consisted of mixing 20  $\mu\text{L}$  of ANS (8.0 mM in 20 mM phosphate buffer, pH 7.4) and 2 mL of the sample, and measuring the relative fluorescence intensities at room temperature using a fluorescence spectrophotometer. Fluorescence intensity was measured using a fluorescence spectrometer (F-4500, Hitachi, Tokyo, Japan) at 390 nm (excitation) and 470 nm (emission), with a constant excitation and emission slit of 5 nm. The protein hydrophobicity was expressed as the initial slope of relative fluorescence intensity vs. protein concentration (mg/mL) (calculated by linear regression analysis).

## 2.7. Circular dichroic spectra

Protein secondary structure was studied using circular dichroism (CD) according to Divsalar, Saboury, Ahmad, and Moosavi-Movahedi (2009). CD of the samples was measured using a MOS-450 spectropolarimeter (French Bio-Logic SAS Co., Claix, France) with a 1.0 mm path length quartz cuvette. Each spectrum was recorded in the wavelength range of 190–250 nm at a scan speed of 100 nm/min, 1.0 nm bandwidth, and four scans. The CD spectra of the solutions of different protein samples were obtained in a sodium phosphate buffer (20 mM, pH 7.4) at room temperature. All observed CD spectra were corrected by subtracted buffer baseline spectrum, and the CD data were expressed as mean residue ellipticity. Structure predictions from CD spectra were analyzed by online Dichroweb software (<http://dichroweb.cryst.bbk.ac.uk/html/process.shtml>).

## 2.8. Enzyme-linked immunosorbent assay (ELISA)

The IgE-binding capacity of  $\beta$ -Lg samples was estimated by an indirect ELISA and inhibition ELISA with modification (Taheri-Kafrani et al., 2009). For indirect ELISA 96-well micro-plates were coated 100  $\mu$ L/well 10  $\mu$ g/mL of  $\beta$ -Lg samples and were incubated overnight at 4 °C. Residual free-binding sites were blocked with 1% fish gelatin in carbonate buffer solution (CBS) for 1 h at 37 °C. The wells of the plates were washed 3 times with PBS/Tween solution (PBST). The wells were added 100  $\mu$ L of human serum diluted 1:30 in PBST, and the plates were then incubated at 37 °C for 2 h. After removing the solutions, the wells were washed thrice with PBST. A solution of 100  $\mu$ L of purified goat anti-human IgE-HRP conjugate diluted 1:200 in PBST was added, and the plates were incubated at 37 °C for 1 h. After washing, 100  $\mu$ L of tetramethylbenzidine solution was immediately added to each well. The reaction was stopped by adding 100  $\mu$ L of 2 M sulfuric acid. The absorbance was measured at 450 nm using the Bio-Rad Microplate Reader.

For inhibition ELISA 96-well micro-plates were coated 100  $\mu$ L/well 5  $\mu$ g/mL of  $\beta$ -Lg samples and were incubated overnight at 4 °C. Residual free-binding sites were blocked with 1% fish gelatin in carbonate buffer solution (PBS) for 1 h at 37 °C. The wells of the plates were washed 3 times with PBS/Tween solution (PBST). Equal volumes of antisera samples (1:10 diluted human sera) and glycosylated  $\beta$ -Lg (inhibitors) were mixed and incubated for 1 h at 37 °C. The wells were added 100  $\mu$ L of each inhibited antisera sample mixture, and the plates were then incubated at 37 °C for 2 h. After removing the solutions, the wells were washed thrice with PBST. A solution of 100  $\mu$ L of purified goat anti-human IgE-HRP conjugate (A9667, Sigma Chemical Co.) diluted 1:5000 in PBST was added, and the plates were incubated at 37 °C for 1 h. After washing, 100  $\mu$ L of tetramethylbenzidine solution was immediately added to each well. The reaction was stopped by adding 50  $\mu$ L of 2 M sulfuric acid. The absorbance was measured at 450 nm using the Bio-Rad Microplate Reader. The inhibition rate was calculated using the following equation: % inhibition =  $(1 - B/B_0) \times 100$ , where B and  $B_0$  are the absorbance values of the well with and without the inhibitor, respectively.  $IC_{50}$  is the concentration of inhibitors that causes a 50% inhibition of antibody binding ( $\mu$ g/mL).

## 2.9. Statistical analysis

All glycation experiments were conducted in triplicate. All glycation samples and control samples were analyzed in triplicate. Statistical analysis was established by ANOVA.

## 3. Results and discussion

### 3.1. IgE-binding capacity analysis

The binding capacity of IgE from patients having CMA on heated  $\beta$ -Lg and  $\beta$ -Lg-galactose conjugates was studied by indirect ELISA and

inhibition ELISA. As shown in Fig. 1, the glycosylated protein showed a decreased IgE-binding capacity (Gruber, Vieths, Wangorsch, Nerkamp, & Hofmann, 2004; Nakamura et al., 2006). The calculated  $IC_{50}$  values of  $\beta$ -Lg-galactose (0 MPa),  $\beta$ -Lg pretreated and  $\beta$ -Lg-galactose mixture processed by DHPM at 80, 120, and 160 MPa, respectively, increased from 2.02 (heated  $\beta$ -Lg) to 3.18, 10.08, 20.36, 24.56, 14.93, 11.41, and 17.25  $\mu$ g/mL, respectively (Fig. 1B). This result may be attributed to the conjugation of masking of some epitopes because of sugars (Niemi et al., 2007; Taheri-Kafrani et al., 2009). The two glycation sites of protein during Maillard reaction are lysine and arginine residues. The major and minor  $\beta$ -Lg epitopes contain one or more lysyl residues (e.g., K47, K60, K83, and K135) (Cocco, Jarvinen, Han, Beyer, & Sampson, 2007; Jarvinen, Chatchatee, Bardina, Beyer, & Sampson, 2001).

When  $\beta$ -Lg was pretreated by DHPM at different pressures, the IgE-binding capacity of  $\beta$ -Lg-galactose conjugates decreased with the pressure increased from 80 MPa to 160 MPa. When  $\beta$ -Lg-galactose mixture was treated by DHPM at different pressures, the IgE-binding capacity of  $\beta$ -Lg-galactose conjugates were initially increased following by decrease as the pressure was increased from 80 MPa to 160 MPa. Glycation reaction is affected by many factors, such as protein conformation, sugar structure, temperature and so on. DHPM has an effect on the structures of milk proteins in aqueous (Bouaouina, Desrumaux, Loisel, & Legrand, 2006). Bunn and Higgins (1981) revealed that the reactivity of sugar was dependent on the extent to which it existed in the open (carbonyl)

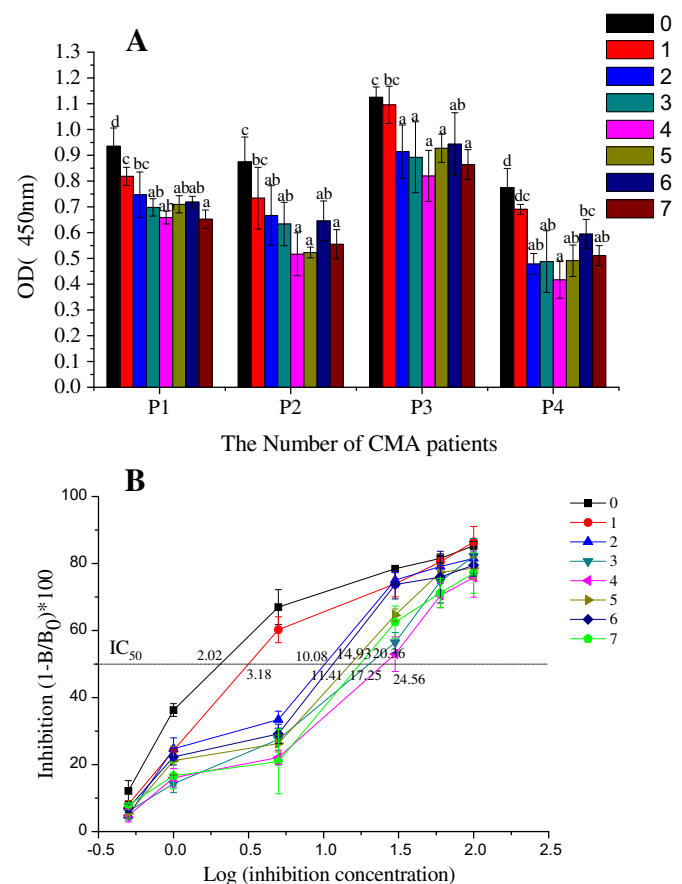


**Fig. 1.** (A) IgE binding ability of heated  $\beta$ -Lg and  $\beta$ -Lg-galactose conjugates was defined by indirect ELISA. (B) IgE-binding capacity of heated  $\beta$ -Lg and  $\beta$ -Lg-galactose conjugates was defined by inhibition ELISA. Anti- $\beta$ -Lg patients' pooled sera (50  $\mu$ L/well) were incubated separately with 0, 1.5, 5, 30, 60 and 100  $\mu$ g/mL (50  $\mu$ L/well) of the glycosylated  $\beta$ -Lg as inhibitors. 0: Heated  $\beta$ -Lg; 1:  $\beta$ -Lg-galactose mixture treated by 0 MPa; 2–4:  $\beta$ -Lg pretreated by DHPM at 80, 120 and 160 MPa, and then galactose was added, respectively; 5–7:  $\beta$ -Lg-galactose mixture treated by DHPM at 80, 120 and 160 MPa, respectively.

structure rather than in the ring structure during Maillard reaction. According to Hayes, Fox, and Kelly (2005),  $\beta$ -Lg of milk was denatured by high pressure homogenization treatment at pressures  $\geq 150$  MPa. Zhong et al. (2012) reported that disaggregation of  $\beta$ -Lg occurred and the structure of  $\beta$ -Lg was partially unfolded when DHPM pressure was increased from 0.1 MPa to 80 MPa; but reaggregation happened when the pressures was above 80 MPa. In current study, when  $\beta$ -Lg was pretreated by DHPM at 80, 120, and 160 MPa, the structure of protein was firstly unfolded and then reaggregated. After glycation, the IgE-binding ability decreased with the pressure increased due to the mask of some epitopes caused by glycoconjugates and reaggregation. When  $\beta$ -Lg-galactose mixture was treated by DHPM at 80, 120, and 160 MPa, the structure of protein and sugar were both altered. The IgE-binding capacity of  $\beta$ -Lg-galactose conjugates processed at 120 MPa was higher than that at 80 and 160 MPa, which could be that the structure of  $\beta$ -Lg was partial unfolded rather than reaggregation (120 MPa) due to the existence of galactose and exposure of epitopes. Meanwhile, the changes of galactose structure may decrease the glycation reaction rate. The reduction of IgE-binding capacity of  $\beta$ -Lg-galactose conjugates at 160 MPa may be resulted from the reaggregation of  $\beta$ -Lg and glycoconjugates. The results in this study showed that DHPM pretreatment and glycation with galactose was a promising approach for reducing the IgE-binding capacity. Zhong et al. (2014) found that antigenicity (IgG binding) of  $\beta$ -Lg-galacto-oligo-saccharides decreased at a relatively low pressure ( $\leq 120$  MPa). They also showed that when the pressure increased to 160 MPa, the antigenicity markedly increased and the value was about two times higher than that of control  $\beta$ -Lg. Glycated  $\beta$ -Lg at a pressure of 160 MPa does not show any increase of IgE-binding capacity as compared with the heated  $\beta$ -Lg. This apparent discrepancy can be explained by the fact that Zhong et al. (2014) used immunized animal, whereas we worked with human sera. This induced two kinds of important sources of variability. First, Zhong et al. studied only  $\beta$ -Lg-specific IgG epitopes, whereas we studied IgE epitopes. Significant differences exist between IgG and IgE epitopes of  $\beta$ -Lg (Jarvinen et al., 2001); second, the epitopes recognized by the animal immune systems are often different from those recognized by the humans. Moreover, Zhong et al. used  $\beta$ -Lg standard solutions (1 mg/mL) prepared in distilled water (pH 7.0), then treated by DHPM. In this study, we used  $\beta$ -Lg standard solutions (1 mg/mL) diluted in 50 mM phosphate buffer at pH 8.0 and then treated by DHPM. In order to better understand the reason for decreasing of IgE-binding capacity of  $\beta$ -Lg by glycation coupled to DHPM treatment, the conformational changes of  $\beta$ -Lg were investigated.

### 3.2. SDS-PAGE

The SDS-PAGE under reducing conditions profiles of  $\beta$ -Lg conjugates with galactose are shown in Fig. 2. Compared with the heated  $\beta$ -Lg, the molecular weight of  $\beta$ -Lg-galactose conjugates increased after the reaction, suggesting that cross-linking reaction occurred between  $\beta$ -Lg and

galactose. Likewise, a number of aggregation bands corresponding to the oligomeric forms were observed, indicating that covalent linkage reactions that involved sugar cross-links occurred. Corzo-Martinez, Moreno, Olano, and Villamiel (2008) observed increased aggregation bands in SDS-PAGE studies of  $\beta$ -Lg incubated with galactose. However, no significant differences were observed in SDS-PAGE among  $\beta$ -Lg-galactose conjugations under different treatment methods and different pressures.

### 3.3. Thermal properties

Differential scanning calorimetry was used to analyze the changes in the structure of  $\beta$ -Lg and  $\beta$ -Lg-galactose conjugates upon heating. Thermal properties of samples were shown in Fig. 3. The denaturation temperature of heated  $\beta$ -Lg was 74.47 °C, and the results were partly in agreement with those of others (Chanasattru, Decker, & McClements, 2007; Verheul, Roefs, & de Kruif, 1998). The denaturation temperature of  $\beta$ -Lg-galactose conjugates at 0 MPa was 96.75 °C. Interestingly, when the  $\beta$ -Lg and  $\beta$ -Lg-galactose were treated by DHPM at different pressures, the denaturation temperature of  $\beta$ -Lg-galactose conjugates slightly decreased, which was still higher than the heated  $\beta$ -Lg. Glycation could enhance the heat stability of protein, whereas DHPM pretreatment did not significantly change the denaturation temperature. Chevalier, Chobert, Dalgarrondo, Choiset, and Haertle (2002) reported that  $\beta$ -Lg glycated with different sugars could induce conformation changes and increase the denaturation temperature of  $\beta$ -Lg. Chen, Chen, Guo, and Zhou (2015) revealed that  $\beta$ -Lg glycated with reducing sugars exhibited higher denaturation temperature than  $\beta$ -Lg. These results demonstrated that the different DHPM treated methods (pretreatment of  $\beta$ -Lg and treatment of  $\beta$ -Lg-galactose mixture) and different pressures (80 MPa, 120 MPa, and 160 MPa) had no significant effects on changing the denaturation temperature of  $\beta$ -Lg-galactose conjugates. This was in accordance with SDS-PAGE results that did not show any noticeable alternation in intensity of the bands.

### 3.4. Intrinsic fluorescence emission spectroscopy

Intrinsic fluorescence is due to the excitation of the tryptophan (Trp)/tyrosine (Tyr) residues or Trp/Tyr specific interactions. When the conformation of  $\beta$ -Lg is altered, the chromophores become exposed to surrounding environment, resulting in decreased fluorescence intensity and a shift of the wavelength of fluorescence emission peak. Intrinsic fluorescence emission spectroscopy of heated and glycated  $\beta$ -Lg after DHPM treatment was presented in Fig. 4. The fluorescence

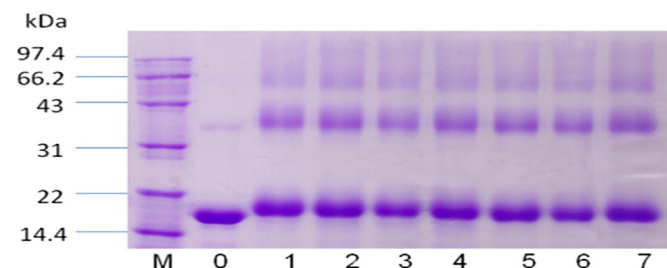


**Fig. 2.** Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of samples. M: molecular weight marker; Lane 0: Heated  $\beta$ -Lg; Lane 1: MPa- $\beta$ -Lg-galactose treated by DHPM at 0 MPa; Lane 2–4:  $\beta$ -Lg pretreated by DHPM at pressures 80, 120, and 160 MPa, and then galactose was added, respectively; Lane 5–7:  $\beta$ -Lg-galactose mixture treated by DHPM at pressure 80, 120, and 160 MPa, respectively.

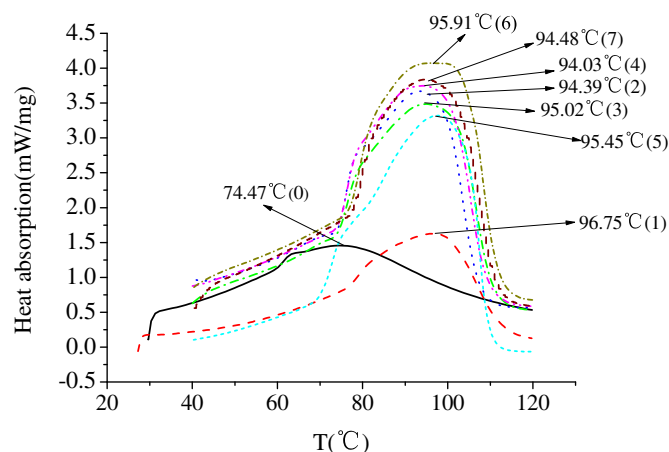


**Fig. 3.** Thermal properties of heated  $\beta$ -Lg and glycated  $\beta$ -Lg. 0: Heated  $\beta$ -Lg; 1:  $\beta$ -Lg-galactose mixture treated by DHPM at 0 MPa; 2–4:  $\beta$ -Lg pretreated by DHPM at pressures 80, 120, and 160 MPa, and then galactose was added, respectively; 5–7:  $\beta$ -Lg-galactose mixture treated by DHPM at pressures 80, 120, and 160 MPa, respectively.

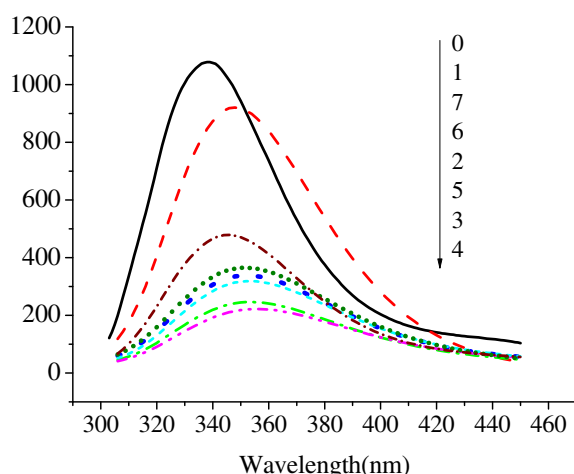


**Fig. 4.** Intrinsic fluorescence intensity of heated  $\beta$ -Lg and glycosylated  $\beta$ -Lg. 0: Heated  $\beta$ -Lg; 1:  $\beta$ -Lg-galactose mixture treated by DHPM at 0 MPa; 2–4:  $\beta$ -Lg pretreated by DHPM at pressures 80, 120, and 160 MPa, and then galactose was added, respectively; 5–7:  $\beta$ -Lg-galactose mixture treated by DHPM at pressures 80, 120, and 160 MPa, respectively.

intensity of  $\beta$ -Lg-galactose (0 MPa),  $\beta$ -Lg pretreated and  $\beta$ -Lg-galactose mixture processed by DHPM at 80, 120, and 160 MPa, respectively, decreased from 1078 (control) to 920.6, 338.1, 246.1, 222, 318.6, 365, and 478.7 respectively, and fluorescence spectrum showed a different degree red shift of the maximum fluorescence emission wavelength compared with  $\beta$ -Lg (control). Zhong et al. (2012) reported that the intensity of intrinsic fluorescence of  $\beta$ -Lg decreased without red shift after DHPM treatment. The results suggested that glycation may have more significant effects on the protein intrinsic fluorescence. In  $\beta$ -Lg-galactose conjugates, the intensity was significantly reduced, which can be attributed to the shielding effect of the galactose bound to  $\beta$ -Lg. The results of red shift indicated that a relatively increased exposure of tryptophan toward a more polar environment and the  $\beta$ -Lg underwent conformational changes around the Trp or Tyr residues (Manderson, Hardman, & Creamer, 1999). Considerable research reported the decrease in fluorescence intensity and a red shift of glycoconjugates after glycation (Jimenez-Castano, Villamiel, & Lopez-Fandino, 2007; Zhong, Tu, Liu, Luo, & Liu, 2015).

### 3.5. Surface hydrophobicity

Protein surface hydrophobicity ( $H_0$ ) is an index of the number of hydrophobic groups on the surface of a protein in contact with polar environment, which greatly influences its functional properties. The surface hydrophobicity of samples was shown in Table 1. The glycosylated conjugates of  $\beta$ -Lg decreased compared with the heated  $\beta$ -Lg. This result is probably due to the blocking of lysine and/or arginine residues in the glycation conjugates because of the increasing hydrophilic groups by conjugation between carbohydrates and the  $\epsilon$ -amino group of lysine during reaction (Corzo-Martinez et al., 2008; Jimenez-Castano et al., 2007). The effect of DHPM treatment was mainly noted at  $\beta$ -Lg-galactose mixture treated at 80, 120, and 160 MPa, respectively. The significant decrease in  $H_0$  suggested that DHPM treatment may induce important structural changes in the 3D configuration of the protein. The varying of  $H_0$  did not relate to the changes of IgE-binding capacity;

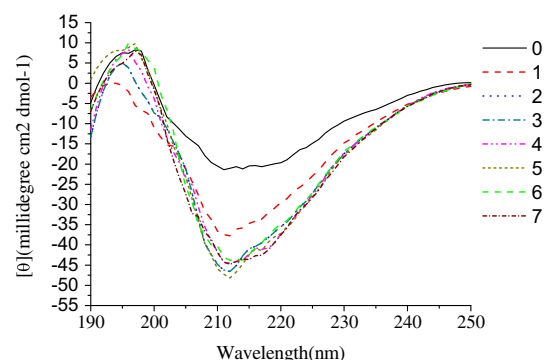


**Fig. 5.** CD spectra of heated  $\beta$ -Lg and  $\beta$ -Lg-galactose conjugates. 0: Heated  $\beta$ -Lg; 1:  $\beta$ -Lg-galactose mixture treated by DHPM at 0 MPa; 2–4:  $\beta$ -Lg pretreated by DHPM at pressures 80, 120, and 160 MPa, and then galactose was added, respectively; 5–7:  $\beta$ -Lg-galactose mixture treated by DHPM at pressures 80, 120, and 160 MPa, respectively.

this could be that surface hydrophobicity may be induced by the cleavage of hydrogen bonds and van der Waals forces and exposure of hydrophilic amino acid residues in the inner site of protein after glycation coupled to DHPM treatment. (Hattori, Ogino, Nakai, & Takahashi, 1997).

### 3.6. CD spectra

CD spectra for the native  $\beta$ -Lg and  $\beta$ -Lg-galactose conjugates are shown in Fig. 5 and Table 2.  $\beta$ -Lg is a protein composed predominantly of  $\beta$ -sheets with a markedly high helical propensity. The secondary structure of heated  $\beta$ -Lg is composed of 35.1%  $\beta$ -sheets, 11.8%  $\alpha$ -helix, 21.7% turns, and 31.5% random coil. The results were in agreement with those of previous studies and X-ray crystal structures (Kelly, Jess, & Price, 2005; Qi et al., 1997). When  $\beta$ -Lg was conjugated with galactose after DHPM treatment, secondary structural changes were observed. A reduction of  $\beta$ -sheets and an increase in  $\alpha$ -helix were induced by glycation and DHPM treatment.

The effect of glycation combined with DHPM treatment on the secondary structure of  $\beta$ -Lg subjected to the sugar used, the level of glycation, reaction time, and water content. Several studies reported that glycation results in a small alteration of the secondary structure of protein (Wong, Day, McNaughton, & Augustin, 2009). However, according to other works, glycation induces the  $\alpha$ -helix and the highly ordered structure content increases, mainly at the expense of  $\beta$ -sheet or random coil content (Liu, Ru, & Ding, 2012). In present study, when  $\beta$ -Lg-galactose conjugates were treated with 0 MPa of DHPM, slight changes were observed on the protein secondary structure. A reduction of  $\beta$ -sheets and an increase in  $\alpha$ -helix content were induced by the glycation with DHPM treatment. When  $\beta$ -Lg and  $\beta$ -Lg-galactose mixture were treated by DHPM at 80, 120, and 160 MPa, the intensity of negative molar residue ellipticity peaks at 212 nm decreased, the spectrum of positive molar residue ellipticity maximums showed a slight changes. Considine, Patel, Singh, and Creamer (2007) reported that the trough at 216 nm broadened and deepened, resulting in an unfolding state under increasing temperature and pressure.

The IgE-binding capacity of  $\beta$ -Lg is related to the 3D structure of protein, and allergic is related to both linear and conformational epitopes of  $\beta$ -Lg (Selo et al., 1998). The major  $\beta$ -Lg allergenic epitopes identified are the fragments of AA102–124, AA41–60, and AA149–162 (Selo et al.,

**Table 1**  
Surface hydrophobicity ( $H_0$ ) of  $\beta$ -Lg (heated) and glycosylated  $\beta$ -Lg.

| Sample                           | 0       | 1       | 2       | 3       | 4       | 5       | 6       | 7       |
|----------------------------------|---------|---------|---------|---------|---------|---------|---------|---------|
| Surface hydrophobicity ( $H_0$ ) | 1970.52 | 1886.23 | 1818.51 | 1960.11 | 1908.69 | 1595.43 | 1552.17 | 1718.46 |

0: Heated  $\beta$ -Lg; 1:  $\beta$ -Lg-galactose mixture treated by DHPM at 0 MPa; 2–4:  $\beta$ -Lg pretreated by DHPM at 80, 120 and 160 MPa, and then galactose was added, respectively; 5–7:  $\beta$ -Lg-galactose mixture treated by DHPM at 80, 120 and 160 MPa, respectively.

**Table 2**  
The percentage content of secondary structure of samples.

| Sample | Secondary structure          |                               |                             |                             |
|--------|------------------------------|-------------------------------|-----------------------------|-----------------------------|
|        | $\alpha$ -Helix(%)           | $\beta$ -Strands(%)           | Turns(%)                    | Unordered(%)                |
| 0      | 11.8 $\pm$ 0.6 <sup>a</sup>  | 35.1 $\pm$ 2.7 <sup>d</sup>   | 21.7 $\pm$ 0.7 <sup>a</sup> | 31.5 $\pm$ 1.5 <sup>a</sup> |
| 1      | 15.2 $\pm$ 0.2 <sup>a</sup>  | 30.0 $\pm$ 1.3 <sup>c</sup>   | 23.9 $\pm$ 0.6 <sup>b</sup> | 31.1 $\pm$ 1.6 <sup>a</sup> |
| 2      | 18.8 $\pm$ 1.6 <sup>c</sup>  | 31.0 $\pm$ 0.6 <sup>c</sup>   | 22.0 $\pm$ 0.4 <sup>a</sup> | 28.1 $\pm$ 1.6 <sup>a</sup> |
| 3      | 20.3 $\pm$ 0.3 <sup>d</sup>  | 26.3 $\pm$ 1.2 <sup>abc</sup> | 22.6 $\pm$ 1.1 <sup>a</sup> | 30.7 $\pm$ 2.1 <sup>a</sup> |
| 4      | 20.4 $\pm$ 0.8 <sup>d</sup>  | 29.4 $\pm$ 2.8 <sup>bc</sup>  | 22.3 $\pm$ 0.2 <sup>a</sup> | 27.9 $\pm$ 3.1 <sup>a</sup> |
| 5      | 20.3 $\pm$ 0.3 <sup>d</sup>  | 25.6 $\pm$ 1.7 <sup>a</sup>   | 22.6 $\pm$ 0.6 <sup>a</sup> | 31.4 $\pm$ 2.1 <sup>a</sup> |
| 6      | 20.0 $\pm$ 0.6 <sup>cd</sup> | 28.1 $\pm$ 1.9 <sup>abc</sup> | 22.8 $\pm$ 0.7 <sup>b</sup> | 29.0 $\pm$ 1.7 <sup>a</sup> |
| 7      | 19.6 $\pm$ 0.1 <sup>cd</sup> | 29.2 $\pm$ 0.9 <sup>bc</sup>  | 22.3 $\pm$ 0.5 <sup>a</sup> | 28.8 $\pm$ 0.7 <sup>a</sup> |

Different letters (a–d) in the same line means significant difference ( $p < 0.05$ ); 0: Heated  $\beta$ -Lg; 1:  $\beta$ -Lg-galactose treated by DHPM at 0 MPa; 2–4:  $\beta$ -Lg pretreated by DHPM at 80, 120 and 160 MPa, and then galactose was added, respectively; 5–7:  $\beta$ -Lg-galactose mixture treated by DHPM at 80, 120 and 160 MPa, respectively.

1999). The AA41–60 and AA102–124 form loops between  $\beta$ -strands C and D, which are stabilized by either hydrogen or disulfide bonds (Wal, 2001). The fragment AA149–162 located at the C terminal and buried in the molecule in the native state forms a flexible turn structure; this fragment is inaccessible to IgE and IgG antibodies unless the structure is denatured (Kleber, Krause, Illgner, & Hinrichs, 2004). Therefore, the IgE binding to epitopes of  $\beta$ -Lg is linked to its secondary or tertiary structure. The results of CD spectra illustrated the alteration of the secondary structure of  $\beta$ -Lg. As the secondary structure changed with the unfolding and aggregation of protein induced by glycation and DHPM treatment, some epitopes may be masked or disrupted after glycation coupled to DHPM treatment, which might explain the weak IgE binding capacity of  $\beta$ -Lg-galactose samples submitted to DHPM treatment.

#### 4. Conclusions

In this study, DHPM treatment and glycation was used to investigate the IgE-binding capacity and conformational changes of  $\beta$ -Lg. The results showed that processing which altered the structure of a protein may have an influence on its capacity to be recognized by antibodies. Alteration of the structure of  $\beta$ -Lg or galactose induced by DHPM treatment, masking of epitopes caused by reaggregation and conjugates after glycation, may explain the decrease in the IgE-binding capacity of  $\beta$ -Lg-galactose conjugations. Different DHPM treatment methods (pretreatment of  $\beta$ -Lg and treatment of  $\beta$ -Lg-galactose mixture), made a different degree of reduction in the IgE-binding capacity. Therefore, pretreatment by DHPM and glycation with galactose was a promising method for eliminating the IgE-binding capacity of  $\beta$ -Lg. Although glycation combined with DHPM can influence the IgE-binding ability, the glycation reaction extent and glycation sites under DHPM treatment needed to be investigated using other methods.

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#### References

- Baez, G. D., Busti, P. A., Verdini, R., & Delorenzi, N. J. (2013). Glycation of heat-treated beta-lactoglobulin: Effects on foaming properties. *Food Research International*, 54(1), 902–909.
- Bouaouina, H., Desrumaux, A., Loisel, C., & Legrand, J. (2006). Functional properties of whey proteins as affected by dynamic high-pressure treatment. *International Dairy Journal*, 16(4), 275–284.
- Bunn, H. F., & Higgins, P. J. (1981). Reaction of monosaccharides with proteins - Possible evolutionary significance. *Science*, 213(4504), 222–224.

- Chanasattru, W., Decker, E. A., & McClements, D. J. (2007). Modulation of thermal stability and heat-induced gelation of beta-lactoglobulin by high glycerol and sorbitol levels. *Food Chemistry*, 103(2), 512–520.
- Chen, Y. J., Chen, X. X., Guo, T. L., & Zhou, P. (2015). Improving the thermostability of beta-lactoglobulin via glycation: The effect of sugar structures. *Food Research International*, 69, 106–113.
- Chen, J., Liang, R. H., Liu, W., Li, T., Liu, C. M., Wu, S. S., et al. (2013). Pectic-oligosaccharides prepared by dynamic high-pressure microfluidization and their in vitro fermentation properties. *Carbohydrate Polymers*, 91(1), 175–182.
- Chevalier, F., Chobert, J. M., Dalgalarondo, M., Choiset, Y., & Haertle, T. (2002). Maillard glycation of beta-lactoglobulin induces conformation changes. *Nahrung - Food*, 46(2), 58–63.
- Cocco, R. R., Jarvinen, K. M., Han, N., Beyer, K., & Sampson, H. A. (2007). Mutational analysis of immunoglobulin E-binding epitopes of beta-casein and beta-lactoglobulin showed a heterogeneous pattern of critical amino acids between individual patients and pooled sera. *Clinical and Experimental Allergy*, 37(6), 831–838.
- Considine, T., Patel, H. A., Singh, H., & Creamer, L. K. (2005). Influence of binding of sodium dodecyl sulfate, all-trans-retinol, palmitate, and 8-anilino-1-naphthalenesulfonate on the heat-induced unfolding and aggregation of beta-lactoglobulin B. *Journal of Agricultural and Food Chemistry*, 53(8), 3197–3205.
- Considine, T., Patel, H. A., Singh, H., & Creamer, L. K. (2007). Influence of binding conjugated linoleic acid and myristic acid on the heat- and high-pressure-induced unfolding and aggregation of beta-lactoglobulin B. *Food Chemistry*, 102(4), 1270–1280.
- Corzo-Martinez, M., Moreno, F. J., Olano, A., & Villamiel, M. (2008). Structural characterization of bovine beta-lactoglobulin-galactose/tagatose Maillard complexes by electrophoretic, chromatographic, and spectroscopic methods. *Journal of Agricultural and Food Chemistry*, 56(11), 4244–4252.
- Croguennec, T., Renault, A., Bouhallab, S., & Pezenne, S. (2006). Interfacial and foaming properties of sulfhydryl-modified bovine beta-lactoglobulin. *Journal of Colloid and Interface Science*, 302(1), 32–39.
- Divsalar, A., Saboury, A. A., Ahmad, F., & Moosavi-Movahedi, A. A. (2009). Effects of temperature and chromium (III) ion on the structure of bovine beta-lactoglobulin-A. *Journal of the Brazilian Chemical Society*, 20(10), 1782–1789.
- Enomoto, H., Nagae, S., Hayashi, Y., Li, C. P., Ibrahim, H. R., Sugimoto, Y., et al. (2009). Improvement of functional properties of egg white protein through glycation and phosphorylation by dry-heating. *Asian-Australasian Journal of Animal Sciences*, 22(4), 591–597.
- Gruber, P., Vieths, S., Wangorsch, A., Nerkamp, J., & Hofmann, T. (2004). Maillard reaction and enzymatic browning affect the allergenicity of Pru av 1, the major allergen from cherry (*Prunus avium*). *Journal of Agricultural and Food Chemistry*, 52(12), 4002–4007.
- Guan, Y. G., Lin, H., Han, Z., Wang, J., Yu, S. J., Zeng, X. A., et al. (2010). Effects of pulsed electric field treatment on a bovine serum albumin-dextran model system, a means of promoting the Maillard reaction. *Food Chemistry*, 123(2), 275–280.
- Haskard, C. A., & Li-Chan, E. C. Y. (1998). Hydrophobicity of bovine serum albumin and ovalbumin determined using uncharged (PRODAN) and anionic (ANS(-)) fluorescent probes. *Journal of Agricultural and Food Chemistry*, 46(7), 2671–2677.
- Hattori, M., Ogino, A., Nakai, H., & Takahashi, K. (1997). Functional improvement of beta-lactoglobulin by conjugating with alginate lyase-lysate. *Journal of Agricultural and Food Chemistry*, 45(3), 703–708.
- Hayes, M. G., Fox, P. F., & Kelly, A. L. (2005). Potential applications of high pressure homogenisation in processing of liquid milk. *Journal of Dairy Research*, 72(1), 25–33.
- Huang, X. Q., Tu, Z. C., Wang, H., Zhang, Q. T., Hu, Y. M., Zhang, L., et al. (2013). Glycation promoted by dynamic high pressure microfluidisation pretreatment revealed by high resolution mass spectrometry. *Food Chemistry*, 141(3), 3250–3259.
- Jarvinen, K. M., Chatchatee, P., Bardina, L., Beyer, K., & Sampson, H. A. (2001). IgE and IgG binding epitopes on alpha-lactalbumin and beta-lactoglobulin in cow's milk allergy. *International Archives of Allergy and Immunology*, 126(2), 111–118.
- Jimenez-Castano, L., Villamiel, M., & Lopez-Fandino, R. (2007). Glycosylation of individual whey proteins by Maillard reaction using dextran of different molecular mass. *Food Hydrocolloids*, 21(3), 433–443.
- Kelly, S. M., Jess, T. J., & Price, N. C. (2005). How to study proteins by circular dichroism. *Biochimica Et Biophysica Acta-Proteins and Proteomics*, 1751(2), 119–139.
- Kleber, N., Krause, I., Illgner, S., & Hinrichs, J. (2004). The antigenic response of beta-lactoglobulin is modulated by thermally induced aggregation. *European Food Research and Technology*, 219(2), 105–110.
- Liu, W., Liu, J. H., Xie, M. Y., Liu, C. M., Liu, W. L., & Wan, J. (2009). Characterization and high-pressure microfluidization-induced activation of Polyphenoloxidase from Chinese pear (*Pyrus pyrifolia* Nakai). *Journal of Agricultural and Food Chemistry*, 57(12), 5376–5380.
- Liu, J. H., Ru, Q. M., & Ding, Y. T. (2012). Glycation a promising method for food protein modification: Physicochemical properties and structure, a review. *Food Research International*, 49(1), 170–183.
- Lopez-Exposito, I., Chicon, R., Belloque, J., Lopez-Fandino, R., & Berin, M. C. (2012). In vivo methods for testing allergenicity show that high hydrostatic pressure hydrolysates of beta-lactoglobulin are immunologically inert. *Journal of Dairy Science*, 95(2), 541–548.
- Manderson, G. A., Hardman, M. J., & Creamer, L. K. (1999). Effect of heat treatment on bovine beta-lactoglobulin A, B, and C explored using thiol availability and fluorescence. *Journal of Agricultural and Food Chemistry*, 47(9), 3617–3627.
- Moreno, F. J., Molina, E., Olano, A., & Lopez-Fandino, R. (2003). High-pressure effects on Maillard reaction between glucose and lysine. *Journal of Agricultural and Food Chemistry*, 51(2), 394–400.
- Nakamura, A., Sasaki, F., Watanabe, K., Ojima, T., Ahn, D. H., & Saeki, H. (2006). Changes in allergenicity and digestibility of squid tropomyosin during the Maillard reaction with ribose. *Journal of Agricultural and Food Chemistry*, 54(25), 9529–9534.
- Niemi, M., Jylha, S., Laukkanen, M. L., Soderlund, H., Makinen-Kiljunen, S., Kallio, J. M., et al. (2007). Molecular interactions between a recombinant IgE antibody and the beta-lactoglobulin allergen. *Structure*, 15(11), 1413–1421.

- Oliver, C. M., Melton, L. D., & Stanley, R. A. (2006). Creating proteins with novel functionality via the Maillard reaction: A review. *Critical Reviews in Food Science and Nutrition*, 46(4), 337–350.
- Papiz, M. Z., Sawyer, L., Eliopoulos, E. E., North, A. C. T., Findlay, J. B. C., Sivaprasadarao, R., et al. (1986). The structure of beta-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature*, 324(6095), 383–385.
- Qi, X. L., Holt, C., McNulty, D., Clarke, D. T., Brownlow, S., & Jones, G. R. (1997). Effect of temperature on the secondary structure of beta-lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: A test of the molten globule hypothesis. *Biochemical Journal*, 324, 341–346.
- Rahaman, T., Vasiljevic, T., & Ramchandran, L. (2015). Conformational changes of beta-lactoglobulin induced by shear, heat, and pH-effects on antigenicity. *Journal of Dairy Science*, 98(7), 4255–4265.
- Rahaman, T., Vasiljevic, T., & Ramchandran, L. (2016). Effect of processing on conformational changes of food proteins related to allergenicity. *Trends in Food Science & Technology*, 49, 24–34.
- Schagger, H., Borchart, U., Machleidt, W., Link, T. A., & Vonjagow, G. (1987). Isolation and amino acid sequence of the 'Rieske' iron sulfur protein of beef-heart ubiquinol: Cytochrome C reductase. *FEBS Letters*, 219(1), 161–168.
- Selo, I., Clement, G., Bernard, H., Chatel, J. M., Creminon, C., Peltre, G., et al. (1999). Allergy to bovine beta-lactoglobulin: Specificity of human IgE to tryptic peptides. *Clinical and Experimental Allergy*, 29(8), 1055–1063.
- Selo, I., Negroni, L., Creminon, C., Yvon, M., Peltre, G., & Wal, J. M. (1998). Allergy to bovine beta-lactoglobulin: Specificity of human IgE using cyanogen bromide-derived peptides. *International Archives of Allergy and Immunology*, 117(1), 20–28.
- Shen, L., & Tang, C. H. (2012). Microfluidization as a potential technique to modify surface properties of soy protein isolate. *Food Research International*, 48(1), 108–118.
- Shi, W. H., Sun, W. W., Yu, S. J., & Zhao, M. M. (2010). Study on the characteristic of bovine serum albumin-glucose model system, treated by ultrasonic. *Food Research International*, 43(8), 2115–2118.
- Taheri-Kafrani, A., Gaudin, J. C., Rabesona, H., Nioi, C., Agarwal, D., Drouet, M., et al. (2009). Effects of heating and glycation of beta-lactoglobulin on its recognition by IgE of sera from cow milk allergy patients. *Journal of Agricultural and Food Chemistry*, 57(11), 4974–4982.
- Tang, C. H., Sun, X., & Foegeding, E. A. (2011). Modulation of physicochemical and conformational properties of kidney bean vicilin (Phaseolin) by glycation with glucose: Implications for structure-function relationships of legume vicilins. *Journal of Agricultural and Food Chemistry*, 59(18), 10114–10123.
- Thomsen, M. K., Olsen, K., Otte, J., Sjöström, K., Werner, B. B., & Skibsted, L. H. (2012). Effect of water activity, temperature and pH on solid state lactosylation of beta-lactoglobulin. *International Dairy Journal*, 23(1), 1–8.
- Verheul, M., Roefs, S. P. F. M., & de Kruif, K. G. (1998). Kinetics of heat-induced aggregation of beta-lactoglobulin. *Journal of Agricultural and Food Chemistry*, 46(3), 896–903.
- Wal, J. M. (2001). Structure and function of milk allergens. *Allergy*, 56, 35–38.
- Wong, B. T., Day, L., McNaughton, D., & Augustin, M. A. (2009). The effect of maillard conjugation of deamidated wheat proteins with low molecular weight carbohydrates on the secondary structure of the protein. *Food Biophysics*, 4(1), 1–12.
- Wong, K. L., Li, H., Wong, K. K. K., Jiang, T., & Shaw, P. C. (2012). Location and reduction of icarapin antigenicity by site specific coupling to polyethylene glycol. *Protein and Peptide Letters*, 19(2), 238–243.
- Zhong, J. Z., Liu, W., Liu, C. M., Wang, Q. H., Li, T., Tu, Z. C., et al. (2012). Aggregation and conformational changes of bovine beta-lactoglobulin subjected to dynamic high-pressure microfluidization in relation to antigenicity. *Journal of Dairy Science*, 95(8), 4237–4245.
- Zhong, J. Z., Tu, Y., Liu, W., Luo, S. J., & Liu, C. M. (2015). Comparative study on the effects of nystose and fructofuranosyl nystose in the glycation reaction on the antigenicity and conformation of beta-lactoglobulin. *Food Chemistry*, 188, 658–663.
- Zhong, J. Z., Tu, Y., Liu, W., Xu, Y. J., Liu, C. M., & Dun, R. Y. (2014). Antigenicity and conformational changes of beta-lactoglobulin by dynamic high pressure microfluidization combining with glycation treatment. *Journal of Dairy Science*, 97(8), 4695–4702.