Antigenicity and conformational changes of β -lactoglobulin by dynamic high pressure microfluidization combining with glycation treatment

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ABSTRACT

The combined effect of previous dynamic high-pressure microfluidization treatment (40, 80, 120, and 160 MPa) and subsequent glycation with galacto-oligosaccharides (GOS) on the antigenicity of β -lactoglobulin (β -LG) was investigated. The antigenicity of β -LG-GOS decreased at relatively low pressure (≤ 120 MPa). Surface sulfhydryl group content of β -LG-GOS increased and surface hydrophobicity of β -LG-GOS decreased. Additionally, protein unfolding in β -LG-GOS samples was reflected by quenching of fluorescence intensity, the red-shift of fluorescence spectra, decreased UV absorption, and circular dichroism analysis, indicating tertiary and secondary structural changes of β -LG. The conformational changes may contribute to the alteration of antigenicity.

Key words: β -lactoglobulin, antigenicity, dynamic high-pressure microfluidization combined with glycation, conformational change

INTRODUCTION

β -Lactoglobulin, the major whey protein, is a globular protein with a molecular mass of about 18.4 kDa, 2 disulfide bridges, and 1 residue of free Cys (Papiz et al., 1986). It is extensively used in food formulations due to its nutritive, biological, and functional properties (Akita and Nakai, 1990; Aguilera and Lillford, 2008). As a functional additive, β -LG is one of the major allergens that causes milk allergy (Exl and Fritsche, 2001). As antigenicity limits the application of β -LG in the food industry, numerous attempts have been made to reduce its antigenicity through physical treatments, such as heat treatment (Iametti et al., 2002) and high hydrostatic pressure treatment (López-Expósito et al., 2012); chemical treatments, such as glycation and phosphorylation (Enomoto et al., 2009; Li et al., 2011); and enzymatic treatments (Kim et al., 2007).

In recent years, a great deal of attention has been focused on glycation to obtain modified proteins. Glycation with polysaccharides, such as dextran (Hattori, 2002) and carboxymethyldextran (Hattori et al., 2000), and monosaccharides, such as galactose (Chevalier et al., 2001b) and glucose (Hiller and Lorenzen, 2010), have been reported, some of which were found to reduce antigenicity effectively. Our research group previously reported that glycation with fructo-oligosaccharides reduced antigenicity of β -LG (Zhong et al., 2013). We also reported that physical treatment with dynamic high-pressure microfluidization (DHPM) altered the antigenicity of β -LG (Zhong et al., 2012). However, little information is known about the effect of DHPM combined with subsequent glycation with galacto-oligosaccharides (GOS) on antigenicity and conformational changes of β -LG. The aim of the current study was to understand the effects of DHPM combined with glycation treatment on the antigenicity of β -LG by indirect competitive ELISA and conformational changes, such as free sulfhydryl content, surface hydrophobicity, UV absorption spectra, intrinsic emission fluorescence, and circular dichroism spectra of β -LG.

MATERIALS AND METHODS

Materials

β -Lactoglobulin standard (L3908), GOS, and anti-rabbit IgG (A6154) were obtained from Sigma Chemical Company (St. Louis, MO). Galacto-oligosaccharides are nondigestible oligosaccharides comprising 2 to 20 molecules of galactose and 1 molecule of glucose. All other reagents were of analytical grade or better.

DHPM Combined with Glycation Treatment

According to our previous report (Zhong et al., 2011), DHPM treatment of β -LG was conducted under the following conditions. β -Lactoglobulin standard solutions (1 mg/mL) were treated to 3 passes at pressures of 0.1, 40, 80, 120, and 160 MPa in a Microfluidizer processor (model M-110EH-30, Microfluidics, Newton, MA). Samples were collected and glycated with GOS immediately.

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According to the methods of Chevalier et al. (2001) and Zhong et al. (2013), with some modifications, glycation with GOS was conducted under the following conditions. The mass ratios of β -LG and GOS (1:4) were dissolved in 600 mL of distilled water and then lyophilized. The mixtures were incubated at 50°C at a relative humidity of 79% for 24 h. After dialysis against distilled water and lyophilization, a crude β -LG-GOS conjugate was obtained. Free oligosaccharides were removed by salting out. The crude sample was dissolved in distilled water, and ammonium sulfate was added to a final concentration of 5 *M*. The precipitate was recovered by centrifugation ($48,400 \times g$ for 30 min) at 20°C. The purified conjugate was obtained by the molecular weight cut-off of dialysis tubing (14,000 Da) from Solarbio Chemical Company (Beijing, China) against distilled water and lyophilization. All glycation experiments were performed in triplicate.

β -Lactoglobulin heated at conjugate conditions without sugar (heated control) was named β -LG (control), and unprocessed β -LG-GOS conjugate was named β -LG-GOS (0.1 MPa). Samples glycosylated with GOS after DHPM treatment at 40, 80, 120, 160 MPa were named β -LG-GOS (40 MPa), β -LG-GOS (80 MPa), β -LG-GOS (120 MPa), and β -LG-GOS (160 MPa), respectively.

SDS-PAGE Analysis

The SDS-PAGE was carried out to confirm conjugate formation, using a 12% acryl amide separating gel and a 4% acryl amide stacking gel, as used by Balti et al. (2009) and Liu et al. (2010). Electrophoresis was run with a 5% (wt/vol) stacking gel and 15% (wt/vol) separating gel, with currents of 15 and 35 mA, respectively. Samples were prepared by mixing β -LG at a 1:1 (vol/vol) ratio with pH 6.8, 0.5 *M* Tris-HCl, 2 mg of SDS, 2 μ L of glycerol, 2 μ L of β -mercaptoethanol, and 0.04 mg of bromophenol blue. Samples were heated at 100°C for 5 to 10 min before electrophoresis. After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 (Sangon Biotech Co., Ltd., Shanghai, China) for at least 30 min in 25% methanol and 10% acetic acid and destained with 5% methanol and 7.5% acetic acid. The molecular weight markers used were 14, 20, 29, 44, 66, and 97 kDa.

Antigenicity Analysis

The antigenicity of different β -LG samples was estimated by an indirect competitive ELISA (Wroblewska et al., 2004; Zhong et al., 2011). Polyclonal IgY antibodies of rabbit, monospecific for β -LG, were applied, detecting the antigenic response of β -LG (Kleber et al., 2004).

Microtiter plates with 96 wells (flat-bottomed; Costar, Corning, Corning, NY) were coated with 100 μ L/well of 1 μ g/mL of antigen and were incubated overnight at 4°C. Residual free binding sites were blocked with 1% pig gelatin in PBS/Tween solution (**PBST**) for 30 min at room temperature. The wells of the plates were washed 6 times with PBST. Competition was initiated by adding 50 μ L of either the standard or a sample solution containing 0.25 to 50 μ g/mL of β -LG in PBST and 50 μ L of anti- β -LG-IgY (10 μ g/mL) to the wells. The plates were incubated overnight at 4°C. After removing the solutions, the wells were washed 6 times with PBST. A solution of 100 μ L of purified anti-rabbit IgG (A6154, Sigma Chemical Co.), diluted 1:10,000 in PBST, was added and the plates were incubated for 2 h at 37°C. After washing, 50 μ L of H₂O₂-urea solution and 50 μ L of tetramethylenbenzidine solution were immediately added to each well. The reaction was stopped by adding 100 μ L of 1 *M* sulfuric acid. Absorption was measured at 450 nm.

The antigenic response of the samples was calculated from a standard curve of β -LG, for which a linear logarithmic correlation was observed in the range of 0.01 to 400 μ g/mL for β -LG. All analyses were carried out in triplicate and the averaged values were converted to concentration equivalents in micrograms per milliliter.

Determination of Surface Sulfhydryl Group Content

Surface sulfhydryl group (**SH**) contents of protein samples were determined using Ellman's reagent (Ellman, 1959; Sava et al., 2005; Zhong et al., 2012). Free SH contents for β -LG were obtained by adding β -LG samples (1 mg/mL) in 0.1 *M*, pH 8.0 sodium phosphate buffer. The above solutions were incubated at 25°C for 1 h, and then 50 μ L of each sample was extracted from the solutions after 1 h and 25 μ L of 0.01 *M* 5,5'-dithiobis-2-nitrobenzoic acid was added to it. The solution was then diluted to 10 mL and UV absorbance at 412 nm was measured at 25°C. The SH contents were calculated by the use of extinction coefficient of 2-nitro-5-thiobenzoate at 412 nm, 13,600 *M* per centimeter, expressed as micromoles per gram of protein.

Determination of Surface Hydrophobicity

Surface hydrophobicity (**Ho**) was determined with the fluorescence probe 1-anilinonaphthalene-8-sulfonate (**ANS**[−]; Sigma Chemical Co.) according to the method of Haskard and Li-Chan (1998) and used by Shen and Tang (2012). Serial dilutions in 0.01 *M* phosphate buffer (pH 7.0) were prepared with the protein samples (stock solutions; 1.5% wt/vol) to a final concentration of 0.004 to 0.02% (wt/vol). The ANS[−] solution (8.0 mM) was

also prepared in the same buffer. Twenty microliters of ANS[−] solution was added to 4 mL of each dilution and fluorescence intensity of the mixture was measured at 370 (excitation) and 470 nm (emission) using an F4500 fluorescence-spectrophotometer (Hitachi, Tokyo, Japan). The initial slope of the fluorescence intensity versus protein concentration (mg/mL) plot (calculated by linear regression analysis) was used as an index of Ho.

Intrinsic Fluorescence and UV Absorption Spectra Analysis

Fluorescence analyses were performed for samples. Fluorescence measurements were carried out in a Hitachi spectrofluorimeter (F-4500, Hitachi) using 10-mm² quartz cells and a protein concentration of 1 mg/mL in phosphate buffer (10 mM, pH 7.0). The excitation wavelength was 280 nm; the emission spectrum was scanned from 300 to 450 nm, using 5-nm bandwidth, with excitation and emission slits of 2.5 and 5.0 nm, respectively, and at a scan speed of 240 nm/min (de la Hoz and Netto, 2008; Zhong et al., 2012). The UV absorption spectra of samples were measured by a UV-VIS 2510PC spectrophotometer (Shimadzu, Kyoto, Japan); UV absorption spectra were scanned from 200 to 360 nm.

Circular Dichroism Analysis

Circular dichroism (CD) spectroscopy analyses were run for different samples according to Divsalar et al. (2009). The CD spectra of samples at 0.2 mg/mL for far-UV in 10 mM sodium phosphate buffer, pH 7.0, were collected using a MOS-450 spectropolarimeter (French Bio-Logic SAS Co., Claix, France), at 22°C. A cylindrical quartz cuvette with a path length of 0.1 cm was used for collecting data in the far-UV (185–250 nm) region. The scanning condition was 1-nm step resolution for far-UV; both scans were performed using a 100-nm/min scan rate, 1.0 nm of bandwidth, and 4 scans, corrected by subtracting the buffer baseline spectrum. Structure predictions from CD spectra were obtained using the Contin LL program (de la Hoz and Netto, 2008; Zhong et al., 2012).

Statistical Analysis

The one-way ANOVA test for significant effects of treatments and assays was determined using SPSS 12.0.1 (SPSS Inc., Chicago, IL). Main effect differences were considered significant at $P \leq 0.05$. Mean separations were determined by Tukey's procedure for multiple comparisons. All experiments (CD analysis 4 times) were analyzed in triplicate.

RESULTS AND DISCUSSION

Changes of Antigenicity of β -LG-GOS

The antigenicity of β -LG-GOS samples were shown in Figure 1. It depicted the antigenicity values of samples obtained through glycation with GOS after DHPM treatment under 40, 80, 120, and 160 MPa. The antigenicity of β -LG (control) was 165.8 μ g/mL. It was showed that after glycation with GOS, the antigenicity of β -LG-GOS (0.1 MPa; 45.2 μ g/mL) decreased remarkably. The observed antigenicity decrease of protein after glycation was similar to that of β -LG modified by acidic oligosaccharides (Hattori et al., 2004), carboxymethyl dextran (Kobayashi, 2001), maltose (Li et al., 2011), and fructo-oligosaccharides (Zhong et al., 2013). Glycation with oligosaccharides was a significantly effective way to reduce antigenicity. The reason for reduced antigenicity of β -LG may be the conjugation of reducing carbohydrates to the ϵ -amino group of Lys during glycation and masking of some epitopes during glycation (Kobayashi, 2001; Hattori et al., 2004; Meltretter et al., 2007).

In our previous research, DHPM-modified β -LG standards showed a remarkable increase in antigenicity when treated to 80 MPa. However, in the current work, DHPM combined with glycation altered antigenicity according to pressure levels. At relatively low pressures (≤ 120 MPa), antigenicity of β -LG-GOS was reduced and reached the greatest degree of reduction in β -LG-GOS at 80 MPa (8.7 μ g/mL), which showed

Figure 1. Antigenicity of β -LG (control), β -LG with galacto-oligosaccharides (GOS; 0.1 MPa), β -LG-GOS (40 MPa), β -LG-GOS (80 MPa), β -LG-GOS (120 MPa), and β -LG-GOS (160 MPa). Different letters (a–d) denote significant differences across different treatments ($P \leq 0.05$).

much lower antigenicity than those obtained by the 40 (18.7 $\mu\text{g/mL}$) and 120 MPa (10.8 $\mu\text{g/mL}$) treatment. But we were surprised to find that when the pressure grew to 160 MPa, the antigenicity increased to 331 $\mu\text{g/mL}$ and the value was about 2 times higher than that of β -LG (control). It could be seen that changes in the β -LG antigenicity might largely depend on the glycation reaction conditions and processing treatments.

Changes of Molecular Weight of β -LG-GOS

The SDS-PAGE gel of samples with β -mercaptoethanol was shown in Figure 2. As it was seen, molecular weight of β -LG (control) was 18.4 kDa. After glycation with GOS, the bands of β -LG-GOS (0.1 MPa), β -LG-GOS (40 MPa), β -LG-GOS (80 MPa), β -LG-GOS (120 MPa), and β -LG-GOS (160 MPa) conjugates moved further (almost 20.1 kDa) in the gel compared with control sample. The band of treated samples with higher molecular weight indicated the formation of β -LG-GOS conjugates compared with control β -LG. Broader and higher molecular weight bands were also observed by Qi et al. (2009) and Zhu et al. (2010) in proteins incubated with dextran. We previously reported that molecular weights of β -LG increased after glycation with fructo-oligosaccharides, too (Zhong et al., 2013). However, no noticeable differences in SDS-PAGE were observed among β -LG-GOS samples under different pressures. It was suggested that DHPM pretreatment mainly changed the conjugation reactions rather than the conjugation numbers between protein and sugar. Meanwhile, the different conjugation reactions may have resulted from different conformational changes of β -LG induced by DHPM pretreatment. In addition, according to Nakamura et al. (2005), antigenicity would be closely related to the conformational changes. To gain a better understanding, the conformational changes of 4 samples among β -LG (control), β -LG-GOS (0.1 MPa), β -LG-GOS (80 MPa), and β -LG-GOS (160 MPa) should be further examined.

Changes of Surface SH Content and Ho of β -LG-GOS

Surface SH content has been evaluated as an index of structural prediction of protein. In this work, the SH group content was found to be $52.44 \pm 1.97 \mu\text{mol/g}$ of protein in β -LG (control; Table 1). The surface SH content decreased to $50.25 \pm 1.93 \mu\text{mol/g}$ when glycated with GOS ($P \leq 0.05$). The result was in accordance with Medrano et al. (2009), who found surface SH content decreased when β -LG was glycated with lactose. In our previous research (Zhong et al., 2012), after DHPM treatment of β -LG, the SH content was increased at 80 MPa and decreased at 160 MPa compared with na-

tive β -LG. It was found that conformational changes of the β -LG molecule occurred in different degrees when treated at 80 and 160 MPa. But the situation was different when samples were treated by DHPM combining with glycation, which resulted in the increase of surface SH content. Surface SH content of β -LG-GOS (80 MPa) and β -LG-GOS (160 MPa) increased to 55.67 ± 0.41 and $58.18 \pm 1.71 \mu\text{mol/g}$, respectively, and values were higher than β -LG (control). The new increased SH group may be the result of cleavage of a disulfide bond into the surface. The increase in SH group content indicated conformational changes of protein according to Monahan et al. (1995).

Also, quantitative analysis of protein surface hydrophobicity is essential for accurate prediction of protein (Leonil et al., 1997). Surface hydrophobicity decreased during glycation. As shown in Table 1, compared with β -LG (control), the Ho of β -LG-GOS (0.1 MPa), β -LG-GOS (80 MPa), and β -LG-GOS (160 MPa) declined from 1,048.5 to 859.81, 727.60, and 880.08, respectively. The result was in agreement with Galazka et al. (1999) and Corzo-Martínez et al. (2008), who also reported that glycated conjugates of β -LG decreased in hydrophobicity. According to van Teeffelen et al. (2005), the decline in Ho may be related with the fact that the conjugation of carbohydrates to the ϵ -amino group of Lys during glycation could bring more hydrophilic groups and lead to the blocking of Lys or Arg residues by GOS in the glycation conjugates (Sava et al., 2005; Gasymov and Glasgow, 2007). In this work, despite the minimum value of Ho found in β -LG-GOS (80 MPa), Ho change did not correlate with the varying of an-

Figure 2. Sodium dodecyl sulfate-PAGE analysis. Lane 1 = standard protein markers of different molecular weights; lanes 2, 3, 4, 5, 6, 7: β -LG (control), β -LG with galacto-oligosaccharides (GOS; 0.1 MPa), β -LG-GOS (40 MPa), β -LG-GOS (80 MPa), β -LG-GOS (120 MPa), and β -LG-GOS (160 MPa).

Table 1. Surface sulfhydryl group (SH) contents and surface hydrophobicity of β -LG (control) and β -LG with galacto-oligosaccharides (GOS)

Sample	β -LG (control)	β -LG-GOS		
		0.1 MPa	80 MPa	160 MPa
Surface SH content ($\mu\text{mol/g}$)	52.44 ± 1.97^c	50.25 ± 1.93^d	55.67 ± 0.41^b	58.78 ± 1.71^a
Surface hydrophobicity (Ho)	1,048.5	859.81	727.60	880.08

^{a-d}Different letters denote significant differences across different treatments ($P \leq 0.05$).

tigenicity because it may be induced by the cleavage of hydrogen bonds and van der Waals forces and the exposure of hydrophilic amino acid residues in the inner site of proteins (Tanaka et al., 1996).

Changes of Intrinsic Fluorescence and UV Absorption Spectroscopy of β -LG-GOS

The intrinsic fluorescence of β -LG is mainly excited from Trp residues. As shown in Figure 3a, after glycation treatment, the fluorescence intensity of β -LG-GOS (0.1 MPa), β -LG-GOS (80 MPa), and β -LG-GOS (160 MPa) decreased from 2,476 (control) to 1,684, 1,479, 1,746, respectively, and fluorescence spectra showed a 4, 6, and 7 nm red shift of the maximum emission wavelength compared with β -LG (control), respectively. In our previous research (Zhong et al., 2012), DHPM treatment contributed to an increase in relative fluorescence intensity, but a red shift of the spectral peak was not observed. It may be noted that glycation contributes more significantly to protein intrinsic fluorescence. Concerning conformational characterization of glycoconjugates, a common decrease in intensity has been observed and red-shift can be seen after glycation (Sun et al., 2004; Wooster and Augustin, 2007; Corzo-Martinez et al., 2008). Generally, the carbohydrate-binding sites in the β -LG were mainly identified to be residue Lys (Hattori et al., 2004). The decrease of fluorescence intensity in the case of glycation conjugates was considered to have been due to a shielding effect by oligosaccharide chain bound to ⁶⁰Lys in this conjugate (Hattori et al., 2004). This red shift on the fluorescence emission spectrum was due to the fact that Trp residues moved from a less-polar environment to a more-polar region (Manderson et al., 1999). Glycation caused a reduction of intensity and a shift of Trp (¹⁹Trp and ⁶¹Trp) emission maximum in fluorescence spectra, which demonstrated protein unfolding (Wooster and Augustin, 2007; Liu et al., 2012).

The results of UV absorption spectra of β -LG (control), β -LG-GOS (0.1 MPa), β -LG-GOS (80 MPa), and β -LG-GOS (160 MPa) are shown in Figure 3b. The maximum absorption peak was at 280 nm, which corresponded mainly to Tyr near 277 nm and an overlapping of Tyr and Trp near 283 nm (Kolakowski et al.,

2001). Glycation with GOS caused the reduction of UV absorption intensity. The absorption intensity of β -LG (control), β -LG-GOS (0.1 MPa), β -LG-GOS (80 MPa), and β -LG-GOS (160 MPa) at 280 nm was 0.47,

Figure 3. (a) Intrinsic fluorescent emission of β -LG (control), β -LG with galacto-oligosaccharides (GOS; 0.1 MPa), β -LG-GOS (80 MPa), and β -LG-GOS (160 MPa). (b) Ultraviolet absorption spectroscopy of β -LG (control), β -LG-GOS (0.1 MPa), β -LG-GOS (80 MPa), and β -LG-GOS (160 MPa), respectively. Color version available in the online PDF.

Table 2. The calculated percentage content of secondary structures of β -LG (control) and β -LG with galacto-oligosaccharides (GOS)

Circular dichroism (%)	β -LG (control)	β -LG-GOS		
		0.1 MPa	80 MPa	160 MPa
Helix	19.9 $\pm$ 0.14 ^a	20.3 $\pm$ 0.63 ^a	10.1 $\pm$ 0.07 ^b	1.6 $\pm$ 0.15 ^c
Sheet	38.0 $\pm$ 0.70 ^b	38.4 $\pm$ 0.28 ^b	44.3 $\pm$ 0.50 ^a	36.6 $\pm$ 0.46 ^c
Turn	24.6 $\pm$ 0.14 ^b	24.0 $\pm$ 0.35 ^b	17.6 $\pm$ 0.29 ^c	33.0 $\pm$ 0.20 ^a
Random	17.5 $\pm$ 0.42 ^b	17.3 $\pm$ 0.56 ^b	28.0 $\pm$ 0.21 ^a	28.8 $\pm$ 0.26 ^a

^{a-c}Different letters denote significant differences across different treatments ($P \leq 0.05$).

0.3534, 0.3116, and 0.3225, respectively. The lowest UV absorption intensity was also found in β -LG-GOS (80 MPa), which was consistent with the analysis of fluorescence emission spectra. According to Housaindokht et al. (2001), the unfolding of protein decreased the UV absorption. Ultraviolet absorption spectra analysis indicated that the tertiary and quaternary structure of β -LG changed after DHPM combining with glycation treatment.

Antigenicity of β -LG depends on the epitopes, which are associated with the protein conformations and conformational flexibility (Kleber et al., 2004). When β -LG unfolded, GOS had more opportunities to bind with ⁶⁰Lys residues, masking ⁶¹Trp and epitopes. That would explain the low fluorescence intensity and the low antigenic response found in β -LG-GOS (80 MPa) samples and not in β -LG-GOS (0.1 MPa) or β -LG-GOS (160 MPa). It may be responsible for conformational changes of the molecule, making the epitopes less accessible for the antibodies. The extent of protein unfolding in β -LG-GOS (80 MPa), caused by pre-DHPM treatment, may facilitate the glycation with GOS, resulting in masking of epitopes, and hence a great decrease of β -LG antigenicity. Moreover, the change of fluorescence intensity of β -LG-GOS (0.1 MPa), β -LG-GOS (80 MPa), and β -LG-GOS (160 MPa) was in accordance with their change of H_0 value. The low fluorescence intensity and great degree of shift in β -LG-GOS (80 MPa) samples indicated that the DHPM combined with glycation treatment was a significant factor affecting β -LG tertiary structure, and the results could be closely related to its low antigenicity.

Changes of CD Analysis of β -LG-GOS

β -Lactoglobulin is predominantly a β -sheet protein, consisting of 9 antiparallel β -strands and 1 major α -helix at the C terminus of the molecule (Shibayama, 2008). As shown in Figure 4, the CD spectra showed a negative extreme around 195 nm and a positive maximum around 213 nm, indicating a high content of β -strands, in accordance with the secondary structure content shown. The secondary structure composition of

β -LG (control) was approximately 19.9%, α -helix; 38%, β -sheet; 24.6%, β -turn; and 17.5%, unordered coil (as calculated; Table 2). The calculated values are in the range of secondary structure content of native β -LG determined by other authors using CD (Qi et al., 1997; Croguennec et al., 2004).

As the secondary structure of protein depends on both the local sequence of amino acids and the interactions between different parts of molecule, the effects of glycation on secondary structure subjected to reaction conditions (glycation extent, reaction time, and the water content) and proteins. Some studies found that the secondary structure of protein was almost maintained after glycation (Hattori et al., 2004; Wong et al., 2011). According to other studies, however, glycation increased the α -helix content and highly ordered structure content, mainly at the expense of β -sheet or random coil content (Liu et al., 2012). In the current work, the spectrum of the β -LG-GOS (0.1 MPa) showed a little increase

Figure 4. Far-UV circular dichroism spectra of secondary structures of samples; (1) β -LG (control), (2) β -LG with galacto-oligosaccharides (GOS; 0.1 MPa), (3) β -LG-GOS (80 MPa), and (4) β -LG-GOS (160 MPa). θ = mean residual ellipticity. Color version available in the online PDF.

in ellipticity and a shift of the positive and negative maximums to a higher wavelength compared with β -LG (control). Little change of α -helix and β -sheet structure content was observed as calculated. With regard to β -LG-GOS (80 MPa), values of negative peaks at 213 nm decreased and shifted to a higher wavelength, accompanied by an increase in percentage content of β -strands, from 38.0 to 44.3%, which generally referred to a continuous stretch of amino acids adopting an extended conformation. It has been found that the trough at 216 nm broadened and deepened, accompanied by the unfolding of β -LG (Considine et al., 2007).

The antigenicity of β -LG depends on the epitopes, which are closely related to protein secondary structures. According to previous literature, the best recognized epitopes of the β -LG were the fragments AA102–124, AA41–60, and AA149–162 (Sélo et al., 1999), of which AA102–124 and AA41–60 formed a protruding loop between β -strands C and D on the surface of the molecule (Wal, 2001). As the content of β -strands increased with the unfolding of the protein, some epitopes may be masked or disrupted after DHPM combined with glycation treatment, which contributed to a reduction of antigenicity in β -LG-GOS (80 MPa) samples. However, we also observed that random coil content increased at a more severe condition (160 MPa), along with a sharp reduction in percentage content of α -helix, from 19.9 to 1.6%, and a loss of β -sheet content, from 38.0 to 36.6%. The sharp increase of antigenicity of β -LG-GOS (160 MPa) samples may be explained by the detection of linear epitopes buried in the turn and random structures and some new epitopes formed by recombination of protein.

CONCLUSIONS

We investigated antigenicity and conformational changes of β -LG by DHPM combined with glycation treatment. We showed that antigenicity of β -LG was reduced at relatively low pressures (≤ 120 MPa), and the lowest antigenicity was found in β -LG-GOS (80 MPa) samples. The DHPM combined with glycation treatment resulted in an increase of the surface SH content and a decrease of Ho. Moreover, quenching of fluorescence intensity, the red-shift of fluorescence spectra, decreased UV absorption, and CD analysis indicated protein unfolding and changes in the secondary structure. All of these conformational changes may contribute to antigenicity changes of β -LG, whereas more profound reasons should be explored further.

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REFERENCES

- Aguilera, J. M., and P. J. Lillford. 2008. Food Materials Science: Principles and Practice. Springer Science & Business Media LLC, New York, NY.
- Akita, E. M., and S. Nakai. 1990. Lipophilization of β -lactoglobulin: Effect on hydrophobicity, conformation and surface functional properties. *J. Food Sci.* 55:711–717.
- Balti, R., A. Barkia, A. Bougatef, N. Ktari, and M. Nasri. 2009. A heat-stable trypsin from the hepatopancreas of the cuttlefish (*Sepia officinalis*): Purification and characterisation. *Food Chem.* 113:146–154.
- Chevalier, F., J. M. Chobert, Y. Popineau, M. G. Nicolas, and T. Haertle. 2001. Improvement of functional properties of β -lactoglobulin glycosylated through the Maillard reaction is related to the nature of the sugar. *Int. Dairy J.* 11:145–152.
- Considine, T., H. A. Patel, H. Singh, and L. K. Creamer. 2007. Influence of binding conjugated linoleic acid and myristic acid on the heat- and high-pressure-induced unfolding and aggregation of β -lactoglobulin B. *Food Chem.* 102:1270–1280.
- Corzo-Martínez, M., F. J. Moreno, A. Olano, and M. Villamiel. 2008. Structural characterization of bovine β -lactoglobulin galactose/tagatose Maillard complexes by electrophoretic, chromatographic and spectroscopic methods. *J. Agric. Food Chem.* 56:4244–4252.
- Croguennec, T., D. Molle, R. Mehra, and S. Bouhallab. 2004. Spectroscopic characterization of heat-induced nonnative β -lactoglobulin monomers. *Protein Sci.* 13:1340–1346.
- de la Hoz, L., and F. M. Netto. 2008. Structural modifications of beta-lactoglobulin subjected to gamma radiation. *Int. Dairy J.* 18:1126–1132.
- Divsalar, A., A. A. Saboury, F. Ahmad, and A. A. Moosavi-Movahedi. 2009. Effects of temperature and chromium (III) ion on the structure of bovine β -lactoglobulin A. *J. Braz. Chem. Soc.* 20:1782–1789.
- Ellman, G. L. 1959. Tissue sulfhydryl groups. *Arch. Biochem. Biophys.* 82:70–77.
- Enomoto, H., Y. Hayashi, C. P. Li, S. Ohki, H. Ohtomo, M. Shiokawa, and T. Aoki. 2009. Glycation and phosphorylation of alpha-lactalbumin by dry-heating: Effect on protein structure and physiological functions. *J. Dairy Sci.* 92:3057–3068.
- Exl, B. M., and R. Fritsche. 2001. Cow's milk protein allergy and possible means for its prevention. *Nutrition* 17:642–651.
- Galazka, V. B., D. Smith, E. Dickinson, and D. A. Ledward. 1999. Effect of high pressure on protein-polysaccharide complexes. Food emulsions and foams: Interfaces, interactions and stability. Pages 104–116 in *Proc. Int. Conf. Food Emulsions and Foams: Interfaces, Interactions and Stability*, Seville, Spain. Royal Society of Chemistry, Cambridge, UK.
- Gasymov, O. K., and B. J. Glasgow. 2007. ANS fluorescence: Potential to augment the identification of the external binding sites of proteins. *Biochim. Biophys. Acta* 1774:403–411.
- Haskard, C. A., and E. C. Y. Li-Chan. 1998. Hydrophobicity of bovine serum albumin and ovalbumin determined using uncharged (PRO-DAN) and anionic (ANS[−]) fluorescent probes. *J. Agric. Food Chem.* 46:2671–2677.
- Hattori, M. 2002. Functional improvements in food proteins in multiple aspects by conjugation with saccharides: Case studies of β -lactoglobulin-acidic polysaccharides conjugates. *Food Sci. Res.* 8:291–299.
- Hattori, M., N. Koichi, and A. Akio. 2000. Functional changes in β -lactoglobulin by conjugation with cationic saccharides. *J. Agric. Food Chem.* 48:2050–2056.

- Hattori, M., S. Miyakawa, and Y. Ohama. 2004. Reduced immunogenicity of β -lactoglobulin by conjugation with acidic oligosaccharides. *J. Agric. Food Chem.* 52:4546–4553.
- Hiller, B., and P. C. Lorenzen. 2010. Functional properties of milk proteins as affected by Maillard reaction induced oligomerisation. *Food Res. Int.* 43:1155–1166.
- Housaindokht, M. R., J. Chamani, A. A. Saboury, A. A. Moosavi-Movahedi, and M. Bahrololoom. 2001. Three binding sets analysis of α -lactalbumin by interaction of tetradecyl trimethyl ammonium bromide. *Bull. Korean Chem. Soc.* 22:145–148.
- Iametti, S., P. Rasmussen, H. Frokiaer, P. Ferranti, F. Addeo, and F. Bonomi. 2002. Proteolysis of bovine β -lactoglobulin during thermal treatment on subdenaturing conditions highlights some structural features of the temperature-modified protein and yields fragments with low immunoreactivity. *Eur. J. Biochem.* 269:1362–1372.
- Kim, S. B., K. S. Ki, and M. A. Khan. 2007. Peptic and tryptic hydrolysis of native and heated whey protein to reduce its antigenicity. *J. Dairy Sci.* 90:4043–4050.
- Kleber, N., I. Krause, S. Illgner, and J. Hinrichs. 2004. The antigenic response of beta-lactoglobulin is modulated by thermally induced aggregation. *Eur. Food Res. Technol.* 219:105–110.
- Kobayashi, K. 2001. Reduced immunogenicity of β -lactoglobulin by conjugation with carboxymethyl dextran differing in molecular weight. *J. Agric. Food Chem.* 49:823–831.
- Kolakowski, P., E. Dumay, and J. C. Cheftel. 2001. Effects of high pressure and low temperature on β -lactoglobulin unfolding and aggregation. *Food Hydrocoll.* 15:215–232.
- Leonil, J., D. Molle, J. Fauquant, L. Maubois, R. J. Pearce, and S. Bouhallab. 1997. Characterization by ionization mass spectrometry of lactosyl β -lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. *J. Dairy Sci.* 80:2270–2281.
- Li, Z., Y. Luo, and L. Feng. 2011. Effects of Maillard reaction conditions on the antigenicity of α -lactalbumin and β -lactoglobulin in whey protein conjugated with maltose. *Eur. Food Res. Technol.* 233:387–394.
- Liu, J. H., Q. Ru, and Y. Ding. 2012. Glycation a promising method for food protein modification: Physicochemical properties and structure, a review. *Food Res. Int.* 49:170–183.
- Liu, W., Z. Q. Zhang, C. M. Liu, M. Y. Xie, Z. C. Tu, J. H. Liu, and R. H. Liang. 2010. The effect of dynamic high-pressure microfluidization on the activity, stability and conformation of trypsin. *Food Chem.* 123:616–621.
- López-Expósito, I., R. Chicón, and J. Belloque. 2012. In vivo methods for testing allergenicity show that high hydrostatic pressure hydrolysates of β -lactoglobulin are immunologically inert. *J. Dairy Sci.* 95:541–548.
- Manderson, G. A., M. J. Hardman, and L. K. Creamer. 1999. Effect of heat treatment on bovine β -lactoglobulin A, B and C explored using thiol availability and fluorescence. *J. Agric. Food Chem.* 47:3617–3627.
- Medrano, A., C. Abirached, L. Panizzolo, P. Moyna, and M. C. Anon. 2009. The effect of glycation on foam and structural properties of β -lactoglobulin. *Food Chem.* 113:127–133.
- Meltretter, J., S. Seiber, and A. Humeny. 2007. Site-specific formation of Maillard, oxidation, and condensation products from whey proteins during reaction with lactose. *J. Agric. Food Chem.* 55:6096–6103.
- Monahan, F. J., J. B. German, and J. E. Kinsella. 1995. Effect of pH and temperature on protein unfolding and thiol-disulfide interchange reactions during heat-induced gelation of whey proteins. *J. Agric. Food Chem.* 43:46–52.
- Nakamura, A., K. Watanabe, and T. Ojima. 2005. Effect of Maillard reaction on allergenicity of scallop tropomyosin. *J. Agric. Food Chem.* 53:7559–7564.
- Papiz, M. Z., L. Sawyer, E. E. Eliopoulos, A. C. T. North, J. B. C. Findlay, R. Sivaprasad, T. A. Jones, M. E. Newcomer, and P. J. Kraulis. 1986. The structure of β -lactoglobulin and its similarity to plasma retinol binding protein. *Nature* 324:383–385.
- Qi, J. R., X. Q. Yang, and J. S. Liao. 2009. Improvement of functional properties of acid-precipitated soy protein by the attachment of dextran through Maillard reaction. *Int. J. Food Sci. Technol.* 44:2296–2302.
- Qi, X. L., C. Holt, D. McNulty, D. T. Clarke, S. Brownlow, and G. R. Jones. 1997. Effect of temperature on the secondary structure of β -lactoglobulin at pH 6.7, as determined by CD and IR spectroscopy: A test of the molten globule hypothesis. *Biochem. J.* 324:341–346.
- Sava, N., I. Van der Plancken, W. Claeys, and M. Hendrickx. 2005. The kinetics of heat-induced structural changes of beta-lactoglobulin. *J. Dairy Sci.* 88:1646–1653.
- Sélo, I., G. Clement, and H. Bernard. 1999. Allergy to bovine β -lactoglobulin: Specificity of human IgE to tryptic peptides. *Clin. Exp. Allergy* 29:1055–1063.
- Shen, L., and C.-H. Tang. 2012. Microfluidization as a potential technique to modify surface properties of soy protein isolate. *Food Res. Int.* 48:108–118.
- Shibayama, N. 2008. Circular dichroism study on the early folding events of beta-lactoglobulin entrapped in wet silica gels. *FEBS Lett.* 582:2668–2672.
- Sun, Y., S. Hayakawa, and K. Izumori. 2004. Modification of ovalbumin with a rare ketohexose through the Maillard reaction: Effect on protein structure and gel properties. *J. Agric. Food Chem.* 52:1293–1299.
- Tanaka, N., Y. Tsurui, and I. Kobayashi. 1996. Modification of the single unpaired sulfhydryl group of β -lactoglobulin under high pressure and the role of intermolecular S-S exchange in the pressure denaturation. *Int. J. Biol. Macromol.* 19:63–68.
- van Teeffelen, A. M. M., K. Broersen, and H. H. J. de Jongh. 2005. Glycosylation of β -lactoglobulin lowers the heat capacity change of unfolding; a unique way to affect protein thermodynamics. *Protein Sci.* 14:2187–2194.
- Wal, J. M. 2001. Structure and function of milk allergens. *Allergy* 56:35–38.
- Wong, B. T., L. Day, and M. A. Augustin. 2011. Deamidated wheat protein-dextran Maillard conjugates: Effect of size and location of polysaccharide conjugated on steric stabilization of emulsions at acidic pH. *Food Hydrocoll.* 25:1424–1432.
- Wooster, T. J., and M. A. Augustin. 2007. Rheology of whey protein-dextran conjugate films at the air/water interface. *Food Hydrocoll.* 21:1072–1080.
- Wroblewska, B., M. Karamac, R. Amarowicz, A. Szymkiewicz, A. Troszynska, and E. Kubicka. 2004. Immunoreactive properties of peptide fractions of cow whey milk proteins after enzymatic hydrolysis. *Int. J. Food Sci. Technol.* 39:839–850.
- Zhong, J. Z., C. M. Liu, and W. Liu. 2011. Effect of dynamic high-pressure microfluidization at different temperatures on the antigenic response of bovine β -lactoglobulin. *Eur. Food Res. Technol.* 233:95–102.
- Zhong, J. Z., W. Liu, and C. M. Liu. 2012. Aggregation and conformational changes of bovine β -lactoglobulin subjected to dynamic high-pressure microfluidization in relation to antigenicity. *J. Dairy Sci.* 95:4237–4245.
- Zhong, J. Z., Y. J. Xu, and W. Liu. 2013. Antigenicity and functional properties of β -lactoglobulin conjugated with fructo-oligosaccharides in relation to conformational changes. *J. Dairy Sci.* 96:2808–2815.
- Zhu, D., S. Damodaran, and J. A. Lucey. 2010. Physicochemical and emulsifying properties of whey protein isolate (WPI)-dextran conjugates produced in aqueous solution. *J. Agric. Food Chem.* 58:2988–2994.