Conformational changes of β -lactoglobulin induced by shear, heat, and pH—Effects on antigenicity

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ABSTRACT

Structural modifications influence the immune-reactivity of food proteins. We investigated effects of pH (3, 5, 7), temperature (80, 100, 120°C), and shear (100, 500, and 1,000 s⁻¹) on conformational changes (monitored by surface hydrophobicity, total thiol content, Fourier transform infrared spectroscopy, and gel electrophoresis) and their relation to antigenicity (determined by indirect ELISA) of β -lactoglobulin (β -LG). Overall, heating at low pH (3) caused unfolding of proteins and fragmentation due to partial acid hydrolysis and thereby exposed β -strands that contributed to appearance of some hidden epitopes, resulting in higher antigenicity. Heating at pH 5 and 7 decreased the allergenic response due to covalently bonded molecular polymerization and aggregation, which destroyed or masked some epitopes. Shear alone had no effect on the antigenic response of β -LG but may have an effect in combination with pH or temperature. Overall, heating β -LG solutions to 120°C at pH 5 with shearing (100–1,000 s⁻¹) resulted in minimal antigenicity. Structural modifications of β -LG via denaturation or disulfide- or thiol-mediated interactions can either enhance or decrease its antigenicity.

Key words: antigenicity, shear, heat, pH, conformation

INTRODUCTION

Cow milk allergy (CMA) is one of the most common childhood food allergies, affecting about 2% of newborns under 2 yr of age (Bossios et al., 2011). Several proteins in cow milk can stimulate allergic reactions, such as caseins, α -LA, β -LG, and BSA. Of these, β -LG is considered the main allergen for children sensitive to milk protein (Bu et al., 2009), being responsible for allergic reactions in 60 to 80% of CMA patients. The resistance of β -LG to proteolysis by gastric enzymes means that it reaches the intestine intact, and thus has

the potential to cause allergic reactions (Zhong et al., 2011). Such allergenicity limits the consumption of milk by certain groups of consumers and is a disadvantage in the manufacturing of infant milk products. Measuring the antigenic response of a protein by ELISA is a common method to measure the allergenicity of a protein ingredient.

Allergic reactions are triggered by parts of protein called epitopes that bind with specific antibodies and provoke adverse immune reactions (Lehrer et al., 1996). In β -LG, both conformational epitopes (discontinuous AA sequences whose function is based on the tertiary or quaternary structure of protein) and linear or sequential epitopes (continuous sequences of AA that determine the primary structure of protein) contribute to the antigenic response (Kleber et al., 2004). Therefore, modifying these epitopes in proteins may modulate the antigenic responses of milk proteins.

Various methods have been adopted to destroy or modify such epitopes in β -LG. These include heat treatment to temperatures above 90°C (Davis and Williams, 1998; Fritsche, 2003), combination of heat and pressure treatments (Zhong et al., 2011), enzymatic hydrolysis (Ena et al., 1995; Heyman, 1999; Wróblewska and Troszyńska, 2005), microwave treatment (Grar et al., 2009), gamma radiation (Lee et al., 2001), and exposure to high-intensity ultrasound (Stanic-Vucinic et al., 2012). All of these studies have attributed changes in antigenicity to alterations in the secondary and tertiary structure of the protein caused by processing. Specifically, the degree of unfolding, content of β -strands, and formation of intermolecular disulfide bonds are linked to increases and decreases of protein antigenicity (Zhong et al., 2012).

Reports to date have focused on some physical as well as enzymatic treatments of β -LG and its subsequent structural deformation associated with antigenic potentiality; however, no reports have outlined the effects of combinations of different conditions, such as pH and temperature, on conformational changes of β -LG and their relation to antigenicity. Further, food proteins are exposed to shear stress during many common manufacturing processes such as centrifugation, mixing, ho-

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mogenization, ultrafiltration, and microfiltration (Yim and Shamlou, 2000). Shear stress is also known to alter the structure of native protein molecules (Bekard et al., 2011), which, in turn, could affect functional properties of proteins and their susceptibility to enzymatic digestion. However, there are no reports on shear-induced conformational changes of β -LG and their effects on antigenicity. Thus, a gap exists in our understanding of the combined effects of various processing conditions, including temperature, pH, and shear, on the structure of β -LG and consequences of changes in processing conditions on its antigenicity. Therefore, the objective of this study was to establish effects of different shear, pH, and temperature conditions on conformational changes of β -LG in relation to perceived antigenicity.

MATERIALS AND METHODS

Preparation of Protein Samples

Samples of β -LG (85% purity) containing 96% protein (as analyzed in the laboratory) were provided by Davisco Co. Ltd. (Le Sueur, MN). A 0.3% (wt/vol) solution of β -LG was prepared in citric acid-phosphate buffer at different pH values (3, 5, or 7). Citric acid-phosphate buffers at pH 3, 5, and 7 were prepared by mixing appropriate volumes of 0.1 M citric acid and 0.2 M Na_2HPO_4 and contained 0.02% sodium azide to prevent microbial growth. A solution of β -LG prepared in milliQ water (Millipore Inc., Billerica, MA) served as the control (pH 7.6). The concentration chosen was similar to the reported concentration of β -LG in milk (Dupont et al., 2013).

Treatment of Samples

Each prepared sample of β -LG was subjected to different shear rates (100, 500, or 1,000 s^{-1}) and temperatures (80, 100, or 120°C) in a pressure cell (CC25/PR-150) of a rheometer (Physica MCR 301 series; Anton Paar GmbH, Ostfildern-Scharnhausen, Germany) with constant pressure of 250 kPa following the method of Liyanaarachchi et al. (2015). Samples at control pH (7.6) not subjected to shear were the controls under each condition of sample preparation. An aliquot of 15.14 mL of each sample at 20°C was poured into the measuring cup of the pressure cell. The samples were then heated at 2°C/min to the required temperature, held for 1 min at that temperature, and then cooled at 2°C/min to 20°C. Fixed shear rate was applied throughout the period. Samples were then taken immediately for Fourier transform infrared (FTIR) analysis. The remaining portion was divided and stored at -80°C for

subsequent analysis. The aliquot for SDS-PAGE analysis was stored in SDS sample buffer.

Determination of Total Thiol Content

Total thiol (SH) content was determined according to the method of Sava et al. (2005). The control and treated samples of β -LG solutions were diluted 10 times with standard buffer (0.086 M Tris, 0.09 M glycine, 4 mM Na_2EDTA , and 8 M urea; pH 8). Ten microliters of Ellman's reagent [5,5'-dithiobis-2-nitrobenzoic acid (DTNB); 4 mg/mL of standard buffer] was added to 1 mL of diluted sample, vortexed, and incubated for 2 min. The absorbance was measured at 412 nm at room temperature with a UV spectrometer (Libra S11, Biochrom, Cambridge, UK), using standard buffer as a blank. The absorbance value was divided by molar extinction coefficient of 13,600 to obtain the thiol content, expressed as micromoles per gram of protein.

Determination of Surface Hydrophobicity

Surface hydrophobicity was determined fluorometrically using 8-anilino-1-naphthalenesulfonic acid (ANS; Sigma Aldrich, St Louis, MO) as a fluorescence probe, according to the method of Alizadeh-Pasdar and Li-Chan (2000) with some modifications. A stock solution of ANS was prepared in 0.1 M phosphate buffer (pH 7.6) and stored in a screw-capped bottle, wrapped with aluminum foil to prevent exposure to light. The treated and untreated samples of β -LG were diluted in respective pH buffer to the concentration range of 0.0002 to 0.0012%. To 4 mL of diluted β -LG solution was added 20 μL of ANS stock solution, and the mixture was vortexed and incubated for 15 min in dark. Relative fluorescence intensity (RFI) was measured at excitation and emission wavelengths of 390 and 470 nm, respectively, using a Shimadzu fluorescence spectrophotometer (model-RF5301PC, Shimadzu Corp., Kyoto, Japan). The RFI of protein dilution blank (without ANS) was also measured and subtracted from that of corresponding protein dilutions with ANS to obtain net RFI. The initial slope of net RFI versus protein concentration was calculated by linear regression analysis and used as an indicator for surface hydrophobicity.

PAGE

Both reducing and nonreducing SDS-PAGE were performed to study the status of protein in control and treated samples. For nonreducing SDS-PAGE, 400 μL of sample was mixed with 600 μL of SDS sample buffer [500 mL of MilliQ water, 125 mL of 0.5 M Tris-HCL

buffer, pH 6.8; 100 mL of glycerol, 200 mL of 10% (wt/vol) SDS solution, and 25 mL of 0.4% (wt/vol) bromophenol blue solution]. For reducing SDS-PAGE, in addition, 50 μ L of β -mercaptoethanol was added to the SDS sample buffer and the solution heated for 5 min at 90°C. A 1% solution of β -LG and BSA in distilled water, suitably diluted with SDS sample buffer, was used as protein standard. Broad-range unstained standard from Bio-Rad Laboratories (Gladsville, NSW, Australia) was used as a molecular marker. Molecular weight markers (15 μ L), β -LG and BSA standards (8 μ L each), and samples (10 μ L) were loaded onto a Mini-Protean TGX Any KD (Bio-Rad Laboratories) precast gel. The gels were placed in a Bio-Rad Protean II xi cell filled with Tris-glycine-SDS buffer. Electrophoresis was performed at 50 mA, 180 V, and 6.5 W. The gels were stained by immersing the gels in staining solution (0.025% Coomassie Brilliant Blue, 40% methanol, 7% acetic acid, and 50% distilled water) overnight. Then, the gels were transferred into destaining solution (40% methanol, 7% acetic acid, and 53% distilled water) for 2 h followed by immersion in distilled water until the background became clear. Gel images were taken using a ChemiDoc imager (Chemidoc MP, Bio-Rad Laboratories)

FTIR

About 15 μ L of each sample was placed between a pair of CaF₂ discs. Sample-containing discs were set on a sample holder assembly that was then put into the sample compartment of FTIR instrument (IRAffinity-1, Shimadzu Corp.). The FTIR spectrum was obtained at room temperature from 20 scans at a resolution of 4 cm⁻¹ using Happ Genzel apodization. The spectrum thus obtained was smoothed by 5 points to reduce the noise and deconvoluted using IRSolution software (Shimadzu Corp.). To observe the effect of treatments, the final spectra were obtained after subtracting the spectra obtained for each of the treated samples from the spectra for corresponding sample buffers.

Determination of Antigenicity

Antigenicity of all samples (control and treated) was determined by sandwich ELISA using the bovine β -lactoglobulin ELISA quantitation kit (Bethyl Laboratories Inc., Webster, TX). Individual wells of a 96-well microtiter plate (Nunc Maxisorp C bottom well) were coated with capture antibody diluted in 0.05 M carbonate-bicarbonate coating buffer. After a 1-h incubation at room temperature, the plate was washed 5 times with ELISA wash solution (50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8). Then, the wells were filled with 200

μ L of blocking solution to block the residual free binding sites in the wells. After a 30-min incubation at room temperature, the blocking solution was drained and the plate was washed 5 times to remove unbound antibody. Thereafter, 100 μ L of standards or samples diluted in sample diluent was added to each well, incubated for 1 h followed by washing the plate. Then, 100 μ L of horseradish peroxidase-conjugated β -LG-detecting antibody was added to each well. After a 1-h incubation and subsequent washing, the wells were filled with 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) substrate and color was allowed to develop for 15 min in the dark. The reaction was stopped by adding 0.18 M H₂SO₄ and absorbance measured on a microplate reader (iMark, Bio-Rad Laboratories). The antigenicity was calculated from the standard curve as equivalent nanograms of protein per milliliter of sample.

Statistical Analysis

The experiment was designed as a randomized, blocked split-plot design with pH as the main plot and shear and temperature as the subplots. This block was replicated with 3 subsamplings. The results of the various determinations were analyzed using the GLM procedure of SAS (SAS Institute, 1996), establishing effects of main factors (pH, temperature, shear) and their interactions. The level of significance was set at $P = 0.05$.

RESULTS AND DISCUSSION

Total Thiol Content

Among the whey proteins of milk, β -LG contains 5 cysteine residues. Four of these, Cys¹⁰⁶-Cys¹¹⁹ and Cys⁶⁶-Cys¹⁶⁰ form 2 disulfide (SS) bonds, whereas Cys¹²¹ remains as a free thiol (SH) group (Sava et al., 2005). The amount of free SH groups present can be influenced by modifying the conditions that influence the structural arrangement of β -LG. Our results indicated that different treatment conditions had varying effects on the total thiol content of the samples (Figure 1). Overall, thiol content was affected not only by pH, temperature, or shear alone ($P < 0.0001$), but also by the interaction of pH with shear and with temperature ($P < 0.0001$) and by the interaction of all 3 parameter ($P = 0.0159$). On average, the total thiol content of freshly prepared solution of β -LG (control, pH 7.6) was 46.5 μ mol/g of protein, which is close to the reported values for native β -LG (50.9 μ mol/g of protein; Sava et al., 2005). Adjusting the solution pH to 7 did not result in any major change in thiol content (46.4 μ mol/g of protein), whereas lowering the pH to 5 or 3 decreased

Figure 1. Thiol (SH) content of β -LG solutions at different pH [pH 7.6 (control), 3, 5, or 7], temperature [room temperature, RT ($\sim 22^{\circ}\text{C}$; control), 80, 100, or 120°C], and shear [S ; 0 (control), 100, 500, or $1,000\text{ s}^{-1}$]. Error bars indicate standard error of the mean ($\text{SEM} = 0.741$).

($P < 0.05$) thiol content to 40.7 and $1.7\text{ }\mu\text{mol/g}$ of protein, respectively (Figure 1). The structural changes that could have occurred due to the low pH may have reverted upon changing the pH during analysis of thiol groups. Therefore, the minimum thiol content observed at pH 3 could be due to lower exposure, slow reactivity, and lesser accessibility of thiols to DTNB at acidic pH (Wada et al., 2006).

Heating β -LG caused variable changes in thiol content depending on the pH (Figure 1). At control pH, the total thiol content decreased ($P < 0.05$) as the temperature increased, with a substantial change occurring between 80 and 100°C . At pH 7, the thiol content declined following the temperature increase to 100°C , after which it remained steady. The trend was similar at pH 5, although no change in thiol content was observed beyond 100°C . Interestingly, the trend was reversed at pH 3: thiol content increased with the increase in temperature. Such an increase in thiol content could be attributed to unfolding as well as fragmentation of β -LG by acid hydrolysis at pH 3

and higher temperature. Dissociation of dimers and partial unfolding of β -LG could have exposed reactive SH groups at pH 7 but not at pH 5 or 3, which could explain, in part, the lower thiol content at a lower pH. Participation of reactive SH groups in thiol-thiol oxidation or thiol-disulfide exchange reactions contributing to the heat-induced aggregation could account for the decrease ($P < 0.05$) in thiol content when the temperature was increased above 80°C at control pH and pH 5 and 7. The reversed trend of changes in thiol group content with temperature observed at pH 3 could be due to exposure of certain thiol groups but lack of participation in thiol-disulfide-mediated polymerization at elevated temperature due to their lowered reactivity in an acidic environment (de la Fuente et al., 2002; Wada et al., 2006). In addition, β -LG has a positive charge at pH 3, which would further hinder interactions between individual molecules due to electrostatic repulsion (de la Fuente et al., 2002). These observations are similar to those of Monahan et al. (1995), Visschers and de Jongh (2005), and Mudgal et al. (2011).

Surface Hydrophobicity

The hydrophobicity of β -LG was markedly affected by different pH values and temperatures (Figure 2), with shear ($P < 0.05$) becoming an important factor at $>80^\circ\text{C}$ and at low (100 s^{-1}) or high ($1,000\text{ s}^{-1}$) shear rates. Interestingly, a decline of average hydrophobicity values coincided with an increase in shear rate under different temperatures and pH (Figure 2). The opposite observation was made at pH 3. Heat-induced initial aggregation through nucleation-mediated coagulation pathway (Dunstan et al., 2009) is modulated by shear. Such a shear-modulated aggregation may lead to a structural rearrangement that would render some hydrophobic binding sites unavailable, resulting in decreased hydrophobicity because of greater shear at pH 5 and 7.

Overall, the highest hydrophobicity was observed in samples adjusted to pH 3, whereas those adjusted to pH 5 exhibited the lowest values, irrespective of temperature. These observations are in line with those

of Shimizu et al. (1985) and Alizadeh-Pasdar and Li-Chan (2000). Denaturation of β -LG molecules leading to exposure of hydrophobic AA residues could explain the greater hydrophobicity of samples adjusted to pH 3. The prevalence of positive charges on β -LG at pH 3 could prevent aggregation due to electrostatic repulsion, which could further account for increased hydrophobicity when the shear rate was increased. In addition, partial acid hydrolysis of β -LG at pH 3 and higher temperature, as shown by SDS-PAGE, might expose new hydrophobic residues, resulting in increased hydrophobicity. In contrast, because pH 5 is near the isoelectric point of β -LG, the tendency of proteins to interact and aggregate could explain the very low values of hydrophobicity observed at this pH.

At a given pH, hydrophobicity generally increased with increasing temperature. However, this observation could not be applied to the samples adjusted to pH 5. Instead, at pH 5, hydrophobicity declined ($P < 0.05$) as the treatment temperature increased—an observation that coincides with the observations of Monahan et al.

Figure 2. Effect of treatment pH [pH 7.6 (control), 3, 5, or 7], temperature [room temperature, RT ($\sim 22^\circ\text{C}$; control), 80, 100, or 120°C], and shear [S; 0 (control), 100, 500, or $1,000\text{ s}^{-1}$] on surface hydrophobicity of β -LG. Error bars indicate standard error of the mean ($\text{SEM} = 1.286$).

Figure 3. Average peak intensity at specific wave numbers (1,610 to 1,685 cm^{-1}) obtained from interferograms for β -LG treated at different pH and temperatures and their corresponding controls. RT = room temperature ($\sim 22^\circ\text{C}$).

(1995), who also observed decrease in hydrophobicity of whey protein isolate heated at pH 5. Extensive intermolecular disulfide bonding of β -LG at higher temperatures and pH 5 (as observed in SDS-PAGE) could have resulted in burial of hydrophobic AA residues, thereby resulting in decreased hydrophobicity values. Although disulfide-mediated polymerization can occur at higher temperatures at both pH 5 and pH 7 (de la Fuente et al., 2002), isoelectric precipitation could have preceded this polymerization and aggregation at pH 5, which explains the lower hydrophobicity values at pH 5 than at pH 7.

FTIR

The effect of pH and temperature on the secondary structures of β -LG was ascertained by deconvoluted FTIR interferograms (Figure 3). Overall, we did not observe any major variations in peak intensities in the amide I range (1,600–1,700 cm^{-1}) that would indicate structural changes as a function of shear. However, marked variations in amide I bands were exhibited

by β -LG samples subjected to different pH and temperatures. Deconvoluted amide I interferograms showed peaks between 1,608 and 1,610 cm^{-1} (corresponding to side-chain residues of AA), 1,626 to 1,627 cm^{-1} (exposed β -sheet), 1,645 to 1,647 cm^{-1} (related to unordered structures), 1,649 to 1,654 cm^{-1} (defining α -helix), 1,681 to 1,685 cm^{-1} (intermolecular β -sheet aggregation), and 1,693 cm^{-1} (representing β -turns and β -bends; Casal et al., 1988; Lefèvre and Subirade, 2000; Bhattacharjee et al., 2005).

Untreated β -LG in milliQ water (control pH) showed 2 major peaks, representing intramolecular β -sheet (1,637 cm^{-1}) and α -helix (1,651 cm^{-1}) structures. Heating at control pH did not show any change in the profile up to 80°C, with only an increase in peak intensity at 80°C indicating minor structural modifications. However, at 100 to 120°C, another peak appeared at 1,626 cm^{-1} related to additional exposure of β -sheets that is indicative of partial unfolding of β -LG. This extra peak at 1,626 cm^{-1} also appeared when solutions of β -LG adjusted to pH 3 were exposed to higher temperatures (80 to 120°C). This finding suggested minimal confor-

mational modifications during heating at pH 3, which further explains the observations of changes in hydrophobicity values (Figure 2).

However, at pH 5, increasing the temperature to 80°C resulted in a similar FTIR spectrum as in control β -LG, only with greater intensity. At 100 and 120°C, however, some additional peaks appeared at 1,608 to 1,610 cm^{-1} (corresponding to side-chain residues of AA), 1,618 to 1,620 cm^{-1} (related to intermolecular cross linking of β -sheet), 1,654 cm^{-1} (indicating α -helix), 1,684 to 1,685 cm^{-1} (related to β turns, as well as antiparallel β -sheet associated with aggregation), and 1,693 cm^{-1} (representing β -turns and β -bends). The appearance of such a wide array of structural features indicated that β -LG passed through more structural changes, denaturation, and aggregation when heated near the isoelectric pH than at any other pH. Similar findings have been reported by Casal et al. (1988). This further supports the observations in our study related to decline of hydrophobicity values during heating at pH 5 (Figure 2). On the other hand, heat treatment at pH 7 gave a spectrum with peaks at 1,626, 1,637, 1,645–1,647, and 1,651–1,654 cm^{-1} when heated to 80°C. When heated to 100 to 120°C, the intensity for β -sheet bands (1,626 and 1,637 cm^{-1}) increased, whereas that for unordered structures (1,645–1,647 cm^{-1}) decreased, indicating extensive denaturation and aggregation. Overall, we observed complex structural modifications, particularly during heating of β -LG solutions at pH 5 compared with that at pH 7; minimal changes were observed at pH 3.

SDS-PAGE

Reducing and nonreducing SDS-PAGE was performed to investigate the extent of covalent aggregation under experimental conditions (Figure 4 A to F). Gel patterns of untreated β -LG samples (lane 10) exhibited monomeric bands under both reducing and nonreducing conditions, along with a faint band corresponding to BSA, indicating the presence of some BSA in the analyzed samples.

The gel patterns obtained by heating β -LG at 80, 100, and 120°C at pH 3 (Figure 4 A and B) were similar under nonreducing conditions, indicating the absence of aggregates formed through thiol interchange and formation of disulfide bonds, which supports our FTIR observation (Figure 3) of relatively high structural stability. This observation is in agreement with that of Wada et al. (2006), who reported the absence of covalently bonded aggregation at low pH, even after heating up to 95°C. The enhanced stability of β -LG to heat denaturation at low pH (Kella and Kinsella, 1988; Wada et al., 2006; Dissanayake et al., 2013) re-

sulted in reduced exposure and minimal reactivity of thiol groups and thereby decreased thiol or disulfide exchange-mediated polymerization. Appearance of some low-molecular-weight bands (Figure 4 A and B) suggest the formation of some peptide fragments as a result of partial acid hydrolysis of β -LG at pH 3 and higher temperature (120°C).

The patterns for samples adjusted to pH 5 and heated to 80°C (Figure 4 C and D) also indicated the absence of thiol- or disulfide-mediated polymerization. However, upon increasing the temperature to 120°C, the typical bands for β -LG did not appear under nonreducing conditions compared with those obtained under reducing conditions, indicating that at high temperatures β -LG created polymer aggregates, which were cleaved into the monomeric form upon dissociation by 2-mercaptoethanol under the reducing conditions of electrophoresis. This observation is supported by the spectral features related to intermolecular cross-linking of β -sheets and related aggregates (Figure 3). The absence of repulsive forces at the isoelectric point could further explain the formation of aggregates at pH 5. This is in line with the observations of Monahan et al. (1995). This further explains why we observed the lowest hydrophobicity at pH 5 (Figure 2).

Antigenicity Related to Conformational Changes

The 3-dimensional structure of β -LG is an important feature that dictates its antigenicity. Previous studies have shown that people exhibit allergic responses to both linear and conformational epitopes of β -LG (Sélo et al., 1998, 2002; Clement et al., 2002). Allergenic epitopes are spread throughout the structure of β -LG. Major potential allergenic epitopes of β -LG identified are located on f(41–60), f(102–124), and f(149–162) (Sélo et al., 1999; Wal, 2001). The peptide f(41–60) forms a loop between β -strands C and D and also f(102–124), which is stabilized by hydrogen and disulfide bonds. Another potential antigenic fragment, f(149–162), is located at the C terminal and forms a flexible turn structure that, in the native state, is buried in the molecule and therefore inaccessible to IgE and IgG antibodies unless denaturation occurs (Kleber et al., 2004). More recently, Niemi et al. (2007) indicated a flat epitope area on the β -strands of β -LG that consisted of 6 different short fragments that bind to all 6 CDR (complementary-determining region) loops of IgE Fab (facilitated allergen binding). Thus, the availability of epitopes in β -LG for antibody binding is linked to its secondary or tertiary structure and can be influenced by conformational changes in response to various treatments.

The antigenicity of β -LG subjected to various pH, temperatures, and shear (Table 1) related well to the

Figure 4. Sodium dodecyl sulfate-PAGE analysis showing the effect of heat and shear on polymerization of β -LG at pH 3 (A = nonreducing SDS-PAGE, B = reducing SDS-PAGE), pH 5 (C = nonreducing, D = reducing), pH 7 (E = nonreducing, F = reducing). For all gels, lane (L) 1 = molecular weight marker, L2 = BSA standard; L3 = β -LG standard; L4 = 80°C, shear 100 s⁻¹; L5 = 80°C, shear 1,000 s⁻¹; L6 = 100°C, shear 100 s⁻¹; L7 = 100°C, shear 1,000 s⁻¹; L8 = 120°C, shear 100 s⁻¹; L9 = 120°C, shear 1,000 s⁻¹; L10 = untreated β -LG (control).

structural modifications observed. In general, the antigenic potential of β -LG was strongly influenced by variations in pH, temperature, and shear. The antigenicity of control samples (pH 7.6) was unaffected ($P > 0.05$) by shear and temperatures up to 80°C. It increased ($P < 0.05$), however, at 100°C and then remained stable with a further increase up to 120°C. Lowering the pH to 3 resulted in increases ($P < 0.05$) in antigenicity as the temperature was increased to 120°C. Interestingly, although shear had a minor effect on the extent of antigenicity, application of shear at pH 3 resulted in reduced ($P < 0.05$) antigenicity when the temperature was increased above 80°C. Exposure of the proteins to increasing temperature at pH 3 caused unfolding of the protein molecules: we observed enhanced hydrophobicity (Figure 2) as well as exposure of β -sheet structures (Figure 3). These structural changes, although limited, suggested the possible exposure of conformational or linear epitopes, which were otherwise buried inside the structure of β -LG molecules, resulting in increased antigenicity (Table 1). Increased antigenicity at pH 3 and elevated temperature could also be attributed to the formation of some peptide fragments (Figure 4A and B) containing exposed linear epitopes. On the other hand, the decrease in antigenicity due to increased shear rate at temperatures above 80°C could be attributed to alterations in the integrity of conformational epitopes brought about by intramolecular SH or SS exchange reactions in a manner that decreased their antibody-binding capability.

We found that the influence of increasing temperature at pH 5 on antigenicity of β -LG was opposite to that observed at pH 3. At pH 5, antigenicity was similar to that of untreated β -LG heated to 80°C. Similar structural features (Figure 3), thiol contents (Figure 1), and hydrophobicity (Figure 2) suggested limited structural rearrangement and denaturation, which resulted in similar antigenicity values. However, when β -LG was heated to 100 or 120°C, heat- and shear-induced aggregation resulted in a reduction ($P < 0.05$) in the antigenic response. This result concurs with the observation of Kleber et al. (2004) and Bu et al. (2009), who reported heat-induced aggregation above 90°C that decreased the antigenicity of β -LG. Such a decrease in antigenicity could be attributed to the destruction of some conformational epitopes due to intermolecular disulfide-mediated aggregation as well as a resultant compacting of structure of β -LG that resulted in some inner epitopes becoming inaccessible. Increasing the temperature from 100 to 120°C further enhanced the aggregation process, as was evident from the electrophoretic patterns (Figure 4 C and D), leading to a further reduction in antigenicity. Additionally, increasing the shear rate that caused a reduction in thiol content (Figure 1) and surface hydrophobicity (Figure 2), indicating greater polymerization and more compactness in the structure, could have contributed to additional masking of some surface epitopes, thereby further lowering the antigenicity.

Table 1. Antigenicity of β -LG (ng/mL) as affected by treatment temperature [room temperature (RT; ~22°C; control), 80, 100, or 120°C], pH [pH 7.6 (control), 3, 5, or 7], and applied shear [0 (control), 100, 500, or 1,000 s⁻¹]

pH	Temperature	Shear, s ⁻¹			
		0	100	500	1,000
Control (7.6)	RT	50.9 ^{a,A}	49.7 ^{a,A}	48.6 ^{a,A}	47.8 ^{a,A}
	80°C	52.9 ^{a,A}	50.6 ^{a,A}	50.4 ^{a,A}	49.3 ^{a,A}
	100°C	62.3 ^{a,B}	60.6 ^{a,B}	59.1 ^{a,B}	59.1 ^{a,B}
	120°C	63.46 ^{a,B}	61.5 ^{a,B}	60.3 ^{a,B}	60.2 ^{a,B}
3	RT	67.2 ^{a,A}	63.7 ^{a,A}	61.4 ^{a,A}	61.0 ^{a,A}
	80°C	70.8 ^{a,A}	69.8 ^{ab,B}	66.1 ^{ab,A}	65.7 ^{ab,AB}
	100°C	81.1 ^{a,BC}	78.7 ^{ab,C}	75.9 ^{b,B}	69.1 ^{c,B}
	120°C	86.0 ^{a,C}	81.3 ^{ab,C}	78.0 ^{b,B}	77.5 ^{b,C}
5	RT	55.9 ^{a,A}	53.2 ^{ab,A}	51.9 ^{ab,A}	49.4 ^{b,A}
	80°C	50.7 ^{a,B}	45.36 ^{b,B}	40.6 ^{bc,B}	39.7 ^{c,B}
	100°C	18.8 ^{a,C}	18.6 ^{a,C}	10.7 ^{b,C}	9.8 ^{b,C}
	120°C	8.8 ^{a,D}	6.6 ^{a,D}	4.5 ^{ab,D}	1.0 ^{b,D}
7	RT	47.5 ^{a,A}	46.0 ^{a,A}	45.7 ^{a,A}	45.8 ^{a,A}
	80°C	60.4 ^{a,B}	57.0 ^{a,B}	55.7 ^{ab,B}	51.8 ^{b,B}
	100°C	19.7 ^{a,C}	20.2 ^{a,C}	15.7 ^{a,C}	15.4 ^{a,C}
	120°C	18.1 ^{a,C}	19.3 ^{a,C}	19.0 ^{a,C}	19.0 ^{a,C}
Pooled SEM		1.79			

^{a-c}Means in the same row at a given pH are different ($P < 0.05$).

^{A-D}Means in the same column at a given shear and pH are different ($P < 0.05$).

¹All values presented are the mean of at least 6 independent observations ($n = 6$).

Increasing the pH of β -LG solutions to 7 resulted in an antigenicity trend opposite to that observed at pH 3. At 80°C, the increased hydrophobicity (Figure 2), indicative of β -LG unfolding, could have exposed hidden epitopes, resulting in increased antigenic potential of protein molecules. Increasing the temperature to 100 or 120°C followed by a decline in total thiol content (Figure 1) due to SH or SS exchange-mediated polymerization (Figure 4 E and F) could have masked some conformational epitopes, resulting in decreased antigenicity. However, because some β -LG molecules persisted as monomers (Figure 4 E and F) at 100 and 120°C, relatively more epitopes could have remained available for binding with antibodies, which might explain the relatively higher antigenicity at pH 7 compared with pH 5. Shear alone did not affect the antigenic potential at pH 7. Although the mechanism remains to be explained, high shear rate might have contributed to stability of the epitopes.

Overall, under defined processing conditions such as heating at 120°C, pH 5, and high shear rate ($1,000\text{ s}^{-1}$), β -LG can undergo substantial structural modifications, resulting in maximum reduction in its antigenicity. However, the stability or further modification of these structures during digestion needs to be investigated to confirm the carryover effect from processing to the physiological level. It remains to be seen whether such changes in antigenicity translate to modified allergenic potential of β -LG.

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