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ARTICLE

Effects of Heating and Glycation of β-Lactoglobulin on Its Recognition by IgE of Sera from Cow Milk Allergy Patients

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β-Lactoglobulin (β-LG) is one of the cow's major milk proteins and the most abundant whey protein. This globular protein of about 18 kDa is folded, forming a β-barrel (or calyx) structure. This structure is stabilized by two disulfide bonds and can be altered by heating above 65 °C. β-LG is also one of the major allergens in milk. Heating is one of the most common technologic treatments applied during many milk transformations. During heating in the presence of reducing sugars, β-LG is also submitted to the Maillard reaction, which at the first stage consists of the covalent fixation of sugars on the ε-amino groups of lysyl residues. The following steps are condensation and polymerization reactions leading to the formation of melanoidins (brown pigments). Despite the frequency of use of heating during milk transformation, the effects of heat-induced denaturation and of glycation of β-LG on its recognition by IgE from cow's milk allergy (CMA) patients are not fully understood. The objectives of our work were to evaluate the effect of heat-induced denaturation of bovine β-LG on binding of IgE from CMA patients and to determine the effect of moderate glycation on the degree of recognition by IgE. We showed that heat-induced denaturation (loss of tertiary and secondary structures) of β-LG is associated with weaker binding of IgE from CMA patients. It was also shown that moderate glycation of β-LG in early stages of Maillard reaction has only a small effect on its recognition by IgE, whereas a high degree of glycation has a clear "masking" effect on the recognition of epitopes. This demonstrates the importance of ε-amino groups of lysines in the definition of epitopes recognized by IgE.

KEYWORDS: Allergy; milk; heating; Maillard reaction; glycation; β-lactoglobulin; protein denaturation; IgE

INTRODUCTION

Food allergies are ranked by the World Health Organization (WHO) as the sixth problem of human health. It is estimated that in the United States, between 4.5 and 8% of the children below 2 years old suffer from food allergies. The number of people with symptoms of allergic reactions to food and the severity of these symptoms have increased continuously during the past several years (*1*−*4*). Food allergy is an adverse reaction to an otherwise harmless food or food component that involves an abnormal response of the immune system to specific protein(s) in foods. The majority of food allergies are mediated by allergen-specific IgE antibodies that cause an immediate type of reaction with symptoms occurring from minutes to a few hours after ingestion of the untolerated foods (*5*). Allergenicity to certain foods or food ingredients affects both children and adults. The most serious sensitivities are those that affect children, particularly infants and babies because of the influence on their growth. Milk being the first food for newborns, cow's milk allergy (CMA), defined as an immunologically mediated reaction against cow's milk antigens (*6*), is an important problem in pediatrics. Childhood CMA is the third most prevalent food allergy in France, with approximately 9% of the total allergies diagnosed (*7*). The most abundant milk proteins are α_{S1}-, α_{S2}-, β-, and κ-caseins, α-lactalbumin, and β-lactoglobulin (β-LG). Caseins, β-LG, and α-lactalbumin are the main allergens in milk (*8*−*10*). Other proteins present in milk in lower amounts such as bovine serum albumin, lactotoferrin, and IgG-heavy chain are also recognized by CMA patients (*9*, *10*).

Nowadays, milk is quite exceptionally consumed in its raw state. Heating (pasteurization), one of the most commonly applied treatments of milk, induces sometimes important protein structural changes. Heating also induces chemical modifications of proteins, which can influence IgE binding and the allergenicity (*11*). Because milk contains significant amounts of lactose and lower quantities of other reducing sugars, milk proteins are modified by so-called Maillard reaction or glycation during

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heating (*12*). Glycation is one of the most frequent chemical modifications during industrial processing (*13*, *14*). It occurs during the heating of proteins in the presence of reducing sugars. This is a complex reaction, leading to the formation of advanced Maillard reaction products (AMPs) or advanced glycated end products (AGEs). The first step of the reaction consists of the creation of Schiff type adducts of reducing sugars with primary amino groups of mainly lysyl residues of the proteins, leading to the formation of Amadori and Heyns products. The reaction does not stop there; the following steps include condensation and polymerization reactions, leading to the formation of brown pigments called melanoidins. In milk, Maillard reaction mainly leads to the Amadori products. The extent of glycation in commercial foods depends on many factors such as the heating temperature, the duration of it, and the concentrations of reducing sugars. The extent of lysine blockage of whey proteins in milk is the same as in other food systems and is well-documented (*12*−*15*). Typical AGEs such as pyrraline or carboxymethyllysine do not play a major role in heated milk when compared to the Amadori product lactuloselysine. Additionally, the conditions of glycation used were specifically set to reduce the amount of AGE or to eliminate them altogether.

Several studies attempted to evaluate the IgE-binding ability of allergens modified by the Maillard reaction (*15*−*20*). In some cases, glycation of allergens increases their recognition by IgE (*15*, *18*), whereas in other cases, glycation reduces IgE binding (*16*, *19*) or has no effect (*16*, *17*). No general effect was observed; the effect seems to be allergen- and sugar-dependent.

β-LG is a small globular protein of 162 amino acid residues with a molecular mass of 18.36 kDa. Three-dimensional (3D) crystallographic studies have shown that the β-LG 3D structure consists of nine antiparallel β-sheet structures forming a so-called β-barrel (or calyx) stabilized by two disulfide bonds (one cysteinyl residue remaining free) (*21*−*23*). A short three-turn α-helix lying on the outer face of the barrel is present at the C-terminal part of the molecule. The interior of the calyx contains a hydrophobic pocket, allowing the binding of small hydrophobic molecules such as retinoids, fatty acids, vitamins, and cholesterol (*24*−*26*). The quaternary structure of β-LG varies according to the temperature, concentration, ionic strength, and pH (*27*). In physiological conditions (pH 5.5−7.5 and concentration < 5 g.L^{-1}), β-LG is mainly a noncovalent dimer stabilized by electrostatic interactions (*28*). At pH > 8, Tanford transition occurs consisting of dimer dissociation and increased exposition to the solvent (*22*, *29*) of tyrosines and tryptophans. At a pH close to the pI (between 4.5 and 5.5), β-LG aggregates in tetramers (*27*), whereas it dissociates in monomers at pH < 3.5. pH variations have only a small effect on the tertiary and secondary structures of β-LG (*21*). Heating to temperatures > 60 °C causes destabilization of β-sheets, unfolding of the β-barrel, and exposition of disulfide bonds and the free cysteine to the solvent. Above 65 °C, denaturation becomes irreversible: Loss of secondary and tertiary structures is associated with new hydrophobic interactions and disulfide bond exchanges, leading to irreversible aggregation (*30*−*33*). All of these structural modifications are likely to induce changes in the antigenic character or immunoreactivity of this important protein (*34*). The antigenicity of β-LG is increased by heating at temperatures of 80−90 °C and decreased after heating above 100 °C (*35*). The denatured structure of the molecule has been shown to modulate the immunologic response of rat and mouse models of allergy (*36*, *37*). The objectives of the present work were (1) to evaluate the effect of heat-induced denaturation of bovine β-LG on binding of IgE from CMA patients and (2) to determine the effect of moderate glycation on the degree of recognition by IgE.

MATERIALS AND METHODS

Sera. A series of 18 sera from CMA patients presenting various symptoms were used. Total milk protein- and β-LG-specific IgE concentrations were determined with the Phadia ImmunoCAP System. All of the sera had total milk protein-specific IgE concentrations from < 0.35 to 57.8 kAU L^{-1}. Thirteen sera had β-LG-specific IgE values from 0.36 to 26.3 kAU L^{-1}, and four had β-LG-specific IgE concentrations < 0.35 kAU L^{-1}. For competitive enzyme-linked immunosorbent assay (ELISA), a pool containing the 14 sera was constituted. Sera were collected in the Laboratory of Immuno-Allergology of Academic Hospital (Angers, France), and their use was approved by the internal Ethical Committee of the hospital.

Preparation of Heated Proteins. Native bovine β-LG (variant A) was purified in Nantes INRA laboratory according to the method of Mailliart and Ribadeau Dumas (*38*) from the milk of cows confirmed as homozygote for β-LG variant A allele. Lyophilized purified native bovine β-LG was diluted in phosphate-buffered saline (PBS), and its concentration was adjusted to 2.7 mg mL^{-1}, corresponding to its concentration in milk, after verification of its protein content with the BCA assay kit (Sigma, St. Quentin-Fallavier, France).

β-LG was heated with a Touchgene gradient thermocycler (Techne Inc., Princeton, NJ) at the rate of 1 °C min^{-1} and incubated for 20 min at 65, 75, 85, and 95 °C. After it was heated, the protein was rapidly cooled (about 1 °C s^{-1}) to 4 °C and used immediately for studies of its recognition by IgE.

Preparation of Glycated Proteins. Purified native bovine β-LG (variant A) was glycated with lactose (β-LG-Lac), ribose (β-LG-Rib), glucose (β-LG-Glu), galactose (β-LG-Gal), arabinose (β-LG-Ara), and rhamnose (β-LG-Rha) (Sigma) as described by Chevalier et al. (*39*, *40*). Briefly, β-LG (0.217 mM) and the different sugars (0.217 M) were dissolved in 0.1 M phosphate buffer, pH 6.5. After filtration on 0.22 μM acetate cellulose filters (Millipore, Bedford, MA), mixtures of protein and sugar were put in well-capped flasks and heated in a water bath at 60 °C for 72 h. This mild heat treatment limited the self-aggregation of β-LG. All experiments were performed under strictly anaerobic and sterile conditions; all media were purged and saturated with N_2. After they were heated, the different fractions were dialyzed against distilled water, freeze-dried, and stored at −20 °C. β-LG heated at 60 °C for 72 h without sugar (β-LG-60 °C) was used as a control. The quantity of available amino groups was determined by the modified *ortho*-phthaldialdehyde (OPA) method (*41*). The OPA reagent was prepared daily by mixing 40 mg of OPA (dissolved in 1 mL of methanol), 50 mL of 0.1 M sodium borate buffer, pH 9.3, 100 mg of *N*-dimethyl-2-mercaptoethylammonium chloride (DMMAC), and 1.25 mL of 20% w/w sodium dodecyl sulfate (SDS) in water. Fifty microliters of protein solution (2 g L^{-1} in 50 mM sodium phosphate buffer, pH 7.8) was added to 1 mL of OPA reagent. The absorbency was read at 340 nm after a minimal delay of 5 min. A calibration curve was obtained by using 0.25−2.00 mM L-leucine as a standard. The degrees of modification of the proteins were as follows: β-LG, 60 °C, 6.2%; β-LG-Lac, 34.4%; β-LG-Rha, 40.6%; β-LG-Glu, 41.2%; β-LG-Gal, 41.9%; β-LG-Ara, 55.0%; and β-LG-Rib, 69.4% (*40*). Lyophilized glycated proteins were diluted in PBS before use (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4), and their concentrations were adjusted to 1 mg mL^{-1}.

Electrophoresis. Proteins were analyzed by 12% SDS-polyacrylamide gel electrophoresis (PAGE). Briefly, proteins were diluted in loading buffer [60 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, and 0.025% (w/v) bromophenol blue] and loaded on 12% polyacrylamide gel containing 0.1% (w/v) SDS. Migration was performed for about 1 h in a buffer containing 0.3% (w/v) Tris and 1.44% (w/v) glycine. Gels were stained with an aqueous solution containing 50% (v/v) ethanol, 10% (v/v) glacial acetic acid, and 0.25% (w/v) Coomassie brilliant blue and destained with a solution containing 10% (v/v) ethanol and 7% (v/v) glacial acetic acid. Electrophoresis was performed under reducing and nonreducing conditions. In reducing conditions, the sample buffer was added with 5% reducing agent β-mercaptoethanol (β-ME).

Circular Dichroism (CD). CD spectra were recorded on a CD6 dichrograph (Jobin Yvon, Longjumeau, France), using cells of the appropriate lengths and a scan time of 1 nm s^{-1}. All measurements were carried out at 20 °C with thermostatically controlled cell holders. The instruments were calibrated with ammonium d-10-camphorsulfonic acid.

The data were expressed as molar residue ellipticity [θ], which is defined as $[\theta] = 100\ \theta_{obs}/cl$, where θ_{obs} is the observed ellipticity in degrees, c is the concentration in residue mol cm^{-3}, and l is the length of the light path in cm. For measuring CD spectra in the far-UV region, the sample concentration was 1 mg mL^{-1} in 100 mM PBS buffer, pH 7.4, and the spectra were recorded between 190 and 260 nm using a quartz cuvette with a 0.1 mm path length. Each spectrum was the accumulation of five successive measurements, and baseline was corrected by subtracting the buffer spectrum.

A protein concentration of 0.5 mg mL^{-1} was used for near-UV CD spectra, and the spectra were recorded between 260 and 350 nm using a quartz cuvette with a 1 cm path length. Four scans were averaged per spectrum. The baseline was corrected by subtracting the corresponding solvent spectrum from the sample spectrum.

Colorimetric ELISA (C-ELISA). Maxisorp bottom flat transparent 96 microtitration plates (Nunc, Roskilde, Denmark) were coated overnight with 100 μL per well native, heated, and sugar-modified β-LG diluted to 5 μg mL^{-1} in PBS, with native β-LG diluted to 5 μg mL^{-1} in 100 mM carbonate buffer, pH 9.6, or with PBS in wells used as negative controls. After they were coated, they were washed three times with PBS containing 0.1% (v/v) Tween-20 (PBS/T) and saturated for 1 h with 250 μL of a solution of PBS/T containing 1% (w/v) polyvinyl alcohol (Sigma) (PBS/T/PVA). Plates were washed three times with PBS/T and incubated for 90 min at room temperature with 100 μL of mouse monoclonal anti-β-LG antibody (IgG) diluted to 1:100 in PBS/T/PVA from hybridoma supernatant. Mouse monoclonal anti-β-LG antibody mAb37 and mAb96, which bind β-LG on two different epitopes independently of secondary and tertiary structures of β-LG, were produced and characterized in Nantes INRA laboratory (*42*). Plates were washed three times with PBS/T and incubated for 1 h at room temperature with a peroxidase-conjugated antimouse IgG (Bio-Rad Laboratories, Hercules, CA) diluted 1:3000 in PBS/T/PVA. The secondary antibody binding was revealed after three washes with PBS/T by addition of 100 μL of OPD (Sigma, 0.4 mg mL^{-1} in 50 mM citrate buffer, pH 5.5). The staining reaction was stopped after 20 min of incubation at room temperature by the addition of 100 μL of 2 M H_2SO_4. The absorbance was measured at 492 nm with the EL_X800 plate reader (BioTek Instruments, Inc., Winooski, VT).

Fluorescent ELISA (F-ELISA). Maxisorp bottom flat white 96 microtitration plates (Nunc) were coated overnight with either 100 μL per well of a mouse monoclonal antihuman IgE antibody ($IgG_{2\beta}$) (Fitzgerald, Concord, United States) diluted to 1:2500 (1.6 μg mL^{-1}) in PBS or with native, heated, and sugar-modified β-LG diluted to 5 μg mL^{-1} in PBS or with native β-LG diluted to 5 μg mL^{-1} in 100 mM carbonate buffer, pH 9.6. After they were coated, they were washed three times with PBS/T and saturated for 1 h with 250 μL of a solution of PBS/T/PVA. Plates were washed three times with PBS/T. Wells coated with the antihuman IgE antibody were incubated with 100 μL of serial dilutions to 1/2 from 160 to 0.08 ng mL^{-1}, plus one dilution at 1 ng mL^{-1}, of the second WHO international reference preparation of human IgE (prepared in PBS/T/PVA). In the wells coated with allergens and in those coated with PBS as a negative control, 100 μL of 1:25 dilutions of sera from patients was added. After an overnight incubation at 4 °C, the plates were washed three times with PBS/T and incubated for 2 h at room temperature with an alkaline-phosphatase-conjugated polyclonal antihuman IgE (Sigma) diluted 1:1000 in PBS/PVA. The secondary antibody binding was revealed, after three washings with PBS/T, by the addition of 4-methylumbelliferyl phosphate (4-MUP) substrate diluted 1:5 in 1 M Tris-HCl, pH 9.8. The fluorescence emission was measured after 90 min of incubation at 37 °C with the FL_X800 plate reader (BioTek Instruments) fitted with a 360 nm excitation filter and a 440 nm emission filter. The reading parameters were adjusted to 45 in sensitivity for a reading from the top. Controls included secondary antibody on capture antibody (no IgE), secondary antibody on IgE (no capture antibody), secondary antibody on allergens (no patient serum), and secondary antibody on serum (no allergen).

To relate the fluorescence intensity to IgE concentrations, a sigmoid four-parameter model [$y = d + (a - d)/1 + (x/c)^b$] was adjusted to standard curve data by nonlinear regression using the Solver option from Microsoft Excel 2000 by minimizing the sum of the squared differences. Limits of detection [mean + three standard deviations (SD)] and quantification (mean + 10 SDs) were calculated from fluorescence data of the eight wells with no IgE. Fluorescence data measured for each antigen and serum were corrected by subtracting the fluorescence of the control with no antigen, and corresponding specific IgE concentrations were calculated from the adjusted standard curve provided that the fluorescence exceeded the quantification limit.

Competitive ELISA. Maxisorp bottom flat white 96 microtitration plates (Nunc) were coated overnight with 100 μL per well of native β-LG diluted to 5 μg mL^{-1} in PBS. F-ELISA was performed as previously described except that sera of patients were replaced by a pool of sera preincubated for 1 h at 37 °C in the presence of increasing concentrations of competitor. The final dilution of the serum was 1:20, and the final concentration of the inhibitor ranged from 0 to 100 μg mL^{-1} (1:10 serum pool dilution and inhibitor solution were mixed v/v to a final volume of 100 μL). Because of the low amount of serum available, the experiment was realized once in triplicate. The concentration of protein (inhibitor) needed to inhibit 50% of IgE binding (IC_{50}) was calculated from the inhibition curves by relating fluorescence intensity to inhibitor concentrations. A sigmoid four-parameter model [$y = d + (a - d)/1 + (x/c)^b$] was adjusted to standard curve data by nonlinear regression using the Solver option from Microsoft Excel 2000.

RESULTS

Characterization of Heated β-LG. Analysis of SDS-PAGE results of heated β-LG (**Figure 1A**) in nonreducing conditions showed that until 65 °C, β-LG is present mostly in a monomeric form in PBS solution at pH 7.4 (band at about 14.4 kDa). A small proportion of β-LG was also dimeric (band slightly higher than 31 kDa). After they were heated at higher temperatures, the proportion of dimers increased, and additional bands corresponding to larger polymers (trimers, tetramers, etc.) appeared. Under reducing conditions (**Figure 1B**), β-LG migrated as a unique band, indicating that heat-induced polymerization involves disulfide bond formation. Those data are in accordance with what is already well-known. Heat-induced denaturation and aggregation of β-LG by the formation of disulfide bonds occur when it is heated at temperatures higher than 70−72 °C (transition temperature) (*43*). However, this allowed checking that in the samples used for ELISA, β-LG structure has been effectively changed. A more accurate characterization of β-LG samples was

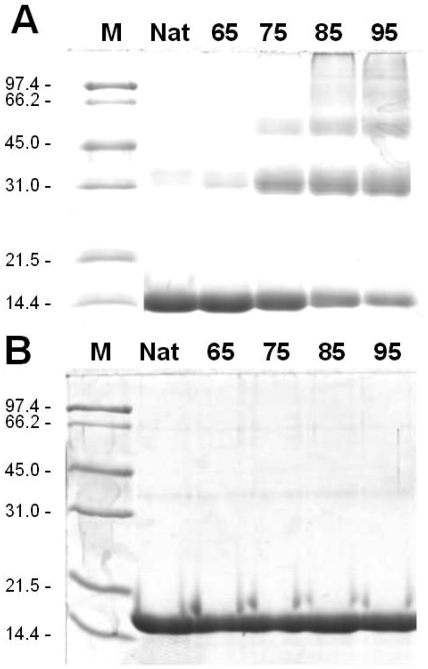

Figure 1. Heat-induced polymerization of β-LG: Native β-LG (line Nat) and β-LG heated for 20 min at 65, 75, 85, and 95 °C (lines 65−95) in PBS buffer were analyzed on 12% SDS-PAGE in nonreducing (**A**) or in reducing (**B**) conditions; M, molecular mass marker (kDa).

achieved by CD spectra measurements, which allowed monitoring of the folding changes of heated β-LG.

The main chromophores in the near-UV CD are tryptophan, tyrosine, and phenylalanine (*44*). Near-UV CD spectra allow us to characterize the tertiary structure of proteins mainly due to the asymmetries in the environment of the aromatic amino acids and to characterize the stability of their conformers. β-LG contains two tryptophans (Trp 19 and Trp 61). It is known that Trp 61 is on the surface of the protein and has considerable rotational freedom, which together with Trp19 is the source of the near-UV CD signals at 286 and 293 nm (*22*, *24*, *45*). As shown in **Figure 2A**, heat treatment of β-LG induces a decrease in the intensity of the deep troughs observed at 286 and 293 nm, confirming other recent findings (*30*, *46*). The changes in [θ] at 286 and 293 nm, which were most probably due to the changes in the environment of Trp 19, were likely to reflect irreversible structural changes that occurred within the calyx of the β-LG molecule as a result of heat treatment.

Far-UV CD spectra have been used to follow the evolution of the secondary structure of β-LG when submitted to heat treatment at pH 7.4 (**Figure 2B**). The trough, with a minimum at 216 nm, gradually broadened and deepened so that the minimum shifted to lower wavelengths, indicating the loss of secondary structure between 65 and 75 °C, as already observed (*33*, *47*); α-helix and β-sheet structures were progressively converted to aperiodic structure. These results confirm that the tertiary and secondary structures of heated β-LG used for assaying IgE binding were progressively altered by heating.

Characterization of Glycated β-LG. Figure 3 illustrates the strong effect of glycation on β-LG. On SDS-PAGE, in nonreducing conditions, a band at about 31 kDa corresponding to β-LG dimers was present. In reducing conditions, this band disappeared in the case of nonglycated β-LG but persisted in the case of glycated β-LG. Consequently, as compared to native β-LG, glycated β-LG is also able to form covalent dimers that do not involve disulfide bond formation. In the case of substitution by ribose and arabinose (sugars inducing the highest degree of modification), a smear was observed in both conditions, indicating that substituted proteins form polymers stabilized by sugar-induced covalent bonds (*39*). Full modifications induced by glycation, including conformational changes, have been analyzed in detail by Chevalier et al. (*39*, *40*).

Response of Patient Sera to Heated β-LG. The binding of IgE from patients having CMA on native and heated β-LG was studied by F-ELISA. Because heating of β-LG causes its polymerization and aggregation, it was essential to check that heated β-LG was able to coat the wells as well as native β-LG. Consequently, in parallel to F-ELISA, the adsorption of heated β-LG on microtitration plates was checked by C-ELISA using two β-LG-specific monoclonal antibodies mAb37 and mAb96, which bind β-LG on two different epitopes independently of secondary and tertiary structures of β-LG (*42*). The result showed that coating of microtitration plate wells with heated β-LG, whatever the temperatures, was identical to what was observed with native β-LG (**Figure 4A**).

For each patient, the specific IgE titer was determined for native and heated β-LG. The experiment was repeated three times. Three of the patients with [IgE] > 0.35 kAU L^{-1} generated values above the detection limit but below the quantification limit; they were eliminated from the study. In contrast, three out of the four patients with [IgE] < 0.35 kAU L^{-1} generated well quantifiable IgE concentration values. For each treatment, specific IgE concentration values were compared to that obtained with native β-LG. The results are expressed as a percentage of the native β-LG-specific IgE titer (**Figure 4B**). Significance of

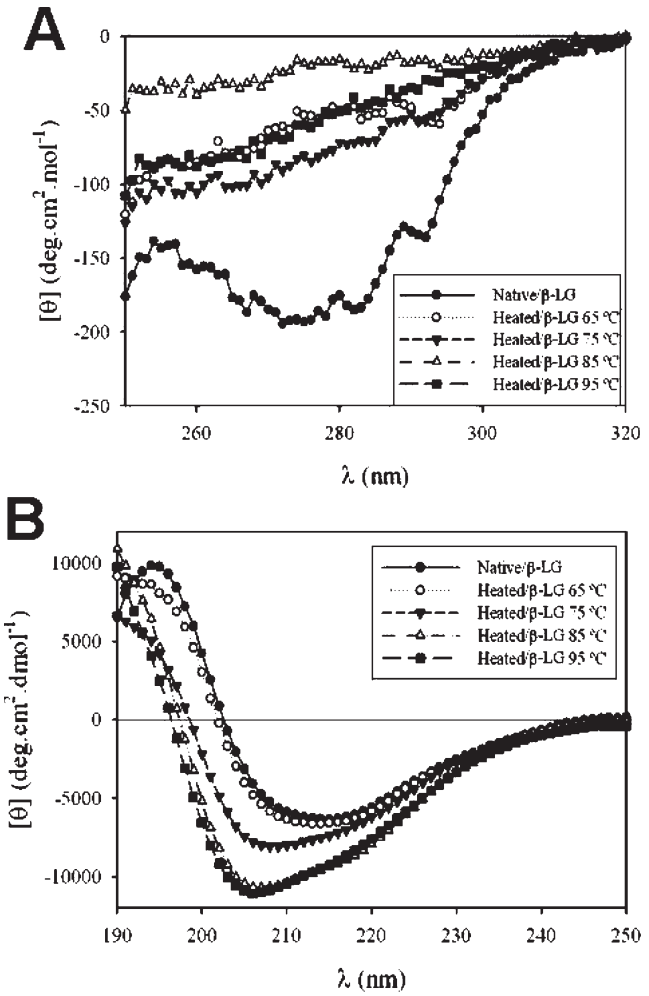

Figure 2. Far-UV (**A**) and near-UV (**B**) circular dichroism spectra of native and heated β-LG.

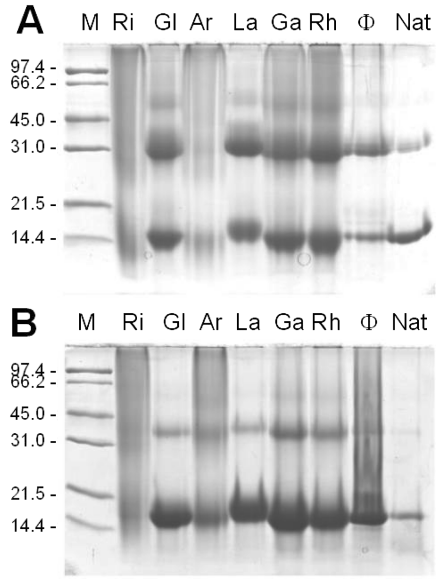

Figure 3. Polymerization of glycated β-LG: β-LG glycated with ribose (Ri), glucose (Gl), arabinose (Ar), lactose (La), galactose (Ga), and rhamnose (Rh) as compared with native β-LG (Nat) and β-LG heated for 72 h at 60 °C in the absence of sugar (Φ) were analyzed on 12% SDS-PAGE in nonreducing (**A**) or in reducing (**B**) conditions; M, molecular mass marker (kDa).

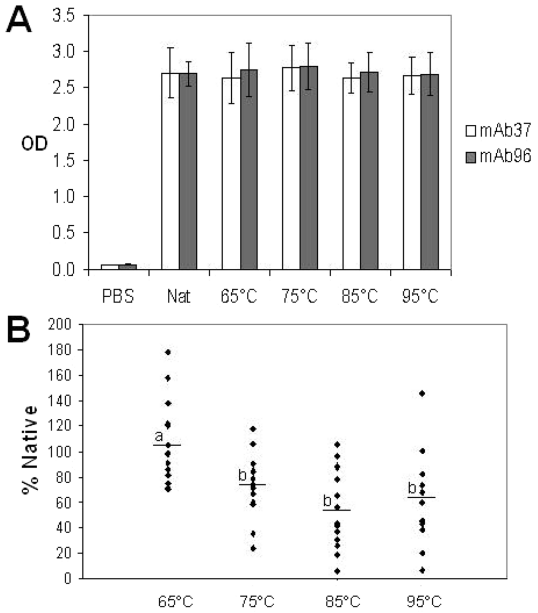

Figure 4. (**A**) Adsorption of heated β-LG on ELISA plate: signal from the binding (OD) of two anti-β-LG monoclonal antibodies in wells coated with native (Nat) and β-LG heated at 65, 75, 85, and 95 °C. (**B**) IgE binding on heated β-LG: binding of IgE from 17 CMA patients on native (Nat) and β-LG heated at 65, 75, 85, and 95 °C. Results are expressed as the percentage of the signal obtained by ELISA on native β-LG. The mean is indicated by an horizontal black bar. Identical letters indicate that means are not significantly different at a level of 5%.

differences between medians was tested by the Kruskal−Wallis test. The result indicated that the differences of means observed between native and β-LG heated at 65 °C (mean 106%) and β-LG heated at 75, 85, or 95 °C (means of 73, 55 and 64%, respectively) were significant ($\alpha = 0.05$). At the opposite, the slight differences observed between means obtained with β-LG heated at 75, 85, and 95 °C were not significant.

A relative heterogeneity of patient responses was observed. This heterogeneity is illustrated in **Figure 5**, which shows the different individual responses of three patients. Patient M95 reacted as the average. Patient M86 had a β-LG-specific IgE response strongly affected by β-LG heating. In contrast, the binding of IgE from patient M88 was greatly reduced on β-LG heated to temperatures higher than 65 °C. Ten out of fourteen patients (70%) had a similar response to patient M95, 6/14 patients (43%) had a similar response to patient M89, and only patient M88 (7%) had a strong decrease in IgE binding to heated β-LG.

Lower binding of β-LG-specific IgE to heated β-LG was confirmed by IgE binding inhibition experiments using a pool of sera constituted by the 17 tested sera (**Figure 6**). The standard deviation was not reported on the graph because it was always < 10%. The calculated IC_{50} value was 0.34 μg mL^{-1} for native β-LG and increased progressively with the heating to reach a maximum of 11.74 and 11.63 μg mL^{-1} for 85 and 95 °C heated β-LG, respectively (**Table 1**), indicating that heated β-LG is less recognized than native β-LG by IgE from CMA patients and that the effect increases progressively with temperature.

Response of Patient Sera to Glycated β-LG. As well as in the case of heated β-LG, the adsorption of glycated β-LG on ELISA plates was checked using monoclonal antibodies mAb37 and mAb96. The results showed that coating of microtitration plate wells was identical with either glycated β-LG or with β-LG incubated at 60 °C for 72 h in the absence of sugars (**Figure 7A**).

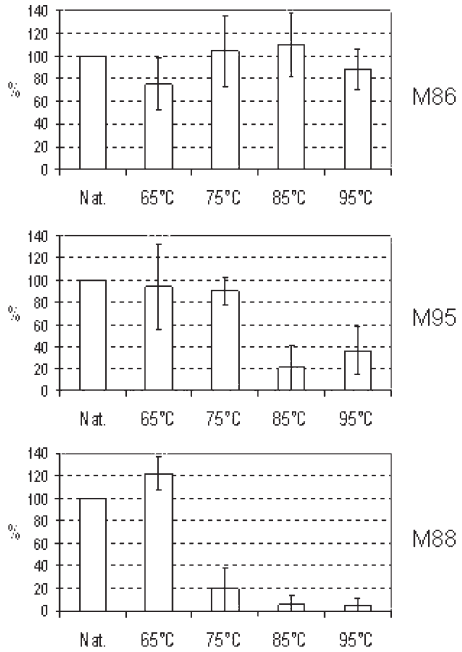

Figure 5. Individual IgE response of CMA patients to heated β-LG: average of the binding of IgE from 17 CMA patients on native (Nat) and β-LG heated at 65, 75, 85, and 95 °C. Results are expressed as the percentage of the signal obtained by ELISA on native β-LG.

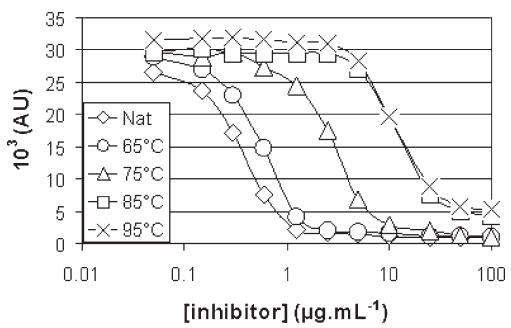

Figure 6. Inhibition of anti-β-LG IgE in serum pool. Comparison between native (Nat) and heat-treated β-LG at 65, 75, 85, and 95 °C. The signal (AU) resulting from the binding of IgE on native β-LG coated plates is shown.

Table 1. Inhibition of IgE Binding to Native β-LG by Native and Heated β-LG[a]

	β-LG treatment				
	native	65 °C	75 °C	85 °C	95 °C
IC_{50} (μg mL^{-1})	0.34	0.52	2.44	11.74	11.63

[a] IC_{50} values are presented.

To evaluate the effect of glycation of β-LG on IgE binding, the mean IgE binding to glycated β-LG was compared with IgE binding to β-LG incubated at 60 °C for 72 h in the absence of sugars (no glycation) (**Figure 7B**). The significance of differences between medians was tested by the Kruskal−Wallis test ($\alpha = 0.05$). The binding of patient's IgE on β-LG-Lac, β-LG-Gal, β-LG-Glu, and β-LG-Rha (means of 99, 98, 95, and 74%, respectively) was not significantly different from the IgE binding to β-LG heated at 60 °C for 72 h. IgE binding to β-LG-Ara and β-LG-Rib was significantly lower. The obtained values represent 50 and 24%, respectively, of the value obtained with control β-LG. The difference between these two values was statistically irrelevant.

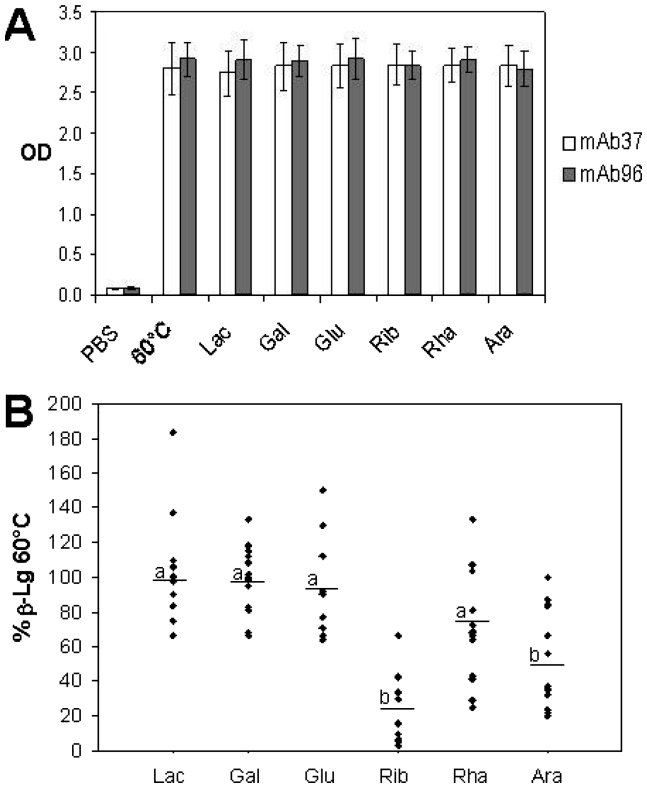

Figure 7. (**A**) Adsorption of glycated β-LG on ELISA plate: signal from the binding (OD) of two anti-β-LG monoclonal antibodies in wells coated with β-LG heated for 72 h at 60 °C and β-LG glycated with lactose (Lac), galactose (Gal), glucose (Glu), ribose (Rib), rhamnose (Rha), and arabinose (Ara). (**B**) IgE binding on glycated β-LG: binding of IgE from 14 CMA patients on β-LG glycated with lactose (Lac), galactose (Gal), glucose (Glu), ribose (Rib), rhamnose (Rha), and arabinose (Ara). Results are expressed as the percentage of the signal obtained by ELISA on β-LG heated for 72 h at 60 °C in the absence of sugar (control). The mean is indicated by an horizontal black bar. Identical letters indicate that means are not significantly different at a level of 5%.

Results obtained by indirect ELISA were confirmed by IgE-binding inhibition experiments using the pool of sera previously described (**Figure 8**). The standard deviation was not reported on the graph because it was always < 10%. The calculated IC_{50} value was 2.12 μg mL^{-1} for β-LG heated for 72 h at 60 °C and 2.50 μg mL^{-1} for lactosylated β-LG (**Table 2**). This difference was not significant. A considerable increase of the IC_{50} (~100 μg mL^{-1}) was observed in the case of ribosylated β-LG, indicating that the recognition of β-LG by IgE from CMA patients is strongly impaired by a high degree of glycation, especially with ribose. Because of the too low amount of available sera, the other glycated β-LGs were not assayed.

DISCUSSION

The binding of IgE from CMA patients to heat-modified β-LG is presented in this study. For nearly 70% of CMA patients sensitized to β-LG, it was observed that moderate heating of β-LG at 75 °C caused a decrease in its recognition by IgE. The maximal effect was observed between 85 and 95 °C (**Figure 4B** and **Figure 6**). This is consistent with the observation of Ehn et al. (*48*) who found, using pools of sera but not individual sera, that heating of β-LG at 74 °C caused a significant decrease in IgE binding, more evident after heating at 90 °C (*48*). Because the denaturation (modification of secondary and tertiary structures) of β-LG begins at 70−72 °C, it seems that its consecutive

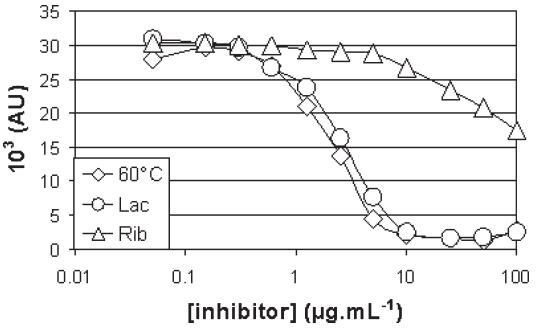

Figure 8. Inhibition of anti-β-LG IgE in serum pool. Comparison between β-LG heated for 72 h at 60 °C in the absence of sugar and in the presence of lactose (Lac) or ribose (Rib). The signal (AU) resulting from the binding of IgE on native β-LG coated plates is shown.

Table 2. Inhibition of IgE Binding to Native β-LG by β-LG Heated for 72 h at 60 °C without the Presence of Sugar or with Lactose or Ribose[a]

	β-LG treatment		
	60 °C	lactose	ribose
IC_{50} (μg mL^{-1})	2.12	2.50	~100

[a] IC_{50} values are presented.

aggregation is responsible for the disappearance of conformational epitopes and, consequently, of reduced binding. Reorganization of protein structure and/or aggregation of β-LG may have a masking effect on the recognition of sequential (or linear) epitopes. This should explain, at least partially, why some CMA patients are more tolerant to boiled milk than to raw milk. Our results are also consistent with the data obtained by Rytkönen et al. (*36*) who observed that rats sensitized with heat-denatured β-LG produce less specific IgE than rats sensitized with native β-LG, despite the fact that it induces a more intense inflammatory response. According to these results, it is clear that heat-induced denaturation of β-LG and/or aggregates generated by heat treatments at moderately high temperatures induced a more or less pronounced decrease of β-LG recognition by IgE from CMA patients. None of the studied sera from allergic patients showed a stronger IgE response against denatured β-LG, indicating that this protein does not contain internal masked linear epitopes becoming exposed only after denaturation.

We found that all of the patients do not react identically to heated β-LG. Mild reduction of IgE binding associated with β-LG heating was observed for the majority (60%) of the patients studied (**Figures 4B** and **Figure 5**). Nevertheless, it was found that one serum among 17 (6% of patients) recognizes only native β-LG and β-LG heated to 65 °C. This patient serum recognizes most likely conformational epitope(s), which are damaged by heating. It appears that in the case of a few sera, heating of β-LG to temperatures higher than 75 °C abrogate near totally its recognition by IgE. Surprisingly, the measured IgE concentration by the β-LG-specific Phadia ImmunoCAP System (F77) was < 0.35 kIA L^{-1}, suggesting that patients sensitized only with native β-LG are not well-detected by this assay. It should be underlined that β-LG adsorbed on the surface of the ImmunoCAP System is probably not in its native state any more. Consequently, for a better diagnosis, it would be important to ensure the use of native β-LG or execute them in solution and not adsorbed on any surface.

Glycation of proteins by the Maillard reaction occurred when they were incubated with the sugar at 60 °C for 72 h. This reaction is slow at room temperature, but its yield increases with temperature. The resulting products are responsible for the browning of

Table 3. Comparison between the Glycation Degree of β-LG and the IgE Binding[a]

sugar	glycation degree (%)	modified amino groups	IgE binding (%)
no	6.2	1.0	100
lactose	34.4	5.5	98
galactose	41.9	6.7	99
glucose	41.2	6.6	95
ribose	69.4	11.1	24
rhamnose	40.6	6.5	74
arabinose	55.0	8.8	50

[a] One hundred percent of binding corresponds to the IgE binding on β-LG heated for 72 h at 60°C in the absence of sugar.

cooked food. Because milk contains a high amount of lactose and because milk proteins are also present in multiple food preparations containing free reducing sugars, milk proteins, including β-LG, are susceptible to glycations. The results presented in this study show that low or moderate glycation of β-LG has no effect on its recognition by IgE, whereas the strongest substitution rates are associated with a decreased recognition of β-LG by IgE (**Figures 6A** and **8** and **Table 3**). This result can be explained by a "masking" effect due to sugars. Modification of β-LG occurs mainly on lysyl residues. All major and minor β-LG epitopes (*49*) contain one or more lysyl residues, and some of them have been identified as critical for IgE binding (e.g., K_{75}, K_{83}, K_{135}, K_{138}, and K_{141}) (*50*). It is clear that substitution of lysyl residues contained in the epitopes weakens or prevents IgE binding. When β-LG is substituted at a low rate, binding to epitopes is not much affected since remaining in the standard error range; hence, they are hardly detectable (**Figures 6** and **8** and **Table 3**). However, when β-LG is highly substituted (with important percentages of lysyl ε-amino groups glycated), IgE encounters less unhindered epitopes, and their binding to the protein is either decreased or nonexistent. In the early 1980s, Otani and Tokita (*51*) found that antigenicity (IgG binding) of heated β-LG decreased when the temperature increased and that this effect was less in the presence of lactose, suggesting that lactosylation by the Maillard reaction increased the antigenicity of denatured β-LG. They also showed that the sugar moiety linked to β-LG can act as a neo-epitope generated during the browning reaction (*52*). The fact that glycated β-LG did not show any increase of IgE binding as compared to the control β-LG indicates that, in the conditions used, the glycation does not lead to the formation of neo-epitopes even stronger recognized by the studied IgE. This apparent discrepancy can be explained by the fact that Otani and Tokita (*51*) used to work with immunized animal, whereas we worked with human sera. This induced two kinds of important sources of variability: (1) Otani and Tokita studied only β-LG-specific IgG epitopes, whereas we studied IgE epitopes, which are different, and their differences in the case of β-LG are well-known (*49*); (2) additionally, the epitopes recognized by animal immune systems are often different from those recognized by the humans. Moreover, the experimental conditions used by Otani and Tokita (*52*) for glycosylation lead to a more AMPs since they refer to browning products. In the present study, lactosylated β-LG was not brown. There are several investigations of the effect of Maillard reaction on protein allergenicity (*15*−*20*). The effects of allergen glycation are variable according to the allergen tested, the sugars used, and the stage of the Maillard reaction. No general effect on IgE binding could be seen. However, only scarce data about the effect of Maillard reaction are available. Consequently, it is not surprising that glycated β-LG shows a lower allergenicity than nonglycated β-LG as it was observed in the case of Pru av 1, the major allergen from cherry (*16*) and squid tropomyosin (*19*).

Despite of these in vitro allergenicity data, Bleumink and Berrens (*52*) observed in the 1960s that incubation of β-LG at 50 °C in the presence of lactose increases 100 times its skin reactivity. In this work, β-LG was heated from 48 to 216 h. The authors did not describe precisely what preparation was used for the intradermal reaction. They probably used β-LG samples more intensively modified than those used in our study, quite probably containing AGE. Additionally, in vivo skin reactivity involves other complex mechanisms than IgE binding on the allergen. It is possible that AGE present in the reaction medium affects these mechanisms, increasing the overall inflammation and allergic reactions without affecting IgE binding. Otani and Tokita (*52*) have reported contradictory results since they found that lactosylated β-LG was able to strongly inhibit the passive coetaneous anaphylaxis test. This result suggests lower allergenicity of glycated β-LG as compared to native β-LG. The human in vivo allergenicity of different glycated β-LG remains to be carefully evaluated.

In conclusion, for most studied sera of the CMA patients, a moderate heat treatment and glycation of β-LG during the moderate stages of the Maillard reaction do not have a drastic influence on the recognition of the protein by IgE. Nevertheless, in the case of rare individuals, heat treatment of β-LG can neutralize near totally its recognition by IgE.

ABBREVIATIONS USED

AGEs, advanced glycated end products; AMPs, advanced Maillard reaction products; AUs, arbitrary units; β-LG, β-lactoglobulin; β-LG-Lac, β-LG-lactose; β-LG-Rib, β-LG-ribose; β-LG-Glu, β-LG-glucose; β-LG-Gal, β-LG-galactose; β-LG-Ara, β-LG-arabinose; β-LG-Rha, β-LG-rhamnose; CMA, cow's milk allergy; CD, circular dichroism; C-ELISA, colorimetric enzyme-linked immunosorbent assay; F-ELISA, fluorescent enzyme-linked immunosorbent assay; OPD, *ortho*-phenylene diamine; 4-MUP, 4-methylumbelliferyl phosphate; SD, standard deviation; WHO, World Health Organization.

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