# Modification of Bovine β-Lactoglobulin by Glycation in a Powdered State or in an Aqueous Solution: Immunochemical Characterization

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Bovine β-LG was modified by glycation with lactose in a powdered state or in an aqueous solution. An immunological characterization was performed using monoclonal antibodies with defined epitopes. The results showed that the structural changes were confined to the AB loop region of the molecules when glycation was conducted in a restricted water environment and had little consequences on the association state of glycated β-LG. The protein conformation was much more extensively modified when glycation was performed in an aqueous solution at 60 °C, despite a lower glycation extent. These structural changes were located at the dimer interface (AB loop, GH loop, β-strand I, and α-helix). These results allowed us to establish a relationship between the conformational changes and the modification of the association state of the glycated protein (formation of disulfide bridges between the free thiol groups of two monomers), previously described.

**Keywords:** *β-Lactoglobulin; glycation; local conformation; monoclonal antibodies*

## INTRODUCTION

The effects of heat on the structure of β-lactoglobulin (β-LG) have received considerable attention since this globular protein is abundant in whey and exhibits excellent nutritional and functional properties. The X-ray crystal structure of β-LG (lattice X) at 1.8 Å resolution has been recently published by Brownlow et al. (1997). The β-LG monomer is composed of nine antiparallel β-strands shaped into a calyx (labeled A to I in Chart 1), one three-turn α-helix, and four short $3_{10}$ helical fragments located in the N-terminal, AB, GH, and C-terminal loops. The protein comprises two disulfide bridges and a single free cysteine (residue 121). The dimeric physiological form of β-LG is stabilized by hydrogen bonds between both the AB loop and β-strand I of two adjacent monomers. The quantitative analysis of the secondary structure of native β-LG in solution near neutral pH at a concentration of 10 mg/mL, using circular dichroism, gave contents of ca. 10, 50, 8, and 35% for helix, sheet, turn, and random structures, respectively (Qi et al., 1997).

The mechanism of heat denaturation and aggregation of purified β-LG has been widely investigated. Several recent reports, performed in water or in low-salt concentrations at relatively low temperatures (60−75 °C) and near neutral pH, have led to similar conclusions (Roefs and De Kruif, 1994; Iametti et al., 1995, 1996; Hoffmann and Van Mil, 1997; Hoffmann et al., 1997; Qi et al., 1995, 1997; Belloque and Smith, 1998). The proposed mechanism has a marked dependence on the protein concentration (Griffin et al., 1993; Matsuura and Manning, 1994; Qi et al., 1995; Iametti et al., 1996). The first step involves the dissociation of the natural dimer. It is followed by conformational changes in the monomer, leading to an increased reactivity of the free thiol group (Cys121). Subsequent aggregation of unfolded protein monomers occurs via hydrophobic interactions and thiol−disulfide interchange reactions, the latter being predominant.

Although several recent reports show that this overall denaturation mechanism is initiated by changes in the secondary and tertiary structure of β-LG, conflicting results exist concerning the nature and the localization of these changes (Qi et al., 1997). These authors have quantified the secondary structure variation of the protein upon heating at a concentration of 10 mg/mL. They have shown that heating between 60 and 70 °C resulted in an almost complete destruction of the α-helix occurred, and a fifth of the β-sheet content disappeared at 70 °C. Kaminogawa et al. (1989) have used monoclonal antibodies to monitor the denaturation of β-LG. When heated at about 70 °C, the first structural changes occurred in the random region containing the fragment 8−19, followed by modifications in the helical region containing the fragment 125−135. These two studies, as well as others (Belloque and Smith, 1998), suggested that the denaturation mechanism is initiated by conformational changes at the dimer interface (N-terminal, α-helix, AB loop, strands A, H, and I), which allowed reassociation of unfolded monomers and a further polymerization step through hydrophobic interactions and thiol−disulfide interchange reactions. However, the occurrence of these sequential events and the nature of the aggregates still give rise to much controversy (Griffin et al., 1993; Matsuura and Manning, 1994; Prabakaran and Damodaran, 1997).

The above structural studies were performed with pure protein. However, in milk and milk products, β-LG

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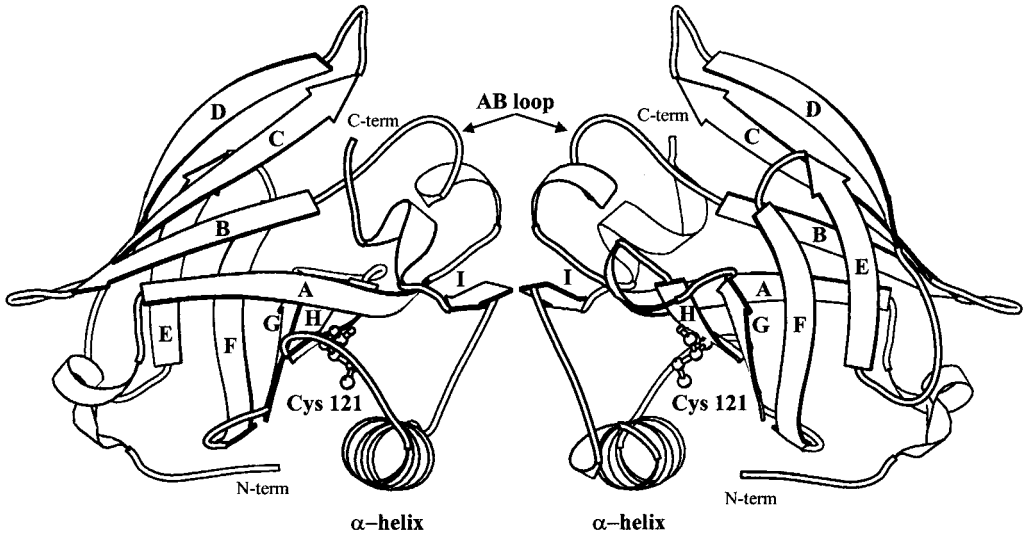
**Chart 1. 3-D Structure of β-LG Dimer Showing the Dimer Interface[a]**




[a] The figure is based on the X-ray crystallographic data of Brownlow et al. (1997) and was produced using MOLSCRIPT (Kraulis, 1991).

**Table 1. Biochemical Properties of Dry-Way and Solution Glycated β-Lactoglobulin (Morgan et al., 1999)**

| properties | glycation in solution | glycation in dry-way | method used |
|---|---|---|---|
| no. of lactose linked | low | high | mass spectrometry |
| association state | increased proportion of monomers; presence of covalent homodimers | no modification | size exclusion chromatography; electrophoresis |
| solubility | important loss | no modification | isoelectric precipitation |
| surface hydrophobicity | important increase | slight increase | ANS binding; pepsin hydrolysis |

can interact with other components. Among them is the Maillard reaction between β-LG and lactose, which is of practical interest since it is promoted by a number of industrial processes (heating, concentration, drying) and during storage of powders (O'Brien, 1995). In a previous study, we detected the presence of a significant amount of lactolated β-LG in commercial whey-protein-based products (Léonil et al., 1997). This reaction can take place under mild heat treatment (60 °C) in solution as well as in a limited water environment and we have determined the glycation sites (Morgan et al., 1997, 1998).

The structural consequences of β-LG modification are of great interest since its functional and biological properties are greatly affected by the protein conformation and association state. We have described the structural changes induced by the glycation of β-LG (Morgan et al., 1999), and the mean results are summarized in Table 1. When glycation was carried out in a dry system ($A_w = 0.65$), the nativelike conformation and association state were preserved. On the contrary, glycation in solution affected the initial steps of the heat-induced denaturation/aggregation process of β-LG. The following reaction scheme was proposed:

$$N_2 \overset{(1)}{\leftrightarrow} N \overset{(2)}{\rightarrow} N^* \overset{(3)}{\rightarrow} [N\text{-}N]^*$$
$$\downarrow \qquad\qquad \downarrow$$
$$\text{Polymerization}$$
$$(4)$$

The first step involves the well-described dissociation of the β-LG dimer ($N_2$) into monomers (N). The combined effect of heating and attachment of lactose induced specific conformational changes, forming the swollen monomer N* (step 2). The reactivity of the free thiol group of this swollen monomer was probably enhanced, and stable covalent homodimers were formed (step 3). Some of the unfolded monomers and homodimers underwent subsequent polymerization into a high molecular weight species, via noncovalent interactions (step 4).

In the present study, the molecular basis of β-LG structural changes was investigated using monoclonal antibodies (MAbs) as conformational probes. A panel of MAbs, raised against nonglycated β-LG (Vénien et al., 1997), was used. The reactivity of MAbs with modified β-LG was investigated using indirect ELISA technique. Distinct conformational forms of β-LG, specifically recognized by different MAbs, were purified by immunoaffinity chromatography and characterized by limited proteolysis. The relationships between glycation, local conformational changes, and overall structural modifications in glycated β-LG were investigated.

## MATERIALS AND METHODS

**Materials.** Fresh bovine raw milk was obtained from the experimental dairy farm (INRA, Rennes, France). β-LG B was prepared from the milk of homozygous cows by a modified method of Fauquant et al. (1988), as previously described (Léonil et al., 1997), in which membrane processes and low temperatures (below 56 °C) are involved. The freeze-dried β-LG B powder contained 93.2% total nitrogen, 2% nonproteic nitrogen, 0.3% ash, 2.2% citrate, 0.6% lactose, and 2% water. β-LG represented 98% of the protein and α-lactalbumin less than 2%, based on reversed-phase HPLC analysis.

Monohydrated lactose was obtained from Merck (Darmstadt, Germany). Porcine pepsin (EC 3.4.23.1), pepstatin, peroxidase-labeled avidin (type VI), dimethyl sulfoxide, and biotinamidocaproate *N*-hydroxysuccinimide ester (B-cap-NHS) were obtained from Sigma Chemical Co. (St. Louis, MO). Peroxidase-labeled goat anti-mouse IgG Fc antibody was purchased from Nordic Immunolog (Tilburg, The Netherlands).

**Monoclonal Antibodies.** Monoclonal antibodies were raised in mice against nonglycated β-LG. Their production and

**Table 2. Experimental Conditions for the Preparation of the Lactose/β-LG Complexes, Corresponding Average Number of Lactose Bound per Monomer (ANLBM), Glycoform Distribution, and Sample Codes**

| treatment | reaction time (h) | lactose | ANLBM | glycoform distribution | sample code |
|---|---|---|---|---|---|
| no treatment | 0 | − | 0.03 | 0 and 1 lactose adducts | native |
| dry-way treatment | 12 | − | 0.03 | 0 and 1 lactose adducts | DW-Ct |
| | 12 | + | 3.81 | from 1 to 7 lactose adducts | DW-Lac |
| solution treatment | 96 | − | 0.03 | 0 and 1 lactose adducts | SOL-Ct |
| | 96 | + | 1.96 | from 0 to 4 lactose adducts | SOL-Lac |

epitopic characterization are described by Vénien et al. (1997). Eleven of these MAbs were purified from ascitic fluids by ion-exchange chromatography as described by Levieux et al. (1995).

**Glycation Experiments and Characterization of the Modified β-LG.** Glycation with lactose was performed in both dry and aqueous systems.

For the dry-way glycation (DW-Lac sample), β-LG B (0.15 mM) was dissolved in a 15 mM lactose solution and the pH was adjusted to 7.2. After the solution was freeze-dried, the protein−sugar powders were kept under 65% relative humidity and 50 °C for 12 h. After such treatment, the powders were dissolved in cooled distilled water and free lactose was removed by extensive dialysis against distilled water at 4 °C (Spectra/Por 1 cellulose membranes, molecular mass cutoff: 6−8 kDa, Spectrum, Laguna Hills, CA). The control sample (DW-Ct) was prepared without added lactose

For solution glycation experiment (SOL-Lac), a solution containing β-LG B (0.15 mM) and lactose (150 mM) was adjusted to pH 7.2, heat-treated at 60 °C for 96 h, and then cooled in an ice−water bath. Dialysis was performed as above. A mild heating (60 °C) was chosen in order to limit thermal denaturation and aggregation effects. Control sample (Sol-Ct) was prepared without added lactose.

Dialyzed samples of control and glycated β-LG were then precipitated at pH 4.6 with 1 M HCl at 23 °C for 1 h. The acidified solutions were filtered on a cellulose acetate 0.45 μm Nalgene filter (Polylabo, Strasbourg, France), and the pH was adjusted to 7.2. After the filtrates were freeze-dried, powders were kept at 4 °C until use. Protein concentration was determined spectrophotometrically at 278 nm using a specific absorption coefficient $A_{1cm}^{1\%}$ of 9.6 (Eigel et al., 1984).

The properties of the glycated β-LG samples and the corresponding controls are shown in Table 2.

**ELISA Experiments (Inhibition of Indirect ELISA).** Flat-bottomed polystyrene microtiter plates (Maxisorp; Nunc, Kamstrup, Denmark) were coated overnight at 4 °C with 100 μL of native β-LG at a concentration of 1 μg/mL in PBS. Plates were washed four times with PBS containing 0.1% v/v Tween 20 (PBS-Tw). MAbs were incubated in test tubes without (Bo value) or with serially diluted solutions of the inhibitors (native or modified β-LG; *B* values) in PBS-Tw containing 1% v/v of rabbit serum (PBS-Tw-RS). After 1 h at room temperature, 100 μL of the MAb/β-LG mixtures was added to the wells for a 1 h supplementary incubation. After the wells were washed, 100 μL of peroxidase-labeled goat anti-mouse antibody diluted in PBS-Tw-RS was added, and the plates were incubated for 1 h in the dark. After washing, 100 μL of *o*-phenylenediamine (0.4 mg/mL) in 0.1 M phosphate/citrate buffer pH 5.0 was added, and the color was allowed to develop in the dark for 30 min. The reaction was stopped by adding 50 μL of 2.5 M $H_2SO_4$, and absorbance was measured at 492 nm using a microplate reader (IEMS; Labsystem, Helsinki, Finland).

**Table 3. Classification of MAbs According to Their Relative Reactivity toward Native and Glycated β-LG**

| class A native > DW-Lac > SOL-Lac | class B native = DW-Lac > SOL-Lac | class C native = DW-Lac = SOL-Lac | class D SOL-Lac > DW-Lac > native |
|---|---|---|---|
| 17 C5 | 49 F11 | I7 G2 | 102 G5 |
| 81 1C3 | 91 E7 | 37 1C10 | |
| 100 B3 | 116 D6 | | |
| 121 C7 | 140 C1 | | |

The determined coefficients of variation were lower than 5%.

Given the fact that the binding capacities of the various MAbs toward native β-LG were not identical, optimal concentrations of the reagents were determined in preliminary experiments. Consequently, to allow the comparison between various experiments, the results are expressed as relative inhibitory effectiveness (RIE) defined as:

$$\mathrm{RIE} = \frac{\text{modified } \beta\text{-LG concentration at 50\% inhibition}}{\text{native } \beta\text{-LG concentration at 50\% inhibition}} \quad (1)$$

**Immunoaffinity Chromatography.** MAbs 91 E7 and 102 G5 were bound to cyanogen bromide-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. About 10 mg of antibody was bound to 3.5 mL of gel. Gel beads were poured into a column (1 cm i.d. × 10 cm; Pharmacia) connected to an FPLC apparatus (Pharmacia). The column was equilibrated in 0.1 M Tris/HCl pH 8.0 buffer (buffer A), at a flow rate of 30 mL/h. Two milligrams of glycated β-LG, dissolved in 200 μL of buffer A, was loaded on the column, and the flow rate was set to 5 mL/h for at least 1 h. The unbound β-LG was eliminated by increasing the flow rate at 30 mL/h. The specific bound protein was then eluted using a 0.1 M glycine/HCl pH 2.2 buffer. The recovered yield varied from 7.5 to 15%. The elution was monitored at 280 nm.

**Characterization of the MAb-Specific Material.** The total and MAb-specific material (protein concentration: 65 μg/mL) were digested by pepsin at an enzyme/substrate molar ratio of 1/40, in 0.1 M glycine/HCl buffer pH 2.2, at 37 °C. The hydrolysis was stopped at different time intervals by cooling and adding a small volume of an ethanolic solution of pepstatin in order to obtain an enzyme/inhibitor molar ratio of 1/10. Samples (200 μL) were analyzed by RP-HPLC on a Vydac C18 column (4.6 mm i.d. × 25 cm; Pierce, Rockford, IL) equilibrated with 95% solvent A (0.106% (v/v) trifluoroacetic acid in Milli-Q water) and 5% solvent B (0.1% (v/v) trifluoroacetic acid in 4:1 (v/v) acetonitrile/Milli-Q water). Elution was performed with a gradient of 5−60% solvent B over 30 min followed by 100% solvent B over 10 min. Separation was achieved at 40 °C at a flow rate of 1 mL/min. The mobile phase was delivered by a SP 8800 pump (Spectra Physics, San Jose, CA), and eluted peaks were detected at 214 nm by an SP 8490 detector (Spectra Physics). The time-course hydrolysis was monitored by the disappearance of the β-LG peak area.

## RESULTS AND DISCUSSION

**Screening of MAbs Reactivity with Modified β-LG.** As a first step, screening of the reactivity of 11 MAbs was done with native and glycated β-LG (DW-Lac and SOL-Lac) using inhibition of indirect ELISA. Four distinct patterns of reactivity were observed. A classification of the MAbs according to these patterns is given in Table 3. Since these antibodies were raised against native β-LG, most of them exhibited either lower or equal reactivity with glycated β-LG. The glycation treatments led to a decrease in the reactivity of class A and B MAbs. The decrease in binding properties for class B MAbs was specifically induced by a treatment of β-LG in aqueous solution. Class C MAbs could not distinguish between native and glycated β-LG. Class D

**Table 4. Localization of Epitopes to the Monoclonal Antibodies on Bovine β-Lactoglobulin (Adapted from Vénien et al., 1997)**

| MAb | method used | epitope region |
|---|---|---|
| 37 1C10 | peptide binding | C-terminal peptide linked to β-strand D (peptide 61−69/70 = 149−162) |
| 91 E7 | cross reactivity with β-LG from other species | close to the GH loop residues 110−118) |
| 100 B3 | cross reactivity with β-LG from other species | delimited by an α-helix and the β-strand I (residues 130−150) |
| 102 G5 | peptide binding | AB loop (peptide 21−40) |

was represented by a unique MAb that bound more strongly to the glycated β-LG than to the native one.

Taken together, these results suggested that glycated β-LG contained regions with a preserved native structure as well as regions that were structurally modified and/or not available for antibody recognition. Furthermore, glycation of β-LG in an aqueous solution induced a more pronounced structural destabilization than in the dry system, despite the lower number of lactose attached. This confirmed our previous results (Morgan et al., 1999).

**Binding Studies with Selected MAbs.** Further analysis was done using selected MAbs, representative of the different binding patterns obtained (102 G5, 37 1C10, 91 E7, and 100 B3). Furthermore, this selection was made with respect to the epitopes recognized by these antibodies, which are located in regions involved in the denaturation of β-LG.

The epitopes recognized by these four antibodies, summarized in Table 4, have been defined by Vénien et al. (1997) on the basis of their ability to react with tryptic peptides of native β-LG or with β-LG from various species. In the latter case, the epitope were deduced from the comparison of the primary sequences of the different β-LG.

In the present study, the reactivity of these MAbs with glycated and control β-LG was measured and compared to that of the native protein, used as a reference. The typical sigmoidal inhibition curves are shown in Figure 1 and the calculated values of the relative inhibitory effectiveness (RIE) of the modified β-LG are given in Table 5. The same results were obtained from another ELISA technique, i.e., competitive ELISA based on the use of biotinylated native β-LG (results not shown).

**MAb 102 G5.** The epitope region to MAb 102 G5 is located in the stand A and AB loop regions of the molecule (Table 4). Its reactivity was promoted after glycation of β-LG (Figure 1 and Table 5), indicating that the epitope region to MAb 102 G5 (AB loop) is more accessible after extensive unfolding of β-LG. The observed reactivity changes were more pronounced in β-LG modified in solution. Glycated protein showed higher reactivity (lower RIE) compared to the control. This suggested that the attachment of lactose to β-LG in solution increased the unfolding induced by heating itself. The epitope region (AB loop) is stabilized by eight hydrogen bonds in the natural dimer (Brownlow et al., 1997), and any disruption in this area could induce dissociation of the dimer. Monomerization was observed upon prolonged glycation in solution but was not observed when glycation was done in the dry system (Morgan et al., 1999). Despite the observed reactivity changes of MAb 102 G5, unfolding of the AB loop may not be extensive enough to cause dimer dissociation.

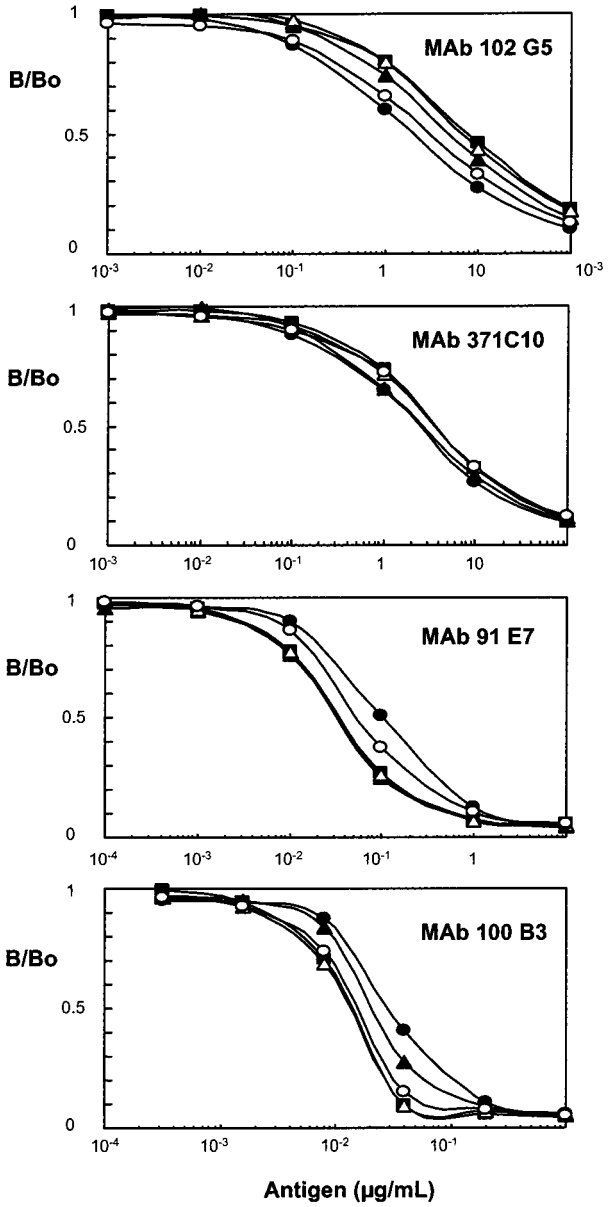



**Figure 1.** Inhibition curves obtained by indirect ELISA technique for four monoclonal antobodies. The inhibitors were as follows: ■, native; ●, ○, SOL-Lac and SOL-Ct; ▲, △, DW-Lac and DW-Ct. The MAb concentration was 10 ng/mL for 37 1C10 and 100 ng/mL for the others. B/Bo: absorbance in the presence of inhibitors/absorbance in the absence of inhibitors.

**Table 5. Relative Inhibitory Effectiveness (RIE) of the Modified β-LG Determined from Inhibition of Indirect ELISA[a]**

| | Mabs | | | |
|---|---|---|---|---|
| | 102 G5 | 37 1C10 | 91 E7 | 100 B3 |
| modified β-LG | | | | |
| DW-Lac | 0.6 | 1 | 1 | 1.4 |
| DW-Ct | 1 | 1 | 1 | 1 |
| SOL-Lac | 0.15 | 1.2 | 3.1 | 2.1 |
| SOL-Ct | 0.4 | 1.2 | 1.5 | 1 |

[a] Inhibition by native β-LG was taken as reference (RIE = 1).

Brownlow et al. (1997) reported that four other hydrogen bonds between β-strand I of adjacent monomers participate in dimer stabilization. The above result is consistent with the fact that β-strand I was probably not extensively modified when β-LG was glycated in the dry state.

**MAb 37 1C10.** MAb 37 1C10 recognizes the C-terminal part of the protein, linked to the β-strand D by the intramolecular disulfide bridge (Cys66-Cys160) (Table 4).

As shown in Figure 1 and Table 5, the reactivity of MAb 37 1C10 remained almost unchanged whatever treatments were used, indicating that no significant modification of the epitope had occurred following β-LG modification. Thus, the disulfide bridge between cysteine 66 and cysteine 160 was preserved in the epitope area and supports our previous findings (Morgan et al., 1999), which showed that no thiol/disulfide exchange reactions occurred upon glycation under the used experimental conditions.

**MAbs 91 E7 and 100 B3.** The region recognized by these two MAbs, i.e., α-helix, β-strand I, and GH loop (Table 4) are exposed and close to each other in the three-dimensional structure of β-LG (Chart 1).

MAb 91 E7 reactivity was not affected after dry-way glycation but was decreased after glycation in solution (Figure 1 and Table 5). The reactivity of this antibody with the control β-LG (SOL-Ct) was only slightly affected, indicating that heating by itself does not affect the structure of the epitope.

MAb 100 B3 reactivity toward the glycated β-LG was considerably decreased compared to the control β-LG (Figure 1 and Table 5). This decrease in reactivity was more pronounced when glycation was performed in solution.

The decrease in the reactivity of these two MAbs with the β-LG glycated in solution could be related to conformational changes in the epitope regions (α-helix, β-stand I, and GH loop). Such changes could lead to an increased accessibility of the free thiol group, normally buried in a hydrophobic pocket delimited by these secondary structure elements. We have previously postulated that the free thiol group played an important role in the denaturation and aggregation mechanism of β-LG glycated in solution, notably by promoting the formation of covalent homodimers through disulfide bond between the two free cysteine residues (Morgan et al., 1999). On the other hand, binding of MAbs 91 E7 and 100 B3 to the formed covalent dimers could be impaired through steric hindrance.

**Pepsin Hydrolysis of the MAb-Specific β-LG.** The results obtained using the ELISA technique showed that MAbs 91 E7 and 100 B3 recognized specific regions mainly in native molecules, while MAb 102 G5 recognized partially unfolded molecules. MAb 37 1C10 could not distinguish between native and unfolded molecules, suggesting that the epitope was not modified. Consequently, β-LG glycated in solution should be considered as a mixture of native and partially unfolded molecules. To verify this assumption, glycated β-LG molecules that react specifically with either MAb 91 E7 or MAb 102 G5 were purified by affinity chromatography and submitted to pepsin digestion.

The time-dependent hydrolysis of MAb-specific materials by pepsin compared to those of the total samples are shown in Figure 2. Under the experimental conditions used, the total SOL-Lac sample was highly susceptible to pepsin hydrolysis compared to the native protein. The domains recognized by MAb 102 G5 in the SOL-Lac sample were readily hydrolyzed by pepsin, whereas those recognized by Mab 91 E7 in the same sample were not. This result supports the fact that the SOL-Lac sample contained both the pepsin-susceptible unfolded molecules (recognized by MAb 102 G5) and the native molecules, which are resistant to the enzyme (recognized by MAb 91 E7).

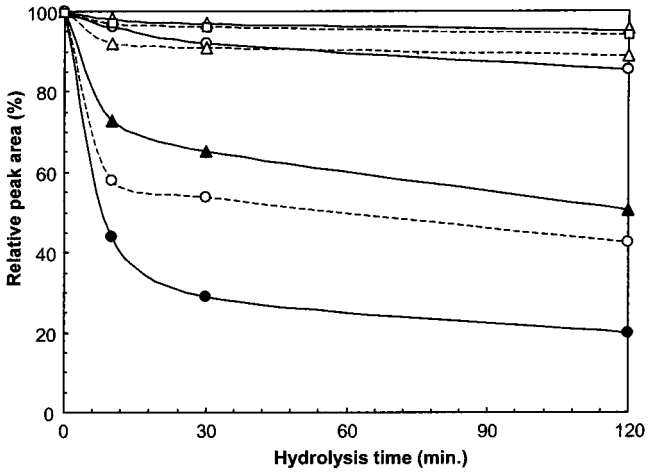



**Figure 2.** Time-course peptic hydrolysis of native, glycated, and MAb-specific β-LG. Dashed lines and open symbols: total material. Solid lines and open symbols: MAb 91 E7-specific material. Solid lines and closed symbols: MAb 102 G5 specific material. □: Native; ●, ○: SOL-Lac; ▲, △: DW-Lac. Values are the mean of two replicate experiments.

The total DW-Lac sample aand the molecules specifically recognized in this sample by MAb 91 E7 were poorly hydrolyzed by pepsin under these experimental conditions. As previously observed, this result showed the specificity of MAb 91 E7 for native structure (resistant to pepsin). On the contrary, the molecules specifically recognized by MAb 102 G5 in the DW-Lac sample were significantly hydrolyzed by pepsin. We have already demonstrated that a conformational change occurred in the AB loop region of the DW-Lac sample. This local unfolding increased the reactivity of the MAb 102 G5 and could explain the observed hydrolysis of the molecules specifically recognized by this MAb in the DW-Lac sample. Since the total DW-Lac sample was almost not hydrolyzed, it was likely that the conformational changes involved only a restricted amount of molecules in this sample, not easily detected using classical biochemical techniques as in our previous work (Morgan et al., 1999).

Furthermore, the obtained results showed that MAb 102 G5 recognized different protein structures in the SOL-Lac and DW-Lac samples as shown by both the rate and extent of hydrolysis of these two fractions (two times higher for the molecules purified from the SOL-Lac sample). The conformational change in the AB loop region was probably a common structural feature of these two samples, but the molecules purified from the SOL-Lac sample using MAb 102 G5 contained other unfolded regions (notably near the dimer interface), which rendered the protein highly susceptible to pepsin. The conformational changes occurring in these regions (AB loop, GH loop, β-strand I and α-helix) were probably the starting point for the modification of the protein association state, described in our previous work (Morgan et al., 1999).

## ABBREVIATIONS USED

β-LG, β-lactoglobulin; Ct, control sample; DW, dry-way glycation; ELISA, enzyme-linked immunosorbent

assay; Lac, glycated sample; MAb, monoclonal antibody; RIE, relative inhibitory effectiveness; SOL, solution glycation.

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