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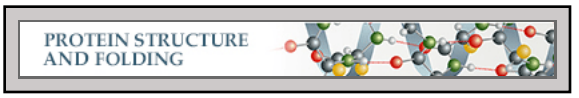

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INVOLVEMENT OF RESIDUES 66–76 OF STRAND D IN THERMAL DENATURED β-LACTOGLOBULIN*

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β-Lactoglobulin (β-LG) is a bovine milk protein sensitive to thermal denaturation. Previously, we demonstrated that such structural change can be detected by a monoclonal antibody (mAb) specific to denatured β-LG. In the present study, we show a dramatic increase in β-LG immunoreactivity when heating raw milk between 70 and 80 °C. To map out the specific epitope of β-LG recognized by this mAb, we used a combined strategy including tryptic and CNBr fragments, chemical modifications (acetylation and carboxymethylation), peptide array containing *in situ* synthesized peptides, and a synthetic soluble peptide for immunoassays. The antigenic determinant we defined was exactly located within the D strand (residues 66–76) of β-LG. Circular dichroic spectral analysis shows that carboxymethylation on β-LG not only resulted in a substantial loss of β-configuration but also exerted a 10 times increase in immunoreactivity as compared with heated β-LG. The result suggests that a further disordered structure occurred in β-LG and thus rendered the mAb recognition. Mutations on each charged residue (three Lys and one Glu) revealed that Lys-69 and Glu-74 were extremely essential in maintaining the antigenic structure. We also show an inverse relationship between the immunoreactivity in heated β-LG and its binding to retinol or palmitic acid. Most interestingly, pH 9–10, which neutralizes the Lys groups of β-LG, not only reduced its immunoreactivity but also its binding to palmitic acid implicating a role of Lys-69. Taken together, we concluded that strand D of β-LG participated in the thermal denaturation between 70 and 80 °C and the binding to retinol and palmitic acid. The antigenic and biochemical roles of mAb specific to D strand are discussed in detail.

Bovine β-lactoglobulin (β-LG)[1] is one of the major proteins in milk consisting of about 10–15% (1). Because of the thermally unstable and molten globule nature, β-LG has been studied extensively for its physical and biochemical properties (2, 3). The protein comprises 162 amino acid residues, with one free cysteine and two disulfide linkages (Fig. 1). According to the three-dimensional crystallographic studies, β-LG is predominantly a β-sheet configuration containing nine antiparallel β-strands from A to I (4–6) (Fig. 1). Topographically, strands A–D form one surface of the barrel (calyx), and strands E–H form the other. The only α-helical structure with three turns is at the COOH terminus, which follows strand H lying on the outer surface of the calyx (7). A remarkable property of the calyx is its ability to bind *in vitro* hydrophobic molecules such as retinol, fatty acids, vitamin D, and cholesterol (8–11). Spectroscopic studies have demonstrated that irreversible modification of the β-LG structure occurs upon thermal treatment above 65–70 °C. Thermodynamic analysis of the calorimetric signal reveals that there are two domains unfolding independently while heating (12). The exact regions involved in the thermal denaturation are still unclear. Whether the subtle unfolding changes can be detected by an immunochemical approach remains a question.

Regardless of intensive research, the biological function of this protein has not yet been satisfactorily resolved. Recently, we immunized the mice with commercially prepared dry milk and produced a panel of monoclonal antibodies (mAb). From the 900 hybridomas screened, a clone specific to dry milk, but not to raw milk, has been selected. Characterization of this dry milk-specific mAb reveals that this antibody recognizes thermally denatured β-LG (13). It suggests that a new antigenic epitope in β-LG is being exposed by a heating process used in the preparation of dry milk. In the present study, we defined the immunoreactive site that was recognized by this specific mAb, and we attempted to relate it to the thermal denaturation properties of β-LG. The strategy for epitope mapping combined several approaches, including tryptic and CNBr fragments, chemical modifications (acetylation and carboxymethylation), peptide array containing *in situ* synthesized peptides (with overlapped regions), and a synthetic peptide in solution for immunoassays. We demonstrate that the epitope was located exactly within the D strand of β-LG (residues 66–76). The immunoreactivity as recognized by this mAb was correlated to the thermal denaturation and conversion of β-sheet to a disordered structure of β-LG. Most interestingly, the D strand is associated with the A–C strands forming one domain at the opening of the calyx (Fig. 1). For this reason, we also studied

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[1] The abbreviations used are: β-LG, β-lactoglobulin; CM-LG, carboxymethylated-β-LG; mAb, monoclonal antibody; ELISA, enzyme linked immunosorbent assay; PBS, phosphate-buffered saline; PDB, Protein Data Bank; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; LDL, low density lipoprotein; CM, carboxymethylated.

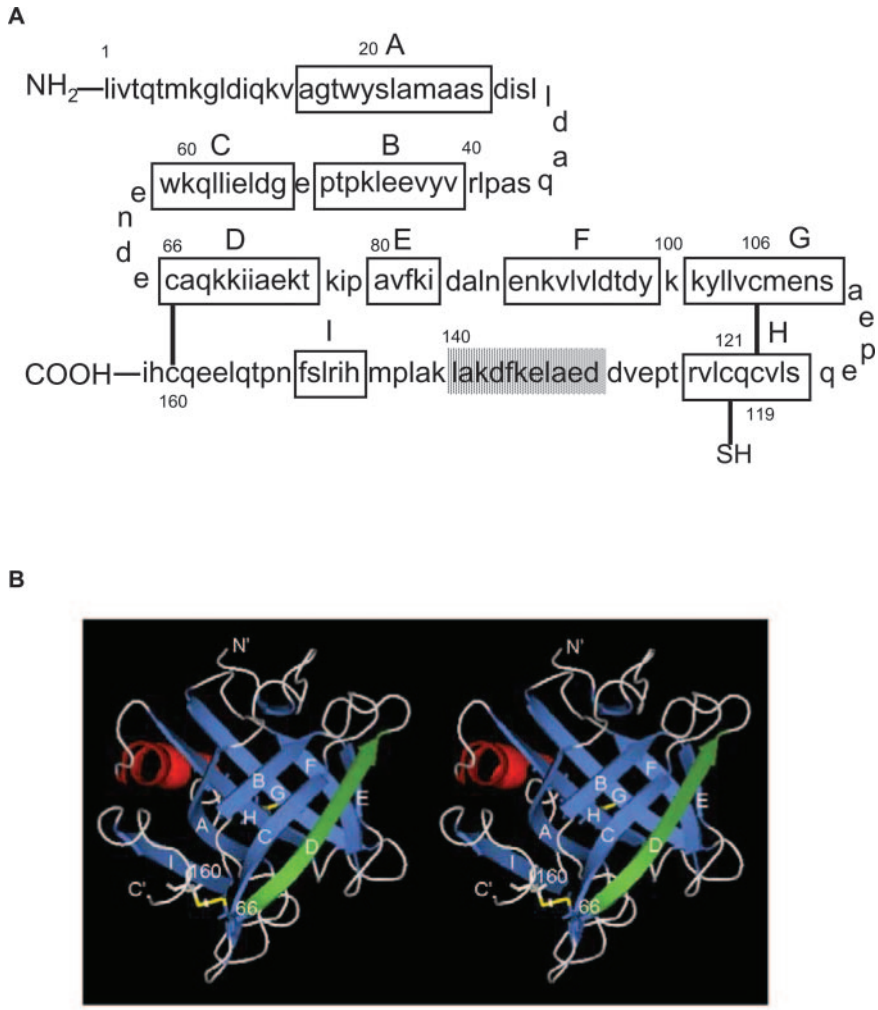


FIG. 1. **Amino acid sequence and three-dimensional structure of β-LG.** *A*, β-LG consisted of 162 amino acids with nine β-sheet strands (*A–I*) and one α-helix (*shadow*). There are two disulfide bonds located between strand D and the carboxyl terminus (Cys-66 and Cys-160) and between strands G and H (Cys-106 and Cys-119), whereas a free buried thio group is at Cys-121. *B*, crystal structure of β-LG determined by Ref. 6 and created by PyMOL (25) (PDB code 1CJ5), shows that strands A–D form one surface of the barrel, and strands E–I form the other. The antigenic site recognized by the mAb is located within strand D (*green*) following thermal denaturation. The two disulfide linkages are also shown (*yellow*). Hydrophobic molecules such as retinol and palmitic acid are bound in the pocket of calyx.

the effect of heating and pH on β-LG binding to retinol and palmitic acid. Further epitope mapping shows that conversion of Glu-74 into either Ala or negatively charged Asp totally abolished its immunoreactivity. A similar result was seen in Lys-69 but not the other Lys residues. Finally, we propose that strand D plays a provocative role in the molten globule state of β-LG as probed by our mAb.

EXPERIMENTAL PROCEDURES

Materials—β-LG was purified from fresh raw milk using 30% saturated ammonium sulfate top fraction followed by a G-150 column chromatography as described previously (13, 14).

Preparation of Monoclonal Antibody Specific to Dry Milk—Monoclonal antibodies were produced according to the standard procedures described previously by us (15, 16), in which dry milk (Nestle Australia Ltd., Sidney, Australia) was used for immunization (13). In brief, the myeloma cell line (FO) was fused with spleen cells from immunized BALB/c mice at a ratio of 1:5. The culture medium (between days 14 and 21 after fusion) was assayed for the production of specific antibodies by a solid-phase ELISA using both raw and dry milk as the respective antigen. Each monoclonal antibody was established by limiting dilutions at least two times (15, 16).

Trypsin and CNBr Fragmentation—For trypsin treatment, 50 μg of β-LG in 100 μl of phosphate-buffered saline (PBS) containing 0.02 M phosphate and 0.12 M NaCl, pH 7.4, were preheated at 100 °C for 10 min. After this, 1 μl of trypsin (0.1 mg/ml) was added and incubated at room temperature for 4 h (13). Trypsinized LG was analyzed on an SDS-PAGE (18% polyacrylamide) followed by a Western blot. For CNBr fragmentation (17, 18), 5 mg of β-LG were first dissolved in 70% (v/v) trifluoroacetic acid with the addition of 10 mg of CNBr in the dark for 24 h at room temperature. After evaporating three times in a Speed Vac (CVE 200D, ELELA, Japan) with the addition of 5× volume of deionized water, the dry material was dissolved in the 10 mM phosphate buffer, pH 7.0. The immunoreactivity of CNBr fragments was then analyzed on an 18% SDS-PAGE, followed by a Western blot.

Acetylation and Carboxymethylation of β-LG—Chemical modification of β-LG by acetylation was conducted by a modification of the procedure described previously by us (19). To 5 mg of β-LG in 2 ml of 50 mM sodium bicarbonate, pH 8.0, containing 6 M urea, 5 μl of acetic anhydride were slowly added into the reaction mixture step by step, while maintaining the pH at 8.0 by using 0.1 M NaOH. After 3 h of incubation at room temperature, the acetylated protein was desalted on Bio-Gel P-2 column eluted by 0.05 M ammonium bicarbonate and lyophilized. For carboxymethylation (19, 20), 5 mg of β-LG were first dissolved in 5 ml of 0.1 M Tris-HCl buffer, pH 8.6, containing 6 M ultra pure urea and 0.02 M dithiothreitol. Following flushing with nitrogen, 20 mg of iodoacetic acid were added into the reaction mixture, while maintaining the pH at 8.6 by the addition of 0.1 M NaOH, and were incubated for another 3 h. Finally, carboxymethylated (CM) β-LG was desalted on a Bio-Gel P2 column eluted by 0.05 M ammonium bicarbonate and lyophilized. By amino acid analysis, the CM-β-LG contained 4.98 residues of CM cysteine per mol of β-LG.

CD Spectrum—The secondary structure of native, heated, or chemically modified β-LG was determined using a computerized Jasco J-715 CD spectropolarimeter. Each protein sample was dissolved in 10 mM phosphate buffer, pH 7.0, with a final concentration of 0.2 mg/ml. About 300 μl of the protein solution were used for analysis within a cuvette of 1-mm path length. The obtained spectra were accumulated for 25 times at a scanning rate of 50 nm/min. All the data were shown as the mean residue molar ellipticity $[\theta]_{MRW}$ (20, 21).

Peptide Array—Twelve synthetic peptides in one nitrocellulose array, each containing 15 amino acid residues, were designed corresponding to residues 25–107 of β-LG or to residues 67–75 within strand D (Fig. 1). The synthetic peptides were prepared under a contract with Genesis Biotech Inc. (Taipei, Republic of China). Briefly, the peptides were directly synthesized *in situ* on a nitrocellulose paper according to the method described previously (22). The nitrocellulose membrane in

0.01 M Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBST) was blocked with 5% (w/v) gelatin in TBST for 2 h at room temperature followed by washing three times. After incubation with mAb for 2 h and three washes, goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) in 0.5% gelatin/TBST was added and incubated. Finally, following the washes, the chemiluminescent substrate (ECL™ Western blotting System, Amersham Biosciences) was added, washed, and immediately developed by exposing onto a film.

Competitive ELISA—In brief, heated β-LG (1 μg in 50 μl of PBS) was first immobilized onto microtiter wells followed by three washes to remove unbound β-LG (13, 23). The wells were then blocked by 3% gelatin in PBS. After three washes, 50 μl of the competitive protein (β-LG, heated β-LG, acetylated or carboxymethylated β-LG, or synthetic peptide residues 67–76) in PBS containing 0.3% gelatin were mixed with 50 μl of mAb and incubated at room temperature for 1 h. Following washes and secondary antibody (goat anti-mouse IgG conjugated with HRP) incubation, the microtiter plate was developed with 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and read at 415 nm.

Effect of pH on β-LG Binding to mAb—β-LG (1 μg/well) was immobilized onto a microtiter plate followed by blocking and washing at neutral pH. The immobilized β-LG was then incubated with mAb at various pH values for 2 h at room temperature. After removing the unbound mAb, the plate was developed according to the standard ELISA procedures at neutral pH (13, 23). Because pH itself can affect the antigen-antibody binding, a control experiment using mouse IgG as immobilized antigen was also conducted at various pH values for a parallel comparison.

Retinol and Palmitic Acid Binding to β-LG—β-LG was reported to be a 1 to 1 binding ratio with retinol or palmitic acid as measured by fluorescence emission techniques (24). In general, binding of retinol to β-LG was measured by extrinsic fluorescence emission of the retinol molecule at 470 nm using excitation at 287 nm. Binding of palmitic acid to β-LG was measured by the fluorescence enhancement of Trp residues of β-LG at 332 nm by using excitation at 287 nm. For the effect of the pH experiment, 5 or 20 μM of native β-LG was instantly incubated with 5 μM of retinol or 20 μM of palmitic acid, respectively, at various pH values at 24 °C. For the effect of the heat experiment, β-LG was preheated at 80 or 100 °C for 5 min and then incubated with retinol or palmitic acid at pH 8.0. Fluorescence spectra were recorded at 24 °C with a fluorescence Spectrophotometer F-4500 (Hitachi High-Tech Corp., Tokyo, Japan).

Three-dimensional Analysis of β-LG Structure—The three-dimensional structure of β-LG used in this context was provided by Protein Data Bank (www.rcsb.org/pdb/), code 1CJ5 (6), with the diagram created by PyMOL (25).

Three-dimensional Analysis of β-LG and Palmitic Acid Complex Structure—The three-dimensional structure of bovine β-LG complexed with palmitic acid used in this context was provided by Protein Data Bank code 1GXA (11), with the diagram created by RasMol (26).

RESULTS

Characterization of the Monoclonal Antibody Specific to Dry Milk—Previous studies (13) show that the monoclonal antibody (mAb) used in this report is specific to processed dry milk but not to raw milk. It only recognizes β-LG, one of the major milk proteins. The mAb is apparently able to discriminate the denatured β-LG from a given milk product (13). Because heating procedures are used to process the dry milk, the finding indicates that β-LG undergoes a conformational rearrangement, which facilitates the binding of this mAb. In the present study, we show a dramatic and sharp increase in β-LG immunoreactivity when raw milk was heated between 70 and 80 °C over time (Fig. 2). It was of interest that the increase in immunoreactivity was concomitant with the reported transition temperature for converting native to denatured β-LG (27). The finding suggests that the immunoreactive site recognized by this mAb lies in the thermal denatured region of β-LG.

Mapping of Antigenic Determinant of Denatured β-LG Utilizing Tryptic Digestion and Acetyl Modification—To initially map out the specific immunoreactive region, heated β-LG was limitedly digested by trypsin. On Western blot, we demonstrated that the immunoreactivity was totally abolished after the trypsin treatment (13), suggesting that Lys, Arg, or both

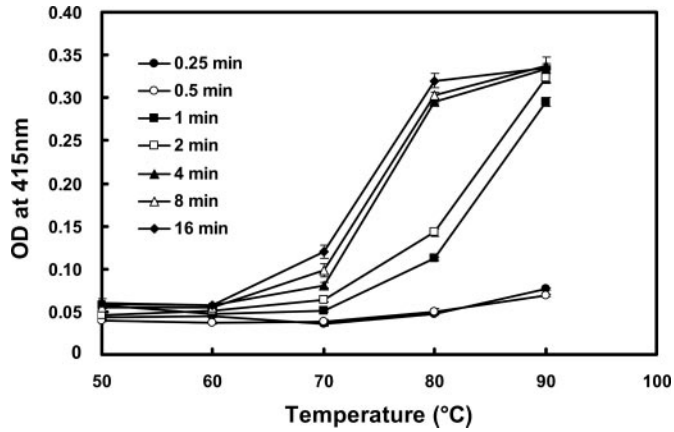
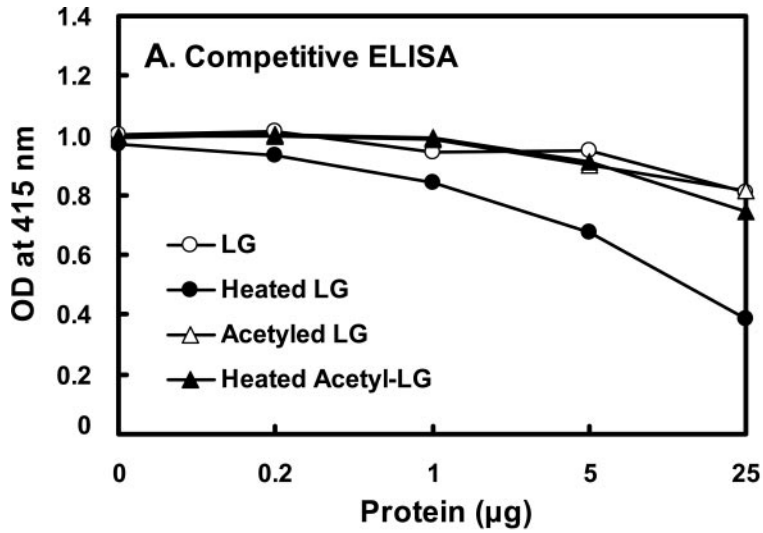


FIG. 2. **Immunoreactivity of β-LG in raw milk heated at different temperatures over time.** Immunoreactivity was monitored using an ELISA on raw milk heated at various temperatures. The increase in immunoreactivity of β-LG assessed by dry milk-specific mAb is correlated to the molten globule state of β-LG with a transition between 70 and 80 °C.

residue(s) were probably involved in maintaining the antigenic structure for β-LG. Chemical modification using acetylation (pH 8.0) on positively charged residues (mostly Lys) attenuated the immunoreactivity of β-LG on a competitive ELISA (Fig. 3*A*). These two experiments support the notion that positively charged amino acids of β-LG attributed for the mAb recognition.

Immunoreactivity of CNBr Fragments of β-LG—To delineate further the major antigenic domain, CNBr cleavage on β-LG was conducted. Western blot analysis shows that there was a major immunoreactive fragment corresponding to a molecular mass of about 9 kDa (Fig. 3*B*). As estimated from its Met cleavage site, this fragment was presumed a peptide containing residues 25–107 (Fig. 1). Subsequently, the amino-terminal sequence of this fragment was determined. The first six amino acid residues (AASDIS) confirmed that the immunoreactive site was located between residues 25 and 107 of β-LG (Fig. 1).

Final Antigenic Mapping Using a Solid-phase Peptide Array—As described above, Lys-enriched areas were assumed to participate in maintaining the antigenic structure. Pro residues are also considered to be involved as they are located at or near the antigenic determinant by forming a loop at the surface of a given protein. By using an EMBOSS program for searching a possible antigenic determinant within residues 25–107 of β-LG (Fig. 4), we predicted that two domains, namely residues 42–56 and 67–81, were most likely to be immunoreactive. Accordingly, a solid-phase peptide array containing the above predicted regions and 10 other overlapped synthetic peptides (each with 15 residues) was prepared. These peptides were directly synthesized on a nitrocellulose membrane (Fig. 4). After binding of mAb and HRP-conjugated secondary antibody, the array was developed by a chemiluminescent agent. We show that only peptides 4 (residues 70–84), 8 (residues 61–75), and 12 (residue 67–81) were immunoreactive (Fig. 4). Peptide 4 gave partial immunoreactivity, suggesting that residues 67–70 were essentially involved in the reactive site (Fig. 4). Because the size of an epitope is relatively small, usually containing 6–9 amino acid residues (16, 23, 28–30), it was possible to narrow down the reactive site from the immunoreactivity in overlapped peptides. We proposed that the reactive site was closely associated with AQKKIIAEK (or nine residues 67–75) (Fig. 4). Notably, this region is highly positive in charges. By observing the high resolution crystal structure of β-LG (6), it is fascinating to see that this proposed region is exactly located within the D strand of surfaced β-sheet (residues 66–76) (Fig.



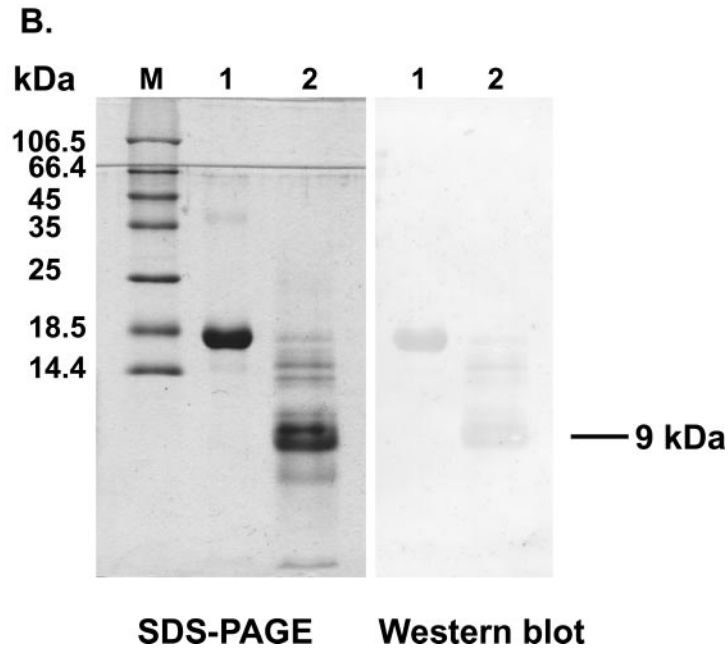


FIG. 3. **Effect of acetylation and CNBr cleavage on immunoreactivity of β-LG.** *A*, competitive ELISA using heated β-LG as an immobilized antigen, while competing with native, heated, acetylated, and heat-acetylated β-LG. Immunoreactivity of β-LG was significantly increased upon heating at 100 °C for 5 min but not of acetylated and heated acetyl-LG. *B*, immunoreactivity of CNBr fragments of β-LG. About 10 μg of native and CNBr cleaved β-LG were used for SDS-PAGE containing β-mercaptoethanol as a reducing agent (*left*) followed by a Western blot (*right*). A major immunoreactive peptide with a molecular mass of about 9 kDa was found to be in residues 25–107 as determined by an amino-terminal sequence analysis. In theory, fragments with a molecular weight greater than 9 kDa represent those incompletely cleaved peptides (Fig. 1). *Lane M*, molecular markers. *Lane 1*, native β-LG. *Lane 2*, CNBr fragments of β-LG.

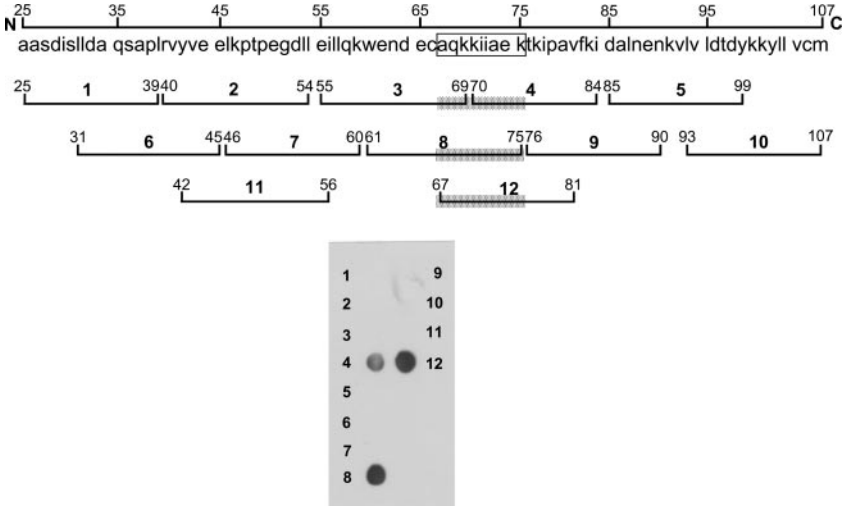


FIG. 4. **Delineation of an epitope recognized by mAb.** Twelve peptides corresponding to a 9-kDa CNBr fragment of β-LG (residues 25–107) were directly synthesized *in situ* on nitrocellulose membrane. Peptides 11 and 12 were prepared due to the presence of Pro residues thought to be potentially antigenic and Lys thought to be involved per our trypsin and acetylation experiments. The entire peptide array was commercially prepared under a contract for customer designing. Binding of antibody was conducted by using HRP-labeled secondary antibody with chemiluminescent agent as a developer. The shed region represents the proposed epitope.

1). We therefore defined this immunoreactive site as an epitope. Another noticed point is that there is a disulfide linkage between strand D (Cys-66) and the carboxyl terminus (Cys-160). This disulfide linkage plays an important role in stabilizing the β-structure by forming antiparallel sheets of β-LG. The proposed epitope (residues 67–75) in its native state is rather ordered with a β-sheet span about 28 Å in length. As such, the orientation in native β-LG may prohibit the binding

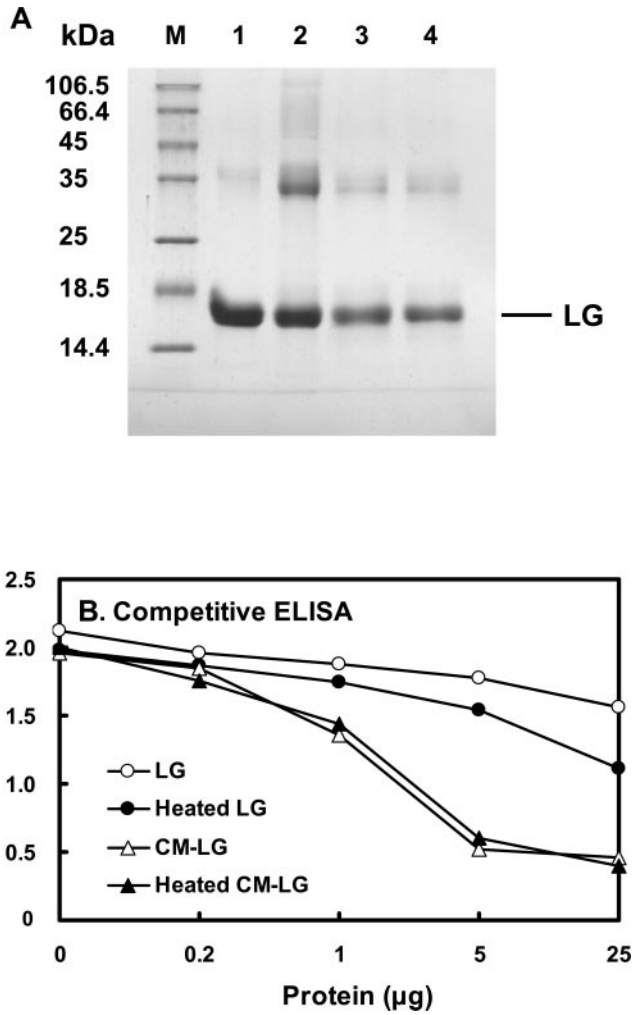


FIG. 5. **SDS-PAGE profile and immunoreactivity of carboxymethylated β-LG (*CM-LG*).** *A*, about 10 μg of β-LG and CM-LG were loaded on 15% SDS-PAGE. *Lane M*, molecular markers. *Lanes 1*, β-LG. *Lane 2*, heated β-LG. *Lane 3*, CM-LG. *Lane 4*, heated CM-LG. Significant increase in β-LG dimer and high molecular forms are seen, while heating at 100 °C for 5 min, but not in CM-LG. *B*, competitive ELISA of native, heated, CM and heated CM-LG. The plate was immobilized with heated β-LG.

of our specific mAb. We hypothesized when the D strand underwent disordered structure, it would then allow the "denatured" mAb binding.

Carboxymethylated β-LG and CD Spectrum—To test the above hypothesis by which the conformational change of the D strand would enhance the binding of our mAb, we chemically modified all the Cys residues to irreversibly block the disulfide linkages within the native β-LG (Fig. 5*A*). By using a competitive ELISA with heated β-LG as a positive control, it revealed that carboxymethylation on β-LG resulted in a striking increase in its immunoreactivity. The increase was about 10× greater than that of heated β-LG (Fig. 5*B*). Meanwhile, analysis of CD spectra on carboxymethylated β-LG further confirmed a significant conformational change by converting a β-sheet (typically at 215 nm) to a more disordered structure than that of heated β-LG (Fig. 6).

Immunoreactivity of a Soluble Synthetic Peptide—Finally, a soluble peptide corresponding to the linear sequence of strand D (residues 67–76 or AQKKIIAEKT) was synthesized. Fig. 7 shows that this linear sequence was able to inhibit completely mAb binding to heated β-LG on a competitive ELISA. Furthermore, this synthetic peptide exhibited a typical disordered structure rather than a β-configuration (Fig. 6).

Role of Charged Residues in Epitope Specificity—To determine which lysines were responsible for the mAb recognition, mutation on each Lys (Lys-69, Lys-70, and Lys-75) with Ala was conducted. As shown in Fig. 8, only Lys-69 was very specific for the mAb binding. Replacement with positively charged Arg did not salvage the immunoreactivity. Glu-74 played a similar role; replacement with Ala or negatively charged Asp failed to show any immunoreactivity. Ile-71 and Ile-72 also played an essential hydrophobic role, although the exact residue has not been identified. Meanwhile, negative control peptides (Fig. 8, *peptides 11–12*) retaining all the Lys residues did not show any binding.

Effect of pH on β-LG Binding to mAb—Because the structural stability of β-LG is pH-dependent (31), we tested whether changes of pH could also induce an increase in β-LG immunoreactivity. Fig. 9*A* shows that the CD structure of β-LG was stable at pH 2 with some changes between 3 and 7, whereas a transition to disorder was seen from 8 to 10. However, such a disordered structure did not facilitate the mAb binding (Fig. 9*B*). Because Lys-69 was essential (Fig. 8) and the overall positive charge of this residue started to become neutralized under pH 8–10, the immunoreactivity was decreased (Fig. 9*B*). A control experiment showing a typical pH-dependent antigen-antibody reaction was performed (Fig. 9*C*), and there was a slight decrease in immunoreactivity at pH 9–10.

Effect of pH and Heating on β-LG Binding to Retinol and Palmitic Acid—To explore the correlation between the structural change of β-LG (at various pH) and its retinol binding, we monitored the extrinsic fluorescent change of retinol upon the binding to β-LG. The optimal binding for retinol appeared to be at pH between 8 and 10 (Fig. 10*A*). Heating β-LG at temperatures greater than 80 °C almost completely abolished its binding for retinol (Fig. 10*B*). The data support the notion that the striking increase in immunoreactivity of the D strand at this temperature (Fig. 2) was negatively correlated to the retinol binding, which requires the integrity of a β-sheet structure of β-LG.

Most interestingly, the binding to palmitic acid was decreased to some extent at pH 9–10 (Fig. 10*C*), which correlated with the binding to mAb (Fig. 9*B*). Because Lys-69 played an very essential role in the antigenic site (Fig. 8), such correlation suggests that the protonated state of this residue might be involved in stabilizing both the mAb binding and β-LG-palmitic acid complex formation (see more details under "Discussion"). Similarly, heating on β-LG substantially reduced the binding for palmitic acid.

DISCUSSION

Molten globules are thought to be general intermediates in protein folding and unfolding (32, 33). β-LG, a major moiety of bovine whey proteins, is one of the most investigated models for understanding the mechanism involved in protein stability upon heating. Although the three-dimensional crystal structure of β-LG has been elucidated, the area involved in thermal denaturation remains unclear. On the other hand the region responsible for Tanford transition (4), occurring at pH from 6.5 to 8.0 is known to be within the residues 85–90 (EF loop). This region opens or blocks the entrance of the calyx (34).

The present study (13) demonstrates that denatured strand D of β-LG was responsible for the binding of our thermally sensitive mAb. Several unique features of the binding are identified. First, heating on native β-LG resulted in a loss of β-sheet to more disordered structure (Fig. 6) in which the immunoreactivity was concomitantly increased (Fig. 5). Second, blocking the disulfide linkage between the D strand (Cys-66) and COOH terminus (Cys-160) of β-LG by carboxymethylation not only produced a disordered structure (Fig. 6) but also markedly

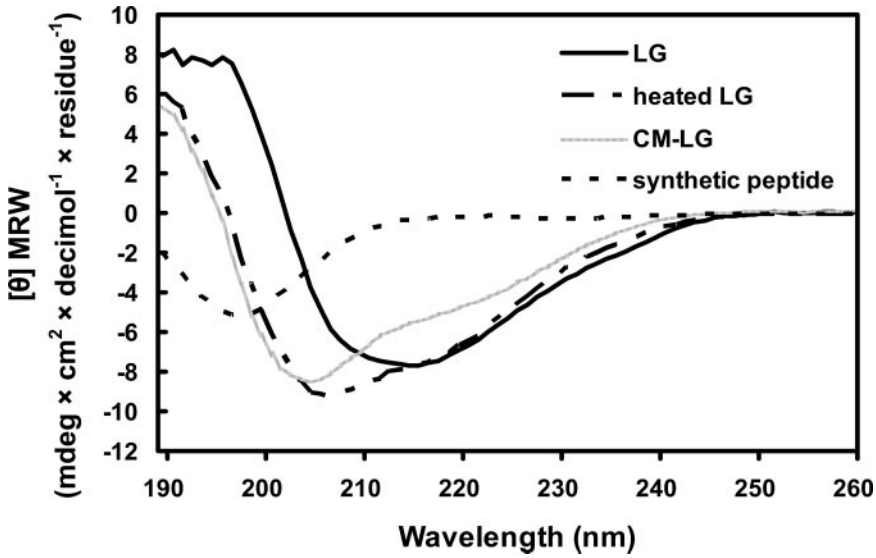


FIG. 6. **Circular dichroic spectra of native β-LG, heated β-LG, carboxymethylated LG (*CM-LG*), and synthetic peptide 67–76.** Samples at a final concentration of 0.2 mg/ml in 10 mM phosphate buffer, pH 7.4, were used for the study. A typical β-structure of native β-LG is shown at 215 nm. β-LG was heated at 100 °C for 5 min.

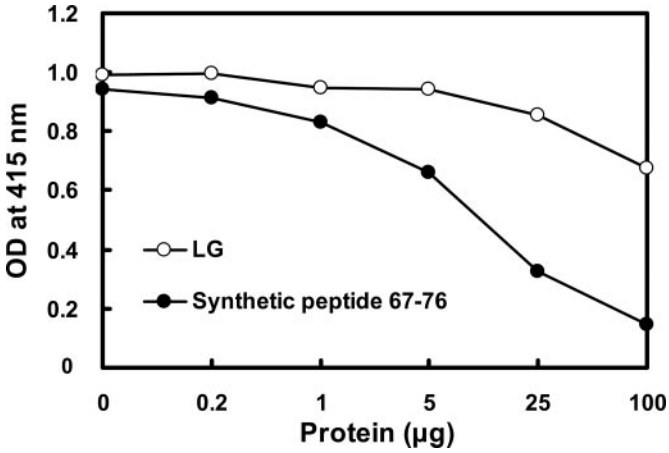


FIG. 7. **Immunoreactivity of the synthetic peptide determined by an ELISA.** A synthetic peptide corresponding to residues 67–76 (AQKKIIAEKT) in strand D was prepared as a soluble form. The plate was immobilized with heated β-LG.

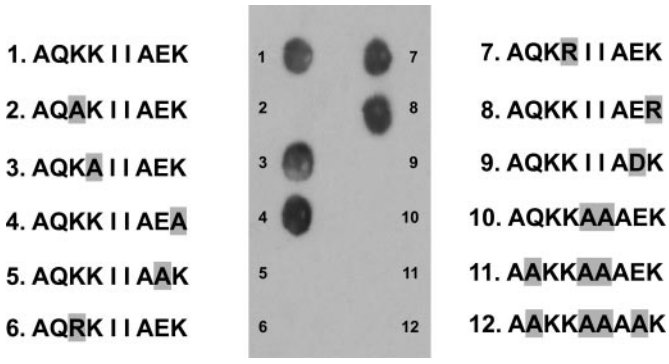


FIG. 8. **Role of charged residues in antigenic specificity determined by a peptide array.** Peptide 1 represents the native sequence 67–75 of β-LG. Substitution of Lys-69 and Glu-74 by Ala or a same charged amino acid, respectively, resulted in a total loss of immunoreactivity (*peptides 2–9*). Hydrophobic residues Ile-71 and Ile-71 were essential in maintaining the antigenic structure, whereas peptides 11 and 12 retaining all the Lys residues were used as randomized negative controls.

enhanced the mAb binding. Further heating on carboxymethylated β-LG did not give more binding. Such enhancement was even greater than heated β-LG (10×) on competitive ELISA (Fig. 5*B*). Presumably, this was because of the augmented degree of freedom of the D strand without the disulfide linkage rendering more antibody binding. It is of interest to point out that the secondary structure of strand D alone, without including strand C, is predicted as 50% random coiled (residues 66–70) and 50% helical (residues 71–76) by using the parameters from three-dimensional PSSM; the folding recognition server at the Imperial Cancer Research Fund (ICRF) (www.sbg.bio.ic.ac.uk/~3dpssm/). However, in the presence of strand C as an anti-parallel orientation, the predicted structure of strand D becomes β-configuration. Obviously, the formation of an anti-parallel β-structure in native β-LG molecule is stabilized through the help of a disulfide linkage (Cys-66 and Cys-160) between strand D and the helical domain at COOH terminus (Fig. 1). Thus, conformational change on strand D played a vital role for the mAb recognition. It is worth mentioning that although severe heating might break the disulfide linkage at Cys-66, it would be immediately "stabilized" via re-oxidation by forming high molecular or self-associated polymers as shown in our previous report (13). Thus, the immunoreactivity of heated β-LG was less than that of carboxymethylated β-LG. Third, the soluble synthetic peptide (residues 67–76) corresponding to strand D (without Cys-66) was able to completely inhibit the binding of mAb to β-LG. Fourth, the D strand is topographically located at the surface of β-LG (Fig. 1), which is agreeable to the general concept of a given antigenic epitope (35). Fifth, the buried side chain of Lys-69 was exposed upon heating and then recognized by the mAb (described below).

With respect to the exact size of the epitope that was recognized by our mAb, we excluded the possibility of Cys-66 as part of the epitope from the D strand. First, peptide 67–81 without Cys-66 gave an almost equal immunoreactivity to that of peptide 61–75 with Cys-66 in a peptide array assay (Fig. 4) suggesting that Cys-66 might not be located in antigenic determinant. Second, carboxymethylation on whole β-LG molecules with Cys-66 included in the modification markedly increased its immunoreactivity. If Cys-66 were involved in the antigenic site, introduction of such a bulky group (carboxymethyl) on this residue would have resulted in a significant loss of immunoreactivity (30). It should be noted here that Cys-66 was only responsible for the conformational restraint by cross-linking Cys-66 and Cys-160; as such it limited the binding for denatured mAb. Third, the length of a linear epitope can be as short as 6–7 residues as demonstrated by our previous work (16, 23, 28) and the work of others (29, 30). The present study shows the involvement of Glu-74 in the epitope (Fig. 8), which is eight residues apart from Cys-66. Finally, our synthetic soluble peptide without Cys-66 could completely inhibit the mAb binding

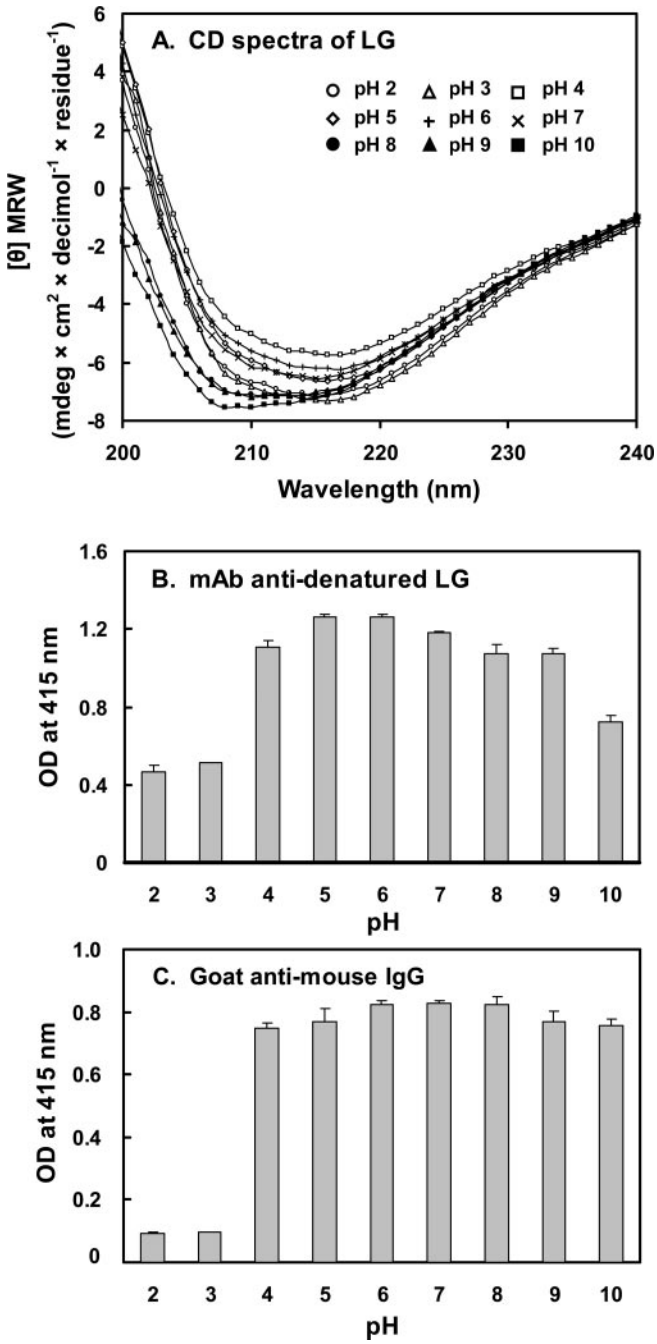


FIG. 9. **Effect of pH on β-LG binding to mAb.** *A*, CD spectra of β-LG at pH from 2 to 10. *B*, binding of mAb to β-LG at various pH values determined by ELISA. The primary mAb binding was conducted at the pH values indicated; all other reactions, including coating, blocking, washing, and secondary antibody binding, were performed at pH 7.4 according to standard procedures. The binding was significantly decreased at pH 8–10. *C*, control experiment evaluating the effect of pH on antigen-antibody interaction. This experiment was designed to study the pH effect on the binding of antigen and antibody in general. Antigen (mouse IgG) was coated on the plate and allowed the binding of HRP-labeled anti-IgG (goat) at various pH values. The plate was then developed after removing unbound antibody using PBS at pH 7.4. The binding was slightly decreased at pH 9–10.

(Fig. 7). Regardless, our antigenic mapping suggests that strand D located on the surface is involved in the molten globule and the unfolding structure of β-LG while heated. Coincidently, the transition temperature of native β-LG was between 70 and 80 °C, which is in agreement with the increase in immunoreactivity for this mAb. On the other hand, the polyclonal antibody raised against the native β-LG did not exhibit such a unique property (13).

The antigenic mapping from this study also provides some interesting insight as to the specificity of antigen-antibody interaction. Lys-69 and Glu-74 in the epitope were found to be extremely essential in maintaining the antigenic structure (Fig. 8), and substituting each with uncharged Ala diminished the mAb binding. Replacing each with the same charged amino acid Arg and Asp, respectively, could not restore the immunoreactivity. However, such point mutation in a given protein between the same charged residues Lys/Arg or Glu/Asp is very common within or among the species, while still maintaining its biological function. Notably, fragment 70–84 (Fig. 4) without Lys-69 gave a partial immunoreactivity, which differed from the mutation experiment (Fig. 8). Although the mechanism involved remains elusive, one possible explanation was the different solution property between the peptide with and without Lys-69. We speculate that the binding was more specific with the increase of chain length of the epitope, such as "lock and key." Another interesting feature is that the span between Lys-69 and Glu-74 is six amino acids, which are sufficient to form an epitope as described previously by us using a mAb against fibrin (16). It is consistent with our observations for the interaction of protein antigens with antibodies, in which six determining residues, for the most part, were involved in binding with antigen (16, 29). We also demonstrate the importance of Ile-72 and Ile-73 within residues 69–74; substituting these two hydrophobic residues by Ala diminished its immunoreactivity. However, a further delineation using point mutation to each residue is needed to draw a final conclusion.

Using mAb as a probe to study the structural and functional relationship of a given protein has been popular and has been reviewed (36). It provides a powerful tool in defining the functional location within the molecule. Previously, we have shown mAb prepared against human hepatic lipase can distinguish between active and inactive forms of lipase (15). Some unique low density lipoprotein (LDL) mAbs have been used for discriminating between patients with and without coronary artery disease (37, 38). Those LDL mAbs have also been utilized for probing the thermal changes of human LDL (39), whereas the immunoreactivity was conversely correlated to the temperature with the optimal binding at 4 °C (38, 39). Because the three-dimensional structures of lipase and LDL were lacking, the exact mechanism involved remains elusive. The epitope of the β-LG we mapped in this study, however, is known and provides a better understanding for its interaction with mAb. We propose that because of a highly ordered β-configuration of the D strand in its native state, the binding to our specific mAb is prohibited. But the D strand underwent a conformational change upon heating, which would then allow the denatured mAb binding.

With respect to physiologic significance, the epitope region we defined is located at one of the critical domains forming a conical central calyx that is responsible for the binding of retinol and fatty acids (10, 11). An *in vivo* experiment shows that β-LG enhances the intestinal uptake of retinol in preruminant calves (40). It has been suggested that conformational changes in the calyx and the exposure of the surface hydrophobic site of β-LG in the molten globule state reduces the retinol affinity (24). The binding specificity may be determined by the dynamic motion of loops between the β-strands (34, 41). It is not known which β-strand(s) is involved for the exposure of the surface hydrophobic site. In this study we show a substantially decreased binding of retinol to β-LG that was preheated at a transition temperature of 80 °C (Fig. 10), with a significant increase in immunoreactivity (Fig. 2). Notably, two hydrophobic residues Ile-71 (buried in native state) and Ile-72 (exposed in native state) are located within the epitope and participate

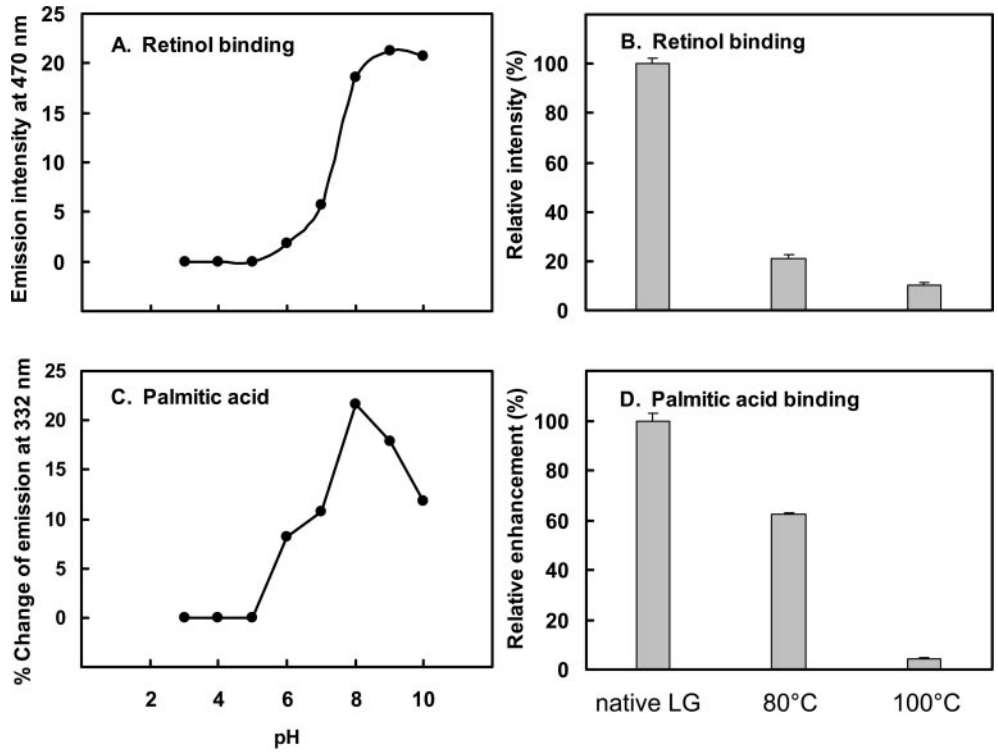


FIG. 10. **Effect of pH and heating on β-LG binding to retinol and palmitic acid.** *A*, fluorescence emission for binding of β-LG to retinol was measured at 470 nm with excitation at 287 nm. Binding was determined by the enhancement of extrinsic fluorescence of retinol at 24 °C. *B*, effect of β-LG heating (5 min) on retinol binding. *C*, fluorescence emission for binding of β-LG to palmitic acid was measured at 332 nm with excitation at 287 nm. Binding was determined by the enhancement of intrinsic fluorescence of β-LG at 24 °C. *D*, effect of β-LG heating (5 min) on palmitic acid binding.

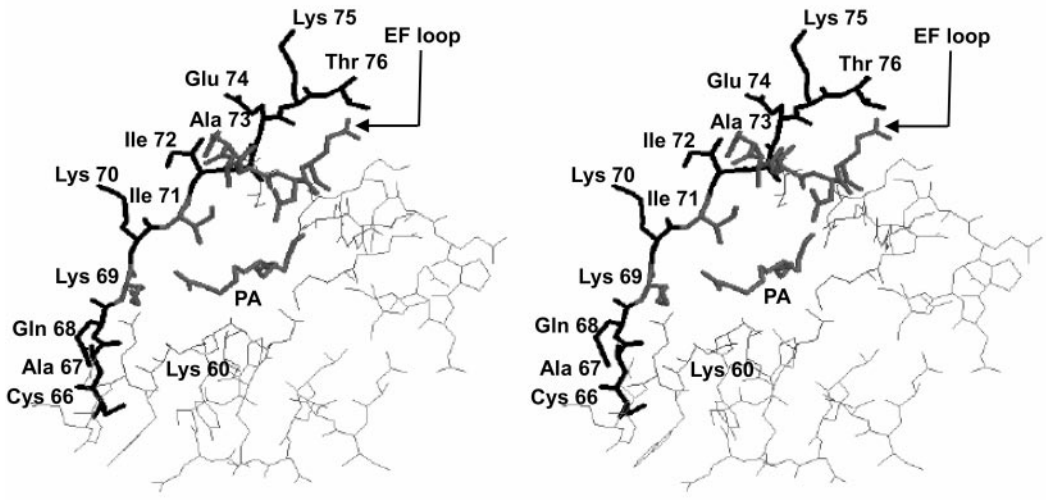


FIG. 11. **Three-dimensional view of β-LG complexed with palmitic acid.** The diagram is constructed according to the RasMol wire frame model (26), showing the D strand (residues 66–76) and other residues (28–43, 60–65, 84–93, and 107–117) (light wire frame) close to the calyx of β-LG. Notably, palmitic acid (*PA*) is almost perpendicularly oriented against the D strand, whereas Lys-69 is capable of interacting with the acidic group of palmitic acid. A hydrophobic interaction between the side chain of Ile-71 and palmitic acid is speculated. The EF loop (85–89), which controls the opening or closing of the calyx, is also seen. During the thermal denaturation, the conformational change of strand D results in the exposure of buried side chains of Lys-69 and Ile-71 and thereby attenuates and enhances the binding to palmitic acid and mAb, respectively.

in the binding to mAb (Fig. 8). One or both of these two residues must be exposed to the surface during thermal transition for rendering mAb binding. Thus, it is conceivable that Ile-71 might have become exposed on the surface during heating. Using co-crystallized β-LG with palmitic acid, the refined structure (at 2.5-Å resolution) reveals that the carboxyl group of palmitic acid binds to both Lys-60 and Lys-69 (9, 10). Fig. 11 shows a stereo view in which Lys-69 lies exactly within the antigenic epitope and orients at the entrance of the binding pocket, whereas the side chain of Ile-71 may interact with the fatty acyl chain. For the retinol binding, there is no obvious contact between the hydroxyl group of retinol and Lys-69 (11), although Lys-69 is closely oriented to the hydroxyl group (42). Again, we show a decreased binding of palmitic acid to β-LG

that was preheated at 80 °C (Fig. 10), and a significant increase in immunoreactivity (Fig. 2).

We demonstrate that the binding of retinol to β-LG was pH-dependent with initial binding at pH 7, while reaching a maximal pH between 8 and 10 (Fig. 10*A*). The data are consistent with the Tanford transition occurring at pH values from 6.5 to 9.5 (43). The calyx opens at pH 7.1–8.2 (4) and closes at pH 2.6 (7). Most interestingly, the binding of palmitic acid was attenuated at pH values from 8 to 10 (Fig. 10*C*). One of the possible explanations is that the protonated state of buried side chain of Lys-69 was neutralized at a pH value above 8 and resulted in weakening the ionic interaction with the carboxyl group of palmitic acid. Of course we could not rule out the other part of β-LG that might also undergo conformational changes at high pH, affecting the interaction with palmitic acid (Fig. 10*C*). We also speculate that the change of protonated state of Lys-69 contributed to the decreased immunoreactivity at pH 8–10 (Fig. 9*B*).

As mentioned above, the immunoreactivity assessed by this mAb was positively correlated to its molten globule state (between 70 and 80 °C in Fig. 3). This epitope in strand D was not only thermal sensitive but was also negatively correlated to retinol and palmitic acid binding. Lys-69 in native state participates in the binding for palmitic acid (10), but in the heat-denatured state it contributes to the binding for mAb. Therefore, this mAb may be used as a probe to study the thermal changes and the physiologic activity of β-LG, such as its binding to fatty acids and retinol.

Much is known about the physicochemical properties of β-LG (3). However, the biological function of this protein in addition to the transport of retinol and fatty acids has not yet been satisfactorily resolved. Recent studies have shown that β-LG produces hypocholesterolemic (44, 45) and antioxidant effects (46, 47) and may also serve as a growth factor for mammalian cells (48). The protein is acid-resistant in the gastrointestinal tract with a superior absorption capability via a receptor-mediated process (49–51). Because β-LG is a major protein consisting of about 10–15% of total milk proteins (1) and is labile to heat treatment by forming large polymers with other milk proteins (13, 52), it is conceivable that overheating should be avoided in order to maintain the physiologic role of β-LG. Therefore, our mAb may be useful for monitoring the immunochemical and biochemical nature of β-LG in heat-processed milk. From a technological standpoint, the monoclonal antibody prepared against the strand D region may be relevant to the design and operation of appropriate processes for thermal sanitation of milk and of other dairy products.

Taken together, we conclude that our mAb was able to discriminate the dry milk from the raw milk. The immunoreactivity of thermal denatured β-LG was correlated to its molten globule state and structural changes from primarily β-sheet to disordered conformation. Epitope mapping reveals that Lys-69, Ile-71, Ile-72, and Glu-74 in denatured D strand were directly involved in binding to mAb. The data suggest that the D strand plays a critical role in β-LG thermal denaturation, in which the buried side chains of Lys-69 and Ile-71 were exposed to the surface of β-LG calyx. At thermal transition temperature 80 °C, the increased immunoreactivity was associated with the decreased retinol and palmitic acid binding. We propose the mAb produced in this study may be used as a probe to study the thermal changes and the physiologic activity of β-LG, such as its binding to fatty acids and retinol. Whether the other β-strands may also participate in this role upon the heating between 70 and 80 °C, remains to be addressed.

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