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生物科技學院
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碩士論文

利用單株抗體探討β-乳球蛋白之結構與功能

Investigation of the structure and function of
β-lactoglobulin by monoclonal antibody

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β-乳球蛋白在牛乳中是一種熱不穩定蛋白。因此β-乳球蛋白易因加熱而導致結構之改變，在先前研究中我們製作出可辨識受熱變性後β-乳球蛋白之專一性單株抗體。於本研究中將利用此抗體深入探討β-乳球蛋白之結構與功能。在研究中發現當生乳加熱溫度達攝氏 70 到 80 度時，此抗體與β-乳球蛋白之結合能力會大幅地增加，證明β-乳球蛋白於加熱至攝氏 70 到 80 度時結構會明顯改變。 為了找出這抗體於β-乳球蛋白所辨識之抗原決定位 (epitope)，我們利用綜合策略之免疫分析法包含: 酵素及化學法分解蛋白質 (胰蛋白酵素及 CNBr)，化學修飾法(acetylation 及 carboxymethylation)，胜肽矩陣 (peptide array)與水溶性胜肽。 其結果準確的分析出抗原決定位於β-乳球蛋白之 D 股上(其胺基酸為 66-76 之位置)。其次於旋光雙級光譜(Circular dichroic spectral analysis)分析中，發現 carboxymethylation 之β-乳球蛋白不僅完全喪失二級結構，且與此單株抗體結合力遠超出經加熱後變性之β-乳球蛋白 10 倍以上，此證明β-乳球蛋白結構形成 disorder 時會被此抗體辨識。於抗體決定位做單點突變，結果發現 Lys-69 和 glu-74 是抗原決定位中與抗體結合之重要胺基酸。此外，我們更證明出結構變性後之β-乳球蛋白不能與疏水性分子(retinol 和 palmitic acid)結合，而此抗原決定位扮演重要的角色，

當此抗原決定位結構改變被單株抗體所辨識，則β-乳球蛋白不能與疏水性分子結合。而當 pH 9~10 時β-乳球蛋白上之抗體決定位的 Lys 呈電中性，會造成其免疫反應能力與疏水性小分子結合能力下降。綜合上述結果，β-乳球蛋白上 D 股為熱變性之區域及參與疏水性分子結合之功能。

Investigation of the structure and function of β-lactoglobulin by monoclonal antibody

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Abstract

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 °C and 80 °C.   To map out the specific epitope of β-LG recognized by this mAb, we used a combined strategies including tryptic digestion 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 x increase in immunoreactivity as compared to 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. Interestingly, at 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 the temperatures of 70 °C 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.
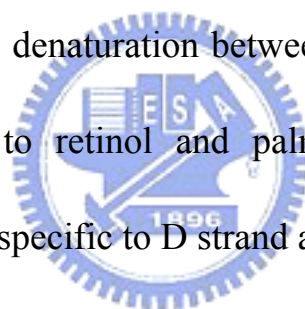
誌謝
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兩年碩士的生涯，讓我不僅在學術研究的領域有學習及發揮的空間，更讓我感受到與人相處及團隊合作的重要性；我喜愛科學但我更熱愛這群一起奮鬥的夥伴。 今天能夠拿到著碩士學位，要感謝的人真的太多了，飲水之恩必當湧泉以報。

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Abbreviations

β-LG	β-Lactoglobulin
CD	Circular dichroism
CM-LG	Carboxymethylated-lactoglobulin
ELISA	Enzyme link immunosorbent assay
HRP	Horseradish peroxidase
LDL	Low density lipoprotein
mAb	Monoclonal antibody
PB	Phosphate buffer
PBS	Phosphate buffered saline
PDB	Protein data bank
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
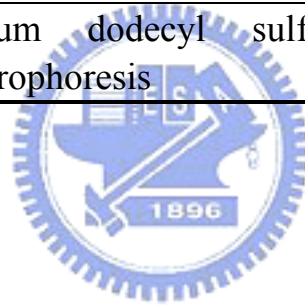
INTRODUCTION

1.1 Structure of β-LG

Bovine β-lactoglobulin (β-LG) is one of the major proteins in milk consisting of about 10-15% (Hambling et al, 1992). The protein comprises 162 amino-acid residues, with one free cysteine and two disulfide linkages (Fig. 1). According to the 3D crystallographic studies, β-LG is predominantly a β-sheet configuration containing nine antiparallel β-strands from A to I (Qin et al, 1998; Qin et al, 1999; Kuwata et al, 1999) (Fig. 2). Topographically, strands A-D form one surface of the barrel (calyx) while strands E-H form the other. The only 3-turn α-helical structure is at the COOH-terminus, which follows strand H lying on the outer surface of the calyx (Uhrinova et al, 2000). It is of interest, the EF loop acts as a gate over the calyx. It forms closed configuration to inhibit the ligand binding while pH < 6, whereas such configuration turns to open status allowing ligands to penetrate into the calyx while pH ≥ 6. The "latch" for this gate is Glu89, the residue implicated in the Tanford transition observed, although not identified, as having an abnormally high pKa (Tanford et al, 1959; Brownlow et al, 1997; Qin et al, 1998). The bovine β-LG is monomeric at pH values below 3, but exists in non-covalent bonding dimeric form at natural pH (Jameson et al., 2002),

1.2 Biochemical Properties of β-LG

Due to the thermally unstable and molten-globule nature, β-LG has been studied extensively for its physical and biochemical properties (Qi et al, 1995; Sawyer and Kontopidis, 2000; Kontopidis et al, 2004). 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 (Fessas et al, 2001). Heat treatment of β-LG at neutral pH causes the dimeric native protein to dissociate, partially unfold, denature, and aggregate; the rates and pathways are known to be dependent on the protein concentration, pH, temperature (Havea et al, 2004). Two major aggregating features are related to hydrophobic association and disulfide-bond interchange reactions (Havea et al, 2004). When a -LG is heated at neutral pH, the equilibrium between the native β-LG dimers and monomers is shifted toward the monomers. At temperatures above 60 °C, the β-LG monomer partially unfolds, with the loss in the helical structure allowing Cys121 to interact with the Cys106-Cys119 disulfide bond, to give a non-native Cys106-Cys121 disulfide bond and Cys119 (Croguennec et al, 2003). While,

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Cys160 is also likely to become involved in interprotein disulfide bonding in whey protein aggregates (Creamer et al, 2004).   Thus, the resultant activated monomers lead to the formation of various intermediate aggregation products.   Involvement of the free thiol group in aggregation has been shown in a number of studies, most notably by site-directed mutagenesis (Cho et al., 1994) where thermal stability was enhanced by incorporating the free thiol into a third disulphide bridge to the neighbouring helix.   Further, the porcine protein has no free thiol and does not form gels under the same conditions as the cow (Gallagher et al., 1996).   However, the exact regions involved in the thermal denaturation are still unclear.   Whether the subtle unfolding changes can be detected by an immunochemical approach remains at question.

1.3 Physicochemical Properties of β-LG

A remarkable physicochemical property of the β-LG is its ability to bind in vitro small hydrophobic molecules such as retinol, fatty acids, vitamin D, and cholesterol (Narayan and Berliner, 1997; Qin et al, 1998; Wu et al, 1999; Kontopidis et al, 2002). β-LG has been postulated to serve as a carrier for retinol in neonates (Sawyer et al, 1985), and it has been proposed that the binding of fatty

acids to β-LG at neutral pH is related to a biological function of this protein in

bovine milk (Perez et al, 1992; Perez and Calvo, 1995).   The binding of fatty acids

to β-LG has been reported to increase the resistance of the protein to proteolytic

degradation (Puyol et al, 1993), thermal degradation (Puyol et al, 1994), and

unfolding in urea solution (Creamer, 1995).

Regardless of intensive research, 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

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 (Chen et al, 2004).   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 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. 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 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.

MATERIALS and METHODS

2.1 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 (Chen et al, 2004; McCreath et al, 1997).

2.2 Preparation of Monoclonal Antibody Specific to Dry Milk

Monoclonal antibodies were produced according to the standard procedures previously described by us (Mao et al, 1988; Mao et al, 1990), in which dry milk (Nestle Australia Ltd, Sidney, Australia) was used for immunization (Chen et al, 2004). 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 a respective antigen. Each monoclonal was established by limiting dilutions at least 2 x (Mao et al, 1988; Mao et al, 1990).

2.3 Trypsin and CNBr Fragmentation

For trypsin treatment, 50 μg of β-LG in 100 μl 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 which time, 1 μl trypsin (0.1 mg/ml) was added and incubated at room temperature for 4 h (Chen et al, 2004). Trypsinized LG was analyzed on a SDS-PAGE (18% polyacrylamide) followed by a Western blot. For CNBr fragmentation (Mao et al, 1975; Mao et al, 1977), 5 mg of β-LG were first dissolved in 70% (v/v) TFA with the addition of 10 mg CNBr in dark for 24 h at room temperature. After 3 x evaporation in Speed Vac (CVE 200D, ELELA, Japan) with the addition of 5 x volume of de-ionized water, the dry material was dissolved in the 10 mM phosphate buffer (PB), pH 7.0. The immunoreactivity of CNBr fragments were then analyzed on an 18% SDS-PAGE, followed by a Western blot.

2.4 Acetylation and Carboxymethylation of β-LG

Chemical modification of β-LG by acetylation was conducted by a modification of the procedure previously described by us (Mao et al, 1980). To 5 mg of β-LG in 2 ml 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 using 0.1 M NaOH. After 3 h 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 (Mao et al, 1980; Tseng et al, 2004), 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 incubation 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 mole of β-LG.

2.5 CD Spectrum

The secondary structure of native, heated or chemically modified β-LG was determined using a computerized Jasco J-715 circular dichroic (CD) spectropolarimeter.   Each protein sample was dissolved in 10 mM phosphate buffer at pH 7 with a final concentration of 0.2 mg/ml.   About 300 µl of the protein solution were used for analyzation 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 $[\theta]_{MRW}$ (Tseng et al, 2004; Chen et al, 1994).
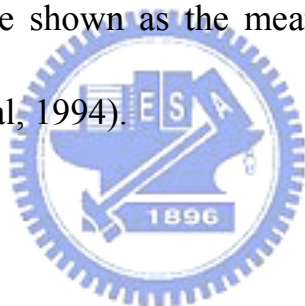
2.6 Peptide Array

Twelve synthetic peptides in one nitrocellulose-array, each containing 15 amino-acid residues, were designed corresponding to the residues 25-107 of β-LG or to the residues 67-75 within strand D (Fig. 1).   The synthetic peptides were prepared under a contract with a local biotechnology company (Genesis Biotech Inc., Taipei, ROC).   Briefly, the peptides were directly synthesized in situ on a nitrocellulose (NC) paper according to the method described (Frank, 1992).   The NC 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 3 x washes.   After incubation with mAb for 2 h and 3 x washes, goat anti-mouse IgG conjugated with horseradish peroxidase (HRP) in 5% gelatin/TBST was added and incubated.   Finally, following the washes, chemiluminescent substrate (ECL™ Western Blotting System, Amersham) was added, washed, and immediately developed by exposing onto a film.

2.7 Competitive ELISA

In brief, heated β-LG (1 μg in 50 μl of PBS) was first immobilized onto microtiter wells followed by 3 x washes to remove unbound β-LG (Chen et al, 2004; Mao et al, 1989).   The wells were then blocked by 3% gelatin in PBS.   After 3 x 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 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-ethylbenz-thiazoline-6-sulfonic acid) and read at 415 nm.

2.8 Effect of pH on β-LG Binding to mAb

Heated 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 for 2 h at room temperature.   After removing the unbound mAb, the plate was developed according to the standard ELISA procedures at neutral pH (Chen et al, 2004; Mao et al, 1989).   Since pH itself can affect the antigen-antibody binding, a control experiment using mouse IgG as immobilized antigen was also conducted at various pH for a parallel comparison.
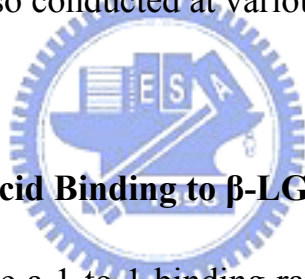
2.9 Retinol and Palmitic Acid Binding to β-LG

β-LG was reported to be a 1-to-1 binding ratio with retinol or palmitic acid as measured by fluorescence emission techniques (Yang et al, 2002).   In general, binding of retinol to β-LG was measured by extrinsic fluorescence emission of retinol molecule at 470 nm using excitation at 287nm.   Whereas, binding of palmitic acid to β-LG was measured by the fluorescence enhancement of Trp residues of β-LG at 332 nm using excitation at 287 nm.   For the effect of the pH experiment, 5 uM or 20 uM of native β-LG was instantly incubated with 5 uM of retinol or 20 uM of palmitic acid, respectively, at various pH at 24 °C.   For the

effect of the heat experiment, β-LG was preheated at 80 °C or 100 °C for 5 min and then incubated with retinol or palmitic acid at pH 8. Fluorescence spectra were recorded at 24 °C with a fluorescence Spectrophotometer F-4500 (Hitachi High-Tech.Cor., Tokyo, Japan).

2.10 Three Dimensional Analysis of β-LG Structure

3D structure of β-LG used in this context was provided by Protein Data Bank (PDB, http://www.rcsb.org/pdb/), code 1CJ5 (Kuwata et al, 1999), with the diagram created by PyMOL (DeLano, 2002).

2.11 Three Dimensional Analysis of β-LG and Palmitic Acid Complex Structure

3D structure of Bovine β-LG complexed with palmitic acid used in this context was provided by Protein Data Bank (PDB, http://www.rcsb.org/pdb/), code 1GXA (Kontopidis et al, 2002), with the diagram created by Rasmol (Sayle et al, 1995).

RESULTS

3.1 Characterization of The Monoclonal Antibody Specific to Dry Milk

Previous studies show that the monoclonal antibody (mAb) used in this report is specific to processed dry milk, but not to raw milk (Chen et al, 2004). 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 (Chen et al, 2004). Since 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 °C and 80 °C over time (Fig. 3). It was of interest that the increase in immunoreactivity was concomitant with the reported transition temperature for converting native to denatured β-LG (de Wit et al, 1980). The finding suggests that the immunoreactive site recognized by this mAb lied in the thermal denatured region of β-LG.

3.2 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 (Chen et al, 2004) suggesting that Lys, Arg, or both residue(s) were probably involved in maintaining the antigenic structure for β-LG (Fig 4). Chemical modification using acetylation (pH 8.0) on positively charged residues (mostly Lys) attenuated the immunoreactivity of β-LG on a competitive ELISA (Fig. 5). These two experiments support the notion that positively charged amino acids of β-LG attributed for the mAb recognition.

3.3 Immunoreactivity of CNBr Fragments of β-LG

To further delineate 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 weight about 9 kDa (Fig. 6). As estimated from its Met cleavage site, this fragment was presumed a peptide containing residues 25-107 (Fig. 1). Subsequently, the N-terminal sequence of this fragment

was determined.   The first six amino-acid residues (AASDIS) confirmed that the

immunoreactive site was located between the residues 25-107 of β-LG (Fig. 1).

3.4 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.   Using an EMBOSS program for searching a

possible antigenic determinant within residues 25-107 of β-LG (Fig. 7), 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 ten other overlapped synthetic peptides (each with 15

residues) was prepared.   These peptides were directly synthesized on a

nitrocellulose membrane (Fig. 7).   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. 7).   Among which peptide 4 gave a partial

immunoreactivity suggesting that residues 67-70 were essentially involved in the

reactive site (Fig. 7). Since the size of an epitope is relatively small usually

containing 6-9 amino-acid residues (Mao et al, 1990; Mao et al, 1989; Bhatnagar

et al, 1983; Davies and Cohen, 1996; Atassi, 1984), 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. 7). Notably, this region is highly positive in charges. Observing

from the high resolution crystal structure of β-LG (Kuwata et al, 1999), it is

fascinating that this proposed region is exactly located within the D strand of

surfaced β sheet (residues 66-76) (Fig. 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 carboxyl terminus (Cys-160).

This disulfide linkage plays an important role to stabilize the β-structure by

forming antiparallel sheets of β-LG. The proposed epitope (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 of our specific mAb. We

hypothesized when the D strain underwent disordered structure, it would then allow

the "denatured" mAb binding.

3.5 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. 8). 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 x greater than that of heated β-LG (Fig. 9).   Meanwhile, analysis of CD spectra on carboxymethylated β-LG further confirmed a significant conformational change by converting β sheet (typically at 215 nm) to a more disordered structure than that of heated β-LG (Fig. 10).

3.6 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.   Figure 11 shows that this linear sequence was able to completely inhibit 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. 10).

3.7 Role of Charged Residues in Epitope Specificity

To determine which Lys(s) 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 extremely 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 (peptides 11-12, Fig. 12) retaining all the Lys residues did not show any binding.
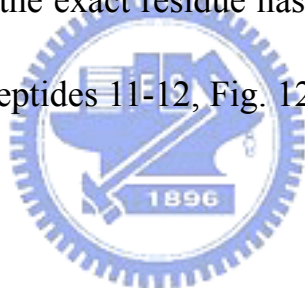
3.8 Effect of pH on β-LG Binding to mAb

Because the structural stability of β-LG is pH dependent (Casal et al, 1988), we tested whether changes of pH could also induce an increase in β-LG immunoreactivity. Fig. 13A shows that the CD structure of β-LG was stable at pH 2 with some changes between 3 and 7, while a transition to disorder was seen from 8 to 10. However, such disordered structure did not facilitate the mAb binding (Fig. 13B). Since Lys-69 was essential (Fig. 12) and the overall positive charge of

this residue started to become neutralized under pH 8-10, the immunoreactivity was decreased (Fig. 13B). A control experiment showing a typical pH dependent antigen-antibody reaction was performed (Fig. 13C), and there was a slight decrease in immunoreactivity at pH 9-10.

3.9 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. 14A). Heating β-LG at temperatures greater than 80 °C almost completely abolished its binding for retinol (Fig. 14B). The data support the notion that the striking increase in immunoreactivity of the D strand at this temperature (Fig. 3) was negatively correlated to the retinol binding, which requires the integrity of a β-sheet structure of β-LG.

Interestingly, the binding to palmitic acid was decreased in some extent at pH 9-10 (Fig. 14C) correlated with the binding to mAb (Fig. 13B). Since Lys-69 played an extremely essential role in the antigenic site (Fig. 12), such correlation

suggests that the protonated state of this residue might be involved in stabilizing both the mAb binding and β-LG-palmate complex formation (please see more detail in Discussion). Similarly, heating on β-LG substantially reduced the binding for palmitic acid.
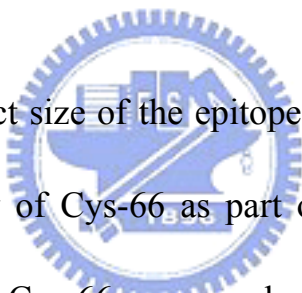
DISCUSSION

Molten globules are thought to be general intermediates in protein folding and unfolding (Ptitsyn et al, 1990; Chang and Li, 2001). 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 3D 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 (Qin et al, 1998), occurring at pH from 6.5 to 8.0, is known to be within the residues 85-90 (E-F loop). This region opens or blocks the entrance of the calyx (Ragona et al, 2003).

The present study demonstrates that denatured strand D of β-LG was responsible for the binding of our thermally sensitive mAb (Chen et al, 2004). 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. 10) in which the immunoreactivity was concomitantly increased (Fig. 9). Second, blocking the disulfide linkage between the D strand (Cys-66) and C-terminus (Cys-160) of β-LG by carboxymethylation not only produced a disordered structure (Fig. 10) but also

markedly 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 x) on competitive ELISA (Fig. 9).   Presumably, this was due to 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) using the parameters from

3D-PSSM: the   folding   recognition   server   at   the   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. 2).

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 (Chen et al, 2004).   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. 2), which is agreeable with the general concept of a given antigenic epitope (Atassi, 1980). Fifth, the buried side chain of Lys-69 was exposed upon the 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. 7) 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 was involved in the antigenic site, introduction of such a bulky group (carboxymethyl) on this residue would have resulted in a significant loss of immunoreactivity (Atassi, 1984). It should be noted here that Cys-66 was only responsible for the conformational

restrain by crossly 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 (Mao et al, 1990; Mao et al, 1989;

Bhatnagar et al, 1983) and others (Davies and Cohen, 1996; Atassi, 1984). The

present study shows the involvement of Glu-74 in the epitope (Fig. 12), which is

eight residues apart from Cys-66. Finally, our synthetic soluble peptide without

Cys-66 could completely inhibit the mAb binding (Fig. 11). 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 70-80 °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 (Chen et al, 2004).

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. 12), 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. 12). 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 previously described by us using a mAb against fibrin (Mao et al, 1990). It is consistent to our observations for the interaction of protein antigens with antibodies, in which six determining residues involved in binding for the most part antigen (Mao et al, 1990; Davies and Cohen, 1996). We also demonstrate the importance of Ile-72 and Ile-73 within residues 69-73; substituting these two hydrophobic residues by Ala diminished its immunoreactivity. Whereas, 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 reviewed (Miller and Cohen, 1991).   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 (Mao et al, 1988).   Some unique low- density-lipoprotein (LDL) mAb have been used for discriminating between patients with and without coronary artery disease (Patton et al, 1983; Mao, et al, 1983).   Those LDL mAb have also been utilized for probing the thermal changes of human LDL (Mao et al, 1982), while the immunoreactivity was conversely correlated to the temperature with the optimal binding at 4 °C (Mao et al, 1983; Mao et al, 1982).   Since the 3D structure of lipase and LDL were lacking, the exact mechanism involved remains elusive.   The epitope of β-LG we mapped in this study, however, is known and provides a better understanding for its interaction with mAb.   We propose that due to highly ordered β-configuration of D strain in its native state, the binding to our specific mAb is prohibited.   But the D chain 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 (Wu et al, 1999; Kontopidis et al, 2002).   In vivo experiment shows that β-LG enhances the intestinal uptake of retinol in preruminant calves (Kushibiki et al, 2001).   It has been suggested that conformational changes in the calyx and the exposure of surface hydrophobic site of β-LG in molten globule state reduces the retinol affinity (Yang et al, 2002). The binding specificity may be determined by the dynamic motion of loops between the β strands (Ragona et al, 2003; Abduragimov et al, 2000).   It is not known which β strand(s) is involved for the exposure of surface hydrophobic site. In this study we show a substantially decreased binding of retinol to β-LG that was preheated at transition temperature 80 °C (Fig. 14), while a significant increase in immunoreactivity (Fig. 3).   Notably, two hydrophobic residues Ile-71 (buried in native state) and Ile-72 (exposed in native state) are located within the epitope and participate in the binding to mAb (Fig. 12).   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 cocrystallized 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 (Qin et al, 1998; Wu et al, 1999). Fig. 15 shows a stereo view in which Lys-69 lies exactly within the antigenic epitope and orients at the entrance of the binding pocket, while 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 (Kontopidis et al, 2002), although Lys-69 is in a proximity oriented to the hydroxyl group (Cho et al, 1994). Again, we show a decreased binding of palmitic acid to β-LG that was preheated at 80 °C (Fig. 14), while a significant increase in immunoreactivity (Fig. 3).

Due to that β-LG and human retinol binding protein both belong to lipocalin superfamily and possess the retinol binding ability (Flower et al, 2000). By comparing their biochermical properties in all respects to realize whether or not we could apply our findings to the human retinol binding protein. To structure, their three dimensional structure are very similar and both have an eight-stranded β-barrel (caylx) accompanied by a short α-helix closing to C terminus (Fig. 2) (Greene et al, 2001 and Qin et al, 1998). However, their primary protein sequences show that there is no significant identity. To heating treatment, they both reveal the

similar thermal transition temperature (70-80 °C) (Muccio et al, 1992), but there is no corresponding thermal sensitive domain as we determined in the human retinol binding protein. Additionally, thermal denaturation is irreversible to β-LG, but not to human retinol binding protein (Greene et al, 2001and Muccio et al, 1992). To ligands binding ability, β-LG is able to bind retinol and fatty acid in the caylx binding pocket, nevertheless the retinol binding protein is able to specifically bind retionol derivatives. The retinol bindinf affinity of β-LG and human retinol binding protein are similar with $K_d = 4.7x\ 10^{-8}M$ and $7.5x\ 10^{-8}M$, respectively (Muccio et al, 1992 and Cho et al, 1994). Taken together, human retinol binding protein is similar to β-LG in some aspects, but our findings in β-LG can not totally fit in that of human retinol binding protein.

We demonstrate that the binding of retinol to β-LG was pH dependent with initial binding at pH 7, while reaching maximal pH between 8 and 10 (Fig. 14A). The data is consistent to the Tanford transition occurring at pH from 6.5 to 9.5 (Tanford et al, 1959). Calyx opens at pH 7.1-8.2 (Qin et al, 1998) and closes at pH 2.6 (Uhrinova et al, 2000). Interestingly, the binding of palmitic acid was attenuated at pH from 8 to 10 (Fig. 14C). One of the possible explanations is that

the protonated state of buried side chain of Lys-69 was neutralized at pH above 8 and resulted in weakening the ionic interaction with the carboxyl group of palmate. 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 palmate. We also speculate that the change of protonated state of Lys-69 contributed in the decreased immunoreactivity at pH 8-10 (Fig. 14C).

As mentioned above, the immunoreactivity assessed by this mAb was positively correlated to its molten globule state (between 70-80 °C in Fig. 3). This epitope in strand D was not only thermal sensitive but also negatively correlated to retinol and palmate binding. Lys-69 in native state participates in the binding for palmitic acid (Wu et al, 1999), but in 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 (Sawyer and Kontopidis, 2000). 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 (Nagaoka et al,
2001; Yamauchi et al, 2003) and antioxidant effects (Zommara et al, 1998;
Chevalier et al, 2001) and may also serve as a growth factor for mammalian cells
(Francis et al, 1995). The protein is acid resistant in the gastro-intestinal tract
with a superior absorption capability via a receptor-mediated process (Guo et al,
1995; Papiz et al, 1986; Caillard and Tome, 1994). Since β-LG is a major protein
consisting of about 10-15% of total milk proteins (Hambling et al, 1992) and is
labile to heat treatment by forming large polymers with other milk proteins (Chen
et al, 2004; Havea et al, 2001), it is conceivable that overheating should be avoided
in order to maintain the physiologic role of β-LG. Our mAb may, therefore, be
useful for monitoring the immunochemical and biochemical nature of β-LG in heat
processed milks. 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 palmate 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 70-80 °C, remains to be addressed.
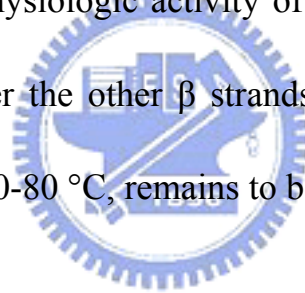
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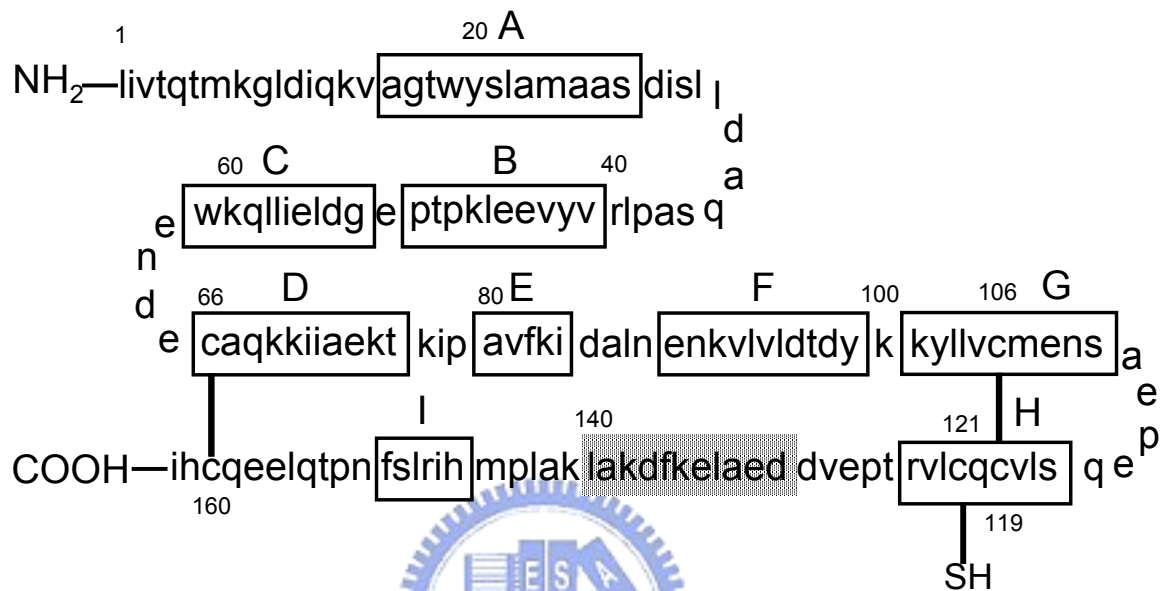
Figure 1: Amino acid sequence of β-LG. β-LG is consisted of 162 amino acids with nine β-sheet strands (A-I) and one α helix (shadow). There are two disulfide bonds located between strand D and carboxyl-terminus (Cys-66 and Cys-160) and between strands G and H (Cys-106 and Cys-119), while a free buried thio group is at Cys-121.
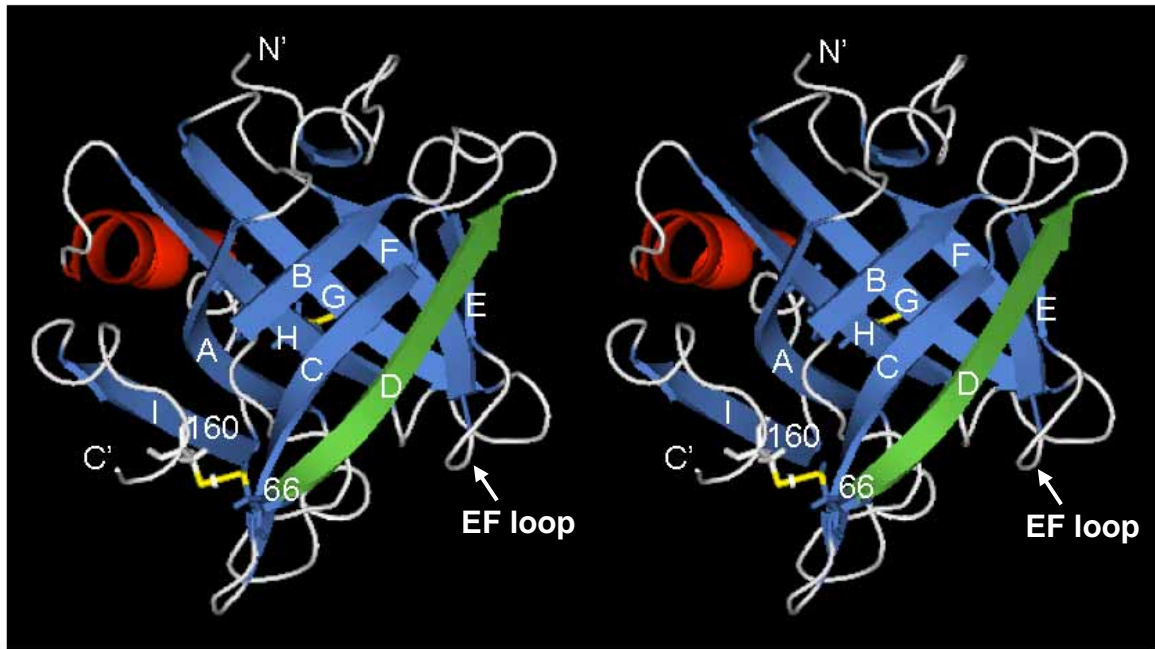
Figure 2: 3D structure of β-LG.   Crystal structure of β-LG determined by (6) and created by PyMOL (25) (PDB code 1CJ5), showing that strands A-D form one surface of the barrel while strands E-I form the other.   The antigenic site recognized by the mAb is located within strand D (green) following thermal denaturation.   The two disulfide linkages are also shown (yellow).   Hydrophobic molecules such as retinol and palmate are bound in the pocket of calyx.
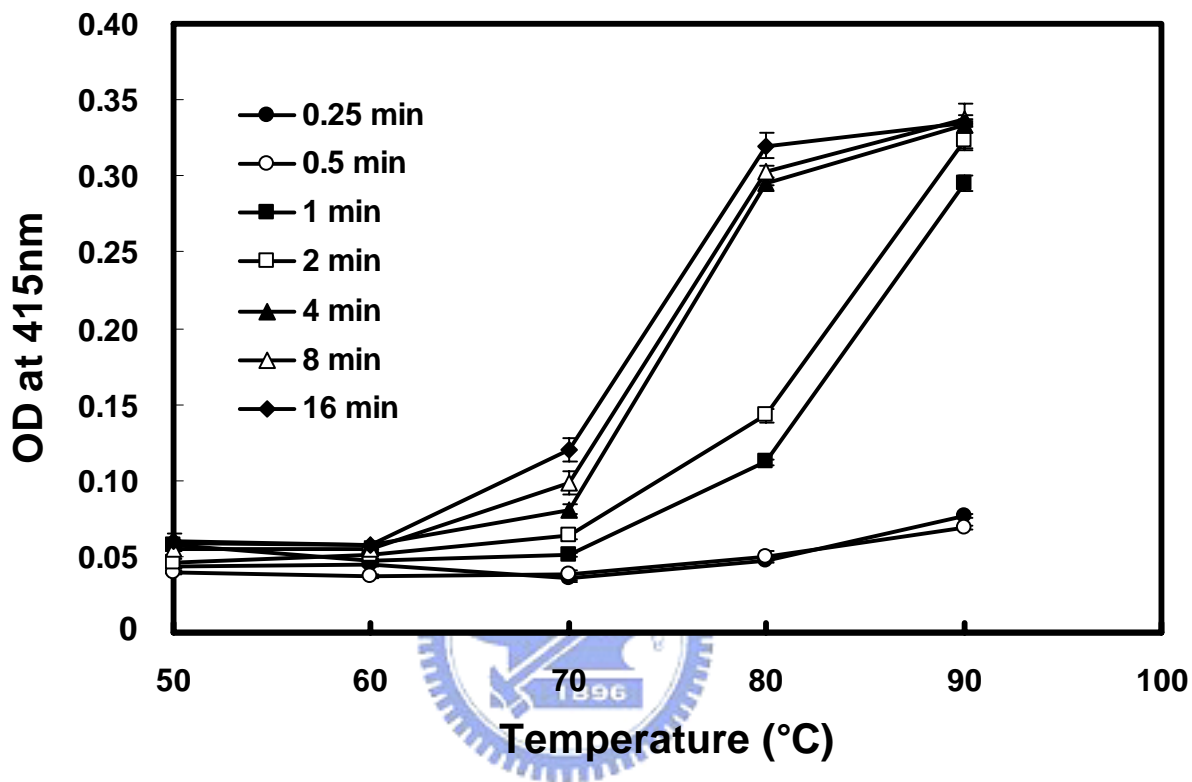
Figure 3: Immunoreactivity of β-LG in raw milk heated at different temperatures over time. Immunoreactivity was monitored using an ELISA on dry milk heated at various temperatures. The increase in immunoreactivity of β-LG assessed by dry milk specific mAb is correlated to the molten globule state of β-LG with a transition between 70-80 °C.

SDS-PAGE   **Western blot**
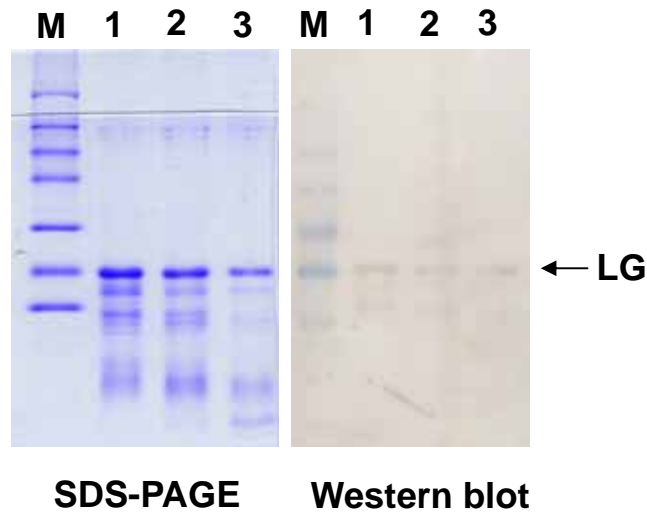
Figure 4: Effect of tryptic digesition on immunoreactivity of β-LG. About 10 μg of tryptic digested LG were used with time for SDS-PAGE containing mercaptoethanol as a reducing agent (left) followed by a Western blot (right). Lane M: molecular markers. Lane 1: tryptic digestion for 5 min.   Lane 2: for 10 min.   Lane 3: for 30 min.
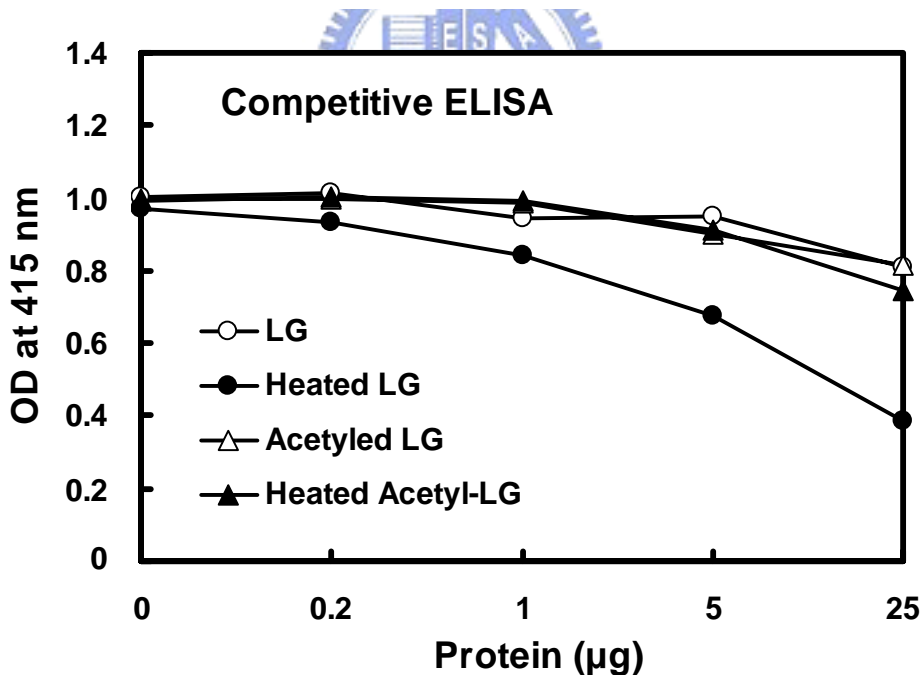
Figure 5: Effect of acetylation on immunoreactivity of β-LG. Competitive ELISA using heated β-LG as an immobilized antigen, while competing with native, heated, acetylated, and heated acetylated β-LG. Immunoreactivity of β-LG was significantly increased upon heating at 100 °C for 5 min, but not of acetylated and heated acetylated β-LG.

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M 1 2   M 1 2

SDS-PAGE   Western blot
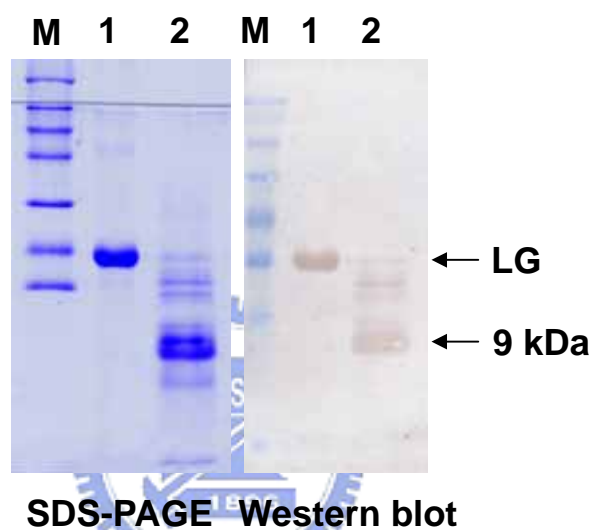
Figure 6: Effect of CNBr cleavage on immunoreactivity of β-LG. About 10 μg of native and CNBr cleaved β-LG were used for SDS-PAGE containing mercaptoethanol as a reducing agent (left) followed by a Western blot (right). A major immunoreactive peptide with a molecular weight about 9 kDa was found to be in residues 25-107 as determined by an amino-terminal sequence analysis. In theory, fragments with 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.
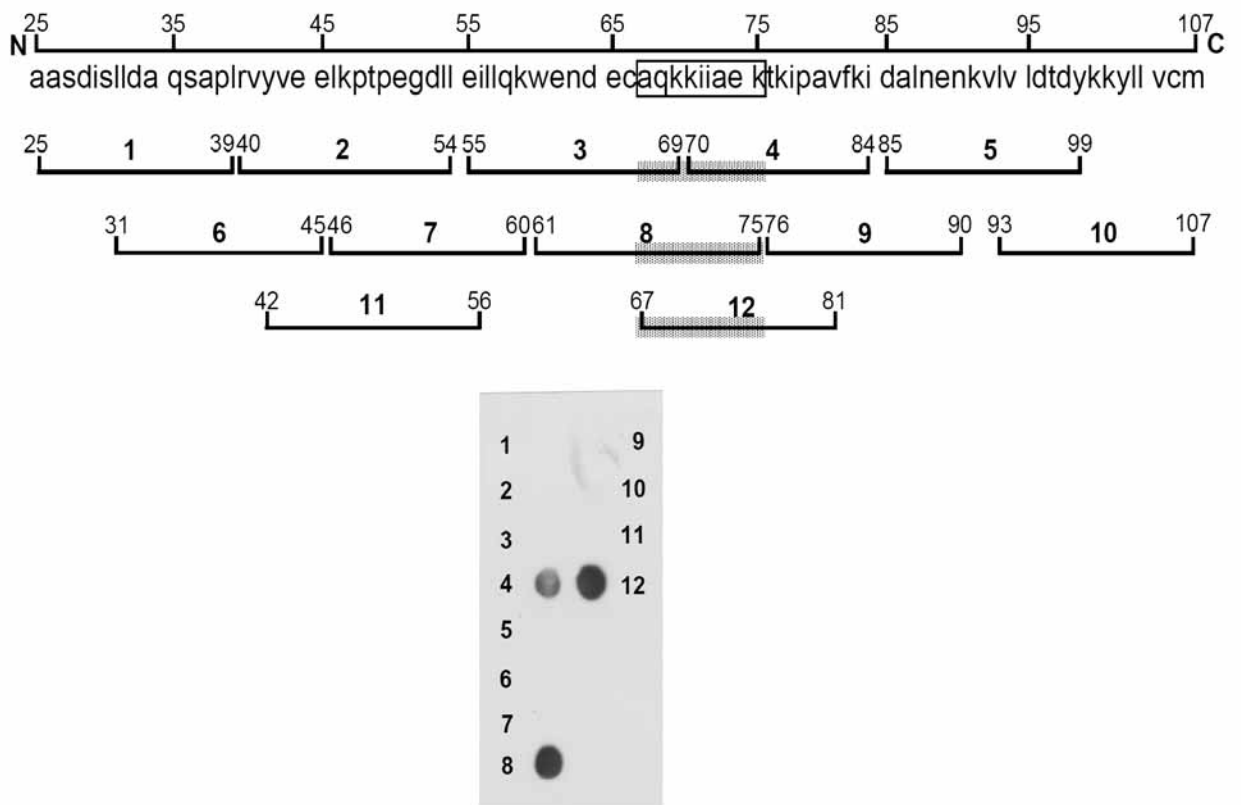
Figure 7: Delineation of an epitope recognized by mAb. Twelve peptides corresponding to a 9 kDa CNBr fragment of β-LG (residues 25-107) were directly synthesized in situ on nitrocellulose membrane. Peptides 11 and 12 were prepared due to the presence of Pro residues thought to be potentially antigenic 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 using HRP-labeled secondary antibody with chemiluminescent agent as a developer. The shed region represents the proposed epitope.
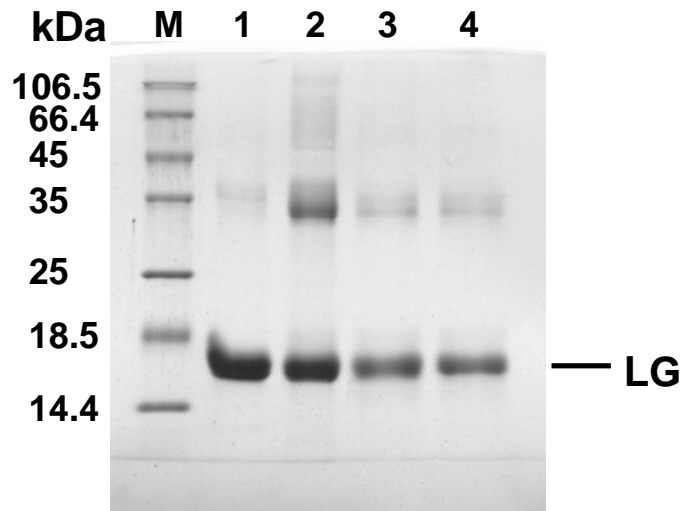
Figure 8 SDS-PAGE profile of carboxymethylated β-LG (CM-LG).
About 10 μg of β-LG and CM-LG were loaded on 15% SDS-PAGE.   Lane
M: molecular markers.   Lanes 1: β-LG.   2: heated β-LG.   3: CM-LG.   4:
heated CM-LG.   Significant increase in β-LG dimer and high molecular
form are seen, while heating at 100 °C for 5 min, but not in CM-LG.
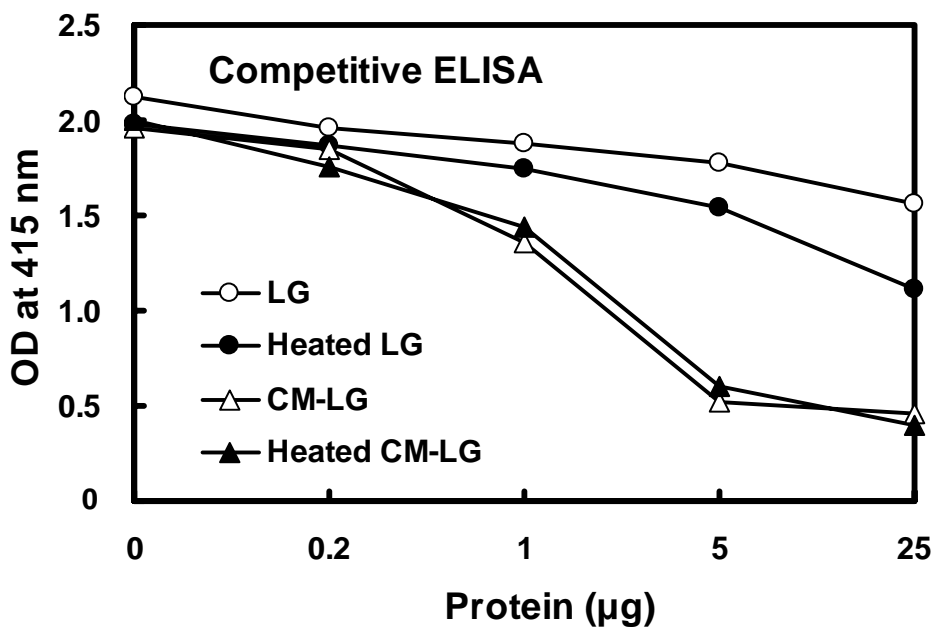
Figure 9: immunoreactivity of carboxymethylated β-LG (CM-LG).
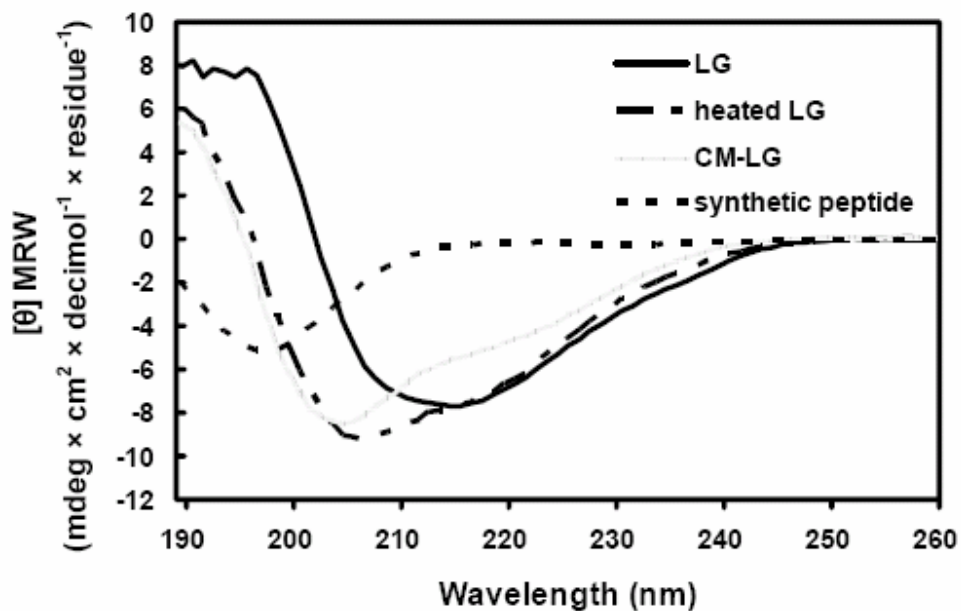Competitive ELISA of native, heated, CM, and heated CM β-LG.   The plate
was immobilized with heated β-LG.

Figure 10. 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.
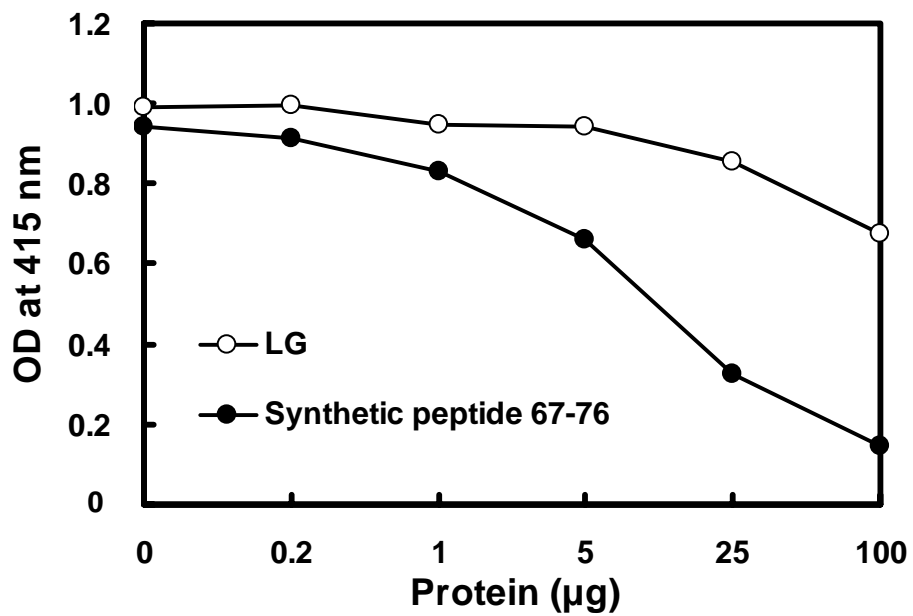
Figure 11: Immunoreactivity of the synthetic peptide determined by an ELISA.
A synthetic peptide corresponding to residues 67-76 (AQKKIIAEKT) in strand D
was prepared as a soluble form.   The plate was immobilized with heated β-LG.
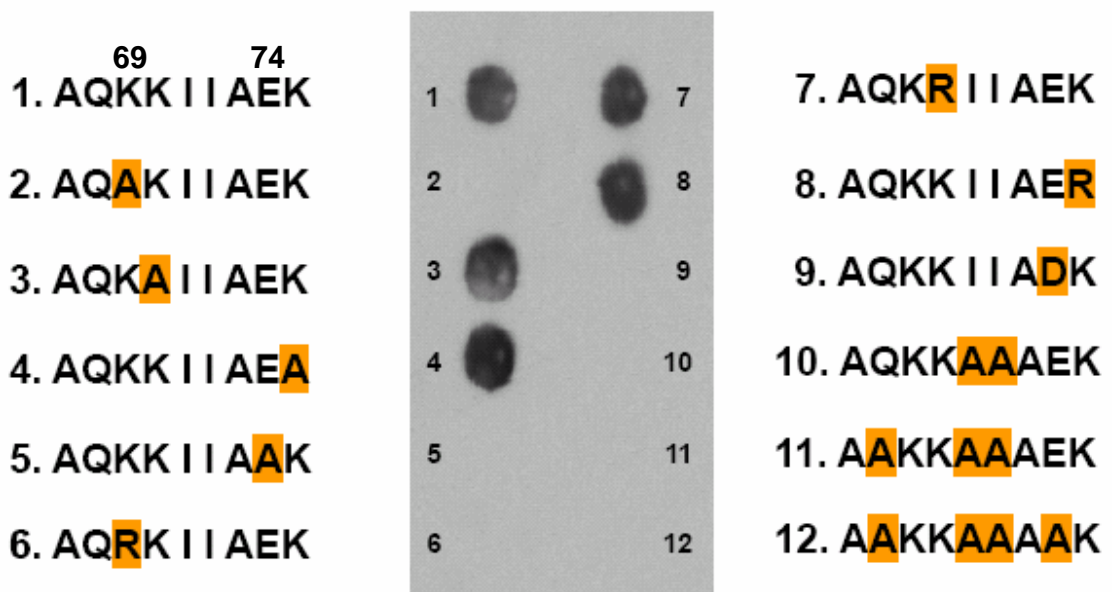
Figure 12: Role of charged residues in antigenic specificity determined by a peptide array. Peptide 1 represents the native sequence 67-75 of β-LG. Substitution of Lys-69 and Glu-74 by Ala or a same charged amino acid, respectively, resulted in a total loss of immunoreactivity (peptide 2-9). Hydrophobic residues Ile-71 and Ile-71 were essential in maintaining the antigenic structure, while peptides 11 and 12 retaining all the Lys residues were used as randomized negative controls.
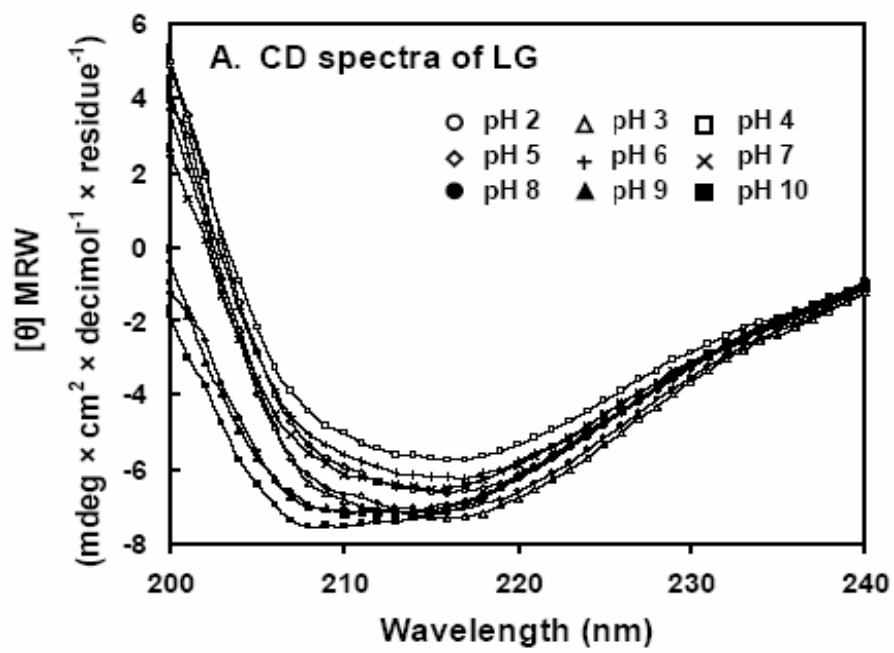
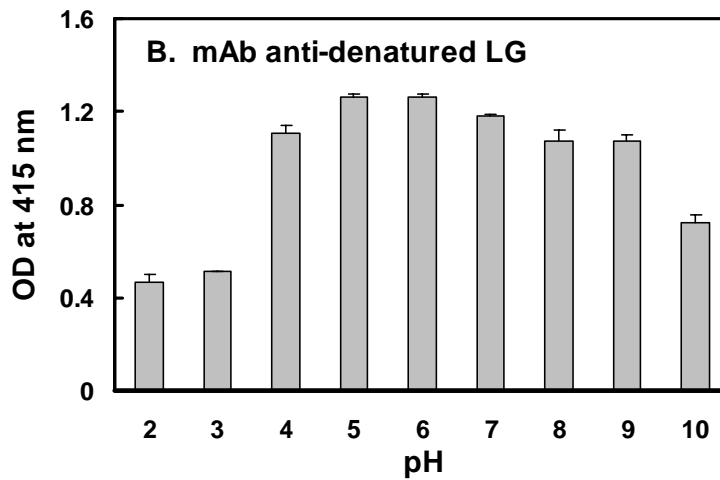**Figure 13 A: Effect of pH on β-LG binding to mAb.** CD spectra of β-LG at pH from 2 to 10.

Figure 13B: Binding of mAb to β-LG at various pH determined by ELISA. The primary mAb binding was conducted at the pH 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.
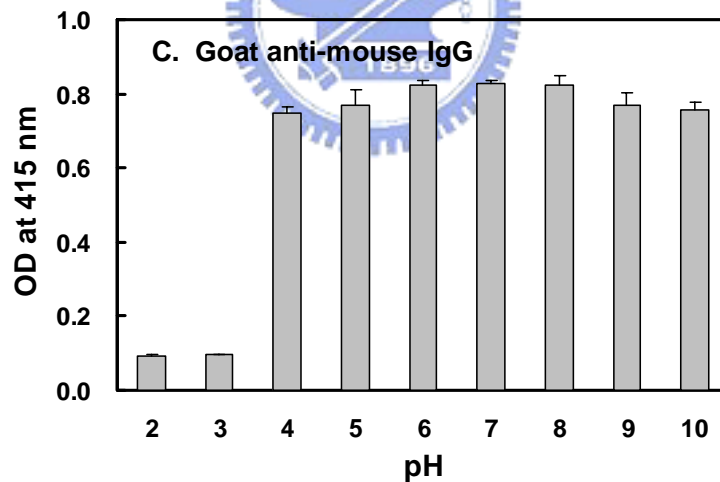

Figure 13C: Control experiment evaluating the effect of pH on antigen-antibody interaction. This experiment was designed to study the pH effect on the binding of antigen and antibody in general. Antigen (mouse IgG) was coated on the plate and allowed the binding of HRP-labeled anti-IgG (goat) at various pH. The plate was then developed after removing unbound antibody using PBS at pH 7.4. The binding was slightly decreased at pH 9-10.
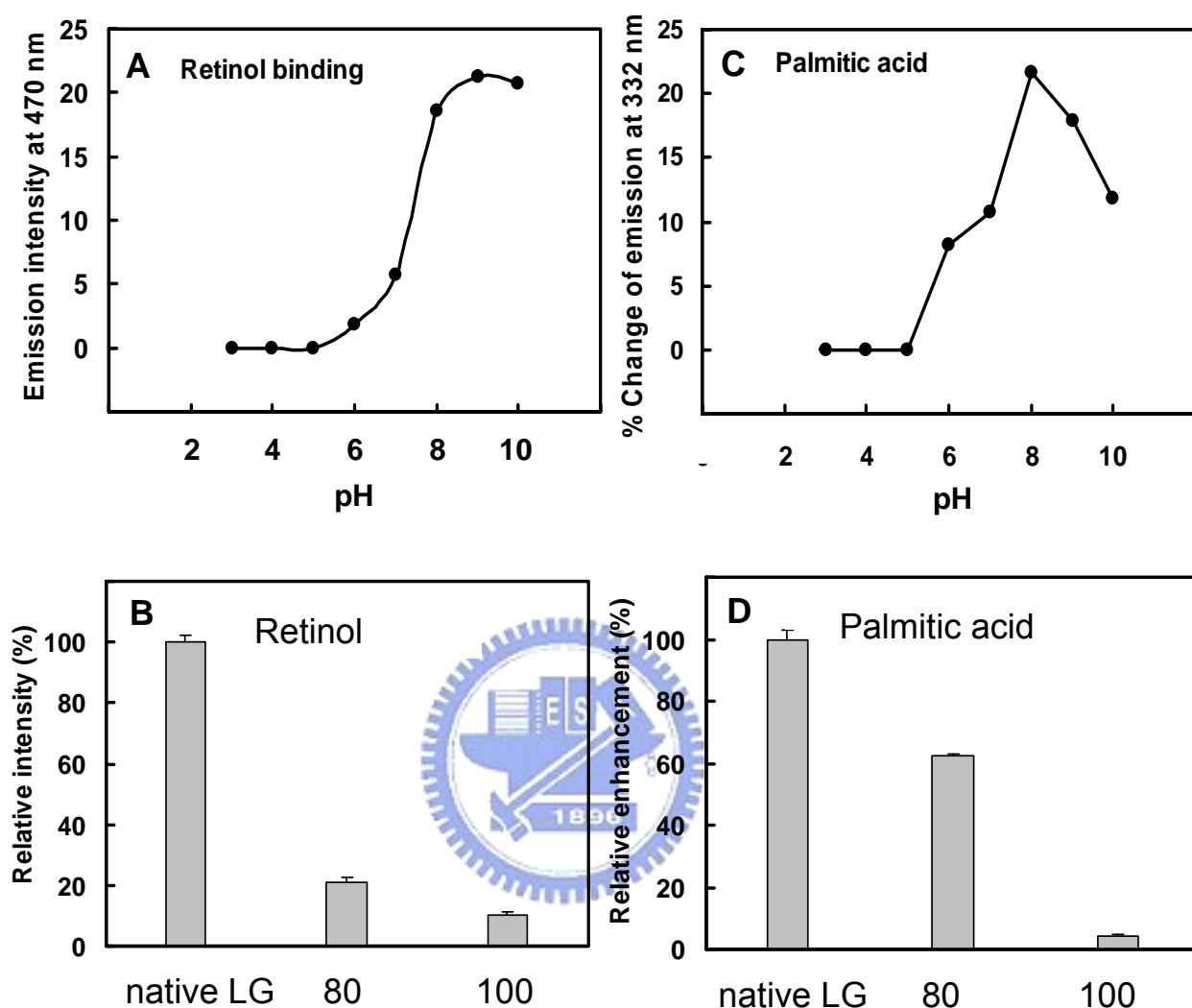
Figure 14: Effect of pH and heating on β-LG binding to retinol and palmitic acid.   A: Fluorescence emission for binding of β-LG to retinol was measured at 470 nm with excitation at 287 nm.   Binding was determined by the enhancement of extrinsic fluorescence of retinol at 24 °C.   B: Effect of β-LG 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.
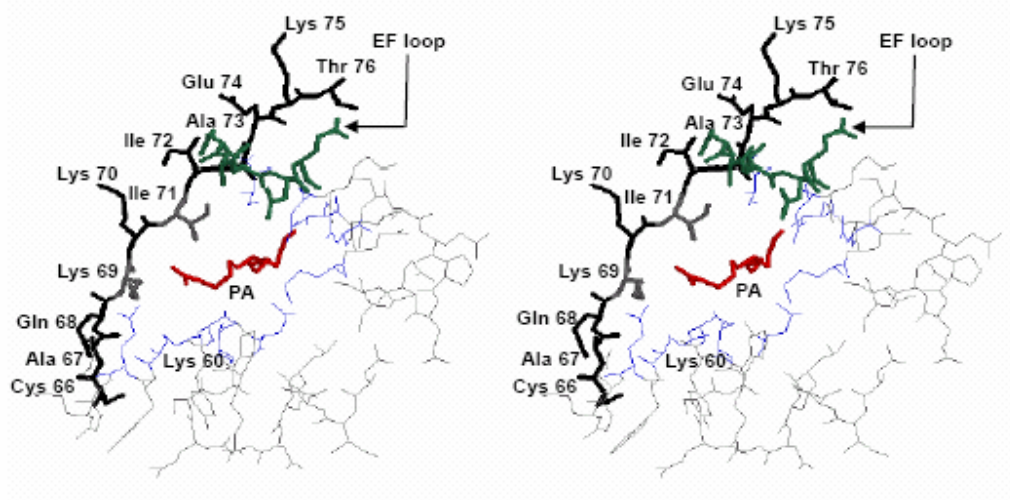
Figure 15: Three dimensional view of β-LG complexed with palmitic acid. The diagram is constructed according to the RasMol wireframe model (26), showing the D strand (residues 66-76) and other residues (28-43, 60-65, 84-93, and 107-117) (light wireframe) nearby the calyx of β-LG. Notably, palmitic acid (PA) is almost perpendicularly oriented against the D strand, while Lys-69 is capable of interacting with the acidic group of palmate. A hydrophobic interaction between the side chain of Ile-71 and palmate is speculated. EF loop (85-89), which controls the open or close of 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.