國立交通大學

生物科技學系

博士論文

利用單株抗體探討牛乳 β-lactoglobulin 之結構及特性

Structural and functional properties of bovine milk β-lactoglobulin as probed by monoclonal antibodies

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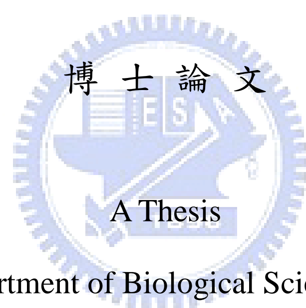

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本授權書所授權之論文為本人在__國立交通__大學(學院)__生物科技學系系所
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指導教授：__毛 仁 淡 院長__ ____________________

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組 95 學年度第 二 學期取得 博 士學位之論文。

論文名稱： 利用單株抗體探討牛乳 β-lactoglobulin 之結構及特性

指導教授： 毛 仁 淡 院長

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利用單株抗體探討牛乳β-lactoglobulin之結構及特性

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摘　　　要

乳製品中加熱殺菌是必要的過程，而蛋白質之結構會因為加熱殺菌過程中改變，在本研究中，我們利用物理、生物化學及免疫學之方式深入探討蛋白質之變化。利用 native-PAGE 分析不同品牌之鮮乳及還原乳發現，部分品牌中之乳蛋白β-lactoglobulin (**β-LG**)結構有 90%改變，但 α-lactoalbumin (**α-LA**)結構並未改變。進一步利用 Western blot 分析加工後之乳製品中之乳蛋白，其結果發現部分結構改變之 β-LG 會形成多種大分子之結構。利用 circular dichroic (CD)分析 β-LG 經不同時間(5-960 秒)及不同溫度(50-95℃)加熱後結構之改變，此加熱條件導致在 ellipticity 在 205 nm 觀察其結構由 β-sheet 轉變成不規則之結構，發現溫度低於 70℃加熱達 480 秒其結構無顯著之改變，但在加熱 80-95℃其結構會隨著加熱時間快速且顯著性變化，在 15 秒即有 50%之 β-LG 結構達到最大改變。此外當 β-LG 加熱超過 80℃結構改變後會完全喪失與 retinol 結合及其特性。因此檢測乳製品中蛋白質結構之改變可達監控乳製品品質之目的。然而現今並無利用免疫化學方式測定鮮乳或還原乳中蛋白質結構之改變。本研究中目的在於製作可區分還原乳及生乳之單株抗體，因此我們將還原乳直接免疫小鼠製作單株抗體。在 900 株 hybridoma 中發現有四株能專一性辯識還原乳，且此單株抗體能直接偵測生乳中

摻雜還原乳，其靈敏度能偵測生乳中僅添加 5%還原乳。有趣的是利用 Western blot 分析此四株單株抗體皆辨識 β-LG 及與 β-LG 結合之大分子結構。其中一株專一性抗體辨認區位於 β-LG D strand (第 66-76 胺基酸)，此外此結構上之第 69 胺基酸 Lys 與 retinol 及 palmitic acid 結合有關，當此區加熱結構改變後即會被此抗體辨識，但結合 retinol 及 palmitic acid 之能力會完全喪失。綜合上述結果 β-LG 為鮮乳中重要之溫度指標。為了評估乳製品之品質，我們另外製作了一株單株抗體僅辨識 native β-LG，當 β-LG 經加熱結構改變後即不會被此抗體辨識。最後我們更證明出當 β-LG 受熱結構改變後會喪失刺激 B-lymphocytes 增生之功能，此外我們發現刺激 B-lymphocytes 增生之功能是經由 receptor-mediate，亦證明出此 receptor 為 IgM。

Structural and Functional Properties of Bovine Milk β-lactoglobulin as Probed by Monoclonal Antibodies

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Abstract

Heating process is necessary for milk manufacture. However, the structures and properties of some protein existed in milk may change during this procedure. In this study, we utilized physical, biochemical, and immunological methods to study such structural changes. Using a native-polyacrylamide gel electrophoresis (**PAGE**), 90% of β-lactoglobulin (**β-LG**) was denatured in some brands of the processed and dry milk, but not α-lactoalbumin (**LA**).

By Western blot, a part of the denatured β-LG formed amounts of multimeric ones in the processed product. The thermal denaturation processes of β-LG and LA were monitored at various temperatures (50-95 °C) and duration (5-960 sec) via a circular dichroic analysis. The heating conditions might influence the ellipticity at 205 nm reflecting the conformational changes from β-structure to disordered structure. There were no significant conformational changes of β-LG at the temperature below 70 °C for as long as 480 sec. However, pronounced and rapid changes occurred between 80-95 °C with a time-dependent fashion. Fifty percent of the maximal changes achieved within 15 sec. The heated β-LG above 80 °C was almost completely lost its binding property with retinol. Nevertheless, there is no

immunochemical method that can be employed for the detection of protein structural change in processed or dry milk. This study aims to develop a sensitive monoclonal antibody (**mAb**) that could distinguish the dry milk from freshly prepared raw milk. Therefore, commercially prepared dry milk was used to immunize mice for production of mAb. From 900 hybridomas, 4 clones were found to be specific to dry milk. The specific mAbs could detect the dry milk spiked into the raw milk as low as 5% in concentration (v/v). Most interestingly, the 4 mAbs were all against β-LG and LG-milk protein conjugates in western blot. Furthermore, the epitope was characterized to be located within the D strand of β-LG (residues 66-76). In this region, lysine (residue 69) was associated with β-LG binding ability of retinol and palmitic acid. After heating process, it completely lost its binding ability. According to these results, β-LG could be regarded as a thermal marker in processed milk. To completely evaluate the quality of dairy products, we also produced a mAb specific against native β-LG. Eventually, we speculated that the conformational changes of β-LG during the heating process leads to the loss of its ability in stimulating B-lymphocytes proliferation.

誌　　謝

研究生涯中在完成此篇論文到達一個轉捩點，從此自己將獨當一面，回想這幾年研究生之生活有甘有苦，曾在中途想放棄，從不曾想過自己會完成博士論文，當在排回同時指導教授毛仁淡院長，利用另一種方式來教導及鼓勵，此種方式無法用言語形容只能用心體會，同時更有家人的支持，使學生在博士班三年級時如夢初醒般，自己尋找到預追求之目標，因此努力追回過去所浪費之時間，也因為如此才能順利完成此論文。成功者的背後，必定付出相當大的努力及所有人的幫助。不敢說自己是成功者，但論文的完成是受到多人的教導、鼓勵與支持。懷抱著感恩的心將此篇論文與幫助我的人分享。此篇論文承蒙畜產試驗所總所王政騰所長、鄭崴教授、張正教授及曾慶平主任，不吝斧正使論文得以更完整。

毛院長是我研究旅途中的領航者，從大學二年級開始即跟隨在他身邊學習，無論在研究領域上或是日常生活上皆盡心盡力教導我，使我在科學領域上能獨當一面，且生活上日漸成熟茁壯，不再畏懼突來的壓力或是事物，有時當然會因為自己停滯不前惹的他滿肚怒氣，但他從不放棄對我的教導，反而一再改變教導方式使我能快速向前邁進。在這幾年的學習中在潛移默化中對於科學的態度，轉變更為積極且充滿好奇心，對於日後研究中有莫大之幫助。對於毛院長感激之心是無法利用簡單之文字來表達，唯有更努力才能報答毛院長之教導。

此外實驗室等同於是我另外一個家，在這大家庭裡充滿許多甜美的回憶，有著日日奮戰到天明的身心的疲乏，更有一同出遊歡樂的記憶，當然亦有被挨罵

時的心酸，這點點滴滴將永遠身藏我心。在這家族成員中有著經驗老到的阿木學長，跟我一同打拼的繼鋒學長，很照顧我的純儀學姐，還有大學時期跟我同時進入院長實驗室奮鬥的靜怡，此外有以祥、明誌、英潔、佳靜、心怡、怡荏、繡文、媛婷、忠義、彰威、盛龍、惠君、俊瑩、宏輝、韶嬋、珮真、文昭、立品、佩茹、中曦、冠佑、威延、小涵、韋廷、佩宇、怡安。這家族中也因為有大家才會創造出許多美好回憶，非常感謝大家，期待未來日子裡能夠與大家相聚。更同時感謝志賢學長及小俞學長在剛進實驗室給予的鼓勵，建龍學長在研究最苦悶時的相互解悶。

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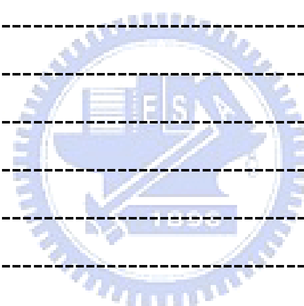

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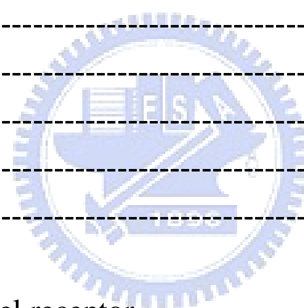

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Overview

Dairy foods provide, in addition to high quality protein, both calcium and phosphorus in a ratio that has evolved to be optimal for skeletal growth and development (Heaney, R. P., 2002., Shamik, J. P., and Yanovsky, J. A., 2003). High calcium intake, especially from dairy foods, along with adequate vitamin D, has been demonstrated to maximize the genetic potential for peak bone mass (McCarron, D. A., and Heaney R. P., 2004) (which is reached during the first 20 to 30 yr of life), to maintain skeletal mass in adulthood, to slow age-related bone loss and to reduce the susceptibility to fracture later in life (Huth et al., 2006). Heating process is necessary for dairy foods when it is manufactured. In general, a distinction can be made between two major classes of heating, i.e., pasteurization and sterilization. Pasteurization is essentially a mild process with minimal changes to the milk that kills spoilage microorganisms and results in the almost complete destruction of pathogenic bacteria initially present in raw milk (Goff, H. D. and Griffiths, M. W., 2006). This process may change the physical and chemical properties of milk while heating; examples may include the browning of milk, the unique flavor obtained by cooking the milk, and the structural change of the protein. The biochemical and nutritional differences between raw and processed milk has posed a dispute for a long time in the milk industry, but the following data may solidity any remaining doubts that may challenge the truth.

1. The composition of the proteins in bovine milk

Bovine milk has about 3.5% protein, 80% of which are caseins and the remaining 20% are whey proteins. Whey proteins contain all the essential amino acids and have the highest protein quality rating among other proteins. Advances in processing technologies have led to the industrial production of different products with varying protein contents from liquid whey. These products have different biological activities and functional properties (Yalcin A. S., 2006). Distribution of milk proteins are described in table 1.

Table 1 Distribution of milk proteins

Component	% of Total protein	Isoionic point	Molecular weight (kDa)
Caseins	**80**		
s1-casein	35	4.92~5.35	23.6
s2-casein	8		25.2
κ-casein	9	5.77~6.07	19
β-casein	24	5.20~5.85	24
γ-casein	4	5.8~6.0	12-21
Whey proteins	**20**		
β-lactoglobulin	10~15	5.53~5.41	18.5
α-lactalbumin	1~2	4.2~4.5	14.2
Serum albumin	1~2	5.13	66.3
Immunoglobulin	1	5.5~6.8	150
Proteose-peptone	2	3.3~3.7	4-41

2. Effect of heating process in whey protein

Heat treatments such as pasteurization may cause heat denaturation of β-lactoglobulin (**β-LG**) and modify its native structure (Chen et al., 2005). Heat denaturation of β-LG makes it susceptible for proteolytic degradation (Reddy et al., 1988., Takagi et al 2003). In addition, heat denaturation of β-LG may expose new antigenic sites (Davis, P. J. and Williams, S. C., 1998) and change the IgE binding properties of the protein (Ehn et al., 2004). Native β-LG passes through the gastrointestinal more efficiently than heat-denatured β-LG. These denaturation reactions, especially which of β-LG, the major whey protein, are of extreme importance to the dairy industry as they can be used to manipulate the physicochemical and functional properties of the resultant dairy products. As a consequence, there have been numerous studies examining the denaturation of whey proteins during the heat treatment of milk, the interactions of the denatured whey proteins with other milk components, and the effect of these reactions on the physical and functional properties of the milk products (Chen et al., 2005 and 2006). Full kinetic and thermodynamic studies on whey protein denaturation have been conducted and thus allows the prediction of the degree of denaturation under most processing conditions (Dannenberg, F. and Kessler, H. G.., 1988, Anema, S. G. and Mckenna, A. B., 1996, Oldfield et al., 2000). Coupling these denaturation studies with those examining the interactions of the denatured whey proteins with other milk components has provided more detailed information that aids in the prediction of the functional properties of milk

products based on the compositional properties of the milk and the processing conditions applied (Song et al., 2005, Anema., et al., 2004, Creamer et al., 2004, Lowe et al., 2004).

3. Structure of β-LG

Bovine β-LG is one of the major proteins in milk which accounts for 10~15% of the total protein concentration. (Hambling et al., 1992). Due to the thermally unstable and molten-globule nature, β-LG has been studied extensively for its physical and biochemical properties (Oi et al., 1995; Sawyer and Kontopidis., 2000). The protein is comprised of 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 and 1999; Kuwata et al., 1999) (Figure. 1). Topographically, strands A-D form one surface of the barrel (calyx) while 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 (Uhrinova et al., 2000). A remarkable property of the calyx is its ability to bind in vitro hydrophobic molecules such as retinol, fatty acids, vitamin D, and cholesterol (Narayan et al., 1997; Qin et al., 1998; Wu et al., 1999; Kontopidis et al., 2002). Utilizing its structural data and its advantage of smallness as a protein molecule, β-LG has also been studied as a model of unfolding (Kaminogawa et al., 1989, Laligant et al., 1994, Dufour et al., 1994, Katou et al.,

2001) and refolding (Hattori et al., 1993, Ragona et al., 1999, Creamer, L. K., 1995, Hamada et al., 1996, Forge et al., 2000). The refolding process of β-LG is unique in that non-native α-helix intermediates appear during an early phase. Elucidation of the β-LG folding mechanism can be used as a model to study the α-helix/β-sheet transition of other proteins such as prion proteins (Ragona et al., 1999, Forge et al., 2000). β-LG is remarkably acid-stable, resisting denaturation at pH 2, (Papiz et al., 1986) and undergoes oligomerization at pH 4-5.(Mckenzie, H. A., 1971) At room temperature and physiological conditions bovine β–LG exists as a dimer in solution due to the electrostatic interactions between monomers, though pH levels between 2 and 3 tend to dissociate them into monomers.(Sawyer, L., and Kontopidis, G., 2000) Within a given species β-LG occurs in several genetic variants; for example, cow milk contains at least nine forms (Godovac-Zimmermann et al., 1990 and 1996) of which phenotypes A and B are the most common, The two differ only at two amino acids, Asp64 is substituted for Gly and Val118 for Ala in variant B.(Qin et al., 1998).

4. β-LG biological function

Much is known about the physicochemical properties of β-LG. However, the biological function of this protein has not yet been satisfactorily resolved despite intensive research on the biochemical structure of β-LG (Jacques et al., 1999; Engfer et al., 2000; Kontopidis et al., 2002, Song et al., 2005). Recent studies have indicated that β-LG is associated with hypocholesterolemic, transportation of retinol (Zsila et al., 2002; Greene et al.,

2003,; Kontopidis et al., 2004), and antioxidant properties (Salvi et al., 2001 Chevalier et al., 2001; Marshall, 2004). Clinical studies have shown that β-LG can be directly absorbed into the circulation of infants through the gastro-intestinal system (Kuitunen et al., 1994). The immunoreactive β-LG is recovered after 2h ingestion. The β-LG is therefore an effective vehicle in providing the essential amino acids.

Dairy industries are interested to know an appropriate heat treatment of milk to control the quality of drinking milk or to control their heating system. On the contrary, the consumers are concerned whether or not the dry milk (powdered milk) has been supplemented to pasteurized raw milk. It happens when particularly the supply of raw milk is not sufficient in the summer where the demanding of consumption increases and the production of cow milk decreases. Since ultra heat treatment (UHT) procedure has been widely used in preparing processed milk, effort using heat-denatured milk proteins as a bioindicator has been cast to estimate such false practice (Relkin, 1996; Sanchez et al., 2002; Steffensen et al., 2002). For example, Recio and Olieman showed in 1996 that the amount of heat-denatured proteins can be estimated by analyzing the casein fraction using a capillary zone electrophoresis. A fluorescent probe using intrinsic basis states analysis has been employed for quantitative estimation of the stability of proteins in aqueous solution as a function of temperature (Tsonev and Hirsh, 2000). Monoclonal antibody prepared against β-LG has been utilized for studying the biological properties of β-LG, such as its interaction

with ligands and hypersensitivity reactions (Venien et al., 1997; Clement et al., 2002; Selo et al., 2002; Kobayashi et al., 2001; Restani et al., 1999; Morgan et al., 1999). Nevertheless, there are no immunochemical methods presently employed for the detection of dry milk mixed in raw milk.

For those reasons, we attempt to investigate some major changes of the milk proteins in the processed milk, with heat treatment being the major influence. In this study, we utilized physical, biochemical, and immunological methods which include native PAGE, SDS-PAGE, Western blot, circular dichroism (CD) and ELISA, to analyze the protein structural change in milk and its biological roles.

The specific aims are to:

1. Explore the major protein alterations in processed milk.
2. Define the structural changes of β-LG upon the heating process.
3. Identify the thermal maker for processed milk.
4. Develop a monoclonal antibody to distinguish dry milk from raw milk.
5. Produce a monoclonal antibody (mAb) specific to recognize to native β-LG and to determine the quality of milk products.
6. Study physiological role of β-LG in B-lymphocytes.

1. Major protein changes in processed bovine milk.

(1) We examined the raw milk and processed milk on a denatured SDS-PAGE using a PAGE procedure without pre-heating and found that β-LG at 18.5 kDa molecular weight had been substantially reduced in processed milk.

(2) Using a native –PAGE, a contrast was established between processed milk and raw milk regarding two acidic proteins. N-terminal sequence analysis of these proteins revealed that they were the isoforms of β-LG.

(3) Purified β-LG collected from HPLC was heated at different temperatures showing that a marked change of β-LG structure occurred at 80℃ for a heating session longer than 60s.

(4) The content of β-LG in commercially processed milk was low, most likely due to the UHT process.

(5) β-LG was cross linked to other milk proteins to form large molecules via disulfide linkages.

2. Structural change of β-LG upon the heating.

(1) The kinetics of thermal denaturation of purified β-LG at various temperatures was studied using a circular dichroic (CD) analysis. β-sheet structures of β-LG became disordered after the heating process.

(2) The maximal change of ellipticity at 205 nm was correlated to the heating temperature

and time. With a change occurring at 80℃ for heating duration over 60s.

(3) We analyzed the structural changes of β-LG after heating by using the circular dichroic (CD) method and mAb. The results showed that the structure of β-LG was severely destroyed but the binding between β-LG and specify what kind, i.e. denature mAb had increased.

3. <u>Production of a mAb that is specific only to dry milk.</u>

To test this possibility, we immunized mice with commercially prepared DMLK and produced a panel of mAb. From 900 hybridomas screened using an ELISA, 4 clones were found to be specific to DMLK; the other 68 clones recognized both DMLK and raw milk. In contrast to polyclonal antibodies, only the specific mAb could detect the DMLK spiked into the raw milk at as low as 5% in concentration (vol/vol). Western blot analysis shows that these specific mAb were all directed against *β*-lactoglobulin (β-LG) and LG-milk protein conjugates. These mAb reacted with raw milk heated at 95° for 15 min; the reaction with β-LG-conjugates, however, was abolished when treated with reducing reagent. Thus, results suggest that a new antigenic epitope was exposed in a heating process, and the thio-group of β-LG cross linked with other protein moiety played a provocative role in mAb recognition. A hypothetical model with respect to the interaction between the mAb and DMLK was proposed and discussed.

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. 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. Epitope mapping reveals that Lys-69, Ile-71, Ile-72, and Glu-74 in denatured D strand were directly involved in the binding to mAb. Most interestingly, the D strand is associated with the A–C strands in forming one domain at the opening of the calyx. For this reason, we also investigated the effect of heating and pH on β-LG binding to retinol and palmitic acid. Circular dichroic spectral analysis shows that carboxymethylation on β-LG not only resulted in a substantial loss of configuration but also exerted a 10 fold 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 showed 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 reduced its binding ability to palmitic acid implicating a role of Lys-69. Integrating the data, 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. Finally, we propose that strand D plays a provocative role in the molten globule state of β-LG as probed by our mAb.

4. <u>Producing a mAb specific only to native β-LG.</u>

We used native *β*-LG as an immunogen to test the hypothesis that a specific mAb against the native *β*-LG could be constructed. As result, a mAb (4H11E8) directed against the native structure of *β*-LG was produced. The antibody did not recognize the heat-denatured form of *β*-LG, such as its dimer or its aggregates. Immunoassay using this "native" mAb showed that the stability of *β*-LG was kept at temperatures ≦70°C. *β*-Lactoglobulin began to deteriorate between 70 and 80°C over time. The denaturation was correlated with the transition temperature of *β*-LG. Further chemical modification of Cys (carboxymethylation) or positively charged residues (acetylation) of *β*-LG totally abolished its immunoreactivity, confirming the conformation-dependent nature of this mAb. Using competitive ELISA, the 4H11E8 mAb could determine the native *β*-LG content in commercially processed milks. Concentrations of native *β*-LG varied significantly among the local brands tested. From a technological standpoint, the mAb prepared in this study is relevant to the designing and

operating of appropriate processes aimed for thermal sanitation of milk and of other dairy products.

5. Studying the physiological role of β-LG in B-lymphocytes.

We have shown that native β-LG possessed a potent activity for cell proliferation of hybridoma lymphocytes, but not thermally denatured β-LG (95 ℃, 5 min). The proliferation activity was also seen in whole milk with a removal of β-LG from milk proteins by an antibody-affinity column, the result being a substantial decrease in activity. To further study the influence of conformational change on β-LG regarding cell proliferation, we chemically modified the β-LG via carboxymethylation and acetylation; a lack of proliferation activity suggested that the specific conformation of β-LG plays a role in inducing cell proliferation. We further demonstrated that this activity was via a receptor-mediated process. Flow cytometry and confocal microscopy analyses revealed that β-LG may bind to a cell surface through a temperature dependant manner. The receptors isolated using both HPLC and an β-LG affinity column showed these they were immunoglobulin in nature. β-LG increased the expression of cyclins A and D but not cyclins B and E, where the latter participates in phase G2 of a cell cycle. Hence β-LG acts as a 'growth factor' in lymphocytes via a receptor-mediated mechanism.

In conclusion, the loss of unique chemical and immunochemical and the conformational changes after heating made β-LG a superior marker for evaluating the thermal processed milk. The detailed thermal denaturation curves of β-LG constructed with respect to its time and temperature in this study provide the dairy industry with a valuable reference. We postulate that heat treatment over 80 °C for 15 sec or longer may induce a significant denaturation of milk β-LG. Using the β-LG conformational change during the heating process, we produced mAbs specific to native β-LG and heated β-LG. The mAb mentioned in this study may provide an efficient probe that may monitor the thermal changes and the physiologic activity of β-LG, such as its denaturation or its binding to fatty acids and retinol. Whether β strands may also participate in this role upon the heating between 70-80 °C, remains to be addressed.

As for physiological function, we focused on demonstrating that β-LG is the most important protein in milk that stimulates cell proliferation; we proved that the cell proliferation activity of thermally denatured β-LG becomes totally suppressed. We demonstrated also that β-LG stimulates cell proliferation via a receptor-mediated mechanism.

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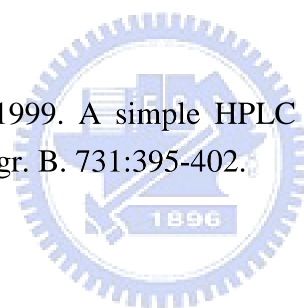
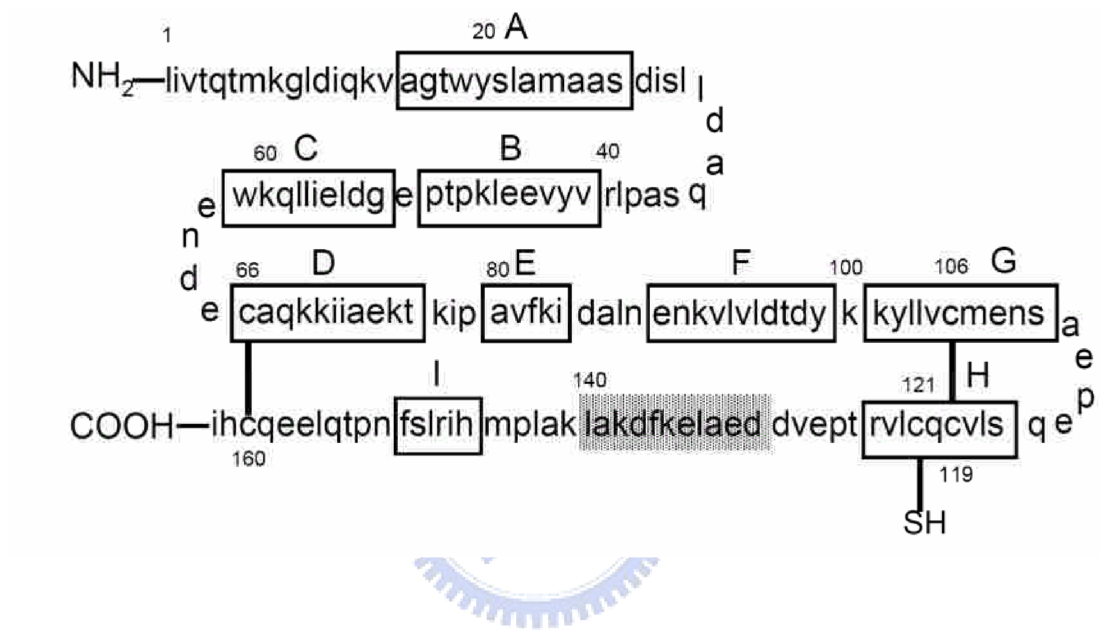
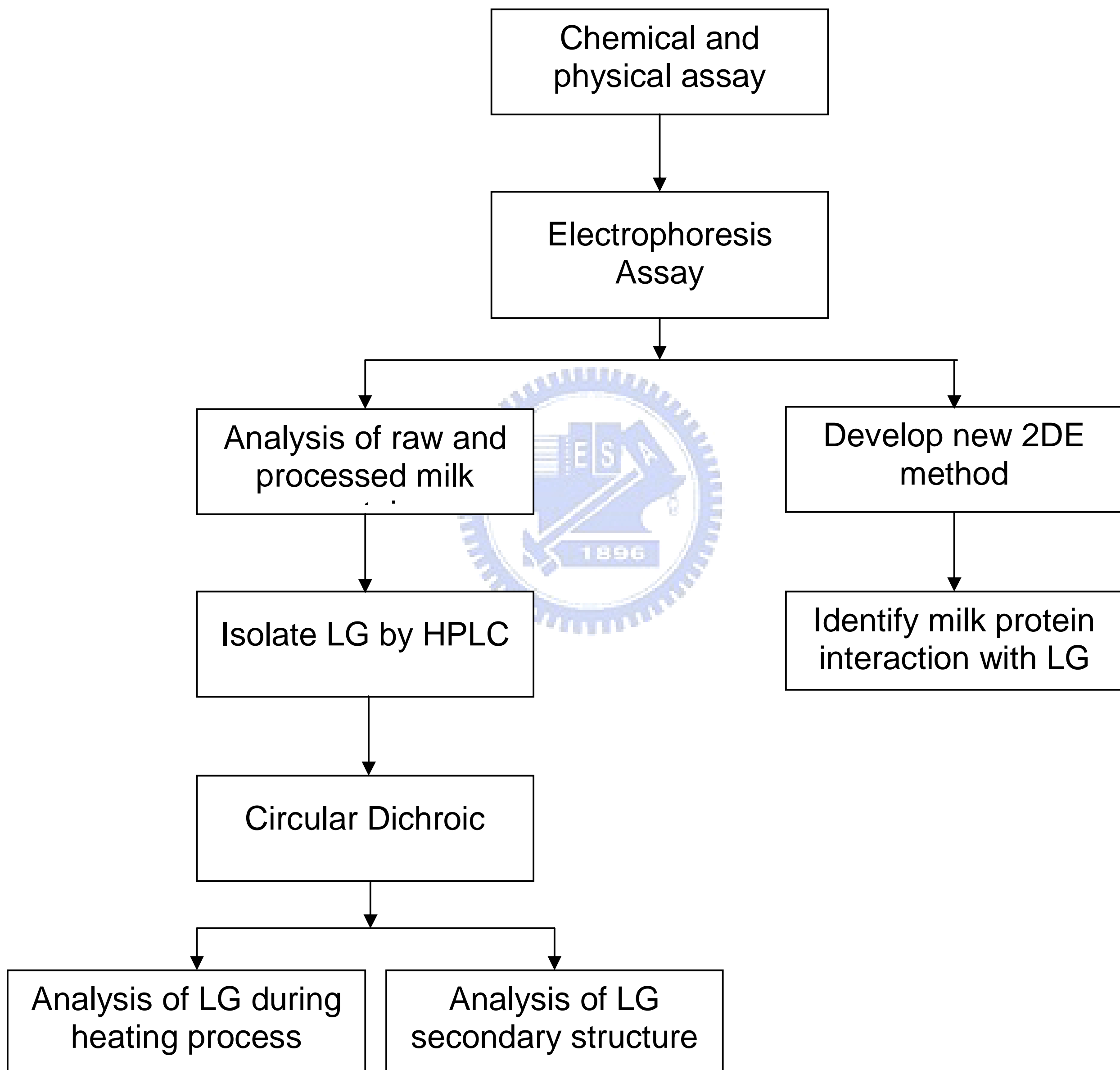
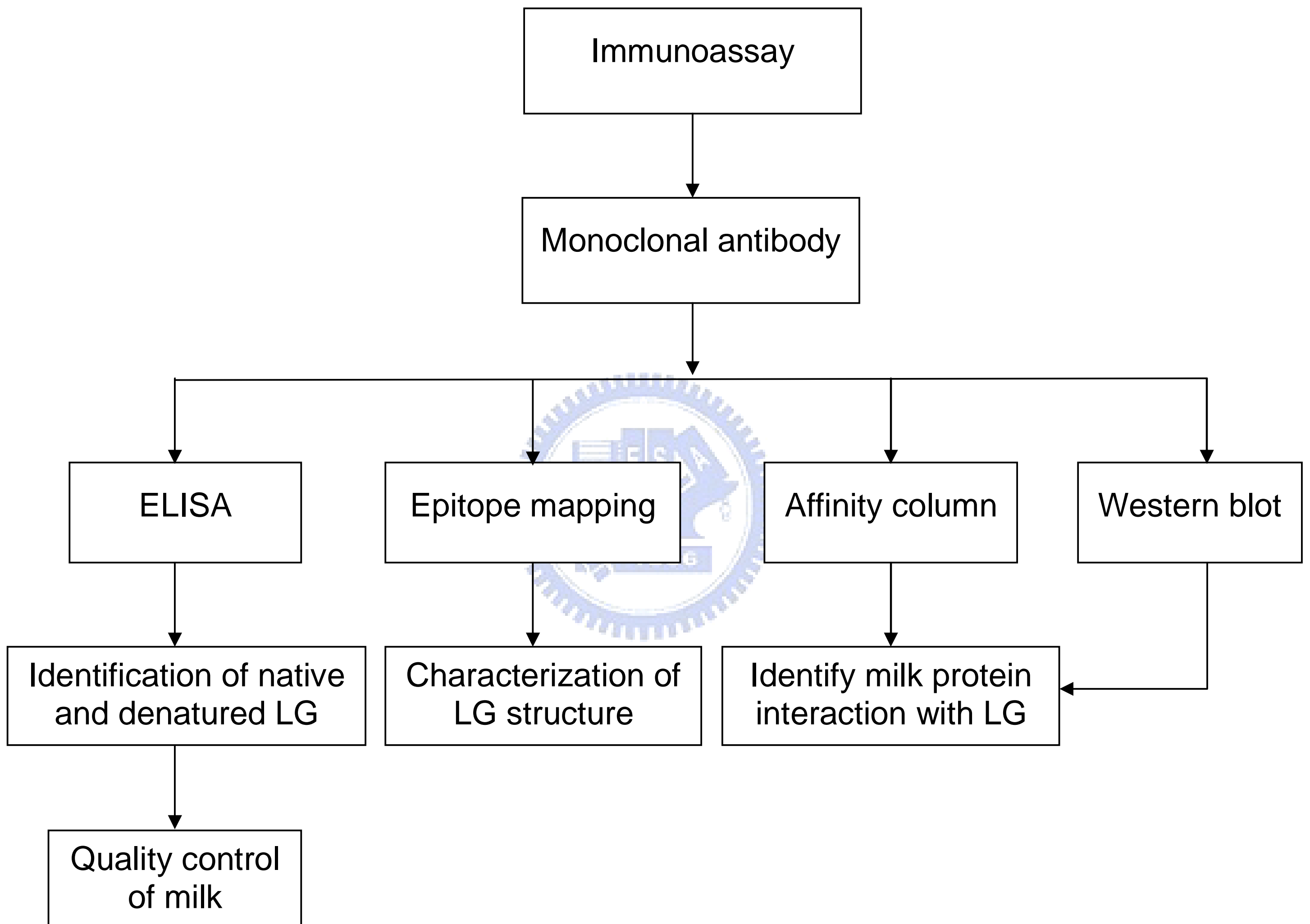


Figure 1



Flow Chart




Immunoassay
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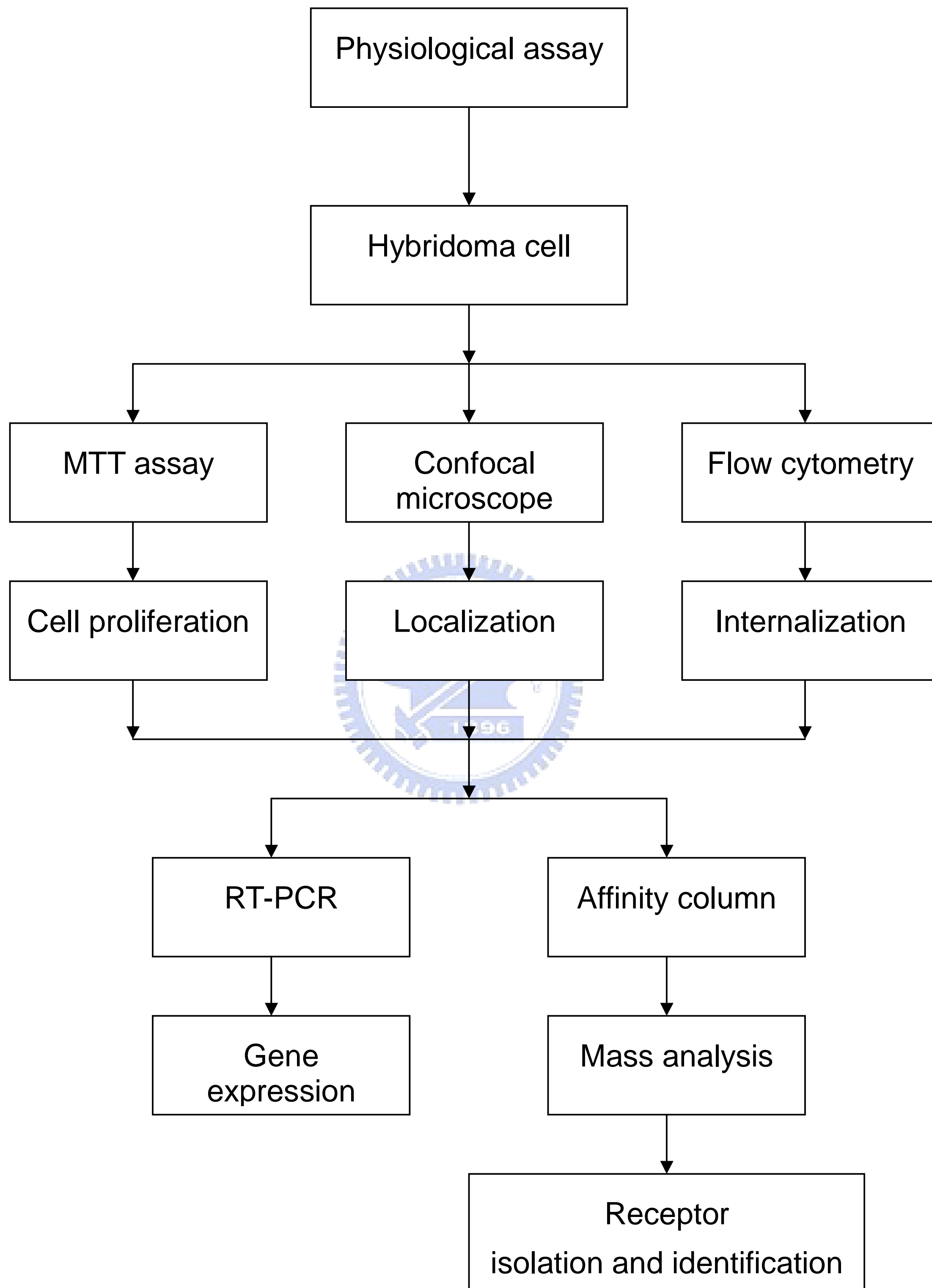
ELISA
Epitope mapping
Affinity column
Western blot
Identification of native and denatured LG
Characterization of LG structure
Identify milk protein interaction with LG
Quality control of milk


Physiological assay
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MTT assay
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Flow cytometry
Cell proliferation
Localization
Internalization
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Affinity column
Gene expression
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Receptor
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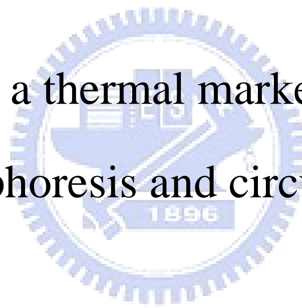


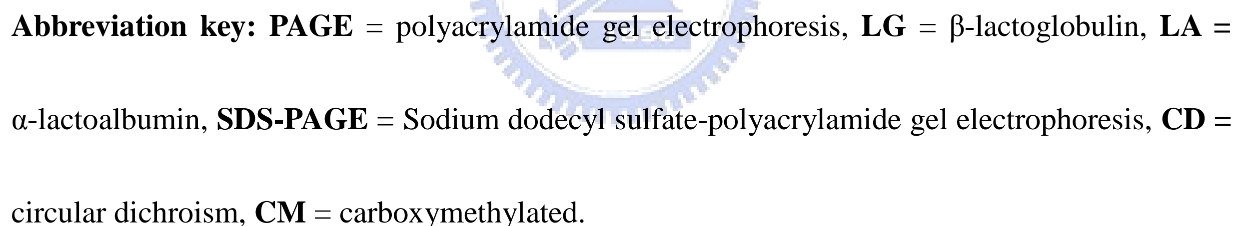
Beta-lactoglobulin is a thermal marker in processed milk as studied by electrophoresis and circular dichroic spectra

ABSTRACT

As much of the process involves heat treatment during the preparation of milk on an industrial scale, the unpredictable measures of the process are an essential issue in determining the quality of the milk. The purpose of the present study was to investigate the major protein change(s) of whey proteins in processed milk and extend its knowledge for future reference in dairy industry. Using a native-polyacrylamide gel electrophoresis (**PAGE**), we show almost a 90% loss and denaturation of β-lactoglobulin (**LG**) in some brands of the processed and dry milks, but not α-lactoalbumin (**LA**). Immunochemical study using Westernblot revealed that part of the loss was attributed to the formation of large multiple forms of LG in the processed product. Such denaturation was presumably associated with the heating procedure used in the process. Essentially, LG was the only major fraction converted to aggregates in milk heated at 95 °C for 30 min on two-dimensional PAGE. The detailed thermal denaturation of purified LG and LA at various temperatures (50-95 °C) and time (5-960 sec) were investigated using a circular dichroic (**CD**) analysis. The maximal changes of ellipticity at 205 nm (converting β-structure to disordered structure) were correlated to the heating temperature and time. There were no significant conformational changes of LG at the temperature below 70 °C for as long as 480 s. Pronounced and rapid changes were between 80-95 °C in a time-dependent fashion. Fifty % of the maximal changes could be reached within 15 sec. In conclusion, the unique

chemical and immunochemical loss and conformational changes made LG as a superior marker for evaluating the thermal processed milk. The detailed thermal denaturation curves of LG constructed with its time and temperature in this study provide dairy industry a valuable reference. We postulate that heat treatment over 80 °C in 15 sec may induce a significant denaturation of milk LG.

(**Key words:** β-lactoglobulin, SDS-PAGE, Native-PAGE, circular dichroic, thermal denaturation, processed milk, structure)



Abbreviation key: PAGE = polyacrylamide gel electrophoresis, **LG** = β-lactoglobulin, **LA** = α-lactoalbumin, **SDS-PAGE** = Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, **CD** = circular dichroism, **CM** = carboxymethylated.

INTRODUCTION

Molten globules are thought to be general intermediates in protein folding and unfolding (Chang et al., 2000; Yang et al., 2001; Croguennec et al., 2004; Song et al., 2005).

α-lactoalbumin (LA) and β-lactoglobulin (LG) are two of the major protein moieties of bovine whey proteins. Both of them are the most investigated models for understanding the mechanism involved in protein stability, folding and unfolding upon the heating. Recent studies have shown that milk LA and LG induce apoptosis in tumor cells (Håkansson et al., 1995; Svensson et al., 1999; Casbarra et al., 2004; Baltzer et al., 2004) and produce immunomodulatory (Wong and Watson, 1995; Cross and Gill, 2000; Filteau, 2001; Brix et al., 2003) and hypocholesterolemic effect (Eichholzer and Stahelin, 1993; Nagaoka et al., 2001). More recently, we have shown that heating markedly reduce the binding of LG to fatty acid and retinol (Song et al., 2005). Carbonaro et al (1999) have shown that the proteins extracted from cooked common beans are more resistant to proteolysis (due to the formation of protein aggregation) than that of raw beans. The iron absorption of heme from beef exposed to prolonged heating was substantially reduced in humans (Martinez et al., 1986). As much of the process involves heat treatment during the preparation of milk on an industrial scale, the unpredictable nature of the process has therefore been an essential issue that may affect the physiologic role of the LA and LG. For this reason, we attempted to investigate some major changes of the whey proteins in the processed milk, while heat treatment is mainly involved. In fact, only limited reports have been documented concerning the processed milk thus far. We have reported that a monoclonal antibody prepared against dry milk can specifically

recognize dry milk, but not fresh raw milk (Chen et al., 2004). Characterization of this monoclonal antibody revealing it was directed towards to milk LG. Therefore LG might provide a provocative marker in milk quality control. In the present study, we demonstrate that there was a substantial loss of LG in processed milk. Since the manufacturers do not disclose the processing procedures of the milk, we established a thermal denaturation curves for the loss of LG and LA with respective to their heating time and temperatures. Furthermore, we studied the overall structural changes of LA or LG by a circular dichroism and correlated these changes over temperature and time with the loss of LG. Such detailed changes, however, have not been reported previously. The present study provides a reference value for dairy industry as to the consideration for preparing high quality products. In addition, the Westernblot analysis on the changes in some molecular form of LG in processed milk is also described.

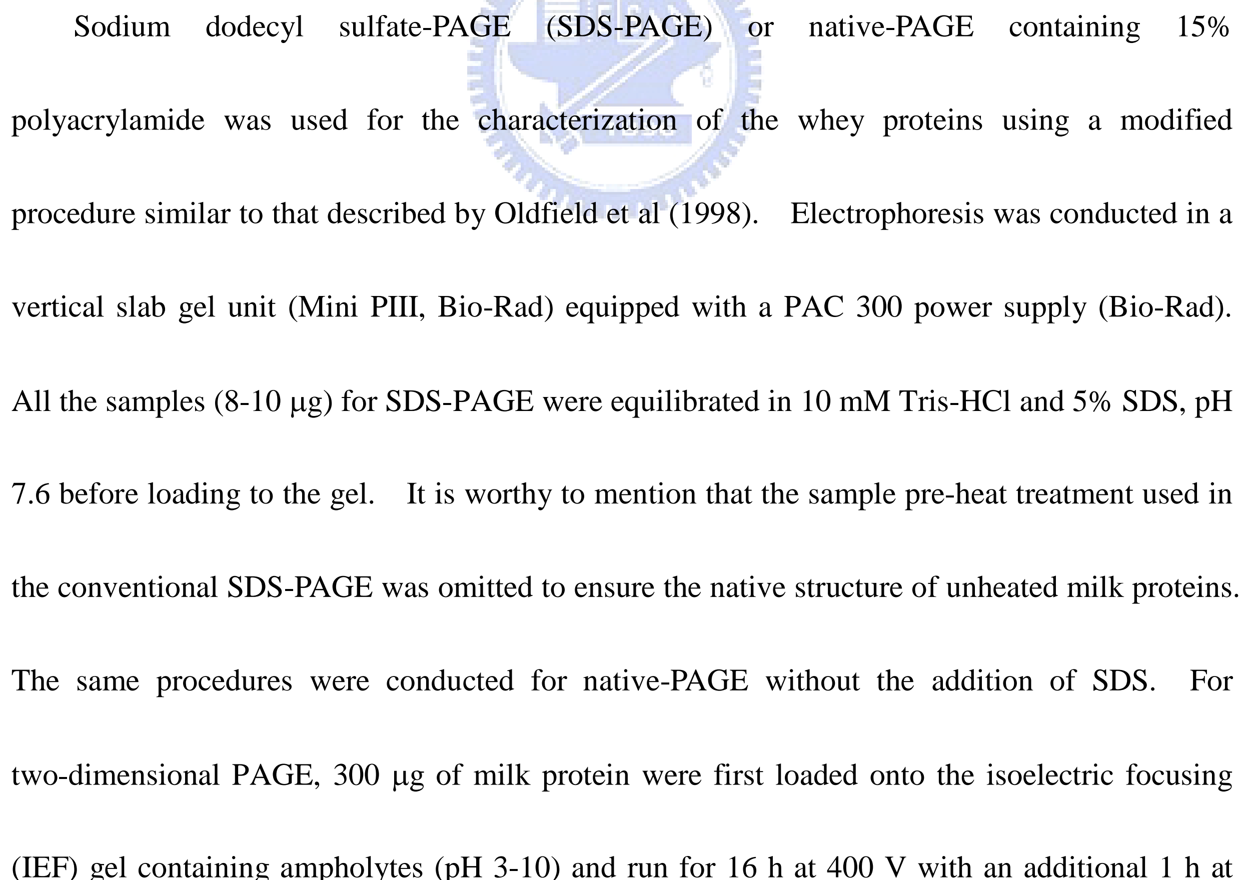
MATERIALS AND METHODS

Preparation of Milk Samples and Whey Protein

Freshly bulked whole raw milk obtained from a local dairy farm was immediately centrifuged at 13,000 rpm (15,500g) for 1h at 4 °C. The top layer in the supernatant was carefully removed, while the remaining fraction (whey protein) containing minimal casein was

used for the analysis of polyacrylamide gel electrophoresis (PAGE) and Westernblot. Five different brand of commercially processed milk, were purchased from the local market and 4 others were from the US market (Kroger, Cincinnati, Ohio; Trauth, New Port, Kentucky; United Dairy Farmers, Cincinnati, Ohio; Weingarden, Loveland, Ohio). Three powdered milk were obtained from the local market imported from Australia (Klim, Nestle Australia Ltd), New Zealand (Anchor, NZ Milk Ltd) and Denmark (Quaker, MD Foods Kjersing A/S).

Gel Electrophoresis



Sodium dodecyl sulfate-PAGE (SDS-PAGE) or native-PAGE containing 15% polyacrylamide was used for the characterization of the whey proteins using a modified procedure similar to that described by Oldfield et al (1998). Electrophoresis was conducted in a vertical slab gel unit (Mini PIII, Bio-Rad) equipped with a PAC 300 power supply (Bio-Rad). All the samples (8-10 μg) for SDS-PAGE were equilibrated in 10 mM Tris-HCl and 5% SDS, pH 7.6 before loading to the gel. It is worthy to mention that the sample pre-heat treatment used in the conventional SDS-PAGE was omitted to ensure the native structure of unheated milk proteins. The same procedures were conducted for native-PAGE without the addition of SDS. For two-dimensional PAGE, 300 μg of milk protein were first loaded onto the isoelectric focusing (IEF) gel containing ampholytes (pH 3-10) and run for 16 h at 400 V with an additional 1 h at

800 V. The IEF gel was then loaded onto a 15% SDS–polyacrylamide slab gel (the second dimension) with a 5% stacking gel as previously established in our laboratory (Yang and Mao, 1999; Wang et al., 2002).

Heat Treatment on Whey Protein Solution Containing LA and LG

To obtain the whey proteins consisting predominantly of LA and LG, whey protein was fractionated in a final concentration of 40% of saturated ammonium sulfate at 4 °C for 30 min. After which time the supernatant was collected by a centrifugation at 4,000 g for 30 min at 4 °C. The sample was then dialyzed against a Tris buffered saline containing 50 mM Tris-HCl and 0.12 M NaCl, pH 7.2 (TBS). Sample solution containing 1mg/ml was subjected to the thermal denaturation at 50, 60, 70, 80, 90, and 95 °C, respectively, over a period from 15 to 960 sec. The reaction was then immediately stopped at 20 °C using a water bath.

Heat Treatment on Purified LA and LG

LA with calcium was purchased from the Sigma (Lot No. 60k700, St. Louis, Mo). While, LG was purified from a HPLC DEAE-column in our laboratory using a method similar to that previously described (McCreath et al., 1997). In brief, freshly prepared whey proteins from raw milk were first fractionated by a 40% of saturated ammonium sulfate. The dialyzed top fraction was then concentrated to protein content at approximately 20 mg/mL. Two ml of the solution

were applied to a 10 x 64 mm DEAE-5 column (Biorad) and separated using a Waters HPLC system equipped with a 600 controller and 996 photodiode array. The sample was then eluted with a 0-0.5M linear NaCl gradient in 0.02 M phosphate buffer, pH 8.0, over 60 min. The flow rate was 1 mL/min. The peak fractions containing LG isoforms were immediately pooled and dialyzed against PBS at 4 °C. Heating experiment for LA or LG was then conducted at various temperatures over time according to the procedures mentioned above.

N-Terminal Amino Acid Sequencing

LG identified on native PAGE was sequenced from the N-terminus by an automatic Edman degradation procedure on an ABI 476A peptide sequencer described previously in our laboratory (Yang and Mao, 1999). Briefly, the protein bands separated on native-PAGE gel were firstly transferred onto a PVDF membrane. The protein bands corresponding to the expected LG were sliced and subjected to an automatic Edman degradation for amino acid sequence determination.

Carboxymethylation of LG

About 5 mg of 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 (Song et al., 2005). 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**) LG was desalted on a Bio-Gel P2 column eluted by 0.05 M ammonium bicarbonate and lyophilized. By amino acid analysis, the CM-LG contained 4.9 residues of CM-cysteine per mole of LG.

CD Measurements

For the CD measurement, each sample heated or unheated at 0.5 mg/mL in a Tris-buffered saline containing 50 mm Tris-HCl and 0.12 M NaCl, pH 7.2 was used (Tseng et al., 2004, Song et al., 2005). The CD spectra were conducted on a Jasco-J715 spectropolarimeter (Jasco, Tokyo, Japan) at 24 ℃ over the wavelength ranges from 200 to 250 nm, and recorded at a 20 nm/min scan speed. All spectra were measured in a cuvette with a path length of 0.1 cm. Each heated sample (100 μL) was treated, respectively, at 50, 60, 70, 80, 90, and 95 °C. At each temperature, the sample was maintained separately over a time period from 15 to 960 sec and instantly stopped at a 20 °C water bath prior to an immediate measurement.

To construct the thermal denaturation curves for LA , % of maximal change= [absolute value of ellipticity of 208 nm at each time point /maximal absolute ellipticity of 208 nm at 95 °C for 16 min] x 100. For LG, the maximal absolute value of ellipticity was based on that of 205 nm at 95 °C for 8 min.

Preparation of Antiserum against β- lactoglobulin

The polyclonal antibody prepared against LG was raised in female Balb/C mice (n=6) by subcutaneous and intraperitoneal injections of purified LG (Chen et al., 2004). In brief, LG was mixed and homogenized with an equal volume of complete Freund's adjuvant by a three-way stopcock. Each animal was initially given a total emulsion of 500 μL containing 200 μg of LG including 6 subcutaneous injections onto the back and an intraperitoneal injection. After ten days, a 500 μL sterile PBS solution containing 200 μg LG without adjuvant was boosted by 2 intramuscular injections. Seven days following the final booster, blood was collected in 0.1% EDTA and plasma was obtained. The titer of this antibody was over 1:20,000 as judged by an ELISA using a method previously described (Huang et al., 1999).

Animal Care and Use

Balb/c mice with 5-7 weeks of age obtained from National Science Council (NSC) of Taiwan were fed in animal facility in Chiao Tung University during the period of immunization. Feed and water were available daily. CO_2 was used as a method of sacrificing and the other management was conducted according to guidelines established by NSC of Taiwan.

Western blot

Following the separation of proteins by SDS-PAGE or native-PAGE, the gel was soaked in a

buffer containing 50 mM Tris-HCl, 50 mM boric acid and 1mM EDTA, pH 8.2 for 30 min (Chen et al., 2004). The gel was then electrotransferred to a nitrocellulose membrane (Hybond-C extra; Amersham, Buckingham, UK at 100 mA for 1 h in a semi-dry transfer cell (Bio-Rad). The membrane was immersed in 1% (w/v) gelatin for 1 h with gentle shaking. Following a wash with 0.05 % Tween 20-containing phosphate buffer saline (TPBS), pH 7.4, for 3 min, the membrane was incubated with a primary antibody (1:2500 dilution in PBS containing 1 % gelatin) for 1 h and washed three times with PBS containing 1 % gelatin. The paper was then incubated with a diluted antiserum conjugated with horseradish peroxidase against mouse IgG for 1 h in PBS containing 1% gelatin. Finally, the membrane was washed and developed with 3-3'-diaminobenzidine (3, 3', 4, 4'-tetra-amino-biphenyl) containing 0.01 % H_2O_2 in PBS.

RESULTS

To identify the possible difference in protein profiles between raw and processed milk, whey proteins from the same batch of the milk before and after manufactured process were analyzed on a native-PAGE. Figure 1 (left panel) shows that there was a marked decrease (at least 90%) in two acidic proteins of processed milk. N-terminal sequence analysis revealed that these proteins were the isoforms of LG with a sequence of LIVTQTMKGLDIQKVAGTWY consistent to the chemical characteristics of LG previously reported (Molloy et al., 1977). In the next experiment,

we examined the whey proteins on a SDS-PAGE using a procedure without heat. Similarly, LG corresponding to molecular weight about 18 kDa was substantially reduced (Figure 1, right panel). Notably, one extra band corresponding to a molecular weight about 25 kDa in the processed milk was observed on the SDS-gel (Figure 1). This protein was not related to LG as assessed by a Westernblot (described below), although its biochemical nature remains elusive. We then determined the feature of whey proteins in five brands of the processed milk from our domestic market. Figure 2 shows that the LG, with some extent of LA, was extensively lost or denatured in those samples (No. 2-6). Three brands of dry milk imported from Australia, Denmark, and New Zealand were also analyzed (No. 7-9), in which one brand not only exhibited large extent of denaturation in LG, but also in LA. However, the LG remained almost intact in 4 randomly chosen brands from the US market (Figure 2). Thus, a simple native-PAGE may be used to impart technological difference involved in processing the milk.

To localize the loss of LG in milk, a polyclonal antibody prepared against native LG was employed for Western blot analysis. The domestic processed- and imported dry-milk, but not the raw milk, revealed large and multiple LG aggregates on both native-PAGE (Figure 3A) and SDS-PAGE (Figure 3B). One single band, but no detectable LG aggregates, was observed in raw milk. There were slight and trace aggregates in the US brands.

To test the hypothesis that the electrophoretic change in LG was caused by a heat treatment

in the milk processing, we heated the purified LG at 95 °C for different time followed by a Western blot. Formation of multiple aggregates of immunoreactive LG were clearly demonstrated on both native- and SDS-PAGE (Figure 4), which was somewhat similar to that in the processed milk (Figure 3). The molecular weight of the smallest LG polymer was about 36 kDa suggesting that at least a dimer was being formed. It is worthy mentioning that the SDS-PAGE was run without heat procedure, some non-covalently linked LG polymers might be existed.

To test whether cysteine residues were responsible for the polymerization upon the heating, chemical modification (carboxymethylation) blocking the thio groups of LG was conducted. Figure 4C shows that there was no detectable cross-linking of carboxymethylated-LG upon the heating.

Although we show the substantial loss of LG in processed milk forming large LG polymers, the sum of these polymers was still not account for the total loss as shown in Figure 1. The processed-milk purchased in the market is usually underwent ultra-heat treatment (UHT) at 135 °C with undisclosed time. In the next experiment, we heated the raw milk at 95 °C for 30 min and analyzed its protein profile on two dimensional (2D) gel electrophoresis. Using Coomassie blue staining, only LG was attenuated, the aggregated polymers were not seen. However, some large molecular forms of LG (either self-associated or other casein-associated) with molecular

weight greater than 500 kDa were observed on Western blot (Figure 5).

We speculated the initial thermal denaturation of LG responsible for its cross-linking to form large molecular forms. We studied the structural change of LG caused by a heat treatment as compared with the LA, another major milk whey protein. First, the isolated LG and LA were heated at 95 °C overtime. On native-PAGE analysis, the acidic property of LG was altered in a time dependent manner (Figure 6). A marked change in heat treatment was observed in LG over time up from 30 s. The major extra-band (Figure 6) from the denaturation was LG as confirmed by a Western blot (data not shown). Whereas, the LA was more resistant (240 s) while compared to LG. We further examined the thermal changes in each purified LG or LA at various temperatures over time using the same native-PAGE. Following the integration using a digital image system from the gel, there was no significant change in LG below 60 °C over a period of 960 s (Figure 7). Some moderate changes occurred at 70-80 °C, but the most pronounced changes occurred above 80 °C and were in a time-dependent fashion. The native form of LG was almost abolished when heating was proceeded for longer than 240 s. Similarly, the thermal denaturation curves of LA were constructed, but the severity was much less than that of LG in both time and temperature responses (Figure 7B).

Therefore, our data suggested that LG suffered more changes in overall structure than did LA upon the heating. To further support this hypothesis, we monitored the structural changes in

LG and LA using a CD spectral measurement. Figure 8 shows that the native LG exhibited primarily a typical β-sheet configuration with a deep at 215 nm. The β-sheet content is about 50% consistent to our previous observation (Song et al., 2005). The structure of LG underwent disordered upon the continuous heating (50, 60, 70, 80, 90, and 95 °C) is shown in Figure 8. Essentially, there were minimal changes in ellipticity, while heating between 50 to 70 °C over the entire period. Significant structural changes (ellipticity at 205-208 nm) were found to be at and above 80 °C. Such changes, however, were not substantial in LA. Extrapolation from the CD studies, thermal denaturation curves for both LA and LG were than constructed based on the ellipticity (or conformational changes) at each time point vs. the maximal changed ellipticity (please see MATERIALS AND METHODS for details). Figure 9 shows that there were no or minimal changes at temperatures below 80 °C for different time intervals. Markedly structural changes were observed when temperatures were above 80 °C. Thus, the data suggest that 80°C is a cutoff temperature in maintaining the intact structure of LG.

DISCUSSION

Although the exact physiologic role of milk whey proteins has not been fully elucidated, recent studies have indicated that LA possesses an immunomodulatory activity, conferring increased resistance to the growth of tumors. On the other hand, LG is associated with

hypocholesterolemic, transport of retinol (Zsila et al., 2002; Greene et al., 2003,; Kontopidis et al., 2004), and antioxidant properties (Salvi et al., 2001 Chevalier et al., 2001; Marshall, 2004). Clinical studies have shown that LG can be directly absorbed into the circulation of infants through the gastro-intestinal system (Kuitunen et al., 1994). The immunoreactive LG is recovered following 2h ingestion of bovine milk. The LG is therefore an effective vehicle in providing the essential amino acids. Thus, the substantial loss of LG forming large polymers in processed milk found in the present study cannot be ignored. In contrast, the LG of four milks from the US market were intact. Our study also suggests that the heating procedures used in domestic milk (Taiwan) were significantly different from those in other countries.

Because almost all-dairy processes require heat treatments, information on the heat stability of milk with a modified protein component becomes an essential subject. It is conceivable that the dairy industry accounts for a large number of the knowledge and nature of molten globules, since the industry is concerned with improving the process of whole milk and concentrates as well as extending the products used for nutritional source (Sawyer and Kontopidis, 2000). With respect to the physiologic significance of denatured LG, we have recently shown that LG overheated above 80 ℃ produce substantially decrease in binding to fatty acid and retinol (Song et al., 2005). This was due to a significant loss of β-structure within residues 67-76 of LG, in which the hydrophobic domain in the pocket of calyx becomes exposed loosing its binding nature

(Song et al., 2005)

In addition to the milk quality in nutritional aspect, it is certainly of worth to avoid the excessive heating. The undesirable effect, such as the formation of deposits in the heating or cooling equipment (De Jong, 1996) and the impaired renneting properties (Lieske, 1997). Detailed knowledge of the denaturation behavior of LG on heating time and temperature is required to promote the positive effects and to minimize the deleterious outcomes (Lieske et al., 1997). In practice, we have found that the deposit of milk remnants did produce the damage on the radiators in some facility of our local dairy manufacturers in Taiwan. Since LG can form clot during the heating process (Bienvenue et al., 2003, Cayot et al., 2003, Guyomarc'h and Law, 2003, Corredig et al., 2004), overheating of the milk during the sterilization may be responsible for such undesired event.

From the thermal denaturation curves (Figure 7) and CD spectra (Figures 8 and 9), we show that the denaturation of LG was much rapid and extensive at and over 80 °C. We suggest that 70-80 °C is a margin. Any treatment above 80 °C, intact LG suffers a large loss (or denaturation) and conformational changes. But, the extent of changes was limited in LA. The relatively weak hydrophobic interactions (Chaplin et al., 1986, Rattray and Jelen, 1998) occur between unfolded LA may explain its high resistant to the thermal changes. The data suggest that the transition temperature of LG occurred between 70-80 °C consisting to our previous finding using

a thermal sensitive monoclonal antibody as a probe (Chen et al., 2004, Song et al., 2005). It is interesting, however, prolonged heating (up to 16 min) at transition temperature did not totally denature the LG with only about 50% loss. Perhaps the denaturation and renaturation occurs simultaneously and eventually reaches equilibrium. Similar case was observed at lower temperature. But, at temperature above the transition, the denaturation is not reversible.

Although the denaturation of whey proteins in milk has been studied by native-PAGE (Havea et al., 2002), no information is available on the detailed kinetics of heat-induced conformational changes in LA and LG using both CD and native-PAGE simultaneously (50-95 °C for 15s to 15 min). The present study provides a detailed and valuable reference for the study of the correlation of conformational changes and electrophoretic properties in both LA and LG.

It is worth mentioning that only the whey proteins, rather than whole milk, were chosen for native-PAGE in the present study. This is due to the lipids (micelles) and casein in whole milk considerably affect the performance of gel electrophoresis as described by the others (Xiong et al., 1993, Hollar et al., 1995, Oldfield et al., 1998a, Oldfield et al., 1998b). Furthermore, since some LG could associate or polymerize with micelle and casein fractions in overheated whole milk (Vasbinder et al., 2004, Guyomarc'h et al., 2003, Guyomarc'h et al., 2003, Vasbinder et al., 2003), this association is responsible for the partial loss of LG (but not all) in whey protein

fraction as indicated in our previous study (Chen et al., 2004). Likewise, the immunoreactive LG blotted as in multiple large forms (Figures 3 and 4) may sorely represent the partial loss of total LG in the processed milk. Nevertheless, we demonstrate that the Western blot technique was relatively sensitive in detecting the thermal changes of LG in the milk including its aggregate forms (Chen et al., 2004).

In conclusion, the present study has observed a severe denaturation of LG, but not the other whey proteins, in local commercially processed and some dry milks. Western blot analysis shows that some of the loss of LG were either self-aggregated or conjugated with other milk proteins. Carboxymethylation on cysteine totally inhibited the LG cross-linking suggesting that cysteines are responsible for the loss of LG upon the heating. The detailed thermal denaturation curves of LG constructed from the circular dichroic spectra may provide a reference value for dairy industry in future preparing milk product. We postulate that heat treatment over 80 °C in 15 sec may induce a significant loss of milk LG.

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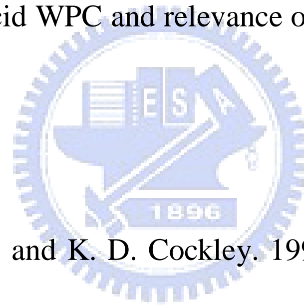

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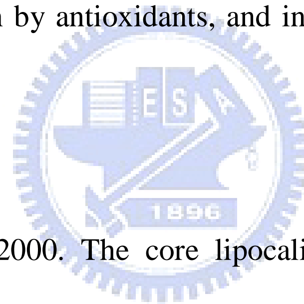

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CAPTIONS AND LEGENDS

Figure 1. Gel electrophoresis of whey proteins obtained from raw and processed milk. Left panel: Native-PAGE. Lane A: raw milk. Lane B: processed milk. Right panel: SDS-PAGE. Lane A: raw milk. Lane B: processed milk. Lane C: purified LG and LA standard. Ten μg of the protein sample were loaded onto each lane. There was a marked decrease in two acidic proteins in processed milk on the native-PAGE. The acidic proteins were eluted from a transfer blot followed by an amino acid sequencing and were subsequently identified as two isoforms of LG with first 20 residues as LIVTQTMKGLDIQKVAGTWY. The processed milk was purchased from the local market.

Figure 2. Native-PAGE analysis on whey proteins obtained from raw, processed, and dry milk. Lane 1: freshly prepared raw milk (from Taiwan); Lanes 2-6: processed milk (5 major brands from local market in Taiwan); Lanes 7-9: powdered milk (imported from Denmark, Australia, and New Zealand, respectively); Lanes 10-13: processed milk (4 brands from USA). Whey proteins loaded on lanes 1 and 2 were freshly prepared from the same batch, and were obtained from a university dairy farm before and after the process.

Figure 3. Western blot analysis on whey proteins obtained from raw, processed, and dry milk. All samples are loaded from the same batch as shown in Figure 2, except lane 10 which represents a typical processed milk from the US market. Notably, the decrease in LG was not as sharp as that characterized on Coomassie blue staining due to the extremely high sensitivity of the immunoblot.

Figure 4. Characterization of heated LG using a Westernblot analysis on native-PAGE (**A**) and SDS-PAGE (**B**). The experiment was carried out by heating isolated LG (1mg/mL) at 95 °C followed by blotting with a polyclonal antibody prepared against LG. Time course is expressed in seconds, while there were no aggregates of LG on the initial sample (time 0). No aggregate was observed forming 25 kDa polymer as that shown in Figure 1. (**C**) Chemical modification using carboxymethylation (CM) on all the cysteine residues abolished the formation of LG aggregates. The gel was stained by coomassie blue. The molecular weight of CM-LG was slightly higher than expected due to the lack of preheating in sample preparation.

Figure 5. Two dimensional gel analysis on raw and heated milk. (**A**) Coomassie blue. (**B**) Western blot using a LG polyclonal antibody. Sample containing 300 μg protein was first run in IEF (pH 3-10 from left to right) followed by a conventional SDS-PAGE.

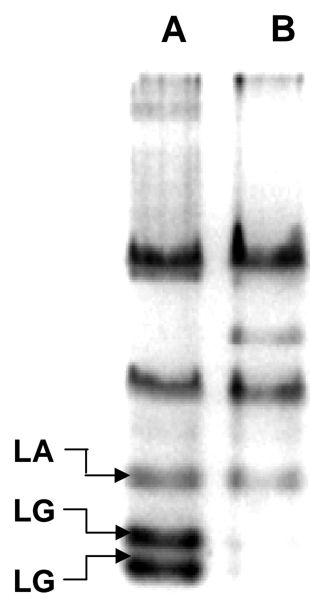
Figure 6. Native-PAGE analysis on isolated LG and LA (1mg/mL) heated at 95 °C over time. Lane 1: Native LG and LA without heat; Lanes 2 to 7: LG and LA heated over time from 30, 60, 120, 240, 480, and 960 seconds, respectively. Ten μg of each protein were loaded on the native gel.

Figure 7. Effect of temperature and time on the loss of LA (**A**) and LG (**B**). The loss of each protein upon the heating was extrapolated from the native-PAGE (Figure 6), while using the sample without heat as 0% loss. The experiment was conducted independently at various temperatures as indicated : 50 (●); 60 (○); 70 (■) ; 80 (□); 90 (▲); and 95 (△) °C.

Figure 8. Effect of temperature and time on circular dichroic spectra of LA and LG. (**A**) LA heated at 50 (—), 60 (-----), 70 (•• •• ••), 80 (— ·), 90 (— · –) and 95 °C (······) for 15 s to 15min; (**B**) LG heated at 50 (—), 60 (-----), 70 (•• •• ••), 80 (— ·), 90 (— · –) and 95 °C (······) for 15 s to 16 min. Notably, the spectrum of unheated was identical to that heated at 50 °C for 15 sec (data not shown). LA appears to be more resistant to heat than that of LG.

Figure 9. Thermal denaturation curves based on the maximal changes of the ellipticity of LA

(**A**) and LG (**B**) heated at 50 (●), 60 (○), 70 (■), 80 (□), 90 (▲) and 95 (△) °C. The data were extrapolated from Figure 8 (please see MATERIALS AND METHODS for details).



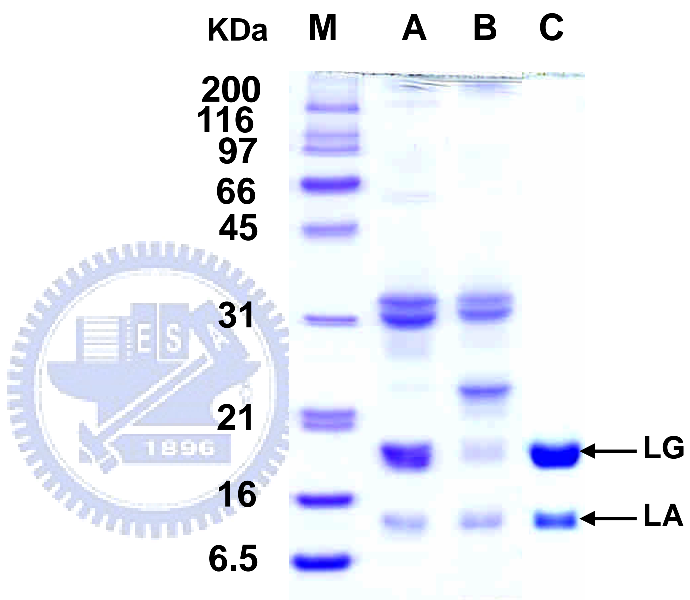


Figure 1

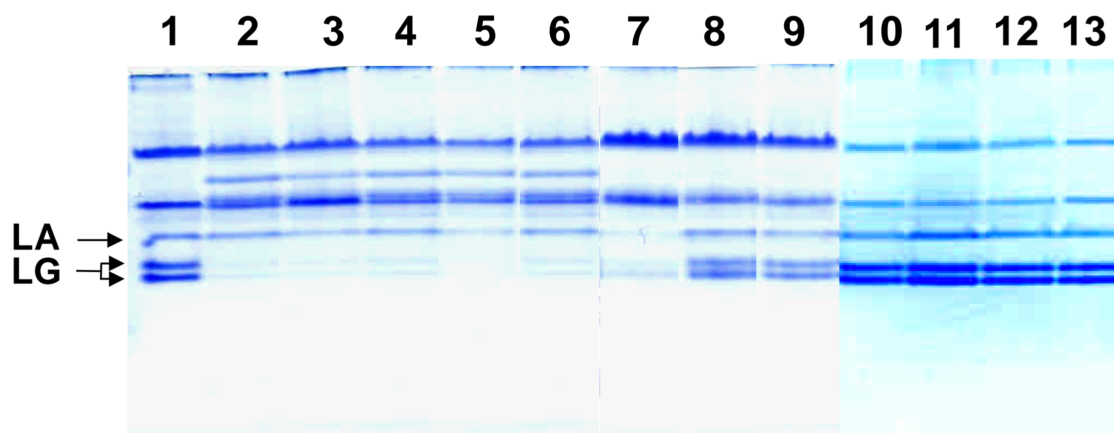
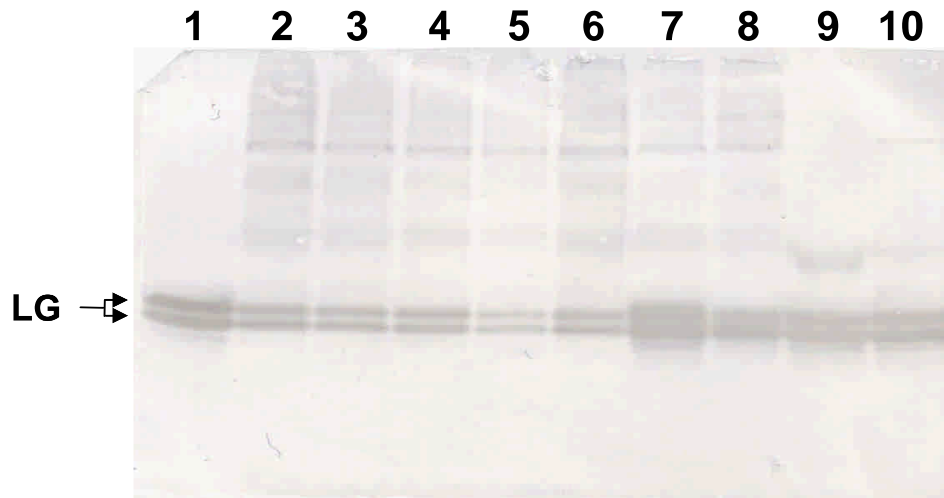


Figure 2

A. Native-PAGE



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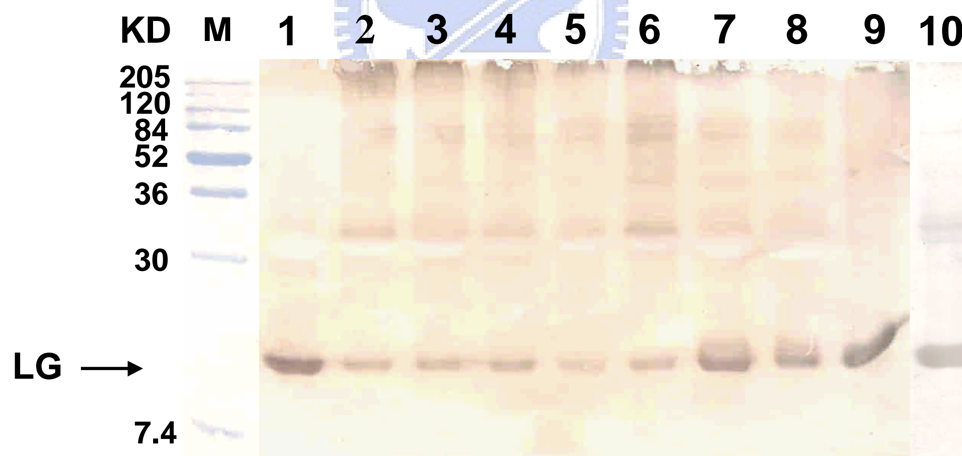
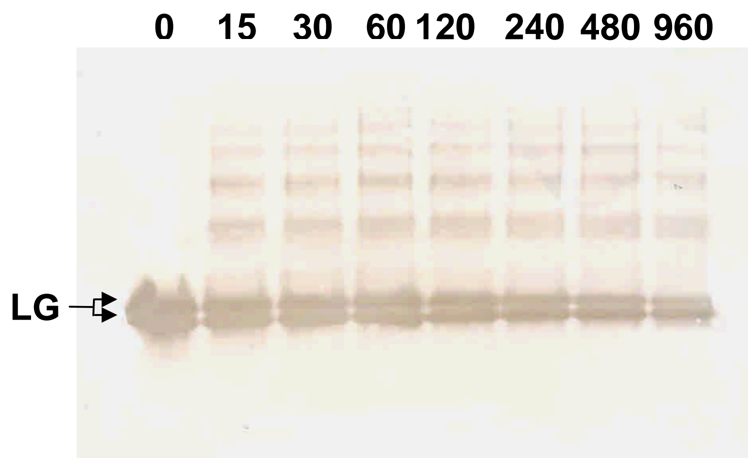
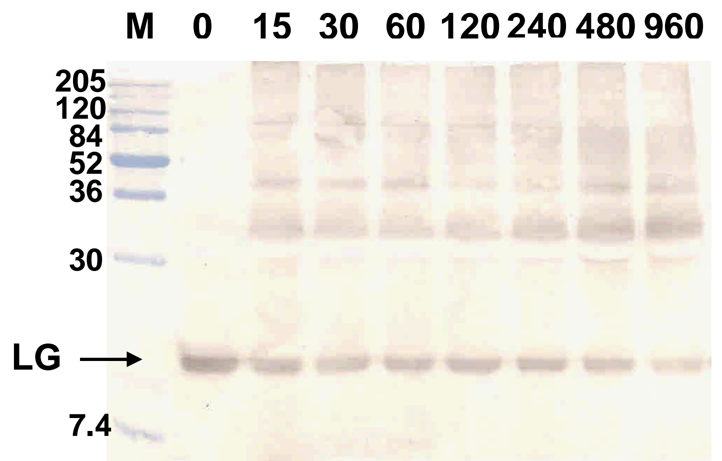


Figure 3

A. Native - PAGE



B. SDS - PAGE



C. SDS – PAGE

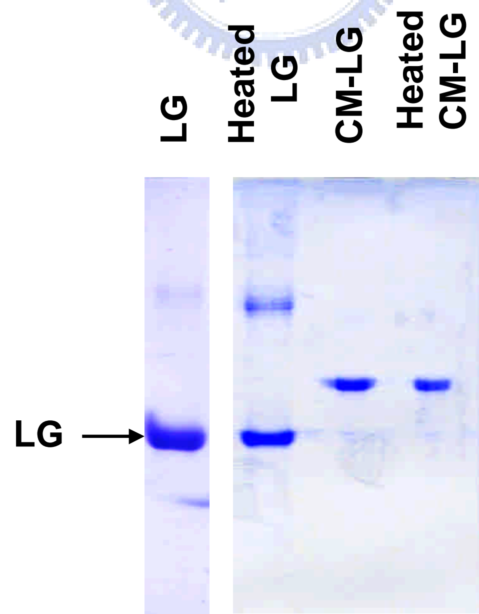
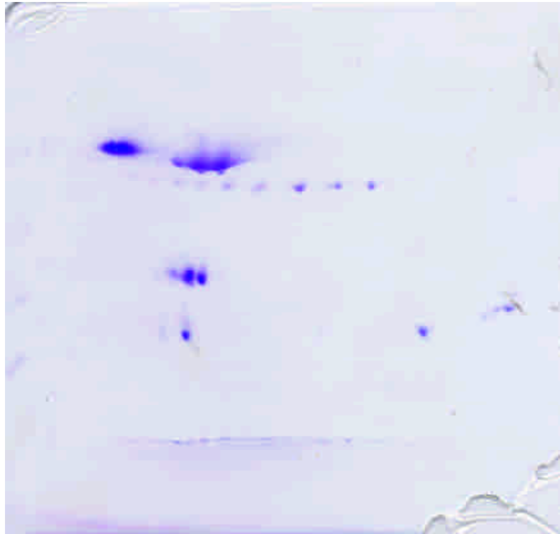


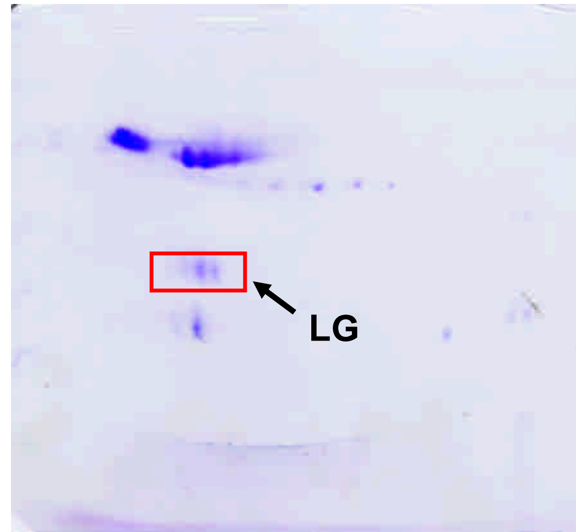
Figure 4

A. Coomassie blue staining

Raw milk

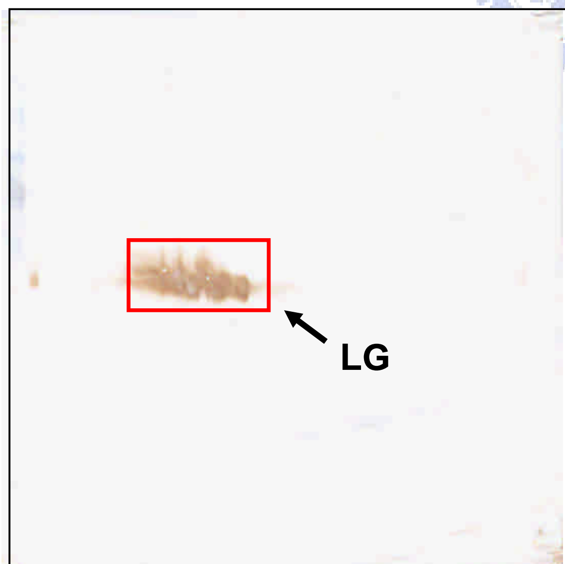


Heated raw milk



B. Western Blot

Raw milk



Heated raw milk

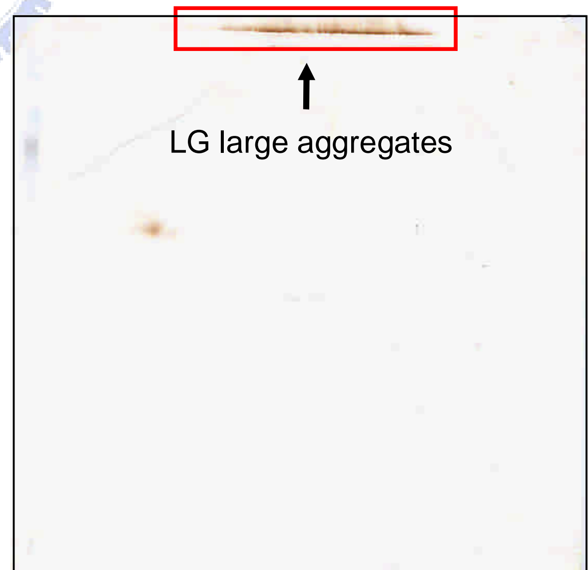


Figure 5

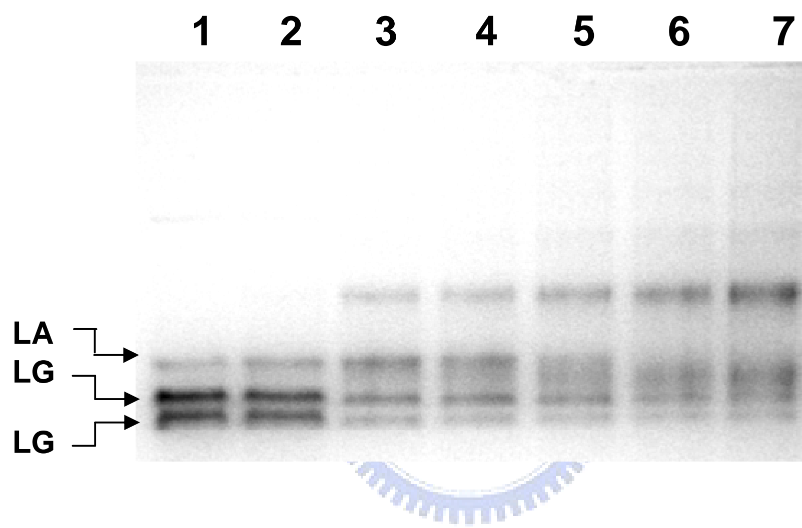
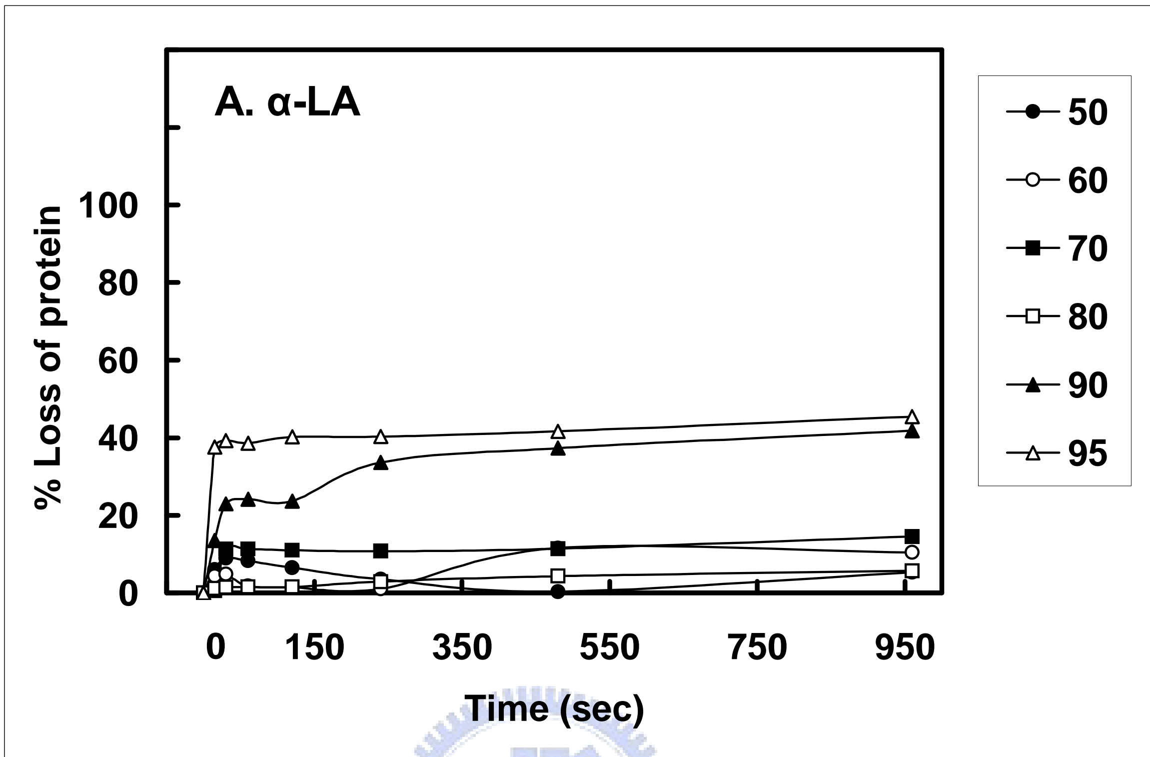


Figure 6



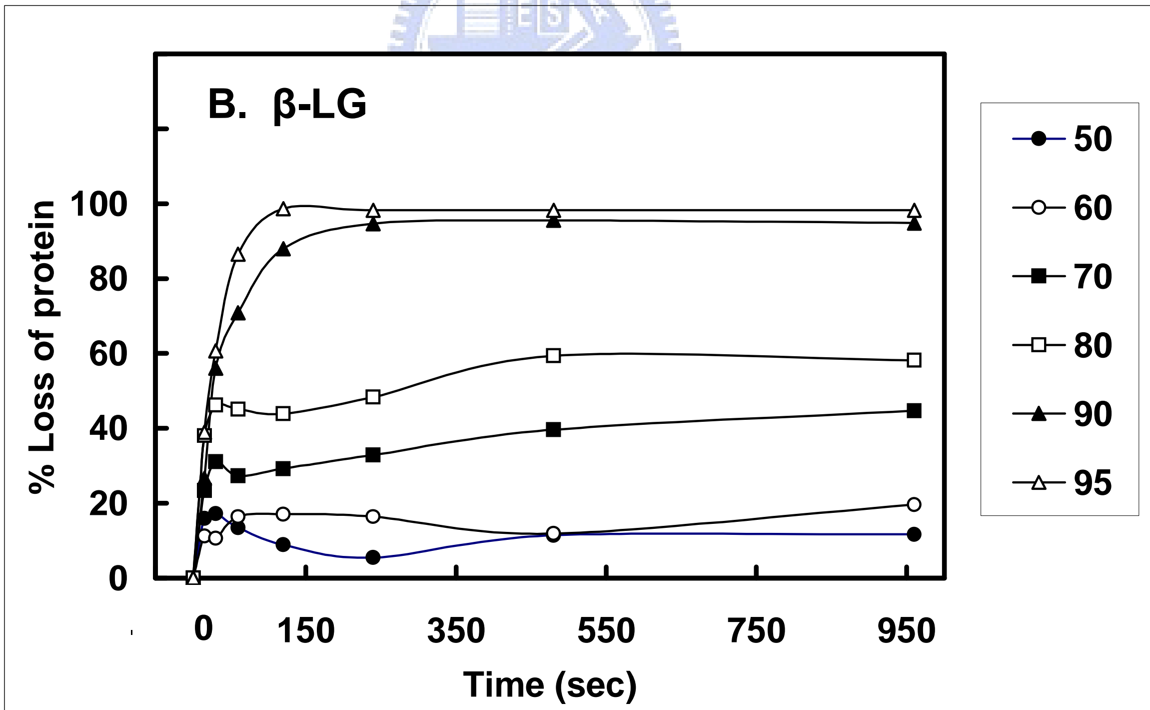


Figure 7

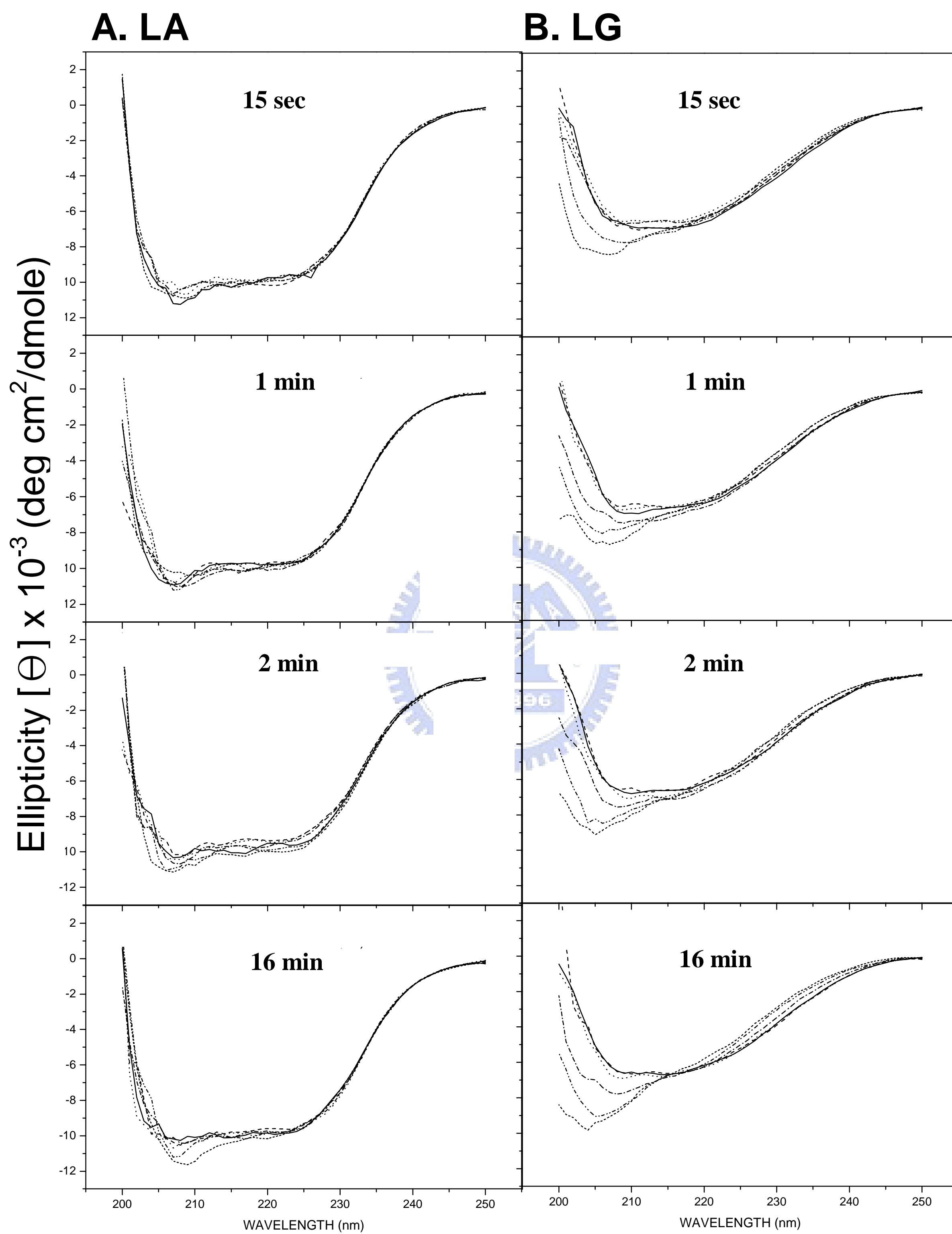
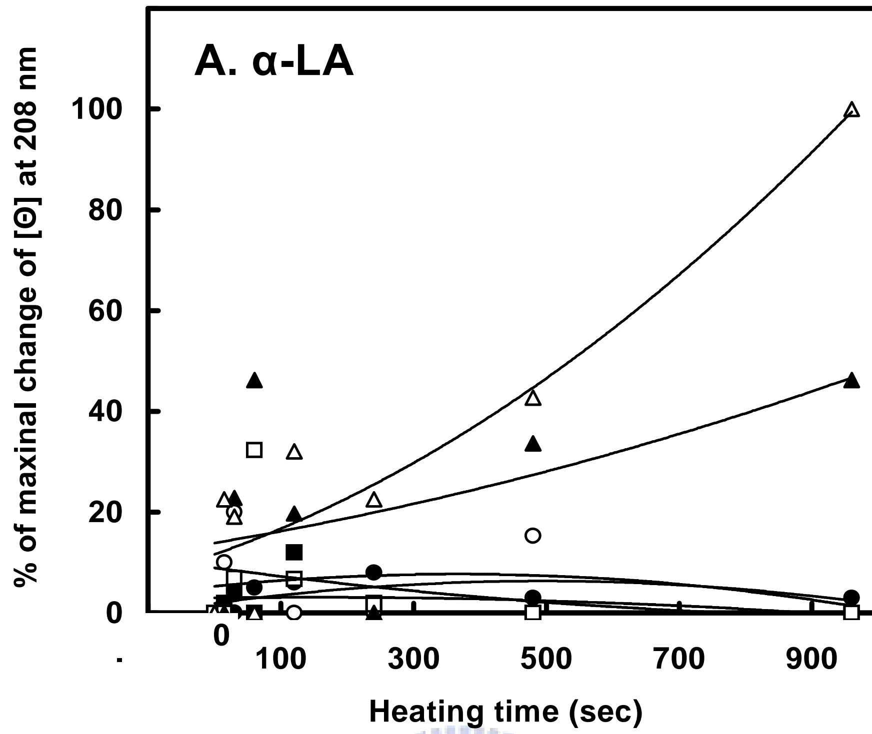


Figure 8



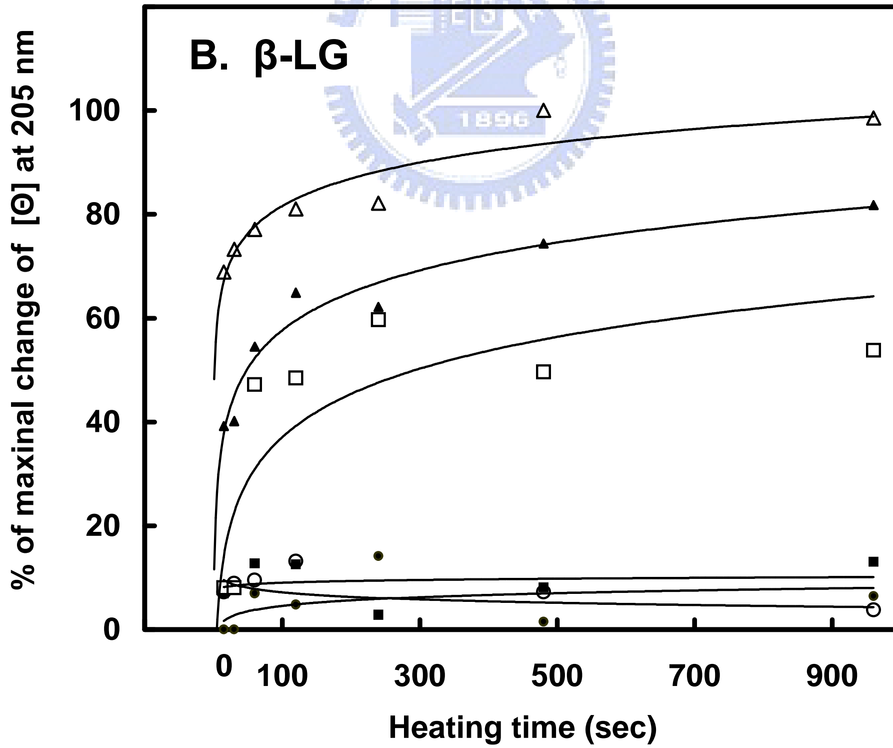


Figure 9

Distinction between dry and raw milk using monoclonal antibodies prepared against dry milk proteins

ABSTRACT

It is well established that the heating process during the preparation of dry milk causes structural changes in some milk proteins. Since such changes are subtle, whether or not they can be detected by an immunochemical approach remaining to be of challenge. The present study attempted to develop a sensitive monoclonal antibody (**mAb**) that might distinguish the dry milk from freshly prepared raw milk. To test this possibility, we immunized the mice with commercially prepared dry milk and produced a panel of mAb. From 900 hybridomas screened using an ELISA, 4 clones were found to be specific to dry milk, while the other 68 clones recognized both dry and raw milk. In contrast to polyclonal antibodies, only the specific mAb could detect the dry milk spiked into the raw milk as low as 5% in concentration (vol/vol). Western blot analysis shows that these specific mAb were all directed against β-lactoglobulin (**LG**) and LG-milk protein conjugates. These mAb reacted with raw milk heated at 95℃ for 15 min, the reaction with LG-conjugates, however, was abolished when treated with reducing reagent. Thus, it suggests that a new antigenic epitope was exposed in a heating process and the thio group of LG crossly linked with other protein moiety played a provocative role in mAb recognition. A hypothetical model with respect to the interaction between the mAb and dry milk is proposed and discussed.

(**Key words:** monoclonal antibody, dry milk, β-lactoglobulin, thermal denaturation)

Abbreviation key: LG = β-lactoglobulin, **mAb** = monoclonal antibody, **ELISA** = enzyme linked immunosorbent assay, **PAGE** = polyacrylamide gel electrophoresis, **SDS-PAGE** =

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, **PBS** = phosphate buffered saline, **DM** = dry milk.

INTRODUCTION

Dairy industries are interested to know an appropriate heat treatment in milk for controlling the quality of drinking milk or to control their heating system. On the contrary, the consumers are concerned whether or not the dry milk (powdered milk) has been supplemented to pasteurized raw milk. It happens when particularly the supply of raw milk is not sufficient in the summer where the demanding of consumption increases and the production of cow milk decreases. Since ultra heat treatment (UHT) procedure has been widely used in preparing milk powder, effort using heat-denatured milk proteins as a bioindicator has been probed to estimate such false practice (Relkin, 1996; Sanchez et al., 2002; Steffensen et al., 2002). For examples, Recio and Olieman in 1996 show that the amount of heat-denatured proteins can be estimated by analyzing the casein fraction using a capillary zone electrophoresis. A fluorescent probe using intrinsic basis states analysis has been employed for quantitative estimation of the stability of proteins in aqueous solution as a function of temperature (Tsonev and Hirsh, 2000). Monoclonal antibody prepared against β-lactoglobulin (**LG**) has been utilized for studying the biological properties of LG, such as its interaction with ligands and hypersensitivity reactions (Venien et al., 1997; Clement et al., 2002; Selo et al., 2002; Kobayashi et al., 2001; Restani et al., 1999; Morgan et al., 1999). Nevertheless, there are no immunochemical methods presently employed for the detection of dry milk mixed in raw milk.

The purpose of the present report was to use dry milk as an antigen(s) to randomly produce a panel of mAb and then select the monoclonals (if any) that were able to discriminate between the

dry and raw milk. Using an enzyme linked immunosorbent assay (**ELISA**), we established 4 monoclonals possessed such a unique property. Further characterization revealed that these mAb were directed toward LG epitope on a Western blot analysis. Meanwhile, we demonstrated that the specificity achieved in these dry milk antibodies was due to, in part, the cross-linking of LG with the other milk proteins. A hypothetical model explaining their specificity is described in details.

MATERIALS AND METHODS

Preparation of Milk Samples

Bulked whole raw milk obtained from a university dairy farm (Tunghi University, Taichung, Taiwan) and dry milk (Nestle Australia Ltd, Sidney, Australia) without further heat or other manipulation (unless specifically mentioned) were used for polyacrylamide gel electrophoresis (**PAGE**), Western blot, and ELISA analyses.

Animal Care and Use

The monoclonal antibody productions were utilized Balb/c mice with 5-7 weeks of age from National Science Council (NSC) of Taiwan. The mice were fed in animal room from Chiao Tung University during the period of immunization. Feed and water were available daily. CO_2. was used as a method of sacrificing and the other management was conducted according to guidelines established by NSC of Taiwan.

Immunization of Mice

Female Balb/c mice, aged 5-7 weeks, were used for immunization according to the method previously described by us (Yang and Mao, 1999). In brief, dry milk protein in sterilized phosphate buffered saline (**PBS**), containing 0.12 *M* NaCl, 0.02 *M* phosphate, pH 7.4, was mixed

and homogenized with an equal volume of incomplete Freund's adjuvant by a three-way stopcock. Each mouse was initially given a total emulsion of 0.5 ml containing 200 μg of protein with 6 subcutaneous injections onto the back and an intraperitoneal injection. At day 7, an identical dose with incomplete adjuvant was given intraperitoneally followed by two intramuscular injections without adjuvant at day 14. Seven days following a final booster, blood was collected in 0.1% (wt/vol) EDTA and plasma was obtained. This plasma was used as a source for conventional polyclonal antibody against dry milk. The titers of this antibody was over 1:8,000 as judged by an ELISA previously established in our laboratory (Huang et al., 1999). The spleen obtained was used for preparing hybridoma fusion.

Production of Monoclonal Antibody

Monoclonal antibodies were produced according to the standard procedures previously described by us (Mao et al., 1990; Mao et al., 1988). In brief, myeloma cell line (FO) was fused with spleen cells from immunized Balb/c mice at a ratio of 1:5. Fusion was carried out within 2 min at 37 ℃ using 1 ml of 50% (wt/vol) polyethylene glycol containing 10% (vol/vol) DMSO (Hybri-Max; Sigma). Cell mixture was then washed and resuspended in HAT medium (Hybri-Max; Sigma) containing approximately 1 x 10^5 FO cells per ml. The suspended cells were distributed as 100 μl per well in 96-well microtiter plates and incubated at 37℃ in a 5% CO_2-incubator followed by an addition of 100 μl of fresh HAT medium after 7 days. Subsequently, culture medium was assayed for the production of specific antibodies, between 14 and 21 days following the fusion, using a solid-phase ELISA described below. After primary screening, desired hybridomas were selected, expanded, and subcloned. Each monoclonal was established by limiting dilutions at least 2 x (Mao et al., 1990; Mao et al., 1988).

Enzyme Linked Immunosorbent Assay

Initially, approximate 1 μg of dry or raw milk protein in 50 μl of PBS was coated on each well of an ELISA plate (Nunc, Roskilde, Denmark) for screening hybridoma antibodies. Unbound proteins were washed with PBS 3 x and subsequently blocked by an addition of 350 μl of 1% (wt/vol) gelatin for 30 min (Mao et al., 1988). Following washes with PBS, 50 μl of hybridoma culture medium (2-3 weeks following the fusion) were added and incubated at room temperature for 60-90 min. Each well was washed 3 x with PBS containing 0.1% gelatin and 0.05% Tween-20. Bound antibodies were detected using a goat anti-mouse IgG conjugated with horseradish peroxidase for 30 min in PBS containing 0.1% gelatin and 0.05% Tween-20. Finally, each well was washed and developed with 0.04% (wt/vol) 2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) containing 0.01% (vol/vol) H_2O_2 in PBS.

Gel Electrophoresis

Sodium dodecyl sulfate-PAGE (**SDS-PAGE**) or native-PAGE containing 15% (wt/vol) polyacrylamide (unless specified) was used for the characterization of the milk proteins using a modified procedure (Yang and Mao, 1999) similar to that described (Oldfield et al.,1998). Electrophoresis was conducted in a vertical slab gel unit (Mini PIII, Bio-Rad) equipped with a PAC 300 power supply (Bio-Rad). All the samples (5-20 μg) for SDS-PAGE were equilibrated in 10 mM Tris-HCl and 5% SDS, pH 7.6, before loading to the gel. It is worthy to mention that pre-heat treatment used in the conventional SDS-PAGE for the tested samples was omitted to ensure the native structure of unheated milk proteins. The same procedures were conducted for native-PAGE without the addition of SDS.

Western Blot Analysis

Following the SDS-PAGE or native-PAGE, the gel was soaked briefly and instantly in a

transfer buffer containing 25mM Tris, 192mM glycine, 20% methanol, 0.0375% SDS (pH8.3) for 30 s. The gel was then immediately electrotransferred to a nitrocellulose membrane (Hybond-ECL extra; Amersham, Buckingham, UK) at 90 mA for 45 min in a semi-dry transfer cell (Bio-Rad). The membrane was immersed in 1% gelatin for 1 h with gentle shaking. Following 3 x washes with PBS for 5 min, the membrane was then treated with mAb or polyclonal antibodies and developed with 3-3'-diaminobenzidine (3,3',4,4'-tetra-amino-biphenyl) according to the method previously described (Yang and Mao, 1999).

Trypsin Treatment on LG and Its Immunoreactivity

For trypsin treatment, 0.6 µl of trypsin (2 mg/ml) were added to 30 µg of LG in 100 µl of PBS and incubated at 37℃ for 40 min. The reaction was then stopped by adding SDS in a final 0.5% concentration and immediately applied onto SDS-PAGE containing 20% polyacrylamide gel followed by a Western blot analysis.

Isotyping of Monoclonal Antibodies

Isotyping of each mAb was conducted according to the instruction provided by manufacturer (Sigma, St. Louis, MO). Briefly, 1 µg LG or powder milk in 50 ul of PBS was coated on an ELISA plate using the method previously established in our laboratory (Yang and Mao, 1999; Mao et al., 1982; Mao and Kottke, 1980). Following incubation with tested mAb, each monoclonal was subtyped by adding specific goat antibodies prepared against mouse IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA, respectively. Finally, 50 µl of HRP-labeled rabbit anti-goat Ig was used to complete the reaction as mentioned above.

RESULTS AND DISCUSSION

It has been well established that some milk proteins are denatured during the process of dry milk (mostly heating is involved) (Oldfield et al., 1998). Identifying of such denatured proteins would be a subject of essential in differentiating dry and freshly prepared raw milks. Since the structural changes are subtle, some complicated physical (Anema and Li, 2003; Ofeil, 1998; Dutta et al., 1991; Havea and Singh, 2001) and biochemical (Jimenez-Guzman et al., 2002; Valkonen et al., 2001; Bertrand-Harb et al., 2002; Chang and Li, 2001; Turner et al., 2002) methods have been employed to monitor such changes. Previously, we have shown that mAb are extremely sensitive for probing the structural changes of human low-density-lipoproteins (Mao et al., 1982) in discriminating the patients with and without coronary artery disease (Mao et al., 1983; Patton et al., 1983; Marcovina et al., 1985; Marcovina et al., 1985). Monoclonal antibodies prepared against human hepatic lipase can even distinguish between active and inactive forms of lipase (Mao et al., 1988). Thus, we anticipated that the mAb might allow us to detect the thermal denaturation of proteins as that occurred in heat processed milk.

Primary Screening

As shown in Table 1, from 900 hybridomas in a primary screening, there were 68 hybridomas reacted equally with dry and raw milk. Remarkably, 8 hybridomas were able to distinguish the dry milk apart from the raw milk. A typical example of the hybridomas displaying the dry milk specificity on ELISA is shown in Figure 1. In general, immunoreactivity of clones specific to dry milk was at least 8-10 times greater than that to raw milk. Finally, 4 monoclonals that distinctly recognized the dry milk (designated as DM-1, -2, -3, and -4; or 1B5F2, 1C10F10, 1D8F8, and 2F2D9, respectively) were established and used in this study (Table 1).

Dose-Responsive Binding Curve Of mAb to Dry Milk

A representative dose-responsive curve for the immunoreactivity of each mAb specific to dry milk (n=4) is shown in Figure 2A. At high dose of mAb, a slight cross-reactivity with raw milk was noticed. We also tested other mAb (n=20) that recognized both raw and dry milk, a typical example using mAb 2B4B4 is shown in Figure 2B. Thus, our results indicated that mAb approach was novel in detecting the subtle structural changes in dry milk.

Effect of Heat on the Immunoreactivity of Raw Milk

Because heating is one of the major processes in preparation of dry milk, we hypothesized that thermal denaturation might have exposed new antigenic epitopes from the raw milk. To test this hypothesis, in the next experiment we heated the raw milk and determined whether or not there were newly formed epitopes that could be probed by our specific mAb (DM-1 to DM-4). Figure 3 reveals that these "new epitopes" were exposed upon the heating on raw milk, in which an increase in immunoreactivity was seen. The data suggest that these mAb were directed toward the heat-sensitive milk proteins. It also demonstrates that the mAb could detect the dry milk as low as 5% (vol/vol) spiked into the raw milk (Figure 4).

Characterization of monoclonals specific to dry milk

To characterize the denatured or thermal sensitive antigen(s) that recognized by these 4 monoclonals, Western blot using dry milk sample in non-heated SDS- and native-PAGE was conducted. We identified that all of these 4 mAb (DM-1 to DM-4) recognized LG (Figures 5 A-C). Meanwhile, they also reacted with high molecular forms of proteins in dry milk (Lanes C-E). Whereas, the immunoreactivity in high molecular forms were not found in raw milk (Lane B). We later demonstrated that they were LG crossly linked with dry milk proteins (described below).

Thus, the data would indicate that LG is a sensitive thermal-denatured component in dry milk as judged by our mAb approach, which was initially not designed for the preparation of mAb against LG.

On the other hand, none of the tested mAb (n=20) that lacking the specificity to dry milk could recognize LG; a typical example with such mAb 2B4B4 is shown in Figure 5D. Most of milk mAb reacted with casein proteins as illustrated in Western blots (Figure 5D) and they did not react with LG as judged by ELISA (data not shown). Although these mAb (n=20) had not been characterized fully thus far, they did not apparently recognize milk lactalbumin, albumin, and immunoglobulin. This was because any monoclonal antibodies initially produced against these proteins would be automatically neutralized by fetal bovine serum (FBS) containing albumin, and immunoglobulin. The FBS was routinely used in the hybridoma cell culture.

Lack of dry milk specificity in polyclonal antibody prepared against LG

To address whether or not the polyclonal antibody prepared against dry milk or LG could also distinguish between the dry and raw milk. Using an ELISA, we show that neither dry milk (panel A) nor LG (panel B) polyclonal antibody was able to detect the difference (Figure 6). Obviously, the populations of polyclonal antibodies prepared against LG or dry milk recognize the multiple epitopes that are commonly shared in both dry and raw milks. The finding substantiates the hypothesis that mAb was specific to a unique LG epitope in dry milk.

Hypothetical model of immunochemical property of LG in dry milk

We demonstrated that all of these 4 randomly prepared mAb recognized LG and its denatured larger molecular form in dry milk (Figure 5). These mAb, however, also recognized LG in raw milk for some extent on Western blot (Figure 5). But on ELISA, these mAb were capable of differentiating dry and raw milk. To explain how these monoclonals effectively bound to LG in

dry milk, but not to that in raw milk; we show a hypothetical model (Figure 7). First, it is probably not so surprising that the ELISA approach was feasible, since the unique mAb were initially identified by the ELISA. One of the possible mechanisms by which the mAb differentially reacted with the dry milk was that the specific LG epitope was either being masked by the polystyrene surface on ELISA plate or interacting with the other milk proteins during the immobilization of milk antigen (Figure 7A). Therefore, the mAb could not bind that specific LG epitope in raw milk. While, in dry milk (Figure 7B) LG was crossly linked to the other milk proteins possibly via disulfide linkages (Figure 5). With such cross linking the LG epitope was emerged again at the surface, which was accessible for the binding of mAb (Figure 7B). To prove that LG was crossly linked with other milk proteins in dry milk via disulfides, we treated the dry milk with a reducing reagent (mercaptoethanol) to eliminate the disulfide linkages (if any). Under this condition, the mAb (DM 1-4) did not react with any cross-linking forms of LG as demonstrated on a Western blot (Figure 8). Furthermore, LG polyclonal antibodies, which did not recognize the unique LG epitope, would bind both raw (Figure 7C) and dry milk (Figure 7D) as mentioned above.

Taking together, we conclude that cross-linking of LG with other milk proteins may play an essential role in our ELISA system by providing the additional epitope for mAb binding. However, we still cannot rule out that the possible conformational change of this LG epitope upon the heating is responsible for the recognition by the mAb. As to the specific epitope that interacts with the plate interface or mAb, we have yet identified at the present time. However, our preliminary data show that this epitope of LG was somewhat sensitive to the trypsin cleavage. A typical example of the immunoreactivity affected by trypsin treatment on Western blot is depicted in Figure 9. Subtyping of IgG class revealed that all the mAb reported in this study

were IgG1 without exceptions (Table 1). Further antigenic mapping of LG is now in progress, which may provide the insight of the surface property of LG and its interaction with mAb.

With respect to the possible mechanism involved in the cross-linking, one can rationalize it as below. LG is a protein consists of 162 amino acids containing 5 cysteines at residues 66, 106, 119, 121, and 160. There are 2 proposed cross-linking disulfide bonds at position Cys 66-160 and Cys 106-119, respectively (Papiz et al., 1986; Cho et al., 1994; Cho et al., 2003). Activation of free Cys 121 by thermal treatment in milk has been thought to induce the disulfide bond formation between LG (dimerization) and K-casein (Kitabatake et al., 2001; Doi et al., 1985; Henry et al., 2002). Our Western blot (Figure 5) showing that LG monoclonals reacted with both LG and LG conjugates (large molecular forms), while mercaptoethanol treatment reversed the immunoreactivity of LG conjugates (Figure 8). The result supports the notion that LG crossly links with other proteins in dry milk (Figure 7). In the present study, at least three milk proteins were found to be involved in such linkages (Figure 5); although we have not identified these proteins yet. Nevertheless, the Western blot technique recognizing the species crossly linked by LG may help us to further delineate the cross-linking between LG and milk proteins.

Finally, as to the effect of heat on the other food proteins, Carbonaro et al., 1999 have shown that the proteins extracted from cooked common beans are more resistant to proteolysis (due to the formation of protein aggregation) than that of raw beans. The iron absorption from heme in beef exposed to prolonged heating was substantially reduced in humans (Martinez-Torres et al., 1986). In egg-allergic patients, heat treatment and disulfide blockage dramatically decrease the antigenicity of ovotransferrin and ovomucoid, but not ovalbumin (Mine, 2002). Because milk LG can be directly absorbed into the gastro-intenstinal tract of human infants (Kuitunen et al, 1994; Kuitunen et al, 1994; Sorva et al, 1994); whether or not the cross-linking formation of over

heated milk may reduce the absorption of amino acid supplement remaining a worthy subject of investigation.

ACKNOWLEDGEMENT

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CAPTIONS AND LEGENDS

Figure 1. ELISA selection of eight primary cultures that predominantly reacted with dry, but not raw milk. None of the culture media were diluted in the assay. Clones 1, 2, 3 and 5 (1B5F2, 2F2D9, 1C10F10, and 1D8F8, respectively), which possessed the high selectivity, were subjected to monocloning by limited dilutions.

Figure 2. Dose-responsive curves of four mAb (1B5F2, 2F2D9, 1C10F10, 1D8F8) specific to dry, but not raw milk (**A**). A typical example of mAb (2B4B4) that recognized both raw and dry milk is also shown (**B**). The initial dilution of each mAb was 1:100.

Figure 3. Effect of heat on the immunoreactivity of raw milk reacted with mAb (1B5F2, 2F2D9, 1C10F10, and 1D8F8) specific to dry milk. Clone 2B4B4 represents that recognized both raw and dry milk. Immunoreactivity was determined using an ELISA. Data suggest a formation of "new epitope" upon the heating on raw milk (95℃ for 15 min).

Figure 4. Immunoreactivity of raw milk when mixed with dry milk. Dry milk with various amounts was spiked into raw milk and assessed by an ELISA using mAb 1D8F8.

Figure 5. Characterization of mAb (1B5F2, 1D8F8, 2F2D9) specific to dry milk and a mAb (2B4B4) recognizing both dry and raw milk using a Western blot analysis. Each lane was loaded with 10 μg of milk protein. Lane A: native LG; lane B: raw milk; lane C: processed "fresh" milk (from Taiwan); lane D: dry milk (from Australia); lane E: heated raw milk at 95℃ for 15 min.

Figure 6. Dose-responsive curves for the binding of LG polyclonal antibody (**A**) and dry milk polyclonal antibody (**B**). ELISA plates were coated with raw milk (○) or dry milk (●).

Figure 7. A hypothetical model explaining the mechanism by which dry milk specific mAb recognizes the epitope of dry milk. (**A**) LG specific epitope is masked by the polystyrene surface on the ELISA plate. Therefore, mAb (DM 1-4) cannot bind to the specific LG epitope in raw milk. (**B**) LG conjugates with other milk protein moiety and exposes the epitope in heat processed dry milk and therefore facilitates mAb to bind LG specific epitope. (**C**) LG polyclonal antibodies recognize many other LG epitopes in raw milk. (**D**) LG polyclonal antibodies recognize many LG epitopes regardless the disulfide cross-linkings. Notably, we still cannot rule out that the possible conformational change of this LG epitope upon the heating is responsible for the recognition by the mAb

Figure 8. Western blot analysis on LG and dry milk treated with reducing reagent. About 10 μg of each respective protein were load on 15 % SDS-PAGE. A final concentration of 0.1% β-mercaptoethanol was used as a reducing reagent.

Figure 9. Effect of trypsin cleavage on the immunoreactivity of LG using dry milk specific mAb. A typical example is shown herein using clone 1D8F8. Left: Coommassie blue staining on 20% SDS-PAGE. Right: Western blot analysis.

Table 1. Designated monoclonal antibodies specific to dry milk and their characterizations

	Hybridoma	ELISA specific to		IgG subclass
		Raw and Dry milk	Dry milk	
Number of primary hybridoma	900	68	8	
Established and Expand monoclonals		20 selected	4	
mAb designated			DM 1（1B5F2） DM 2（1D8F8） DM 3（2F2D9） DM 4（2B3D11）	IgG1 IgG1 IgG1 IgG1
Reacted with LG on Western blot		none	all	

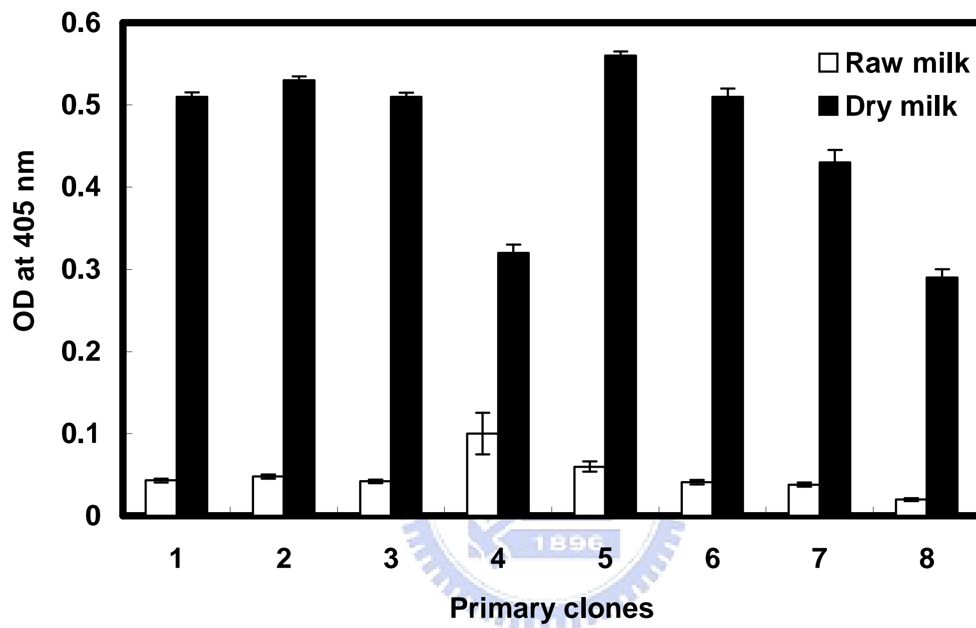


Figure 1

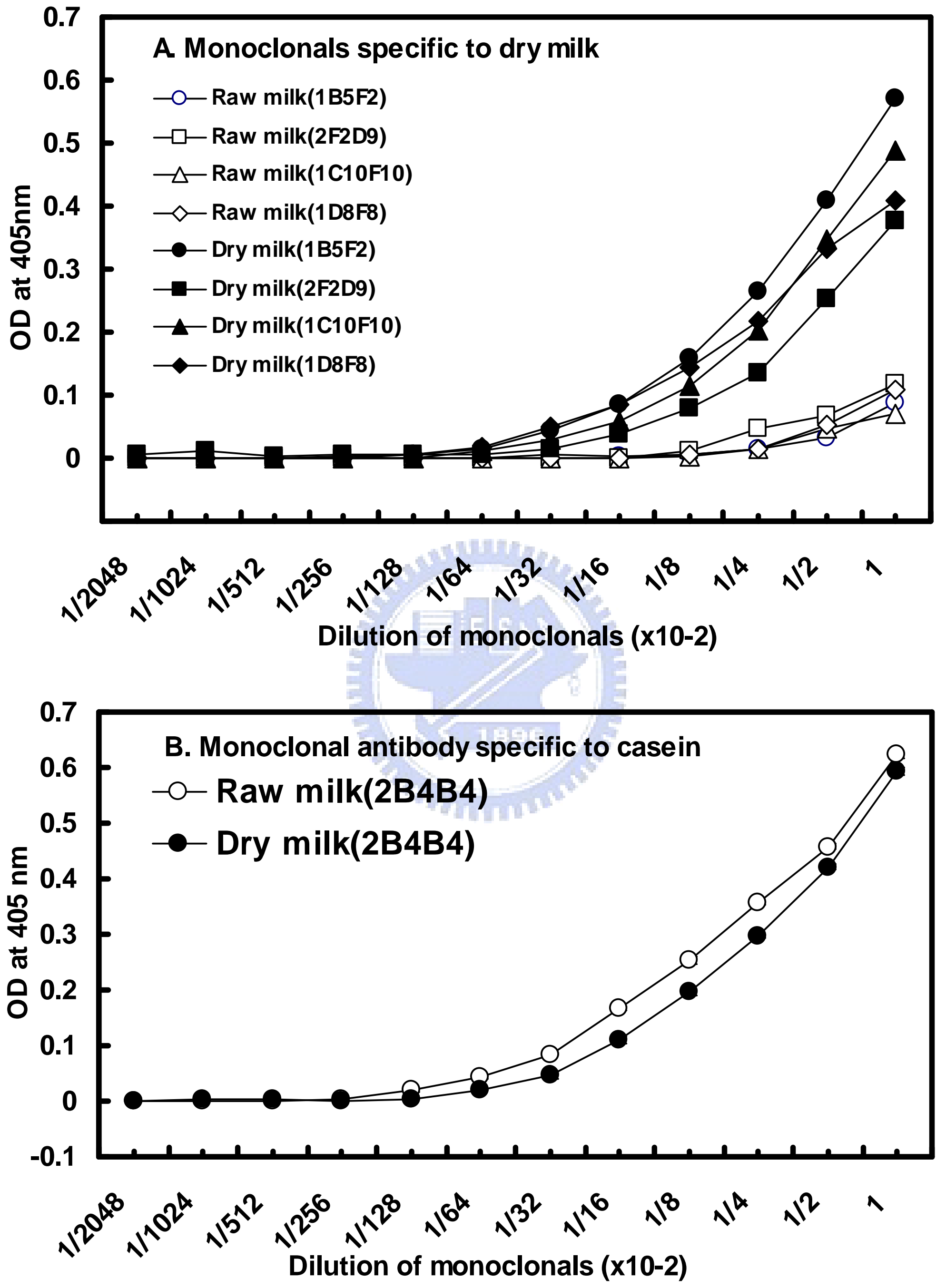


Figure 2

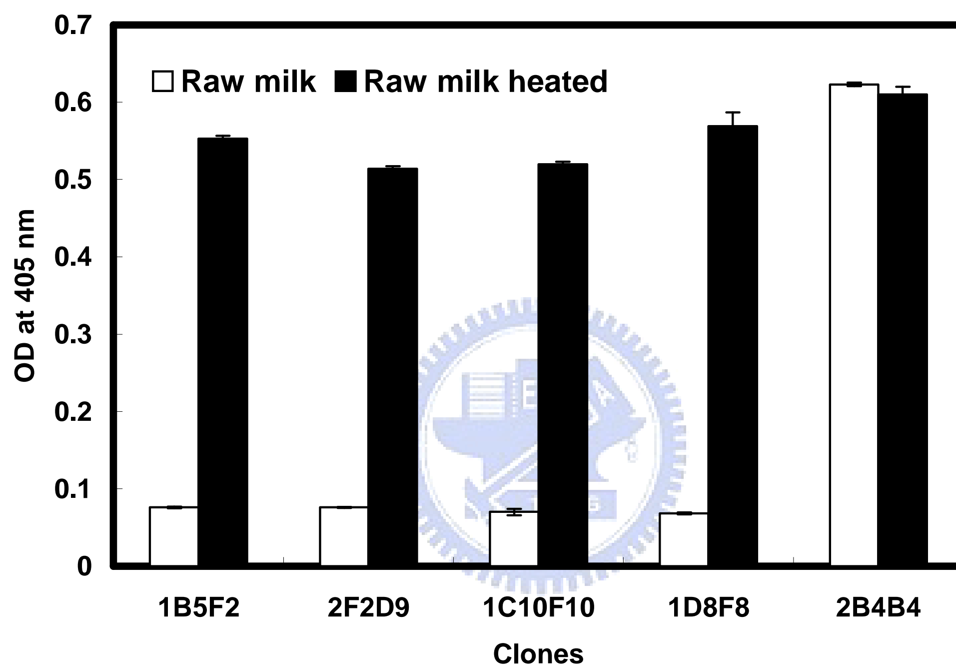
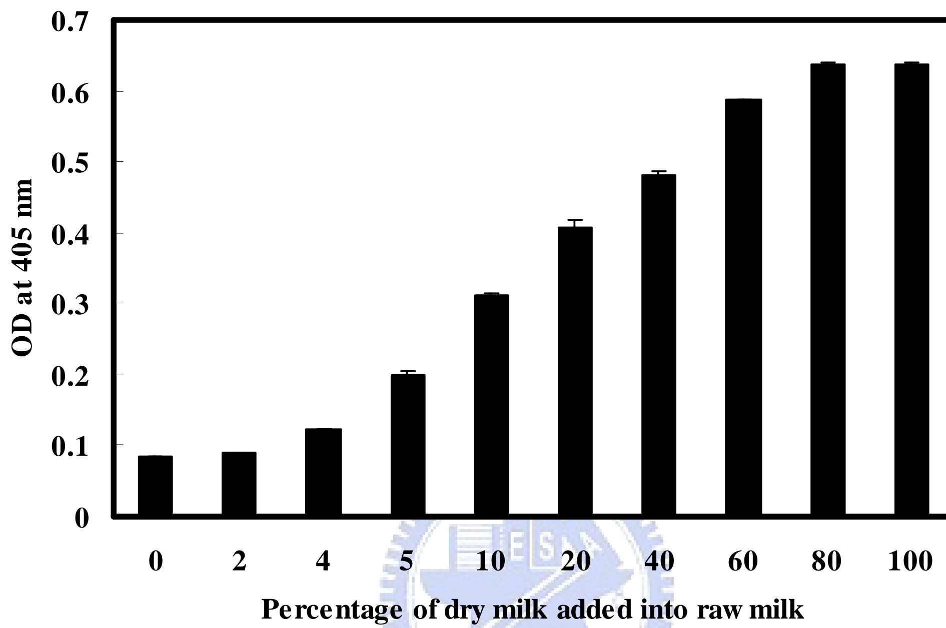


Figure 3



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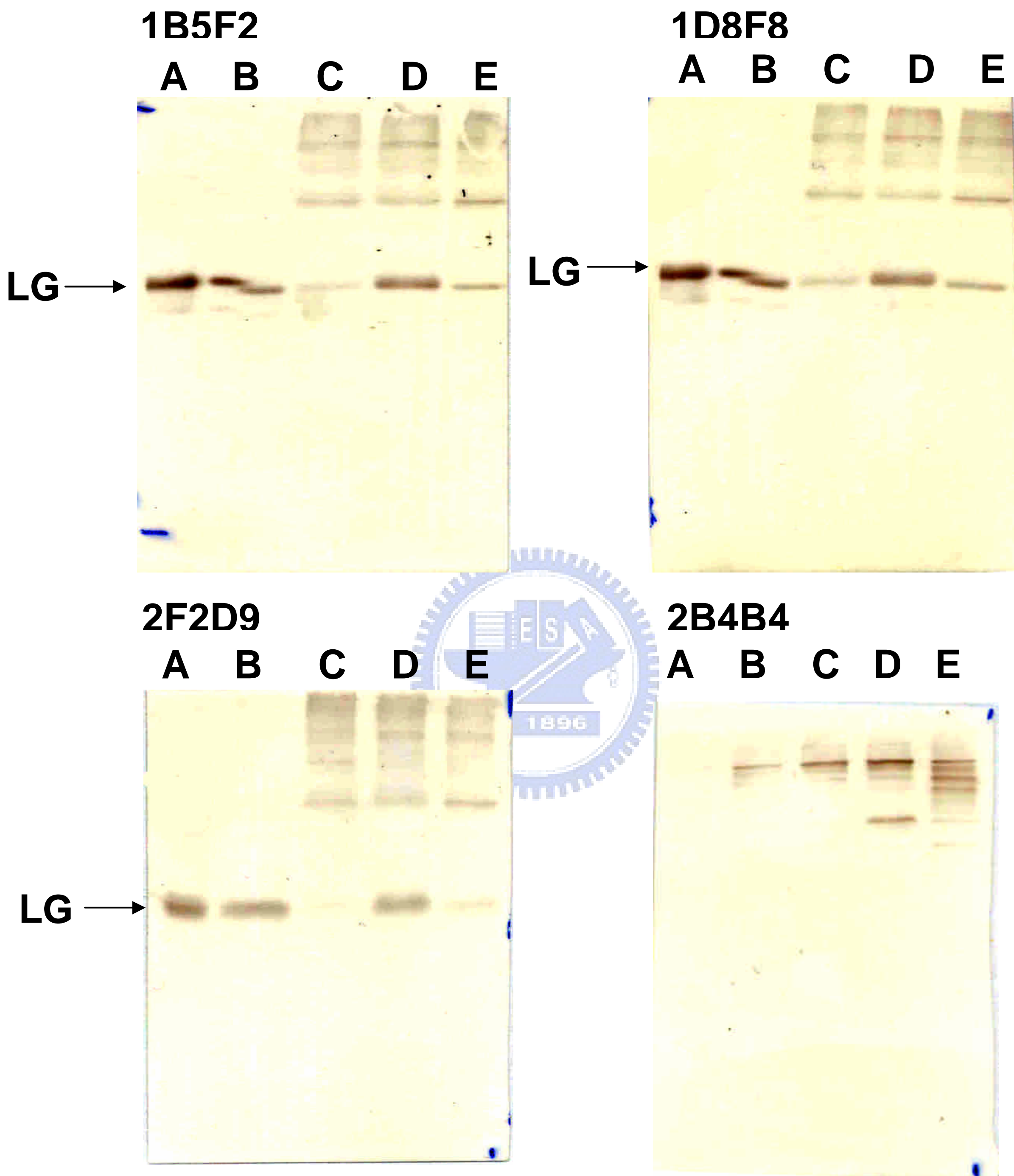
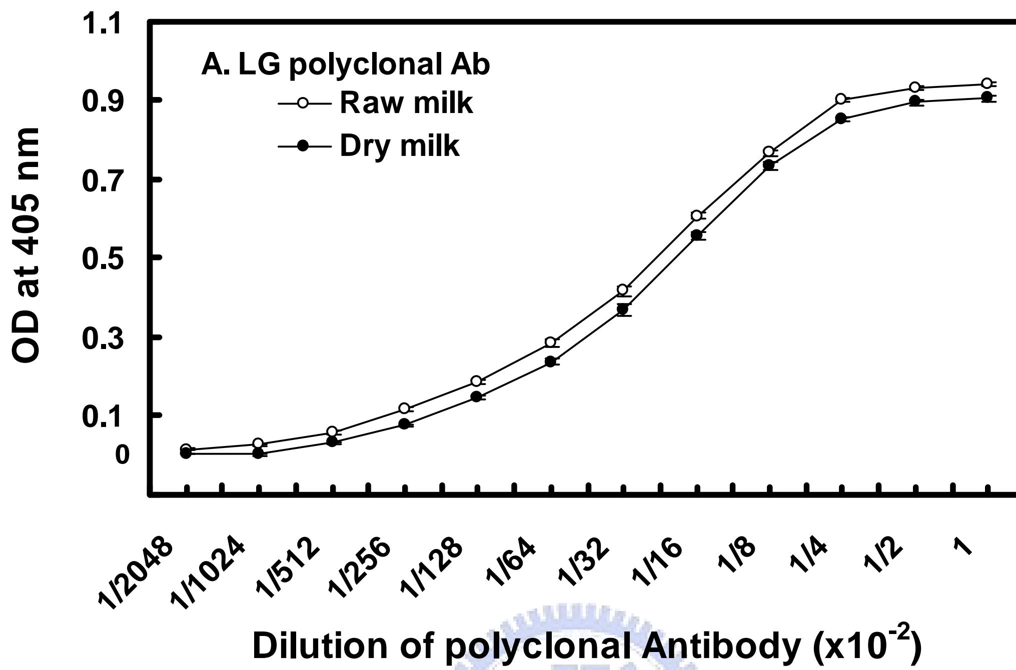


Figure 5



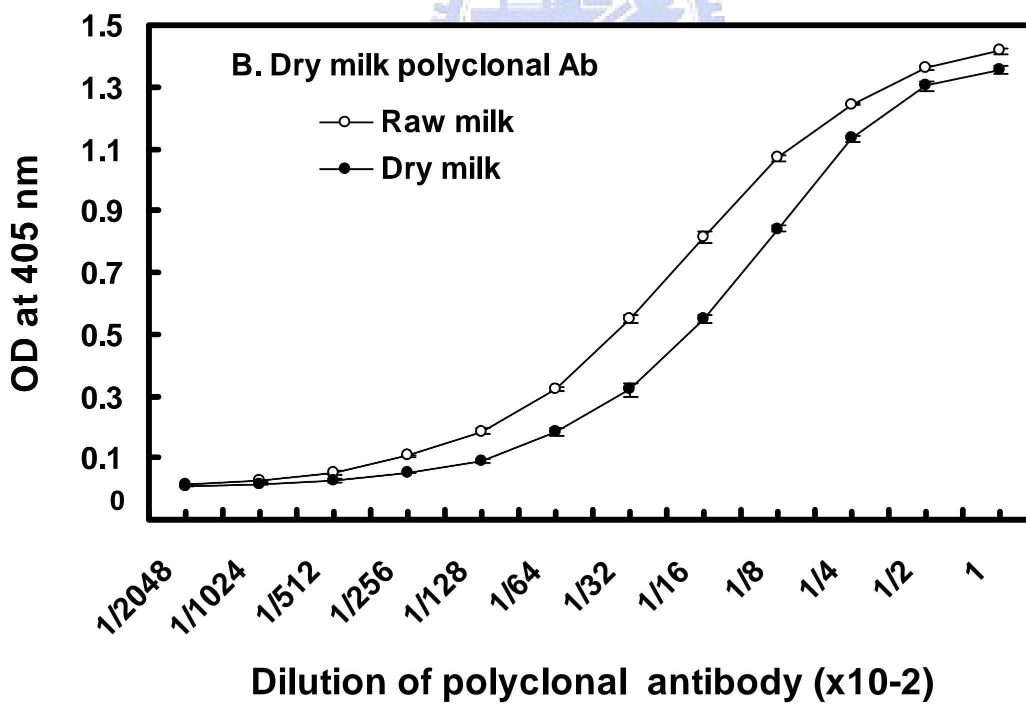


Figure 6

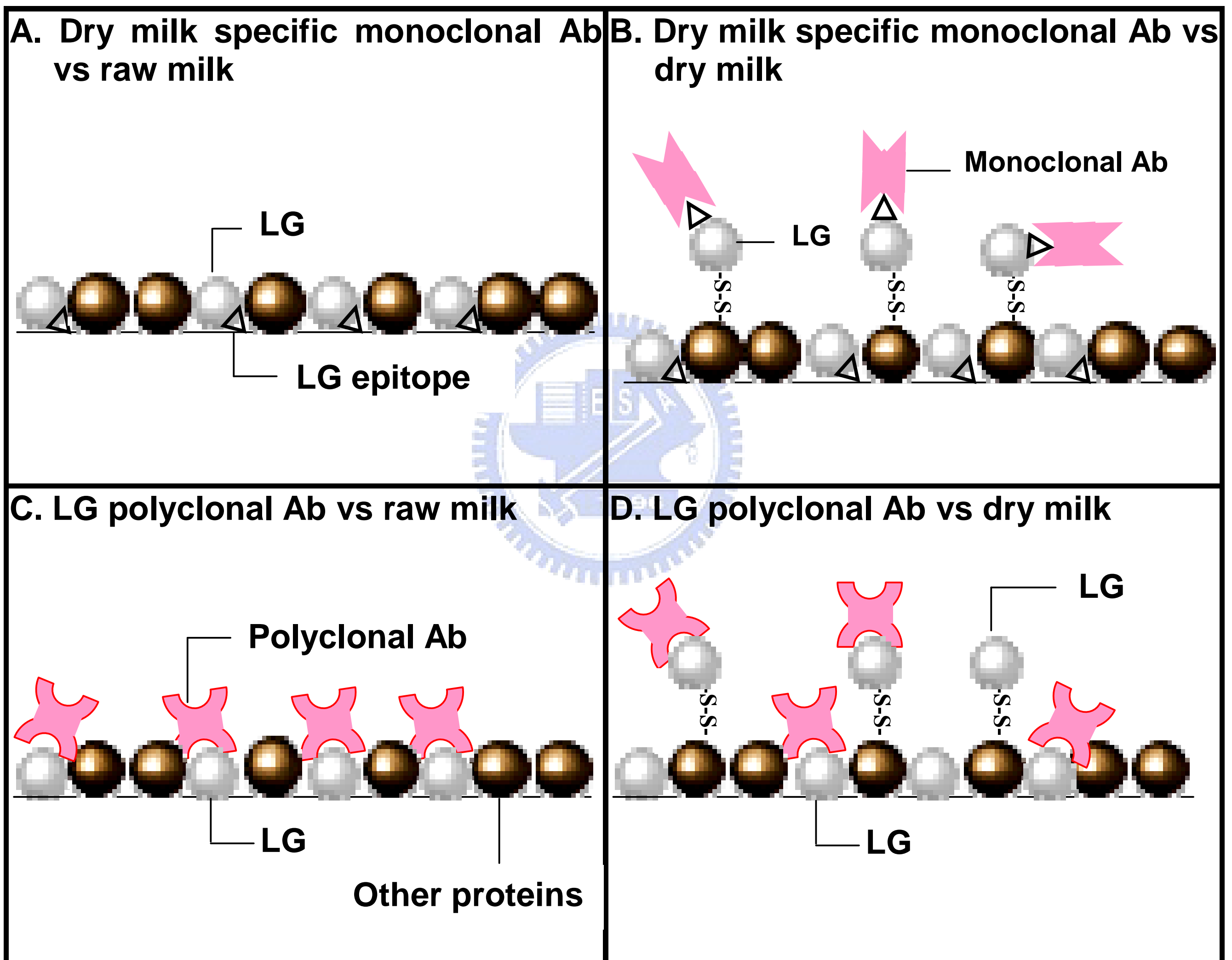


Figure 7

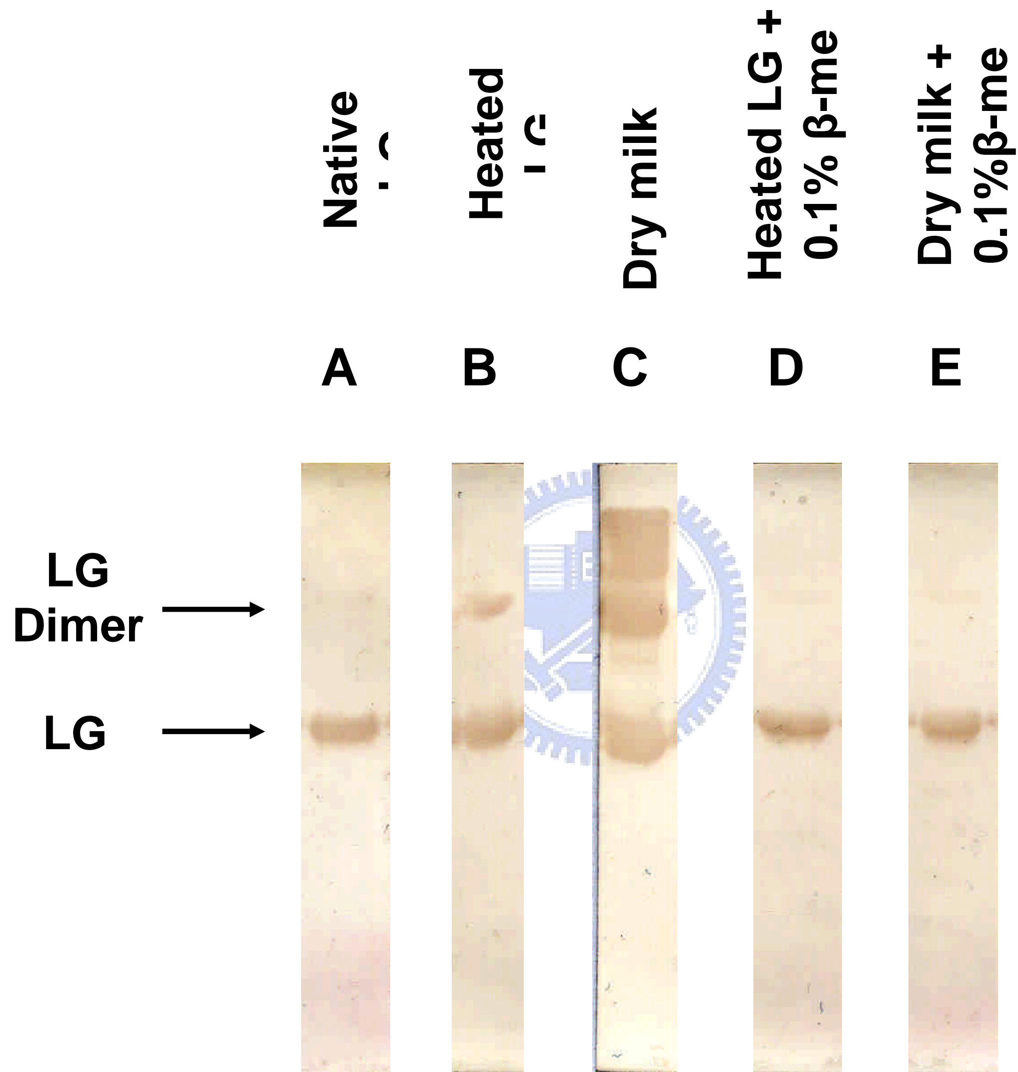


Figure 8

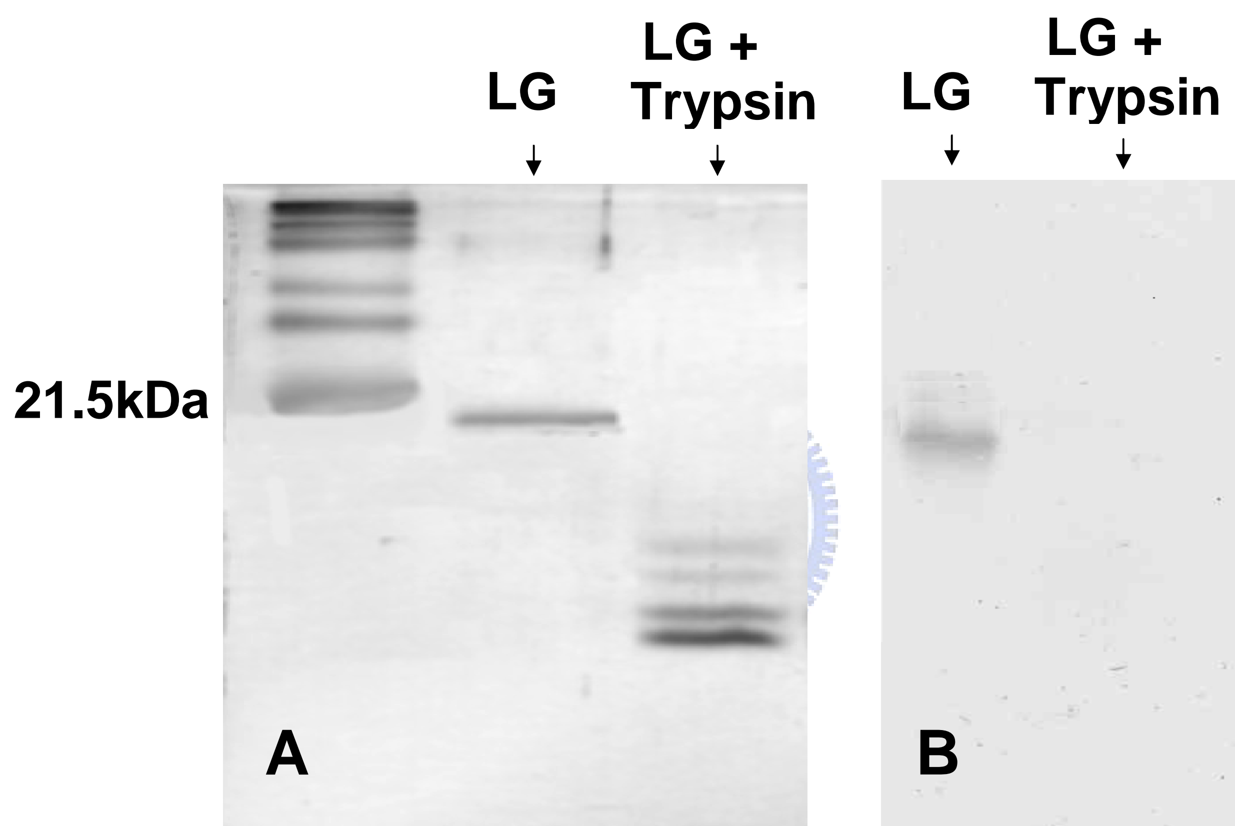
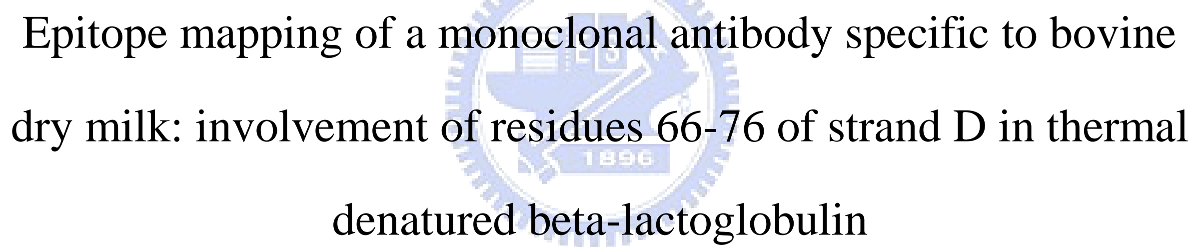


Figure 9



Epitope mapping of a monoclonal antibody specific to bovine dry milk: involvement of residues 66-76 of strand D in thermal denatured beta-lactoglobulin

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 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 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.

INTRODUCTION

Bovine β-lactoglobulin (β-LG)[1] is one of the major proteins in milk consisting of about 10-15% (Hambling et al., 1992). Due to the thermally unstable and molten-globule nature, β-LG has been studied extensively for its physical and biochemical properties (Oi et al., 1995; Sawyer and Kontopidis., 2000). 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 and 1999; Kuwata et al., 1999) (Fig. 1). Topographically, strands A-D form one surface of the barrel (calyx) while 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 (Uhrinova et al., 2000). A remarkable property of the calyx is its ability to bind in vitro hydrophobic molecules such as retinol, fatty acids, vitamin D, and cholesterol (Narayan et al., 1997; Qin et al., 1998; Wu et al., 1999; Kontopidis et al., 2002). 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). 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.

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

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).

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 and 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 and 1990).

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 and 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.

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.

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).

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 R., 2002). 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.

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.

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.

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).

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 (6), with the diagram created by PyMOL (DeLano WL., 2002).

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 and Milner-White., 1995).

RESULTS

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. 2). 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 and Swinkels., 1980). The finding suggests that the immunoreactive site recognized by this mAb lied 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 (Chen et al., 2004) suggesting that Lys, Arg, or both 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. 3A). 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 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. 3B). 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).

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. 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 ten 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). Among which peptide 4 gave a partial immunoreactivity suggesting that residues 67-70 were essentially involved in the reactive site (Fig. 4). Since the size of an epitope is relatively small usually containing 6-9 amino-acid residues (Mao et al., 1990 and 1989; Bhatnagar et al., 1983; Davies and Cohen., 1996; Atassi MZ., 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. 4). 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.

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. 5A). 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. 5B). 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. 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 7 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. 6).

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. 8) 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 (Casal et al., 1988), we tested whether changes of pH could also induce an increase in β-LG immunoreactivity. Fig. 9A 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. 9B). Since 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. 9B). A control experiment showing a typical pH dependent antigen-antibody reaction was performed (Fig. 9C), 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. 10A). Heating β-LG at temperatures greater than 80 °C almost completely abolished its binding for retinol (Fig. 10B). 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.

Interestingly, the binding to palmitic acid was decreased in some extent at pH 9-10 (Fig. 10C) correlated with the binding to mAb (Fig. 9B). Since Lys-69 played an extremely 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-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 (4), 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. 6) in which the immunoreactivity was concomitantly increased (Fig. 5). 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. 6) 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. 5B). 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. 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 (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. 1), which is agreeable with the general concept of a given antigenic epitope (Atassi MZ., 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. 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 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 MZ 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 1989 and 1990; Bhatnagar et al., 1983) and others (Davies and Cohen., 1996). 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. 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 70-80 °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. 8), 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 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 Cohen1991). 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., 1982 and 1983). 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. 10), while 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 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 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. 11 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. 10), while 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 maximal pH between 8 and 10 (Fig. 10A). 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. 10C). 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. 10C).

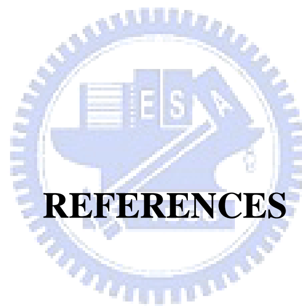
As mentioned above, the immunoreactivity assessed by this mAb was positively correlated to its molten globule state (between 70-80 °C in Fig. 3). This epitope in strand D was not only thermal sensitive but also negatively correlated to retinol and palmate binding. Lys-69 in native state participates 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 Tom., 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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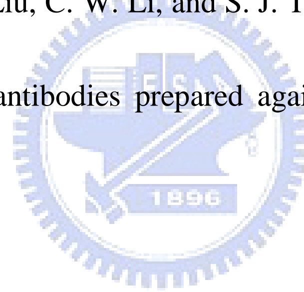

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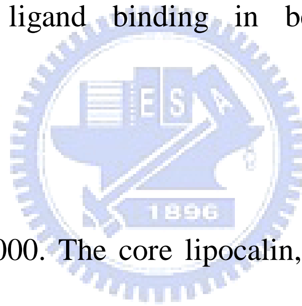

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FOOTNOTES

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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; CD, circular dichroism; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PB, phosphate buffer; PDB, Protein Data Bank.

CAPTIONS AND LEGENDS

FIG. 1. **Amino acid sequence and 3D structure of β-LG.** A: 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. B: 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.

FIG. 2. **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.

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 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. B: immunoreactivity of CNBr fragments 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.

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 using HRP-labeled secondary antibody with chemiluminescent agent as a developer. The shed region represents the proposed epitope.

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. 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. B: Competitive ELISA of native, heated, CM, and heated CM β-LG. The plate was immobilized with heated β-LG.

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 (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.

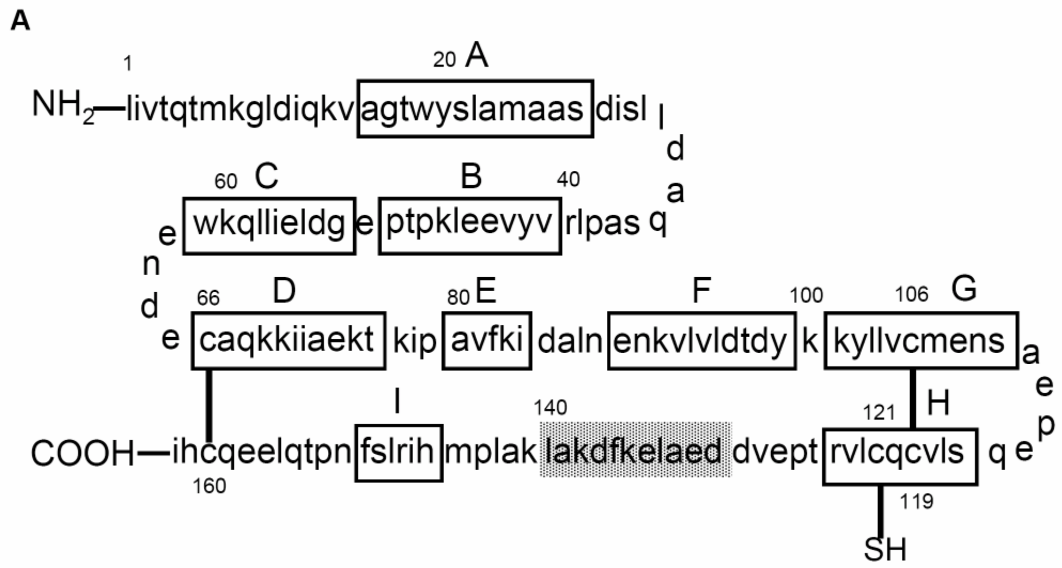
FIG. 9. **Effect of pH on β-LG binding to mAb.** A: CD spectra of β-LG at pH from 2 to 10. B: Binding of mAb to β-LG at various pH determined by ELISA. The primary mAb binding was conducted at the pH indicated; all other reactions, including coating, blocking, washing, and secondary antibody binding, were performed at pH 7.4 according to standard procedures. The binding was significantly decreased at pH 8-10. 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. 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. 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 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.

Figure 1



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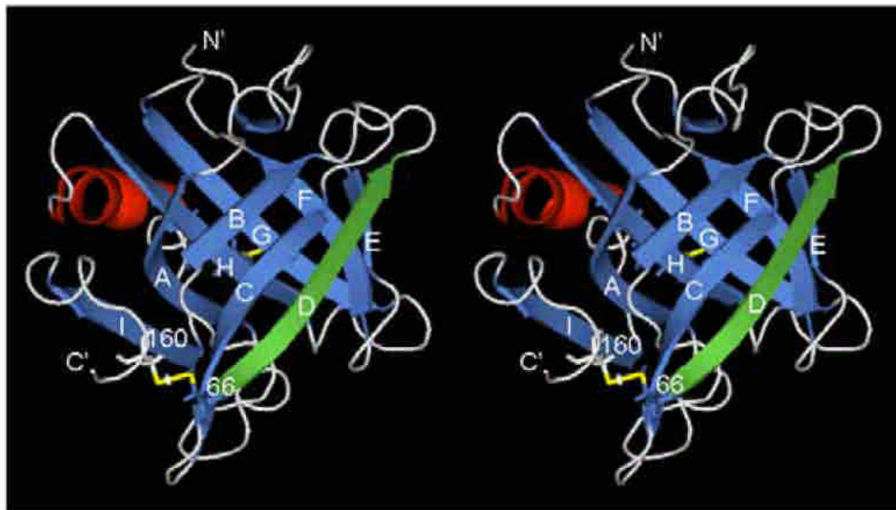


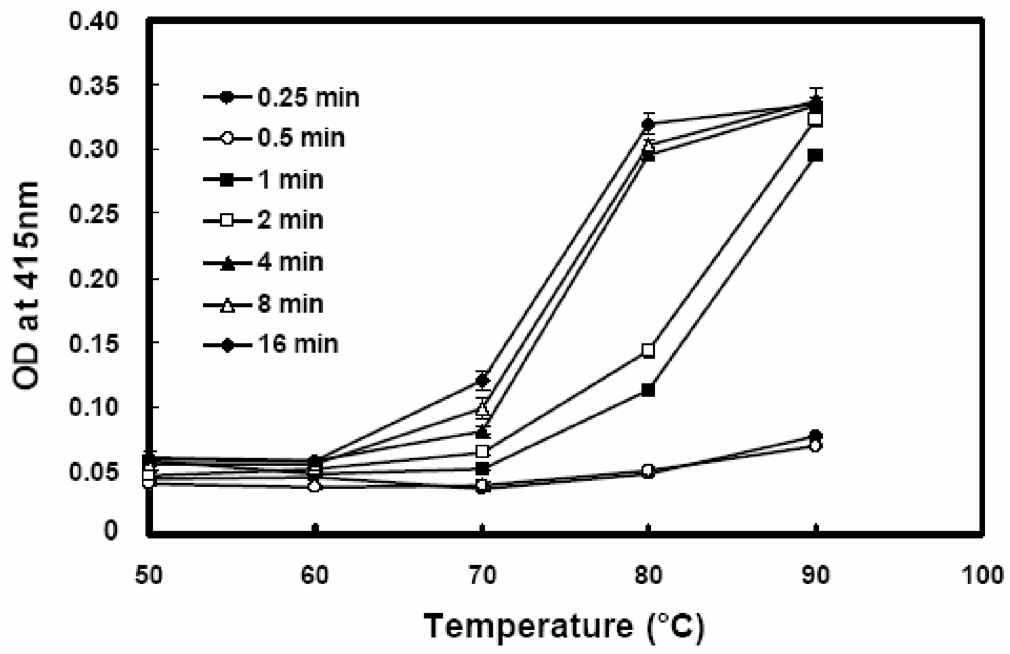
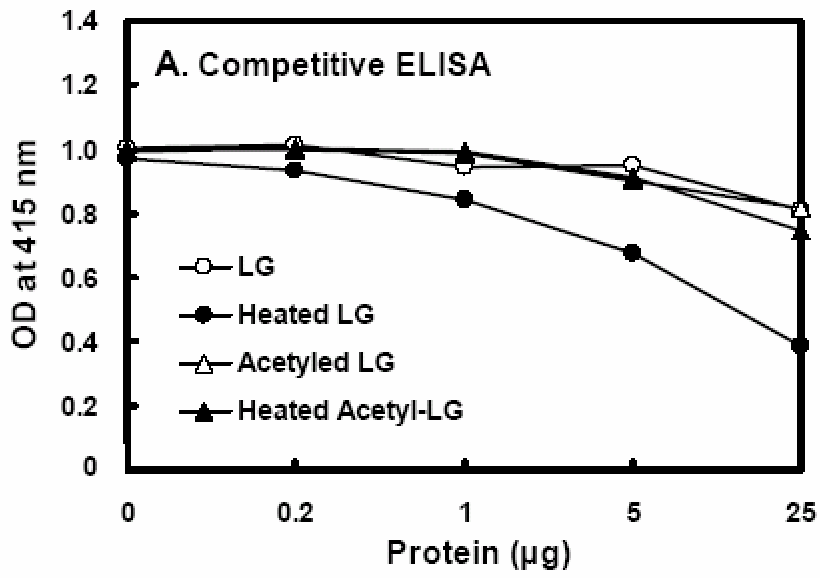


Figure 2
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0
OD at 415nm
0.25 min
0.5 min
1 min
2 min
4 min
8 min
16 min
50
60
70
80
90
100
Temperature (°C)

1896

Figure 3



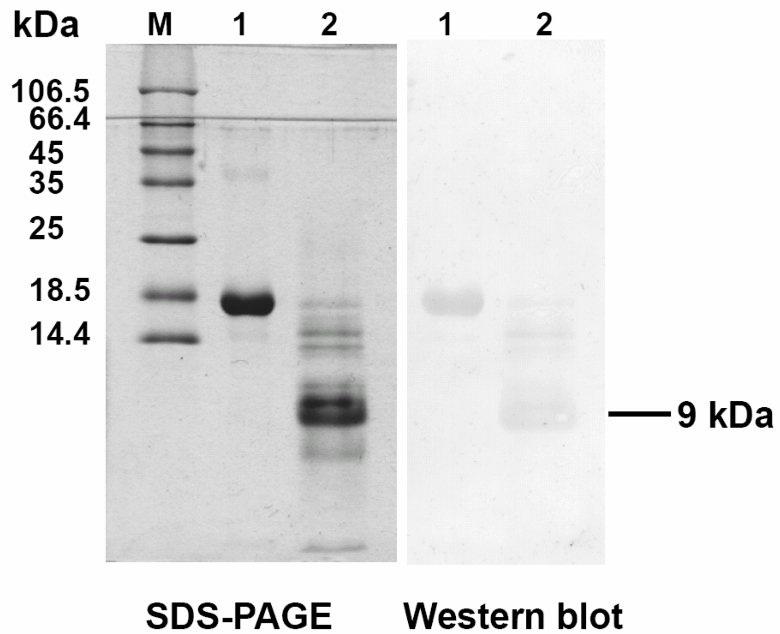


Figure 4

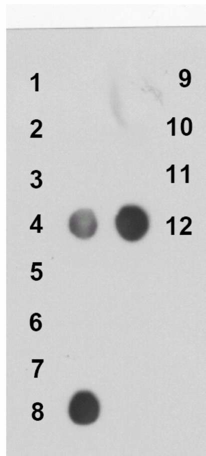
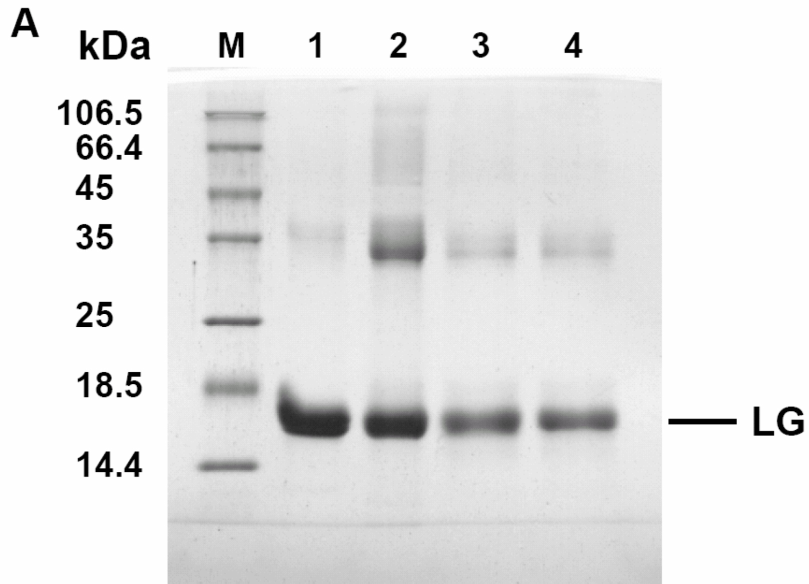


Figure 5



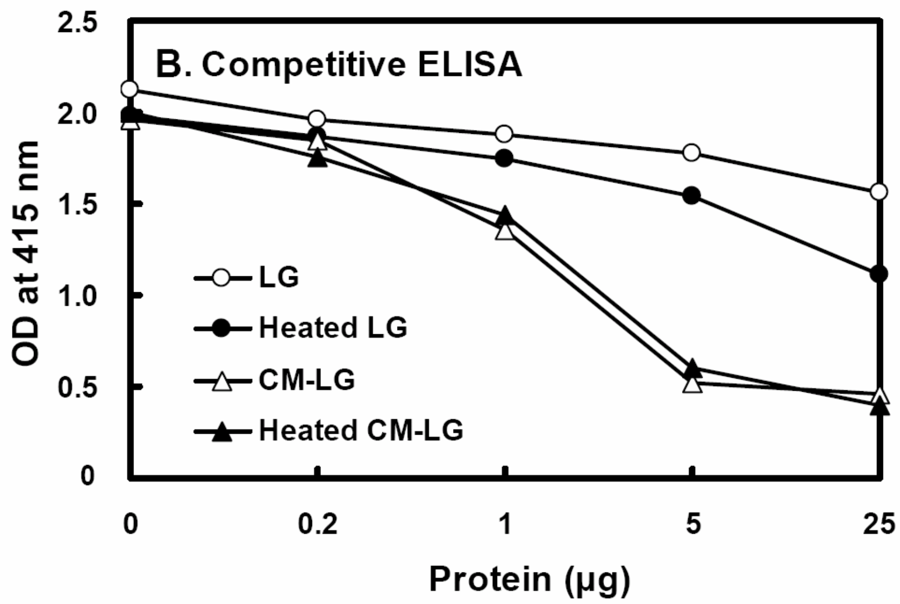


Figure 6

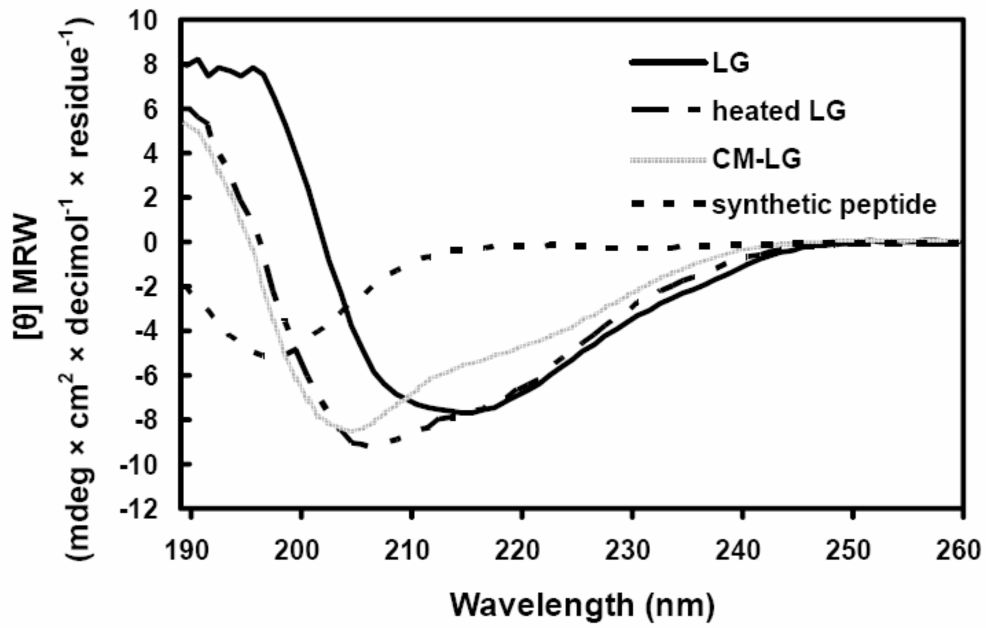


Figure 7

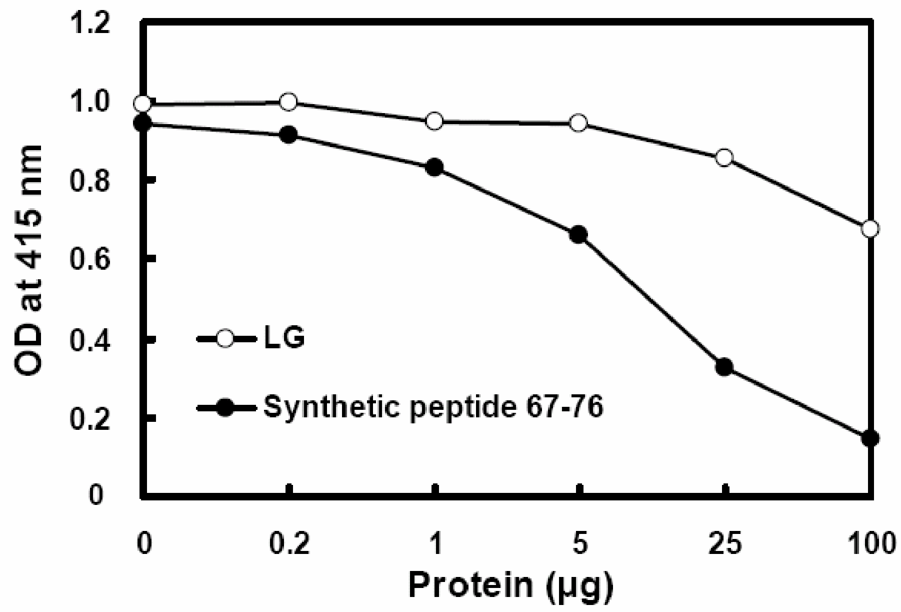
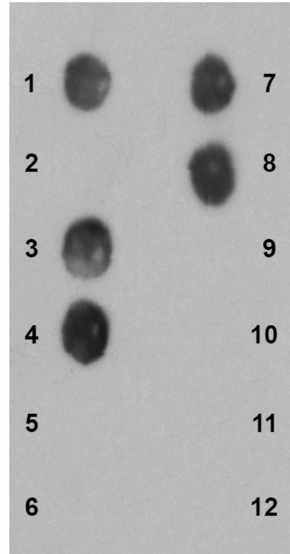


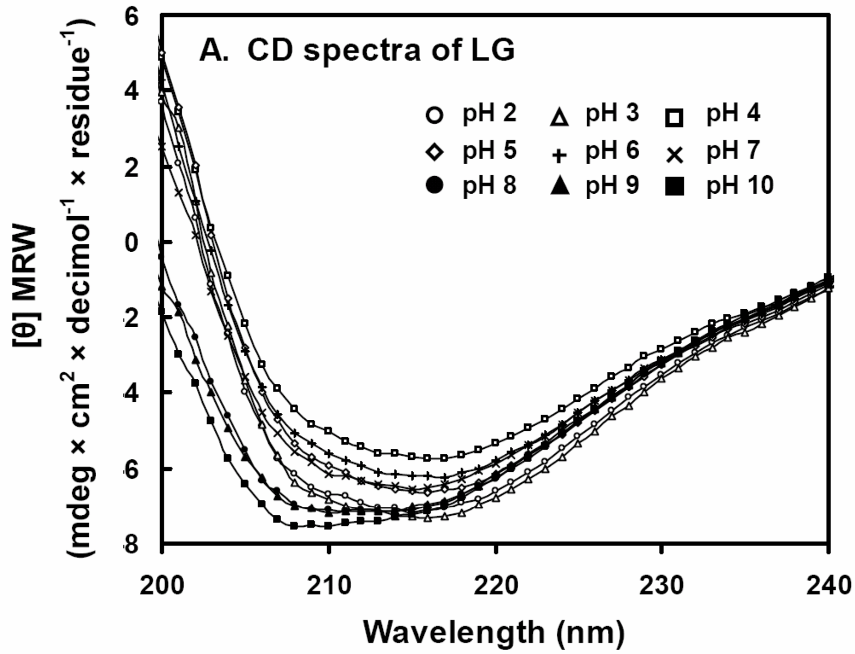
Figure 8

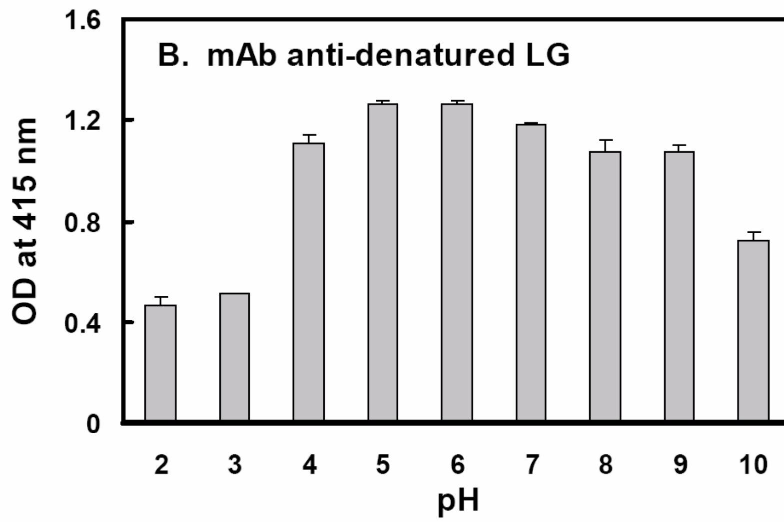
1. AQKK I I AEK
2. AQAK I I AEK
3. AQKA I I AEK
4. AQKK I I AEA
5. AQKK I I AAK
6. AQRK I I AEK



7. AQKR I I AEK
8. AQKK I I AER
9. AQKK I I ADK
10. AQKKAAAEK
11. AAKKAAAEK
12. AAKKAAAAK

Figure 9





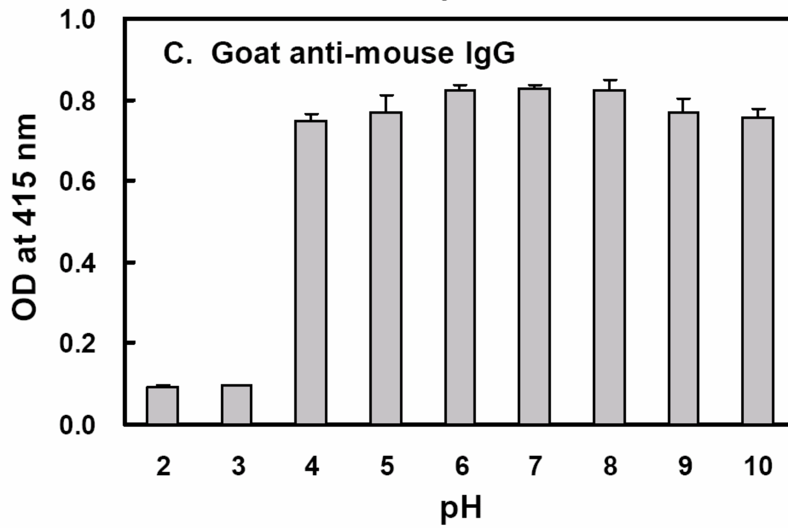


Figure 10

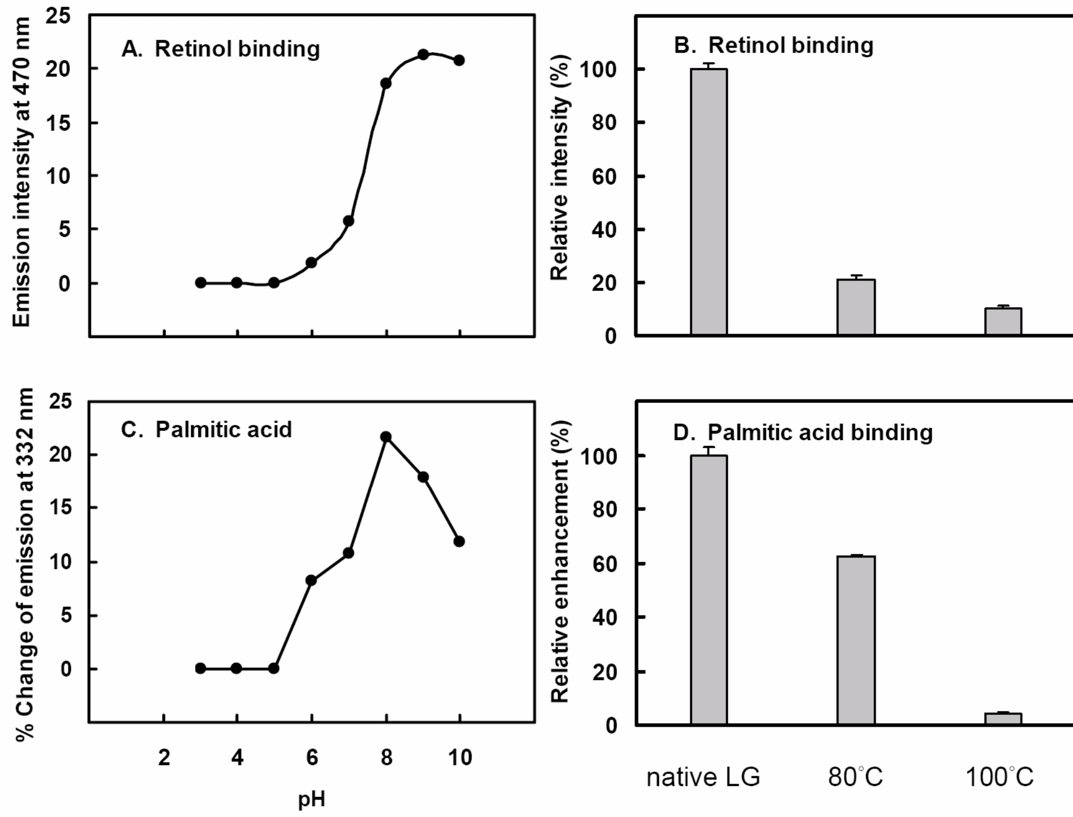
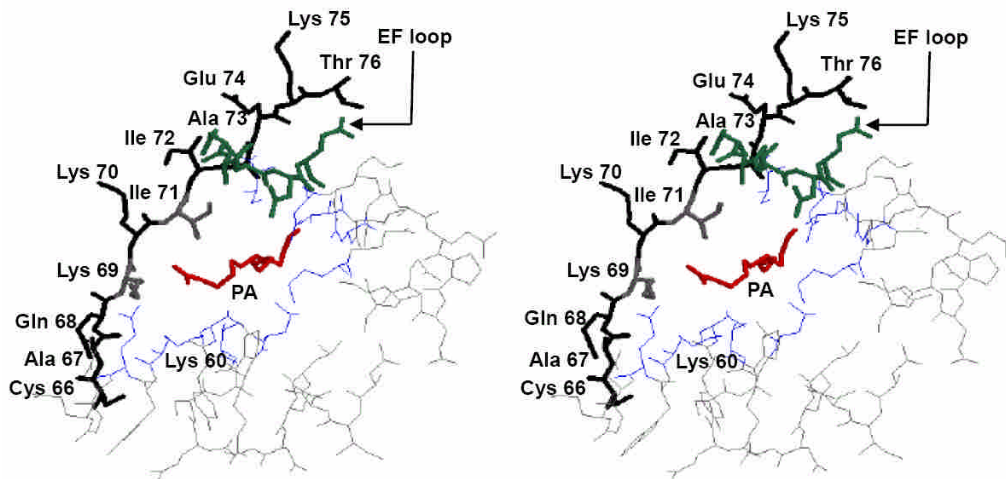
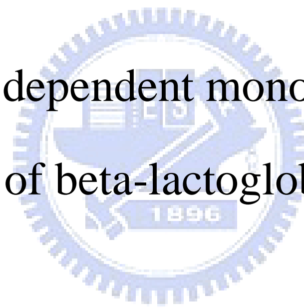


Figure 11





A novel conformation-dependent monoclonal antibody specific to the native structure of beta-lactoglobulin and its application

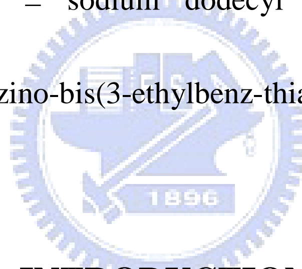
ABSTRACT

Molten globules are thought to be general intermediates in protein folding and unfolding. β-lactoglobulin (**β-LG**) is one of the major bovine whey proteins consisting of about 10-15% of that total milk. We have recently identified that β-LG is a superior marker for evaluating the thermally processed milk. Strand D of β-LG participates in irreversibly thermal unfolding as probed by a monoclonal antibody (**mAb**) specific to thermally denatured β-LG. On the contrary, in the present study we used native β-LG as an immunogen to test the hypothesis that a specific mAb against the native β-LG could be established. As result, a mAb (4H11E8) directed against native structure of β-LG was made. The antibody did not recognize heat denatured form of β-LG, such as its dimer and aggregates. Immunoassay using this "native" mAb showed that the stability of β-LG was at the temperature ≤ 70 °C. β-LG began to deteriorate between 70 and 80 °C over time. The denaturation was correlated with the transition temperature of β-LG. Further chemical modification of cysteine (carboxymethylation) or on positively charged residues (acetylation) of β-LG totally abolished its immunoreactivity, confirming the conformation-dependent nature of this mAb. Using competitive ELISA, the 4H11E8 mAb could determine the native β-LG content in commercially processed milks. Concentrations of native β-LG varied significantly among the local brands tested. From a technological standpoint, the mAb prepared in this study is relevant to the design and operation of appropriate processes

for thermal sanitation of milk and of other dairy products.

(**Key words:** β-lactoglobulin structure, immunoassay, native monoclonal antibody, thermal denaturation, processed milk)

Abbreviation key: β-LG = β-lactoglobulin, **mAb** = monoclonal antibody, **ELISA** = enzyme-linked immunosorbent assay, **PAGE** = polyacrylamide gel electrophoresis, **PBS** = phosphate buffered saline, **SDS** = sodium dodecyl sulfate**, Cyc** = cysteine, **CM** = carboxymethylated, **ABTS** = 2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)



INTRODUCTION

Molten globules are thought to be general intermediates in protein folding and unfolding (Song et al., 2005; Chen et al., 2005; Croguennec et al., 2004; Yang et al., 2001; Chang et al., 2000). α-lactoalbumin and β-LG are two of the major protein moieties of bovine whey proteins, in which β-LG is consist of 50 % of the whey or about 10-15% of total milk proteins (Chen et al., 2005; Wang and Lucey, 2003; de Jongh et al., 2001; Braunschweig et al., 2000). Both of them are the most investigated models for understanding the mechanism involved in protein stability, folding and unfolding upon heating.

β-LG is comprised of 162 amino-acid residues, with one free cysteine and two disulfide linkages (Sava et al., 2005; Pérez and Calvo, 1995). According to 3D crystallographic studies, β-LG is predominantly a β-sheet configuration containing nine antiparallel β-strands from A to I (Forge et al., 2000; Qin et al., 1999). Topographically, strands A-D form one surface of the barrel (calyx) while 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 (Uhrinova et al., 2000). A remarkable property of the calyx is its ability to bind in vitro hydrophobic molecules such as retinoids, fatty acids, vitamin D, and cholesterol (Kontopidis et al., 2002; Wu et al., 1999; Qin et al., 1998b). We have recently identified that strand D of β-LG participates in the irreversibly thermal unfolding (Song et al., 2005), of which a new antigenic epitope in β-LG is being exposed between the amino-acid residues 66 to 76 when heating above 70 °C (Chen et al., 2005; Song et al., 2005). The unique structural and conformational changes made β-LG as a superior marker for evaluating the thermal processed milk (Chen et al., 2005).

In the present study, a conformation-dependent mAb (4H11E8) directed against native β-LG was prepared. The decreased immunoreactivity of β-LG as recognized by this "native" mAb was correlated to the thermal denaturation and conversion of β-sheet to disordered structure of β-LG. Chemical modification of **Cys** and positively charged residues totally abolished the immunoreactivity of β-LG confirming the nature of conformational dependent of this mAb.

Using competitive enzyme-linked immunosorbent assay (**ELISA**), the mAb was able to determine the native β-LG content in commercially processed milk. We show that the native β-LG concentrations varied significantly among the brands. The utility of this native mAb as to monitoring the quality dairy products and the biological consequences is discussed in detail.

MATERIALS AND METHODS

Preparation of milk samples and β-LG

Fresh bulked whole raw milk obtained from a local dairy farm (Chyayi, Taiwan) (other manipulations unless specifically mentioned) was used for polyacrylamide gel electrophoresis (**PAGE**), Western blot, and ELISA analyses. For the heating experiments, each milk sample was heated at each temperature for the indicated time and immediately cooled down in an ice-bath before the study. β-LG was purified from freshly skimmed milk using 40% saturated ammonium-sulfate top fraction followed by a β-LG-antibody affinity column chromatography (Chen et al., 2005; Chen et al., 2004).

Animal care and use

Balb/c mice were purchased from the National Animal Center of National Science Council (NSC) of Taiwan. The mice were maintained and fed in light-cycled animal rooms with the

facility and management according to guidelines established and approved by the NSC.

Immunization of mice

Female Balb/c mice, aged 5-7 weeks, were used for immunization according to the method previously described by us (Chen et al., 2005; Chen et al., 2004; Yang and Mao, 1999). In brief, native β-LG of 200 μg in 200 μL of phosphate buffered saline (**PBS**) containing 0.02 M phosphate and 0.12 M NaCl, pH 7.4 was homogenized with an equal volume of complete Freund's adjuvant by a three-way stopcock for the first injection. The other two injections were then followed using incomplete Freund's adjuvant at days 7 and 14. Seven days following a final booster, plasma was obtained from blood collected in 0.1% ethylenediamine tetraacetate (EDTA) (wt/vol) and was then used as a source for conventional β-LG polyclonal antibody. The titers of this antiserum were typically over 1:10,000 as judged by an ELISA (Chen et al., 2004). The spleen obtained was used for preparing hybridoma fusion.

Production of monoclonal antibody

Monoclonal antibodies were produced according to the standard procedures previously described (Chen et al., 2004; Mao et al., 1990; Mao et al., 1988). In brief, myeloma cell line (FO) was fused with spleen cells from immunized mice at a ratio of 1:5. Fusion was carried out within 2 min at 37 °C using 1 mL of 50% (wt/vol) polyethylene glycol containing 10% (vol/vol) dimethyl sulfoxide (DMSO) (Hybri-Max; Sigma). Cell mixture was then washed and

resuspended in a hypoxanthine-aminopterin-thymidine (HAT) medium (Hybri-Max; Sigma) containing approximately 1 x 10^4 FO cells per 100 μL. The suspended cells were distributed as 100 μl per well in 96-well microtiter plates and incubated at 37 °C in a 5% CO_2-incubator followed by an addition of 100 μL of fresh HAT medium after 7 days. Subsequently, culture medium was assayed for the production of specific mAb, between 14 and 21 days following the fusion, using a solid-phase ELISA described below.

Screening of mAb specific for native β-LG using ELISA

About 0.5 μg of raw or dry milk protein in 50 μL of PBS was coated onto a respective microtiter plate (Nunc; Roskilde, Denmark). Unbound β-LG was washed and subsequently blocked by an addition of 350 μL of 1% gelatin (wt/vol) for 30 min (Chen et al., 2005; Song et al., 2005; Chen et al., 2004; Mao et al., 1988). Following washes with PBS, 50 μL of hybridoma culture medium (2-3 weeks following the fusion) were added and incubated at room temperature for 60-90 min. Each well was then washed 3 x with gelatin-PBS containing 0.1% gelatin and 0.05% Tween-20. Bound β-LG antibodies were detected using a goat anti-mouse IgG conjugated with HRP in gelatin-PBS for 30 min. Finally, each well was washed and developed with 0.04% (wt/vol) 2,2-Azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (**ABTS**) in PBS containing 0.01% H_2O_2 (vol/vol). For primary screening, mAb that differentially reacted with

native β-LG were selected, while that those reacted equally with native and thermally denatured β-LG were ignored. The selected hybridomas were expanded and subcloned by limiting dilutions for at least 2 x to establish the monoclonals (Mao et al., 1990; Mao et al., 1988). The specificity of the mAb that recognized the native structure of β-LG was further confirmed by Western blot using native and heated β-LG (95 °C for 5 min).

Gel electrophoresis

Sodium dodecyl sulfate-PAGE (**SDS-PAGE**) or native-PAGE containing 15% (wt/vol) polyacrylamide (unless specified) was used for the characterization of the milk proteins using a modified procedure (Chen et al., 2005; Yang and Mao, 1999) similar to that described (Chen et al., 2005; Oldfield et al., 1998). All the samples (5-20 μg) for SDS-PAGE were equilibrated in 10 mM Tris-HCl and 0.1% SDS, pH 7.6, before loading to the gel. It is worth mentioning that a pre-heat treatment used in the conventional SDS-PAGE was omitted in this study to ensure the native structure of unheated β-LG or milk proteins. The same procedures were conducted for the native-PAGE.

Western blot analysis

Following the SDS-PAGE or native-PAGE, the gel was soaked instantly and briefly in a transfer buffer containing 25 mM Tris-HCl, 192 mM glycine, 20% methanol, 0.0375% SDS (pH

8.3) for 30 s (Chen et al., 2005; Chen et al., 2004). The gel was then immediately electrotransferred to a nitrocellulose membrane (Hybond-ECL extra; Amersham, Buckingham, UK) at 90 mA for 45 min in a semi-dry transfer cell (Bio-Rad). The membrane was immersed in 1% gelatin for 1 h with gentle shaking. Following 3 x washes with PBS for 5 min, the membrane was incubated with tested mAb (with appropriate dilution in PBS containing 0.1% gelatin and 0.05% Tween-20) for 1 h and followed by 3 x washes and incubation with HRP conjugated goat anti-mouse IgG for 1 h. Finally, the membrane was developed with 0.1 mg/ml of 3-3'-diaminobenzidine (3,3',4,4'-tetra-amino-biphenyl) containing 0.01 % H_2O_2 in PBS.

Trypsin and CNBr Fragmentation

For trypsin treatment, 50 μg of β-LG in 100 μL PBS were incubated with 1 μL of trypsin (0.1 mg/ml) at room temperature for 4 h (Song et al., 2005; Chen et al., 2004). Trypsinized β-LG was analyzed on a SDS-PAGE (18% polyacrylamide) followed by a Western blot. CNBr fragmentation was conducted according to the method previously described with some modifications (Song et al., 2005; Mao et al., 1977). In general, 5 mg of β-LG were first dissolved in 70% (v/v) TFA as that described (Andrews et al., 1992; Caprioli et al., 1991) with the addition of 10 mg CNBr in dark for 24 h at room temperature. After 3 x evaporation in a lyophilizer 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.

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 (Song et al., 2005; 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 (Song et al., 2005; Tseng et al., 2004; Mao et al., 1980), 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 2-mercaptoethanol. 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 4.9 residues of CM-cysteine per mole of β-LG.

Standard displacement curve of milk samples and determination of β-LG concentration

In brief, β-LG (0.5 μg in 50 μL of PBS) was first immobilized onto each microtiter well

followed by washes and blocking as that described for ELISA. After washes, 50 μL of the milk samples (raw or commercially processed milks) at various dilutions in 0.1% gelatin-PBS were mixed with 50 μL of the 4H11E8 mAb and incubated at room temperature for 1 h. Following washes, a secondary antibody (goat anti-mouse IgG conjugated with HRP) was added, incubated, washed, and then developed with ABTS. To determine the native β-LG content of each milk sample, the β-LG concentration was based on the equivalent immunoreactivity extrapolated from that using purified β-LG as a standard. The mean value of each unknown sample was obtained from triplicated determinations with an intra-assay variation of less than 8%.

Milk samples for native-PAGE

Different brands of commercially processed milk were purchased from the local market and one was from the US market (Kroger, Cincinnati, Ohio). Freshly bulked whole raw milk was obtained from a local dairy farm. Samples were immediately centrifuged at 13,000 rpm (15,500g) for 1h at 4 °C. The top layer in the supernatant was carefully removed, while the remaining fraction (whey protein) containing minimal casein was used for the analysis of PAGE and Western blot.

RESULTS

Using dry milk as an immunogen, we have recently produced monoclonal antibodies (mAb) that can distinguish dry milk from raw milk (Chen et al., 2004). Antigenic mapping shows that the epitope is located at residues 66-76 in strand D of thermally denatured β-LG (Song et al., 2005). When heated above 80 °C, the epitope region of β-LG is exposed and bound by the mAb (3E7G7). The antibody is therefore defined as "denatured" mAb. In the present study, we tested the hypothesis as to whether a native β-LG mAb could be prepared when native β-LG was used for immunization. Approximately 500 hybridoma clones were screened, among which one monoclonal was established (4H11E8) that specifically reacted with the native β-LG, but not heat denatured β-LG. A typical example is shown in Figure 1, mAb 4H11E8 bound primarily to native β-LG in contrast to denatured mAb that bound to heated β-LG.

Western Blot Analysis

Using SDS-PAGE and Western blot analysis, mAb 4H11E8 only recognized the native monomeric β-LG of raw and dry milk, but not denatured β-LG dimer or polymers of dry milk (Figure 2). The denatured β-LG was almost not detectable by Coomassie Blue staining. Such minimal denatured forms, however, were observed using a denatured mAb prepared previously (Figure 2; right panel) (Chen et al., 2005; Chen et al., 2004). To demonstrate the specificity and

sensitivity of the 4H11E8 mAb, we heated raw milk at 95 °C over time. Following a native-PAGE and Western blot analysis, β-LG was found to be severely denatured after heating of milk at 95 °C for longer than 2 min (Figure 3A). Formation of large polymers from either self-associated β-LG or β-LG conjugates (with other milk proteins) were seen by a denatured mAb (Figure 3B). Since more β-LG aggregates formed after heating for longer than 4 min, less denatured β-LG could run into the gel; such that some of the sample became undetectable by the denatured mAb on the blot (Figure 3B). It was obvious that mAb 4H11E8 only recognized the native β-LG. We herein defined it as "native" mAb.

Effect of heat on the immunoreactivity of raw milk

Quantitative ELISA was then used to analyze immunoreactivity of native mAb against raw milk heated at different temperature over time (Figure 4). The β-LG thermal denaturation curve shows that in general β-LG was stable at the temperatures $\leq$ 70 °C. It began to deteriorate at between 70 and 80 °C. Most interestingly, the thermal denaturation curve determined by the immunoassay in this study is almost identical to that determined by the structural change of β-LG using physical-chemical approach: circular dichroic (CD) spectrum (Chen et al., 2005). A severe decrease in immunoreactivity was observed when heating was over 80 °C. The finding demonstrated that the structural loss of native β-LG could be probed by the native 4H11E8 mAb.

Effect of conformational change on the immunoreactivity of β-LG

We anticipated that the overall structure of β-LG might determine the specificity nature of this native mAb. To test this hypothesis, we chemically modified β-LG by carboxymethylation to break up the disulfide-linkage in altering its overall structure. As shown in Figure 5A, the native mAb reacted with native β-LG, but not that carboxymethylated using a Western blot analysis. Notably, an extra band was present in the modified β-LG (Coomassie blue staining), this might be generated during the modification by an unknown mechanism. Additional acetylation on the lysyl residues, limited trypsin treatment, and CNBr fragmentation also diminished the immunoreactivity (Figure 5B-D). It should be noted here that both of these two chemical modifications were carried out in the presence of 6 M urea. The modified β-LG was dialyzed against PBS prior to the Western blot to remove the urea. After dialysis, the immunoreactivity of β-LG treated with 6 M urea was retained due to the renaturation of the conformation. Also, sample treated with TFA alone (without the presence of cleavage reagent CNBr) did not show the loss of immunoreactivity after removing the TFA by lyophilization. However, β-LG treated with 8 M urea without dialysis resulted in a complete loss of the immunoreactivity on a nitrocellulose using a dot-blot assay (data not shown). Taken together, these results suggest that the native mAb was not sequentially dependent.

Determination of native β-LG in processed milk from local market

Using competitive ELISA by native mAb, we monitored the immunoreactivity of native β-LG in processed milk. A typical example for the standard displacement curves resulting from the samples purchased from the local market is shown in Figure 6. Optical density is inversely correlated with the expression of native β-LG in tested milks. The raw milk shows a superior β-LG immunoreactivity to most of the brands tested (Figure 6), and was almost super imposed with one brand claimed to be pasteurized at below 65 °C for 30 min (low temperature and long time or LTLT). Using a standard curve of native β-LG (data not shown), the extrapolated β-LG content in each brand is listed in Table 1. The β-LG left over in each processed brand varied significantly. Interestingly, the native β-LG values in a US brand (No. 3) and domestic brand (No. 2; LTLT pasteurized) were essentially identical to that in raw milk. Finally, we characterized the native β-LG content in processed milk using a native-PAGE. B-LG was found severely denatured in some of the brands (Figure 7) and was almost consistent with the quantitative ELISA using the native mAb (Table 1).

DISCUSSION

It is well established that the heating process during the preparation of dry milk causes structural changes in some milk proteins (Chen et al., 2004; Needs et al., 2000). Although such changes are subtle, we have demonstrated that the denatured mAb can distinguish the minor differences between the dry and raw milk (Chen et al., 2004). Subsequently, β-LG was found to be the component responsible for the specificity of that denatured mAb (Chen et al., 2004). Following an antigenic mapping using synthetic peptides, we have recently delineated the epitope to be located in residues 66-76 of the denatured D strain of β-LG (Song et al., 2005). Thus, it suggests that β-LG is a sensitive thermal-marker that can be probed by a mAb specific to denatured β-LG. Conversely, it predicts that a mAb specific to native β-LG can be made following an appropriate immunization and screening. For this reason, in the present study we used native β-LG as an immunogen to test the hypothesis that a specific mAb against the native structure of β-LG could be established.

Since those monoclonals that crossly reacted with denatured β-LG had already been eliminated in the initial screening, the remaining clones that specific to native β-LG should be most likely directed against a conformational dependent epitope. Several lines of evidence support this possibility. First, from the known 3D structure, Cys residues are responsible for stabilizing the

overall structure of β-LG by crossly linking the positions strand D Cys-66 and carboxyl terminus Cys-160 (Song et al., 2005; Creamer et al., 2004). Our chemical modification (carboxymethylation) on Cys residues not only resulted in the conformational change of β-LG as shown in our previous study (Song et al., 2005.), but also completely abolished the immunoreactivity in the present study (Figure 4). Second, acetylation that neutralizes the positively charged lysyl residues (at pH 10) resulted in a total loss of the immunoreactivity of β-LG (Figure 5B). These charged residues are also crucial in maintaining the overall 3D structure of a given protein or β-LG (Song et al., 2005). Third, in contrast to the denatured β-LG mAb (Song et al., 2005; Chen et al., 2004), this native mAb did not recognize the CNBr fragment (Figure 4). It is conceivable that this mAb may not be a sequence-dependent. It was tempting to speculate that the entire β-LG molecule was required in maintaining the antigenic structure, but we could not rule out that such fragmentation might directly cleave the epitope resulting in a loss of the immunoreactivity. We then denatured the β-LG using 8 M urea and demonstrated it was not immunoreactive using a dot-blot assay (data not shown). Thus, it further supports the notion that conformation of β-LG plays a role for this native mAb. With respect to the CNBr cleavage, we used 70% TFA instead of formic acid. Although formic acid is commonly used due to its good solubility and denaturation for a given protein, it may damage tryptophan and tyrosin residues (Morrison et al., 1990). It may also result in formylation and so

increase in peptide mass (Beavis and Chait., 1990). On the other hand, TFA is preferable to formic acid giving a satisfactory result (Andrews et al., 1992; Caprioli et al., 1991), but the reaction time is somewhat slower than that of formic acid.

Taken together, our study reveals that this native mAb is conformationally dependent on the overall structure of β-LG. It is difficult for us to map out the specific antigenic determinant at the present time, but it is very possible that Cys-121 or its neighboring residues might play an essential role for the recognition of the native mAb. Because the free Cys-121 residue is known to be involved in the simplest form of covalently linked β-LG dimer upon the heating (Farrell et al., 2004; Kontopidis et al., 2004; Panick et al., 1999), such dimerization causes the loss of our mAb binding (figure 2). The covalent linkage may eventually block the 4H11E8 binding via steric hindrance (Figure 2). Furthermore, the Cys-121 is also responsible for cross linking with β-LG aggregates or with other milk proteins upon the heating (Chen et al., 2005; Chen et al., 2004; Creamer et al., 2004; Croguennec et al., 2004; Anema, 2004). We have attempted to map out the antigenic structure for this native mAb using the peptide array technique previously established in our laboratory (Song et al., 2005.), but failed to show any immunoreactivity against the synthetic peptides. Therefore, we can only speculate that Cys-121 (or its neighboring residues) is essential for the mAb recognition, but the antigen-antibody interaction may still need the integrity of the native structure of β-LG. Final conclusive interpretation of the involvement

of Cys-121 with respect to the native mAb recognition probably lies on the use of recombinant β-LG with a single mutation on Cys-121, this experiment is now in progress in our laboratory using site-directed mutagenesis. In addition, the acetylation experiment conducted in this study (Figure 5) tended to suggest the possible involvement of lysyl residues in the antigenic determinant similar to that found previously (Song et al., 2005), in which the positively charged residues are frequently expressed on a surface epitope.

As to the biological function of β-LG, studies have shown that it produces hypocholesterolemic (Nagaoka et al., 2001) and antioxidant effects (Hernandez-Ledesma et al 2005; Wong et al., 2003; Peña-Ramos et al., 2001) and may also probably serve as a growth factor for mammalian cells (Feuermann et al., 2004). It can transport and complex with retinol and fatty acids via its hydrophobic binding pocket "calyx" of β-LG (Song et al., 2005; Wang et al., 1999). Interestingly, β-LG is not only acid resistant in gastro-intestinal tracts, but also possesses a superior absorption capability via a receptor-mediated process (Dew et al., 1997). Our recent study demonstrates the loss of this binding capability when β-LG is denatured by heating above 80 °C. We have further suggested that it is due to the exposure of hydrophobic region (residues 66-76) of the D strand to a polar environment, which destabilizes the calyx structure and complex formation with retinol and palmitic acid (Song et al., 2005). Since β-LG is also labile to heat treatment by forming large polymers with other milk proteins (Chen et al.,

2005; Chen et al., 2004), it is tempting to speculate that heat may reduce the extent of absorption of β-LG through the gut. Conceivably, additional overheating should be avoided in order to maintain the physiologic role of β-LG. Our mAb may provide a novel mean for monitoring the native structure of β-LG in heat processed milks. It is of interest to note that the reported transition temperature of native β-LG (between 70 and 80 °C) is almost completely agreeable with the decrease in immunoreactivity for the native mAb (Figure 4). From a technological standpoint, this mAb may be relevant to the design and operation of appropriate processes for thermal sanitation of milk and of other dairy products. For example, in a standard Ohio State University method of manufacturing whey protein curd, milk is short-time pasteurized (about 72 °C for 30 seconds) and held overnight at 40 °C. In the next morning the mixture is cooled to 30 °C, inoculated with a lactic acid culture, and incubated for 30 min. Rennet extract is added and the mixture is stirred, resulting in coagulation of curd (Marshall, 2004). According to our curve for thermal denaturation constructed by the mAb (Figure 4), β-LG is likely intact using the standard procedures mentioned above. The present study shows the use of native mAb in determining the native β-LG concentration in processed milk (Table 1), but not of polyclonal antibodies as they recognized both native and denatured β-LG (Chen et al., 2005). Table 1 illustrates that the β-LG content varied significantly among the brands we tested. Although the status for the manufactured process is not disclosed and yet not readily known, our study indicate

the remarkable difference among the procedures for thermal sterilization and pasteurization as previously indicated (Douglas et al., 1981). It is therefore recommended that the heating process should be eventually standardized to avoid unnecessary overheating in our domestic manufacturers. In conclusion, the present study provides a notion that a novel conformation-dependent β-LG mAb can be prepared via an appropriate immunization strategy and screening. Such native mAb can be used as a unique reagent to routinely monitor the quality of dairy products, when β-LG is considered to be an essential ingredient.

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CAPTIONS AND LEGENDS

Figure 1. Typical example for mAb that differentially reacted with native and heated β-LG. Immunoreactivity was monitored using an ELISA, while equal amount of native and heated β-LG protein (1 μg) was coated onto the microtiter plate. β-LG was heated at 95 °C for 5 min prior to the coating. Each bar represents the mean ± SEM of triplicate determinations.

Figure 2. Characterization of native mAb 4H11E8 using Western blot. Left: Raw and dry milk on a 15% SDS-PAGE followed by coomassie blue staining. Right: Raw and dry milk on

SDS-PAGE followed by Western blot using native and denatured mAb. The data show that native mAb only recognizes the native form of β-LG, but not β-LG dimer, polymers, or aggregates. The denatured forms of β-LG can not be seen by Coomassie staining.

Figure 3. Thermal denaturation of β-LG characterized by native mAb 4H11E8 and denatured mAb on native-PAGE. The experiment was carried out by heating raw milk at 95 °C over time, followed by a Western blot using mAb (A) and denatured mAb (B). Two isoforms of β-LG were seen on native-PAGE. The native mAb does not recognize the thermally denatured β-LG.

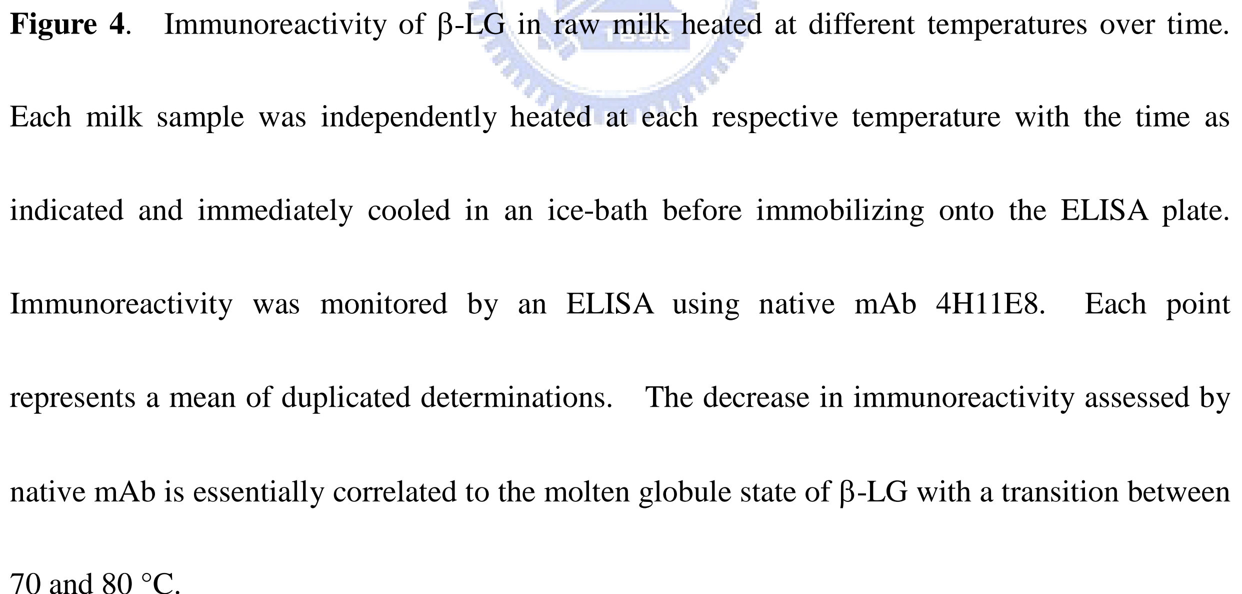


Figure 4. Immunoreactivity of β-LG in raw milk heated at different temperatures over time. Each milk sample was independently heated at each respective temperature with the time as indicated and immediately cooled in an ice-bath before immobilizing onto the ELISA plate. Immunoreactivity was monitored by an ELISA using native mAb 4H11E8. Each point represents a mean of duplicated determinations. The decrease in immunoreactivity assessed by native mAb is essentially correlated to the molten globule state of β-LG with a transition between 70 and 80 °C.

Figure 5. Role of disulfide-linkage in maintaining the antigenic structure of β-LG and effect of

chemical modifications on its immunoreactivity. A: β-LG was irreversibly reduced by carboxymethylation. Lane I: Native β-LG. Lane II: Carboxymethylated β-LG. Left: 15% SDS-PAGE by Coomassie blue staining. B: β-LG was modified before (I) and after (II) acetylation at pH 10. C: β-LG was treated before (I) and after (II) trypsin cleavage. D: β-LG was treated before (I) and after (II) CNBr-cleavage. The left of each panel represents the analysis of 20% SDS-PAGE using Coomassie Blue staining. While, the right of each panel represent the Western blot analysis using native mAb 4H11E8. The data suggest that the immunoreactivity is dependent on the overall structure of β-LG (A and B) and the epitope is likely not located in the β-LG fragments (C and D).

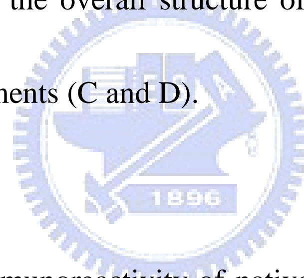


Figure 6. Typical example of immunoreactivity of native β-LG in raw and processed milk as determined by a competitive ELISA. Native β-LG was used as an immobilized antigen, while competing the binding of raw and processed milks for native mAb 4H11E8. A: Raw milk. B: Processed milk from a local market pasteurized at 60 °C for 30 min. C-G: Processed milks purchased from the local market with undisclosed heating procedures. Apparently, the immunoreactivity of β-LG in most of the processed milk was attenuated as compared with the raw milk.

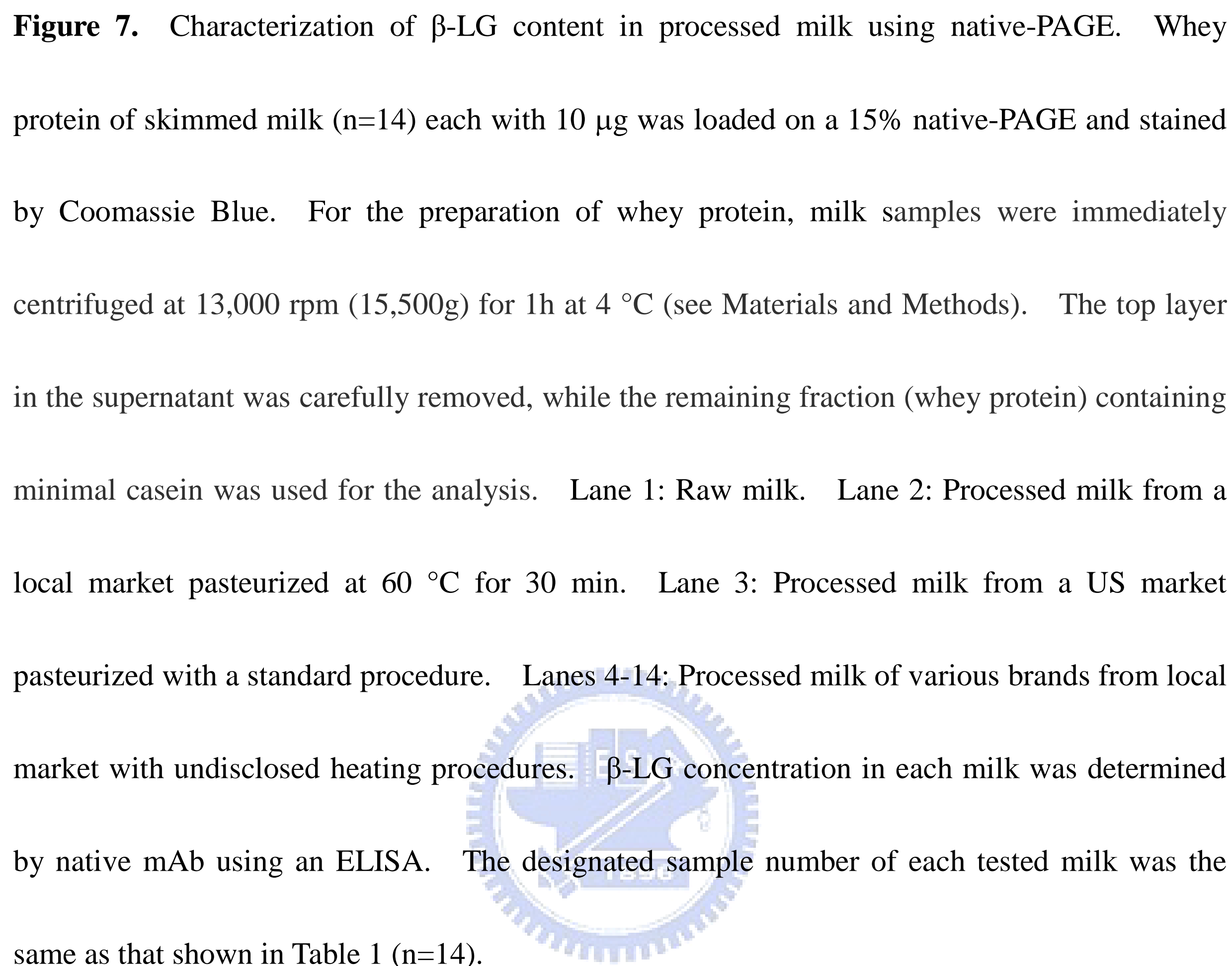


Figure 7. Characterization of β-LG content in processed milk using native-PAGE. Whey protein of skimmed milk (n=14) each with 10 μg was loaded on a 15% native-PAGE and stained by Coomassie Blue. For the preparation of whey protein, milk samples were immediately centrifuged at 13,000 rpm (15,500g) for 1h at 4 °C (see Materials and Methods). The top layer in the supernatant was carefully removed, while the remaining fraction (whey protein) containing minimal casein was used for the analysis. Lane 1: Raw milk. Lane 2: Processed milk from a local market pasteurized at 60 °C for 30 min. Lane 3: Processed milk from a US market pasteurized with a standard procedure. Lanes 4-14: Processed milk of various brands from local market with undisclosed heating procedures. β-LG concentration in each milk was determined by native mAb using an ELISA. The designated sample number of each tested milk was the same as that shown in Table 1 (n=14).

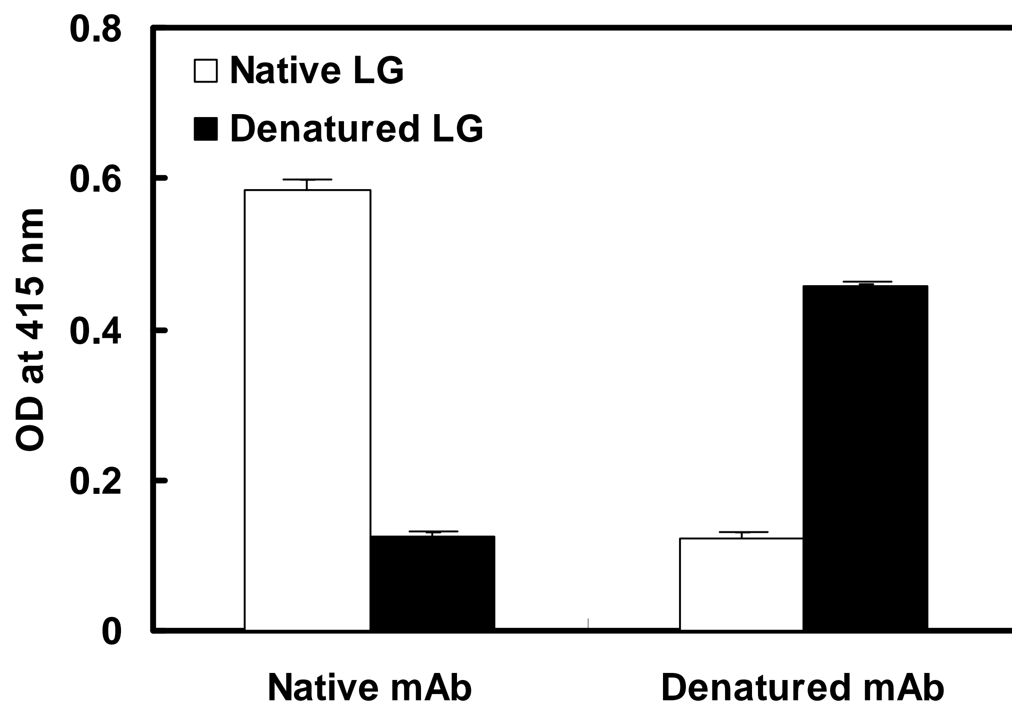
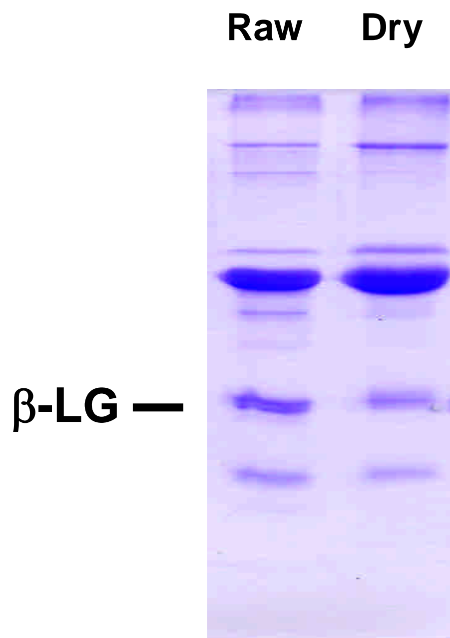


Figure 1



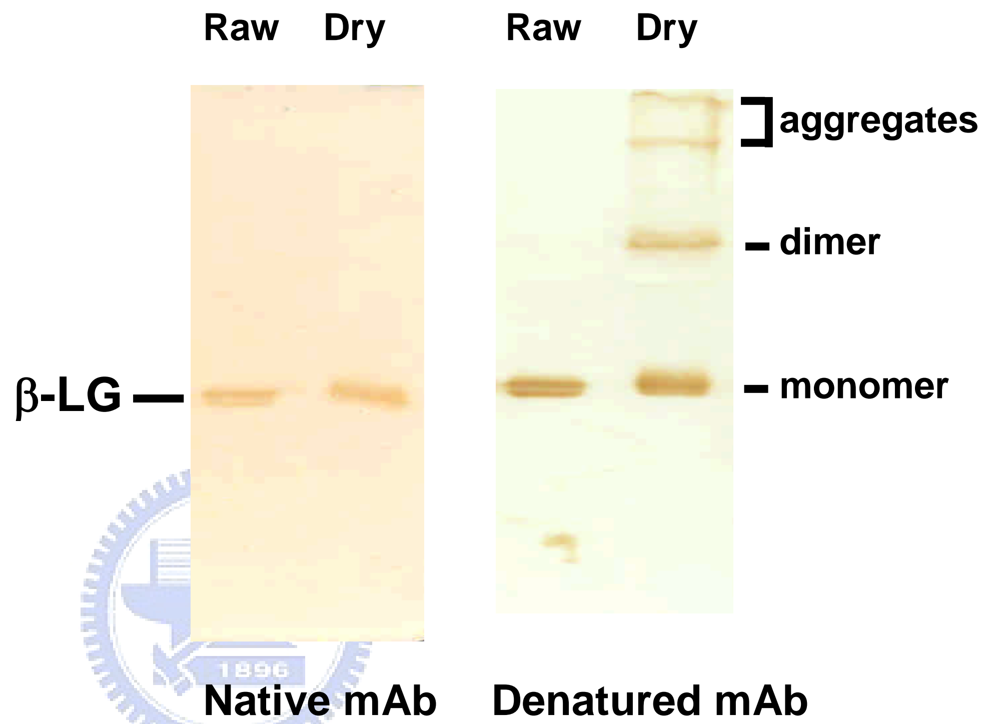
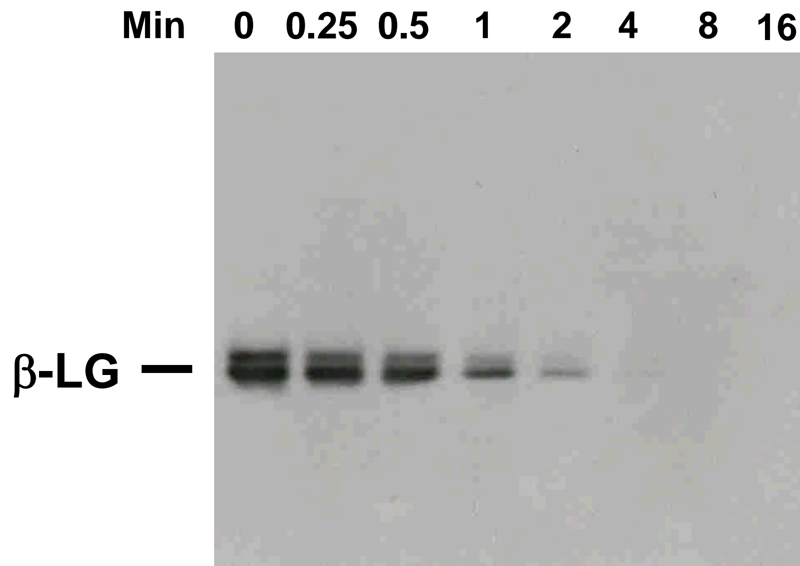


Figure 2

Western Blot

A. Native mAb



B. Denatured mAb

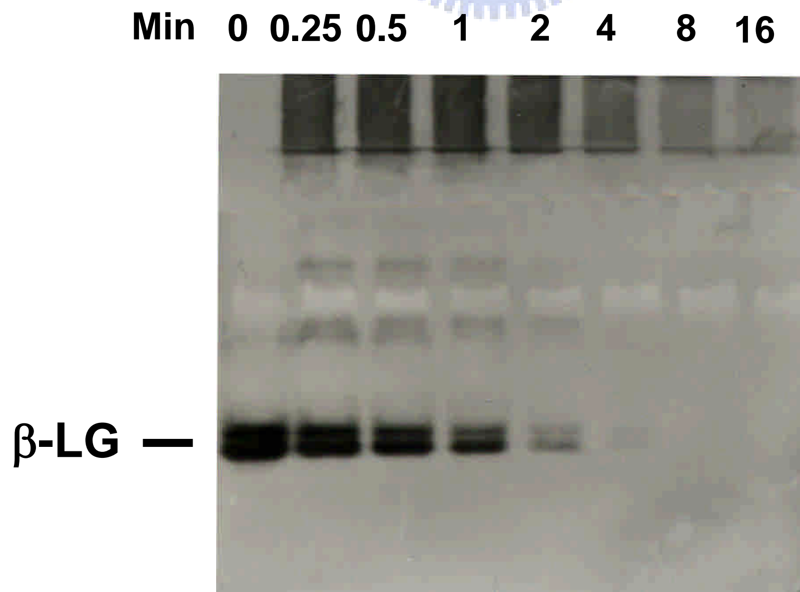


Figure 3

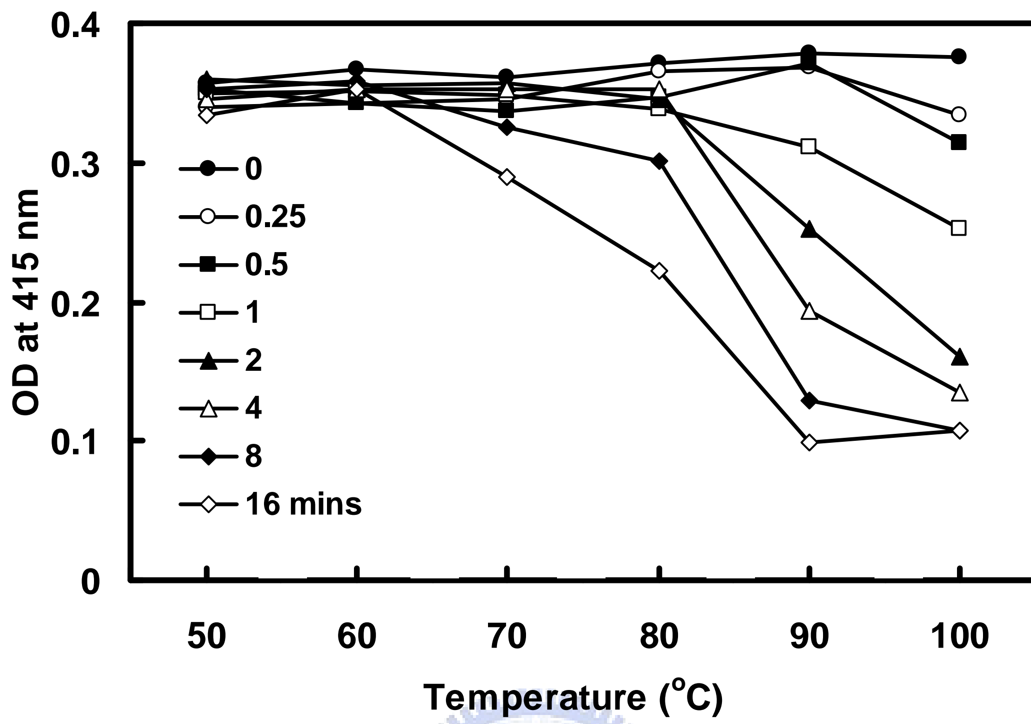
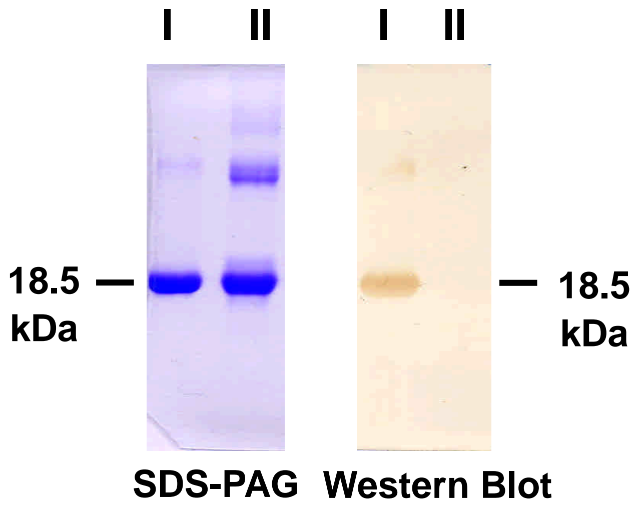
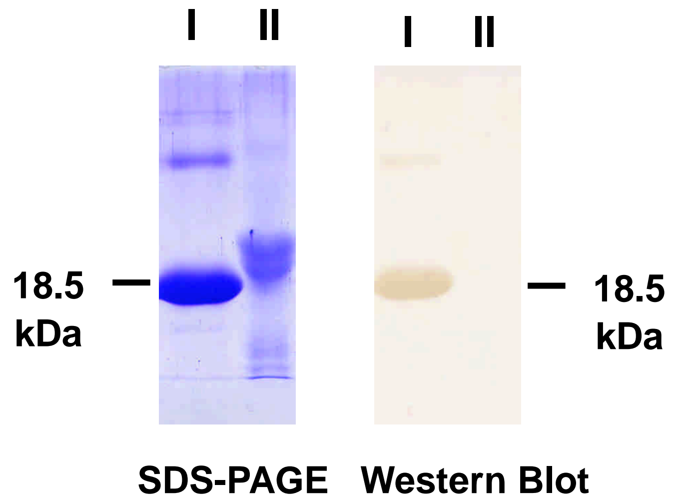


Figure 4

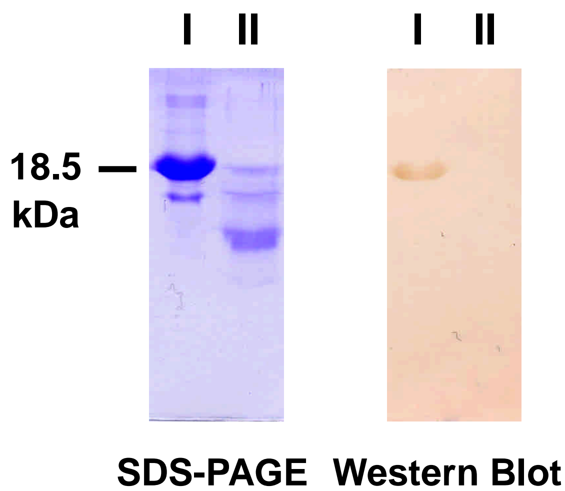
A. Carboxymethylation



B. Acetylation



C. Trypsin treatment



D. CNBr cleavage

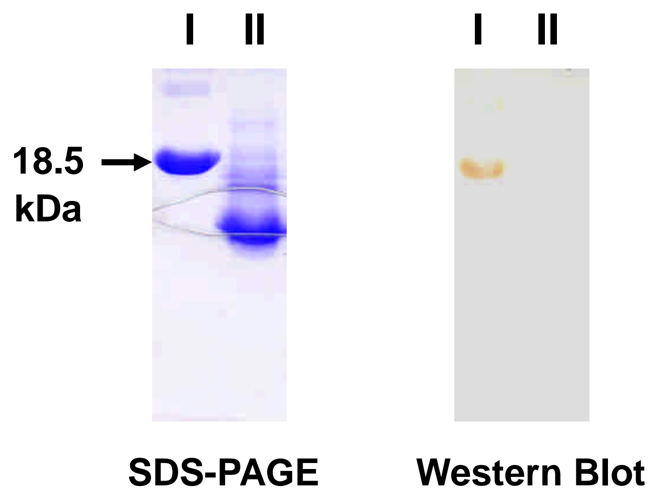


Figure 5

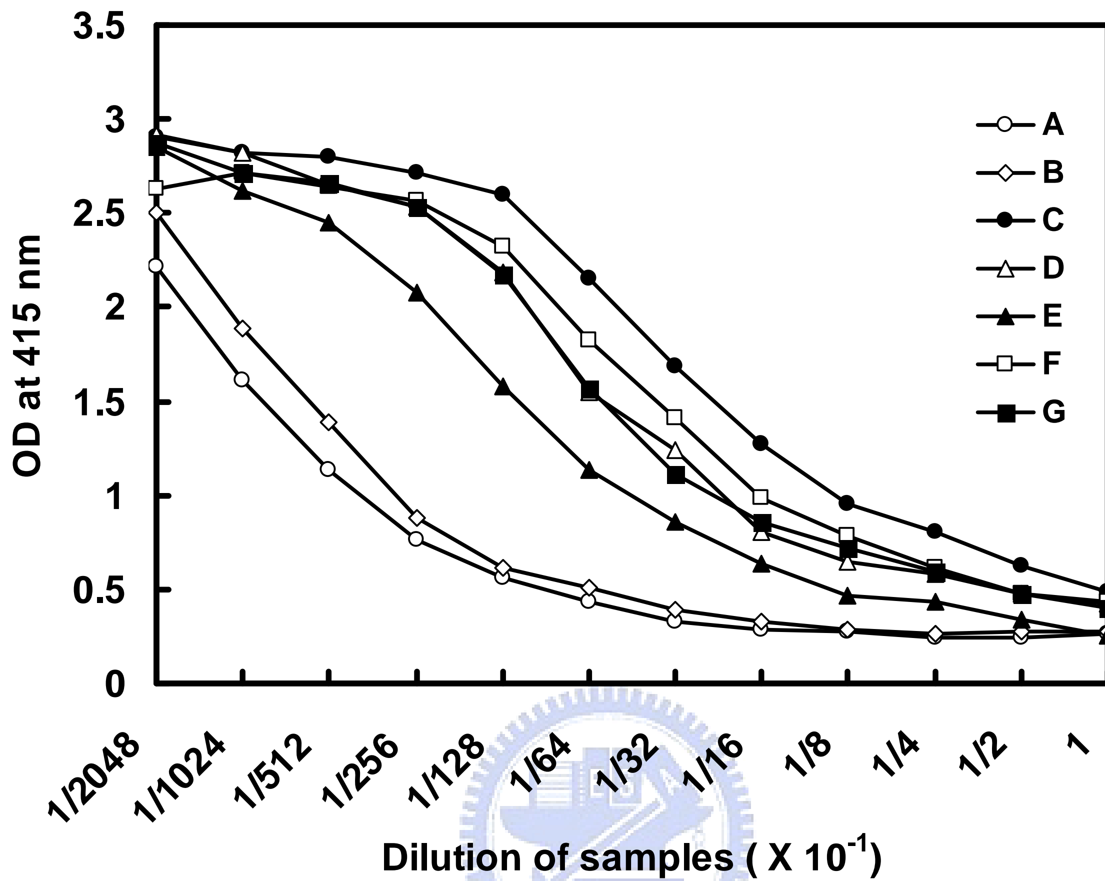


Figure 6

Table 1. Determination of native β-LG from commercially processed milk

Processed milk from market[1]	Amount of native β-LG (mg/mL)[2]
1	5.50 ± 0.06
2	5.35 ± 0.15
3	5.40 ± 0.12
4	2.98 ± 0.27
5	0.60 ± 0.08
6	0.36 ± 0.02
7	0.55 ± 0.08
8	1.84 ± 0.36
9	0.39 ± 0.03
10	0.34 ± 0.08
11	0.68 ± 0.18
12	1.05 ± 0.16
13	0.49 ± 0.04
14	0.48 ± 0.05

[1]Samples 1, 2, and 3 represent a raw milk, processed milk of a local market pasteurized at 60 °C for 30 min, and processed milk of a US market pasteurized with a standard procedure, respectively. Samples 4-13 represent various processed milks of local market with an undisclosed heating procedure.

[2]Determination of β-LG by competitive ELISA. Each value represents the mean ± SEM of triplicate determinations

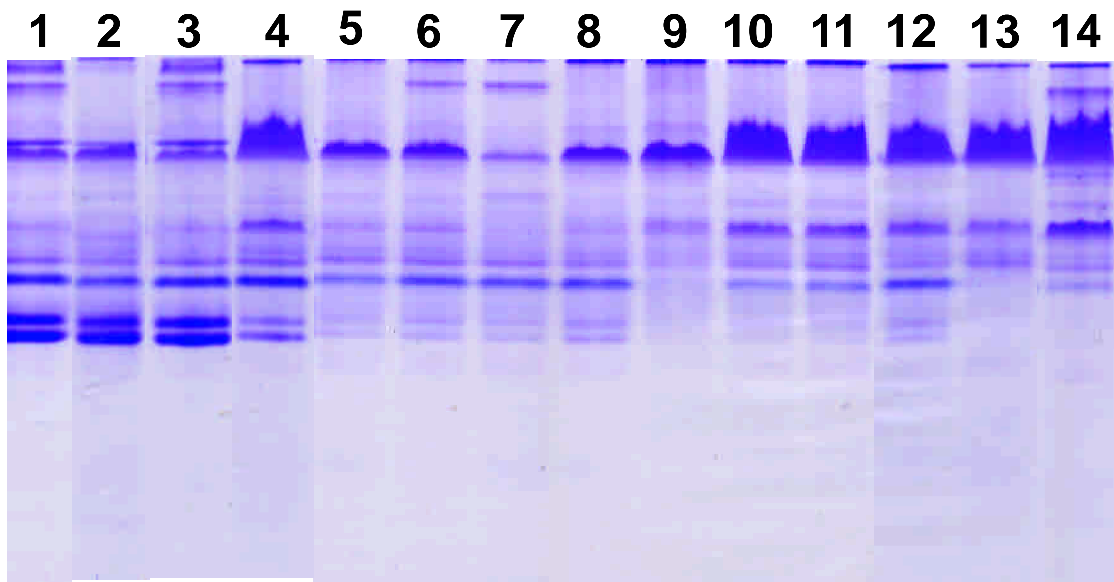


Figure 7

Beta-lactoglobulin and its novel receptor

ABSTRACT

β-lactoglobulin (β-LG) is a major protein moiety of bovine whey proteins; β-LG comprises about half the whey or about 10~15 % of total milk proteins. β-LG is sensitive to thermal denaturation, forming dimer and polymers upon heating beyond 80 ℃ over time. We previously investigated some major physical and biochemical properties of heated β-LG, but its physiological effects have not been elucidated. Here we show that β-LG possesses a potent activity for cell proliferation of hybridoma lymphocytes, but thermally denatured β-LG shows no such effect. After removal of β-LG from milk proteins on an antibody-affinity column, the proliferation activity of β-LG-deficient milk was much less than that activity of whole milk. To study further the influence of β-LG conformation on cell proliferation, we modified chemically, through carboxymethylation and acetylation, the β-LG to disrupt the disulfide linkages; the proliferation activity was not observed. Hence the conformation of β-LG plays a key role in inducing cell proliferation. To demonstrate that β-LG might stimulate cell proliferation via a receptor-mediated process, using flow cytometry and confocal microscopy we showed that β-LG binds to a cell surface. We also isolated and identified the β-LG receptor using both HPLC and an β-LG affinity column. β-LG increased the cyclin A and D2 expression, which participates in phase G2 of a cell cycle. Hence β-LG acts as a 'growth factor' in lymphocytes via a receptor-mediated

mechanism.

INTRODUCTION

The biological function of β-LG has not been studied profoundly (Qi et al., 1995; Sawyer et al., 2000; Chen et al., 2005). According to previous work, β-LG is capable of binding with vitamins A and D, palimic acid and other hydrophobic compounds (Song et al., 2005; Qiwu et al., 1997; Kontopidis et al., 2004; Narayan et al., 1997; Wu et al., 1999; Qin et al., 1998b) because of its calyx structure that forms a central hydrophobic cavity (Sawyer et al., 1985). According to the three-dimensional crystallographic measurements, β-LG contains nine antiparallel β-strands from A to I, and one α-helix (Qin et al., 1998, 1999; Kuwata et al., 1999); topographically, strands A–D form one surface of the barrel (calyx), and strands E–H form the other. The only α-helix structure with three turns occurs at the COOH terminus, which follows strand H lying on the outer surface of the calyx (Uhrinova et al., 2000). Chen et al. showed that β-LG undergoes conformational changes on being overheated (Chen et al., 2004, 2005). Song et al. proved a large increase in β-LG immunoreactivity when raw milk was heated between 70 and 80 °C. The structure of β-LG deteriorated totally after heating for one minute, and the ligand-binding ability was also eliminated (Song et al., 2005). Our immunoassay using a monoclonal antibody specific to native β-LG. proves that there is

no native β-LG present after heating at 95 °C for 2 min. The reason is that, apart from self aggregation, β-LG forms aggregates with casein, α-lactoalbumin and other milk proteins (Chen et al., 2006). The aggregation of β-LG caused by heat was confirmed to be mediated by a hydrophobic effect and a disulfide bond linkage (Havea et al., 2002).

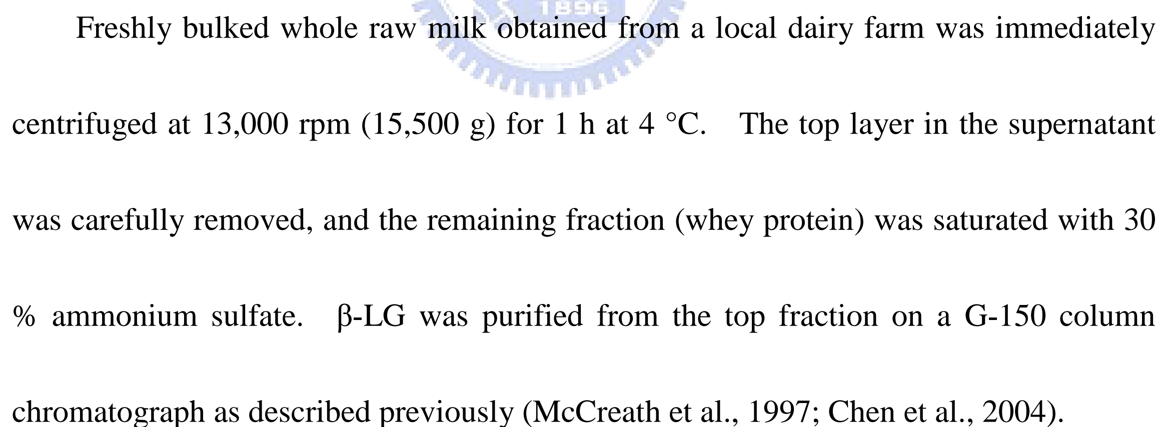
The medium used to culture cells contains basic ingredients to ensure cell survival (carbohydrates, minerals salts, ions, amino acids and vitamins). Fetal calf serum (FCS) or bovine calf serum (BCS) is generally added to promote cell growth, but both FCS and BCS are expensive. For this reason, many serum substitutes have been tested, including milk fractions and dairy by-products. Several authors have shown that bovine whey stimulates DNA synthesis leading to short-term proliferation of hybridoma immunoglobulin secretion, and long-term cryoconservation (Damerji et al., 1988; Guimont et al., 1997; Derouiche et al., 1989; Sereni and Basergha, 1981). Belford et al demonstrated that bovine whey is a source of potent growth-promoting activity for all mesodermal-derived cells tested, including human skin and embryonic lung fibroblasts, Balb/c 3T3 fibroblasts, and rat L6 myoblasts (Belford et al., 1995). To substitute for serum, Ramirez and Derouiche used bovine milk and bovine whey proteins to culture hybridoma cells and showed that bovine milk has the function to stimulate cell proliferation; milk is thus regarded as a potent substitute for serum (Derouiche et al., 1990; Ramirez et al., 1990). Although casein, α-lactoalbumin and β-LG are major

proteins in milk, Derouiche et al. showed that whey proteins are the most important ingredients for milk to stimulate cell proliferation (Derouiche et al., 1990), and β-LG is the major protein in whey proteins. In the present work we therefore focused on demonstrating that β-LG is the most important protein in milk to stimulate cell proliferation; we proved that the cell proliferation activity of thermally denatured β-LG becomes totally suppressed. We demonstrated also that β-LG stimulates cell proliferation via a receptor-mediated mechanism.

MATERIALS AND METHODS

Materials

Freshly bulked whole raw milk obtained from a local dairy farm was immediately centrifuged at 13,000 rpm (15,500 g) for 1 h at 4 °C. The top layer in the supernatant was carefully removed, and the remaining fraction (whey protein) was saturated with 30 % ammonium sulfate. β-LG was purified from the top fraction on a G-150 column chromatograph as described previously (McCreath et al., 1997; Chen et al., 2004).



Acetylation and Carboxymethylation of β-LG

β-LG was chemically modified by acetylation similar to a previously described method (Song et al., 2005; Mao et al., 1980). β-LG (2 mL) in aqueous sodium

bicarbonate (50 mM, pH 8.0) containing urea (6 M) and acetic anhydride (5 μL) were slowly added into the reaction mixture, while maintaining the pH at 8.0 using NaOH (0.1 M). After incubation for 3 h near 23 °C, the acetylated protein was desalted on a column (Bio-gel P-2), eluted with ammonium bicarbonate (0.05 M) and lyophilized.

For carboxymethylation (Song et al., 2005; Tseng et al., 2004; Mao et al., 1980), β-LG (5 mg) was first dissolved in Tris-HCl buffer (5 mL, 0.1 M, pH 8.6) containing ultra-pure urea (6 M) and 2-mercaptoethanol (0.02 M). After flushing with nitrogen, iodoacetic acid (20 mg) was added into the reaction mixture, while maintaining the pH at 8.6 through addition of NaOH (0.1 M); incubation followed for another 3 h. Carboxymethylated (CM) β-LG was desalted on a column (Bio-Gel P2) eluted with ammonium bicarbonate (0.05 M) and lyophilized. According to analysis of amino acids, the CM-β-LG contained 4.9 residues of CM-cysteine per mole of β-LG.

Cell culture

The cells used were hybridoma cells against Prostatic-specific Antigen. Cells were cultured in DMEM medium containing L-glutamine (10 %, Boehringer, M12-702, Mannheim, Germany), BCS (10 %, Jacques Boy, Reims, France), HT supplement (10 %), penicillin (100 U/mL) and streptomycin (100 U/mL) at 37 °C in a CO_2 (5 %) atmosphere at 99 % humidity. The culture medium was changed every two days, and

cells in the exponential phase of growth, from cultures with a minimum density 1×10^6 cells/mL, were collected.

MTT assay

Cells were treated with protein of each kind (β-LG, carboxymethylated β-LG, acetylated β-LG, and heated β-LG), in addition to culture medium. Cells were seeded at a density 1×10^4 cells/well on each coated well. The plates were incubated at 37 ^{o}C in a humidified atmosphere (CO_2, 5 %). After predetermined periods, cell layers were rinsed with PBS, and 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution (50 mL, 12mM) was added to each well. After incubation for 4 h at 37 ^{o}C, the MTT solution was removed, and the insoluble formazan crystals that formed were dissolved in dimethylsulfoxide (DMSO, 150 mL). The absorbance of the formazan product was measured at 540 nm using a 96-well plate spectrophotometer (Pariente et al., 2000; Zange et al., 1998).

Preparation of FITC-(β-LG) conjugate

β-LG (Sigma, St. Louis, MO, USA) was dissolved in aqueous sodium bicarbonate (0.1 M, pH 9.6). FITC (Sigma, St. Louis, MO, USA) was dissolved in dimethylsulfoxide. The β-LG and FITC solutions were mixed (final volume 2 mL) and incubated for 90 min

near 23 °C in the dark with constant stirring. The FITC-(β-LG) conjugate was separated from free FITC by dialysis over phosphate-buffered saline (PBS, pH 7.4, Pharmacia, Sweden) and fractions (2 mL) were collected.

Flow cytometry

Samples (1×10^7 cells) were incubated with FITC-(β-LG) at various concentrations (0.0625, 0.125, 0.25, 0.5, and 1 mg) at a designed temperature for 30 min, followed by analysis with a flow cytometer (XL, Coultronics-Margency, France) equipped with an argon laser set at 488 nm. Green fluorescence was processed with a filter (bandpass 520–530 nm). Fluorescence was displayed as a monoparametric histogram (256 channels logarithmic scale) and is expressed as the mean intensity of fluorescence (MIF): $\mathrm{MIF} = e^{[(\ln 1000:256)x]}$; x is the mean peak channel on a logarithmic scale. In total, 1×10^4 cells were analyzed for each assay. Viable cells were selected using the biparametric histogram FLS × 90LS (size × granulometry).

Confocal Microscopy

Samples (1×10^7 cells) were incubated with FITC-(β-LG) (1 mg) at a designed temperature for 30 min. The cells were centrifuged and washed with PBS (pH 7.4) three times. The sample was fixed with paraformaldehyde (4 %) for 30 min and

washed with PBS (pH 7.4). Samples were then analyzed with a confocal microscope; optical sections were obtained with an epifluorescence inverted microscope (Olympus IX-70), equipped with a cell scan (EPR™ system, Scanalytics, Billerica, MA) (Isenberg et al., 1996) and a water immersion apochromatic lens (60 × PSF: 1.2-NA, Olympus, France). Scanning along the optical axis was performed with a piezoelectric *z*-axis focus device (*z* spacing 0.25 mm). Images were collected on a cooled charge-coupled device camera (12 bits, Princeton Instruments, USA). With a filter set (WIB cube, Olympus) we selected the fluorescence excitation (460–490 nm) and the integral part of the emission spectrum (BA515). An image intensity-calibration kit (InSpeck, Molecular Probes, Eugene City, OR) served for calibration. A blank image of the detector dark current and the background were removed from each image acquired pixel by pixel.

β-LG receptor purification

The murine hybridoma cell line used in this work was against Prostatic-specific Antigen. These hybridoma cells were routinely cultured in DMEM supplemented with Bovine Calf Serum (10 %, vol/vol). The plasma membrane-enriched preparations were obtained from the cultured hybrid cells. Approximately 1×10^6 cells/mL were disrupted in PBS (ice-cold, pH 7.4) containing PMSF (1 %) and tween-20 (1 %). The

homogenate was sonicated to facilitate release of membrane protein, followed by centrifugation (5 min at 10000 g), and the supernatant was collected. This cell lysate was purified with HPLC using ammonium bicarbonate (50 mM) as solvent system. β-LG receptor was then purified on an affinity column coated with β-LG.

Gel electrophoresis

Sodium dodecyl sulfate-PAGE (SDS-PAGE) containing polyacrylamide (15 % mass/vol) was used to characterize the receptor using a modified procedure (Chen et al., 2005; Yang and Mao, 1999) similar to that described (Chen et al., 2005; Oldfield et al., 1998). All samples (5 μg) for SDS-PAGE were mixed with loading buffer (12 mM Tris-HCl, pH 6.8, glycerol 5 % vol/vol, bromophenol blue 0.02% mass/vol) with or without 2-mercaptoethanol (2.88 mM) and run for approximately 1.5 h at 120 V.

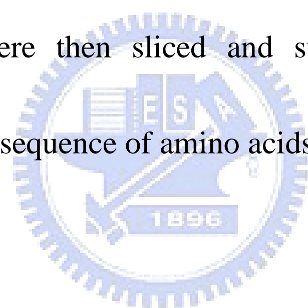
CD Spectrum

The secondary structure of β-LG and its receptor was determined with a computerized spectropolarimeter (JASCO J-715 CD). Each protein sample was dissolved in phosphate buffer (10 mM, pH 7.0) with a final concentration 0.2 mg/mL. Protein solution (about 300 μL) was used for analysis within a cuvette (path length 1 mm). The obtained spectra were accumulated 16 times at a scanning rate 50 nm/min.

All data are shown as the mean residual molar ellipticity [Θ] MRW (Tseng et al., 2004; Chen et al., 1994).

N-Terminal Amino Acid Sequencing

β-LG receptor identified on SDS-PAGE was sequenced from the N-terminus with an automatic Edman degradation procedure on a peptide sequencer (ABI 476A) described previously (Yang and Mao, 1999). The protein bands separated on SDS-PAGE gel were transferred onto a PVDF membrane; the protein bands corresponding to the expected β-LG receptor were then sliced and subjected to an automatic Edman degradation to determine the sequence of amino acids.



Total RNA isolation and RT-PCR

Total RNA was isolated from cultured hybridoma cells grown to confluency in Petri dishes (60 mm). TRIzol reagent (Invitrogen) was used according to the manufacturer's instructions as an improvement to the single-step RNA-isolation method developed by Chomczynski and Sacchi (1986). RNA samples were incubated for 15 min at 29 °C with DNAse I mixture in the presence of RNase inhibitor to digest any contaminating genomic DNA. The RNA samples were then transferred onto ice, and EDTA (25 mM, pH 8.0) was added to each tube. After incubation (5 min, 75 °C), the PCR tubes were

immediately placed on ice again. A concentrated reagent mixture, containing PCR buffer (10x), dNTP (10 mM), DTT (0.1 M), RNAse inhibitor and random hexamers (5 ng/μL), was added to the reaction tubes. The reaction mixture was heated for 3 min at 42 °C. Super-reverse Transcriptase (50 U, Invitrogen) was added, and incubation continued for another 60 min. The enzyme was then inactivated on heating the reaction mixture for 10 min at 65 °C. The RT reaction products were stored at -20 °C until use in PCR.

We used the previously published sequences of oligonucleotide primers for cyclin A-E. Aliquots (10 μL) containing 5' and 3' primers (10 pmol of each) were added to the RT reaction mixture (10 μL) and overlaid with mineral oil (30 μL). The reaction tubes were placed in a thermocycler block (iCycler, BioRad, Hercules, CA), and heated for 2 min at 94 °C. The mixture (5 μL) containing dNTPs and *Taq* polymerase (1.5 U, Invitrogen) was then added. The final concentrations of all components were as follows: PCR buffer without Mg^{2+} (1x, Roche), each dNTP (200 μM, Invitrogen), $MgCl_2$ (2.0 mM), each primer (1 μM), and *Taq* polymerase (30 mU/μL). Reactions were set for 38 cycles. Denaturation temperature was set at 94 °C, elongation temperature 72°C, and annealing temperature 60°C.

The PCR products (9 μL each) were separated on an agarose gel (2 %) preloaded with ethidium bromide (1 μg/mL), and visualized under ultraviolet light. The molecular

masses of the PCR products were compared to a 100-bp DNA ladder (Invitrogen). Identities of the PCR products were confirmed by sequencing. Controls for the DNA contamination were as described above but excluding the reverse transcriptase from the reaction mixture.

RESULTS

The biological function of β-LG has not been studied profoundly. We demonstrated previously that the structure of β-LG was totally deteriorated after heating above 80℃ for 1 min (Chen et al., 2005, 2006). β-LG is capable of binding with vitamins A and D, palimic acid and other hydrophobic substances, but thermally denatured β-LG loses its ligand-binding ability (Song et al., 2005). In the present work we utilized β-LG to treat hybridoma cell and proved that β-LG can stimulate cell proliferation; thermally denatured β-LG lacked this effect.

β-LG can stimulate cell proliferation

To prove that β-LG has the ability to stimulate cell proliferation, we treated hybridoma cells with β-LG at various concentrations, and evaluated the cell number using MTT assay after incubation for 72 h. The results appear in figure 1, which indicates that, with increasing concentration of β-LG treatment, the cell growth numbers correlated with the dosage. Furthermore, thermally denatured β-LG (heated at 95 ℃ for 5 min)

showed no cell growth-promoting activity. The maximum dose of β-LG to stimulate cell proliferation is 5 mg/mL (figure 2); the cell number increases with increasing concentration of β-LG, but the maximum number of cells was attained at a β-LG concentration 5 mg/mL. These data prove that β-LG has the ability to stimulate hybridoma cell proliferation.

β-LG is the major protein in milk capable of stimulating cell proliferation

The protein content of bovine milk is relatively stable; it contains about 85 % casein, which is the major protein in milk, and about 10 % β-LG, the dominant protein in milk whey (Chen et al., 2005; Wang and Lucey, 2003; de Jongh et al., 2001; Braunschweig et al., 2000). To demonstrate that β-LG is the major protein in milk that acts as a potent substance to stimulate cell proliferation, we used an affinity column coated with anti-β-LG antibody to remove β-LG from milk. Following treatment of hybridoma cells with normal milk and milk deprived of β-LG, the results showed that, even though milk deprived of β-LG can stimulate cell proliferation, the ability was greatly inferior to milk with β-LG (Figure 3): β-LG is thus the major protein in milk to stimulate cell proliferation.

β-LG with altered conformation (carboxymethylation and acetylation) lost its

ability to stimulate cell proliferation

According to our previous research, β-LG might lose its biological function on alteration of its conformation. To demonstrate also that conformationally altered β-LG loses its ability to stimulate cell proliferation, we used carboxymethylated and acetylated β-LG to treat hybridoma cells. The results show that the growth-promoting activity of chemically modified β-LG was totally eliminated. This result correlates with thermally denatured β-LG, thus proving that conformation plays an important role in stimulating cell proliferation (Figure 4). We thus suggest that β-LG stimulates cell proliferation by binding with its membrane receptor through conformational interactions, so inducing signal transduction in a series resulting in cell proliferation. Acetylated β-LG typically replaced its lysine group with an acetyl group, thus neutralizing the positive charge of its lysine group. Acetylated β-LG thus loses its ability to bind with a receptor because of ionic factors, and the cell growth-promoting activity also deteriorated. Hence β-LG and receptor are bound through a charge interaction.

Hybridoma cells process β-LG receptor on the membrane surface

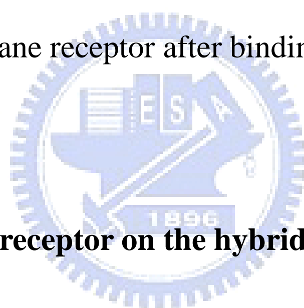
To verify the hypothesis that hybridoma cells process β-LG receptor on its membrane surface, we used a confocal microscope to observe and to analyze the binding of β-LG and its receptor. FITC-labeled (1 mg/mL) β-LG was incubated with hybridoma cells at

4 ℃, and was found to assemble on the cell surface (Figure 5). We applied the same strategy to analyze CHO cells, but the FITC-labeled β-LG did not assemble on the cell surface as it did on hybridoma cells (data not shown). Separately, we incubated hybridoma cells with FITC-labeled β-LG at varied doses, and analyzed the cells using flow cytometry; as the treatment dose increased, the fluorescence intensity detected from the hybridoma cells likewise increased (Figure 6). This result verifies that β-LG can definitely bind with its receptor on hybridoma cells, and was transported into cells by this receptor. To delineate further the localization of this receptor, we purified β-LG receptor from a cell membrane fraction, and immunized a rabbit with this receptor to develop a polyclonal antibody specific to β-LG receptor. Following labeling of this antibody with FITC, we analyzed the localization using a confocal microscope. These data indicate that this antibody binds on the cell membrane surface (Figure7). These data confirm that β-LG can bind with its receptor located on the cell membrane surface.

β-LG transports into cell by a receptor-mediated pathway

To confirm that β-LG transport into hybridoma cell occurs by a receptor-mediated pathway, we utilized the characteristic that cells undergo an abated metabolic activity at low temperature. FITC-labeled β-LG was incubated with hybridoma cells at various temperatures; we then adopted both a confocal microscope and flow cytometry for

analysis. The results show that, when hybridoma cells were incubated with FITC-labeled β-LG at 4 ℃ for 30 min, the fluorescence showed neither on the cell surface nor in the cytoplasm; when the hybridoma cells were first incubated with FITC-labeled β-LG at 37 ℃ for 5 min, and then incubated at 4℃ for another 25 min, the fluorescence showed both on the cell membrane and in the cytoplasm but at a small intensity; when the Hybridoma cells were incubated with FITC-labeled β-LG at 37 ℃ for 30 min, the fluorescence showed mainly in the cytoplasm with large intensity (Figure 8). These results indicate that FITC-labeled β-LG was transported into the cell cytoplasm through its membrane receptor after binding with the receptor.



Purification of membrane receptor on the hybridoma cells

After demonstrating that hybridoma cells process β-LG receptor on the membrane with both a confocal microscope and flow cytometry, we developed an affinity column coated with β-LG to purify this receptor. Following analysis of this purified receptor with SDS-PAGE (Figure 9), the data showed a protein with molecular mass above 150 kDa. A sample treated with a reducing agent (β-ME) that breaks disulfide bonds shows two molecular masses, 50 and 25 kD, similar to the heavy and light chains, respectively, of IgG. The secondary structure of the β-LG receptor according to circular dichroic spectra (Figure 10) is predicted to be 8 % α-helix and 48 % β-sheet from parameters of

three-dimensional PSSM (the folding recognition server at the Imperial Cancer Research Fund, ICRF; www.sbg.bio.ic.ac.uk/_3dpssm). We analyzed this purified protein with mass spectrometry; these data reveal a membrane IgM after alignment.

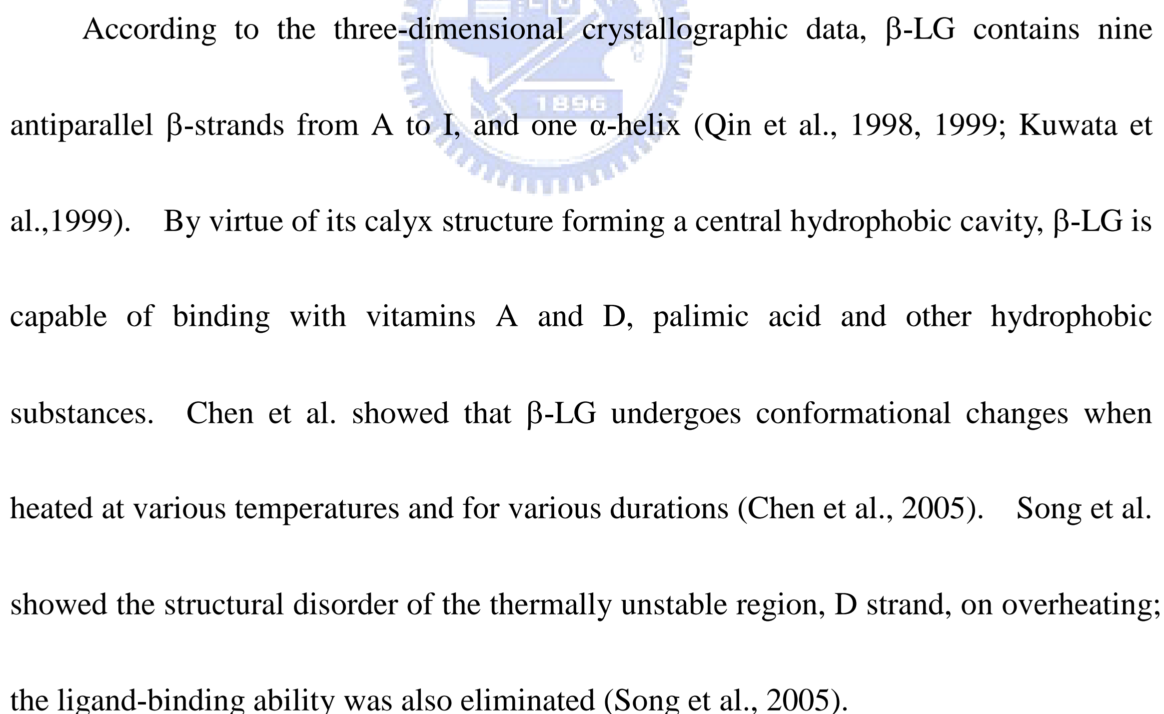
β-LG stimulates cyclin A gene expression in hybridoma cells

The cell cycle is distinguishable into four main phases – G1, S, G2 and M. Among these phases, the major protein cyclin D dominates the G1 phase; cyclin E prevails in the S phase, cyclin A in the G2 phase, and cyclin B in the M phase. We thus identify the cell phase by analyzing the cyclin gene expression (Figure11); the results show that only the expression of cyclins A and D2 increased in correlation with the treatment dose of β-LG. These data thus reveal that β-LG stimulates cyclin D2 gene expression that initiates the cell cycle and activates cyclin A expression rendering the cells into the G2 phase, thus resulting in cell proliferation.

DISCUSSION

The protein content of bovine milk is relatively stable; it contains about 85 % casein, which is the major protein in milk, and about 10 % β-LG, the dominant protein in milk whey (Barbano et al., 2006; Meisel, 2005). In previous research, bovine whey protein was used to substitute for serum to culture cells, so showing that milk protein has

the function of stimulating cell proliferation, so that milk might be regarded as a potent substitute for serum (Xu et al., 2005; Wong et al., 1998). As the major whey protein, β-LG might be the major protein in milk whey to stimulate cell proliferation. We have here demonstrated that β-LG has the function of stimulating cell proliferation (Figure 1), and we further proved that β-LG is the major protein in milk responsible for cell proliferation (Figure 3). With increased treatment dose of β-LG, the cell proliferation phenomena become significant. (Figure 2) Previous research has claimed that β-LG can substitute for the function of IgG in a bovine calf after the early post-partum period (Levieux et al., 1999; Kilshaw et al., 1981).



According to the three-dimensional crystallographic data, β-LG contains nine antiparallel β-strands from A to I, and one α-helix (Qin et al., 1998, 1999; Kuwata et al.,1999). By virtue of its calyx structure forming a central hydrophobic cavity, β-LG is capable of binding with vitamins A and D, palimic acid and other hydrophobic substances. Chen et al. showed that β-LG undergoes conformational changes when heated at various temperatures and for various durations (Chen et al., 2005). Song et al. showed the structural disorder of the thermally unstable region, D strand, on overheating; the ligand-binding ability was also eliminated (Song et al., 2005).

In our current research, we showed that β-LG has an activity to promote the growth of hybridoma cells, and thermally denatured β-LG loses this ability. (Figure 1)

Disrupting the tertiary structure of β-LG with carboxymethylation to modify the SH group to form a carboxyl group and thus interfering with the β-LG native calyx structure, we proved that conformation is an important factor for β-LG to stimulate cell proliferation (Figure 4). Hence the structure of β-LG is an important factor for both binding with hydrophobic substances and stimulating cell proliferation.

Cell proliferation is caused mainly by signal transduction once its receptor is bound by comparable ligands. β-LG employs the same strategy in stimulating hybridoma cells (Figure 5, 6). Using a confocal microscope and flow cytometry, we showed that FITC-labeled β-LG binds on the cell surface, thus demonstrating hybridoma cells to process the β-LG receptor on the cell surface. We developed an antibody specific to the β-LG receptor, which also shows on the cell surface (Figure 7), and adopted acetylation to replace a lysine group with an acetyl group, so to neutralize the positive charges of the lysine group. This result shows that acetylated β-LG loses its binding ability with receptor because of being unable to maintain an interaction with a negatively charged receptor (Figure 4). β-LG and the cell membrane receptor might associate through a positively charged lysine group in β-LG and negative charges carried by the receptor, because of the diminution of positive charge on β-LG that renders it to lose its ability to stimulate cell proliferation. According to figure 5, β-LG is transported into a cell by a receptor-mediated pathway after binding with its receptor. As the treatment

dose of β-LG increases, the amount of β-LG in the cytoplasm increases correspondingly. This phenomenon explains how β-LG proliferates the uptake of hydrophobic substances because of its ligand-binding ability and the receptor-mediated transport. Darren et al. indicated that β-LG can increase the retinal uptake through the intestine of a mature rat, and also detected intact β-LG in the blood of children two hours after milk feeding (Darren et al., 2000). β-LG therefore not only binds on the cell membrane receptor but also undergoes internalization proliferated by its receptor. This receptor-mediated mechanism of transport was also adopted by LDL and LDL receptor (Meddings et al., 1987; Habenicht et al., 1992; Wyne et al., 1992; Rodenburg et al., 2005).

As the β-LG receptor has not yet been isolated, we used an affinity column coated with β-LG to purify and to identify this receptor (Figure 9); this practice is previously unreported. This receptor was identified as the membrane IgM according to its mass spectrum. Generally, B lymphocytes adopt IgM or IgD as an antigen receptor. Interaction between these receptors and their ligands might cause a series of biochemical reactions, such as initiating gene transcription, anchorage of a receptor to the cytoskeleton, receptor endocytosis, antigen presentation, differentiation and cellular proliferation. Hybridoma cells are fused by FO cells and spleenocyte; hybridoma cells thus carry B the lymphocyte receptor. Taken together, β-LG stimulates cell proliferation through binding on the membrane IgM that initiates internalization and then

promotes cell growth.

A cell cycle has four main stages – G1, S, G2 and M phases. At each stage, cells accompany specific proteins that process their own function. In phase G1, there exists a restrictive point that acts as a checkpoint for cell division. This point is regulated by two major proteins – an etinoblastoma susceptibility protein (pRb) and a family of essential transcription factors (E2F) (Lomazzi et al., 2002; Willers et al., 2000). Interaction of these two proteins with target promoters turns off many genes and blocks the cell cycle progression. To pass the restrictive point, the cell must phosphorylate pRb, which then dissociates E2F (Seville et al., 2005). This action allows E2F to activate cyclin E and cyclin A gene expression and to facilitate the cell cycle progression. Any cdk-cyclin pair involved in the cell cycle progression can phosphorylate pRb, but in phase G1 pRb is activated mainly by a Cdk4/6-cyclin D complex (Ouyang et al., 2005; Walker et al., 2005). In phase S, cyclin E is highly expressed and results in stimulating the transcription genes involved in DNA replication. Further, in the S/G2 transition, cyclin A accumulates and renders the cell cycle into phase G2 (Ji et al., 2005). In this phase G2, there exists another checkpoint to monitor the completion of DNA replication. After the confirmation, cells are allowed to enter phase M that is regulated by a cyclin B-Cdk1 complex (Visanji et al., 2005; Li et al., 2005). Hence cell proliferation is caused by initiating a cell cycle, and the most important protein involved in that cell

cycle is cyclin D, which is capable of initiating a cell cycle and activating other cyclin expression. We showed that β-LG stimulates cyclin D2 gene expression (Figure 11), and then starts the cell cycle and activates cyclin A gene expression. Glassford et al. demonstrated that the interaction of antigen and B cell receptor induces the cyclin D2 expression by signaling cascades dependent on Ca^{2+} and PKC that result in an initiating call cycle. We thus confirm that β-LG interacts with the hybridoma cell membrane receptor and then initiates the cell cycle resulting in cell proliferation.

We have demonstrated that β-LG is capable of stimulating cell proliferation, and proved that conformation-disrupted β-LG severely loses its function. We showed also that β-LG is the major protein in milk to stimulate cell proliferation. We incubated cells with FITC-labeled β-LG at various temperatures and used a confocal microscope to demonstrate that β-LG employs receptors to become transported into a cell. As for the mechanism, we proved that β-LG is transported into a cell after binding with its receptor, and promotes cell growth by activating cyclin D to trigger the cell cycle. We purified and analyzed this receptor that we regard as membrane IgM. We have demonstrated that β-LG binds with its receptor through an ionic interaction.

ACKNOWLEDGEMENT

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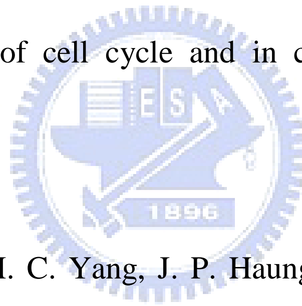

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CAPTIONS AND LEGENDS

Figure 1: Use of MTT assay to analyze the cell proliferation effect of β-LG, thermally denatured β-LG (HLG) and BSA. Treatment of hybridoma cells with various doses of β-LG, HLG and BSA, and analysis of cell proliferation effect with MTT assay after incubation for 72 h. These data indicate that only β-LG stimulates cell proliferation whereas HLG or BSA lacks this ability.

Figure 2: Dose-response curve of β-LG in stimulating cell proliferation. Treatment of

hybridoma cell with various doses of β-LG, and evaluation of the cell proliferation effect using MTT assay. β-LG can stimulate cell proliferation as a dose-dependent effect.

Figure 3: β-LG is the major protein in milk that stimulates cell proliferation. Treatment of hybridoma cells with various doses of raw milk and β-LG deficient milk, and then measurement of the cell proliferation effect using MTT assay. β-LG was removed from raw milk using an anti-LG affinity column.

Figure 4: Effect of thermally denatured (HLG), acetylated and carboxymethylated β-LG on cell proliferation; treatment of hybridoma cell with HLG (100 μg), acetylated LG and carboxymethylated LG, and then analysis of the cell proliferation effect using MTT assay. The data show that only β-LG can stimulate cell proliferation, whereas chemically modified β-LG shows no effect. The structure of β-LG plays an important role in stimulating cell proliferation.

Figure 5: Using a confocal microscope to demonstrate that β-LG binds to the cell surface. (A) Negative control. (B) LG (1 mg) was conjugated with FITC and bound to a cell membrane at 4℃ after incubation for 30 min.

Figure 6: Cell fluorescence histograms obtained with various concentrations of LG-FITC. The cells were incubated with LG-FITC at 0, 0625, 0.125, 0.25 and 0.5 mg and 37 ℃ for 30 min, before analysis of its fluorescence intensity using flow cytometry.

Figure 7: Localization of β-LG receptor using specific antibody. Hybridoma cells were incubated with antibody (1 mg) (A) purified from rabbit normal serum; (B) rabbit polyclonal antibody specific to β-LG receptor. Each antibody was conjugated with FITC and bound to the cell membrane at 4 ℃ after incubation for 30 min. The data show that β-LG receptor is located on the membrane surface.

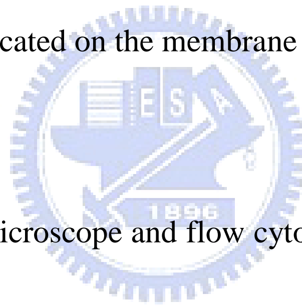


Figure 8: Using a confocal microscope and flow cytometry to analyze the internalization of the LG receptor-ligand complex. (A) Hybridoma cell was incubated with LG-FITC at 4 ℃ for 30 min. The fluorescence showed on the cell membrane. (B) Hybridoma cell was incubated with LG-FITC at 37 ℃ for 5 min; the incubation temperature was changed to 4 ℃ for another 25 min. The fluorescence showed both on the cell membrane and in the cytoplasm. (C) Hybridoma cell was incubated with LG-FITC at 37 ℃ for 30 min: the fluorescence showed in the cytoplasm.

Figure 9: SDS-PAGE profile of purified receptor. The receptor was purified using

LG-affinity column. (M) Marker. (A) Cell lysate. (B) LG receptor. (C) LG receptor. (with reducing agent)

Figure 10: Circular dichroic spectra of β-LG, β-LG receptor, and mouse IgG. Samples (final concentration 0.1 mg/mL) in phosphate buffer (10 mM, pH 7.4) were used for this test.

Figure 11: Cyclin A and cyclin D2 gene expression in β-LG inducted by RT-PCR. The cell was incubated with 0.125, 0.25, 0.5 and 1 mg of LG for 72 h before analysis of (A) cyclin A and (B) cyclin D2 gene expression with RT-PCR. The data show that, with increasing dose of β-LG, both the cyclin A and cyclin D2 gene expression increase.

Figure 1

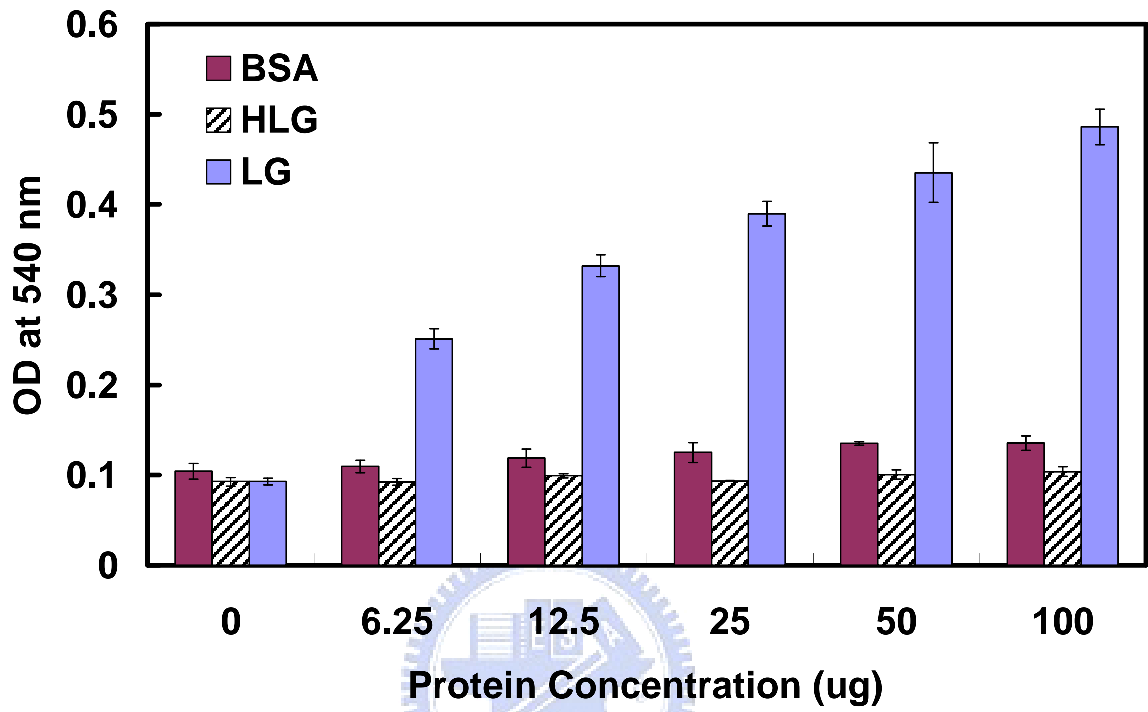


Figure 2

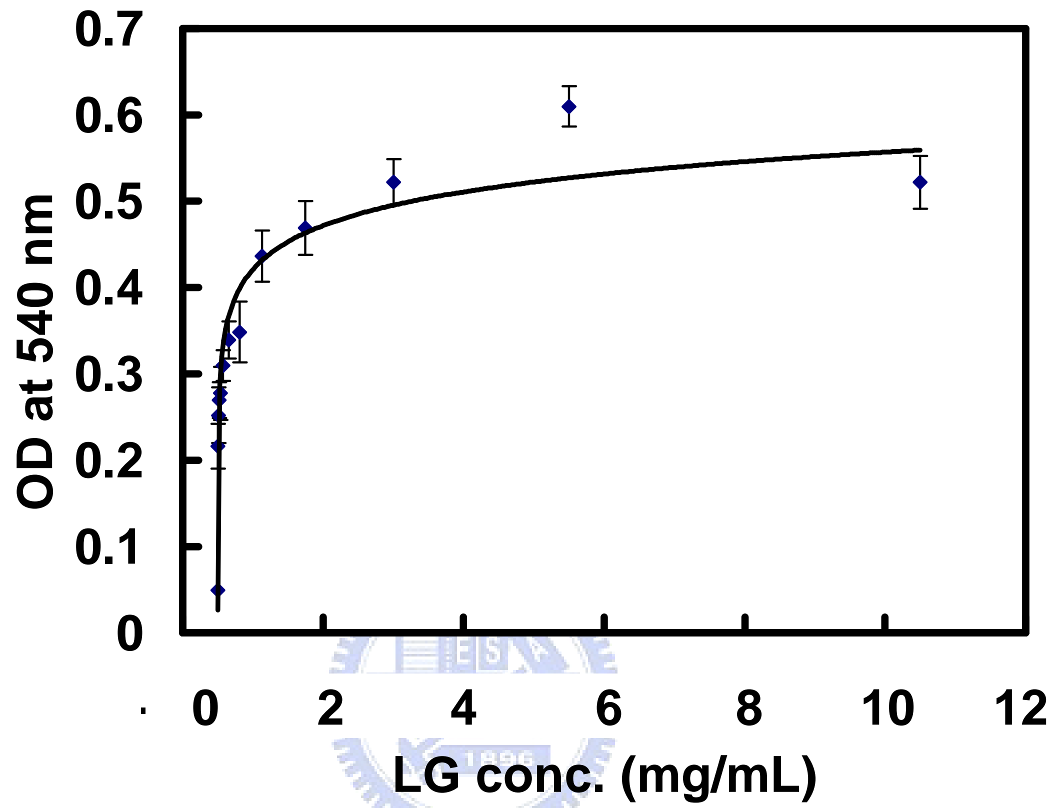


Figure 3

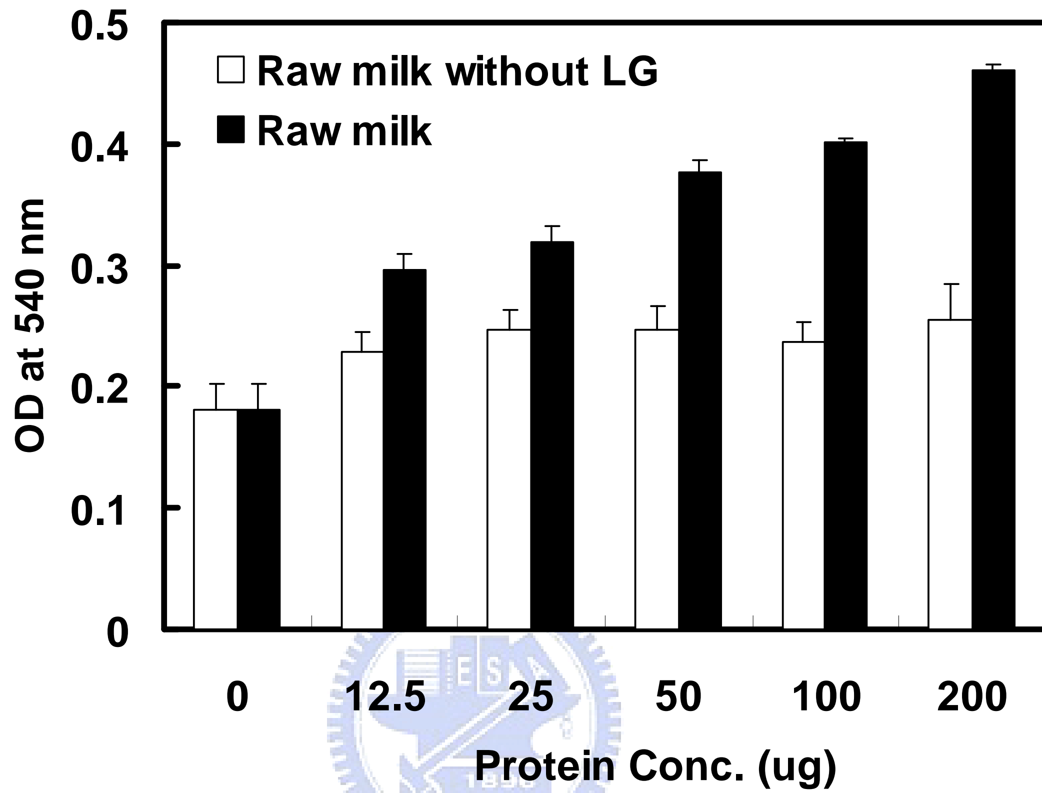


Figure 4

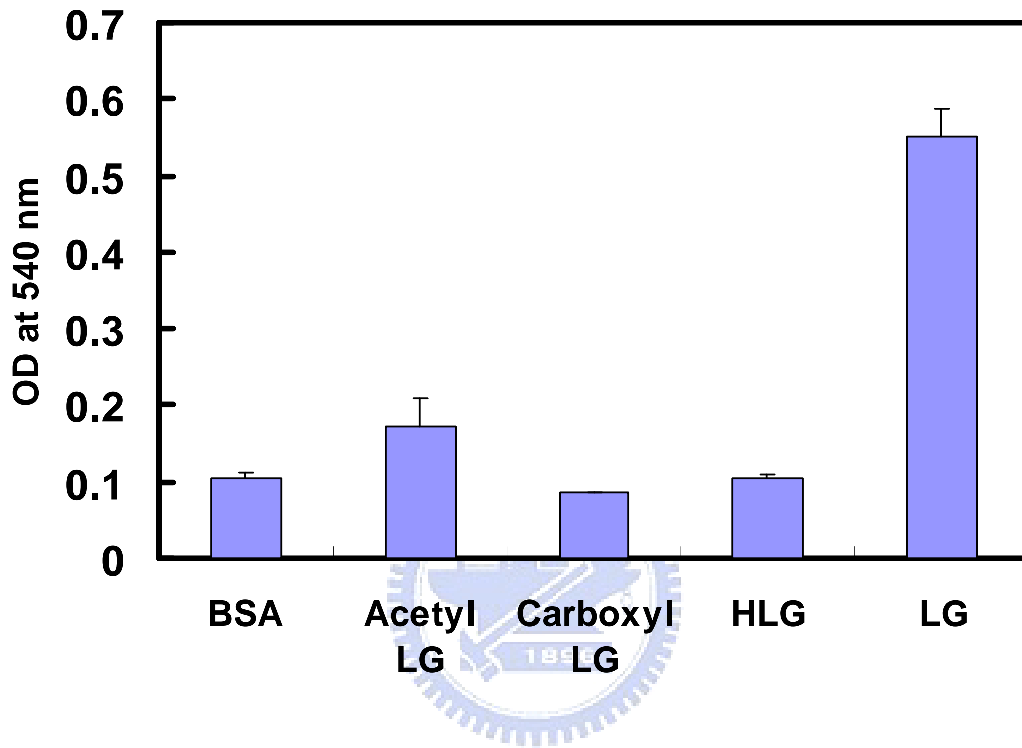
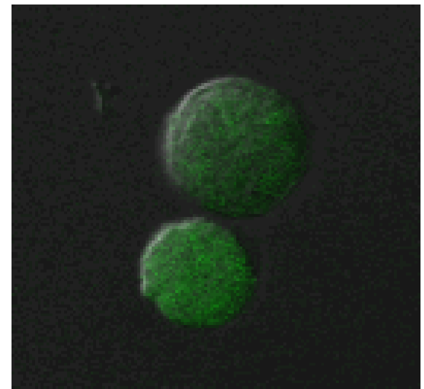


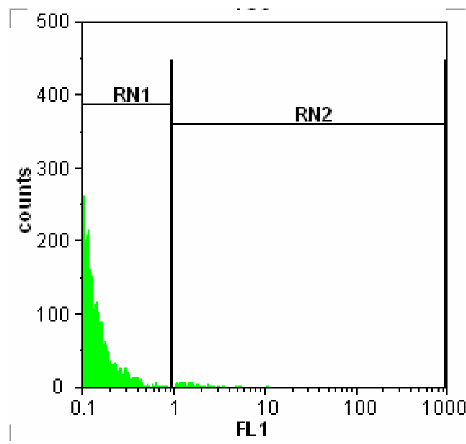
Figure 5



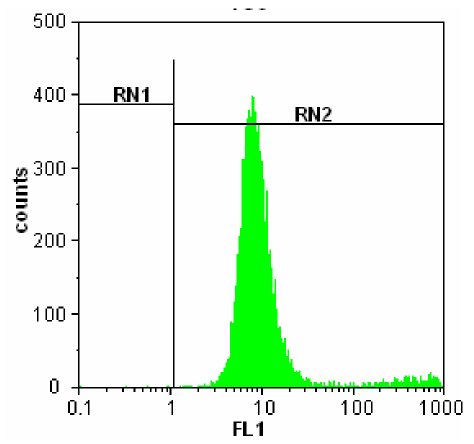
A. Negative control

B. Binding with LG conjugate FITC

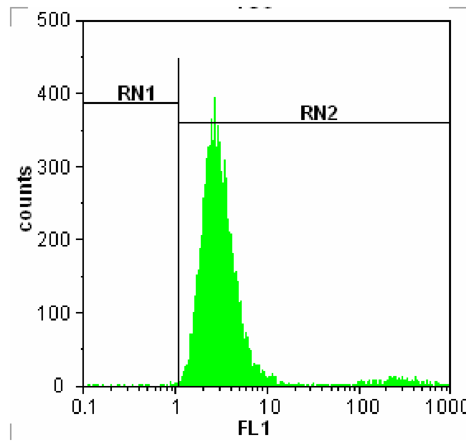
Figure 6



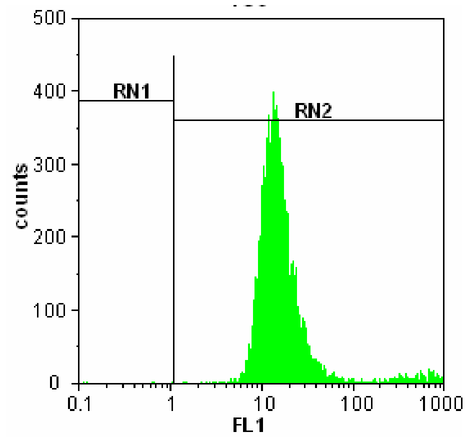
0 mg



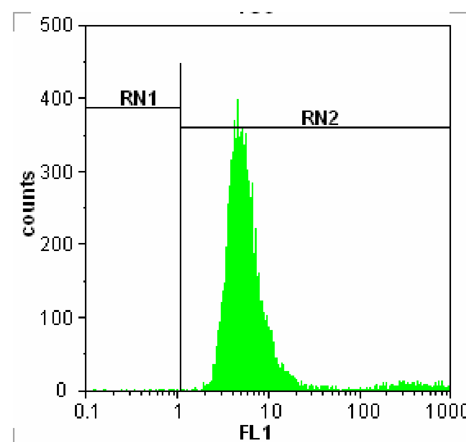
0.25 mg



0.0625 mg

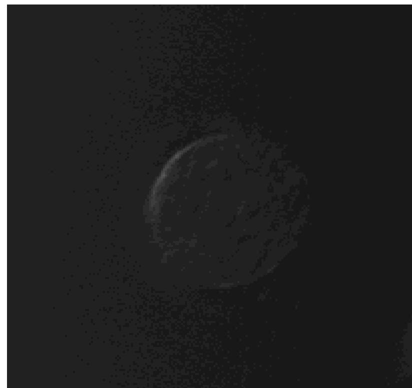


0.5 mg

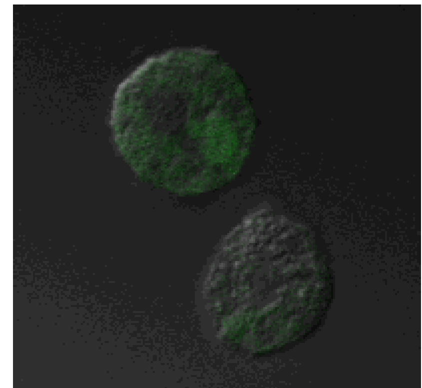


0.125 mg

Figure 7



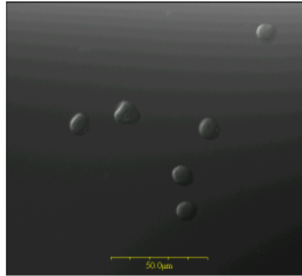
A. Normal IgG



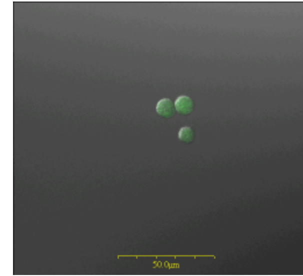
B. Anti-β-LG receptor IgG

Figure 8

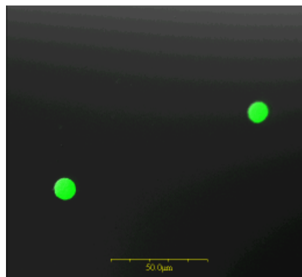
A. Confocal microscope



Incubated at 4 ℃

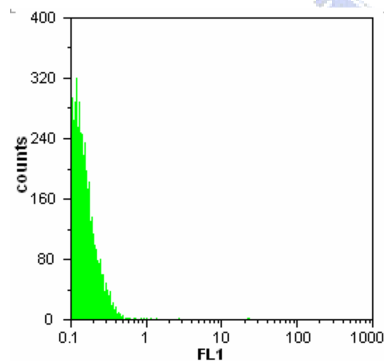


Incubated at 4 to 37 ℃

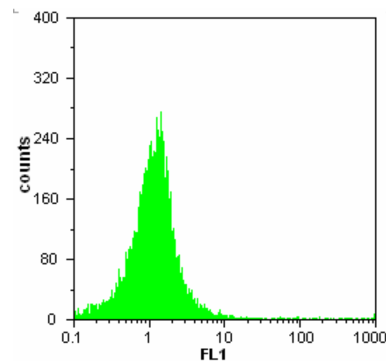


Incubated at 37 ℃

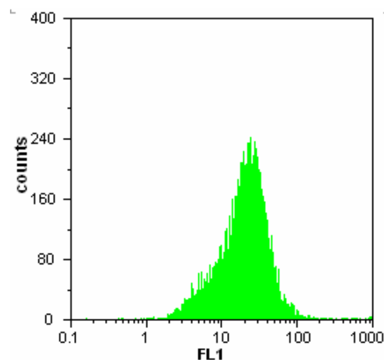
B. Flow cytometry



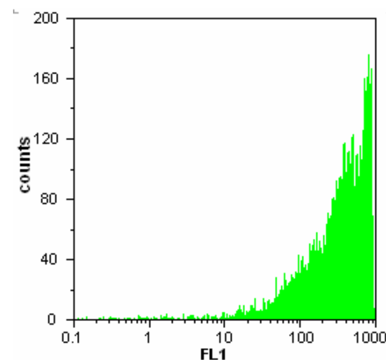
Negative control



Incubated at 4 ℃



Incubated at 4 to 37 ℃



Incubated at 37 ℃

Figure 9

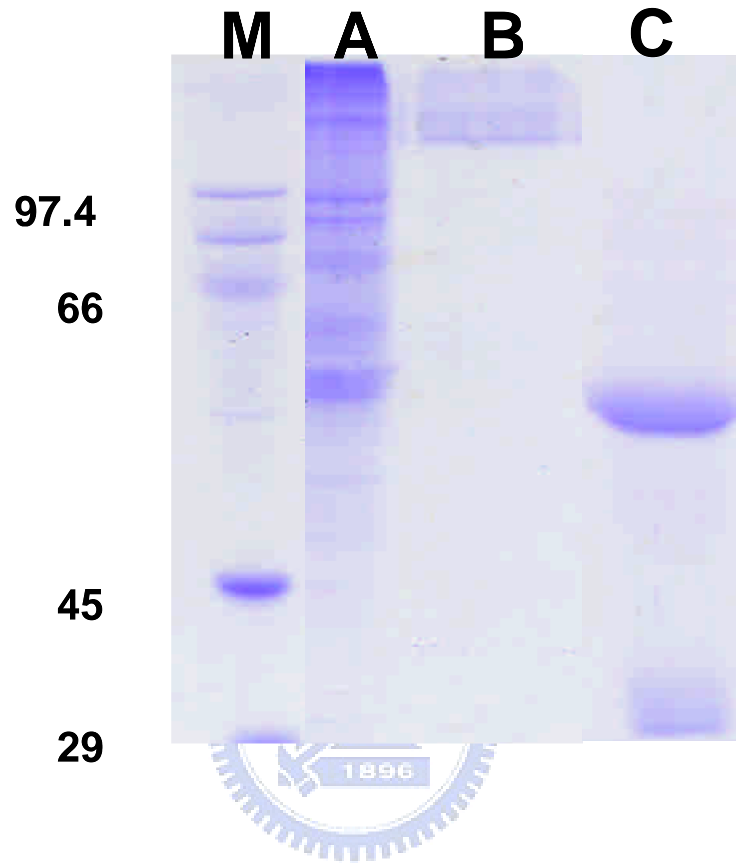


Figure 10

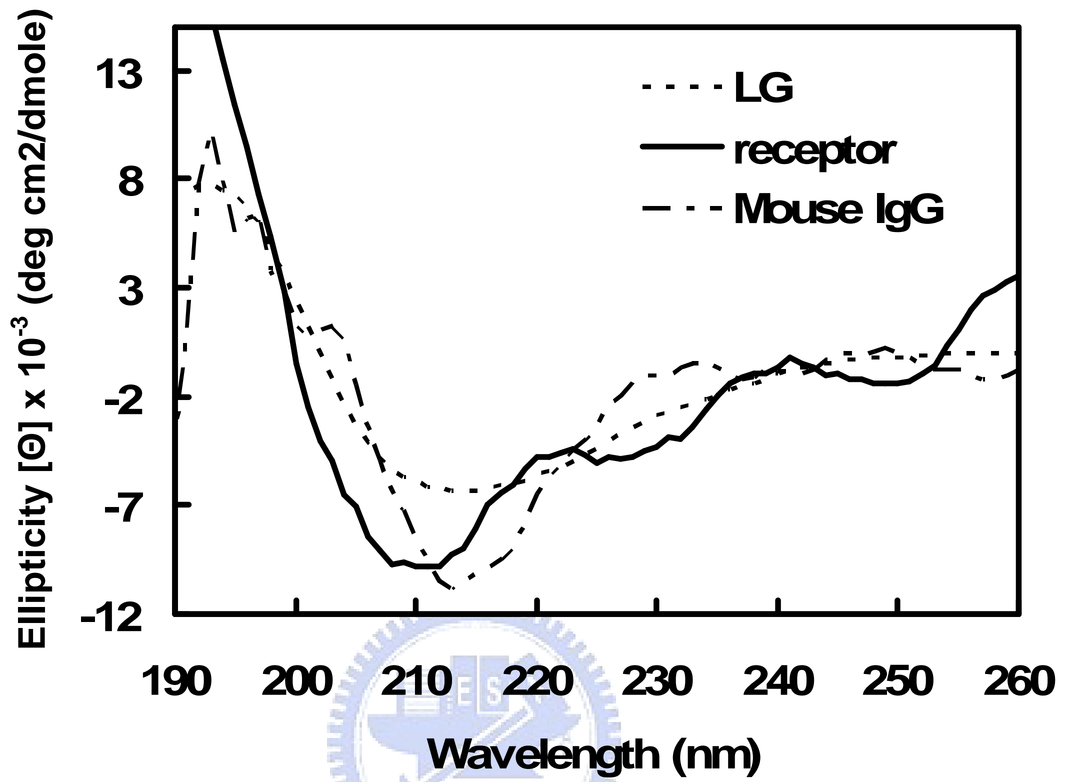
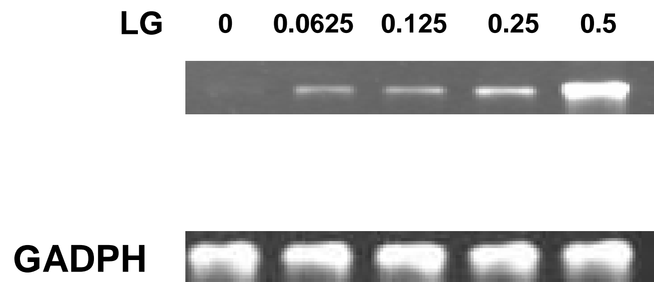
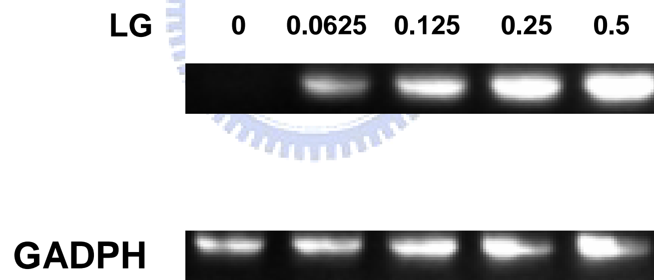


Figure 11

(A) Cyclin A



(B) Cyclin D2



Curriculum vitae

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EDUCATION

B.S.: Depsrtment of Animal Science, Tung Hai University 1996-2000

M.S.: College of Biological Science and Technology, Chiao Tung University 2000-2001

Ph.D.: College of Biological Science and Technology, Chiao Tung University 2001-2006

PUBLICATIONS

1. Liau CY, Chang TM, Pan JP, **Chen WL**, Mao SJT. Purification of human plasma haptoglobin by hemoglobin-affinity column chromatography. **J Chromatogr B**. 2003, 25;790:209-216. **(IF = 2.18, Subject categories: 19 / 70)**

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7. Yueh CH, Lai YA, Hsu HH, **Chen WL**, Mao SJT. Improved method for haptoglobin 1-1, 2-1, 2-2 purification using monoclonal antibody affinity chromatography in the presence of sodium dodecyl sulfate. 2006. **J Chromatogr B**. (Accepted) **(IF = 2.18, Subject categories: 19 / 70)**
8. Yang MC, Guan HH, Liu MY, Yang JM, **Chen WL**, and Mao SJT. Crystal structure of a secondary vitamin D3 binding site of milk beta-lactoglobulin. **PDB structure**, NO. 2GJ5.
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1. **Chen WL**, and Mao SJT. (2000) A simple method for the determination of plasma fibrinogen using a microtiter plate. **The fifteenth joint annual conference of biomedical sciences.** Programs & abstracts. P232

2. **Chen WL**, Ho JC, Hoang KC, and Mao SJT. (2001) Differentiation between fresh and powdered milk using chemical, physical, and immunochemical analyses. **The sixteenth joint annual conference of biomedical sciences.** Programs & abstracts.

3. **Chen WL**, Mao SJT. (2002) Analyses of β-lactoglobulin as a thermal marker in raw and commercially processed milk using a native gel electrophoresis and circular dichroic spectrum. **Experimental Biology 2002 New Orleans, LA, USA.** FASEB J. 16:A1187.

4. Ko YC, **Chen WL**, Liau CY, Kao WS, and Mao SJT. (2002) Preparation of colloidal gold for immunoassay. A simple and stabilized procedure. **The seventeenth joint annual conference of biomedical sciences.** Oral presentation.

5. Liau CY, **Chen WL**, Wu YJ, Lin CC, and Mao SJT. (2002) Differentiation of Milk Proteins Using Phage Displayed Antibodies. **The seventeenth joint annual conference of biomedical sciences.** Oral presentation.

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10. Song CY, **Chen WL,** Wang SS, Mao SJT. (2004) Thermal denaturation of milk β-lactoglobulin. **The nineteenth joint annual conference of biomedical sciences.** Programs & abstracts. P

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12. **Chen WL**, Huang MT, Li CW, Liu HC, Mao SJT. (2004) Thermal denaturation of β-lactoglobulin as probed by an monoclonal antibody. **Experimental Biology 2004 Washington, DC, USA.** FASEB. 18:A144.

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14. Yang MC, **Chen WL**, Mao SJT. (2005) Ligand binding ability of β-lactoglobulin in native and thermal denatured state. **The twentieth joint annual conference of biomedical sciences.** Oral presentation. P230

15. **Chen W L**, Huang MT, and S. J. T. Mao. (2005) A novel two-dimentional gel electrophoresis for studing the cress-linking between β-Lactoglobulin and milk proteins. **2005 ADSA-ASAS-CSA joint annual meeting**. **Cincinnati, OH.** Programs & abstracts. P144.

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17. **Chen WL**, Liu WT, Yang MC, Huang MT, Mao SJT. (2006) β-lactoglobulin plays a provocative role in lymphocyte proliferation. **Fourteenth Symposium on Recent Advances in Cellular and Molecular Biology.** Programs & abstracts. P159

18. **Chen WL**, Liu WT, Yang MC, Huang MT, Mao SJT. (2006) β-lactoglobulin plays a provocative role in lymphocyte proliferation. **Experimental Biology 2006. San Francisco, CA.** Programs & abstracts.

19. **Chen WL**, Liu WT, Yang MC, Huang MT, Mao SJT. (2006) β-lactoglobulin plays a provocative role in lymphocyte proliferation. **The twenty-first joint annual conference of biomedical sciences.** Programs & abstracts. P120.

20. Yueh CH, **Chen WL**, Huang PC, Mao SJT. (2006) Development of a two epitope immunoassay in measuring free PSA and PSA-ACT complex for postate cancer. **The twenty-first joint annual conference of biomedical sciences.** Oral presentation**.** P68.

21. Hsu HH, Yueh CH, **Chen WL**, Huang PY, Mao SJT. (2006) Structure of human plasma haptoglobin α1 probed by a unique monoclonal antibody. **The twenty-first joint annual conference of biomedical sciences.** Programs & abstracts. P167

22. Yueh CH, Lai YA, Hsu HH, **Chen WL**, Mao SJT. (2006) Improved method for haptoglobin 1-1, 2-1, 2-2 purification using monoclonal antibody affinity chromatography in the presence of sodium dodecyl sulfate. **10th International Symposium on Biochromatography. Lille, France.** Programs & abstracts.