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牛乳中β-lactoglobulin之生理功能(1/2)

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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 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. On the other hand, we were to determine the antioxidant role of β-LG in the milk and its possible mechanism involved. We show that β-LG was a mild antioxidant with potency less than that of vitamin E and probucol, the latter has been clinically used for antioxidant therapy. Using anti-LG antibody affinity column to deplete β-LG from the milk, the total antioxidant activity was lost by 50% suggesting that β-LG was a major antioxidant moiety contributing about 50% of the total activity. Either heating (100℃ for 2 min) or chemically modifying β-LG by carboxymethylation for the blockage of free thio group resulted in a total loss of the antioxidant property of β-LG. Furthermore, we show that the conversion of β-LG monomer to dimer was responsible in part for the mode of antioxidant action as assessed by a Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (**SDS-PAGE**). The data suggest that once the β-LG was dimerized, the β-LG lost its antioxidant activity. Accordingly, we conclude that β-LG is a major antioxidant moiety of milk and a free cysteine at residue 121 plays a protective role in preventing lipid oxidation. The significance of the finding and its role in commercially processed milk are also discussed.

(**Key words:** β-lactoglobulin structure, cell proliferation, antioxidant activity, thermal denaturation, β-LG receptor)

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.

Bovine milk is frequently heated in the dairy industry including pasteurization (62.5 degrees C for 30 minutes) and sterilization. This process may induce oxidative losses of proteins, unsaturated lipids, vitamins, active enzymes and immunologic factors (Fidler et al., 1998; Cross and Gill, 1999; Riezzo et al., 2003). β-lactoglobulin (β-LG), α-lactalbumin, immunoglobulin, albumin, and glycomacropeptide are the major milk whey components, in which β-LG accounts for 50-55% of the total whey proteins (Mckenna and O'Sullivan, 1971). 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). β-LG comprises two disulfide bonds at residues C106-C119 and C66-C160 with a free -SH group at C121 (**Figure 1**) (Monaco et al.,1987; Qin et al., 1998, Hernandez-Ledesma et al., 2006). The molecule is covalently linked to form a dimer, polymer, or conjugated with other milk proteins when heated above 80°C, in which intermolecular disulfide bonds play an important role (Roefs and De Kruif, 1994; Chen et al., 2004 and 2005, Song et al., 2005). Such formation of aggregates is stabilized by disulfide bonds and hydrophobic interactions (Roefs and De Kruif., 1994; Surroca et al., 2002). The protein is shown to possess antioxidant activity (Elias et al., 2005), but its antioxidant potency and the extent present in the whole milk has not been fully investigated. Further, although the molecular remodeling of β-LG occurs during the heating process, the cross-linking with respect to antioxidant property has not been elucidated.

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.

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).

β-LG purification

β-LG was purified from a HPLC DEAE-column using a method similar to that previously described (Chen et al., 2004; Schlattere et al., 2004). In brief, freshly prepared whey proteins extracted from raw milk were first fractionated by a 40% 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-column (BioRad, St. LA, USA) using a Waters HPLC system equipped with a 600 controller and a 996 photodiode array detector (Waters, St. Massachusetts, USA). 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 at a flow rate of 1 mL/min. Peak fractions containing β-LG were immediately pooled and dialyzed against a buffer containing 0.12M NaCl, 0.02M phosphate, pH 7.4, (PBS) at 4 °C.

Reduction and carboxymethylation of β-LG

Carboxymethylation was conducted similar to that previously described by us (Song et al., 2005; Chen et al., 2006). Five mg of β-LG were first dissolved in 3 mL of 0.01 M Tris-HCl buffer (pH 8.6) containing 5.4 M urea, and 1% (v/v) β-mercaptoethanol. The reaction mixture was flushed with nitrogen at room temperature for 15 min. After which time, 20 mg of iodoacetic acid were added stepwise, while maintaining the pH at 8.6 using 1 M NaOH within a period of 30 min. The reaction was preceded for another 60 min by incubation at room temperature. Using this procedure, more than 92% of cysteines of β-LG were modified as determined by amino acid analysis. Finally, carboxymethylated (CM) β-LG was desalted on a Bio-Gel P2 column eluted with 0.05 M ammonium bicarbonate and lyophilized. Greater than 90% of the modified β-LG were monomers as judged by electrophoresis.

Gel electrophoresis and Western blot

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Laemmli's method (Laemmli, 1970) with modification by using 5% polyacrylamide as a stacking gel. Samples (typically 5 μg) for SDS-PAGE were mixed with a loading buffer [12 mM Tris-HCl, pH 6.8, 0.4% (w/v) SDS, 5% glycerol, 0.02% bromphenol blue] and run for 1.5 h at 100 V and stained with

Coomassie brilliant blue R-250. For Western blot, gel containing the separated proteins was electro-transferred to a nitrocellulose membrane (Hybond-ECL extra, Amersham, Buckingham, UK) at 90 mA for 1 h in a semi-dry transfer cell (BioRad, St. LA, USA). The transferred membrane was then immersed in 1% gelatin in PBS for 1 hour at room temperature, while shaking gently. After washing with PBS for 3 x, the membrane was incubated with a bovine β-LG monoclonal antibody [1:10,000 dilution in PBS containing 0.1% (w/v) gelatin and 0.05% (v/v) Tween-20 for 1 hour] at room temperature and washed 3 x. The membrane was then incubated with 1:10,000 diluted goat anti-mouse IgG conjugated with horseradish peroxidase for 1 h. Finally, the membrane was developed using 3,3'-diaminobenzidine (DAB) (Yang and Mao, 1999; Liau et al., 2003).

Preparation of anti-β-LG affinity column

A rabbit polyclonal antibody prepared against β-LG was first fractionated by a 50% saturated ammonium-sulfate. After repeating the procedure 2 x, the redissolved pellet was exhaustively dialyzed against PBS followed by a final dialysis in a coupling buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3. The crude IgG fraction was then coupled to CNBr-activated Sepharose-4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's procedures with some modification (Liau et al., 2003). Briefly, 3.33 g of freeze-dried Sepharose were swollen and suspended in

20 mL of 1 mM HCl and immediately washed with 20 x volume of the same solution within 15 min on a sintered glass filter (Liau et al., 2003). The gel was then washed with coupling buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3, and degassed. About 5.5 mL of ammonium-sulfate fraction of polyclonal antibody (26.8 mg/mL) in coupling buffer were added into the gel (in 15 mL), while gently stirring by a magnetic bar for 1 h. After coupling, the gel was washed with 10 x volume of PBS to remove unbound materials via a sintered glass filter. The gel was then treated with a blocking solution containing 0.1 M Tris-HCl and 0.5 M NaCl, pH 8.0, for 2 h to saturate the remaining reactive-sites. The degassed gel was then washed with 3 cycles of blocking buffer and a 0.15 M NaCl solution of pH 11.0 (adjusted by ammonium). Finally, the gel was equilibrated in PBS and packed onto a 1.5 x 20 cm column (Liau et al., 2003).

Preparation of milk samples

Bulked whole raw milk was obtained from a local dairy farm (Chen et al., 2004), while processed (purchased from the local market) and dry (imported from Nestle Australia Ltd, Sidney, Australia; Kroger, Cincinnati, USA; KLIM, Sidney, Australia; Anchor, New Zealand) milks without further heat or other manipulation (unless specifically mentioned) were used for PAGE, Western blot, and antioxidant analyses (Chen et al., 2004). Dry milk was defined as powdered form that was reconstituted

by dilution with deionized water to give a final protein concentration equal to that of raw milk. All the milk samples were skimmed by centrifugation at 3500 x *g* for 30 min at 4 °C prior to the experiments.

Preparation of raw milk devoid of β-LG

β-LG of raw milk was removed by the affinity column as prepared above. One 1 mL of raw milk (about 40 mg/mL protein) was loaded onto the affinity column equilibrated with PBS, while the pass-through fractions confirmed without β-LG on SDS-PAGE were pooled and used for the analysis antioxidant activity. When comparing the antioxidant activity between the β-LG devoid and raw milk, raw milk was slightly diluted with PBS to give a final protein concentration equal to milk devoid of β-LG.

LDL purification

Human LDL (d. 1.021-1.063) was isolated from freshly collected normolipidemic plasma by ultracentrifugation. Briefly, plasma was first adjusted to a density of 1.02 kg/L by NaBr followed by a centrifugation at 45,000 rpm for 12 h at 4 °C to remove the very low-density and intermediate-density lipoproteins (Barr et al., 1981; Yates et al., 1992). The bottom fraction was then adjusted to a density of 1.063

kg/L and subjected to another centrifugation at 45,000 rpm for 16 h. The top LDL fraction was immediately dialyzed in a buffered solution containing 0.12 M NaCl, 0.02 M phosphate, pH 7.4 (PBS) and passed through a 0.45 μ filter before use (Yates et al., 1992).

Antioxidant assay using thiobarbituric acid-reactive substances (TBARS)

The entire assay was conducted using a method previously established in our laboratory (Mao et al., 1991a; Mao et al., 1991b; Yates et al., 1992; Mao et al., 1994; Tseng et al., 2004). In brief, LDL in PBS was incubated with 20 μM Cu_2SO_4 (pH 7.0) in the presence of various amount of β-LG, heated β-LG, probucol, vitamin E, or bovine serum albumin in a final volume of 100 μL for 2 h in a 37 °C water bath (Teng et al., 2005). After which time, 250 μL of 20% TCA were added and vortexed, followed by the addition of 250 μL of 0.1% thiobarbituric acid. The mixture was then incubated in an 80 °C water bath for 30 min followed by a centrifugation for 3 min at 3,000 rpm. The top chromogenic solution in pink (250 μL) of TBARS was read at 540 nm in an ELISA plate (Mao et al., 1994).

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): 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.

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 ^{o}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 2, 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 3); 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 4): 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 5). 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 6). 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 7). 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 (Figure 8). 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 9). 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 10), 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 (Figure12); 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.

Antioxidant activity of β-lactoglobulin

To determine whether β-LG is an antioxidant molecule, we used LDL as a model system previously established by us (Mao et al., 1994; Tseng et al., 2004). Figure 13 shows β-LG possessing a mild antioxidant activity. As judged by IC_{50} value, the antioxidant activity of β-LG was lower than that of probucol and vitamin E (Mao et al., 1994). While bovine albumin (present in milk) did not exhibit any antioxidant activity within the concentration ranges we tested. Although it was a mild antioxidant, considering the amount of daily intake (about 4 g/L of β-LG), the antioxidant nature of β-LG therefore cannot be ignored.

Relationship between antioxidant activity and disulfide linkage of β-LG

In the next experiment, we addressed the possible mechanism by which β-LG inhibited LDL oxidation. First, we show LDL oxidation induced by Cu^{2+} to be a time-dependent manner (Figure 14A). Using a dose of β-LG (60 μM) that inhibited about 40% of LDL oxidation (extrapolated from the IC_{50} curve of Figure 13), the degree of inhibition was maintained within the first 25 h (Figure 14A). Next, we show that the antioxidant activity of β-LG was at the expense of its native structure (18.5 kDa) on a SDA-PAGE analysis (Figure 14B). Some of them formed covalently linked dimer of β-LG (37 kDa). This was further confirmed by a Western blot using a specific β-LG monoclonal antibody (Figure 14C). The data suggest that free thio group of β-LG may participate a crucial role in protecting of LDL against oxidation.

Effect of carboxymethylation and heating on the antioxidant activity of β-LG

To further test a hypothesis that the free thio group of β-LG participated in its antioxidant nature of β-LG, chemical modification using carboxymethylation to block the thio groups of β-LG was conducted. As shown in Figure 15, carboxymethylation on β-LG substantially abolished the protection of LDL from oxidation. It should be noted here that this chemical modification was carried out in the presence of 6 M urea.

The modified β-LG was dialyzed against PBS to remove the urea prior to the antioxidant assay. For the control experiment, the urea treatment did not alter the antioxidant activity of β-LG (data not shown). We anticipated that the free thiol group of residue Cys-121 (Figure 1) would form cross-linking in a red-ox reaction to scavenge free radicals, while protecting LDL from oxidation. Because heat may result in cross-linking of Cys-121 (Burova et al., 1998: Chen et al., 2004, 2005, and 2006), we expected that the heated β-LG could also attenuate its antioxidant potency. In the next experiment, we heated β-LG at 100 °C for 2 min to produce the β-LG dimer (37 kDa) and other polymers as evidenced by SDS-PAGE and Western blot (Figure 16). A marked and dose-dependent decrease in antioxidant activity of β-LG upon the heating is shown in Figure 12. Notably, heated β-LG only retained partial antioxidant activity as compared with native LG. One of the explanations was due to the fact that the heating procedure under our experimental condition did not fully denature β-LG (Figure 16). Because such dimerization was reversible in the presence of a reducing reagent (data not shown), it indicated that the cross-linking was via disulfide linkage, which was consistent with the previous reports (Burova et al., 1998: Chen et al., 2004, 2005, and 2006).

Antioxidant activity of whole milk devoid of β-LG

To address how much antioxidant activity in raw milk is attributed by β-LG, we

depleted β-LG from the skimmed milk using a β-LG antibody affinity-column. Fractions devoid of β-LG were collected. Figure 6A demonstrates that the antibody effectively removed the β-LG, when analyzed on 15% SDS-PAGE. Figure 6B shows that at an equivalent protein concentration, the total antioxidant activity was decreased by about 50% in β-LG depleted milk (judged at IC_{40}) (Figure 17B). Thus, β-LG is a major milk component that accounted for about 50% of the total milk antioxidant capacity.

Antioxidant activity of heated milk

We investigated the difference between heated (100 ℃ for 2 min) and unheated milk for their antioxidant activity. Under this heating condition, β-LG was almost totally denatured either aggregated or conjugated with other milk proteins as shown in a native-PAGE (Figure 18). Most interestingly, heat treatment resulted in a dramatic loss in antioxidant activity (Figure 18). The data reveal that heating not only denature the β-LG but also severely attenuated the antioxidant nature of milk.

Antioxidant activity in commercially processed milk and dry milk

To evaluate the antioxidant activity of processed and dry milks purchased from the market, milk samples at various protein concentrations were assessed for the

antioxidant activity using IC_{50} as a criterion. As shown in Figure 19A, all the processed milks (brands coded from 1 to 6) suffered a significant loss in its antioxidant activity while compared to the raw milk. A similar result was also observed in the dry milks (coded as letters). To correlate whether or not the loss of antioxidant activity was due to the denaturation of β-LG, each milk sample was then analyzed on a native-PAGE (Figure 19B). In general, the loss of activity was in close agreement with the denaturation of native β-LG. For example, processed milk 4 possessed lowest activity (as judged by IC_{50}) in which the β-LG was almost completely denatured.

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 2), 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 3) 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 2) 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 5). 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 6, 7). 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 8), 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 5). 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 6, 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 10); 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 12), 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.

Today, whey is a popular dietary protein supplement purported to provide antimicrobial activity (Wang et al., 2004), immune modulation, improved body composition, and to prevent cardiovascular disease and osteoporosis (Marshall., 2004).

With respect to cardiovascular disease, we have shown that probucol, a potent antioxidant, can prevent Cu^{2+} induced LDL oxidation in vitro and attenuate the atherosclerosis in vivo (Mao et al., 1991a, 1991b, and 1994).

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). The antioxidant property of β-LG has not yet been brought much attention to the public domain. In the present study, we demonstrate that β-LG was an antioxidant molecule that could directly prevent LDL from oxidation. The formation from monomer to dimer was responsible for its antioxidant property. Several lines of the evidence suggest that β-LG plays a key antioxidant role in milk. First, β-LG was a major antioxidant constituent in raw milk contributing about 50% of the total antioxidant activity in milk (Figure 17A). Second, SDS-PAGE and Western blot analyses revealed that prevention of LDL oxidation was at the expense of native β-LG followed by a dimer cross-linking (Figure 14). Third, heated β-LG and carboxymethylated β-LG (with thio-group blocked) lacking of free thio-group resulted in loss of antioxidant activity of β-LG (Figures 13 and 16). Fourth, heated raw milk, while β-LG denatured, also exhibited a striking loss in antioxidant activity that was consistent to that of β-LG

depleted milk (Figure 18).

Nitric oxide (NO) is a gaseous radical with high affinity for various molecules, such as hemeproteins, thiols, and related radicals (Yates et al., 1992; Inoue et al., 2000). In patients with *Helicobacter pylori* (*H. pylor*) infection, the inducible nitro oxide synthase (iNOS) gene in gastric epithelial cells is upregulated and overexpressed (Gobert et al., 2001). It has been suggested that the upregulation and subsequent NO production are mediated through MAP kinase within the epithelial cells, in which production of NO and IL-8 plays a role in the tissue inflammation (Kim et al., 2002). These patients are often associated with stomach ulcer, milk has been widely used as one of the therapeutic approaches (Stromqvist et al., 1995; Hirmo et al., 1998). Recent studies even demonstrate that intact β-LG is acid resistant and can cross the epithelium cells of GI tract with a super permeability (Makinen-Kiljunen and Palosuo, 1992; Lovegrove et al., 1993). β-LG can be easily identified in the plasma of subjects within 1-2 h following an oral ingestion of milk (Makinen-Kiljunen and Palosuo, 1992). We speculate that β-LG attributed antioxidant activity in the milk may therefore play an essential role in protection of the ulcer from oxidative stress through a mechanism in scavenging the level of NO or free radical production. In fact, a potential therapeutic effect has been tested with known antioxidant agents such as alpha-tocopherol, ubiquinone, thio-compound

(mesna), and substances derived from the ergoline (6-hydroxynicotinic acid and 4-hydroxypyridine) (Stetinova and Grossmann, 2000). Allicin, (thio-2-propene-1-sulfinic acid S-allyl ester) a main biologically active component of garlic clove extracts, has been thought as another alternative in which the therapeutic efficacy is attributed to either antioxidant activity or thiol disulfide exchange (Rabinkov et al., 1998). β-LG possessing antioxidant activity may therefore improve the uncomfortable symptom in patients infected by H. pyroli.

Previously, we have shown the milk purchased from the U.S. market to be superior with β-LG almost completely intact (Chen et al., 2004 and 2006). Again, in this study we show the difference in "antioxidant quality" among the commercially processed milks (Figure 19A). The antioxidant activity in the milk appears to be correlated to the intactness of the β-LG. Therefore, a choice of oral intake with less β-LG denatured milk would be essential. Regardless, β-LG suffered a significant loss in dry milk (in some brands) and heated milk (Figure 18A). It is conceivable that additional heat should be avoided in order to maintain the antioxidant nature from purchased milk.

CAPTIONS AND LEGENDS

Figure 1. Primary structure of β-LG. β-LG is comprised of 162 amino acid including 5 cysteine residues. Two disulfide linkages are located at residues C106-C119 and C66-C160. One free cysteine is at position 121.

Figure 2: 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 3: 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 4: 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 5: 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 6: 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 7: 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 8: 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 9: 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 10: 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 11: 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 12: 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 13. Antioxidant activity of β-LG. The antioxidant activity was estimated by the degree of inhibition of Cu^{2+}-induced formation of thiobarbituric acid-reactive substances (TBARS) from LDL. LDL (60 μg) was incubated with 20 μM Cu^{2+} in the presence of β-LG, heated β-LG, probucol, vitamin E, or BSA at 37℃ for 2 h with a final volume of 100 μL in PBS. After which time, 250 μl 20% of TCA and 250 μl of 0.1% of thiobarbituric acid were added followed by incubation at 80°C water bath for 30 min and the supernatant (300 uL) was read at 540 nm on a ELISA plate.

Figure 14. Changes of β-LG in Cu^{2+}-induced LDL oxidation over time. LDL was

incubated with 20 μM Cu^{2+} in the presence of 60 μM β-LG (IC_{50}) at 37℃ as that described in Fig 2. TBARS formation from LDL was with a time-dependent fashion (**A**). β-LG was gradually denatured, in which some of them formed β-LG dimer as analyzed by a 15% SDS-PAGE (**B**) and further confirmed a Western blot (**C**).

Figure 15. Western blot analysis of heated β-LG on 15% SDS-PAGE. Heated β-LG in PBS was heated at 100℃ for 20 min, followed by a 15% SDS-PAGE (Left) and Western blot (Right). Land M: molecular weight markers. Land A: native β-LG. Land B: heated β-LG.

Figure 16. Effect of carboxymethylation (CM) on antioxidant activity of β-LG. Carboxymethylation was conducted to explore the role of disulfide linkages of β-LG in antioxidant activity. The antioxidant activity was determined similar to that described in Fig 1. Each bar represents the mean ± SD of triplicates. The overall antioxidant activity of β-LG was significantly greater then that of CM β-LG ($P<0.001$).

Figure 17. Characterization of β-LG depleted raw milk on SDS-PAGE and its antioxidant activity. β-LG depleted raw milk was obtained from an antibody affinity

column without further manipulation. The volume of raw milk was slightly adjusted as equivalent to β-LG depleted milk per protein concentration. Each point represents mean± SD of triplicates. Land M: molecular weight markers. Land A: β-LG depleted raw milk. Land B: raw milk.

Figure 18. Native-PAGE of heated raw milk and its antioxidant activity. Heated raw milk was prepared by heating at 100 ℃ for 2 min. The possess of isoforms A and B (two bands) of β-LG is shown. The β-LG was substantially lost in heated raw milk by forming large aggregated with milk proteins.

Figure 19. Antioxidant activity of commercially processed fresh and dry milk. To evaluate the antioxidant activity of processed milks, IC_{50} was determined as the expression of antioxidant potency (Top). The moiety of β-LG in each brand of milk (No. 1-6) and dry milk (brand A, K, N and Kr) were also analyzed by a native-PAGE (Bottom).

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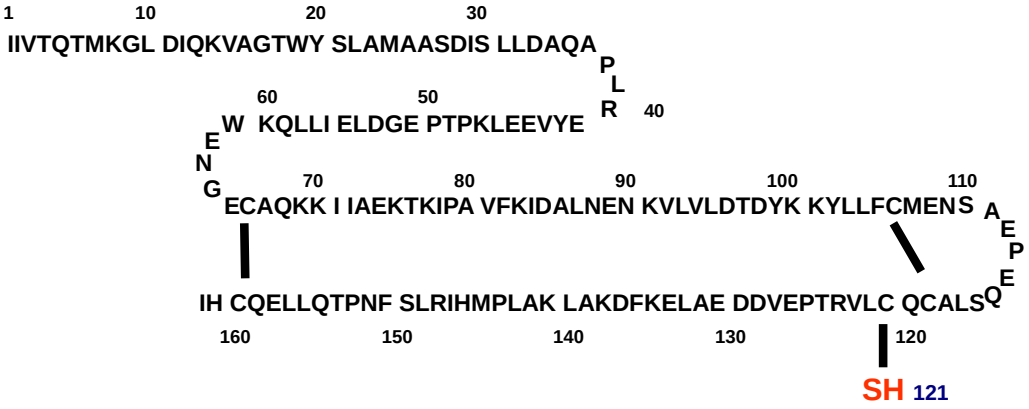
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Figure 1

Figure 2

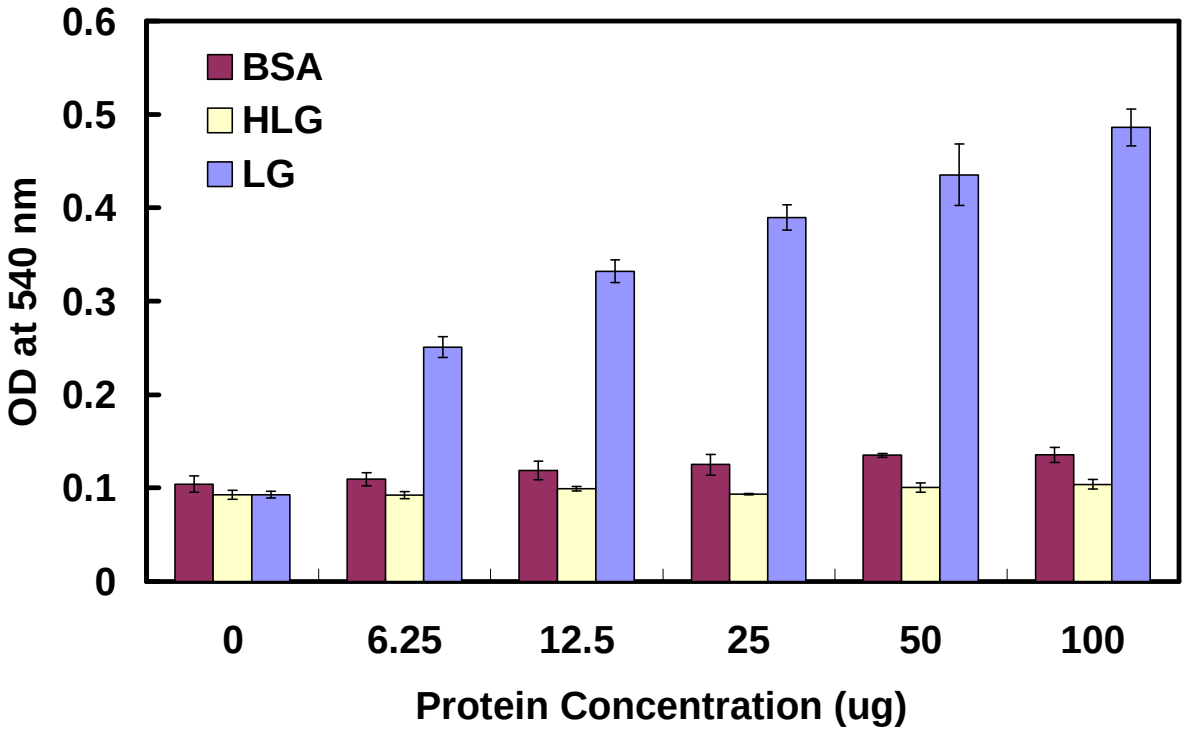

0.6
0.5
0.4
0.3
0.2
0.1
0
OD at 540 nm
BSA
HLG
LG
0
6.25
12.5
25
50
100
Protein Concentration (ug)

Figure 3

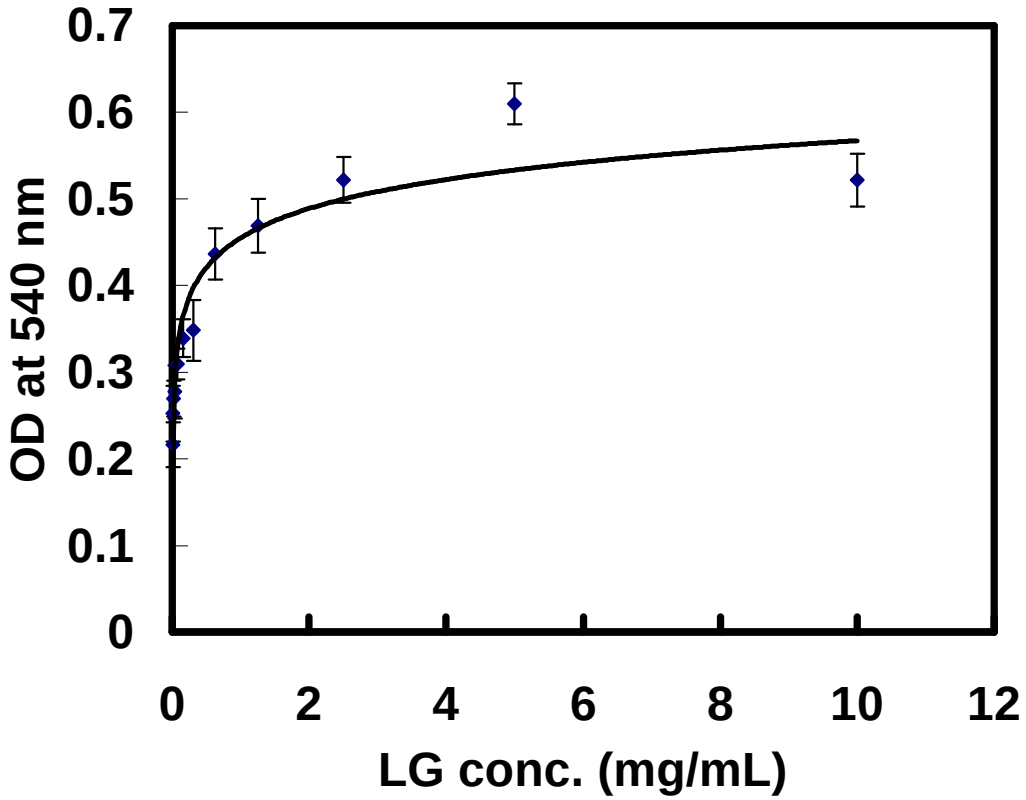


Figure 4

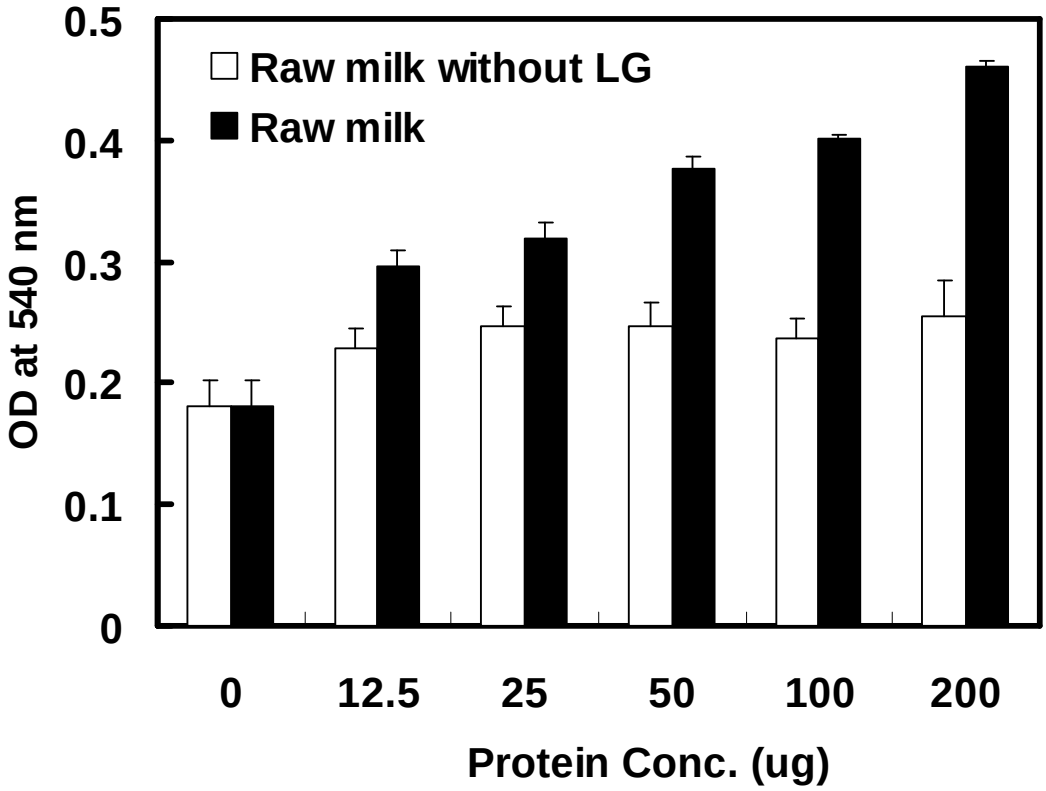
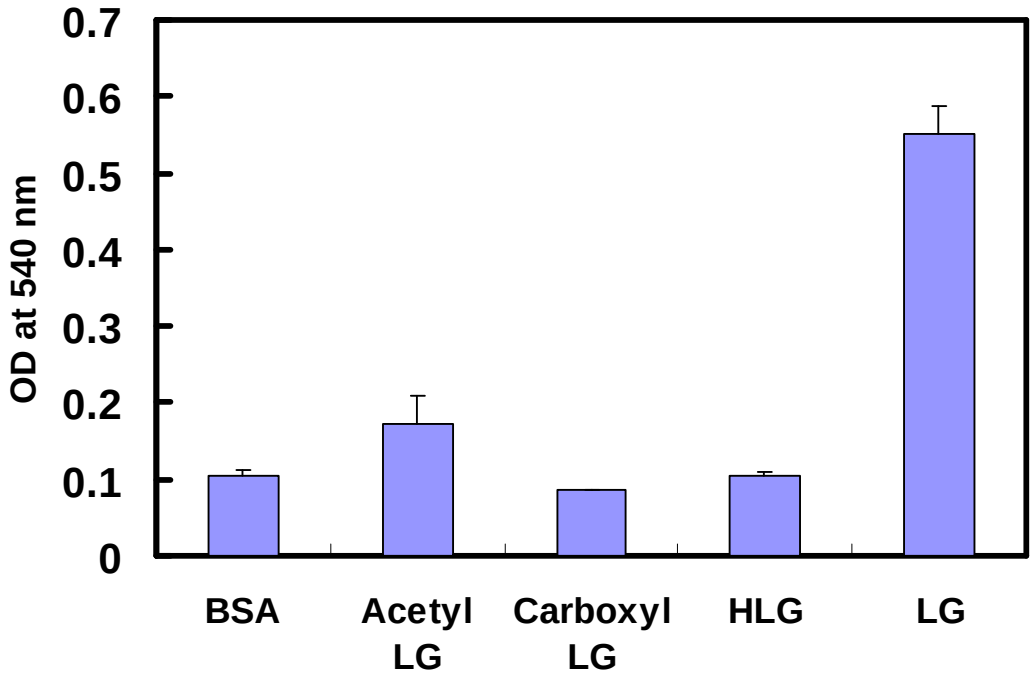

0.5
0.4
0.3
0.2
0.1
0
OD at 540 nm
Raw milk without LG
Raw milk
0
12.5
25
50
100
200
Protein Conc. (ug)

Figure 5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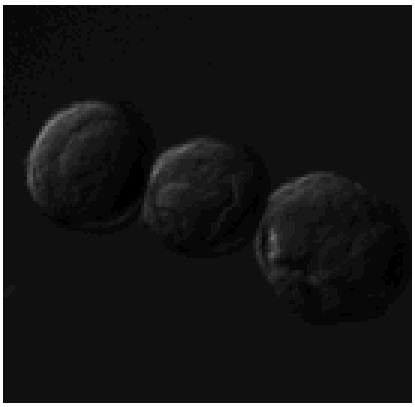
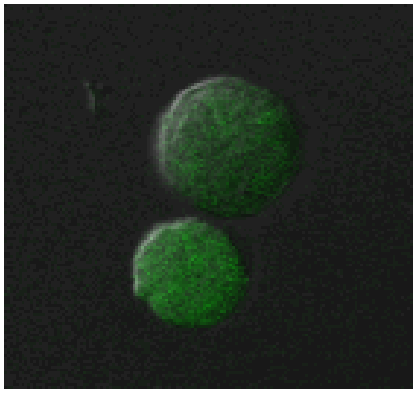
0.1
0
OD at 540 nm
BSA
Acetyl LG
Carboxyl LG
HLG
LG

Figure 6

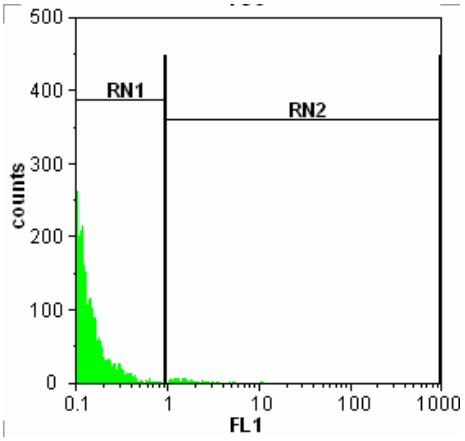


A. Negative control



B. Binding with LG conjugate FITC

Figure 7



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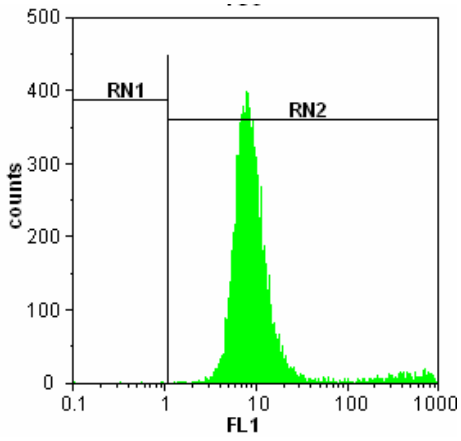

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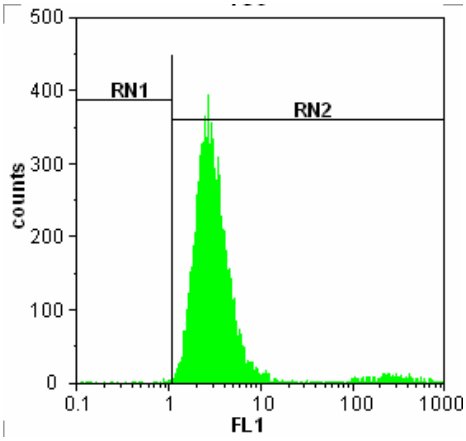

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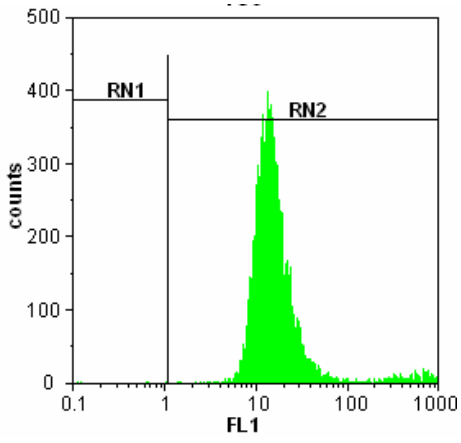

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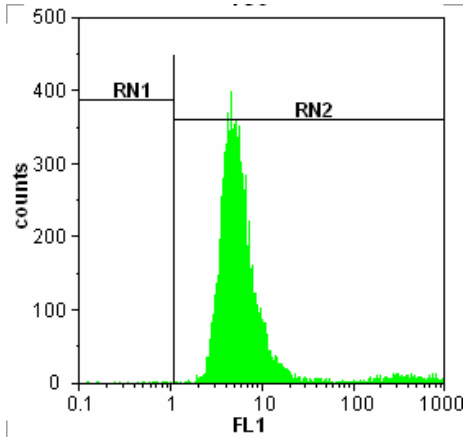

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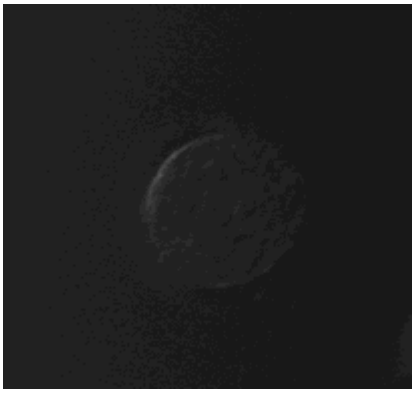
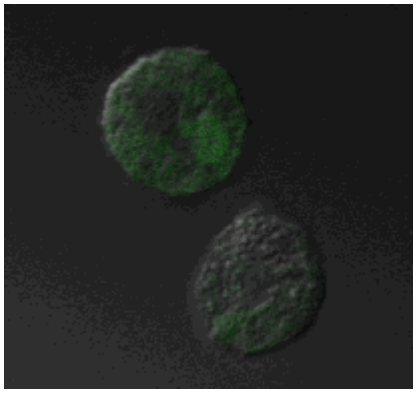
Figure 8



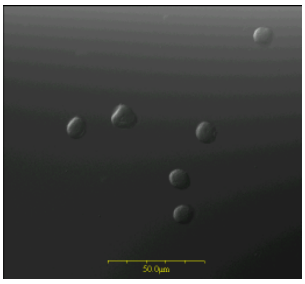
A. Normal IgG



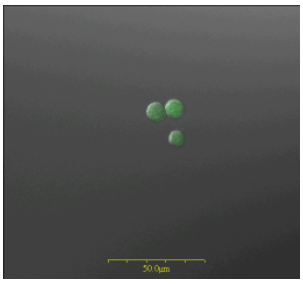
B. Anti-β-LG receptor IgG

Figure 9

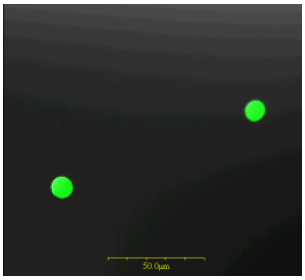
A. Confocal microscope



Incubated at 4 ℃

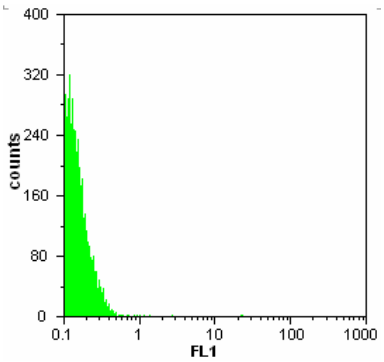


Incubated at 4 to 37 ℃

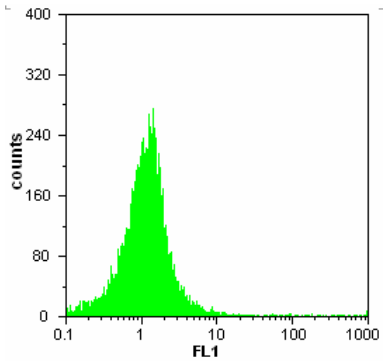


Incubated at 37 ℃

B. Flow cytometry



Negative control



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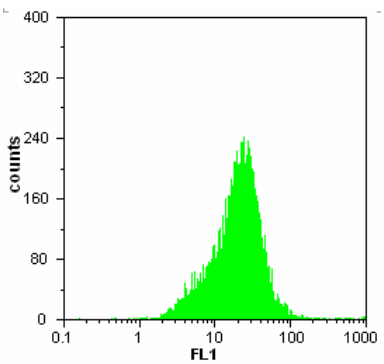
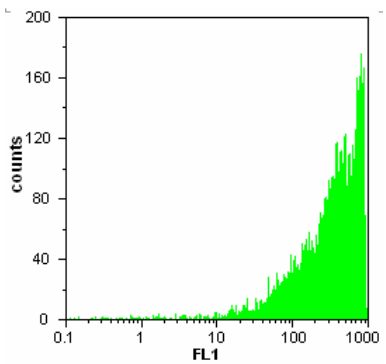


Incubated at 4 to 37 ℃



Incubated at 37 ℃

Figure 10

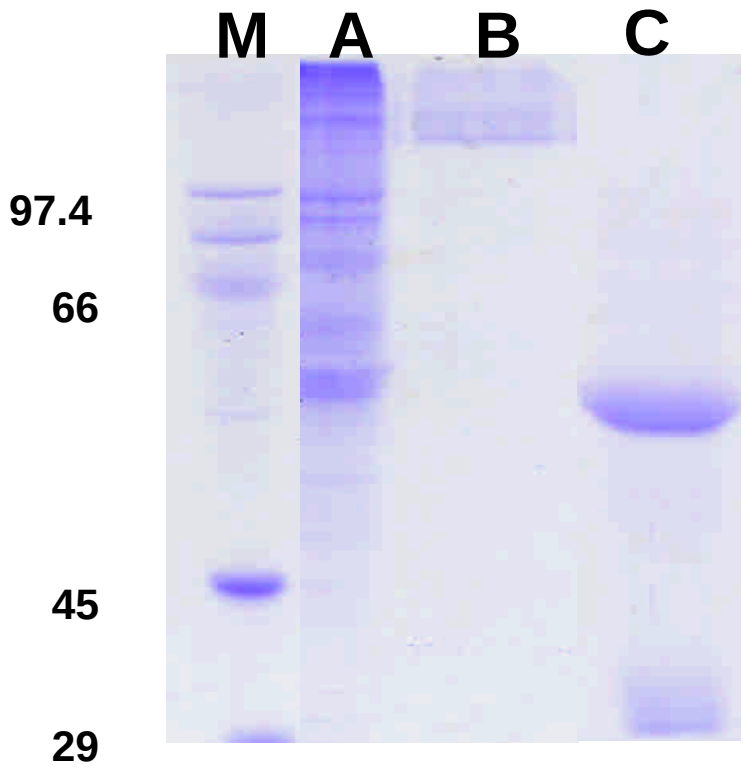


Figure 11

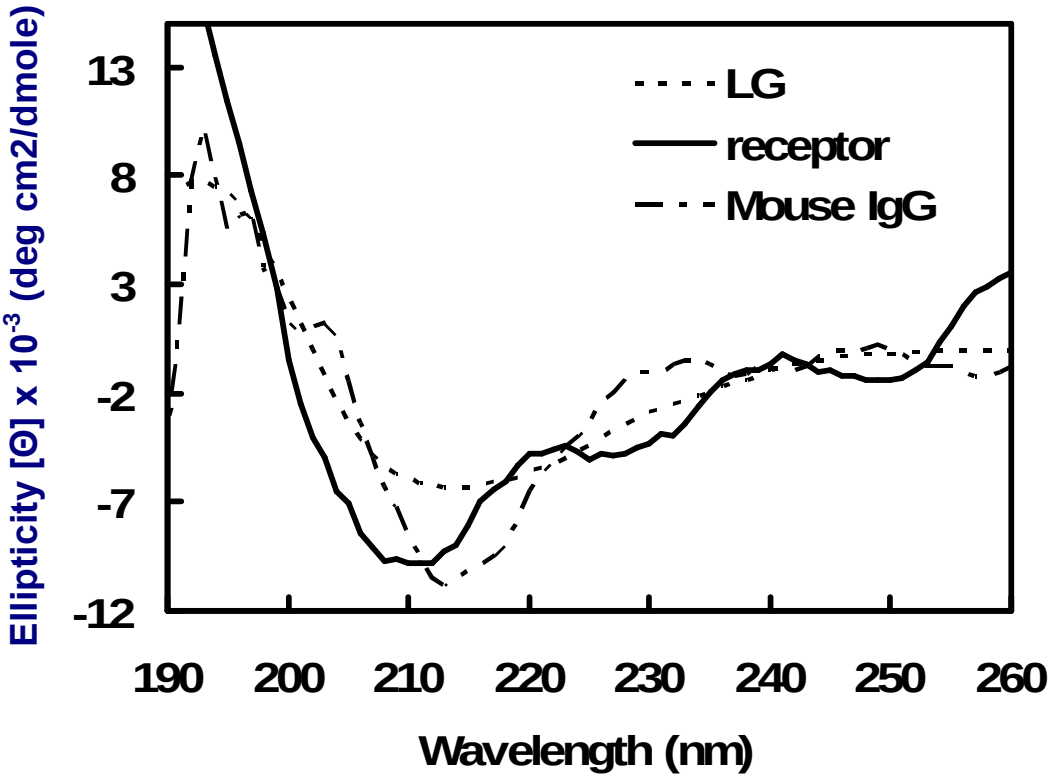
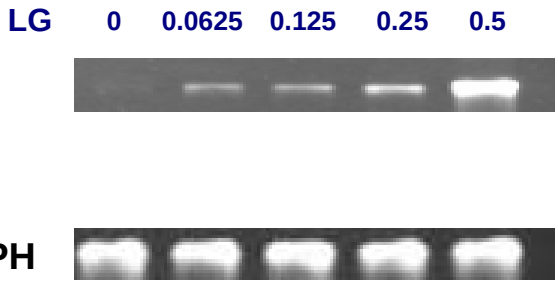
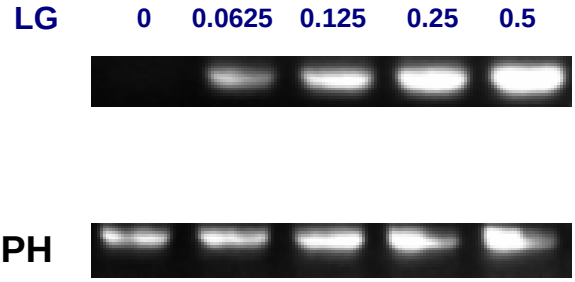

Ellipticity [Θ] x 10-3 (deg cm2/dmole)
13
8
3
-2
-7
-12
190
200
210
220
230
240
250
260
Wavelength (nm)
LG
receptor
Mouse IgG

Figure 12

(A) Cyclin A



(B) Cyclin D2



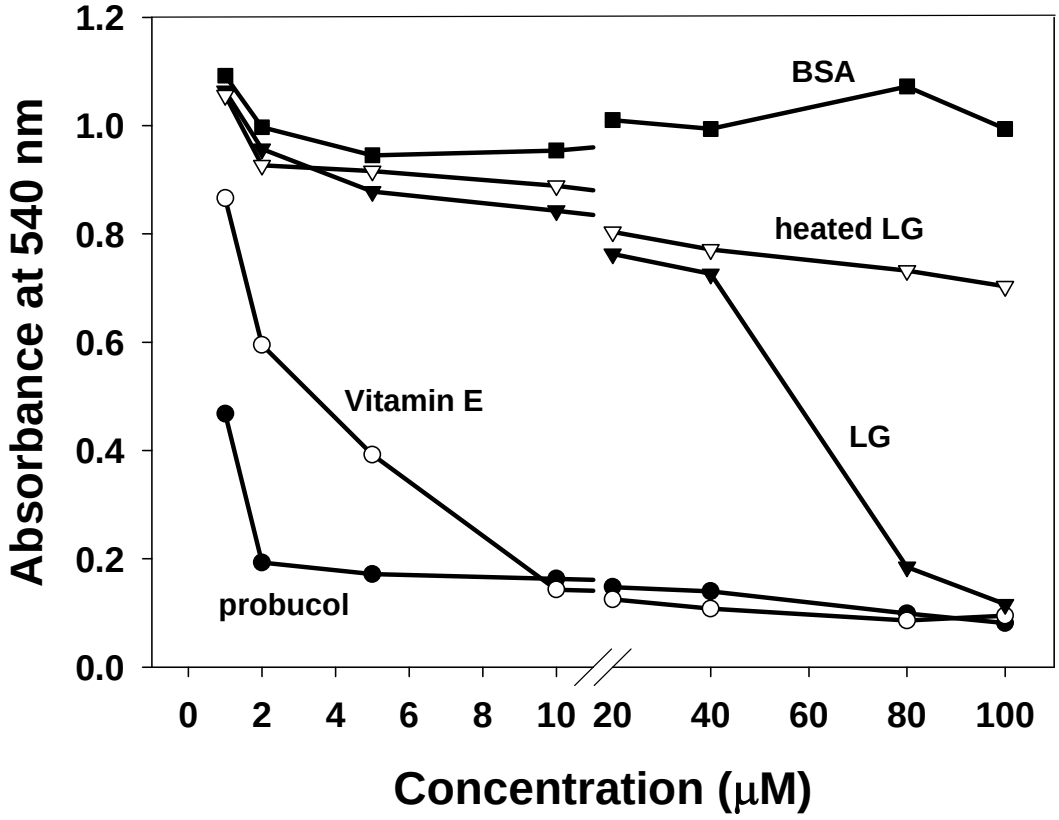
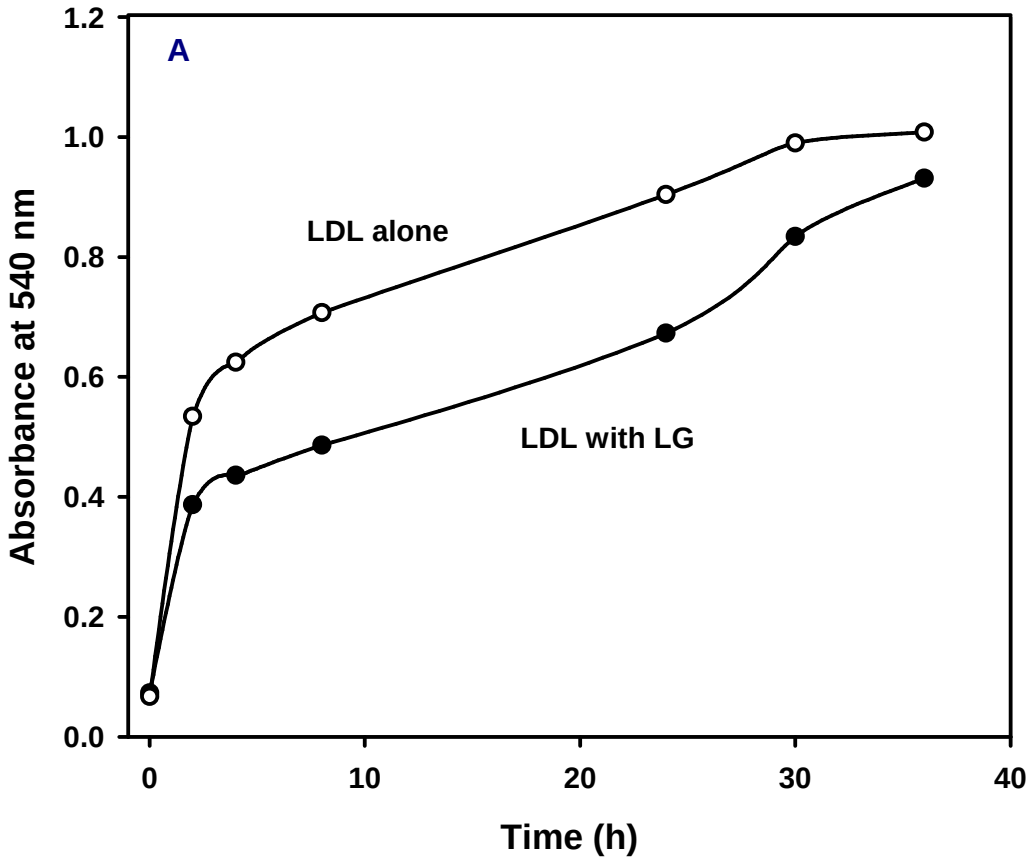
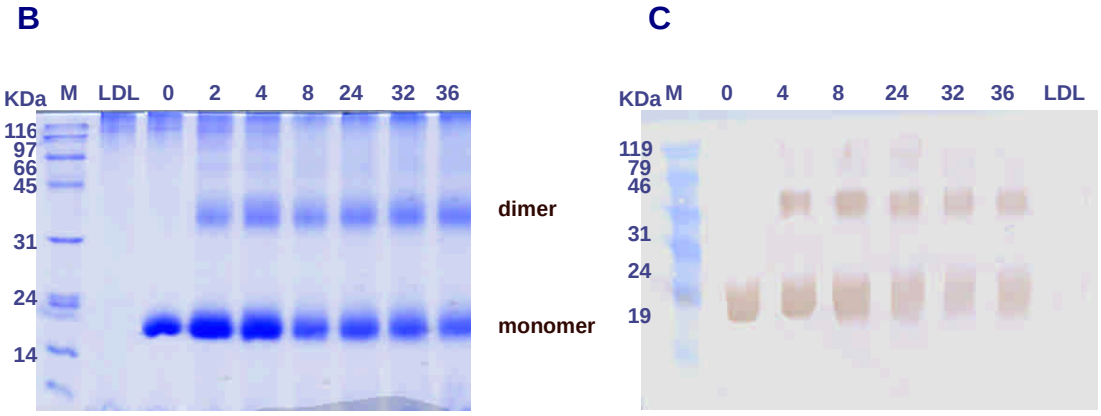

Absorbance at 540 nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
BSA
heated LG
Vitamin E
LG
probucol
0
2
4
6
8
10
20
40
60
80
100
Concentration (μM)

Figure 13





SDS-PAGE

Western blot

Figure 14

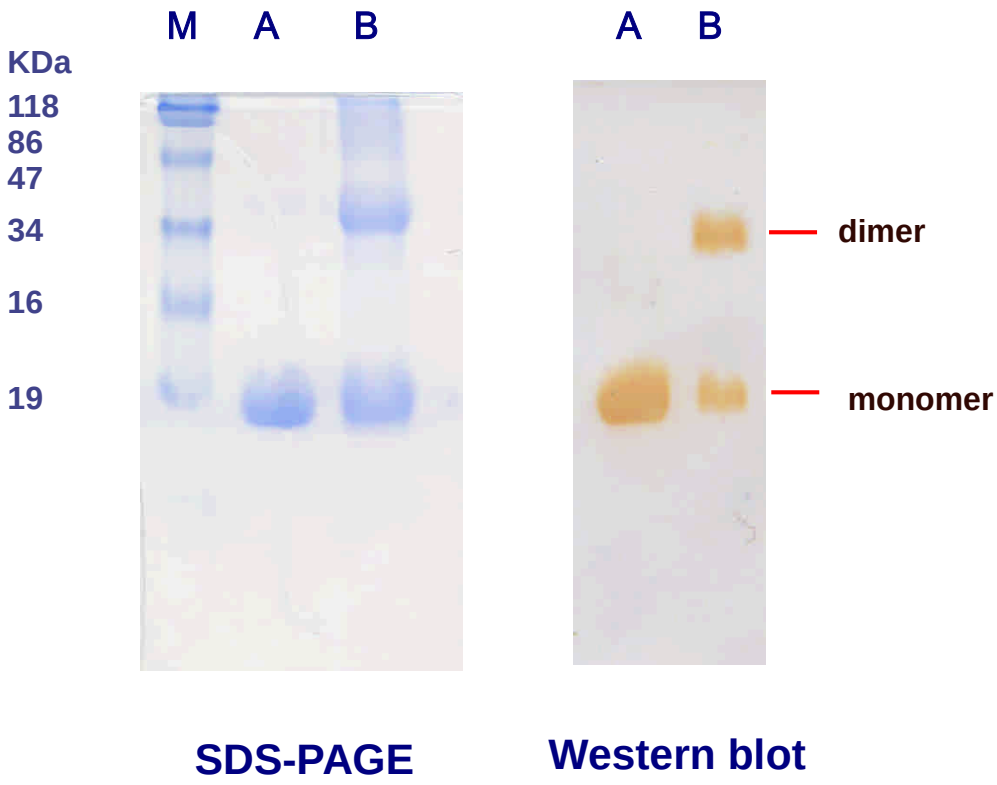

M
A
B
KDa
118
86
47
34
16
19
SDS-PAGE
A
B
dimer
monomer
Western blot

Figure 15

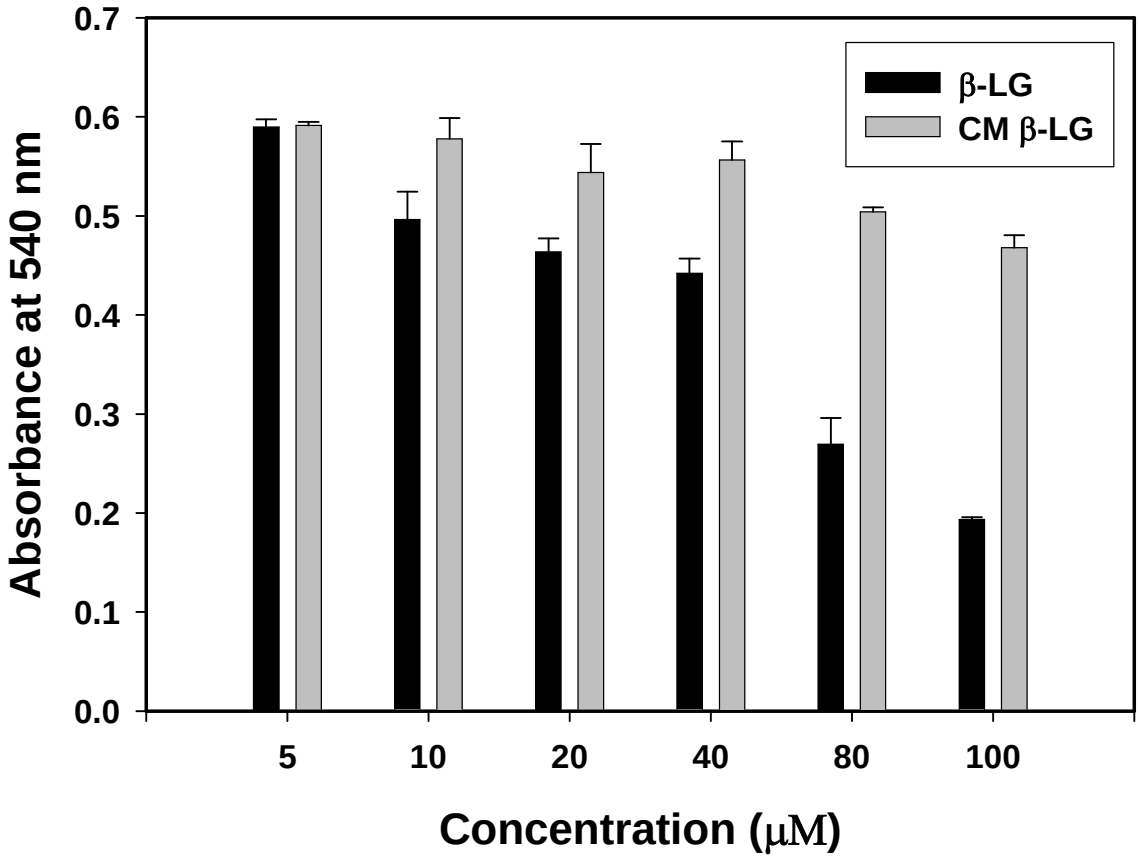

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Absorbance at 540 nm
β-LG
CM β-LG
5
10
20
40
80
100
Concentration (μM)

Figure 16

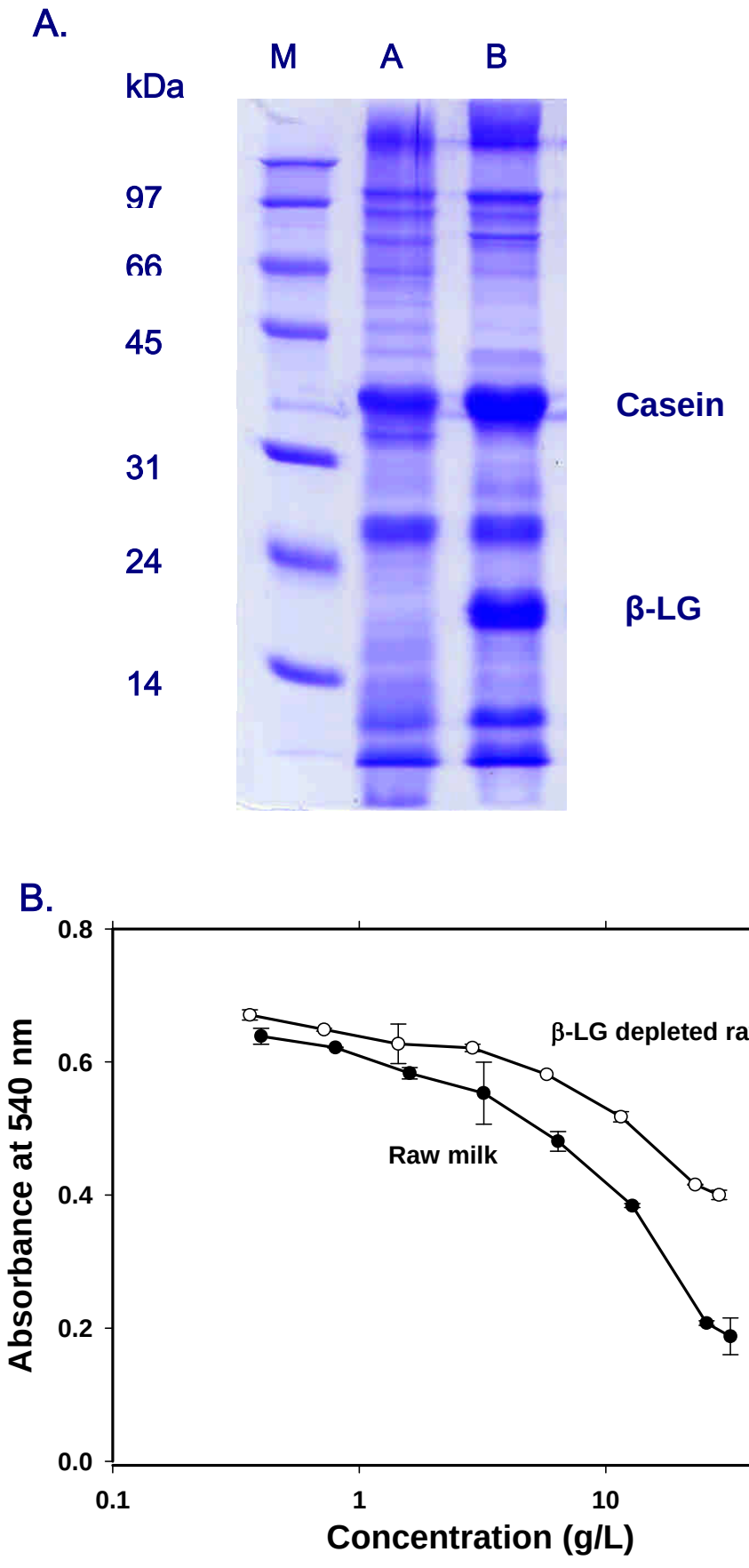

A.
M
A
B
kDa
97
66
45
31
24
14
Casein
β-LG
B.
Absorbance at 540 nm
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
Concentration (g/L)
β-LG depleted raw milk
Raw milk

Figure 17

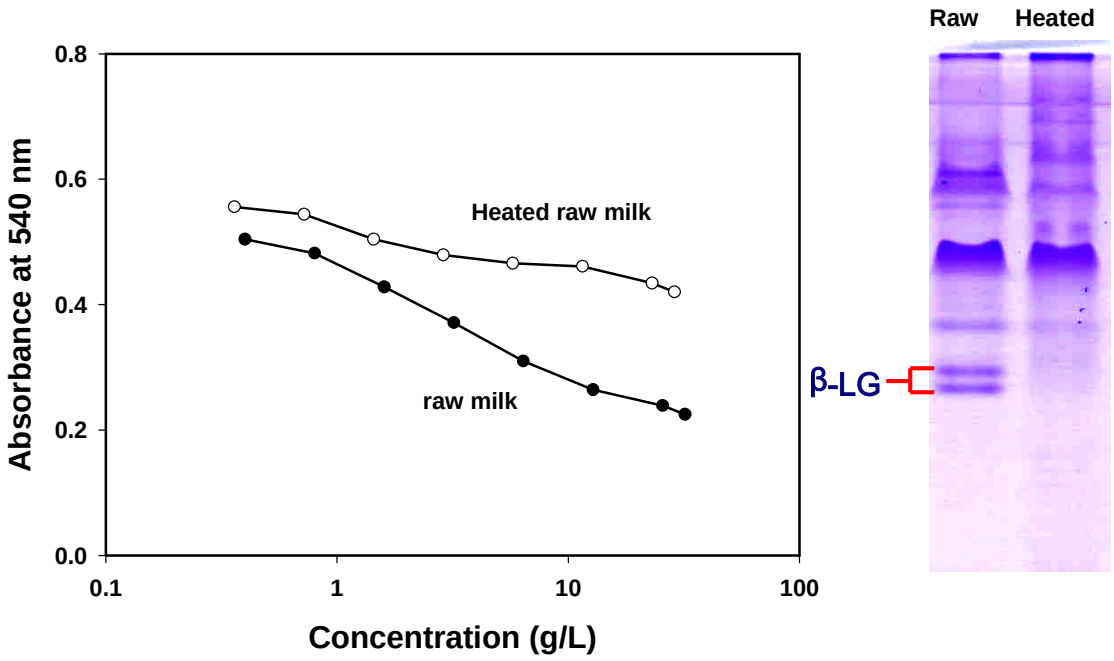

Absorbance at 540 nm
0.8
0.6
0.4
0.2
0.0
0.1
1
10
100
Concentration (g/L)
Heated raw milk
raw milk
Raw
Heated
β-LG

Figure 18

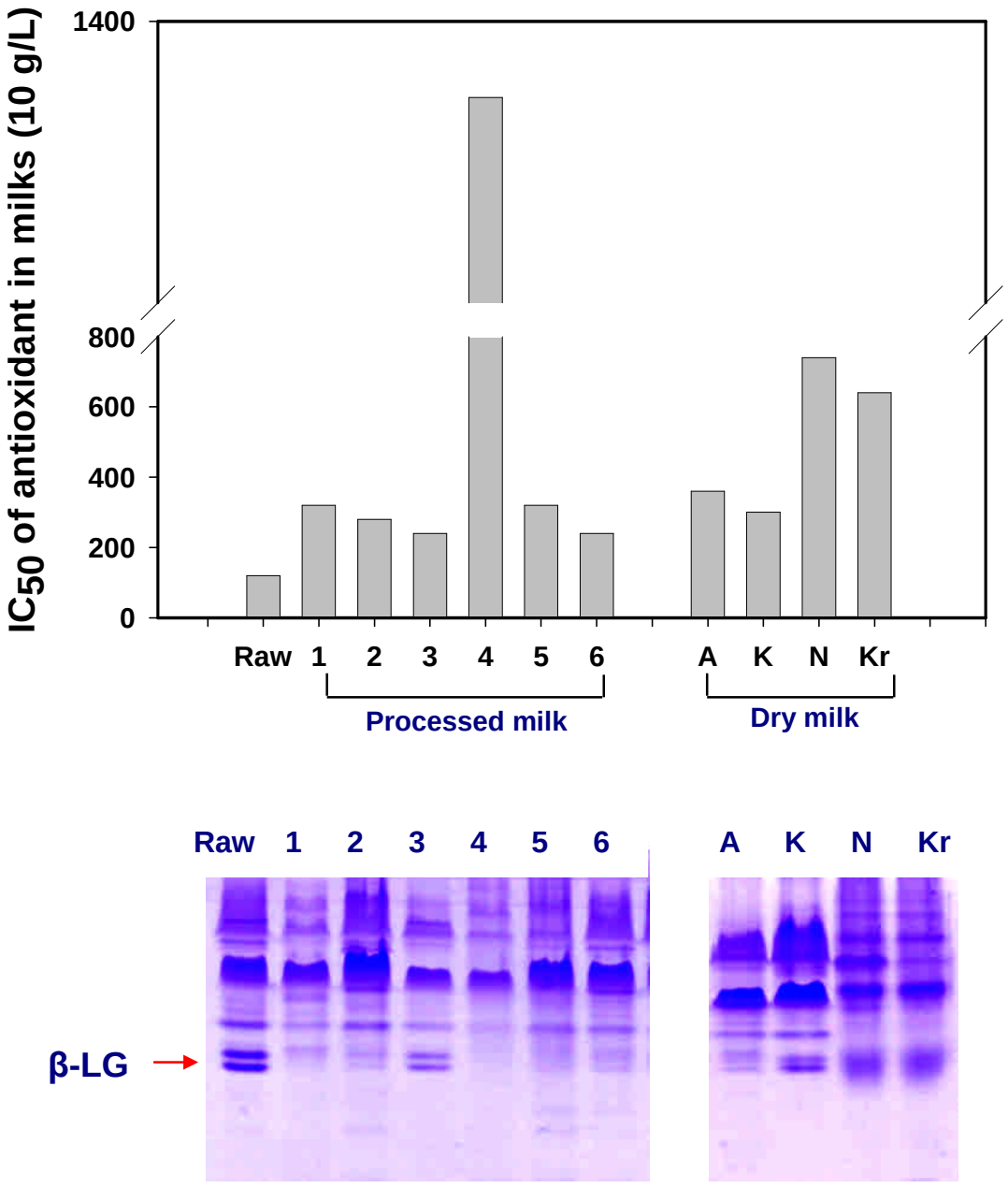

IC50 of antioxidant in milks (10 g/L)
1400
800
600
400
200
0
Raw
1
2
3
4
5
6
A
K
N
Kr
Processed milk
Dry milk
Raw
1
2
3
4
5
6
A
K
N
Kr
β-LG

Figure 19