Antioxidant Nature of Bovine Milk β -Lactoglobulin

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ABSTRACT

Heating is necessary for processing milk in the dairy industry, which evidently produces a conformational change in β -lactoglobulin (β -LG). β -Lactoglobulin, a major protein that accounts for approximately 10 to 15% of total milk proteins, is a globular protein consisting of 162 AA with a relative molecular mass of 18.4 kDa. The purpose of the present study was to determine the antioxidant role of β -LG in milk and the possible mechanism involved. We showed that β -LG is a mild antioxidant whose potency is less than that of vitamin E and probucol (the latter being an antioxidant used for clinical therapy). The conversion of the β -LG monomer to dimer was responsible, in part, for the mode of action in protecting low-density lipoproteins against copper-induced oxidation. Cross-linking the free thiol groups of β -LG by heating (100°C for 2 min), or chemically modifying the β -LG by carboxymethylation to block the thiol groups resulted in a substantial loss of antioxidant activity. The data suggest that Cys-121 plays an essential role in the antioxidant nature of β -LG. By using an anti-LG antibody affinity column to deplete the β -LG from milk, we observed from the lost antioxidant activity that β -LG contributes approximately 50% of the total activity. Because β -LG is extremely sensitive to thermal denaturation, to maintain its antioxidant nature, dairy products consumed daily should not be overheated in order to maintain its antioxidant nature.

Key words: β -lactoglobulin, antioxidant activity, mechanism, milk

INTRODUCTION

In the dairy industry, bovine milk is frequently heated for pasteurization (62.5°C for 30 min) and sterilization. This heating process may induce oxidative losses of proteins, unsaturated lipids, vitamins, active enzymes, and immunological factors (Fidler et al., 1998;

Cross and Gill, 1999; Riezzo et al., 2003). β -Lactoglobulin, α -LA, immunoglobulin, albumin, and glycomacropptide are the major milk whey components, in which β -LG accounts for 50 to 55% of the total whey proteins (McKenna and O'Sullivan, 1971). β -Lactoglobulin consists of 2 disulfide bonds from residue Cys-106 to Cys-119 and from Cys-66 to Cys-160, with a free thiol group at Cys-121 (Figure 1; Monaco et al., 1987; Qin et al., 1998). The molecules covalently link to form dimers, polymers, or conjugates with other milk proteins upon heating above 80°C, whereas intermolecular disulfide bonds play an important role in stabilizing the polymerization (Roefs and De Kruif, 1994; Surroca et al., 2002; Chen et al., 2004, 2005; Song et al., 2005). We recently showed that the aggregation can be detected by a specific monoclonal antibody (Chen et al., 2004, 2006) and postulated that β -LG is a superior marker for evaluating the thermal processing of milk (Chen et al., 2004, 2005). β -Lactoglobulin loses the ability to bind palmitic acid, retinol, and vitamin D after overheating beyond its transition temperature (70 to 80°C; Song et al., 2005). Residues 66 to 76 of the D-strand of the β -structure appear to be involved in the thermal denaturation (Song et al., 2005).

β -Lactoglobulin is one of the proteins in milk shown to possess antioxidant activity (Stadtman, 1993; Chevalier et al., 2001; Sharp et al., 2004; Elias et al., 2005; Hernández-Ledesma et al., 2006), but its antioxidant potency and its respective role in the total level of antioxidant activity of milk has not been fully investigated. Further, although the molecular remodeling of β -LG occurs during the heating process, the effect of cross-linking on its antioxidant properties has not been elucidated. The purposes of the present study were to compare the antioxidant potency of β -LG with clinically used antioxidants, such as probucol (Mao et al., 1994) and vitamin E, to determine the antioxidant capacity of β -LG in milk and to explore the possible mechanism involved in the antioxidant activity of β -LG.

MATERIALS AND METHODS

β -LG Purification

β -Lactoglobulin was purified from a HPLC-DEAE column using a method similar to that previously de-

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Figure 1. Primary structure of β -LG. β -Lactoglobulin comprises 162 AA, including 5 Cys residues. Two disulfide linkages are located at residues Cys-106 to Cys-119 and Cys-66 to Cys-160. One free Cys is at position 121.

scribed (Chen et al., 2004). In brief, freshly prepared whey proteins of raw milk were first fractionated by 40% saturated ammonium sulfate. The top fraction was dialyzed against 4 L of 0.02 M phosphate buffer, pH 8.0, at 4°C for 24 h using a Spectra membrane with a 12,000 to 14,000 molecular weight cutoff (Spectrum Lab; Rancho Domingues, CA), which included 3 changes of the buffer. The final equilibrium of the dialyzed sample was examined by conductivity using a conductivity meter and was concentrated to about 20 mg/mL. Two milliliters of the solution was applied to a Bio-Rad (Hercules, CA) HPLC-DEAE column (10 × 64 mm) using a Waters system equipped with a 996 photodiode array detector (Waters, Boston, MA). The sample was then eluted with a 0 to 0.5 M linear NaCl gradient in 0.02 M phosphate buffer, pH 8.0, over 60 min at a flow rate of 1 mL/min. Samples corresponding to a single peak of β -LG, identified by gel electrophoresis and immunoassay, were pooled immediately and dialyzed against a buffer containing 0.12 M NaCl and 0.02 M phosphate, pH 7.4 (PBS), at 4°C for 24 h with 3 changes of buffer. Homogeneity of the isolated β -LG (>90% purity) was determined using SDS-PAGE.

Gel Electrophoresis and Western Blot

Sodium dodecyl sulfate-PAGE or native PAGE was performed according to the method of Laemmli (1970), modified by utilizing a vertical slab gel (15% polyacrylamide) with stacking on the top (5% polyacrylamide). Electrophoresis was conducted in a mini-gel apparatus (Mini PIII; Bio-Rad, Hercules, CA) equipped with a PAC 300 power supply (Bio-Rad). Samples (typically 5 μ g) for SDS-PAGE were mixed with a loading buffer containing 12 mM Tris-HCl, 0.4% SDS, 5% glycerol, and 0.02% bromphenol blue, pH 6.8, and run for 1.5 h at

100 V, followed by staining with Coomassie brilliant blue R-250. For native PAGE, similar procedures were conducted, but without the addition of SDS and β -mercaptoethanol.

The Western blot performed was similar to that described previously (Yang and Mao, 1999; Liau et al., 2003; Chen et al., 2004). In brief, the gel obtained from SDS-PAGE or native PAGE was electrotransferred in a semidry transfer cell (Bio-Rad) to a nitrocellulose membrane (Hybond-ECL extra; Amersham) at 90 mA for 1 h. The transferred membrane was then immersed in PBS containing 1% gelatin (wt/vol) for 1 h at room temperature. After 3 washes with PBS, the membrane was incubated for 1 h at room temperature with a mouse monoclonal antibody (5C7G3) prepared against β -LG (Chen et al., 2004) at 1:10,000 dilutions in PBS containing 0.1% gelatin and 0.05% (vol/vol) Tween-20 and washed 3 times with the same dilution buffer. The membrane was then incubated with a commercially available goat antimouse IgG conjugated with horseradish peroxidase (Chemcon, Temecula, CA) at 1:3,000 dilutions for 1 h. Finally, the membrane was developed using 3,3'-diaminobenzidine as a developer.

Reduction and Carboxymethylation of β -LG

Carboxymethylation was conducted in a manner previously described (Song et al., 2005; Chen et al., 2006). Five milligrams of β -LG was first dissolved in 3 mL of 0.01 M Tris-HCl buffer (pH 8.6) containing 5.4 M urea, and 1% (vol/vol) β -mercaptoethanol. The reaction mixture was flushed with nitrogen at room temperature for 15 min. Twenty milligrams of iodoacetic acid was added stepwise to the reaction mixture while maintaining the pH at 8.6 by adding 1 M NaOH within a period of 30 min. The reaction proceeded for another

60 min at room temperature. By using this procedure, more than 92% of the Cys residues were modified, as determined by AA analysis (Mao et al., 1987), which monitored the disappearance of the Cys content in the chromatographic profile (Mao et al., 1980). Finally, carboxymethylated β -LG was desalted on a BioGel P2 column eluted with 0.05 M ammonium bicarbonate and lyophilized. More than 90% of the modified β -LG was noncovalent monomers, judged by an SDS-PAGE as described previously (Song et al., 2005).

Preparation of Rabbit Polyclonal Antibody Against β -LG

A rabbit polyclonal antibody against β -LG was prepared according to the procedures described previously (Chen et al., 2005). In brief, β -LG dissolved in sterilized PBS was mixed and homogenized with an equal volume of complete Freund's adjuvant by a 3-way stopcock. Each rabbit was initially given a total emulsion of 1 mL (containing 1 mg of β -LG) by 10 subcutaneous injections onto the back. After 10 d, doses were boosted with 2 intramuscular injections using 1 mL of PBS containing 1 mg of β -LG without adjuvant. Seven days after the final booster injection, blood was collected in 0.1% EDTA to obtain the plasma. The titer of this antibody was over 1:12,800, as judged by an ELISA (Chen et al., 2004, 2005).

Preparation of the β -LG Antibody Affinity Column

The rabbit antiserum prepared against β -LG was first fractionated by a 50% saturated ammonium sulfate solution. The pellet was redissolved in a PBS buffer and then refractionated 2 times, followed by an exhaustive dialysis against 5 L of PBS (with 3 changes) for 24 h at 4°C. After reaching equilibrium with PBS (as described above), the crude IgG was dialyzed against a coupling buffer containing 0.1 M NaHCO₃ 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 modifications (Liau et al., 2003). Briefly, about 3.3 g of freeze-dried Sepharose was swollen and suspended in 20 mL of 1 mM HCl, followed by immediate (within 15 min) washes with 400 mL of 1 mM HCl on a sintered glass filter. The gel was then washed twice with 100 mL of the coupling buffer and degassed to minimize the air to be trapped inside the resin. About 250 mg of crude IgG fraction in 5.5 mL of coupling buffer was added to 15 mL of CNBr-activated Sepharose while gently stirring with a magnetic bar for 1 h. After coupling, the gel was washed with 1.5 L of PBS to remove unbound

materials. 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 a blocking buffer and a 0.15 M NaCl solution, pH 11.0 (adjusted by 0.1 M NH₄OH). Finally, the gel was equilibrated in PBS and packed into a 1.5 × 20 cm column with a 10-mL bed volume (Liau et al., 2003).

Preparation of Raw Milk Devoid of β -LG

Bulk whole raw milk was obtained from a local dairy farm (Chen et al., 2004) and was skimmed by centrifugation at 3,500 × g for 30 min at 4°C prior to the experiments. β -Lactoglobulin in the skim raw milk was removed by the affinity column, prepared as described above. One milliliter of skim raw milk (about 40 mg/mL of protein) was loaded into the affinity column equilibrated with PBS, whereas the pass-through fractions without β -LG (confirmed by SDS-PAGE) were pooled and used for the analysis of antioxidant activity.

Low-Density Lipoprotein Purification

Human low-density lipoprotein (LDL; density 1.021 to 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, then centrifuged at 148,742 × g 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 148,742 × g for 16 h. The top LDL fraction was immediately dialyzed in a buffered solution containing 0.12 M NaCl and 0.02 M phosphate, pH 7.4 (PBS), and passed through a 0.45- μ m filter before use (Yates et al., 1992).

Antioxidant Assay Using Thiobarbituric Acid-Reactive Substances

The thiobarbituric acid-reactive substance (TBARS) assay was conducted using a method previously established in our laboratory (Mao et al., 1991a,b; Yates et al., 1992; Mao et al., 1994; Tseng et al., 2004b). In a typical assay, LDL (60 μ g) was first incubated with various amounts of β -LG, heated β -LG, probucol, vitamin E, or other antioxidant candidates in a test tube containing a total volume of 80 μ L of PBS at room temperature for 10 min. Bovine serum albumin was included as a negative control. To the reaction mixture was added 20 μ L of 100 μ M Cu₂SO₄ (pH 7.0; 20 μ M final) to make a final volume of 100 μ L, and the mixture was incubated in a 37°C water bath for 2 h (Tseng et

Figure 2. Antioxidant activity of β -LG and other antioxidants. The antioxidant activity was estimated by the degree of inhibition of Cu^{2+} -induced formation of thiobarbituric acid-reactive substances. The assay was conducted using 100 μg of low-density lipoprotein in a final 100 μL of reaction mixture (see the Materials and Methods section). Each point represents the mean of duplicate determinations.

al., 2004b). To stop the reaction, 250 μL of 20% TCA was added to the mixture and vortexed briefly. Finally, another 250 μL of 0.1% thiobarbituric acid was added, and the mixture was incubated in an 80°C water bath for 30 min. Each tube was centrifuged at $1,500 \times g$ for 3 min. The upper chromogenic solution (250 μL) in pink was read at 540 nm using an ELISA plate (Mao et al., 1994).

Preparation of Heated β -LG and Others

β -Lactoglobulin (1 mg/mL) in PBS was heated at 100°C for 2 min and immediately cooled to 10°C in an iced water bath. Protein concentrations were determined by a modified Lowry assay using BSA as a standard (Peterson et al., 1977).

RESULTS

Antioxidant Activity of β -LG

Using lipid-in-water emulsions, Elias et al. (2005) demonstrated that β -LG is an antioxidant. In the present study, we used LDL, which was previously established in our laboratory (Mao et al., 1994; Tseng et al., 2004b) as a lipid source, to investigate the antioxidant activity of β -LG. Figure 2 shows β -LG as possessing a mild antioxidant activity. As evaluated by IC_{50} (50% inhibitory concentration) values, the antioxidant activity of β -LG was lower than that of probucol and vitamin E (Mao et al., 1994). In contrast, BSA (present in the

milk) did not exhibit any antioxidant activity within the concentration ranges tested.

Relationship Between Antioxidant Activity and Dimerization of β -LG

Figure 3A shows that the Cu^{2+} -induced LDL oxidation occurred in a time-dependent manner. Next, to observe the possible structural change in β -LG while protecting the LDL against lipid peroxidation, 60 μM of β -LG was added to the experiment. At this dose, we showed that the extent of inhibition was maintained within the first 24 h (Figure 3A). By SDS-PAGE analysis, we observed that some, but not all, of the β -LG formed covalently linked dimers of β -LG (37 kDa; Figure 3B). This observation was further confirmed by Western blot analysis using a β -LG monoclonal antibody (Figure 3C). However, all of these dimers converted to β -LG monomers after a treatment of β -mercaptoethanol (data not shown). This suggests that free thiol groups of the β -LG might participate in protecting the LDL against oxidation.

Effects of Carboxymethylation and Heating on the Antioxidant Activity of β -LG

To further confirm the above hypothesis, chemical modification was conducted using carboxymethylation, which blocked the free thiol groups of β -LG. The modified β -LG was observed as primarily monomers, similar to that described in our previous report (Chen et al., 2006). As shown in Figure 4, the antioxidant activity of carboxymethylated β -LG was significantly diminished as compared with that of native β -LG. This further supports the view that free thiol groups might be involved in the antioxidant activity of β -LG. It should be noted that this chemical modification was carried out in the presence of 5.4 M urea. The urea treatment alone did not alter the antioxidant activity of β -LG (data not shown).

Because heat results in the cross-linking of Cys-121 (Burova et al., 1998; Chen et al., 2004, 2005, 2006), we expected that heating might also attenuate the antioxidant activity of β -LG. To minimize the high molecular aggregation of β -LG (covalently linked) upon heating, we heated β -LG at 100°C for 2 min to produce β -LG dimers (37 kDa). Under this condition, β -LG formed mostly, but not all, dimers, as assessed by SDS-PAGE and Western blot analyses (Figure 5). A marked decrease in the antioxidant activity of β -LG occurred upon heating, as shown in Figure 2, but it still retained some antioxidant activity as compared with native LG. This partial activity could be because the heating procedure did not fully denature the β -LG under our experimental

Figure 3. Changes in β -LG in Cu^{2+} -induced low-density lipoprotein (LDL) oxidation over time. The antioxidant activity was determined in the absence or presence of $60 \mu\text{M}$ of β -LG (A). The assay was conducted using $60 \mu\text{g}$ of LDL in a final 100 mL of reaction mixture. Notably, the formation of thiobarbituric acid-reactive substances was about half of that depicted in Figure 2. Each point represents the mean of duplicate determinations. β -Lactoglobulin was gradually oxidized, and some formed β -LG dimers, as analyzed by a 15% SDS-PAGE (B) and a Western blot (C). Lane M: molecular weight marker.

Figure 4. Effect of carboxymethylation on the antioxidant activity of β -LG. Carboxymethylation was conducted to explore the role of disulfide linkages in β -LG for their antioxidant activity. The overall inhibitory activity against low-density lipoprotein ($60 \mu\text{g/mL}$) oxidation of native β -LG was significantly greater than that of carboxymethylated (CM) β -LG ($P < 0.001$). Each bar represents the mean $\pm$ SD of triplicates.

conditions. Such heat-induced dimerization was reversible in the presence of a reducing reagent (data not shown), consistent with previous reports (Burova et al., 1998; Chen et al., 2004, 2005, 2006). The data suggest that free Cys are involved in the antioxidant properties of β -LG.

Figure 5. Characterization of heated β -LG using PAGE and Western blot. β -Lactoglobulin in PBS (1 mg/mL) was heated at 100°C for 2 min. Left: 15% SDS-PAGE. Right: Western blot analysis. Lane M: molecular weight markers; lane A: native β -LG; lane B: heated β -LG. Notably, dimers covalently linked through disulfide linkages were reversible via the addition of β -mercaptoethanol (data not shown).

Figure 6. Sodium dodecyl sulfate-PAGE of skim raw milk with and without β -LG depletion and the effects on antioxidant activity. (A) β -Lactoglobulin-depleted milk was obtained from a β -LG rabbit polyclonal antibody affinity column by collecting the pass-through fraction without further manipulation. Lane M: molecular weight markers; lane 1: β -LG-depleted raw milk; lane 2: raw milk. (B) The antioxidant assay was conducted using 60 μ g of low-density lipoprotein in a final 100-mL reaction mixture. Each point represents the mean $\pm$ SD of triplicates.

Antioxidant Activity of Whole Milk Devoid of β -LG

To define the extent of antioxidant activity caused by β -LG in skim raw milk, we depleted β -LG from the milk using a β -LG antibody affinity column and collected fractions devoid of β -LG. Figure 6A demonstrates the effective removal of the antibody of β -LG via a SDS-PAGE analysis. Figure 6B shows that at an equivalent protein concentration, the total relative antioxidant activity was decreased by about 50% in β -LG-depleted milk in comparison with raw milk (using an optical

density unit at 0.5; Figure 6B). Thus, the β -LG found in milk accounts for approximately 50% of the total antioxidant capacity of raw milk, when evaluated by Cu^{2+} -induced LDL oxidation.

Antioxidant Activity of Heated Milk

We investigated the fundamental differences between heated (100°C for 2 min) and unheated milk for their antioxidant activities. Under this specific heating condition, the β -LG in milk was almost totally denatured, and either aggregated or conjugated with other milk proteins (Figure 7). Most interestingly, heat treatment resulted in a dramatic loss of antioxidant activity (Figure 7). The data revealed that heating not only denatured the β -LG but also severely attenuated the antioxidant nature of the milk.

DISCUSSION

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 into its biochemical structure (Jacques et al., 1999; Engfer et al., 2000; Kontopidis et al., 2002; Song et al., 2005). One of the remarkable features is the binding of vitamin D to β -LG. Recent studies indicated that increased serum vitamin D₃ concentrations were associated with decreased incidences of breast, ovarian, prostate, and colorectal cancers and osteoporotic fractures (Huth et al., 2006). Nearly all fluid milks in the United States and Canada are fortified with vitamin D; fluid milk is a predominant food source of vitamin D.

Another important feature of β -LG is its antioxidant activity. Several studies have described the antioxidant activity of proteins from animal and plant sources, including milk proteins (Cervato et al., 1999; Tong et al., 2000). Enhancement of the body's antioxidant defenses through dietary supplementation would seem to be a reasonable and practical approach to reduce the level of oxidative stress, and increasing evidence exists to support the effectiveness of such a strategy in vitro (Finkel and Holbrook, 2000).

A wide range of in vitro methods are currently used to assess the antioxidant activity of potential compounds, all of which have certain advantages and limitations (Halliwell et al., 1995). One of the popular approaches is to use the TBARS method to measure the malondialdehyde produced from lipid peroxidation of LDL (Mao et al., 1994) when hydroxyl free radicals generated from Cu^{2+} via a Fenton reaction (Fenton, 1894) are used as the radical source. Using this model system, we have shown that probucol and its analogs

Figure 7. Antioxidant activity of skim raw milk with and without heat. Heated milk was prepared by heating at 100°C for 2 min. (A) The antioxidant assay was conducted using 60 μ g of low-density lipoprotein in a final 100-mL reaction mixture. Each point represents the mean $\pm$ SD of triplicates. (B) Native PAGE of raw milk with and without heating. β -Lactoglobulin was substantially lost in heated raw milk by forming β -LG polymers or large aggregates with milk proteins.

can prevent Cu^{2+} -induced LDL oxidation in vitro and further attenuate atherosclerosis in vivo (Mao et al., 1991a,b, 1994). For this reason, we adapted a similar TBARS assay to measure the antioxidant activity of β -LG in this study (Figure 2). Other researchers using oil-in-water emulsions and a fluorescent probe (Elias et al., 2005; Hernández-Ledesma et al., 2005) have also concluded that β -LG is an antioxidant. However, the

antioxidant potency of β -LG, as compared with other antioxidants, has not been reported. Figure 2 shows that the antioxidant activity of β -LG was lower than that of probucol and vitamin E (Mao et al., 1994). Apparently, β -LG is considered to be a mild antioxidant. But considering the amount of daily intake with 500 mL of milk per day (about 4 g/L of β -LG) suggested by the new Dietary Guidelines of 2005 for Americans (Huth et al., 2006), the antioxidant nature of β -LG cannot be ignored. It is of interest that the glycation of β -LG by adding sugar molecules can enhance its activity (Chevalier et al., 2001).

Several lines of evidence suggest that β -LG plays a key antioxidant role in milk. First, the β -LG-depleted milk possessed about 50% less antioxidant activity than that of whole milk (Figure 6). Second, SDS-PAGE and Western blot analyses revealed that LDL oxidation was prevented at the expense of native β -LG, followed by a dimer cross-linking (Figure 3). Third, heated raw milk with denatured β -LG (Chen et al., 2004, 2005; Song et al., 2005) exhibited a striking loss of antioxidant activity, which was consistent with that of the β -LG-depleted milk (Figure 7). Most interestingly, the loss of activity of heated milk was more severe than that of heated β -LG. Although the explanation is not readily known, it is possible that the free thiol groups in β -LG or in other milk proteins act as a reducing agent to regenerate other antioxidants in the raw milk.

The exact mechanism by which a given protein displays antioxidant activity remains unknown. These proteins are thought to encompass free radical scavenging by AA residues and by chelation of prooxidative transition metals. For a given AA residue to be an antioxidant, it must be oxidized before the polyunsaturated fatty acids are attacked by free radicals in lipid oxidation. Amino acid residues vary greatly with regard to their individual oxidative stability (Stadtman, 1993). The ability of these residues to interact with lipid-derived free radicals and Cu^{2+} -derived hydroxyl radicals affects the overall antioxidant activity of the protein. For example, the relative reactivity of amino side chains oxidized by hydroxyl radicals has been reported to be Cys > Trp > Tyr > Met > Phe > His > Ile > Pro (Sharp et al., 2004). A recent evaluation of the activity of Cys, Trp, and Met of β -LG using oil-in-water emulsions suggests that free Cys and Trp are involved, but not Met (Elias et al., 2005). An early study indicated that the antioxidant effect of β -LG was associated with the free sulfhydryl group at Cys-121 (Allen and Wrieden, 1982). Cysteine is thought to act as an antioxidant by donating a hydrogen from its sulfhydryl group. The involvement of Cys in the antioxidant activity of β -LG is consistent with the present report. Because Cys-121 is partially buried within the protein core, whether it undergoes

total oxidation during lipid peroxidation of LDL remains curious. The time course in the dimerization of β -LG (Figure 3) suggests that some, but not all, of the Cys-121 was oxidized during radical scavenging. Thus, this may explain the weak antioxidant nature of β -LG.

The involvement of Tyr residues in the activity of β -LG is unknown. A β -LG fragment (Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile) containing one Tyr exhibits radical scavenging higher than hydroxyanisole (i.e., BHA; Hernández-Ledesma et al., 2005). However, the crucial role of Tyr and the relative potency of the fragment, as compared with the full length of β -LG, were not reported. To address the role of Tyr directly, one possible approach is to use the mutant of recombinant β -LG. That experiment is currently in progress in our laboratory. Our recent report showed that plasma haptoglobin is an extremely potent antioxidant molecule against LDL oxidation, with a potency much greater than probucol and vitamin E (Tseng et al., 2004b). Mutation on Tyr residues using a recombinant antioxidant fragment of haptoglobin did not alter its antioxidant property (I. H. Lai, C. F. Tseng, W. Y. Lai, and S. J. T. Mao, Coll. Biol. Sci. Technol., National Chiao Tung University, Taiwan, Republic of China; unpublished data). Remarkably interesting chemical modification of Cys residues for the dissociation of disulfide linkages resulted in an approximate 5-fold increase in haptoglobin activity. Because the conformation of haptoglobin was drastically altered to a disordered structure, Tseng et al. (2004b) suggested that the surface "antioxidant domain" was further exposed. Thus, the overall conformation of a protein antioxidant also participates in free radical scavenging.

Previously, we showed that milk purchased on the US market was superior to others from our local market, and that the β -LG of the former was almost completely intact (Chen et al., 2004, 2006). The present study showed that the antioxidant activity of milk was correlated with the intactness of the β -LG. We conclude that dairy products with minimally denatured β -LG would be preferable and essential for nutrient uptake. It is advised that additional heat be avoided to maintain the antioxidant nature of purchased milk.

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