# Functional Changes in β-Lactoglobulin upon Conjugation with Carboxymethyl Cyclodextrin

Makoto Hattori,* Yoko Okada, and Koji Takahashi

Department of Applied Biological Science, Faculty of Agriculture, Tokyo University of Agriculture and Technology, Tokyo 183-8509, Japan

Bovine β-lactoglobulin-carboxymethyl cyclodextrin (β-LG−CMCyD) conjugate was prepared using water-soluble carbodiimide, in an effort to improve the functional properties of the protein. The molar ratio of β-LG to CMCyD in the conjugate was 1:2. The isoelectric point of the conjugate was 4.1−5.8. Spectroscopic studies indicated that the global conformation of the conjugate was similar to that of native β-LG. Structural analyses with monoclonal antibodies indicated that there was a conformational change around $^{15}$Val-$^{29}$Ile (β-sheet). The denaturation temperature of the conjugate was about 77 °C, which is about 4 °C higher than that of native β-LG. The β-LG−CMCyD conjugate maintained retinol-binding activity as strong as that of β-LG. The emulsifying activity of β-LG was much improved and the antioxidative activity of β-LG was maintained after conjugation with CMCyD.

**Keywords:** *β-Lactoglobulin; neoglycoconjugate; functional improvement; cyclodextrin; protein conjugation; emulsification; retinol-binding; lipocalin.*

## INTRODUCTION

β-Lactoglobulin (β-LG), a major whey protein, is a globular protein of $M_r$ 18 400 with two disulfide bridges and a free cysteine, containing plenty of essential amino acids (McKenzie, 1971). The results of X-ray crystallography (Papiz et al., 1986; Brownlow et al., 1997) and protein sequencing (Pervaiz and Brew, 1985) have shown that there is remarkable similarity between β-LG and plasma retinol binding protein. β-LG has a calix fold, and it is categorized as a member of the lipocalin superfamily (Flower, 1996). The function of β-LG is tentatively considered to be the binding and transportation of small hydrophobic ligands such as retinol, fatty acids and so on (Pérez and Calvo, 1995). It is also well-known that β-LG has various functional properties such as gelling (Mulvihill and Kinsella, 1987; Foegeding et al., 1992), foaming, and emulsifying properties (Shimizu et al., 1985; Waniska and Kinsella, 1988). Although β-LG is considered to be a valuable protein in terms of its nutritional feature, β-LG is known to be a potent allergen responsible for milk allergy, about 82% of milk allergy patients are sensitive to β-LG (Spies, 1973). The fact that β-LG is stable at low pH and resisistant to proteolysis is considered to be one of the reasons β-LG is allergenic (Miranda and Pelissier, 1983). Hence, it is very desirable to develop new means of decreasing the allergenicity and enhancing the functional properties of β-LG. To achieve this, we have been studying neoglycoconjugates of β-LG. Many studies on neoglycoconjugates of proteins have been performed during the past 20 years, and various improvements in the functional properties of proteins have been achieved. As far as β-LG is concerned, several studies on conjugates of β-LG have been reported, focusing on improvement of its solubility, heat stability, foaming properties, and emulsifying properties (Akita and Nakai, 1990a,b; Bertrand-Harb et al., 1990; Creuzenet et al., 1992; Kitabatake et al., 1985; Mattarella and Richardson, 1983; Mattarella et al., 1983; Waniska and Kinsella, 1988). We have reported that conjugates of β-LG and acidic saccharides such as carboxymethyl dextran and alginic acid oligosaccharide showed enhanced heat stability, improved emulsifying properties and reduced immunogenicity (Hattori et al., 1994b, 1996, 1997, 2000; Nagasawa et al., 1996a,b). In the present study, we chose β-cyclodextrin as a useful saccharide for conjugation with β-LG to improve the functional properties of the protein. Cyclodextrins are cyclic carbohydrates which form inclusion complexes with various hydrophobic molecules and they are widely used in food and pharmaceutical applications (Szejtli, 1998). Cyclodextrins are used in order to stabilize unstable materials and to solubilize insoluble or poorly soluble materials. We prepared a β-LG−carboxymethyl cyclodextrin (CMCyD) conjugate using a water-soluble carbodiimide. Here, we describe the unique properties of the β-LG−CMCyD conjugate and the conformational changes in β-LG brought about through its conjugation with CMCyD.

## MATERIALS AND METHODS

**Materials.** β-Cyclodextrin was supplied by Ensuiko Sugar Refining Co., Ltd. (Yokohama, Japan) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) hydrochloride was purchased from Dojindo (Kumamoto, Japan).

**Preparation of β-LG and RCM-β-LG.** Fresh milk from a Holstein cow of genotype AA for β-LG was supplied by the dairy farm of Tokyo University of Agriculture and Technology (Tokyo, Japan). Crude β-LG was isolated according to the method of Armstrong et al. (1967), and was purified by ion-exchange chromatography using a DEAE-Sephacel column (2.5 ID x 50 cm) under the following conditions: eluent, a linear gradient of a 0−500 mM NaCl in 0.05 N imidazole buffer at pH 6.7; flow rate, 1.0 mL/min; detection, absorbance

* To whom correspondence should be addressed. Tel.: +81-42-367-5879. Fax: +81-42-360-8830. E-mail: makoto@cc.tuat.ac.jp.

10.1021/jf000204z CCC: $19.00 
Published on Web 08/06/2000

at 280 nm. The major fraction was dialyzed against distilled water and then lyophilized. The purity of β-LG was confirmed by PAGE according to the method of Davis (1964). RCM-β-LG was prepared by reduction of the disulfide bonds in β-LG with 2-mercaptoethanol followed by carboxymethylation of the free sulfhydryl groups with sodium iodoacetate as described previously (Kaminogawa et al., 1989).

**Carboxymethylation of β-Cyclodextrin.** β-Cyclodextrin was carboxymethylated by a method previously used for carboxymethylation of dextran (Hattori et al., 1994a,b), with some modifications. In brief, monochloroacetic acid (9 g) was dissolved in 48 mL of methanol, and 10.5 mL of NaCl solution (1 g/mL) was added with gentle stirring. After adding methanol (12 mL), β-cyclodextrin (3 g) was added to the solution, and the mixture was incubated at 45 °C for 48 h. The pH of the reaction mixture was adjusted to 6.5 with acetic acid after cooling to room temperature. After washing with 70% methanol, filtration and lyophilization, carboxymethyl cyclodextrin (CMCyD) was obtained. The degree of modification as determined by NMR (Silverstein et al., 1991) was about 6.9 carboxyl groups attached per molecule of cyclodextrin.

**Preparation of the β-LG−CMCyD Conjugate.** The β-LG−CMCyD conjugate was prepared using water-soluble carbodiimide by referring to the method described previously (Hattori et al., 1994a,b, 2000; Nagasawa et al., 1996a,b).

In brief, β-LG (300 mg) and CMCyD (1,800 mg) were dissolved in 37.5 mL of 0.9% NaCl solution, the pH was adjusted to 4.75 with 0.1 N HCl and 0.1 N NaOH, and EDC solution (276 mg/1.38 mL of distilled water) was added gradually over a period of 30 min, during which the pH was maintained at 4.75 by addition of 0.1 N HCl and 0.1 N NaOH. The reaction mixture was incubated at 25 °C for 3 h. Gly solution (135 mg/1.35 mL) was added to inactivate the residual EDC, and the solution was incubated for 3 h. The reaction was stopped by adding 1.3 mL of 2 M sodium acetate buffer, pH 5.5. After dialyzing against distilled water and lyophilization, crude β-LG−CMCyD conjugate was obtained.

**Purification of the β-LG−CMCyD Conjugate.** Free CMCyD was removed by salting-out. The crude conjugate was dissolved in distilled water at a protein concentration of 10 mg/mL, and the proteinaceous component was salted out with ammonium sulfate at a final concentration of 5 M. The precipitate was recovered by centrifugation at 12,000 rpm for 30 min at 20 °C and dissolved in distilled water (30 mL). After dialyzing against distilled water and lyophilization, the β-LG−CMCyD conjugate without free CMCyD was obtained.

Free β-LG containing polymerized β-LG was removed by ion-exchange chromatography. A DEAE-Toyopearl 650S column (2.2 i.d. × 20 cm, Tosoh, Tokyo, Japan) was equilibrated with 0.1 M phosphate buffer, pH 7.5, at a flow rate of 4.0 mL/min. The β-LG−CMCyD conjugate, after salting-out (11 mg/3 mL), was applied to the column and eluted with a linear gradient of NaCl from 0 to 1 M. Protein was detected by monitoring the absorbance at 280 nm. To detect CMCyD, the absorbance was monitored at 490 nm after coloring by the phenol−sulfuric acid method (Dubois et al., 1956).

**Size-Exclusion Chromatography (SEC).** The molecular weight of the β-LG−CMCyD conjugate was measured by SEC. A TSKgel G3000SW$_{XL}$ column (7.8 i.d. × 300 mm, Tosoh, Tokyo, Japan) was equilibrated with a 0.07 M phosphate buffer containing 4 M guanidine hydrochloride at pH 6.7. The β-LG−CMCyD conjugate (100 μg of protein/50 μL) was applied to the column and eluted at a flow rate of 0.5 mL/min. The absorbance was monitored at 280 nm.

**Isoelectric Focusing.** Isoelectric focusing of the β-LG−CMCyD conjugate was performed by using the Pharmacia-Phast System (Kramlova et al., 1986). Protein bands were detected by staining with Coomassie Brilliant Blue.

**Measurement of the Retinol-Binding Activity of the β-LG−CMCyD Conjugate.** The retinol-binding activity of the β-LG−CMCyD conjugate was measured by fluorescence titration (Cogan et al., 1976; Futterman and Heller, 1972; Hattori et al., 1993). Retinol in ethanol was added to a 2.0 mL solution in a cuvette containing 1.0 mg of protein in PBS. Small increments (5 μL at a time) of the retinol in ethanol at 2.64 × $10^{-4}$ M were added to the cuvette with a micropipet. The mixture was thoroughly mixed and then allowed to equilibrate for 1 min before recording the fluorescence intensity. The fluorescence was measured using a Shimadzu RF-5300PC instrument (Kyoto, Japan) with excitation at 330 nm and emission at 470 nm.

**Analysis of the Conformation of the β-LG−CMCyD Conjugate by Spectroscopic Methods.** Far UV CD spectra of the β-LG−CMCyD conjugate (0.02% as the protein concentration) in PBS (phosphate-buffered saline; 0.11 M phosphate buffer, pH 7.1, containing 0.04 M NaCl and 0.02% $NaN_3$) were recorded at 25 °C with a JASCO J-720WI (Tokyo, Japan) automatic recording spectro-polarimeter in cells of 1.0 mm path length. The intrinsic fluorescence of the β-LG−CMCyD conjugate dissolved in PBS at 0.001% (as the protein concentration) was measured at an excitation wavelength of 283 nm using a Shimadzu RF-5300PC (Kyoto, Japan) fluorescence spectrophotometer.

**Competitive Enzyme-Linked Immunosorbent Assay (ELISA).** Aliquots of β-LG solution (100 μL; 0.01% β-LG in PBS) were added to the wells of a polystyrene microtitration plate (Maxisorp, Nunc, Roskilde, Denmark) and the plate was incubated at 25 °C for 2 h. The β-LG solution was then removed, the wells were washed three times with 125 μL of PBS-Tween, and 125 μL of 1% ovalbumin solution was added. The plate was again incubated at 25 °C for 2 h and washed. β-LG, RCM-β-LG or β-LG−CMCyD conjugate at various concentrations (50 μL) was added to the wells, then mAb (50 μL) was immediately added, and the plate was further incubated at 25 °C for 2 h. After removing the solution and washing the wells, 100 μL of alkaline phosphatase-labeled goat anti-mouse immunoglobulin diluted with PBS-Tween was added, and the plate was incubated at 25 °C for 2 h. After removing the solution and washing, 100 μL of a 0.1% solution of *p*-nitrophenyl phosphate disodium salt in 1 M diethanolamine-hydrochloride buffer (pH 9.8) was added as the alkaline phosphatase substrate, and the plate was incubated at 25 °C for 30 min. The reaction was stopped by adding 20 μL of 5 M sodium hydroxide, and the absorbance was read at 405 nm. The equilibrium constant ($K_{AS}$) for the interaction between the mAb and the β-LG tested was calculated by the method of Hogg et al. (1987), based on the results of competitive and noncompetitive ELISA.

**Differential Scanning Calorimetry (DSC).** The β-LG−CMCyD conjugate was dissolved in PBS (pH 7.0) at a protein concentration of 50 mg/mL. A 50 μL portion of this solution was sealed in a silver DSC cell, and DSC was conducted to determine the denaturation temperature and enthalpy as described previously (Takahashi et al., 1988). DSC curves were recorded on a Seiko SSC-5020 DSC-100 instrument (Tokyo, Japan) programmed at a heating rate of 2 K/min in the temperature range from 20 to 100 °C. The reference sample was distilled water.

**Evaluation of the Emulsifying Ability of the β-LG−CMCyD Conjugate.** The β-LG−CMCyD conjugate was dissolved in McIlvaine buffer (pH 3.0, 4.0, 5.0, 6.0, 7.0, and 8.0) or in McIlvaine buffer (pH 7.0) containing 0.2 or 0.5 M NaCl to give a concentration of 0.1% (as protein). 0.5 mL of oleic acid was emulsified with 2 mL of this β-LG−CMCyD conjugate solution at 25 °C by a Polytron PTA-7 (Kinematica, Switzerland) for 1 min at 24 000 rpm. The emulsion stability was measured by determining the absorbance at 500 nm of the emulsion diluted 100-fold with 0.1% SDS solution (Pearce and Kinsella, 1978). The emulsifying activity of the β-LG−CMCyD conjugate was evaluated by spectroturbidity according to the method of Pearce and Kinsella (1978). The emulsifying activity is expressed as emulsifying activity index (EAI), calculated using the formula $EAI = 2T/\phi c$, where $T$ (turbidity) $= 2.3A/l$ ($A$ is the absorbance at 500 nm and $l$ (light path) $= 10^{-2}$ m), and $c$ is the concentration of protein ($10^3$ g/m$^3$), with $\phi$ (oil phase volume) $= 0.2$.

**Evaluation of the Antioxidative Activity of the β-LG−CMCyD Conjugate.** The antioxidative activity of the β-LG−CMCyD conjugate was evaluated by the ferric thiocyanate method (Chen et al., 1995, Hattori et al., 1998).

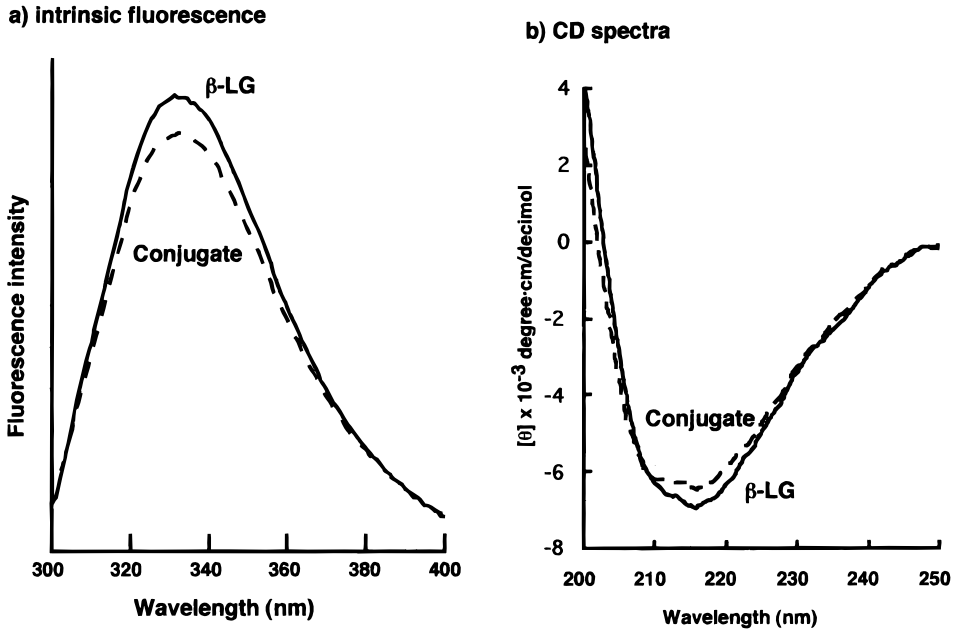


**Figure 1.** Spectroscopic analysis of the β-LG−CMCyD conjugate: (a) intrinsic fluorescence; (b) CD spectra. β-LG (solid line), β-LG−CMCyD conjugate (broken line).

The β-LG−CMCyD conjugate (1 mg) was dissolved in 1.5 mL of 50 mM phosphate buffer (pH 7.0) and 1 mL of 50 mM linoleic acid in ethanol was added. The solution was kept at 60 °C. At intervals, aliquots of the reaction mixture were removed for measurement of the extent of oxidation by the ferric thiocyanate method. In brief, 2.35 mL of 75% ethanol, 50 μL of 30% ammonium thiocyanate, and 50 μL of 20 mM ferrous chloride solution in 3.5% HCl were added to 50 μL of the reaction mixture while stirring the mixture. After 5 min, the absorbance at 500 nm was measured. δ-Tocopherol (1 mg) was used as a positive control.

## RESULTS AND DISCUSSION

**Structural Features of the β-LG−CMCyD Conjugates.** The molar ratio of β-LG to CMCyD in the conjugate was 1:2. The molecular weight of the conjugate was estimated to be 43,000. These results indicate that the conjugate was composed of 2 molecules of β-LG and 4 molecules of CMCyD. The denaturation temperature of the β-LG−CMCyD conjugate as determined by DSC was 77.4 °C which was higher than that of β-LG (73.3 °C).

The fluorescence emission spectrum of the β-LG−CMCyD conjugate is shown in Figure 1a. In the case of β-LG, the fluorescence intensity has been shown to increase with red-shift of the wavelength for maximum emission as the conformation changes (Kaminogawa et al., 1989; Hattori et al., 1993). The maximum wavelength for β-LG−CMCyD (332 nm) was similar to that for native β-LG (332 nm). Hence, the conformation around the Trp residues ($^{19}$Trp and $^{61}$Trp) of the β-LG−CMCyD conjugate was considered to be the same as that in native β-LG. The fluorescence intensity of the conjugate was lower than that of native β-LG, which indicates that CMCyD shielded the area around the Trp residues (Hattori et al., 1994b) and this finding suggests that binding of CMCyD occurs at $^{60}$Lys in the conjugate. The CD spectrum of the β-LG−CMCyD conjugate is shown in Figure 1b, was found to be similar to that of native β-LG. The secondary structure of β-LG is considered to be maintained after conjugation with CMCyD.

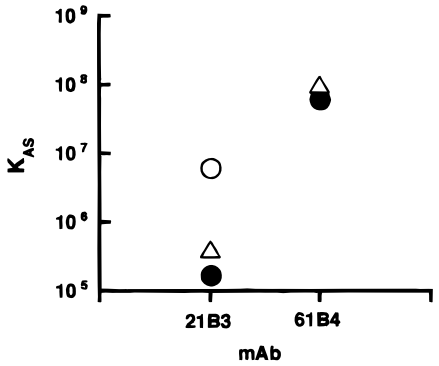


**Figure 2.** Equilibrium constants ($K_{AS}$) of the β-LG−CMCyD conjugate in binding to anti-β-LG mAbs. β-LG (●); RCM-β-LG (○); β-LG−CMCyD conjugate (△). $K_{AS}$ values were calculated from the results of competitive and noncompetitive ELISA according to the procedure of Hogg et al. (1987).

Local conformational changes in β-LG after conjugation with CMCyD were evaluated by competitive ELISA using anti-β-LG mAbs as probes (Figure 2). MAbs are useful to detect subtle conformational changes in local areas within a protein molecule during unfolding and refolding (Kaminogawa et al., 1987; 1989; Hattori et al., 1993) and after conjugation with saccharides (Hattori et al., 1994b; 1996; 1997; 2000; Nagasawa et al., 1996b) by determining the affinity change. The epitope regions for mAbs 21B3 and 61B4 are $^{15}$Val-$^{29}$Ile (β-sheet region) and $^{125}$Thr-$^{135}$Lys (α-helix region), respectively. MAb 61B4 reacts preferentially with native β-LG, whereas mAb 21B3 reacts more strongly with the denatured form of β-LG (RCM-β-LG). The reactivity of the mAbs 21B3 and 61B4 with β-LG−CMCyD was similar to that with native β-LG. Hence, the conformation around $^{15}$Val-$^{29}$Ile (β-sheet region) and $^{125}$Thr-$^{135}$Lys (α-helix region) in the β-LG−CMCyD conjugate is considered to be the same as that in the native form of β-LG.

The retinol-binding activity of the conjugate was evaluated by fluorescence titration. The solution remained clear throughout the entire range of investigated concentrations, in all titrations. As shown in

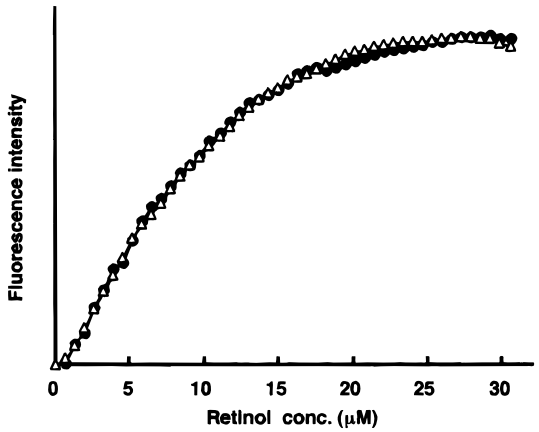


**Figure 3.** Retinol-binding activity of the β-LG−CMCyD conjugate. Mixture (mixture of β-LG and CMCyD in the same ratio as that in the β-LG−CMCyD conjugate) (●); β-LG−CMCyD conjugate (△).

Figure 3, the β-LG−CMCyD conjugate showed retinol-binding activity as strong as that of β-LG. The structure of the retinol binding site (adjacent to $^{19}$Trp and $^{70}$Lys) is considered to be similar to that of the native form of β-LG. Thus, the β-LG−CMCyD conjugate prepared retained the native protein structure of β-LG with improved heat stability.

**Emulsifying Ability of the β-LG−CMCyD Conjugate.** The emulsifying ability of the β-LG−CMCyD conjugate at pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0 was evaluated as the emulsifying activity index (EAI) of an emulsion of oleic acid and the β-LG−CMCyD conjugate (Figure 4a). The β-LG−CMCyD conjugate showed better emulsifying activity the native material under all pH conditions tested. Improved emulsifying ability of β-LG by conjugation with CMCyD is considered to be brought about due to the following: 1) since CMCyD has a substantial net charge, the β-LG−CMCyD conjugate covering oil droplets may inhibit their flocculation by electrostatic repulsion. 2) by conjugating β-LG with CMCyD, the β-LG portion may become more susceptible to surface denaturation than native β-LG.

The emulsifying ability of the β-LG−CMCyD conjugate in the presence of NaCl at pH 7.0 was evaluated as the EAI value of an emulsion of oleic acid and the β-LG−CMCyD conjugate (Figure 4b). The β-LG−CMCyD conjugate showed better emulsifying activity than β-LG, since the conjugate has a substantial net charge which serves a strong protective function against shielding of the electrical charge in the emulsifier.

**Antioxidative Activity of the β-LG−MCyD Conjugate.** The antioxidative activity of the β-LG−CMCyD conjugate was evaluated by the ferric thiocyanate method (Figure 5), with δ-tocopherol used as a positive control. The period during which the $A_{500}$ remained below 0.05 was regarded as the induction period. The induction period in the case of the control sample was 11 days, and it was extended 25, 29, and 29 days in the presence of δ-tocopherol, the mixture of β-LG and CMCyD and the conjugate, respectively. The antioxidative activity of the conjugate was stronger than that of δ-tocopherol and as strong as that of the mixture of β-LG and CMCyD. The β-LG−CMCyD conjugate showed the antioxidative activity as strong as that of β-LG in a water/alcohol system.

**Concluding Remarks.** In this study, we prepared a β-LG−CMCyD conjugate with modified functionality.

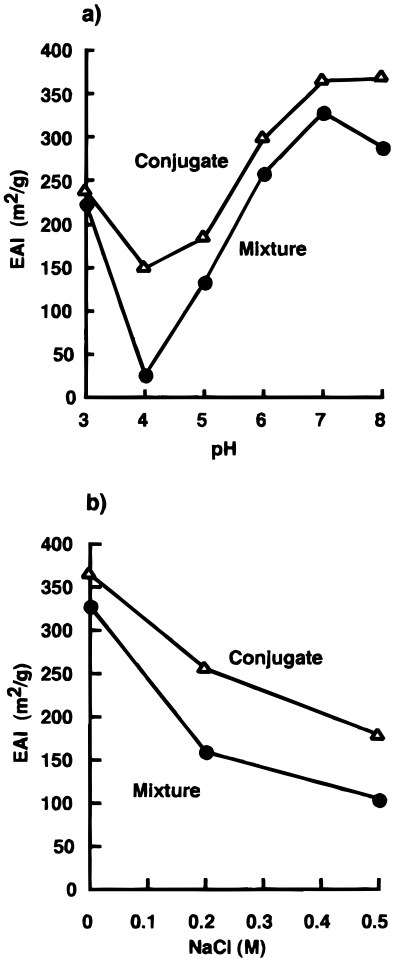


**Figure 4.** Emulsifying activity index of the β-LG−CMCyD conjugate under different pH conditions (a) and in the presence of NaCl (b). Mixture (mixture of β-LG and CMCyD in the same ratio as that in the β-LG−CMCyD conjugate) (●); β-LG−CMCyD conjugate (△).

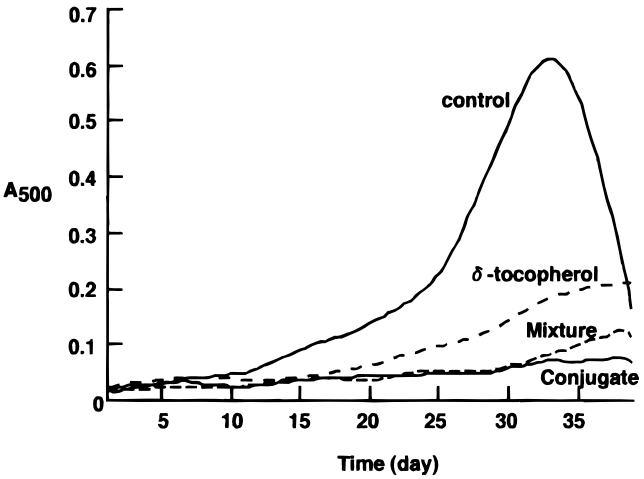


**Figure 5.** Antioxidative activity of the β-LG−CMCyD conjugate. The antioxidative activity of the β-LG−CMCyD conjugate was evaluated by the ferric thiocyanate method. Amounts: β-LG−CMCyD conjugate, 1 mg; δ-tocopherol, 1 mg.

A large change in pI occurred in the β-LG−CMCyD conjugate with little conformational change. The conjugate maintained retinol-binding activity as strong as that of native β-LG. The conjugate was more heat-stable than native β-LG. By conjugation with CMCyD, the emulsifying properties of β-LG under various pH condi-

tions and in the presence of salt were much improved. The conjugate showed the same antioxidative activity as β-LG in a water/alcohol system. This study demonstrates a new possibility of modifying protein functionality by conjugating with an acidic saccharide without drastic changes in conformation.

Abbreviations used: β-LG, β-lactoglobulin; RCM-β-LG, reduced and carboxymethylated β-lactoglobulin; CyD, cyclodextrin; CD, circular dichroism; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PBS-Tween, PBS containing 0.05% Tween 20.

ACKNOWLEDGMENT

We are very grateful to Professor Shuichi Kaminogawa of The University of Tokyo for his help in ELISA experiments. We are also very grateful to Ensuiko Sugar Refining Co., Ltd., for the gift of β-cyclodextrin.

LITERATURE CITED

Akita, E. M.; Nakai, S. Lipophilization of β-lactoglobulin: Effect on hydrophobicity, conformation and surface functional properties. *J. Food Sci.* **1990**, *55*, 711−717.

Akita, E. M.; Nakai, S. Lipophilization of β-lactoglobulin: Effect on allergenicity and digestibility. *J. Food Sci.* **1990**, *55*, 718−723.

Armstrong, J. M.; McKenzie, H. A.; Sawyer, W. H. On the fractionation of β-lactoglobulin and α-lactalbumin. *Biochim. Biophys. Acta* **1967**, *147*, 60−72.

Bertrand-Harb, C.; Charrier, B.; Dalgalarrondo, M.; Cobert, J. M.; Haertlé, T. Condensation of glycosidic and aromatic structures on amino groups of β-lactoglobulin B *via* reductive alkylation: Solubility and emulsifying properties of the protein derivatives. *Lait* **1990**, *71*, 205−215.

Brownlow, S.; Morais, C. J.; Cooper, R.; Flower, D. R.; Yewdall, S. J.; Polikarpov, I.; North, A. C.; Sawyer, L. Bovine β-lactoglobulin at 1.8 Å resolution—still an enigmatic lipocalin. *Structure* **1997**, *5*, 481−495.

Chen, H. M.; Muramoto, K.; Yamauchi, F. Structural analysis of antioxidative peptides from soybean β-conglycinin. *J. Agric. Food Chem.* **1995**, *43*, 574−578.

Cogan, U.; Kopelman, M.; Mokady, S.; Shintzky, M. Binding Affinities of Retinol and Related Compounds to Retinol Binding Proteins. *Eur. J. Biochem.* **1976**, *65*, 71−78.

Creuzenet, C.; Touati, A.; Dufour, E.; Choiset, Y.; Chobert, J. M.; Heartlé, T. Acylation and alkylation of bovine β-lactoglobulin in organic solvents. *J. Agric. Food Chem.* **1992**, *40*, 184−190.

Davis, B. J. DISC ELECTROPHORESIS − II: Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **1964**, *121*, 404−427.

Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350−356.

Flower, D. R. The lipocalin protein family: structure and function. *Biochem. J.* **1996**, *318*, 1−14.

Foegeding, E. A.; Kuhn, P. R.; Hardin, C. C. Specific divalent cation-induced changes during gelation of β-lactoglobulin. *J. Agric. Food Chem.* **1992**, *40*, 2092−2097.

Futterman, S.; Heller, J. The enhancement of fluorescence and decreased susceptibility to enzyme oxidation of retinol complexed with bovine serum albumin, β-lactoglobulin, and the retinol-binding protein of human plasma. *J. Biol. Chem.* **1972**, *247*, 5168−5172.

Hattori, M.; Ametani, A.; Katakura, Y.; Shimizu, M.; Kaminogawa, S. Unfolding/refolding studies on bovine β-lactoglobulin with monoclonal antibodies as probes: Does a renatured protein completely refold? *J. Biol. Chem.* **1993**, *268*, 22414−22419.

Hattori, M.; Imamura, S.; Nagasawa, K.; Takahashi, K. Functional changes of lysozyme by conjugating with carboxymethyl dextran. *Biosci. Biotech. Biochem.* **1994a**, *58*, 174−177.

Hattori, M.; Nagasawa, K.; Ametani, A.; Kaminogawa, S.; Takahashi, K. Functional changes in β-lactoglobulin by conjugation with carboxymethyl dextran. *J. Agric. Food Chem.* **1994b**, *42*, 2120−2125.

Hattori, M.; Ogino, A.; Nakai, H.; Takahashi, K. Functional improvement of β-lactoglobulin by conjugating with alginate lyase-lysate. *J. Agric. Food Chem.* **1997**, *45*, 703−708.

Hattori, M.; Yamaji-Tsukamoto, K.; Kumagai, H.; Feng, Y.; Takahashi, K. Antioxidative activity of soluble elastin peptides. *J. Agric. Food Chem.* **1998**, *46*, 2167−2170.

Hattori, M.; Nagasawa, K.; Ohgata, K.; Sone, N.; Fukuda, A.; Matsuda, H.; Takahashi, K. Reduced immunogenicity of β-lactoglobulin by conjugation with carboxymethyl dextran. *Biocojugate Chem.* **2000**, *11*, 84−93.

Hogg, P. J.; Johnson, S. C.; Bowles, M. R.; Pond, S. M.; Winzor, D. J. Evaluation of equilibrium constants for antigen−antibody interaction by solid-phase immunoassay: The binding of paraquant to its elicited mouse monoclonal antibody. *Mol. Immunol.* **1987**, *24*, 797−801.

Jennes, R. Comparative aspects of milk proteins. *J. Dairy Res.* **1979**, *46*, 197−210.

Kaminogawa, S.; Hattori, M.; Ando, O.; Kurisaki, J.; Yamauchi, K. Preparation of monoclonal antibody against bovine β-lactoglobulin and its unique affinity. *Agric. Biol. Chem.* **1987**, *51*, 797−802.

Kaminogawa, S.; Shimizu, M.; Ametani, A.; Hattori, M.; Ando, O.; Hachimura, S.; Nakamura, Y.; Totsuka, M.; Yamauchi, K. Monoclonal antibodies as probes for monitoring the denaturation process of bovine β-lactoglobulin. *Biochim. Biophys. Acta* **1989**, *998*, 50−56.

Kitabatake, N.; Cuq, J. L.; Cheftel, J. C. Covalent binding of glycosyl residues to β-lactoglobulin: effects on solubility and heat stability. *J. Agric. Food Chem.* **1985**, *33*, 125−130.

Kramlova, M.; Pristoupil, T. I.; Fricova, V.; Kraml, J. First experience with the use of the Pharmacia Phastsystem for the characterization of hemoglobins by isoelectric focusing. *J. Chromatogr.* **1986**, *367*, 443−445.

Mattarella, N. L.; Richardson, T. Physicochemical and functional properties of positively charged derivatives of bovine β-lactoglobulin to form positively charged proteins. *J. Agric. Food Chem.* **1983**, *31*, 972−978.

Mattarella, N. L.; Creamer, L. K.; Richardson, T. Amidation or esterification of bovine β-lactoglobulin to form positively charged proteins. *J. Agric. Food Chem.* **1983**, *31*, 968−972.

McKenzie, H. A. β-Lactoglobulins. In *Milk Proteins: Chemistry and Molecular Biology;* McKenzie, H. A. Ed.; Academic Press: New York, 1971; Vol. 2, pp 257−330.

Mulvihill, D. M.; Kinsella, J. E. Gelation characteristics of whey proteins and β-lactoglobulin. *Food Technology* **1987**, *41*(9), 102−111.

Nagasawa, K.; Takahashi, K.; Hattori, M. Improved emulsifying properties of β-lactoglobulin by conjugating with carboxymethyl dextran. *Food Hydrocolloids* **1996a**, *10*, 63−67.

Nagasawa, K.; Ohgata, K.; Takahashi, K.; Hattori, M. Role of the polysaccharide content and net charge on the emulsifying properties of β-lactoglobulin-carboxymethyl dextran conjugates. *J. Agric. Food Chem.* **1996b**, *44*, 2538−2543.

Papiz, M. Z.; Sawyer, L.; Eliopoulos, E. E.; North, A. C. T.; Findlay, J. B. C.; Sivaprasadrao, R.; Jones, T. A.; Newcomer, M. E.; Kraulis, P. J. The structure of β-lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **1986**, *324*, 383−385.

Pearce, K. N.; Kinsella, J. E. Emulsifying properties of proteins: Evaluation of a turbidimetric technique. *J. Agric. Food Chem.* **1978**, *26*, 716−723.

Perez, M. D.; Calvo, M. Interaction of β-Lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein. *J. Dairy Sci.* **1995**, *78*, 978−988.

Pervaiz, S.; Brew, K. Homology of β-lactoglobulin, serum retinol binding protein, and Protein HC. *Science* **1985**, *228*, 335−337.

Szejtli, J. Introduction and over view of cyclodextrin chemistry. *Chem. Rev.* **1998**, *98*, 1743−1753.

Shimizu, M.; Saito, M.; Yamauchi, K. Emulsifying and Structural Properties of β-lactoglobulin at different pHs. *Agric. Biol. Chem.* **1985**, *49*, 189−194.

Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. In *Spectrometric Identification of Organic Compounds*, (5th ed.); John Wiley & Sons: New York, 1991; p 419.

Spies, J. Milk Allergy. *J. Milk Food Technol.* **1973**, *36*, 225−231.

Takahashi, K.; Shirai, K.; Wada, K. Melting Behavior of Gels Prepared from Isolated Subunits of Collagen. *J. Food Sci.* **1988**, *53*, 1920−1921.

Waniska, R. D.; Kinsella, J. E. Foaming and emulsifying properties of glycosylated β-lactoglobulin. *Food Hydrocolloids* **1988**, *2*, 439−449.

Received for review February 15, 2000. Revised manuscript received May 30, 2000. Accepted May 30, 2000. This work was supported in part by the grant from The Iijima Foundation for The Promotion of Food Science and Technology.

JF000204Z