Functional Changes in β-Lactoglobulin by Conjugation with Carboxymethyl Dextran†

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Two bovine β-lactoglobulin–carboxymethyl dextran (β-LG–CMD) conjugates (conjugates 10A and 10B) were prepared to improve the protein function by using water-soluble carbodiimide. The molar ratios of β-LG to CMD in the conjugates (Conj) were 7:2 (Conj 10A) and 1:1 (Conj 10B). The isoelectric point of each conjugate was 4.7–4.8, which is lower than that of β-LG. Spectroscopic studies suggested that the conformation around had not changed in either conjugate but the α-helix content of Conj 10A had markedly decreased as compared with that of β-LG. Structural analyses with monoclonal antibodies indicated the conformational change of 125Thr–135Lys (α-helix) in Conj 10A and of 15Val–29Ile (β-sheet) in Conj 10B. The denaturation temperature of each conjugate was about 89 °C, which is much higher than that of native β-LG. Each conjugate maintained retinol binding activity as strong as that of native β-LG. The emulsifying activity of β-LG at neutral pH was much improved by conjugation with CMD.

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

INTRODUCTION

β-Lactoglobulin (β-LG), the predominant whey protein, is a globular protein of M_r = 18 400 with two disulfide bridges as well as free cysteine containing plenty of essential amino acids (McKenzie, 1971). Many physicochemical and physiological studies on β-LG have been carried out: higher ordered structure (Townend et al., 1967; Papiz et al., 1986; Gu and Brady, 1992), denaturation mechanism (Alexander and Pace, 1971; Sawyer et al., 1971), polymerization and gelation behavior (Mulvihill and Kinsella, 1987; Laligant et al., 1991; Foegeding et al., 1992), foaming and emulsifying properties (Shimizu et al., 1985; Waniska and Kinsella, 1988), immunological response (Kurisaki et al., 1982, 1985; Takahashi et al., 1988a,b, 1990; Tsuji et al., 1993; J. Kurisak, K. Mizumachi, N. M. Tsuji, S. Kaminogawa, 1994, personal communication), and so on. Results from X-ray crystallography (Papiz et al., 1986) and protein sequence determinations (Pervaiz and Brew, 1985) have shown remarkable similarity between β-LG and plasma retinol binding protein. The function of β-LG is tentatively thought to be binding and transportation of retinol, and it is categorized as a member of the lipocalin super family (Åkerstrom and Lögdberg, 1990). It is well-known that β-LG has good emulsifying properties above pH 5 (Shimizu et al., 1985). However, the emulsifying activity of β-LG decreases in the acidic pH region. Although β-LG is thought to be a valuable protein according to nutritional science, β-LG is known as a potent allergen of milk allergy; about 82% of milk allergy patients are sensitive to β-LG (Spies, 1973).

Hence, it is strongly desirable to develop a new method that would decrease the allergenicity and enhance the functional properties of β-LG. To achieve this, we planned the preparation of a neoglycoconjugate of β-LG. Studies on neoglycoconjugates of proteins have been widely investigated in this decade, and various improvements to the functional properties of proteins have been reported. As far as β-LG is concerned, several studies on the conjugates of β-LG with low molecular weight molecules have been carried out, focusing on the improvement of solubility, heat stability, foaming properties, and emulsifying properties (Kitabatake et al., 1985; Waniska and Kinsella, 1988; Betrand-Harb et al., 1990; Mattarella and Richardson, 1983; Mattarella et al., 1983; Akita and Nakai, 1990a,b; Creuzenet et al., 1992). However, little is known about the improvement of proteins with polymers. Conjugation of a protein with a polymer is thought to improve the protein functions more effectively than conjugation with low molecular weight components. In particular, multiple improvements to protein functions can be expected by conjugation with a charged polymer due to the difference in molecular weight or charge of the chemical species conjugated. To achieve low allergenicity, conjugation of a protein with polysaccharides is thought to be more effective than conjugation of a protein with low molecular weight molecules because it can be expected that polysaccharides will cover the epitopes of the allergens more effectively than low molecular weight molecules (Sehon, 1982).

Thus, we planned improvements to the functional properties of β-LG by conjugation with an acidic polysaccharide. We chose chemically modified dextran as the most appropriate acidic polysaccharide, since dextran is homogeneous in molecular weight and sequence. We have previously established the method for conjugation between hen egg lysozyme and carboxymethyl dextran

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(CMD; Hattori et al., 1994), and we aimed to conjugate β-LG with CMD by using water-soluble carbodiimide.

Our purpose was to create a novel β-LG–CMD conjugate with high heat stability and improved emulsifying ability while maintaining the retinol binding activity without inducing allergenicity. In the present paper, we describe the structural features of a β-LG–CMD conjugate and its unique functional properties.

MATERIALS AND METHODS

Materials. Dextran T10 was purchased from Pharmacia LKB (Uppsala, Sweden), and 1-ethyl-3-[(3-dimethylamino)-propyl]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 in a DEAE-Sephacel column (2.5 i.d. × 50 cm) under the following conditions: eluent, a 0.05 N imidazole buffer at pH 6.7 with a 0–500 mM NaCl linear gradient; flow rate, 1.0 mL/min; detection, absorbance 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 reducing the disulfide bonds in β-LG with 2-ME and then by carboxymethylating the free sulfhydryl groups with sodium iodoacetate as described previously (Kaminogawa et al., 1989).

Carboxymethylation of Dextran. Dextran was carboxymethylated by applying the method of carboxymethylation for the starch that has been described previously (Hattori et al., 1994; Suzuki et al., 1961). In brief, dextran (1 g, Pharmacia) was dissolved in 4.7 mL of a 15% monochloroacetic acid solution containing 0.7 g of sodium hydroxide and then incubated at 40 °C for 48 h. The reaction mixture was neutralized to pH 6.5 with acetic acid after cooling to room temperature. After dialysis against distilled water and lyophilization, carboxymethyl dextran (CMD) was obtained. The degree of modification was determined by hydrochloride–methanol titration (Smith, 1967), and it was clarified that about 25 carboxyl groups were attached to one molecule of dextran.

Preparation of the β-LG–CMD Conjugates. The β-LG–CMD conjugates were prepared as described previously (Hattori et al., 1994) (method 1) and by referring to the method of Kitabatake et al. (1985) (method 2).

Method 1. CMD (1 g) was dissolved in 100 mL of distilled water, and 100 mL of an EDC solution (10 mg/mL) was added. After the pH was adjusted to 5.5 with 1 N HCl, 100 mL of a β-LG solution (10 mg/mL) was added. The reaction mixture was incubated at 4 °C for 6 h while gently stirring. The reaction was stopped by adding 18 mL of acetic acid, and the solution was dialyzed against distilled water to form a separated oily phase of a coacervate. Crude β-LG–CMD conjugate 1 (Conj 10A) was obtained after lyophilization of the coacervate.

Method 2. β-LG (240 mg) and CMD (1050 mg) were dissolved in 30 mL of distilled water and adjusted to pH 4.75 with 1 N HCl, and EDC solution (413 mg/mL of distilled water) was added gradually during 30 min while maintaining the pH at 4.75 with 1 N HCl. The reaction mixture was incubated at 25 °C for 3 h. The reaction was stopped by adding 2 mL of a 2 M sodium acetate buffer at pH 5.5. After dialysis against distilled water and lyophilization, crude β-LG–CMD conjugate 2 (Conj 10B) was obtained.

Purification of the β-LG–CMD Conjugates. Free CMD was removed by salting-out. The crude conjugates were dissolved in a 0.067 M phosphate buffer at pH 7.0 at a concentration of 5 mg/mL, and the proteinaceous component was salted-out with ammonium sulfate to a final concentration of 5 M. The precipitate, which was recovered by centrifuging at 18 000 rpm for 15 min at 25 °C and washing with the 0.067 M phosphate buffer at pH 7.0, containing 5 M ammonium sulfate, was next dissolved in the 0.067 M phosphate buffer at pH 7.0. After dialysis against distilled water and lyophilization, the β-LG–CMD conjugates without free CMD were obtained.

Free β-LG containing polymerized β-LG was removed by ion-exchange chromatography. A DEAE-Toyopearl 650S column (Tosoh, 2.2 i.d. × 20 cm) was equilibrated with the 0.067 M phosphate buffer at pH 7.0 and at a flow rate of 4.0 mL/min. Each β-LG–CMD conjugate after salting-out (30 mg/3 mL) was applied to the column and eluted with a linear gradient from 0 to 1 M NaCl concentration. To detect the protein and CMD, the absorbance was monitored at 280 and 490 nm after coloring according to the phenol–sulfuric acid method (Dubois et al., 1956), respectively.

Size Exclusion Chromatography (SEC). The molecular weight of each β-LG–CMD conjugate was measured by SEC. A TSKgel G3000SW$_{XL}$ column (7.8 i.d. × 300 mm) was equilibrated with a 0.067 M phosphate buffer containing 0.3 M NaCl at pH 7.0. Each β-LG–CMD conjugate (100 μg of protein/50 μL) was applied to the column and eluted at a flow rate of 1.0 mL/min. The absorbance was monitored at 280 nm.

Isoelectric Focusing. Isoelectric focusing of the β-LG–CMD conjugates was performed by using the Pharmacia Phast System (Kramlova et al., 1986). The electrophoresed protein bands were detected by staining with Coomassie Brilliant Blue.

CD Spectra. CD spectra of the β-LG–CMD conjugates (0.1% as the protein concentration) in PBS (phosphate-buffered saline; a 0.11 M phosphate buffer at pH 7.1 containing 0.04 M NaCl and 0.02% NaN_3) were recorded at 25 °C with a JASCO J-20 (Tokyo, Japan) automatic recording spectropolarimeter in cells of 1.0 mm path length.

Fluorescence Measurement. Fluorescence was measured by a Shimadzu RF-510 (Kyoto, Japan) fluorescence spectrophotometer. The intrinsic fluorescence of each β-LG–CMD conjugate dissolved in PBS at 0.001% (as the protein concentration) was measured under an excitation wavelength of 283 nm.

Preparation and Purification of the Monoclonal Antibodies (mAbs). Ascites containing four kinds of mAb were obtained as described previously (Kaminogawa et al., 1985, 1987, 1989; Hattori et al., 1993). Each mAb was purified in an Ultra Pack ABx column (Bakerbond, 8 × 250 mm). The ascites were diluted with four parts of 10 mM MES at pH 5.6, filtered through a 0.2 μm membrane, and applied to the column. Elution was conducted with a linear gradient from 10 mM MES (pH 5.6) to 250 mM KH_2PO_4 (pH 6.8).

Competitive Enzyme-Linked Immunosorbent Assay (ELISA). A β-LG solution (100 μL at 0.01%) dissolved in PBS was added to a polystyrene microtitration plate (Nunc), before the plate was incubated at 25 °C for 2 h. After the β-LG solution had been removed and the well washed three times with 125 μL of PBS–Tween, 125 μL of a 1% ovalbumin solution was added. The plate was again incubated at 25 °C for 2 h and washed. β-LG, RCM-β-LG, or a β-LG–CMD conjugate at various concentrations (50 μL) was added to the well just before an mAb (50 μL) was, and the plate was further incubated at 25 °C for 2 h. After removal of the solution and washing, 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 more. After removal of the solution and washing, 100 μL of a 0.1% solution of *p*-nitrophenyl phosphate disodium salt in a 1 M diethanolamine hydrochloride buffer (pH 9.8) was added as a substrate, and the plate was incubated for the last time at 25 °C for 30 min. The reaction was stopped by adding 6 M sodium hydroxide, and an assay was performed by reading the absorbance at 405 nm. The equilibrium constant for the interaction between mAb and tested β-LG was calculated according to the method of Hogg et al. (1987), based on the competitive and noncompetitive ELISA results.

Measurement of the Retinol Binding Activity of the β-LG–CMD Conjugates. The retinol binding activity of the β-LG–CMD conjugates was measured by fluorescence titration (Cogan et al., 1976; Futterman and Heller, 1972; Hattori et al., 1993). In brief, retinol in ethanol was added to a 2.0 mL solution in a cuvette containing 2.0 mg (109 nmol) of protein

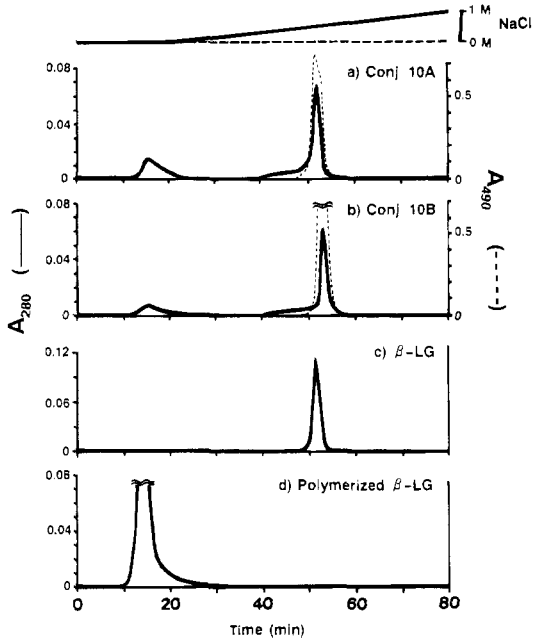

Figure 1. DEAE chromatographic patterns of β-LG–CMD conjugates purified by salting-out. The reaction product after salting-out (30 mg/3 mL) was applied to a DEAE-Toyopearl 650S column (Tosoh, 2.2 i.d. × 20 cm) and eluted by using a linear gradient of 0–1 M NaCl concentration in a 0.067 M phosphate buffer (pH 7.0). To detect the protein, absorbance was monitored at 280 nm, while CMD was detected by the phenol–sulfuric acid method (Dubois et al., 1956).

in PBS. Small increments (5 μL at a time) of the retinol in ethanol at 2.26×10^{-4} M were added to the cuvette with a micropipet. The mixture was thoroughly mixed and then allowed to equilibrate for 5 min before the fluorescence intensity was recorded. The fluorescence was measured by a Shimadzu RF-510 instrument (Kyoto, Japan) with excitation at 330 nm and emission at 470 nm. At the end of the various titrations, the ethanol concentration amounted to less than 7% and is assumed to have had no effect on the measurement system. The solution remained clear throughout the entire range of investigated concentrations.

Differential Scanning Calorimetry (DSC). Each β-LG–CMD conjugate was dissolved in PBS (pH 7.0) at a protein concentration of 50.0 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., 1988a). 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–CMD Conjugates. A β-LG–CMD conjugate was dissolved in a 0.05 M acetate buffer at pH 7.0 to give a concentration of 0.1% (as a protein); 0.5 mL of oleic acid was emulsified with 2 mL of this β-LG–CMD conjugate solution at 25 °C by a Polytron PTA-7 (Kinematica, Switzerland) for 5 min at full speed. The emulsion stability was measured by the absorbance at 500 nm of the emulsion 100-fold diluted with a 0.1% SDS solution (Pearce and Kinsella, 1978).

RESULTS AND DISCUSSION

Isolation of the β-LG–CMD Conjugates. The DEAE chromatographic pattern for each salted-out product is shown in Figure 1. The elution pattern monitored at 280 nm indicates a relatively small peak for the passing fractions and a sharp peak for the adsorbed fractions. The passing fractions are thought to have been polymerized β-LG. The fractions eluted at 0.5–0.6 M sodium chloride concentration were collected, because they indicated strong absorption based on saccharides by the phenol–sulfuric acid method. After dialysis against distilled water and lyophilization, the purified β-LG–CMD conjugates were isolated. Recoveries of the conjugates were about 20% for Conj 10A and about 12% for Conj 10B based on the amount of β-LG used.

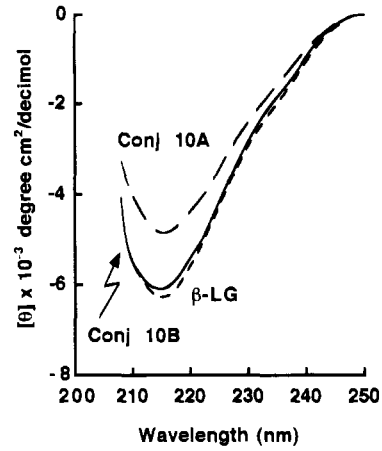

Figure 2. CD spectra of the β-LG–CMD conjugates in PBS: (—) β-LG; (- - -) Conj 10A; (—) Conj 10B.

Isoelectric focusing and SDS–PAGE were carried out to confirm covalent bonding between β-LG and CMD. Each conjugate had a relatively distributed isoelectric point (p*I*), the p*I* values for the main component of Conj 10A and 10B being 4.7 and 4.8, respectively, which are much lower than that of native β-LG (p*I* = 5.2). For SDS–PAGE, Conj 10A and 10B were stained with both Coomasie Brilliant Blue and Alucian Blue, as larger molecule than β-LG and CMD (data not shown). These results strongly suggest that β-LG and CMD were covalently bound to each other. The composition of the two kinds of conjugates determined by their absorbance at 280 nm and by the phenol–sulfuric acid method indicated that the molar ratios of CMD to β-LG were about 7:2 for Conj 10A and 1:1 for Conj 10B. The molecular weight of each β-LG–CMD conjugate was evaluated to be 450 000 and 240 000 for Conj 10A and 10B, respectively, by SEC. Since the molecular weights of β-LG and CMD are 18 400 and 13 000, respectively, Conj 10A and 10B were thought to be composed of 21 molecules of β-LG and 6 molecules of CMD and 8 molecules of β-LG and 8 molecules of CMD, respectively.

Changes in the Conformation of β-LG by Conjugation with CMD. The far-UV CD spectra of the β-LG–CMD conjugates is shown in Figure 2. The α-helix content of native β-LG estimated according to the method of Chen et al. (1972) was 9.3%, corresponding to the results of X-ray crystallography (Papiz et al., 1986). However, the α-helix contents of Conj 10A and 10B were 5.7% and 9.1%, respectively. The secondary structure of Conj 10B was thought to be similar to that of the native material, while that of Conj 10A was rather destroyed. The marked decrease in α-helix content in Conj 10A suggests that CMD was bound to Lys within the α-helix region (135Lys or 138Lys) or its neighbor (141Lys) of β-LG (Papiz et al., 1986).

The fluorescence emission spectra of the β-LG–CMD conjugates are shown in Figure 3. When excited at 283 nm, native β-LG exhibited a fluorescence emission maximum at 336 nm. It has previously been clarified that the fluorescence intensity increases with red shift of the wavelength for maximum emission as the conformation of β-LG changes (Kaminogawa et al.,1989; Hattori et al., 1993). In this experiment, each conjugate also exhibited the same emission maximum wavelength.

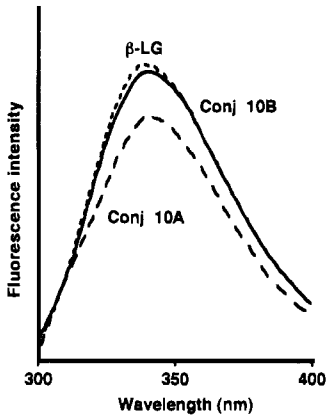

Figure 3. Intrinsic fluorescence of β-LG–CMD conjugates. The emission spectrum of the β-LG–CMD conjugate was measured with an excitation wavelength of 283 nm: (–) β-LG; (- - -) Conj 10A; (—) Conj 10B.

Hence, the conformation around Trp residues (19Trp and 61Trp) of the conjugates seems to have been the same as that of native β-LG. However, the fluorescence intensity of each conjugate was lower than that of native β-LG. This decrease is thought to have been due to the shielding effect by the polysaccharide chain bound to β-LG in each conjugate (Hattori et al., 1994). This shielding effect on the Trp fluorescence was especially prominent in Conj 10A.

The conformational difference between β-LG–CMD conjugates and native β-LG was evaluated by using anti-β-LG mAbs as probes. We have previously shown that mAb can detect the subtle conformational change in local areas within a protein molecule during unfolding and refolding by determining the affinity change (Kaminogawa et al., 1989; Hattori et al., 1993). The properties of mAb used in this experiment are summarized in Table 1. The epitope regions for mAb 21B3, 31A4, and 61B4 are 15Val–29Ile (β-sheet region), 8Lys–19Trp (β-sheet and random coil region), and 125Thr–135Lys (α-helix region), respectively. MAb 61B4 and 62A6 react preferentially to native β-LG, while mAb 21B3 and 31A4 react more strongly to RCM-β-LG (the denatured form of β-LG).

Binding constants for these mAb to the β-LG–CMD conjugates are shown in Figure 4. The reactivity of mAb 21B3 and 31A4 to Conj 10A was a little stronger than that to the native material, while the reactivity of mAb 61B4 and 62A6 to Conj 10A was weaker than that to the native material. In the case of Conj 10A, the conformations of 15Val–29Ile (β-sheet region) and 8Lys–19Trp (β-sheet and random coil region) are thought to have been changed slightly from the native form, while 125Thr–135Lys (α-helix region) and the neighboring region (the epitope for mAb 62A6) are thought to have been changed in conformation. The weak affinity of mAb 61B4 and 62A6 to Conj 10A also infers the possibility of steric hindrance by conjugated CMD in Conj 10A. This ELISA result corresponds with the results of spectroscopic analyses. It thus seems like that the α-helix structure was rather destroyed by conjugating with CMD. Since this α-helix region is thought to be one of the major epitopes in β-LG (Tsuji et al., 1993; Kurisaki et al., 1994, personal communication), it can be expected that Conj 10A would have low allergenicity. The reactivity of mAb 21B3 and 31A4 to Conj 10B was stronger than that to the native material, while the reactivity of mAb 61B4 and 62A6 to Conj 10B was similar to that to the native material. In the case of Conj 10B, 15Val–29Ile (β-sheet region), which exists internally in the native material (Papiz et al., 1986), is thought to have been exposed, while the conformation of the 125Thr–135Lys (α-helix region) and the neighboring region (the epitope for mAb 62A6) is thought to have been similar to that of the native form. 15Val–29Ile is considered to be a hydrophobic region by adopting the hydrophilicity value proposed by Hopp and Woods (1983) to β-LG. It can be expected that Conj 10B would be more hydrophobic than native β-LG and may have better emulsifying activity.

Retinol Binding Activity of the β-LG–CMD Conjugates. To investigate the functionality of the β-LG–CMD conjugates, the retinol binding activity was measured. The β-LG dimer binds two molecules of retinol; this retinol binding does not show pH dependence, the binding site of β-LG being hydrophobic (Fugate and Song, 1980). The results are shown in Figure 5. Each β-LG–CMD conjugate showed retinol binding activity as strong as that of the native material. Thus, the structure of the β-LG–CMD conjugates concerned with retinol binding activity is thought to have been similar to that of the native form.

Thermal Stability of the β-LG–CMD Conjugates. The thermal stability of the β-LG–CMD conjugates was evaluated by DSC. Since CMD was thermally inactive in the temperature range for the present DSC run, it can be said that these DSC curves detected the thermal denaturation of β-LG in the conjugate. The thermal characteristics of the β-LG–CMD conjugates are summarized in Table 2. The denaturation temperature (T_p) and denaturation enthalpy (ΔH) for the mixture of β-LG and CMD in the ratio of the β-LG–CMD conjugates are similar to those for native β-LG. T_p for both conjugates was about 89 °C, which is much higher than that of native β-LG. Kitabatake et al. (1985) have reported that covalent binding of gluconate to amino groups of β-LG enhanced the solubility of β-LG at high temperatures. Conjugation of saccharides to a protein is thought to bring about an improvement in the heat stability of a protein. However, ΔH for Conj 10A decreased to about 40% of the value for native β-LG. This decrease in ΔH is thought to have mainly been due to the decrease in secondary structure such as α-helix in β-LG by conjugation with CMD. ΔH for Conj 10B is similar to that for β-LG, which corresponds with the results of other structural analyses. It is strongly suggested that the β-LG molecule in Conj 10B maintained its higher-order structure.

Emulsifying Ability of the β-LG–CMD Conjugates. The emulsifying ability of the β-LG–CMD conjugates in the neutral pH range was evaluated as the emulsion stability (ES) of an emulsion of oleic acid and each β-LG–CMD conjugate. Figure 6 shows ES values for the emulsion of oleic acid and β-LG–CMD conjugates as a function of time. The emulsions with each β-LG–CMD conjugate were more stable than that with β-LG. In particular, the emulsion with Conj 10B was very stable. The half-reduction time of A_{500} for β-LG is about 10 min, while that for each conjugate was 3 times greater (about 30 min). Since CMD, polymerized β-LG (β-LG cross-linked by EDC), and a mixture of β-LG and CMD showed poor emulsifying ability (data not shown), the enhanced emulsifying ability of the β-LG–CMD conjugates is thought to have been caused by the covalently bound β-LG–CMD conjugates themselves. That is, conjugation with CMD was thought to induce the exposure of hydrophobic region(s) and enhanced flexibility of β-LG molecule that enabled the interaction with oleic acid when emulsification was carried out. The former possibility is supported by the

Table 1. Properties of the Monoclonal Antibodies

mAb	epitope	structure	binding affinity
21B3	15Val–29Ile	β-sheet	native β-LG ≪ RCM-β-LG
31A4	8Lys–19Trp	β-sheet, random coil	native β-LG ≪ RCM-β-LG
61B4	125Thr–135Lys	α-helix	native β-LG ≫ RCM-β-LG
62A6	close to the epitope for mAb 61B4		native β-LG ≫ RCM-β-LG

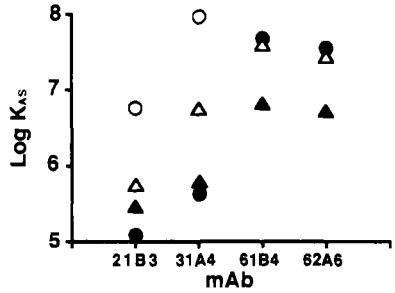

Figure 4. Binding constants of anti-β-LG mAb to β-LG–CMD conjugates: (●) β-LG; (▲) Conj 10A; (△) Conj 10B; (○) RCM-β-LG.

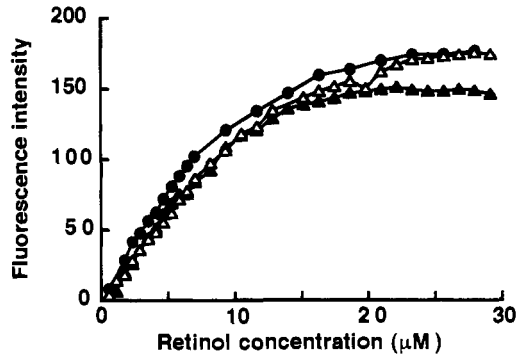

Figure 5. Retinol binding activity of the β-LG–CMD conjugates: (●) β-LG; (▲) Conj 10A; (△) Conj 10B.

Table 2. Thermal Characteristics of the β-LG–CMD Conjugates

	denaturation temp[a] (°C)			denaturation enthalpy (mJ/mg)
	T_o	T_p	T_c	
Conj 10A	79.4	89.0	101.6	5.0
Conj 10B	77.7	89.0	97.7	12.5
β-LG	67.1	72.6	76.7	12.6
β-LG + CMB (mixture 1)[b]	67.5	72.8	78.3	10.3
β-LG + CMD (mixture 2)[c]	69.4	73.6	78.3	10.6

[a] T_o, onset temperature; T_p, peak temperature; T_f, final temperature. [b] β-LG + CMD (mixture 1): mixture of β-LG and CMD at the ratio of Conj 10A. [c] β-LG + CMD (mixture 2): mixture of β-LG and CMD at the ratio of Conj 10B.

results of our structural analysis that the highly hydrophobic region in β-LG (15Val–29Ile) was exposed at the surface by conjugation with CMD, this possibility being particularly plausible for Conj 10B. For the latter possibility, Shimizu et al. (1985) have suggested that the denaturability (flexibility) plays an important role in the emulsifying ability of β-LG. By conjugating with CMD, the β-LG portion is thought to be more susceptible to surface denaturation than native β-LG due to the high mobility of CMD (Hattori et al., 1994).

Concluding Remarks. In this study, we prepared β-LG–CMD conjugates with modified functionality. A large change in p*I* occurred in the β-LG–CMD conjugates with little conformational change. The conjugates maintained retinol binding activity as strong as that of native β-LG. The conjugates were more stable to heating than native β-LG. By conjugation with CMD, the emulsifying properties of β-LG in the neutral pH region were much improved. This study provides a new possibility for modifying protein functionality by conjugation with acidic polysaccharide without drastic changes in conformation. It is expected that β-LG–CMD conjugates prepared in this study may have reduced allergenicity and good emulsifying properties under various environmental conditions. Further experiments on the allergenicity and emulsifying properties of β-LG–CMD conjugates should be carried out.

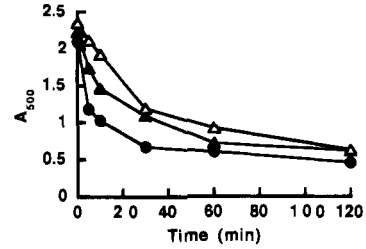

Figure 6. Emulsion stability of O/W emulsions prepared with the β-LG–CMD conjugates at pH 7.0: (●) β-LG; (▲) Conj 10A; (△) Conj 10B. Oleic acid (0.5 mL) was emulsified with 2 mL of each β-LG–CMD conjugate solution (0.01% as a protein) at 25 °C. Emulsion stability was measured by the turbidity at 500 nm of the emulsion 100-fold diluted with a 0.1% SDS solution (Pearce and Kinsella, 1978).

ABBREVIATIONS USED

β-LG, β-lactoglobulin; RCM-β-LG, reduced and carboxymethylated β-lactoglobulin; CD, circular dichroism; mAb, monoclonal antibody; MES, 2-(*N*-morpholino)-ethanesulfonic acid; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PBS–Tween, PBS containing 0.05% Tween 20; UV, ultraviolet; 2-ME, 2-mercaptoethanol; Conj, conjugate.

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