Functional Changes in β-Lactoglobulin by Conjugation with Cationic Saccharides

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Bovine β-lactoglobulin (β-LG) was conjugated to each of three cationic saccharides [glucosamine (GlcN), chitopentaose (CPO), and chitosan (CHS)] by means of a water-soluble carbodiimide or by the Maillard reaction in an effort to improve the functional properties of β-LG. The molar ratios of β-LG to the cationic saccharide in the β-LG−GlcN, β-LG−CPO, and β-LG−CHS conjugates were 2:1, 2:5, and 2:1, respectively. Fluorescence studies indicated that the conformation around Trp had changed in each conjugate and that the surface of each of the conjugates was covered with a saccharide chain. Structural analysis using monoclonal antibodies indicated that the conformation around ^{15}Val−^{29}Ile (β-sheet region) in β-LG−GlcN and β-LG−CPO had changed but that in β-LG-CHS was maintained, whereas the conformation around ^{125}Thr−^{135}Lys (α-helix region) in the conjugates had changed. The emulsifying activity of β-LG was improved by conjugation with CPO or CHS, and aggregation of β-LG was suppressed by conjugation with CHS. Reduction of the antigenicity and immunogenicity of β-LG was achieved by conjugation with CHS.

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

INTRODUCTION

β-Lactoglobulin (β-LG), a major whey protein, is a globular protein of M_r 18400 with two disulfide bridges and one free cysteine residue, containing abundant essential amino acids (McKenzie, 1971). The results of X-ray crystallography (Papiz et al., 1986; Brownlow et al., 1997) and protein sequence determinations (Pervaiz and Brew, 1985) have shown that there is remarkable similarity between β-LG and plasma retinol-binding protein. β-LG has a calyx fold, and it is categorized as a member of the lipocalin superfamily (Åkerstrom and Lögdberg, 1990). The function of β-LG is tentatively considered to be the binding and transportation of small hydrophobic ligands such as retinol and fatty acids (Pérez and Calvo, 1995). It is also well-known that β-LG has various functional properties such as gelling (Foegeding et al., 1992) and foaming and emulsifying properties (Shimizu et al., 1985; Waniska and Kinsella, 1988). Although β-LG is considered to be a valuable protein in view of nutritional science, β-LG is known to be a potent allergen responsible for milk allergy; ∼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 have been studying neoglycoconjugates of β-LG. Various studies on neoglycoconjugates of proteins have been conducted in the past 20 years, and various improvements to the functional properties of proteins have been reported. As far as β-LG is concerned, several studies on conjugates of β-LG have been carried out, focusing on improvement of the 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 Rechardson, 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., 1994, 1996, 1997, 2000; Nagasawa et al., 1996a,b). However, almost no information is available on conjugates of proteins and cationic saccharides. Conjugation with cationic saccharides is likely to affect the structure and function of β-LG, and the improvement of the functional properties of β-LG is expected. Thus, we attempted to prepare neoglycoconjugates of β-LG and cationic saccharides. We chose chitosan and its components, chitopentaose and glucosamine, for conjugation with β-LG in an effort to clarify how a difference in the molecular weight of the cationic saccharides affects the functional improvements of β-LG. Chitosan is a natural mucopolysaccharide found in fungi, yeast, and the shells of crabs and shrimps, and it has many useful properties such as antimicrobial activity, antitumor activity, hypocholesterolemic and hypolipidemic effects, and ion exchange properties. Chitosan is widely utilized for medical, cosmetic, agricultural, and food-related purposes (Winterowd and Sandford, 1995).

In the present study, we prepared β-LG−cationic saccharide conjugates according to the Maillard reaction and by means of a water-soluble carbodiimide. We describe the unique properties of β-LG−cationic saccharide conjugates and the conformational changes in β-LG brought about through conjugation of this protein with cationic saccharides.

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MATERIALS AND METHODS

Materials. Glucosamine and chitopentaose were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Chitosan acetate (MW 8000, degree of deacetylation = 99.8%) was supplied by Saneigen FFI (Osaka, Japan). 1-Ethyl-3-[(3-dimethylamino)propyl]carbodiimide (EDC) was purchased from Dojindo (Kumamoto, Japan).

Preparation of β-LG and RCM-β-LG. β-LG (genotype AA) was isolated from fresh milk of a Holstein cow, supplied by the dairy farm of the Tokyo University of Agriculture and Technology (Fuchu, Japan), according to the method of Armstrong et al. (1967) and was purified by ion-exchange chromatography using a DEAE-Sepharose Fast Flow column (3.0 i.d. × 40 cm; Pharmacia, Uppsala, Sweden). Crude β-LG was applied to the column and eluted with a linear gradient of 0−1 M NaCl in 0.05 N imidazole buffer (pH 6.7) at a flow rate of 5.0 mL/ min. The eluted protein was detected by monitoring the absorbance at 280 nm. The major β-LG-containing fraction was dialyzed against distilled water and lyophilized. The purity of β-LG was confirmed by polyacrylamide gel electrophoresis (PAGE) performed according to the method of Davis (1964). RCM-β-LG was prepared by reduction of the disulfide bonds in β-LG with 2-mercaptoethanol (2-ME) followed by carboxymethylation of the free sulfhydryl groups with sodium iodoacetate as described previously (Kaminogawa et al., 1989).

Preparation of Chitosan Hydrochloride (CHS). Because the competitive consumption of EDC by reaction with acetic acid in chitosan acetate and by reaction with carboxyl groups in β-LG would decrease the efficiency of conjugate formation when the β-LG−chitosan conjugate was prepared, the chitosan acetate was converted into CHS. The chitosan acetate (20 g) was suspended in distilled water (333.3 mL), and HCl (66.6 mL) was added gradually. The mixture was stirred at 25 °C until the solution became turbid. After the addition of ethanol (1600 mL), the precipitate was recovered by centrifugation at 5000 rpm for 10 min at 25 °C. The precipitate was washed successively with ethanol three times and diethyl ether three times. The CHS was recovered by air-drying.

Preparation of the β-LG—Cationic Saccharide Conjugates. β-LG−glucosamine (β-LG−GlcN) conjugate and β-LG−chitopentaose (β-LG−CPO) conjugate were prepared according to the Maillard reaction by referring to the method described previously (Hattori et al., 1997). GlcN (44 mg) or CPO (200 mg) was dissolved in β-LG solution (200 mg/5 mL of 0.9% NaCl solution), and the pH of the solution was adjusted to 7.0 by addition of 0.2 N NaOH. After lyophilization, the mixture was incubated in a desiccator at 50 °C for 27 h at a relative humidity of 79% in the presence of a saturated KBr solution. Each reaction product was dissolved in 0.067 M phosphate buffer (pH 7.0) and then dialyzed against the same buffer at 4 °C for subsequent purification.

β-LG−chitosan (β-LG−CHS) conjugate was prepared with EDC by referring to the method described previously (Hattori et al., 1994; Nagasawa et al., 1996a,b). β-LG (300 mg) and CHS (3600 mg) were each dissolved in 0.9% NaCl solution (40 mL), and the pH of the solution was adjusted to 4.75 by addition of 0.5 N HCl. EDC (90 mg/5 mL of 0.9% NaCl) was added over a period of 1 h while the pH was maintained at 4.75 by addition of 0.5 N NaOH. The reaction mixture was incubated at 25 °C for 6 h, and then 3 mL of acetic acid was added gradually over a period of 30 min to stop the reaction. The reaction product was dialyzed against 0.05 N imidazole buffer (pH 6.7) at 4 °C for subsequent purification.

Purification of the β-LG—Cationic Saccharide Conjugates. Crude β-LG−CHS was applied to a DEAE-Sepharose Fast Flow column (3.0 i.d. × 40 cm; Pharmacia) to remove free β-LG and polymerized β-LG. Elution was carried out with a linear gradient of 0−1 M NaCl in 0.05 N imidazole buffer (pH 6.7) at a flow rate of 5.0 mL/min. For detection of protein, the absorbance was monitored at 280 nm. For detection of saccharides, the absorbance was monitored at 492 nm after color development performed according to the indole−hydrochloric acid method (Dische and Borenfreund, 1950). Crude β-LG−GlcN and β-LG−CPO were purified in a similar manner, except that 0.067 M phosphate buffer (pH 7.0) was used instead of imidazole buffer. The purity of the conjugates was confirmed by SDS−PAGE (Laemmli, 1970).

Chemical Analysis of the β-LG—Cationic Saccharide Conjugates. The amount of protein in each of the β-LG−cationic saccharide conjugates was measured by determining the amount of Asp by amino acid analysis using a Hitachi model 835 amino acid analyzer (Tokyo, Japan). The amount of each cationic saccharide bound to β-LG was measured according to the indole−hydrochloric acid method (Dische and Borenfreund, 1950).

Size Exclusion Chromatography (SEC). The molecular weight of each of the β-LG−cationic saccharide conjugates was measured by SEC. A TSKgel G3000SW$_{XL}$ column (7.8 i.d. × 300 mm; Tosoh, Tokyo, Japan) was equilibrated with 0.02 M phosphate buffer (pH 6.7) containing 4 M guanidine hydrochloride. Each β-LG−cationic saccharide conjugate (100 μg of protein/50 μL) was applied and eluted at a flow rate of 0.5 mL/ min. The absorbance was monitored at 280 nm.

Fluorescence Measurement. The intrinsic fluorescence of each of the β-LG−cationic saccharide conjugates dissolved in phosphate-buffered saline (PBS) [0.11 M phosphate buffer (pH 7.0) containing 0.04 M NaCl and 0.02% NaN_3] at a protein concentration of 0.001% was measured under excitation at 283 nm with a Shimadzu RF-510 instrument (Kyoto, Japan).

Measurement of the Retinol-Binding Activity of the β-LG—Cationic Saccharide Conjugates. The retinol-binding activity of each of the β-LG−cationic saccharide conjugates was measured by fluorescence titration (Futterman and Heller, 1972; Cogan et al., 1976; Hattori et al., 1993, 1994, 1996, 1997). Each conjugate was dissolved in PBS (pH 7.0) at a protein concentration of 0.1%, and 2 mL of the solution was put into a cuvette. Small increments (5 μL at a time) of retinol in ethanol at 2.33×10^{-4} M were added to the cuvette with a micropipet. The fluorescence was measured using a Shimadzu RF-510 instrument (Kyoto, Japan) with excitation at 330 nm and emission at 470 nm.

Enzyme-Linked Immunosorbent Assay (ELISA). Noncompetitive ELISA was carried out as follows. β-LG and each conjugate dissolved in PBS at a protein concentration of 0.01% (100 μL) were added to the wells of a polystyrene microtitration plate (Maxisorp, Nunc, Roskilde, Denmark), and the plate was incubated at 4 °C overnight to coat the wells with each antigen. After removal of the solution, each well was washed three times with 125 μL of PBS−Tween (PBS containing 0.05% Tween 20). A total of 125 μL of 1% ovalbumin /PBS solution was added to each well, the plate was incubated at 25 °C for 2 h, and then the wells were washed. A total of 100 μL of antibody [antiserum obtained after secondary immunization or monoclonal antibody (mAb)] was added to each well, and the plate was incubated at 25 °C for 2 h. After washing, 100 μL of alkaline phosphatase-labeled goat anti-mouse immunoglobulin (DAKO A/S, Glostrup, Denmark) diluted with PBS−Tween was added to each well. The plate was incubated at 25 °C for 2 h, and then the wells were washed. One hundred microliters of 0.1% sodium *p*-nitrophenyl phosphate disodium/diethanolamine-hydrochloric acid buffer (pH 9.8) was added to each well, and the plate was incubated at 25 °C for 30 min. The reaction was stopped by adding 5 M sodium hydroxide solution (20 μL) to each well, and the absorbance at 405 nm was measured using a microplate reader (MPR A4i, Tosoh, Tokyo, Japan).

Competitive ELISA was carried out to investigate the local conformational changes in β-LG after conjugation with cationic saccharides, using anti-β-LG mAbs (mAbs 21B3 and 61B4) as probes, and to evaluate the antigenicity of the β-LG−cationic saccharide conjugates as described previously (Hattori et al., 1993, 1994, 1996, 1997; Kaminogawa et al., 1987, 1989; Nagasawa et al., 1996a,b). The equilibrium constants (K_{AS}) for the interaction of mAb with β-LG, RCM-β-LG, or β-LG−cationic saccharide conjugates were calculated according to the method of Hogg et al. (1987), based on the results of competitive and noncompetitive ELISA.

Evaluation of the Aggregation Properties of the β-LG—Cationic Saccharide Conjugates. The β-LG−cationic saccharide conjugates were each dissolved in distilled water at a protein concentration of 2 mg/mL. The absorbance at 500 nm was measured after the pH of the solution had been adjusted by addition of dilute HCl or NaOH (0.1 or 0.02 N). The solubility of the β-LG−cationic saccharide conjugates was evaluated by measuring the absorbance at 500 nm after compensating for the concentration of the solution.

Evaluation of the Emulsifying Activity of the β-LG—Cationic Saccharide Conjugates. The conjugates were each dissolved in 0.1 N propionate buffer (pH 3.0), 0.1 N acetate buffer (pH 5.0), 0.05 N imidazole buffer (pH 7.0), 0.1 N ammonium−formic acid buffer (pH 9.0), or 0.1 N propionate buffer (pH 3.0) containing 0.2 or 0.5 M NaCl at a protein concentration of 1 mg/mL. To prepare O/W emulsions, a mixture of 2 mL of the protein solution and 0.5 mL of oleic acid was homogenized with a Polytron PTA-7 (Kinematica, Switzerland) at 24000 rpm for 1 min at 25 °C. The absorbance at 500 nm of the emulsion diluted 100-fold with 0.1% SDS solution was measured. The emulsifying activity was evaluated in terms of the emulsifying activity index (EAI). The EAI was calculated by means of the following equation:

$$\mathrm{EAI} = 2T/\phi C$$

where T (turbidity) = 2.3 A/L [A is the absorbance at 500 nm at 0 min and L is the light path (10^{-2} m)], ϕ (oil phase volume) = 0.2, and C is the concentration of protein (10^3 g/m^3) (Pearce and Kinsella, 1978).

Immunization. Female C57BL/6 mice (Charles River Japan Inc., Yokohama, Japan) at 6 weeks of age (five mice/group) were immunized intraperitoneally with 100 μg (as protein) of β-LG and the β-LG−cationic saccharide conjugates emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). Fourteen days after the primary immunization, the mice were boostered with 100 μg of the protein in Freund's incomplete adjuvant (Difco Laboratories). Blood samples were collected 7 days after the primary and secondary immunizations. Blood from five mice of each group was pooled and stored at 4 °C for 24 h to form a clot. Antiserum was prepared from each blood sample after clot formation.

RESULTS AND DISCUSSION

Structural Features of the β-LG—Cationic Saccharide Conjugates. Conjugation of β-LG and the cationic saccharides was confirmed by the coincidence of protein and saccharides in stained bands following SDS−PAGE (data not shown). The composition of each of the conjugates was determined by amino acid analysis (protein moiety) and by the indole−hydrochloric acid method (saccharide moiety). The molar ratios of β-LG to saccharide in the conjugates were 2:1, 2:5, and 2:1, respectively, for β-LG−GlcN, β-LG−CPO, and β-LG−CHS. The molecular weights of the conjugates were evaluated to be 34800, 42000, and 45800, respectively, for β-LG−GlcN, β-LG−CPO, and β-LG−CHS.

The fluorescence emission spectra of the conjugates are shown in Figure 1. It has previously been clarified that the fluorescence intensity increased with red shift of the wavelength for maximum emission as the conformation of β-LG changed (Kaminogawa et al., 1989; Hattori et al., 1993). The fluorescence emission spectra indicated that the emission maximum wavelength of β-LG−GlcN or β-LG−CHS was higher than that of native β-LG. Hence, the conformation around the Trp residues (^{19}Trp and ^{61}Trp) of β-LG−GlcN and β-LG−CHS was considered to have changed from that of native β-LG. In addition, the fluorescence intensity of these conjugates was lower than that of native β-LG. This decrease is considered to have been due to the shielding

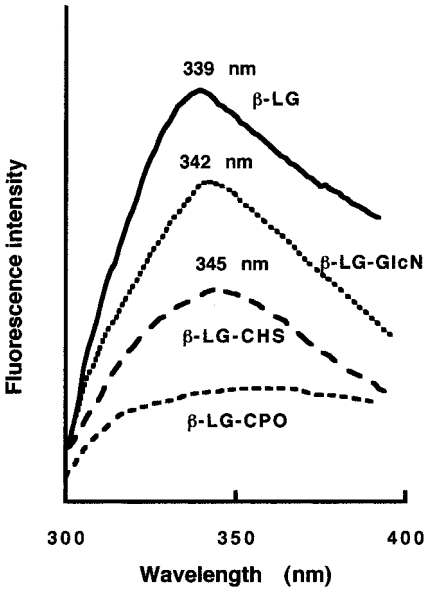

Figure 1. Intrinsic fluorescence of the β-LG−cationic saccharide conjugates. The emission spectra of the β-LG−cationic saccharide conjugates were measured with an excitation wavelength of 283 nm.

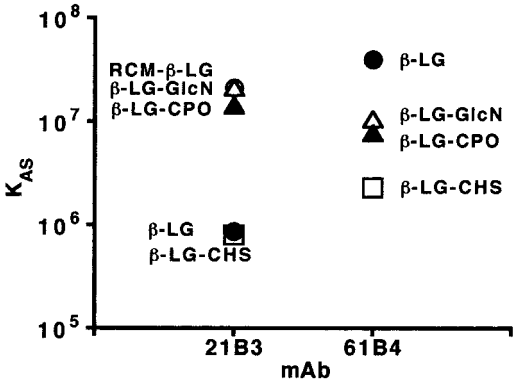

Figure 2. Equilibrium constants (K_{AS}) of anti-β-LG mAbs in interaction with the β-LG−cationic saccharide conjugates: (●) β-LG; (△) β-LG−GlcN conjugate; (▲) β-LG−CPO conjugate; (□) β-LG−CHS conjugate; (○) RCM-β-LG.

effect of the saccharide chain bound to β-LG in the conjugates (Hattori et al., 1994, 1996, 1997). The fluorescence emission spectrum of β-LG−CPO showed very low intensity without any clear maximum emission, which is indicative of conformational changes in β-LG and shielding by the bound CPO.

Local conformational changes in β-LG after conjugation with cationic saccharides were evaluated by competitive ELISA using anti-β-LG mAbs as probes (Figure 2). MAbs can be used to detect the 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., 1994, 1996, 1997; Nagasawa et al., 1996a) by determining the change in affinity. 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 mAb 21B3 with β-LG−GlcN or β-LG−CPO was stronger than that in the case of native β-LG. The reactivity of mAb 61B4 with β-LG−GlcN or β-LG−CPO was weaker

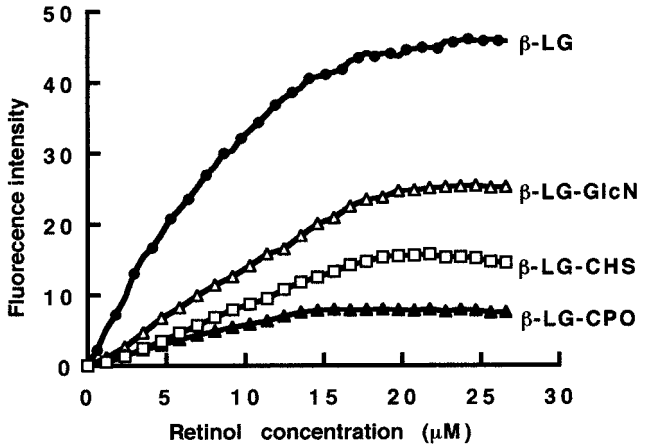

Figure 3. Retinol binding activity of the β-LG−cationic saccharide conjugates: (●) β-LG; (△) β-LG−GlcN conjugate; (▲) β-LG−CPO conjugate; (□) β-LG−CHS conjugate.

than that in the case of native β-LG. In β-LG−GlcN and β-LG−CPO, the region around ^{15}Val−^{29}Ile (β-sheet region), which is apparently inside the molecule in the native state (Brownlow et al., 1997), is considered to have become exposed, whereas the conformation of the region ^{125}Thr−^{135}Lys (α-helix region) is considered to differ from that in native β-LG. The reactivity of mAb 21B3 with β-LG−CHS was similar to that in the case of native β-LG, whereas the reactivity of mAb 61B4 with β-LG−CHS was weaker than that in the case of native β-LG. In β-LG−CHS, the conformation around ^{15}Val−^{29}Ile (β-sheet region) is considered to have maintained the native structure, whereas the conformation of ^{125}Thr−^{135}Lys (α-helix region) is considered to have changed from the native form. The weak affinity of mAb 61B4 to the conjugates also infers the possibility of steric hindrance by bound saccharides. Such conformational differences between the conjugates are thought to have been brought about by the difference in the species of bound saccharides and the binding sites.

The retinol-binding activity of the conjugates was evaluated by fluorescence titration (Futterman and Heller, 1972; Cogan et al., 1976; Hattori et al., 1993). Because the retinol-binding activity of β-LG was lowered by conjugation with cationic saccharides (Figure 3), the structure of the retinol-binding site in the β-LG−cationic saccharide conjugates is considered to have changed from the native form.

Improvements in Functional Properties of β-LG by Conjugation with Cationic Saccharides. Changes in the aggregation properties of β-LG that occurred as a result of conjugation with cationic saccharides were investigated by varying the pH (Figure 4). β-LG and β-LG−GlcN each showed aggregation at pH ∼5.0, whereas β-LG−CPO showed aggregation at pH 6.0. β-LG−CHS did not show any aggregation under acidic conditions, but slight aggregation occurred above neutral pH. The aggregation properties of β-LG under acidic conditions were improved by conjugation with CHS, and this is considered to be due to steric hindrance and electrostatic repulsion brought about by the conjugated CHS. The slight aggregation of β-LG−CHS above neutral pH is considered to be due to the low solubility of CHS above neutral pH.

The effect of pH on the emulsifying activity of the β-LG−cationic saccharide conjugates was evaluated on the basis of the emulsifying activity index (EAI) of emulsions prepared with oleic acid and each of the conjugates (Figure 5a). The emulsifying activity of β-LG−GlcN was slightly higher than that of native β-LG at pH 5.0 and was similar to that of native β-LG at pH 3.0, 7.0, and 9.0. Although the emulsifying activity of β-LG−CPO was lower than that of native β-LG above pH 7.0, β-LG−CPO showed constant emulsifying activity under all pH conditions tested and the EAI of β-LG−CPO was higher under acidic conditions than that of β-LG. Because β-LG−CPO formed aggregates at pH 6.0 (Figure 4), the emulsifying activity of β-LG−CPO may decrease at pH 6.0. The emulsifying activity of β-LG−CHS was markedly high under acidic conditions, whereas it decreased above neutral pH. This decrease is considered to be due to the low solubility of chitosan bound to β-LG in β-LG−CHS above neutral pH. Conjugation with cationic saccharides might have induced the exposure of hydrophobic region(s) or might have enhanced the flexibility of the β-LG molecule, which would facilitate interaction with oleic acid at the time of emulsification. The former possibility is supported by the results of our structural analysis indicating that the most hydrophobic region in β-LG (^{15}Val−^{29}Ile) was exposed at the surface of β-LG−GlcN and β-LG−CPO. Dufour et al. (1998) have shown that the β-LG region protruding into the oil phase at the oil/water interface is ^{15}Val−^{40}Arg. Therefore, ^{15}Val−^{29}Ile in β-LG is considered to be an important region for interaction between oil and this protein. As for the latter possibility, Shimizu et al. (1985) have suggested that denaturability (flexibility) plays an important role in the emulsifying activity of β-LG. Upon conjugation of β-LG with cationic saccharides, the β-LG portion is considered to become more susceptible to surface denaturation than native β-LG due to the high mobility of the cationic saccharides (Hattori et al., 1994). In addition to these factors, we have found that the increase in saccharide content and net charge resulting from conjugation with a high molecular weight polysaccharide is very effective in improving the emulsifying properties under unfavorable conditions (Nagasawa et al., 1996a). The cationic saccharides used in the present study, in particular CPO and CHS, have a high net charge, so the conjugates covering the oil droplets might inhibit their coalescence by electrostatic repulsion.

The emulsifying activity of the conjugates in the presence of NaCl at pH 3.0 was evaluated on the basis of the EAI values of emulsions prepared with oleic acid and each of the conjugates (Figure 5b). β-LG−CPO and β-LG−CHS showed better emulsifying activity than β-LG; both β-LG−CPO and β-LG−CHS have a high net charge, which displays a strong protective effect against shielding of the electrical charge in the emulsifier.

Immunological Properties of β-LG—Cationic Saccharide Conjugates. The antigenicity of the conjugates was evaluated by competitive ELISA (Figure 6). Although anti-β-LG antiserum was obtained by immunizing C57BL/6 mice with native β-LG, the anti-β-LG antiserum showed a high affinity to the denatured form of β-LG (RCM-β-LG). Hence, conformationally altered forms of β-LG are considered to have high antigenicity. β-LG−GlcN and β-LG−CPO both showed stronger binding to anti-β-LG antiserum than native β-LG, whereas β-LG−CHS showed weaker binding. The high antigenicity of both β-LG-GlcN and β-LG-CPO is considered to be due to conformational changes and little shielding of epitopes by the saccharide chain bound

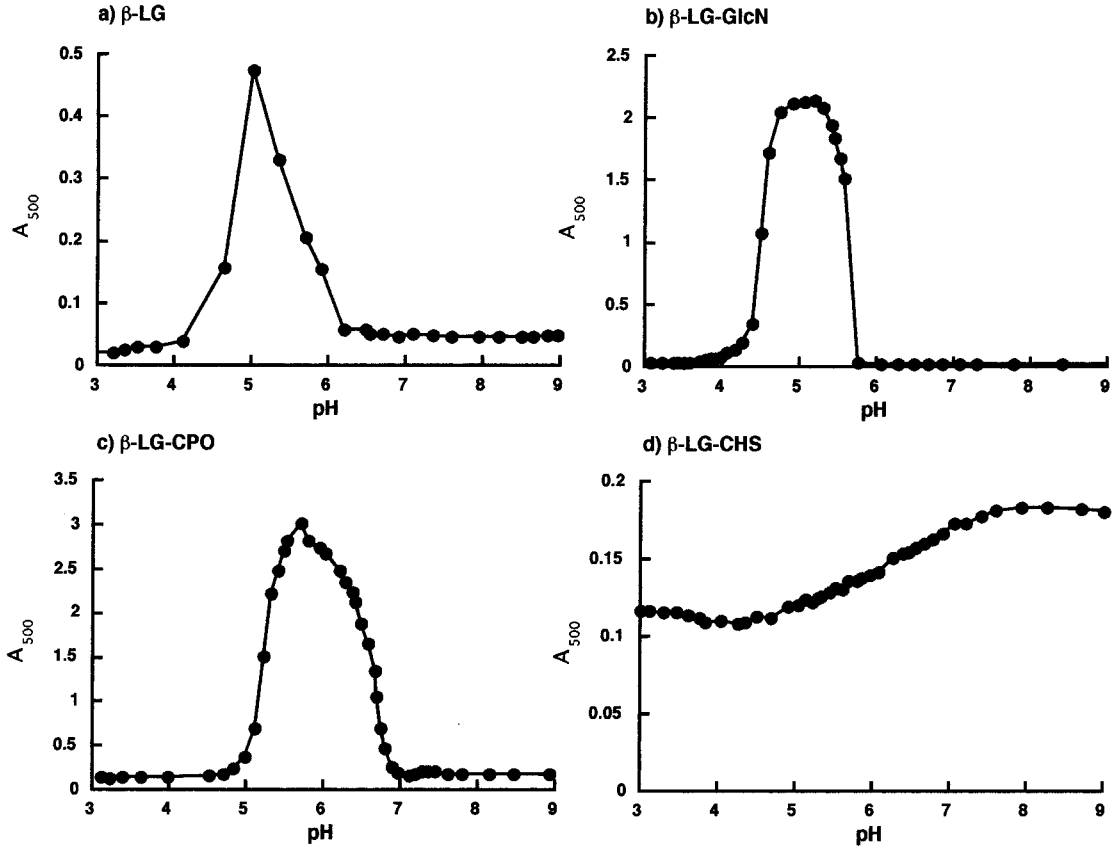

Figure 4. Aggregation properties of the β-LG−cationic saccharide conjugates: (a) β-LG; (b) β-LG−GlcN conjugate; (c) β-LG−CPO conjugate; (d) β-LG−CHS conjugate.

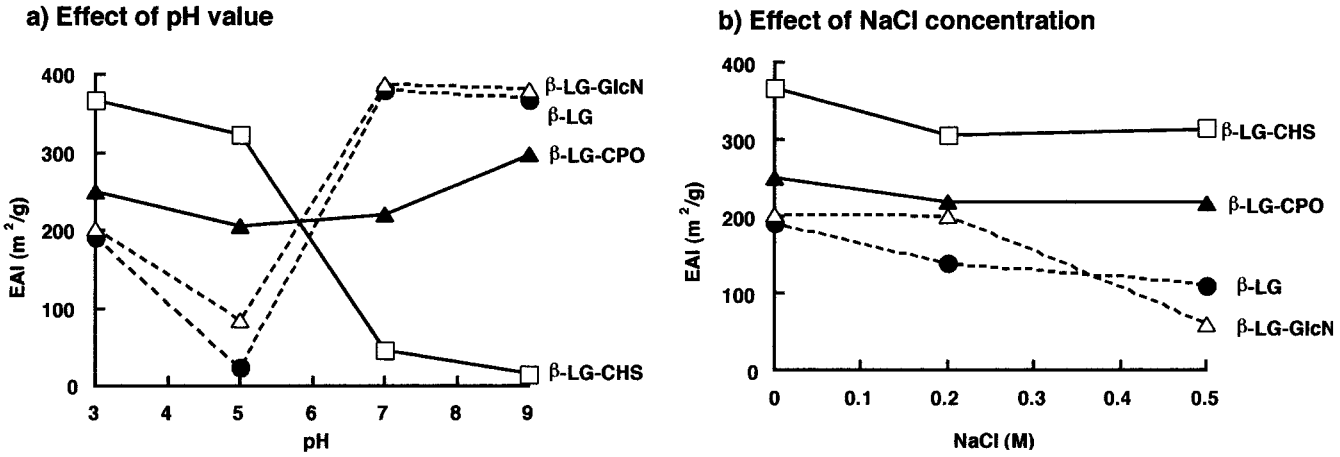

Figure 5. Emulsifying activity of the β-LG−cationic saccharide conjugates: (●) β-LG; (△) β-LG−GlcN conjugate; (▲) β-LG−CPO conjugate; (□) β-LG−CHS conjugate.

to each conjugate, whereas the low antigenicity of β-LG−CHS is considered to be due to substantial shielding of epitopes by the chitosan moiety bound to β-LG. Our findings indicate that cationic saccharides with a high molecular weight are effective in reducing the antigenicity of proteins.

The immunogenicity of the conjugates was evaluated by noncompetitive ELISA. Figure 7a shows the anti-β-LG activity of the anti-conjugate antibodies. Anti-β-LG−GlcN antiserum reacted with β-LG as strongly as anti-β-LG antiserum, whereas the reactivity of anti-β-LG−CPO antiserum with β-LG was stronger than that of anti-β-LG. This finding concerning β-LG−CPO is thought to be due to increased production of antibodies directed toward certain epitopes in the protein moiety of β-LG−CPO exposed by conjugation with CPO. On the other hand, β-LG−CHS did not elicit the production of anti-β-LG antibodies. This remarkable finding is considered to be due to effective shielding of the epitopes in β-LG as a result of conjugation with CHS. Lee et al. (1981) have reported that suppression of IgE production upon immunization with an ovalbumin−poly(ethylene glycol) (PEG) conjugate was due to the induction of regulatory T cells. In the case of the β-LG−CHS conjugates prepared in the present study, there is a possibility that induction of regulatory T cells upon immunization of the mice with the conjugate may contribute to the diminished anti-β-LG antibody response. In addition to this

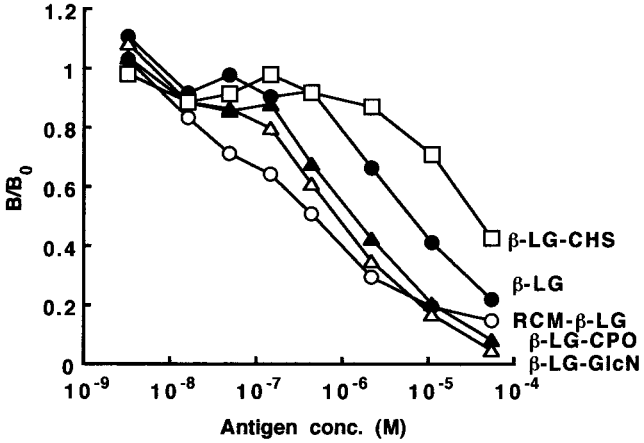

Figure 6. Antigenicity of the β-LG−cationic saccharide conjugates. The reactivity of anti-β-LG antiserum [obtained after secondary immunization of C57BL/6 mice) with β-LG (●), β-LG−GlcN conjugate (△), β-LG−CPO conjugate (▲), β-LG−CHS conjugate (□), and RCM-β-LG (○)] was evaluated by competitive ELISA. B/B_0 is the ratio of the absorbance in the last step of the ELISA in the presence of a competitive antigen at one of various concentrations to the absorbance in the absence of the competitive antigen.

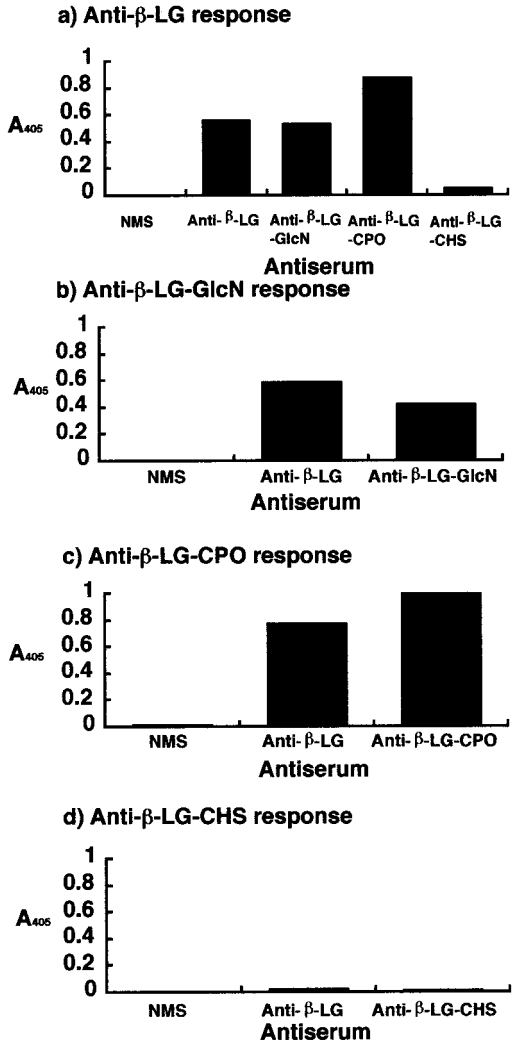

Figure 7. Immunogenicity of the β-LG−cationic saccharide conjugates: anti-β-LG response (a), anti-β-LG−GlcN conjugate response (b), anti-β-LG−CPO conjugate response (c), and anti-β-LG−CHS conjugate response (d) after the secondary immunization of C57BL/6 mice were evaluated by noncompetitive ELISA.

mechanism, So et al. (1996) reported that a lysozyme−PEG conjugate with reduced immunogenicity showed resistance to proteolysis in the course of antigen processing. In the case of β-LG−CHS, it seems that the latter mechanism might also be involved in the diminished anti-β-LG antibody response.

The possibility of the emergence of novel immunogenicity after conjugation with the cationic saccharides was also investigated by evaluating the anticonjugate activity in antiserum by noncompetitive ELISA (Figure 7b−d). The emergence of novel immunogenicity to a slight degree was observed in the case of both β-LG−GlcN and β-LG−CPO. Conformational changes upon conjugation with GlcN or CPO might have induced the emergence of novel epitopes in these conjugates. On the other hand, the emergence of novel immunogenicity was not observed in the case of the β-LG−CHS conjugate. The epitopes in β-LG are considered to be effectively shielded by CHS. Conjugation with CHS was very effective in reducing the immunogenicity of β-LG.

Concluding Remarks. In this study, we prepared three β-LG−cationic saccharide conjugates by means of a water-soluble carbodiimide and by the Maillard reaction. Although conformational changes in β-LG occurred to some extent as a result of conjugation, the aggregation properties and the emulsifying activity of β-LG were improved. In particular, β-LG−CHS was notable in that it showed low antigenicity and low immunogenicity as well as other improved functional properties. Our results indicate that conjugation with high molecular weight cationic saccharides is effective in achieving multiple functional improvements of proteins. It is strongly hoped that the β-LG−CHS prepared in this study will contribute to development of a new method to prepare novel proteins with reduced allergenicity and improved functional properties.

ABBREVIATIONS USED

β-LG, β-lactoglobulin; RCM-β-LG, reduced and carboxymethylated β-lactoglobulin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PBS−Tween, PBS containing 0.05% Tween 20; 2-ME, 2-mercaptoethanol; GlcN, glucosamine; CPO, chitopentaose; CHS, chitosan; PEG, poly(ethylene glycol).

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