Reduced Immunogenicity of β-Lactoglobulin by Conjugation with Carboxymethyl Dextran Differing in Molecular Weight

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To reduce the immunogenicity of β-lactoglobulin (β-LG), two β-LG−carboxymethyl dextran (CMD) conjugates (Conj. 40 and Conj. 162) were prepared by using water-soluble carbodiimide (EDC). The molar ratios of β-LG to CMD in Conj. 40 and Conj. 162 were 8:1 and 7:1, respectively. Each conjugate maintained ∼50% of the retinol binding activity of β-LG. Structural analyses by intrinsic fluorescence, CD spectra, and ELISA with monoclonal antibodies indicated that the surface of β-LG in each conjugate was covered by CMD without great disruption of native conformation. By conjugation with CMD, the antibody response to β-LG was reduced in BALB/c, C3H/He, and C57BL/6 mice, which was eminent in Conj. 162. The results of B cell epitope scanning using overlapping synthesized peptides showed that the linear epitope profiles of the conjugates were similar to those of β-LG, whereas the antibody response to each epitope was reduced, which was eminent in Conj. 162. It was concluded that conjugation with CMD of higher molecular weight is effective in reducing the immunogenicity of β-LG and that masking of epitopes by CMD is responsible for the reduced immunogenicity.

Keywords: *β-Lactoglobulin; carboxymethyl dextran; lipocalin; protein conjugation; reduced immunogenicity; synthetic peptides; B cell epitopes; PEPSCAN assay*

INTRODUCTION

The incidence of allergies to food and dietary components has shown a tendency to increase in advanced countries. Iikura et al. (*1*) reported that 12.6% of children in Japan had experienced an allergic reaction within 1 h after food intake and that the most common sources of food allergens involved were egg (52.3% of food allergy cases), milk (31.8%), and seafoods (10.6%). Although the basic therapy for food allergy is a food elimination diet, a prolonged period of food elimination affects children's growth (*1*). In particular, milk is a basic food component, and it plays a predominant role in the first years of life. The most important allergen in cow's milk is β-lactoglobulin (β-LG), and ∼82% of milk allergy patients are sensitive to β-LG (*2*). β-LG, a major whey protein, is a globular protein of MW 18400 with two disulfide bridges and one free cysteine (*3*). Although the function of β-LG remains unclear, it is tentatively considered to be the binding and transportation of small hydrophobic ligands such as retinol and fatty acids, and β-LG is categorized as a member of the lipocalin superfamily (*4*). β-LG has a β-barrel structure (*5*, *6*), which is a common feature among the lipocalins, and it shows a high allergenic potential. Many major allergens of animal origin belong to the lipocalin superfamily (*7*).

β-LG is considered to be a valuable protein in terms of food science because the protein has various useful functional properties such as emulsifying, foaming, and gelling properties (*8*−*10*) as well as plenty of essential amino acids (*3*). Hence, it is highly desirable to develop a new method that would decrease the allergenicity of β-LG. Reduction of the allergenicity of proteins has been attempted by enzymatic digestion and denaturation (*11*−*14*). However, these methods might destroy the physiological functions of the proteins and bring about problems with their taste. On the other hand, the conjugation of proteins with materials having low allergenicity can achieve improved functions (thermal stability, solubility, emulsifying ability, and so on) as well as reduced allergenicity of the proteins (*15*−*18*).

Many attempts to reduce the antigenicity and the immunogenicity of proteins by conjugation have been reported. Polyethylene glycol (PEG) conjugates of recombinant IL-2 have been reported to elicit 100−1000-fold lower levels of antigen-specific IgG antibody production than recombinant IL-2 (*19*). High lipophilization of β-lactoglobulin (β-LG) has shown to be effective to reduce the antigenicity of β-LG and the production of anti-β-LG IgG and IgE (*20*). Conjugates of monoclonal and polyclonal antibodies with oxidized dextrans of low molecular weight have been reported to have optimal immunoreactivity, in vivo pharmacokinetics, and tumor localization properties, as well as low immunogenicity in vivo (*21*). However, the mechanisms involved remain unclear despite the many studies on the reduced allergenicity of protein conjugates that have been reported.

Our purpose was to create novel β-LG−carboxymethyl dextran (CMD) conjugates without allergenicity while maintaining the retinol binding activity and to elucidate the mechanism involved. In particular, we focused on the structure and B cell epitope profiles of β-LG after conjugation with CMD. Although many studies on

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reduced allergenicity of proteins by conjugation methods have been reported, almost no information is available on the structure of conjugates. Although many studies on the B cell epitope profiles (IgG or IgE) of proteins including β-LG (*22*−*27*) have been performed, the changes in B cell epitope profiles of a protein after conjugation with polysaccharides have not been clarified so far. In our previous study (*16*), we achieved reduced immunogenicity of β-LG, while maintaining the retinol binding activity, through conjugation of β-LG with CMD having a mass of 10 kDa. Our findings showed that a difference in the saccharide content of the conjugates affects the immunogenicity and the B cell epitope profiles of β-LG.

In this study, to further evaluate the influence of the molecular weight of the polysaccharides on the immunological properties of such conjugates, we prepared covalently bonded β-LG−CMD conjugates having similar saccharide contents using CMD of different molecular weights (M_r = about 40 and 162 kDa). After exploring the structures of these conjugates, we investigated the immunogenicity profiles of β-LG and the conjugates in detail.

MATERIALS AND METHODS

Materials. Dextran T40 was purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK) and Dextran 162 was purchased from Sigma Chemical Co. (St. Louis, MO). 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 (Tokyo, Japan), according to the method of Armstrong et al. (*28*), and then purified by ion-exchange chromatography, using a DEAE-Sepharose Fast Flow column (3.0 i.d. × 40 cm; Amersham Pharmacia Biotech) by referring to the method described previously (*15*). The purity of β-LG was confirmed by polyacrylamide gel electrophoresis (PAGE), performed according to the method of Davis (*29*). 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 (*30*).

Carboxymethylation of Dextran. Each dextran was carboxymethylated with monochloroacetic acid under alkaline conditions as described previously (*15*−*18*). The degree of modification was determined by ^{1}H NMR (400 MHz JEOL AL-400, Japan). The numbers of carboxyl groups attached to one molecule of dextran were 39 and 162, respectively, for CMD 40 and CMD 162.

Preparation of the β-LG—CMD Conjugates. β-LG−CMD conjugates were prepared by referring to the method described previously (*15*−*18*). β-LG (1 g) and each CMD (CMD 40, 8.48 g; CMD 162, 9.375 g) (amino groups of β-LG: carboxyl groups of CMD = 1:10 as the molar ratio) were dissolved in distilled water (125 mL), and the pH of the solution was adjusted to 4.75 with 1 M HCl. EDC solution (1.67 g/10 mL) (amino groups of β-LG:EDC = 1:10 as the molar ratio) was added in 30 min, and the pH was kept at 4.75 by the addition of 1 M HCl. The reaction mixture was incubated at 25 °C for 3 h, and then 2 M acetate buffer at pH 5.5 (10 mL) was gradually added over a period of 10 min to stop the reaction. After dialysis against distilled water at 4 °C and lyophilization, the crude β-LG−CMD conjugates were obtained.

Purification of the β-LG—CMD Conjugates. Hydrophobic chromatography was applied to remove free CMD from the crude β-LG−CMD conjugates. Each crude conjugate in 0.067 M phosphate buffer containing 1.5 M ammonium sulfate at pH 7.0 (8.5 g/430 mL) was mixed with Butyl-Toyopearl 650S (Tosoh, Tokyo, Japan) gel slurry equilibrated with the same buffer (850 mL). After stirring for 30 min, the gel slurry was packed into a column (6.0 i.d. × 30 cm; Amersham Pharmacia Biotech). Free CMD was eluted with 0.067 M phosphate buffer containing 1.5 M ammonium sulfate, pH 7.0, at a flow rate of 4 mL/min, and then the crude conjugate was eluted with 0.067 M phosphate buffer at pH 7.0. To detect the protein and CMD, the absorbance was monitored at 280 and 490 nm after coloring by the phenol−sulfuric acid method (*31*), respectively. After dialysis against distilled water at 4 °C and lyophilization, the crude conjugate without free CMD was obtained.

Free β-LG and polymerized β-LG in each crude conjugate without free CMD were removed by anion-exchange chromatography. Each crude conjugate in 0.067 M phosphate buffer containing 0.1 M NaCl at pH 7.0 (1 g/100 mL) was mixed with QA-Cellulofine Q-800-m (Seikagaku Industry Co., Tokyo, Japan) gel slurry equilibrated with the same buffer (100 mL). After stirring for 30 min, the gel slurry was packed into a column (2.8 i.d. × 45 cm). Free β-LG and polymerized β-LG were eluted with 0.067 M phosphate buffer containing 0.1 M NaCl, pH 7.0, at a flow rate of 4 mL/min, and then the conjugate was eluted with 0.067 M phosphate buffer containing 0.5 M NaCl at pH 7.0. To detect the protein and CMD, the absorbance was monitored at 280 and 490 nm after coloring by the phenol−sulfuric acid method (*31*), respectively. After dialysis against distilled water at 4 °C and lyophilization, the purified β-LG−CMD conjugate was obtained. The purity of each of the conjugates was confirmed by SDS-PAGE, performed according to the method of Laemmli (*32*).

Chemical and Structural Analysis of the β-LG—CMD Conjugates. The amount of protein in each of the β-LG−CMD conjugates was determined by measuring the absorbance at 280 nm. The amount of CMD bound to β-LG was determined by measuring the absorbance at 490 nm after coloring by the phenol−sulfuric acid method (*31*). Isoelectric focusing (IEF) of the β-LG−CMD conjugates was performed using the Pharmacia-Phast System (*33*). The protein bands and the saccharide bands were detected by staining with Coomassie Brilliant Blue and Schiff reagent, respectively.

The retinol binding activity of the β-LG−CMD conjugates was measured by fluorescence titration (*16*, *34*−*37*). Each conjugate was dissolved in PBS (pH 7.1) 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 1.46×10^{-5} M retinol in ethanol were added to the cuvette using a micropipet. The fluorescence was measured by means of an RF-5300PC instrument (Shimadzu, Kyoto, Japan) with excitation at 330 nm and emission at 470 nm.

The intrinsic fluorescence of the β-LG−CMD conjugates dissolved in PBS at a protein concentration of 0.001% was measured under excitation at 283 nm by means of an RF-5300PC instrument (Shimadzu, Kyoto, Japan).

The CD spectrum for each β-LG−CMD conjugate was measured with a J-720WI spectropolarimeter (Jasco, Tokyo, Japan), using a cell with a 1.0 mm path length. Samples were dissolved in PBS (0.11 M phosphate buffer at pH 7.1 containing 0.04 M NaCl and 0.02% NaN_3) at a protein concentration of 0.02%.

In the above structural analysis, β-LG obtained by incubation at room temperature for 30 min in PBS containing 6 M guanidine hydrochloride was used as denatured β-LG.

Immunization. Female BALB/c, C3H/He, and C57BL/6 mice (Charles River Japan Inc., Yokohama, Japan) at 6 weeks of age (seven to eight animals per group) were immunized intraperitoneally with β-LG or each β-LG−CMD conjugate (100 μg as protein) emulsified in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). Fourteen days after the primary immunization, the mice were boostered with 100 μg of protein emulsified in Freund's incomplete adjuvant (Difco Laboratories). Blood samples were collected from mice of each group 7 days after the primary and secondary immunizations and stored at 4 °C for 24 h to form a clot. Antiserum was prepared from each blood sample after clot formation. This study was performed in conformance with the guidelines for the care and use of experimental animals established by the ethics committee of the Tokyo University of Agriculture and Technology.

Enzyme-Linked Immunosorbent Assay (ELISA). 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 A/S, Roskilde, Denmark), and the plate was incubated at 4 °C overnight to coat the wells with each antigen. After the removal of the solution, each well was washed three times with 125 μL of PBS-Tween (PBS containing 0.05% Tween 20). One hundred and twenty-five microliters of 1% ovalbumin/PBS solution was added to each well, the plate was incubated at 25 °C for 2 h, and then the plate was washed three times. One hundred microliters of antibody [antisera or monoclonal antibody (mAb)] diluted with PBS was added to each well, and the plate was incubated at 25 °C for 2 h. After three washings, 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 three times. One hundred microliters of 0.1% sodium *p*-nitrophenyl phosphate disodium/diethanolamine hydrochloride buffer (pH 9.8) was added to each well, and the plate was incubated at 25 °C for 30 min. After the addition of 5 M sodium hydroxide solution (20 μL) to each well to stop the reaction, the absorbance at 405 nm was measured with a microplate reader (MPR-A4i, Tosoh, Tokyo, Japan).

For the determination of the immunogenicity of the β-LG−CMD conjugates, a standard curve was made with serially diluted anti-β-LG antiserum pooled from other C3H/He mice. The antibody titer was calculated from the standard curve and expressed as relative concentration.

A competitive ELISA was carried out to investigate the local conformational changes in β-LG after conjugation with CMD, using anti-β-LG mAbs (mAbs 21B3, 31A4, 61B4, and 62A6) as probes as described previously (*15*−*18*, *30*, *35*−*38*). The equilibrium constants (K_{AS}) for the interaction of mAb with β-LG, RCM-β-LG, or β-LG−CMD conjugates were calculated according to the method of Hogg et al. (*39*), based on the results of competitive and noncompetitive ELISA. RCM-β-LG was used as the denatured form of β-LG.

Epitope Scanning. A series of overlapping 15-mer peptides moving one amino acid residue at a time in accordance with the amino acid sequence of β-LG were synthesized on activated polyethylene gears in each pin using a five-in-one B cell and T cell epitope scanning kit (Chiron Mimotopes, Clayton, VIC, Australia) as described previously (*16*). B cell epitopes were scanned by ELISA using the overlapping synthetic peptides on each pin as the antigen on a solid phase as described previously (*16*), except that anti-β-LG, anti-Conj. 40, and anti-Conj. 162 antisera were each diluted with precoat buffer [0.01 M PBS at pH 7.2, containing 2% (w/v) ovalbumin, 0.1% (v/v) Tween 20, and 0.1% (w/v) NaN_3]. The pins were reused after the bound antibodies had been stripped off according to the procedure described in the manual for the epitope scanning kit.

For the determination of the immunogenicity profiles, the following criteria were used to evaluate whether the observed response was significant. Peptides adopted as positive were (1) those which showed reactivity greater than the mean value plus three standard deviations below the median absorbance in the case of peptides binding to antibodies in anti-β-LG antisera (*22*), (2) those which showed positive reactivity with at least two consecutive overlapping peptides (*40*), and (3) those which showed positive reactivity in two of three individual experiments. The common amino acid sequences among the peptides that fulfilled these criteria were identified as the epitopes according to the method of Gammon et al. (*41*).

RESULTS

Structural Features of the β-LG—CMD Conjugates. The covalent binding of CMD to β-LG was confirmed by IEF and by the coincidence of the stained protein and saccharide bands observed upon analysis by SDS-PAGE (data not shown). The molar ratios of β-LG to CMD in the conjugates were 8:1 (Conj. 40) and 7:1 (Conj. 162).

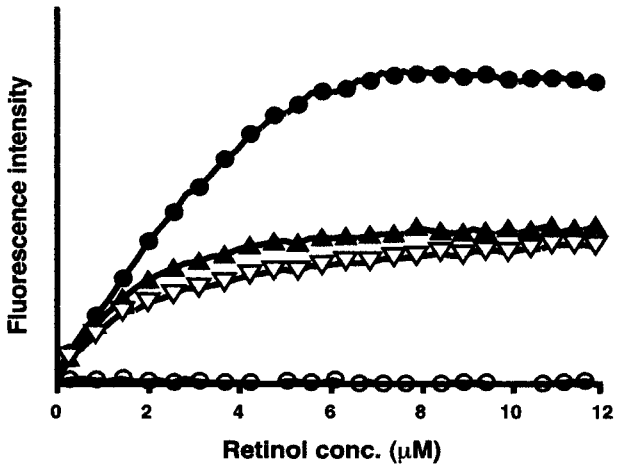

Figure 1. Retinol binding activity of the β-LG−CMD conjugates: β-LG (●); Conj. 40 (▲); Conj. 162 (▽); denatured β-LG (○).

The retinol-binding activity of each β-LG−CMD conjugate was ~50% of that of β-LG (Figure 1). This result indicates that the region involved in retinol binding in the β-LG−CMD conjugates differed from that in the native form.

The intrinsic fluorescence spectra of the β-LG−CMD conjugates were measured to evaluate the conformational changes around the Trp residues (^{19}Trp and ^{61}Trp) of β-LG (Figure 2a). The fluorescence emission maximum wavelength for β-LG was 332 nm, whereas those for Conj. 40 and Conj. 162 were 332 and 333 nm, respectively. It has previously been clarified that the fluorescence intensity increases with red shift of the wavelength for maximum emissions as the conformation of β-LG changes (*30*, *35*). As only a slight red shift was observed in the analysis of the conjugates, the conformation around the Trp residues of the β-LG−CMD conjugates is considered to be maintained in the native form. The fluorescence intensity of each β-LG−CMD conjugate was lower than that of native β-LG, which indicates that the saccharide chain shielded the area around the Trp residues. This effect of shielding of the Trp fluorescence was most prominent in the case of Conj. 162.

The CD spectra of the β-LG−CMD conjugates are shown in Figure 2b. The CD spectrum of native β-LG showed a negative maximum at 217 nm, indicating that β-LG is rich in β-sheet structure, whereas that of each β-LG−CMD conjugate showed a broad negative maximum with a blue shift. The β-sheet regions in β-LG are considered to have changed as a result of conjugation with CMD.

Local conformational changes in β-LG after conjugation with CMD were evaluated by competitive ELISA with four anti-β-LG mAbs (21B3, 31A4, 61B4, and 62A6) as probes. These mAbs can serve to detect the subtle conformational changes in local areas within the β-LG molecule during unfolding and refolding (*30*, *35*, *38*) and after conjugation with saccharides (*15*−*18*, *36*, *37*) by determining the affinity change. The epitope regions for mAbs 21B3, 31A4, 61B4, and 62A6 are ^{15}Val−^{29}Ile (β-sheet), ^{8}Lys−^{19}Trp (random coil, β-sheet, and short helix), ^{125}Thr−^{135}Lys (α-helix), and the regions close to the epitope for 61B4, respectively. MAb 61B4 and 62A6 bind preferentially to native β-LG, whereas mAb 21B3

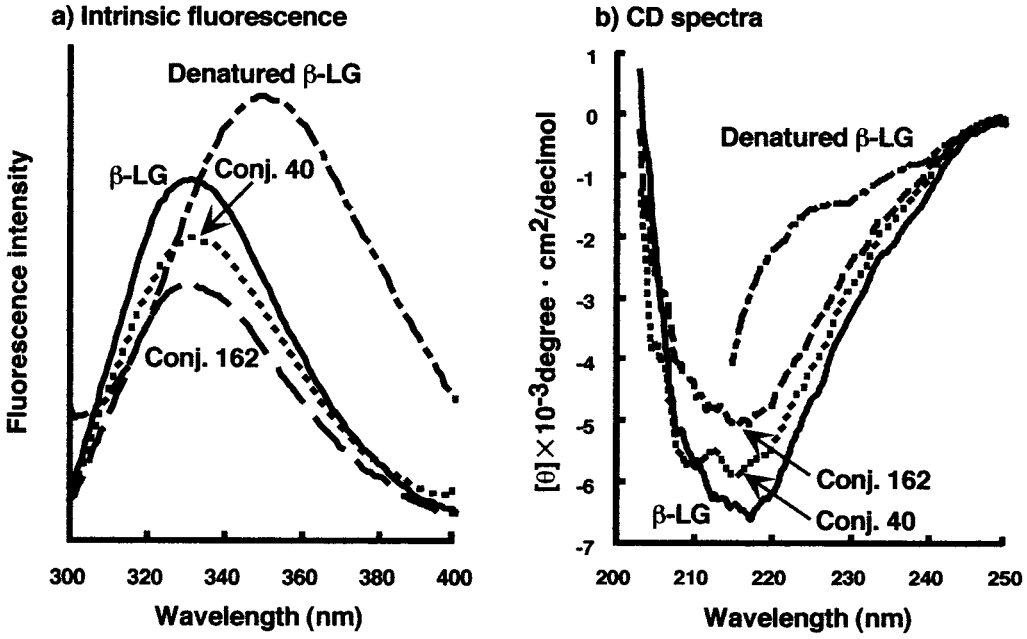

Figure 2. Spectroscopic analysis of the β-LG−CMD conjugates: intrinsic fluorescence (a); CD spectra (b); β-LG (—); Conj. 40 (· · ·); Conj. 162 (- - -); denatured β-LG (-·-·-·).

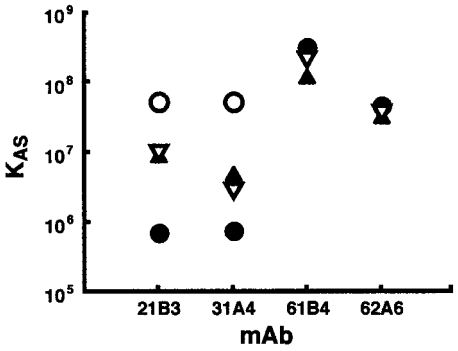

Figure 3. Equilibrium constants (K_{AS}) of the β-LG−CMD conjugates in binding to anti-β-LG mAbs: β-LG (●); Conj. 40 (▲); Conj. 162 (▽); RCM-β-LG (○). K_{AS} values were calculated from the results of competitive and noncompetitive ELISA according to the procedure of Hogg et al. (*39*).

and 31A4 bind more strongly to RCM-β-LG (the denatured form of β-LG). The equilibrium constants (K_{AS}) for binding of mAb 21B3 and 31A4 to the β-LG−CMD conjugates were larger than that for binding to native β-LG, whereas the K_{AS} values for binding of mAb 61B4 and 62A6 to the β-LG−CMD conjugates were similar to that for binding to native β-LG (Figure 3). Hence, the conformation around ^{125}Thr−^{135}Lys (α-helix) and the regions close to the epitope for 61B4 is considered to have maintained its native form, whereas the conformations around ^{15}Val−^{29}Ile (β-sheet) and ^{8}Lys−^{19}Trp (random coil, β-sheet, and short helix) are considered to have changed as a result of conjugation with CMD.

Immunogenicity of the β-LG—CMD Conjugates. The immunogenicity of each β-LG−CMD conjugate in BALB/c, C3H/He, and C57BL/6 mice was evaluated by measuring the reactivity of 5000-fold diluted antisera with antigen (β-LG, Conj. 40, and Conj. 162) adsorbed to the solid phase of a microtitration plate by noncompetitive ELISA (Figure 4). The anti-β-LG antibody response was low in BALB/c, C3H/He, and C57BL/6 mice immunized with each β-LG−CMD conjugate (Figure 4a,d,g). The reduction of immunogenicity was more effective in the case of Conj. 162 than in the case of Conj. 40. The level of production of antibody specific for each conjugate was evaluated in an assay in which each β-LG−CMD conjugate was applied as a coating on the solid phase (Figure 4b,c,e,f,h,i). Although both anti-Conj. 40 and anti-Conj. 162 antisera showed an antibody titer similar to or slightly higher than that of the anti-β-LG antisera, no statistically significant difference was observed. Hence, novel immunogenicity in β-LG after conjugation with CMD is considered not to have emerged.

Scanning of Epitopes in the β-LG—CMD Conjugates. The B cell epitope profiles of β-LG and the β-LG−CMD conjugates were analyzed by ELISA using overlapping 15-mer multipin peptides. The results obtained with BALB/c mice are shown in Figure 5a−c. The horizontal axis indicates the number of the N-terminal amino acid residue of each peptide corresponding to the position in the β-LG sequence, and the vertical axis shows the reactivity of each 15-mer peptide with each antiserum. The epitopes identified according to the method of Gammon et al. (*41*) are summarized in Figure 6, in which the horizontal axis indicates the sequence number in β-LG and the line thickness indicates the response to each epitope.

Anti-β-LG antiserum from BALB/c mice (Figure 5a) reacted with the peptides 1−5, 6−12, 21−30, 40−53, 61−69, 71−80, 119−131, and 135−138 (the number indicates residue number of N terminus of the peptides). The linear B cell epitopes of β-LG recognized in BALB/c mice were determined to be ^{5}Gln−^{15}Val, ^{12}Ile−^{20}Tyr, ^{30}Ser−^{35}Gln, ^{53}Asp−^{54}Leu, ^{69}Lys−^{75}Lys, ^{80}Ala−^{85}Asp, ^{131}Glu−^{133}Leu, and ^{138}Lys−^{149}Leu, the dominant epitope being ^{30}Ser−^{35}Gln, which is located in a loop region of the β-LG molecule (Figure 6). As for the anticonjugate response in BALB/c mice (Figure 5b,c), the epitope distribution did not change, whereas the decrease in response was marked through the entire amino acid sequence as compared with the anti-β-LG antiserum. The B cell epitopes of conjugates recognized in BALB/c mice were determined to be ^{2}Ile−^{15}Val, ^{11}Asp−^{20}Tyr, ^{28}Asp−^{35}Gln, ^{49}Thr−^{55}Glu, ^{72}Ile−^{77}Lys, ^{79}Pro−^{88}Asn,

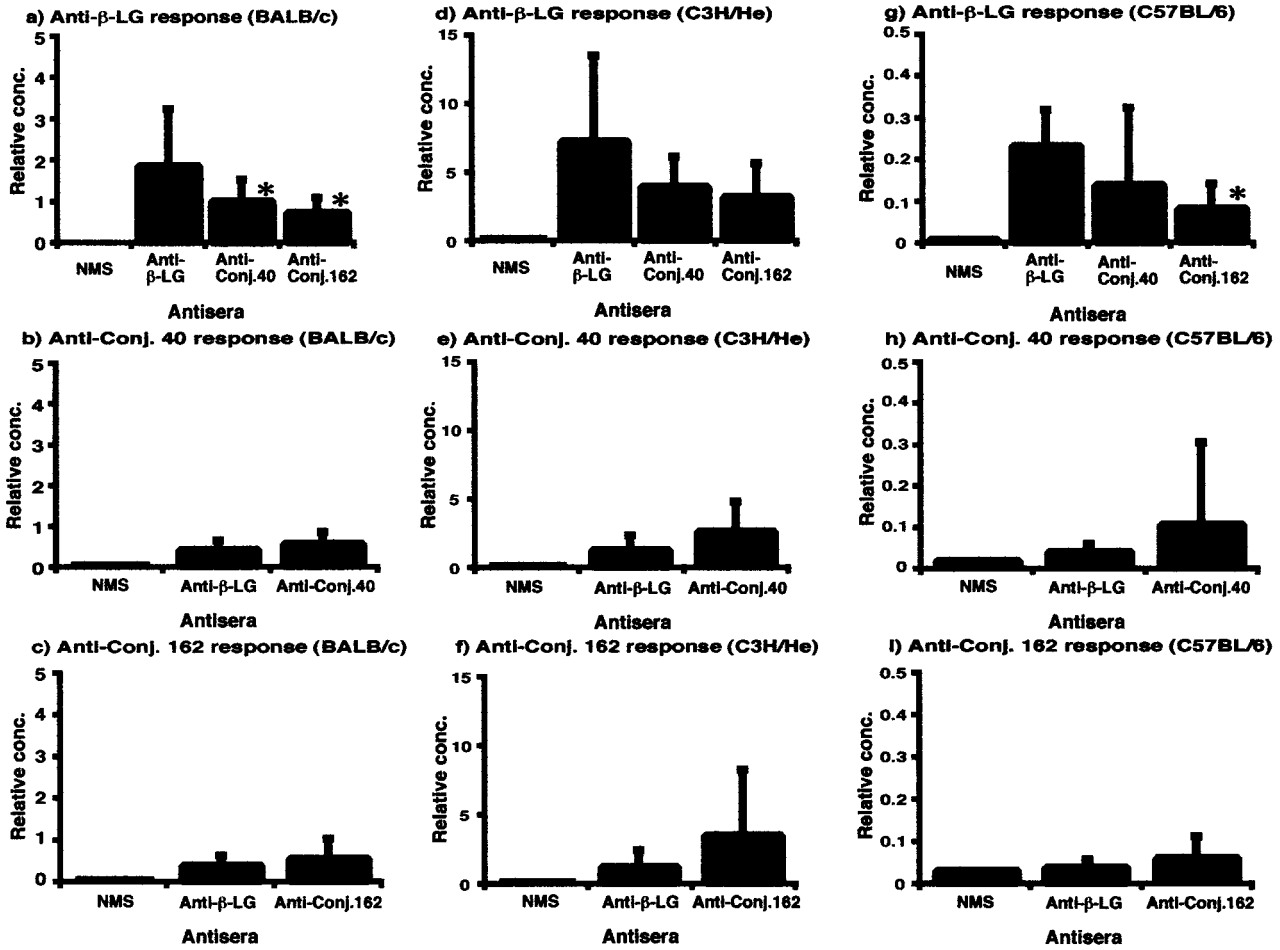

Figure 4. Immunogenicity of the β-LG−CMD conjugates in three strains of mice. The anti-β-LG response, anti-Conj. 40 response, and anti-Conj. 162 response after the secondary immunization in BALB/c (a−c), C3H/He (d−f), and C57BL/6 (g−i) were evaluated by noncompetitive ELISA. A significant difference ($p < 0.05$) as determined by Student's t test is indicated by an asterisk.

and ^{143}Leu−^{156}Leu for Conj. 40 and ^{2}Ile−^{15}Val, ^{11}Asp−^{20}Tyr, ^{28}Asp−^{35}Gln, ^{50}Pro−^{54}Leu, ^{69}Lys−^{76}Thr, and ^{143}Leu−^{155}Gln for Conj. 162 (Figure 6). Reduced immunogenicity of β-LG through conjugation with CMD was achieved in the case of each conjugate as far as the linear B cell epitopes were concerned, and this was most prominent in the case of Conj. 162.

The results obtained with C3H/He and C57BL/6 mice are shown in Figure 5d−i, and B cell epitope profiles of β-LG and the conjugates were determined as shown in Figure 6. The B cell epitopes of β-LG recognized in C3H/He mice were determined to be ^{11}Asp−^{22}Leu, ^{34}Ala−^{39}Leu, ^{51}Glu−^{56}Ile, ^{66}Cys−^{76}Thr, ^{78}Ile−^{82}Phe, ^{93}Leu−^{96}Asp, ^{131}Glu−^{133}Leu, and ^{138}Lys−^{146}His, the dominant epitopes being ^{51}Glu−^{56}Ile, ^{93}Leu−^{96}Asp, and ^{131}Glu−^{133}Leu, which were located in β-sheet and α-helix regions of the β-LG molecule (Figure 6). With respect to the anticonjugate response in C3H/He mice, the epitope distribution was similar to that of β-LG, and the response was less throughout the entire amino acid sequence than the anti-β-LG response (Figure 5d−f). Reduction of the immunogenicity of β-LG was effectively achieved through conjugation with CMD in the case of each conjugate so far as the linear B cell epitopes were concerned, and this was most prominent in the case of Conj. 162. The B cell epitopes of the conjugates recognized in C3H/He mice were determined to be ^{33}Asp−^{39}Leu, ^{51}Glu−^{59}Gln, ^{78}Ile−^{84}Ile, ^{93}Leu−^{102}Tyr, and ^{137}Asp−^{138}Lys for Conj. 40 and ^{33}Asp−^{39}Leu, ^{49}Thr−^{59}Gln, ^{78}Ile−^{84}Ile, and ^{135}Lys−^{138}Lys for Conj. 162 (Figure 6).

The B cell epitopes of β-LG recognized in C57BL/6 mice were determined to be ^{12}Ile−^{20}Tyr, ^{23}Ala−^{35}Gln, ^{33}Asp−^{38}Pro, ^{51}Glu−^{58}Leu, ^{82}Phe−^{92}Val, ^{93}Leu−^{102}Tyr, ^{129}Asp−^{131}Glu, and ^{138}Lys−^{145}Met (Figure 6). The dominant epitope was ^{12}Ile−^{20}Tyr, half of which is located in a loop region of the β-LG molecule (Figure 6). Regarding the anticonjugate response in C57BL/6 mice, the epitope distribution was also similar to that of β-LG, and, for each peptide, the signal obtained with the anticonjugate antisera was weaker than that with the anti-β-LG antiserum (Figure 5g−i). Reduction of the immunogenicity of β-LG in C57BL/6 mice was achieved in the case of each conjugate as far as the linear B cell epitopes were concerned, and this was most prominent in the case of Conj. 162. The B cell epitopes of the conjugates recognized in C57BL/6 mice were determined to be ^{12}Ile−^{20}Tyr, ^{33}Asp−^{39}Leu, ^{51}Glu−^{60}Lys, ^{82}Phe−^{91}Lys, and ^{93}Leu−^{106}Cys for Conj. 40 and ^{12}Ile−^{20}Tyr, ^{30}Ser−^{35}Gln, ^{48}Pro−^{61}Trp, ^{82}Phe−^{92}Val, and ^{94}Val−^{104}Leu for Conj. 162 (Figure 6).

DISCUSSION

To evaluate the influence of the molecular weight of polysaccharides used in the preparation of conjugates on the immunological properties of proteins after conjugation, we prepared conjugates of β-LG and CMD with

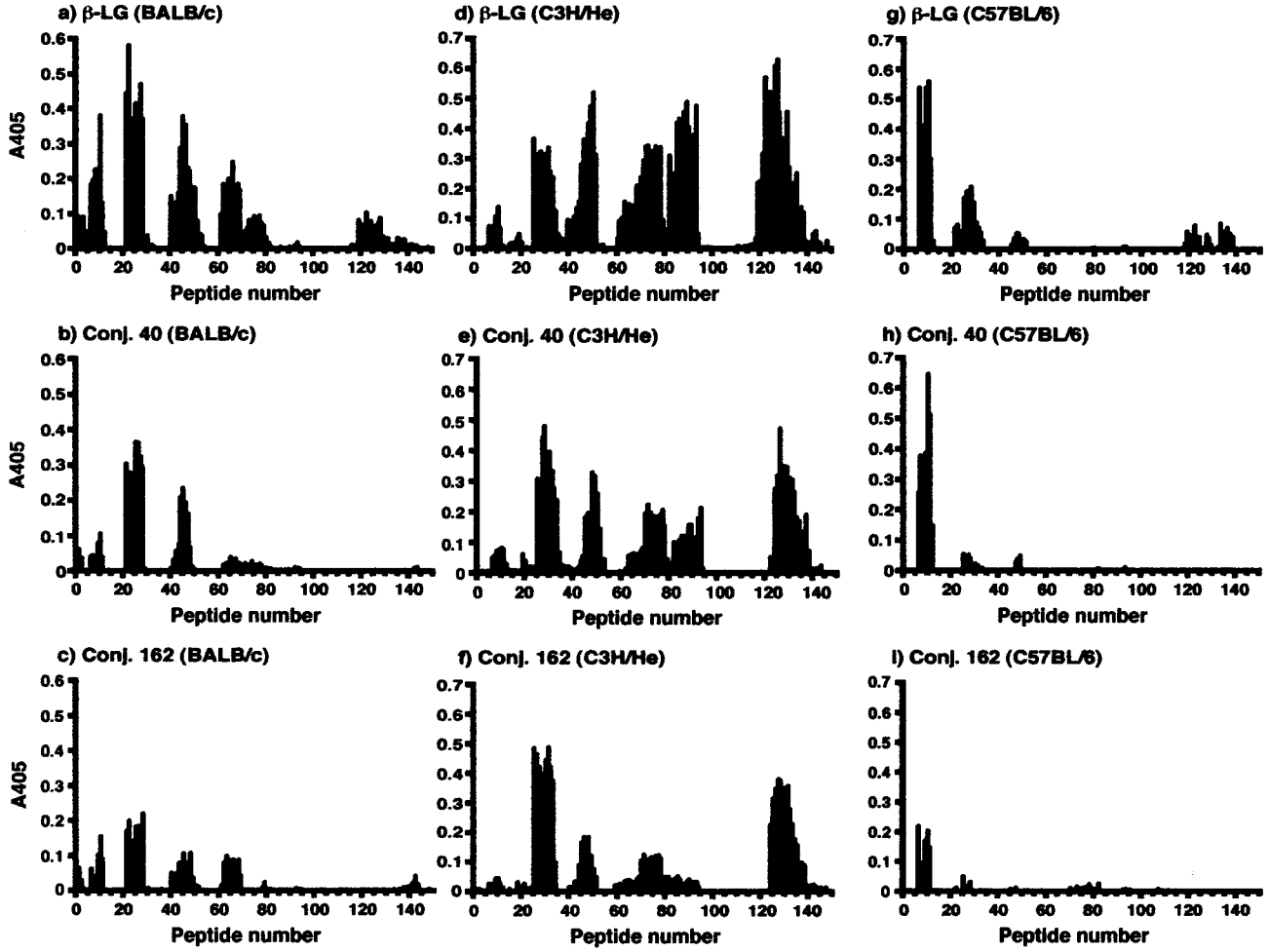

Figure 5. Reactivity of anti-*β*-LG, anti-Conj. 40, and anti-Conj. 162 antisera with overlapping 15-mer peptides covering the amino acid sequence of *β*-LG. The reactivity of anti-*β*-LG (a, d, g), anti-Conj. 40 (b, e, h), and anti-Conj. 162 (c, f, i) antisera obtained after the secondary immunization from BALB/c (a−c), C3H/He (d−f), and C57BL/6 mice (g−i) with multipin peptides was evaluated by ELISA. Representative epitope profiles in three individual experiments are shown.

similar saccharide contents. The results of structural analyses of the *β*-LG−CMD conjugates indicated that the surface of *β*-LG in each conjugate was covered by CMD without great disruption of native conformation (Figures 1−3). The anti-*β*-LG antibody response was low in the three strains of mice (BALB/c, C3H/He, and C57BL/6) immunized with each *β*-LG−CMD conjugate (Figure 4a,d,g). The immunogenicity of *β*-LG was more effectively reduced in the case of Conj. 162 than in the case of Conj. 40. Because shielding of the *β*-LG molecule by CMD was more marked with Conj. 162 than with Conj. 40, as described under Structural Features of the *β*-LG−CMD Conjugates, it is considered that masking of the epitopes by CMD was more effectively achieved in Conj. 162 than in Conj. 40. These results strongly suggest that conjugation with polysaccharides of higher molecular weight is effective to reduce the immunogenicity of proteins. In contrast to the results in our previous study (*16*) suggesting the possibility of emergence of novel immunogenicity upon the conjugation of *β*-LG with CMD having a mass of 10 kDa, the conjugates prepared in the present study did not show novel immunogenicity (Figure 4b,c,e,f,h,i). Conjugation with polysaccharides of higher molecular weight is considered to be an effective method to overcome the emergence of new epitopes that would occur as a result of exposure of the hydrophobic region-(s), buried in the native state, upon conjugation with CMD.

Anti-*β*-LG antisera showed higher affinity to the denatured form of *β*-LG (RCM-*β*-LG) than to the native material in the case of all mouse strains tested, although they were elicited by immunization with native *β*-LG (data not shown). Selo et al. (*23*) also showed that the immunoreactivity of IgE from milk-allergic patients was similar between native *β*-LG and RCM-*β*-LG. In studies on the unfolding/refolding of *β*-LG using mAbs as probes, Hattori et al. (*35*) found that a region of *β*-LG within the molecule was easily exposed during the denaturation process and was hard to refold. Such a structural feature of *β*-LG is likely to be responsible for triggering the production of antibodies that preferentially react with the unfolded molecule. Therefore, it is considered that anti-*β*-LG antiserum recognizes linear epitopes rather than conformational epitopes. Thus, it is important to clarify the linear epitope profiles of *β*-LG and the *β*-LG−CMD conjugates and to investigate how conjugation with CMD leads to reduced immunogenicity of *β*-LG. We analyzed the B cell epitopes of *β*-LG and the *β*-LG−CMD conjugates by ELISA using overlapping 15-mer multipin peptides (Figures 5 and 6). Although the B cell epitope profiles of *β*-LG obtained in this study showed a difference in reactivity of each epitope as compared with our previous result (*16*), the epitope

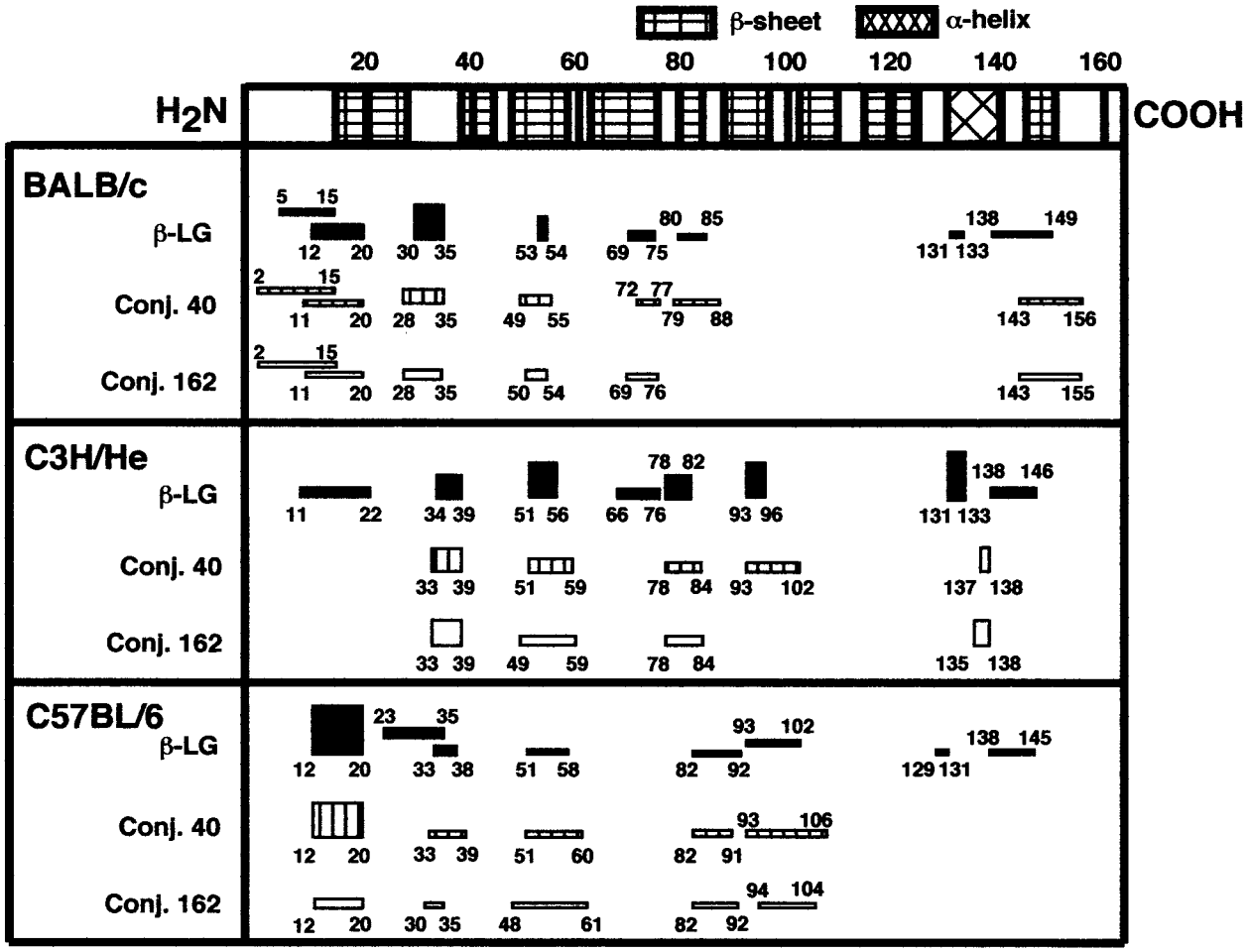

Figure 6. B cell epitope profiles of β-LG and β-LG−CMD conjugates. The common regions of at least two overlapping peptides which showed reactivity greater than the average value plus three standard deviations below the median absorbance in reactions between anti-β-LG antisera and the peptides, and which showed reproducibility in two of three individual experiments, were identified as epitopes according to the method of Gammon et al. (*41*).

distribution was almost the same. The results of epitope scanning showed that the linear epitope profiles of the conjugates were similar to those of β-LG in the three strains of mice, whereas the antibody response to the epitopes was reduced, and reduction of the immunogenicity of β-LG by conjugation with CMD was clearly demonstrated. Although the precise mechanism responsible remains unclear, a plausible one is that shielding of the epitopes in β-LG by CMD allows them to escape from recognition by the immune system. In fact, the immunogenicity of Conj. 162 prepared by conjugation of β-LG with CMD of higher molecular weight was lower than that of Conj. 40. We also previously showed that conjugates with a high saccharide content showed low immunogenicity owing to effective shielding by CMD and that the response to the epitope regions around the carbohydrate-binding sites in the conjugates was reduced in each strain of mice (*16*). The carbohydrate-binding sites in β-LG after conjugation with CMD having a mass of 10 kDa were ^{47}Lys, ^{60}Lys, ^{101}Lys, and ^{138}Lys. Other investigators (*42*−*44*) have reported that sites of preferential lactosylation of β-LG by the Maillard reaction were ^{47}Lys and/or ^{100}Lys. In addition, it is possible that ^{77}Lys may be glycosylated because ^{77}Lys has high surface accessibility to the solvent (*43*) and it is reported to be lactosylated after 24 h of the Maillard reaction (*45*). The response to the epitope regions around these Lys residues in each conjugate prepared in this study was lowered in each strain of mice. In particular, the response to ^{53}Asp−^{54}Leu, ^{69}Lys−^{75}Lys, and ^{80}Ala−^{85}Asp in BALB/c mice and the response to ^{51}Glu−^{56}Ile, ^{66}Cys−^{76}Thr, ^{78}Ile−^{82}Phe, and^{93}Leu−^{96}Asp in C3H/He mice were dramatically reduced (Figure 6). As for the human IgE response to β-LG, ^{97}Thr−^{108}Glu (*22*) and ^{95}Leu−^{113}Pro (*26*) are reported to be major epitopes as determined using synthetic peptides. ^{41}Val−^{60}Lys, ^{102}Tyr−^{124}Arg, and ^{149}Leu−^{162}Ile are also reported to be major allergenic epitopes in β-LG as determined using tryptic peptides (*24*). Because the carbohydrate-binding sites mentioned above are sites neighboring these IgE epitopes, the β-LG−CMD conjugates prepared in this study may elicit low-level IgE production in human.

Although many studies on the reduced allergenicity of protein conjugates have been reported, only a limited number of studies have addressed the mechanisms involved. Lee et al. (*46*) investigated the mechanism of suppression of IgE antibody production upon immunization with ovalbumin−polyethylene glycol conjugate and suggested that the induction of regulatory T cells would bring about the suppressive effect. Takata et al. (*47*) reported that regulatory T cells and a soluble factor derived from these cells suppressed the growth of spleen cells from mice sensitized by treatment with antigen. On the contrary, So et al. (*48*) have reported that the reduced immunogenicity of PEG−lysozyme conjugates was not due to the induction of lysozyme-specific suppressor T cells but to low susceptibility to endosomal/lysosomal enzymes in antigen-presenting cells (APC).

In addition, So et al. (*49*) showed that the low susceptibility of lysozyme derivatives to endosomal/lysosomal enzymes led to a decrease in the generation of T cell epitopes. The induction of regulatory T cells upon immunization of the mice and the decrease in generation of T cell epitopes as a result of low susceptibility to endosomal/lysosomal enzymes in APC, as well as the masking of B cell epitopes by CMD, may be involved in the reduced immunogenicity of the β-LG−CMD conjugates. Further studies focusing on these aspects should be carried out. We intend to clarify the change in T cell response to β-LG after conjugation with CMD.

In conclusion, we were able to clearly demonstrate that conjugation with CMD of higher molecular weight was effective in reducing the immunogenicity of β-LG without inducing novel immunogenicity and that the effective shielding of epitopes by CMD was responsible for the reduced immunogenicity. Together with our previous results (*16*), conjugation with high saccharide content using polysaccharides of higher molecular weight is considered to be the most effective in reduced immunogenicity of proteins. This conjugation method is superior to other hypoallergenic methods in that the reduced immunogenicity of proteins can be achieved irrespective of the haplotype of the mice used. In addition, various functional improvements of proteins can be achieved by this method. In fact, we have reported that Conj. 40 and Conj. 162 showed excellent emulsifying properties with high thermal stability (*18*). Our work is the first study to clarify the changes in the B cell epitope profiles of a protein after conjugation with polysaccharides differing in molecular weight. Further studies on the mechanism responsible for the reduced immunogenicity by this method would lead to practical use of protein conjugates. β-LG−CMD conjugates prepared in this study have a potential for hypoallergenic formulas and hypoallergenic emulsifying agents, and it is strongly hoped that such conjugates will contribute to the development of novel foods with low allergenicity.

ABBREVIATIONS USED

APC, antigen-presenting cells; β-LG, β-lactoglobulin; RCM-β-LG, reduced and carboxymethylated β-lactoglobulin; CD, circular dichroism; CMD, carboxymethyl dextran; mAb, monoclonal antibody; PBS, phosphate-buffered saline; EDC, 1-ethyl-3-[(3-dimethylamino)propyl]carbodiimide; ELISA, enzyme-linked immunosorbent assay; PBS-Tween, PBS containing 0.05% Tween 20; PEG, polyethylene glycol.

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LITERATURE CITED

(1) Iikura, Y.; Imai, Y.; Imai, T.; Akasawa, A.; Fujita, K.; Hoshiyama, K.; Nakura, H.; Kohno, Y.; Koike, K.; Okudaira, H.; Iwasaki, E. Frequency of immediate-type food allergy in children in Japan. *Int. Arch. Allergy Immunol.* **1999**, *118*, 251−252.

(2) Spies, J. Milk allergy. *J. Milk Food Technol.* **1973**, *36*, 225−231.

(3) 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.

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

(5) 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.

(6) Brownlow, S.; Cabral, J. H. M.; Cooper, R.; Flower, D. R.; Yewdall, S. J.; Poolikarpov, I.; North, A. C. T.; Sawyer, L. Bovine β-lactoglobulin at 1.8 Å resolution—still an enigmatic lipocalin. *Structure* **1997**, *5*, 481−495.

(7) Virtanen, T.; Zeiler, T.; Rautiainen, J.; Mäntyjärvi, R. Allergy to lipocalins: a consequence of misguided T-cell recognition of self and nonself? *Immunol. Today* **1999**, *20*, 398−400.

(8) Shimizu, M.; Saito, M.; Yamauchi, K. Emulsifying and structural properties of β-lactoglobulin at different pHs. *Agric. Biol. Chem.* **1985**, *49*, 189−194.

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

(10) 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.

(11) Ishizaka, K.; Okudaira, H.; King, T. Immunogenic properties of modified antigen E. II. Ability of urea-denatured antigen and a polypeptide chain to prime T cells specific for antigen C. *J. Immunol.* **1975**, *114*, 110−115.

(12) Kurisaki, J.; Nakamura, S.; Kaminogawa, S.; Yamauchi, K. The antigenic properties of β-lactoglobulin examined with mouse IgE antibody. *Agric. Biol. Chem.* **1982**, *46*, 2069−2075.

(13) Watanabe, M.; Suzuki, T.; Ikezawa, Z.; Arai, S. Controlled enzymatic treatment of wheat proteins for production of hypoallergenic flour. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 388−390.

(14) del Val, G.; Yee, B. C.; Lozano, R. M.; Buchanan, B. B.; Ermel, R. W.; Lee, Y. M.; Frick, O. L. Thioredoxin treatment increases digestibility and lowers allergenicity of milk. *J. Allergy Clin. Immunol.* **1999**, *103*, 690−697.

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

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

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

(18) 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.* **1996**, *44*, 2538−2543.

(19) Katre, N. Immunogenicity of recombinant IL-2 modified by covalent attachment of polyethylene glycol. *J. Immunol.* **1990**, *144*, 209−213.

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

(21) Fagnani, R.; Hagan, M. S.; Bartholomew, R. Reduction of immunogenicity by covalent modification of murine and rabbit immunoglobulins with oxidized dextrans of low molecular weight. *Cancer Res.* **1990**, *50*, 3638−3645.

(22) Ball, G.; Shelton, M. J.; Walth, B. J.; Hill, D. J.; Hosking, C. S.; Howden, M. E. H. A major continuous allergenic epitope of bovine β-lactoglobulin recognized by human IgE binding. *Clin. Exp. Allergy* **1994**, *24*, 758−764.

(23) Selo, I.; Negroni, L.; Creminon, C.; Yvon, M.; Peltre, G.; Wal, J. M. Allergy to bovine β-lactoglobulin: Specificity of human IgE using cyanogen bromide-derived peptides. *Int. Arch. Allergy Immunol.* **1998**, *117*, 20−28.
(24) Selo, I.; Clement, G.; Bernard, H.; Chatel, J. M.; Creminon, C.; Peltre, G.; Wal, J. M. Allergy to bovine β-lactoglobulin: specificity of human IgE to tryptic peptides. *Clin. Exp. Allergy* **1999**, *29*, 1055−1063.
(25) Williams, S. C.; Badley, R. A.; Davis, P. J.; Puijk, W. C.; Meloen, R. H. Identification of epitopes within beta lactoglobulin recognized by polyclonal antibodies using phage display and PEPSCAN. *J. Immunol. Methods* **1998**, *213*, 1−17.
(26) Heinzmann, A.; Blattmann, S.; Spuergin, P.; Forster, J.; Deichmann, K. A. The recognition pattern of sequential B cell epitopes of β-lactoglobulin does not vary with the clinical manifestations of cow's milk allergy. *Int. Arch. Allergy Immunol.* **1999**, *120*, 280−286.
(27) Miller, K.; Meredith, C.; Selo, I.; Wal, J. M. Allergy to bovine β-lactoglobulin: specificity of immunoglobulin E generated in the Brown Norway rat to tryptic and synthetic peptides. *Clin. Exp. Allergy* **1999**, *29*, 1696−1704.
(28) 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.
(29) Davis, B. J. Disc Electrophoresis—II: Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* **1964**, *121*, 404−427.
(30) 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.
(31) 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.
(32) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(33) Kramlova, M.; Pristoupil, T.; Fricova, V.; Kraml, J. First experience with the use of the Pharmacia Phast System for the characterization of hemoglobins by isoelectric focusing. *J. Chromatogr.* **1986**, *367*, 443−445.
(34) 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.
(35) 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.
(36) Hattori, M.; Aiba, Y.; Nagasawa, K.; Takahashi, K. Functional improvements in alginic acid by conjugating with β-lactoglobulin. *J. Food Sci.* **1996**, *61*, 1171−1176.
(37) 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.
(38) 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.
(39) 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.
(40) Kohri, E.; Maurice, L.; Bourgeois, C.; Bour, J. B.; Pothier, P. Epitope mapping of the major inner capsid protein of group A rotavirus using peptide synthesis. *Virology* **1993**, *194*, 110−116.
(41) Gammon, G.; Geysen, H. M.; Apple, R. J.; Pickett, E.; Palmer, M.; Ametani, A.; Sercarz, E. E. T cell determinant structure: cores and determinant envelopes in three mouse major histocompatibility complex haplotypes. *J. Exp. Med.* **1991**, *173*, 609−617.
(42) Leonil, J.; Molle, D.; Fauquant, J.; Maubois, J. L.; Pearce, R. J.; Bouhallab, S. Characterization by ionization mass spectrometry of lactosyl β-lactoglobulin conjugates formed during heat treatment of milk and whey and identification of one lactose-binding site. *J. Dairy Sci.* **1997**, *80*, 2270−2281.
(43) Fogliano, V.; Monti, S. M.; Visconti, A.; Randazzo, G.; Facchiano, A. M.; Colonna, G.; Ritieni, A. Identification of a β-lactoglobulin lactosylation site. *Biochim. Biophys. Acta* **1998**, *1388*, 295−304.
(44) Siciliano, R.; Rega, B.; Amoresano, A.; Pucci, P. Modern mass spectrometric methodologies in monitoring milk quality. *Anal. Chem.* **2000**, *72*, 408−415.
(45) Morgan, F.; Leonil, J.; Molle, D.; Bouhallab, S. Nonenzymatic lactosylation of bovine β-lactoglobulin under mild heat treatment leads to structural heterogeneity of the glycoforms. *Biochem. Biophys. Res. Commun.* **1997**, *236*, 413−417.
(46) Lee, W. Y.; Sehon, A. H.; Åkerblom, E. Suppression of reaginic antibodies with modified allergens. IV. Induction of suppressor T cells by conjugates of polyethylene glycol (PEG) and monomethoxy PEG with ovalbumin. *Int. Arch. Allergy Appl. Immunol.* **1981**, *64*, 100−114.
(47) Takata, M.; Maiti, P. K.; Kubo, R. T.; Chen, Y. H.; Holford, S. V.; Rector, E. S.; Sehon, A. H. Cloned suppressor T cells derived from mice tolerized with conjugates of antigen and monomethoxypolyethylene glycol. Relationship between monoclonal T suppressor factor and the T cell receptor. *J. Immunol.* **1990**, *145*, 2846−2853.
(48) So, T.; Ito, H.; Koga, T.; Ueda, T.; Imoto, T. Reduced immunogenicity of monomethoxypolyethylene glycol-modified lysozyme for activation of T cells. *Immunol. Lett.* **1996**, *49*, 91−97.
(49) So, T.; Ito, H.; Koga, T.; Watanabe, S.; Ueda, T.; Imoto, T. Depression of T-cell epitope generation by stabilizing hen lysozyme. *J. Biol. Chem.* **1997**, *272*, 32136−32140.

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