

Reduced Immunogenicity of β -Lactoglobulin by Conjugation with Acidic Oligosaccharides

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Bovine β -lactoglobulin (β -LG) was conjugated with the acidic oligosaccharides, alginic acid oligosaccharide (ALGO) and phosphoryl oligosaccharides (POs) by the Maillard reaction to reduce the immunogenicity of β -LG. The molar ratios of β -LG to ALGO and POs in the conjugates were 1:6 and 1:8. The carbohydrate-binding sites in the β -LG–ALGO conjugate were partially identified to be ⁶⁰-Lys, ⁷⁷Lys, ¹⁰⁰Lys, ¹³⁸Lys, and ¹⁴¹Lys. The isoelectric point of each conjugate was lower than that of β -LG. CD spectra indicated that the secondary structure of β -LG was almost maintained after conjugation. The results of fluorescence studies indicated that the conformation around Trp had not changed in each conjugate and that the surface of each conjugate was covered with a saccharide chain. Structural analyses with monoclonal antibodies indicated that the conformation around ⁸Lys–¹⁹Trp (β -sheet, random coil, short helix) in the conjugates had changed, whereas the native structure was maintained around ¹⁵Val–²⁹Ile (β -sheet) and ¹²⁵Thr–¹³⁵Lys (α -helix). The β -LG–ALGO and β -LG–POs conjugates maintained 77 and 70% of the retinol binding activity of β -LG. Conjugation with ALGO and POs substantially enhanced the thermal stability of β -LG. The anti- β -LG antibody response was markedly reduced after immunization with both conjugates in BALB/c, C57BL/6, and C3H/He mice. B cell epitopes of β -LG and the conjugate recognized in these mice were determined with 15-mer multipin peptides, and the linear epitope profiles of the conjugates were found to be similar to those of β -LG, whereas the antibody response to each epitope was dramatically reduced. In particular, effective reduction of the antibody response was observed in the vicinity of the carbohydrate-binding sites. Conjugation of β -LG with these acidic oligosaccharides was effective in reducing the immunogenicity of β -LG. The conjugates obtained in this study are edible, so they would be very useful for food application.

KEYWORDS: β -Lactoglobulin; alginic acid; alginate lyase; neoglycoconjugate; functional improvement; acidic saccharide; protein conjugation; emulsification; solubility; retinol binding; lipocalin

INTRODUCTION

β -Lactoglobulin (β -LG) is a major whey protein of 18 kDa found in mammalian species. Many physicochemical and physiological studies on this protein have been carried out, and its structure has been well characterized. β -LG consists of nine antiparallel β -sheets and one α -helix to form a calyx-shaped β -barrel structure, and it is categorized as a member of the lipocalin superfamily (1, 2), possessing two disulfide bridges as well as 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 (4). β -LG is a valuable protein in view of food science because it contains plenty of essential amino acids (3) and has such various useful functional properties as emulsifying, foaming, and gelling properties (5–7). However, β -LG has some defects, the greatest being that it is a potent allergen of milk allergy; ~82% of milk allergy patients are sensitive to β -LG (8). With respect to its functional properties, β -LG loses its emulsifying properties in the acidic pH region and in the presence of salt.

It is particularly desirable to develop new methods that would reduce the allergenicity and improve the functional properties of β -LG. We have been studying the neoglycoconjugates of β -LG to achieve such multiple functional improvements. Studies

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on the neoglycoconjugates of proteins have been widely investigated for over 20 years (9), and various improvements to the function of proteins have been reported. We have shown that the conjugate of β -LG and carboxymethyl dextran showed improved emulsifying properties (10, 11) and immunogenicity (12–14). β -LG conjugated with alginic acid and alginate lyase-lysate (alginic acid oligosaccharide, ALGO) by the Maillard reaction showed improved thermal stability, emulsifying properties, and aggregating property (15, 16). Similar functional improvements to β -LG have also been achieved by conjugation with cationic saccharides and carboxymethyl cyclodextrin (17, 18).

Conjugation of a protein with polysaccharides is generally considered to be more effective than conjugation of a protein with low molecular weight molecules to achieve low allergenicity, because it can be expected that a polysaccharide will cover the epitopes of the allergens more effectively than a low molecular weight molecule (19). In fact, we have found that one of the mechanisms responsible for the reduced immunogenicity of β -LG–carboxymethyl dextran (CMD) conjugates was shielding of the epitopes in β -LG by CMD, which allowed the epitopes to escape from recognition by the immune system (13). However, because the area of the epitope is not so large [$\sim 1000 \text{ \AA}^2$, six to eight amino acids in length (20)], effective shielding can be expected with covalent binding of an oligosaccharide. In addition, changes in the conformational stability of a protein after its conjugation with other molecules has been reported to affect the quality of the immune response (21). We postulate that conjugation with an oligosaccharide could reduce the immunogenicity of a protein.

In the present study, we prepared edible conjugates of β -LG with acidic oligosaccharides [ALGO and phosphoryl oligosaccharides (POs)] by the Maillard reaction and evaluated the immunological properties of these conjugates.

MATERIALS AND METHODS

Preparation of β -LG and RCM- β -LG. β -LG (genotype AA) was isolated from fresh milk of a Holstein cow supplied by our dairy farm (Tokyo University of Agriculture and Technology, Fuchu, Japan) according to the method of Armstrong et al. (22) and was purified by ion-exchange chromatography in a DEAE-Sepharose Fast Flow column (2.5 i.d. $\times$ 50 cm; Amersham Pharmacia Biotech, Buckinghamshire, U.K.). Crude β -LG was applied to the column and eluted by a 0–500 mM NaCl linear gradient in a 0.05 M imidazole buffer at pH 6.7 with a flow rate of 1.0 mL/min. The eluted protein was detected by the absorbance at 280 nm. The major fraction was dialyzed against distilled water and lyophilized. The purity of β -LG was confirmed by polyacrylamide gel electrophoresis (PAGE) according to the method of Davis (23). RCM (reduced and carboxymethylated)- β -LG was prepared by reducing the disulfide bonds in β -LG with 2-mercaptoethanol and then by carboxymethylating the free sulfhydryl groups with sodium iodoacetate as described previously (24).

Acidic Oligosaccharides. Alginate lyase-lysate (ALGO, DP = 4) was supplied by Meiji Seika Co. Ltd. (Tokyo, Japan). Phosphoryl oligosaccharides (POs, DP = 4) were prepared from potato starch as described previously (25).

Preparation and Purification of the β -LG–Acidic Oligosaccharide Conjugates. The β -LG–acidic oligosaccharides conjugates were prepared by the Maillard reaction according to the method described previously (16). In brief, ALGO (1 g) and β -LG (1 g) were dissolved in 600 mL of distilled water and lyophilized. The mixture was incubated at 50 °C at a relative humidity of 79% for 24 h. After dialysis against distilled water and lyophilization, a crude β -LG–ALGO conjugate was obtained.

The β -LG–POs conjugate was prepared by adding POs (1.5 g), β -LG (1.5 g), and 25 mL of penicillin–streptomycin (5000 units/mL, Life

Technologies, Carlsbad, CA) to 600 mL of distilled water and then lyophilizing. The mixture was incubated at 50 °C at a relative humidity of 79% for 480 h. After dialysis against distilled water and lyophilization, a crude β -LG–POs conjugate was obtained.

Free oligosaccharides were removed by salting-out. The crude sample was dissolved in distilled water, and ammonium sulfate was added to a final concentration of 5 M. The precipitate was recovered by centrifuging (20000 rpm for 30 min) at 20 °C. The purified conjugate was obtained after dialysis against distilled water and lyophilization.

Size Exclusion Chromatography (SEC). The molecular weight of the β -LG–acidic oligosaccharides conjugates was measured by SEC. A TSKgel G3000SW_{XL} column (7.8 i.d. $\times$ 300 mm, Tosoh, Tokyo, Japan) was equilibrated with a 0.067 M phosphate buffer containing 4 M guanidine hydrochloride at pH 7.0. Each conjugate (80 μ g) in 80 μ L was applied to the column and eluted at a flow rate of 0.5 mL/min. The absorbance was monitored at 280 nm.

Isoelectric Focusing. Isoelectric focusing of the β -LG–acidic oligosaccharides conjugates was performed by using the Pharmacia-Phast System as described previously (16). The protein bands were detected by staining with Coomassie Brilliant Blue.

Conformational Analysis of the β -LG–Acidic Oligosaccharides Conjugates by Spectroscopic Methods. The CD spectra for β -LG–acidic oligosaccharides conjugates were measured with a J-720 spectropolarimeter (Jasco, Tokyo, Japan), using a cell with a 1.0-mm path length. Each sample was dissolved in phosphate-buffered saline (PBS; a 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%.

The intrinsic fluorescence of each β -LG–acidic oligosaccharides conjugate dissolved in PBS at a 0.001% (as the protein concentration) was measured at an excitation wavelength of 283 nm by means of an RF-5300PC fluorescence spectrophotometer (Shimadzu, Kyoto, Japan).

Measurement of the Retinol-Binding Activity of the β -LG–Acidic Oligosaccharides Conjugates. The retinol-binding activity of each β -LG–acidic oligosaccharides conjugates was evaluated by fluorescence titration with the RF-5300PC instrument (Shimadzu) as described previously (16).

Differential Scanning Calorimetry (DSC). The β -LG–acidic oligosaccharides conjugates were dissolved in PBS (pH 7.0) at a protein concentration of 5.0%. A 50 μ L 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 (10, 16, 26). DSC curves were recorded by an SSC-5020 DSC-100 instrument (Seiko, Tokyo, Japan) programmed at a heating rate of 2 K/min in the temperature range of 20–100 °C. The reference sample was distilled water.

Determination of the Carbohydrate-Binding Site in the β -LG–ALGO Conjugate. β -LG and β -LG–ALGO conjugate (100 mg as a protein) were reduced with 2-mercaptoethanol and carboxymethylated with sodium iodoacetate as described previously (24). Each reduced and carboxymethylated sample was dissolved in an ammonium carbonate buffer (pH 8.0) at a protein concentration of 1.0 mg/mL and digested with TPCK–trypsin (EC 3.4.21.4; Sigma, St. Louis, MO) at 37 °C for 24 h (S:E = 100:1). After digestion and lyophilization, each sample was redissolved in water and submitted to ultrafiltration with Centricon (Amicon, Beverly, MA) to remove TPCK–trypsin and the nondigested protein. The digested fragments were separated by reverse-phase HPLC (ODS-120T column, 4.6 i.d. $\times$ 250 mm; Tosoh, Tokyo, Japan). The peptides were detected by their absorbance at 230 nm, whereas the saccharides were detected by a reagent containing aniline (1.2 mL) and diphenylamine (1.2 g) in phosphate (9.0 mL) and acetone (60 mL) after spotting on a TLC plate (silica gel 60, Merck, Darmstadt, Germany). The peaks containing peptide and saccharide were further purified by reverse-phase HPLC, and the amino acid sequence was determined by a 470A peptide sequencer (Perkin-Elmer Applied Biosystems, Foster, CA).

Immunization. Female BALB/c, C57BL/6, and C3H/He mice were purchased from Clea Japan (Tokyo, Japan). Seven mice of each group at 6 weeks of age were immunized intraperitoneally with 100 μ g of protein in Freund's complete adjuvant (Difco Laboratories, Detroit, MI). The mice were administered a booster consisting of 100 μ g of protein in Freund's incomplete adjuvant (Difco Laboratories) 14 days after the

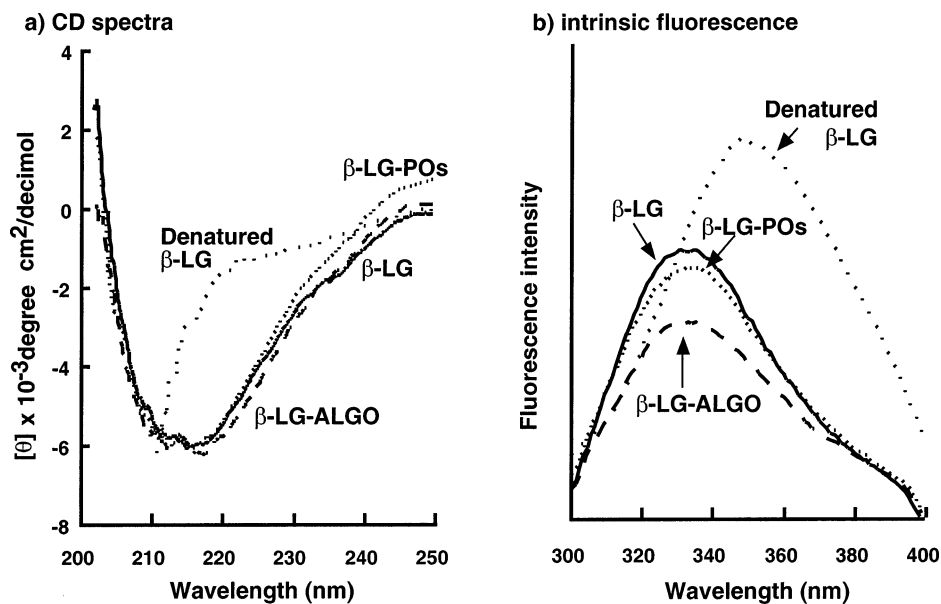


Figure 1. Spectroscopic analysis of the β -LG-acidic oligosaccharides conjugates: (a) CD spectra; (b) intrinsic fluorescence; (solid line) β -LG; (broken line) β -LG-ALGO; (dotted line) β -LG-POs; (low-density dotted line) denatured β -LG.

primary immunization. The mice were bled 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, and antisera were collected from each blood sample after the clot had formed. This study was performed in conformity with the guidelines for the care and use of experimental animals established by the ethics committee of Tokyo University of Agriculture and Technology.

Enzyme-Linked Immunosorbent Assay (ELISA). Noncompetitive ELISA was carried out as follows (12, 13). β -LG and each conjugate were dissolved in PBS at a protein concentration of 0.01% (100 μ L) and added to the wells of a polystyrene microtitration plate (Maxisorp; Nunc, Roskilde, Denmark), and the plate was incubated overnight at 4 °C to coat the wells with each antigen. After removal of the solution, each well was washed three times with 125 μ L of PBS-Tween (PBS containing 0.05% Tween 20). A 125 μ L amount of a 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 with 125 μ L of PBS-Tween. A 100 μ L amount of an antibody [antisera or monoclonal antibody (mAb)] diluted with PBS was added to each well, and the plate was incubated for 2 h at 25 °C. After three washings, 100 μ L 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 for 2 h at 25 °C, and then the wells were washed three times. A 100 μ L amount of 0.1% sodium *p*-nitrophenyl phosphate disodium/diethanolamine hydrochloride buffer (pH 9.8) was added to each well, and the plate was incubated for 30 min at 25 °C. After the addition of a 5 M sodium hydroxide solution (20 μ L) to each well to stop the reaction, the absorbance at 405 nm was measured with an MPR-A4i microplate reader (Tosoh, Tokyo, Japan).

In the determination of the immunogenicity of the β -LG-acidic oligosaccharides conjugates, a standard curve was constructed for the serially diluted anti- β -LG antiserum pooled from other C3H/He mice. The antibody titer was calculated from the standard curve and is expressed as the relative concentration.

A competitive ELISA with anti- β -LG mAbs (mAbs 21B3, 31A4, 61B4, and 62A6) was performed to investigate the local conformational changes in β -LG after conjugation with acidic oligosaccharides as previously described (10–13, 15, 16, 24, 27, 28). The equilibrium constants (K_{AS}) for the interaction of mAb with β -LG, RCM- β -LG, or β -LG-acidic oligosaccharides conjugates were calculated according to the method of Hogg et al. (29), based on the results of competitive and noncompetitive ELISA. RCM- β -LG was used as the denatured form of β -LG.

Epitope Mapping. B cell epitopes were investigated by ELISA with a series of overlapping 15-mer multipin peptides (Chiron Mimotopes,

Clayton, Victoria, Australia) as described previously (11, 13). In the determination of the immunogenicity profiles, certain criteria were used to evaluate whether the observed response was significant. The 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 (30), (2) those which showed positive reactivity with at least two consecutive overlapping peptides (31), 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. (32).

RESULTS AND DISCUSSION

Structural Features of the β -LG-Acidic Oligosaccharides Conjugates. Isoelectric focusing was carried out to confirm covalent binding between β -LG and the acidic oligosaccharides. The *pI* value of each conjugate was relatively distributed and lower (*pI* < 4.6) than that of β -LG (*pI* = 5.2). This result strongly suggests that β -LG and the acidic oligosaccharides were covalently bound to each other. The composition of the conjugate determined according to the Bradford (33) and phenol-sulfuric acid methods (34) indicated that the molar ratios of β -LG to ALGO and POs in the conjugate were 1:6 and 1:8. The molecular weight was evaluated by SEC to be 22000 for both conjugates. The carbohydrate-binding sites in the β -LG-ALGO conjugate were partially identified to be ⁶⁰-Lys, ⁷⁷Lys, ¹⁰⁰Lys, ¹³⁸Lys, and ¹⁴¹Lys.

CD spectra for the conjugates are shown in **Figure 1a**. The spectra for both conjugates are similar to the spectrum for β -LG, which indicates that the secondary structure of β -LG was almost completely maintained after its conjugation with these acidic oligosaccharides. The fluorescence emission spectra of the conjugates are shown in **Figure 1b**. When excited at 283 nm, native β -LG exhibited a fluorescence emission maximum at 336 nm. The fluorescence intensity increases with red shift of the wavelength for maximum emission as the conformation of β -LG changes (24, 28). In the present experiment, both conjugates exhibited the emission maximum wavelength of 336 nm. Hence, the conformation around the Trp residues (¹⁹Trp and ⁶¹Trp) in the conjugates maintained the native form. The fluorescence intensity of the β -LG-POs conjugate was similar to that of native β -LG, whereas that of the β -LG-ALGO conjugate was

Table 1. Equilibrium Constants (K_{AS})^a of the β -LG–Acidic Oligosaccharides Conjugates in Binding to Anti- β -LG MAbs

mAb	K_{AS}			
	β -LG	β -LG–ALGO	β -LG–POs	RCM- β -LG
21B3	7.32×10^6	6.56×10^6	5.87×10^6	3.92×10^7
31A4	1.89×10^6	1.77×10^7	9.23×10^6	3.27×10^7
61B4	9.18×10^7	1.01×10^8	1.22×10^8	
62A6	6.06×10^7	1.09×10^8	9.17×10^7	

^a K_{AS} values were calculated from the results of competitive and noncompetitive ELISA according to the procedure of Hogg et al. (29).

lower than that of native β -LG. This decrease in fluorescence intensity in the case of the β -LG–ALGO conjugate is considered to have been due to the shielding effect by the oligosaccharide chain bound to ⁶⁰Lys in this conjugate. In the case of the β -LG–POs conjugate, binding of POs to ⁶⁰Lys seems not to have occurred.

The local conformational difference between the conjugates and native β -LG was evaluated by using anti- β -LG mAbs as probes. The mAbs used in this study could detect the subtle conformational differences in local areas within the β -LG molecule during unfolding and refolding (24, 27, 28) and after conjugation with saccharides (10–13, 15, 16) by determining the affinity change.

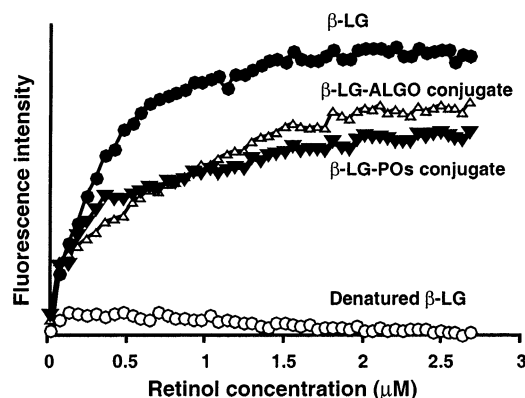
The epitope regions for mAbs 21B3, 31A4, 61B4, and 62A6 are ¹⁵Val–²⁹Ile (β -sheet), ⁸Lys–¹⁹Trp (random coil, β -sheet, and short helix), ¹²⁵Thr–¹³⁵Lys (α -helix), and the regions close to the epitope for 61B4, respectively. MAbs 61B4 and 62A6 bind preferentially to native β -LG, whereas mAbs 21B3 and 31A4 bind more strongly to RCM- β -LG (the denatured form of β -LG).

The equilibrium constants of these mAbs to the conjugates are shown in **Table 1**. The reactivities of mAbs 21B3, 61B4, and 62A6 to the conjugates were similar to that to the native material. In both conjugates, the conformation around the epitopes for these mAbs is considered to have maintained the native form. On the other hand, the reactivity of mAb 31A4 to the conjugates was similar to that to RCM- β -LG, which indicates that the conformation around the epitope for mAb 31A4 was rather exposed after conjugation with ALGO and POs.

Each β -LG–acidic oligosaccharides conjugate thus maintained the native-like protein structure, although a little collapse in the structure was observed.

Retinol-Binding Activity of the β -LG–Acidic Oligosaccharides Conjugates. β -LG belongs to the lipocalin superfamily and can bind retinol and fatty acids (4). The β -LG dimer can actually bind two molecules of retinol (35). Changes in the retinol-binding activity of β -LG after conjugation with acidic oligosaccharides were investigated by fluorescence titration (28, 36, 37). Although the retinol-binding activity of β -LG was reduced a little by conjugation with ALGO and POs, the conjugates did possess this activity (**Figure 2**). The β -LG–ALGO and β -LG–POs conjugates maintained 77 and 70% of the retinol-binding activity of β -LG. The difference in this activity is considered to have been brought about by the subtle conformational changes in the retinol-binding site of β -LG that were induced by conjugation with ALGO and POs.

Thermal Stability of the β -LG–Acidic Oligosaccharides Conjugates. The thermal stability of the conjugates was evaluated by DSC. The thermal characteristics of the conjugates are summarized in **Table 2**. ALGO and POs were thermally inactive throughout the temperature range used for the present DSC run (data not shown). The denaturation temperature (T_p)

**Figure 2.** Retinol-binding activity of the β -LG–acidic oligosaccharides conjugates: (●) β -LG; (○) denatured β -LG; (Δ) β -LG–ALGO; (▼) β -LG–POs.**Table 2.** Thermal Characteristics of the β -LG–Acidic Oligosaccharides Conjugates

	denaturation temp (°C)			denaturation enthalpy (mJ/mg)
	T_0^a	T_p^a	T_c^a	
β -LG	69.0	74.9	80.1	13.3
β -LG–ALGO	75.8	92.7	103.1	23.2
β -LG–POs	75.8	89.6	105.6	17.0

^a T_0 , onset temperature; T_p , peak temperature; T_c , conclusion temperature.

and denaturation enthalpy (ΔH) for a mixture of β -LG and the acidic oligosaccharides in the same ratio as that in the conjugates were similar to the values for native β -LG. The peak temperature (T_p) was ~ 93 °C for the β -LG–ALGO conjugate and ~ 90 °C for the β -LG–POs conjugate, these values being much higher than that of native β -LG. Conjugation of these acidic oligosaccharides to a protein is considered to bring about an improvement in the thermal stability of the protein. ΔH for the conjugate increased to about 174% (β -LG–ALGO) and 128% (β -LG–POs) of the value for native β -LG. These increases in ΔH , which indicate the wide range of thermal transition in β -LG, are considered to have mainly been due to the increased structural changes in the conjugates by heating.

Immunogenicity of the β -LG–Acidic Oligosaccharides Conjugates. The immunogenicity of the β -LG–acidic oligosaccharides conjugates in BALB/c, C57BL/6, and C3H/He mice was evaluated by measuring the reactivity of 10000-fold diluted antisera with the antigen (β -LG, RCM- β -LG, β -LG–ALGO), and β -LG–POs) adsorbed to the solid phase of a microtitration plate by noncompetitive ELISA (**Figure 3**). As described in our previous papers (12, 13), the anti- β -LG antisera in our immunization system reacted strongly to the denatured form of β -LG, so evaluation of the reactivity of each antiserum with the denatured material (RCM- β -LG) is also considered to be important for examining immunogenicity. The anti- β -LG antibody response was low in those three strains of mice immunized with each conjugate (**Figure 3a,e,i**). In particular, the immunogenicity of the β -LG–POs was significantly lower. The antibody response to the denatured form of β -LG (RCM- β -LG) was significantly lower in the BALB/c and C57BL/6 mice after immunization with both β -LG–acidic oligosaccharides conjugates (**Figure 3b,j**). When each β -LG–acidic oligosaccharides conjugate was used as the coating antigen on the solid phase to evaluate the productivity of the specific antibody for each conjugate (**Figure 3c,d,g,h,k,l**), both the anti- β -LG–ALGO antisera and anti- β -LG–POs antisera showed an antibody titer similar to or lower than that of the anti- β -LG antisera. These results indicate that there was no emergence of novel immu-

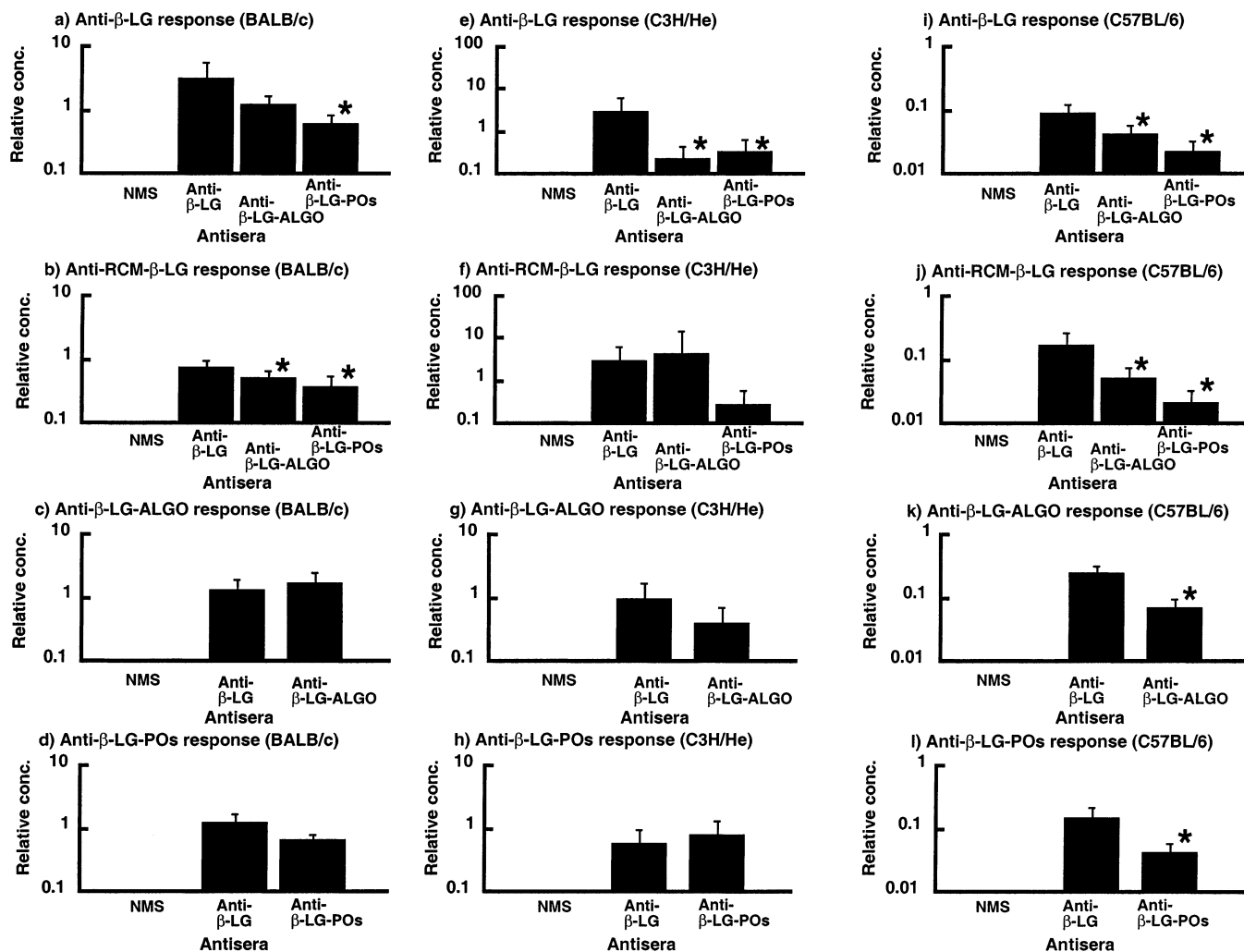


Figure 3. Immunogenicity of the β -LG-acidic oligosaccharides conjugates in three strains of mice. The anti- β -LG response, anti-RCM- β -LG response, anti- β -LG-ALGO response, and anti- β -LG-POs response after the secondary immunization in BALB/c (a–d), C57BL/6 (e–h), and C3H/He mice (i–l) were evaluated by noncompetitive ELISA. A significant difference ($p < 0.05$) as determined by Student's *t*-test is indicated with an asterisk.

nogenicity in β -LG after its conjugation with ALGO and POs. Thus, the conjugation of ALGO and POs with β -LG is considered to be effective for decreasing the immunogenicity of β -LG. In particular, conjugation with POs was very effective for reducing the immunogenicity of β -LG.

Scanning of Epitopes in the β -LG-Acidic Oligosaccharides Conjugates. The B cell epitope profiles of β -LG and the β -LG-acidic oligosaccharides conjugates were analyzed by ELISA, using overlapping 15-mer multipin peptides, to clarify how conjugation with acidic oligosaccharides leads to the reduced immunogenicity of β -LG. The results obtained with BALB/c mice are shown in **Figure 4a–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 indicates the reactivity of each 15-mer peptide with each pooled antiserum. The epitopes were identified by determining the common amino acid sequence among the immunoreactive peptides according to the method of Gammon et al. (32) as summarized in **Figure 5**, in which the horizontal axis indicates the sequence number in β -LG and the line thickness indicates the response to each epitope.

The anti- β -LG antiserum from BALB/c mice (**Figure 4a**) reacted with peptides 1–5, 18–20, 21–30, 38–43, 44–47, 48–51, 52–55, 61–67, 68–69, 70–74, 75–83, and 118–124 (these are the residue numbers of the N terminus of the peptides). The linear B cell epitopes of β -LG recognized in BALB/c mice were

determined to be ^5Gln – ^{15}Val , ^{20}Tyr – ^{32}Leu , ^{30}Ser – ^{35}Gln , ^{43}Val – ^{52}Gly , ^{47}Lys – ^{58}Leu , ^{51}Glu – ^{62}Glu , ^{55}Glu – ^{66}Cys , ^{67}Ala – ^{75}Lys , ^{69}Lys – ^{82}Phe , ^{74}Glu – ^{84}Ile , ^{83}Lys – ^{88}Asn , and ^{124}Arg – ^{132}Ala , the dominant epitopes being ^{30}Ser – ^{35}Gln , ^{47}Lys – ^{58}Leu , ^{51}Glu – ^{62}Glu , ^{67}Ala – ^{75}Lys , and ^{69}Lys – ^{82}Phe (**Figure 5**). As for the anti-conjugate response in BALB/c mice (**Figure 4b,c**), the epitope distribution of each conjugate was similar to that of β -LG. Although ^{84}Ile – ^{93}Leu and ^{93}Leu – ^{100}Lys for β -LG-ALGO and ^{82}Phe – ^{93}Leu for β -LG-POs showed stronger signals than those for β -LG, the decrease in response was marked with both anti-conjugates antisera through the entire amino acid sequence as compared with the anti- β -LG antiserum. The B cell epitopes of the conjugates recognized in BALB/c mice were determined to be ^4Thr – ^{15}Val , ^{28}Asp – ^{35}Gln , ^{48}Pro – ^{55}Glu , ^{54}Leu – ^{63}Asn , ^{67}Ala – ^{75}Lys , ^{69}Lys – ^{82}Phe , ^{78}Ile – ^{85}Asp , ^{84}Ile – ^{93}Leu , ^{93}Leu – ^{100}Lys , and ^{124}Arg – ^{132}Ala for β -LG-ALGO and ^6Thr – ^{15}Val , ^{30}Ser – ^{35}Gln , ^{43}Val – ^{55}Glu , ^{46}Ile – ^{59}Gln , ^{51}Glu – ^{61}Trp , ^{67}Ala – ^{75}Lys , ^{69}Lys – ^{82}Phe , ^{82}Phe – ^{93}Leu , and ^{123}Val – ^{136}Phe for β -LG-POs (**Figure 5**). The reduced immunogenicity of β -LG through conjugation with acidic oligosaccharides 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 β -LG-POs.

The results obtained with C3H/He and C57BL/6 mice are shown in **Figure 4d–i**, and B cell epitope profiles of β -LG and the conjugates were determined as shown in **Figure 5**. The

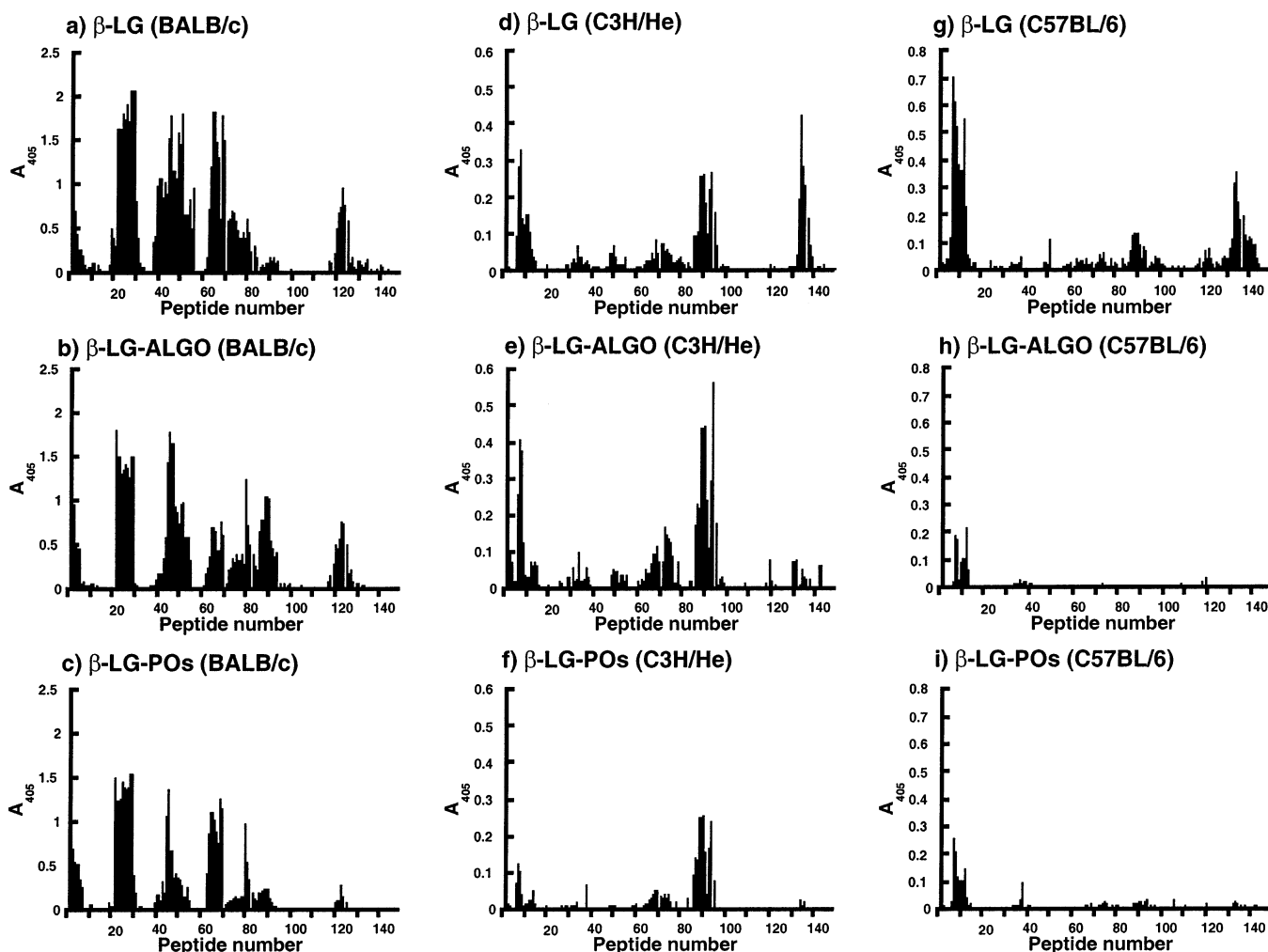


Figure 4. Reactivity of anti- β -LG, anti- β -LG-ALGO, and anti- β -LG-POs antisera with overlapping 15-mer peptides covering the amino acid sequence of β -LG. The reactivity of anti- β -LG (a, d, g), anti- β -LG-ALGO (b, e, h), and anti- β -LG-POs (c, f, i) antisera after the secondary immunization from BALB/c (a–c), C57BL/6 (d–f), and C3H/He mice (g–i) with multipin peptides was evaluated by ELISA.

B cell epitopes of β -LG recognized in C3H/He mice were determined to be ^9Gly – ^{19}Trp , ^{14}Lys – ^{24}Met , ^{38}Pro – ^{44}Glu , ^{52}Gly – ^{61}Trp , ^{69}Lys – ^{77}Lys , ^{76}Thr – ^{84}Ile , ^{80}Ala – ^{91}Lys , ^{90}Asn – ^{99}Tyr , ^{95}Leu – ^{105}Phe , and ^{138}Lys – ^{145}Met , the dominant epitopes being ^9Gly – ^{19}Trp , ^{90}Asn – ^{99}Tyr , ^{95}Leu – ^{105}Phe , and ^{138}Lys – ^{145}Met (Figure 5). With respect to the anti-conjugate response in C3H/He mice, the epitope distribution was similar to that of β -LG. In the case of the β -LG-ALGO, although the response to ^{75}Lys – ^{85}Asp , ^{91}Lys – ^{99}Tyr , and ^{94}Val – ^{106}Cys was a little higher than that for β -LG, the entire response was similar to the results obtained with β -LG, whereas the response to ^{138}Lys – ^{145}Met , which is one of the major epitopes, dramatically decreased. The B cell epitopes for the β -LG-ALGO conjugate recognized in C3H/He mice were determined to be ^3Val – ^{15}Val , ^9Gly – ^{18}Thr , ^{37}Ala – ^{44}Glu , ^{54}Leu – ^{61}Trp , ^{64}Asp – ^{75}Lys , ^{70}Lys – ^{79}Pro , ^{75}Lys – ^{85}Asp , ^{91}Lys – ^{99}Tyr , ^{94}Val – ^{106}Cys , ^{98}Asp – ^{110}Ser , ^{119}Cys – ^{131}Glu , ^{130}Asp – ^{143}Ala , and ^{136}Phe – ^{147}Ile (Figure 5). In the case of the β -LG-POs, a loss in antibody response was prominent through the entire sequence of β -LG and reduction of the immunogenicity of β -LG was effectively achieved. The B cell epitopes of the β -LG-POs conjugate recognized in C3H/He mice were determined to be ^{21}Ile – ^{15}Val , ^8Lys – ^{19}Trp , ^{14}Lys – ^{24}Met , ^{70}Lys – ^{79}Pro , ^{75}Lys – ^{85}Asp , ^{91}Lys – ^{99}Tyr , and ^{94}Val – ^{106}Cys (Figure 5).

The B cell epitopes of β -LG recognized in C57BL/6 mice were determined to be ^8Lys – ^{18}Thr , ^{14}Lys – ^{23}Ala , ^{93}Leu – ^{100}Lys , ^{99}Tyr – ^{111}Ala , ^{122}Leu – ^{133}Leu , ^{130}Asp – ^{141}Lys , ^{138}Lys – ^{145}Met , and ^{142}Ala – ^{153}Pro , the dominant epitopes being ^8Lys – ^{18}Thr , ^{14}Lys – ^{23}Ala , and ^{138}Lys – ^{145}Met (Figure 5). In the case of the conjugates, the antibody response was effectively reduced and the signals were found only in the N-terminal region (Figure 4g–i). Reduction of the immunogenicity of β -LG in C57BL/6 mice was effectively achieved in both conjugates as far as the linear B cell epitopes were concerned. The B cell epitopes of the conjugates recognized in C57BL/6 mice were determined to be ^7Met – ^{20}Tyr and ^{12}Ile – ^{23}Ala for β -LG-ALGO and ^7Met – ^{20}Tyr and ^{12}Ile – ^{22}Leu for β -LG-POs (Figure 5).

As just described, we could achieve reduced immunogenicity of β -LG by its conjugation with acidic oligosaccharides. Although the precise mechanism responsible for the reduction of proteins by conjugation with saccharides remains unclear, it is plausible that shielding of the epitopes in β -LG by conjugated saccharides allows them to escape from recognition by the immune system. In fact, in our previous studies (12, 13), we have found that β -LG-CMD conjugates with high saccharide content showed reduced immunogenicity and that conjugation with CMD of higher molecular weight was also effective to reduce the immunogenicity of β -LG. In the present work, we partially identified the carbohydrate-binding sites in the β -LG-ALGO conjugate to be ^{60}Lys , ^{77}Lys , ^{100}Lys , ^{138}Lys , and ^{141}Lys . In the three strains of mice (BALB/c, C3H/He, and C57BL/6), effective reduction of the anti- β -LG antibody response was found in the vicinity of these carbohydrate-binding sites after conjugation with the acidic oligosaccharides. We conclude that

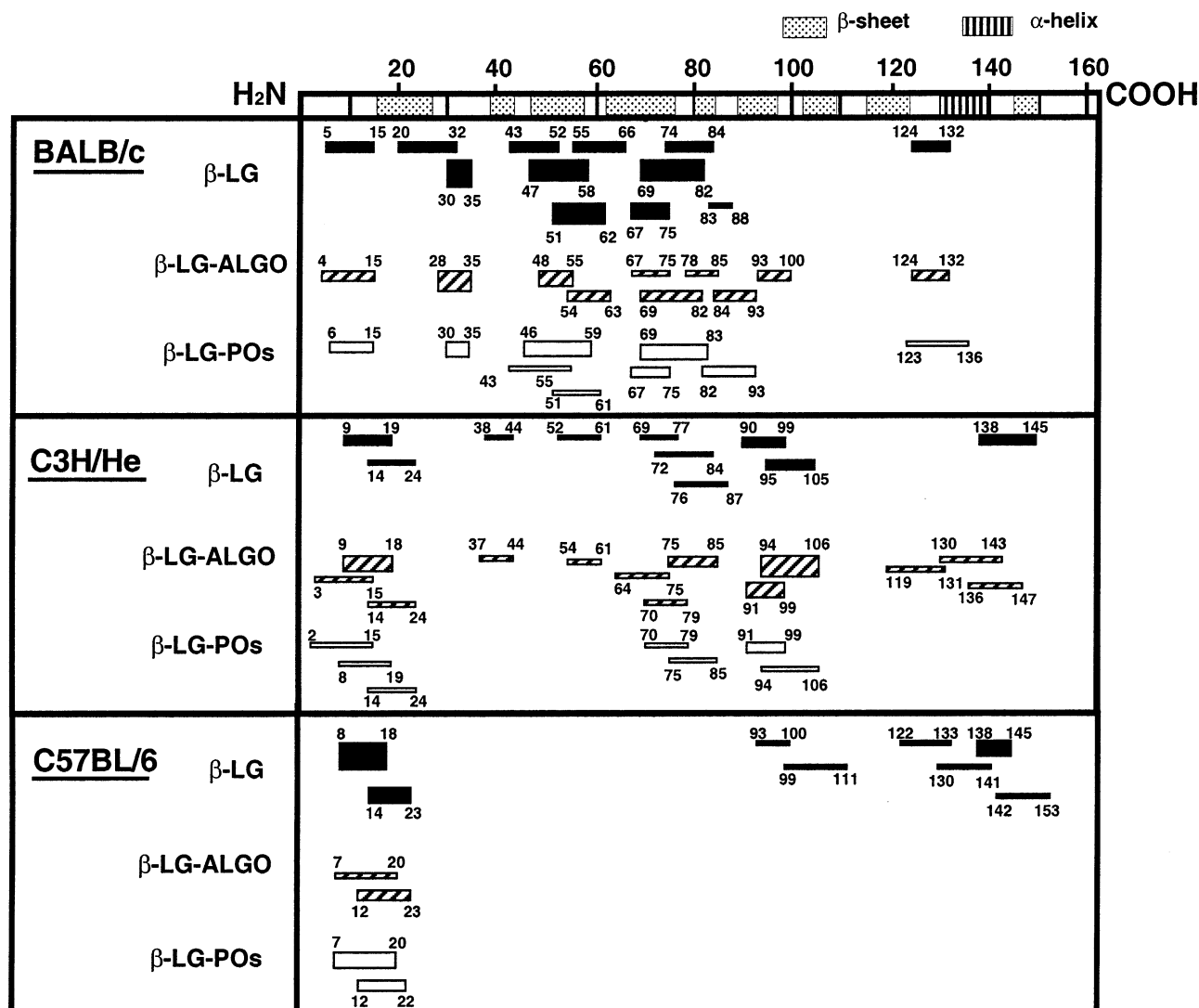


Figure 5. B cell epitope profiles of β -LG and the β -LG-acidic oligosaccharides conjugates. The common regions of at least two overlapping peptides that showed reactivity greater than the average value plus three standard deviations below the median absorbance in reactions between anti- β -LG antisera 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. (32).

one of the mechanisms responsible for the low immunogenicity of the β -LG-acidic oligosaccharides was shielding of the epitopes resulting from the conjugation. When we compare the difference in the immune response to the β -LG-ALGO conjugate and the β -LG-POs conjugate, the β -LG-POs conjugate showed lower immunogenicity in all three strains of mice. The lower immunogenicity of the β -LG-POs conjugate is ascribed to a greater content of saccharides bound to the β -LG molecule than in the β -LG-ALGO conjugate. In addition, the different nature of the conjugated saccharides may have affected the quality of the immune response. Other mechanisms for the reduced immunogenicity of the conjugates, particularly the induction of regulatory T cells (38) and the reduced susceptibility of the conjugates to endosomal/lysosomal enzymes in antigen-presenting cells (39, 40), would be important. Further studies focusing on these aspects should be carried out. We intend to clarify the changes in T cell response to β -LG and the enzymatic digestibility by endosomal/lysosomal enzymes after conjugation with these acidic oligosaccharides.

Concluding Remarks. We prepared in this study β -LG-acidic oligosaccharide conjugates by using the Maillard reaction. Although some conformational changes in β -LG were apparent after conjugation with acidic oligosaccharides, the obtained

conjugates maintained the retinol-binding activity of β -LG, showed enhanced thermal stability, and had reduced immunogenicity without novel immunogenicity. The β -LG-acidic oligosaccharides conjugates prepared in this study are especially valuable in that they are edible. It is strongly hoped that these conjugates will contribute to developing the utilization of useful food proteins such as β -LG as food additives.

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