Effect of Chemical and Genetic Attachment of Polysaccharides to Proteins on the Production of IgG and IgE

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To investigate the effect of polysaccharide attachment to proteins on the production of IgG and IgE, the genetic attachment of polysaccharide to lysozymes (G49N and R21T) using the yeast expression system (*Saccharomyces cerevisiae* AH 22) and the Maillard-type polysaccharide attachment to native lysozyme and soybean P34 protein were attempted. The production of IgG and IgE was investigated by using mice immunized with the protein−polysaccharide conjugates or native proteins. The attachment of polysaccharide to lysozyme using the yeast expression system greatly suppressed the production level of IgG and IgE. The attachment of polysaccharide to native lysozyme and soybean P34 protein using the Maillard-type reaction was also found to be effective in reducing the production level of IgE compared to IgG.

Keywords: *G49N; R21T; galactomannan; lysozyme; P34 protein; IgG; IgE*

INTRODUCTION

The incidence of allergy to food proteins such as egg and soybean proteins is gradually increasing with the growing consumption of processed foods containing egg proteins and soybean products all over the world. A number of patients with atopic dermatitis are considered to be suffering from food hypersensitivity mediated by allergen-specific IgE antibodies, and the incidence of atopic dermatitis is increasing, mainly among children.

Soybean is a principal foodstuff used as a protein source, and soy protein isolate prepared from defatted soybean is also widely utilized as an ingredient in many processed foods. However, soybean is known to be one of the typical allergenic foods with at least 15 allergenic proteins (*1*). It has been reported (*2*) that ∼65% of patients atopic to soybeans suffered from the 34 kDa protein (P34). Thus, the P34 protein has been recognized as a major allergen in soy protein. A P34 protein is one of the four major soybean oil body proteins observed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis of organic solvent-extracted oil bodies from mature seeds (*3*). On the other hand, egg white contains proteins of high nutritional value and better functional properties and, therefore, is widely used in the food industry. Egg white was found to contain allergic proteins such as ovalbumin, ovomucoid, and lysozyme. Lysozyme allergenicity was observed among all patients suffering from egg white allergy (*4*).

It has been reported that intraperitoneal administration of proteins such as bovine serum albumin (BSA), ovalbumin (OVA), or *Aspergillus oryzae* lipase induced vigorous IgG antibody responses. However, OVA and *A. oryzae* lipase stimulate strong IgE antibody responses, whereas BSA provoked low-titer IgE. It would appear, therefore, that the stimulation of IgG antibody responses is a reflection of protein immunogenicity, whereas protein allergenicity is associated with the induction of strong IgE responses (*5*).

The development of methods to reduce the allergenicity of proteins is urgently required. Several methods to reduce the allergenicity of protein allergen are being examined, including enzymatic methods (*6*), physical treatment, or development of soybean seeds that lack the allergenic protein (*7*). Immunological properties of allergens chemically modified with a synthetic copolymer of *N*-vinylpyrrolidone and maleic anhydride showed that when the protein conjugates were injected intraperitoneally into mice, they were found to induce IgG and did not induce significant quantities of IgE (*8*). Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhanced its immunogenicity and reduced its allergenicity (*9*). We suggest that masking of the allergenic structure could be effective in decreasing the allergenicity of the protein. A novel method of conjugating the ϵ-amino groups in the protein with the reducing end carbonyl group in the polysaccharide by a spontaneous Maillard reaction in a controlled dry-heating conditions has been developed (*10*−*13*). The polysaccharide attachment was found to be a very efficient method to reduce the allergenicity in vitro when soybean was conjugated with galactomannan through the Maillard reaction (*6*). However, there is no information on the in vivo production of IgG and IgE by immunizing test animals with polysaccharide−protein conjugates. Therefore, this paper describes the effect of the method of polysaccharide attachment (Maillard reaction products versus genetically modified protein products) to proteins on the allergenicity of the proteins as measured by the production of IgG and IgE.

MATERIALS AND METHODS

Materials. The yeast expression plasmid PYG-100 was supplied by Dr. Matsubara, Osaka University. Restriction enzymes were purchased from Takara Shuzo (Kyoto, Japan). CM-Toyopearl 650M resin was from Tosoh (Tokyo). Male mice (C3H/He), 7 weeks old, were purchased from Japan SLC, Co., Ltd. (Shizuoka, Japan). ^{125}I-Labeled antihuman IgE (1.7×10^4

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Bq/mL) was obtained from Pharmacia Co. (Uppsala, Sweden). Tween 20 and 3,3′-diaminobenzidine were purchased from Sigma Chemical Co. (St. Louis, MO). Peroxidase-conjugated goat anti-mouse IgG from Organon Teknika (West Chester, PA), goat anti-rabbit IgG−peroxidase conjugate, and Freund's complete and incomplete adjuvants were also purchased from Sigma Chemical Co. Biotinylation reagents were purchased from Amersham International, England. Unless otherwise stated, all reagents used in this study were of reagent grade.

Expression of Mutant Hen Egg White Lysozyme (HEWL). Two mutant glycosylated HEWLs, the molecular surface amino acid sequences of which were replaced to create the potential N-linked glycosylation site (Asn-X-Ser/Thr) (*G49N* in which *Gly-49* was substituted with *Asn* and *R21T* in which *Arg-21* was substituted with *Thr*), were produced as described by Nakamura et al. (*14*) and Kato et al. (*15*), respectively. For construction of the yeast plasmids, the mutant HEWL cDNAs were inserted into the *Sal*I site of PYG-100 as described by Kato et al. (*16*). The expression vectors were introduced into *Saccharomyces cerevisiae* AH22 (MAT a, *leu2, His4, Cir*$^{+}$) according to the lithium acetate procedure (*17*). Leu^{+} transformants were screened by subculturing in the modified Burkholder minimum medium plates supplemented with histidine (20 mg/mL) at 30 °C. After cultivation, well-growing colonies were then replica-cultivated in the yeast medium on a small scale (5 mL), and the overexpression subclones with the highest levels of lysozyme activity were screened and propagated from single colonies. The overexpression colonies were directly subcultured on a large scale in the yeast minimum medium at 30 °C for 5 days.

Purification of Mutant HEWLs. The growth medium of the host cells (4 L) was centrifuged at 6000*g* for 15 min to remove the cells at 4 °C. The supernatant was directly applied to a CM-Toyopearl 650M column (1.5 × 5 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5). The column was washed with the same buffer until the washing solution was free from proteins. The adsorbed lysozyme was eluted with 0.5 M NaCl in 50 mM Tris-HCl buffer (pH 7.5). The fraction containing the protein was applied to a Sephacryl S-300 HR column (1.5 × 100 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5) and eluted with the same buffer. The protein content in each fraction (3 mL/tube) was detected by measuring the absorbance at 280 nm.

Carbohydrate Composition. Carbohydrate content of the glycosylated lysozymes was estimated using the phenol−sulfuric acid reaction employing mannose as a standard. The carbohydrate composition of glycosylated lysozymes consisted of ∼300 mol of mannose residues (*14*).

Enzymatic Assay. Lysozyme activity was measured by a lysis and glycolysis assay using *M. lysodeikticus* cells (*14*).

Preparation of Acid-Precipitated Soy Protein (APP). APP was prepared according to the method of Iwabuchi et al. (*18*). A sample of defatted meal (100 g) was extracted once with 2 L of 0.03 M Tris-HCl buffer (pH 8) containing 10 mM 2-mercaptoethanol (2-ME) at 20 °C. After centrifugation (8000*g*), the supernatant was acidified to pH 4.8 with 2 N HCl and then recentrifuged. The precipitates were dissolved with water at 4 °C, and the pH was adjusted to 8. After centrifugation (8000*g*), the clear supernatant was dialyzed against distilled water for 24 h at 4 °C and then freeze-dried.

Preparation of Soybean P34 Protein. Soybean P34 protein was prepared as described by Ogawa et al. (*2*) using crude 7S globulin fraction prepared from defatted meal of soybean protein (APP). The crude 7S globulin fraction was treated with ammonium sulfate (40%), and the precipitate was collected by centrifugation at 10000*g* for 20 min and dissolved in a minimum volume of 35 mM potassium phosphate buffer (pH 7.6) containing 0.4 M NaCl (buffer A). The solution was dialyzed against the same buffer for 20 h at 4 °C. The dialysate was put on a Sepharose 6B column previously equilibrated with buffer A, and the proteins were eluted with the same buffer. All fractions were examined by SDS-PAGE and immunoblot methods. The fractions containing the protein (P34) were pooled and then put on a ConA Sepharose 4B column previously equilibrated with buffer A to remove some 7S globulin components (*β*-conglycinin). After the column was washed with the same buffer, the allergen adsorbed on the column was eluted with the same buffer containing 20 mM α-methylmannose. The fractions containing the allergen were pooled, and the protein was recovered by precipitation with ammonium sulfate (40%). The protein was then dissolved in a minimum volume of 0.1 M sodium phosphate buffer (pH 7.6), and the solution was dialyzed against the same buffer for 20 h at 4 °C. The solution was treated with the same volume of 4% SDS solution containing 10% 2-mercaptoethanol and heated at 98 °C for 10 min. The SDS-treated solution was put on a Sephacryl S-200 column previously equilibrated with 0.1 M sodium phosphate buffer (pH 7.6) containing 1% SDS and 10 mM 2-mercaptoethanol and chromatographed with the same buffer. The fractions containing the allergen were combined and dialyzed against distilled water for 20 h at 4 °C and then lyophilized.

Galactomannan Conjugation. Lysozyme− or soybean P34 protein−galactomannan powder mixtures in the weight ratio of 1:4 were dissolved in water at 10% (w/v) and freeze-dried. Powdered protein−galactomannan mixtures were dry-heated at 60 °C under 65% relative humidity (RH) in a desiccator containing saturated KBr solution in the bottom for 2 weeks.

Purification of Protein—Polysaccharide Conjugates. To remove native proteins, the protein−polysaccharide conjugates were purified by ion-exchange chromatography using CM-Toypearl 650 column equilibrated with 50 mM Tris-HCl buffer (pH 7.5). Elution was done with a linear gradient of NaCl (0−0.5 M). Target peak for each conjugate was collected and dialyzed against distilled water and then lyophilized.

Biotinylation of the Proteins. Protein−polysaccharide conjugates and native proteins were biotinylated according to the manufacturer protocol (Amersham International, England). The protein was diluted to 1.0 mg/mL in a bicarbonate buffer (pH 8.6) to a suitable volume of 2.5 mL, and then 40 μL of biotinylation reagent for each milligram of protein was added. The mixture was incubated at room temperature for 1.0 h with constant agitation. To purify the biotinylated proteins, a Sephadex G25 column was used as described in the protocol. The purified protein was eluted in 5 mL of phosphate-buffered saline (PBS, pH 7.5), and the fractions were collected.

Changes in Free Amino Groups. Changes in free amino groups of 0.1% protein solutions of lysozyme− or soybean P34−galactomannan conjugates were determined by spectrophotometric assay (A_{340}) using *O*-phthaldialdehyde (OPD) (*19*).

SDS—Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was carried out using the method of Laemmli (*20*) with a 15% acrylamide separating gel and a 3% acrylamide stacking gel containing 0.1% SDS. A 0.2% sample (20 μL) was prepared in a Tris−glycine buffer at pH 8.8 containing 1% SDS. Electrophoresis was done at a current of 10 mA for 5 h in electrophoretic Tris−glycine buffer (pH 8.8) containing 0.1% SDS. After electrophoresis, the gel sheets were stained for proteins and carbohydrate with 0.2% Coomassie brilliant blue R250 and 0.5% periodate−fuchsin solution (*21*), respectively. Protein stain was destained with 10% acetic acid containing 20% methanol.

Production of Antibody against the Proteins and Protein—Polysaccharide Conjugates in Mice. Nine mice (C3H/He, 6−8 weeks old) were each injected intraperitoneally with lysozyme or P34 protein (10 μg/mL) before and after polysaccharide attachment dissolved in PBS and adsorbed onto 2 μg of alum (proteins−alum; 0.5 mL/mouse) according to the method of Aramaki et al. (*22*). One week later each mouse was injected with the same antigen (booster injection). As controls, three mice were injected intraperitoneally with a mixture of alum and PBS. The collected antisera (heart bleeding) were stored at −20 °C before use.

Determination of IgG Production Levels. To determine IgG levels, an ELISA was carried out according to the Huang and Chu method (*23*) with a slight modification. For the serum, lysozyme or soybean P34 protein before and after polysaccharide attachment was used separately as a solid-phase test

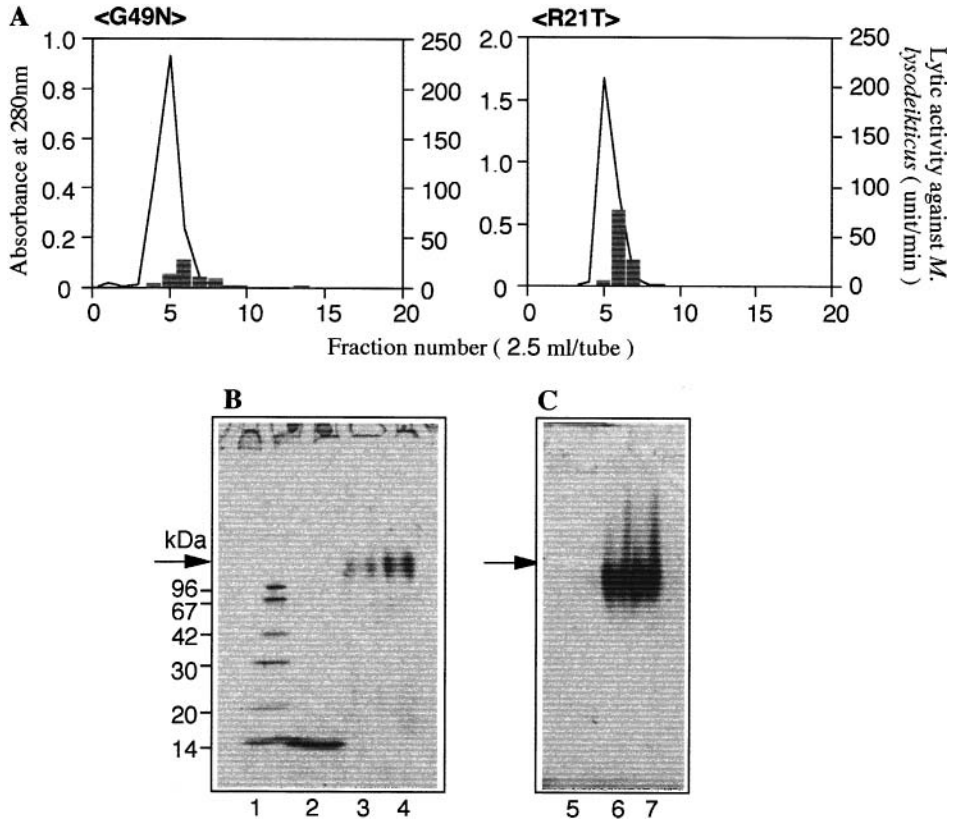

Figure 1. (A) Isolation of glycosylated lysozyme secreted from *S. cerevisiae* carrying G49N or R21T lysozyme cDNA with CM-Toyopearl columun. (B, C) SDS-PAGE pattern of G49N and R21T mutant lysozyme [(B) protein staining; (C) carbohydrate staining]: (lane 1) molecular weight makers; (lanes 2 and 5) native lysozyme; (lanes 3 and 6) G49N; (lanes 4 and 7) R21T. Arrows indicate the boundary between the stacking (upper) and separating (lower) gels.

antigen. One hundred microliters of each antigen (100 μg/mL) in 50 mM sodium carbonate buffer (pH 9.5) was added to each well of a 96-well ELISA microtiter plate (Nunc plate 2-69620; Nunc, Roskilde, Denmark). The plate was kept at 4 °C overnight. After the solution had been removed, the wells were washed four times (0.35 mL/well) with PBS−Tween buffer [0.01 M phosphate saline buffer (pH 7.5) with 0.5% Tween 20]. This was followed by incubation with 0.15 mL of 1.0% BSA in 0.01 M PBS at 37 °C for 1 h. The plate was washed four times with PBS−Tween to remove the excess BSA. To each well was added 0.1 mL of various dilutions of the sera; the plate was gently mixed and incubated at 37 °C for 2 h. After the plate was washed four times with PBS−Tween, 0.1 mL of goat anti-rabbit IgG−HRP at 1:4000 dilution in 0.01 M PBS was added to each well, and the mixtures were incubated at 37 °C for 1.5 h. The plate was washed with PBS−Tween buffer, and 0.1 mL of freshly prepared OPD substrate solution [10 mg of OPD plus 13 μL of 30% hydrogen peroxide in 25 mL of 0.05 M of citrate−phosphate buffer (pH 5.0)] was added. Twenty minutes after incubation at room temperature in the dark, the reaction was terminated by adding 0.1 mL of 4 N H_2SO_4. The absorbance at 490 nm was determined using an automatic microplate reader (Bio-Rad Laboratories, model 450, Hercules, CA).

Determination of IgE Production Levels. To determine IgE levels, an ELISA was carried out using the biotin−avidin method (Amersham International, England). Fifty microliters of anti-IgE antibody (10 μg/mL) in 50 mM sodium carbonate buffer (pH 9.5) was added to each well of a 96-well ELISA microtiter plate (Nunc plate 2-69620). The plate was kept at 4 °C overnight. After the solution had been removed, the wells were washed four times (0.35 mL/well) with PBS−Tween buffer [0.01 M phosphate saline buffer (pH 7.5) with 0.5% Tween 20]. This was followed by incubation with 0.15 mL of blocking buffer supplied by the manufacturer in 0.01 M PBS at 37 °C for 1 h. The plate was washed four times with PBS−Tween to remove the buffer. To each well was added 50 μL of various dilutions of the serum; the plates were gently mixed and incubated at 37 °C for 2 h. After the plates were washed four times with PBS−Tween, 50 μL of biotinylated proteins (1.0 μg/mL) in PBS−Tween was added to each well. After the plates were washed, 50 μL of streptavidine−horseradish peroxidase (2−5 μg/mL) in 0.01 M PBS−Tween was added to each well, and the mixtures were incubated at 37 °C for 1 h. The plate was washed with PBS−Tween buffer, and 50 μL of freshly prepared OPD substrate solution [40 mg of OPD plus 40 μL of 30% hydrogen peroxide in 25 mL of 0.05 M of citrate−phosphate buffer (pH 5.0)] was added. Twenty minutes after incubation at room temperature in the dark, the reaction was terminated by adding 0.1 mL of 4 N H_2SO_4. The absorbance at 490 nm was determined using an automatic microplate reader (Bio-Rad Laboratories, model 450).

RESULTS AND DISCUSSION

Purification of Polymannosyl Lysozymes Secreted in the Yeast Expression System. Two mutant polymannosyl lysozymes, the molecular surface amino acid sequences of which were replaced to create the potential N-linked glycosylation site (Asn-X-Ser/Thr) (*G49N*, in which *Gly-49* was substituted by *Asn*, and *R21T*, in which *Arg-21* was substituted by *Thr*), were expressed in yeast (*S. cerevisiae* AH 22). The mutant polymannosyl lysozymes secreted in the yeast medium were collected and purified by two steps of cation-exchange chromatography. Figure 1 shows the elution profiles of mutant polymannosyl lysozyme (G49N and R21T) using CM-Toyopearl column (panel A) and the SDS-PAGE pattern of the purified proteins (panel B). The hyperglycosylated lysozymes were separated from the normally glycosylated (oligomannosyl) and non-glycosyl lysozyme by using CM-Toyopearl column. The peak obtained for each mutant was detected by SDS-

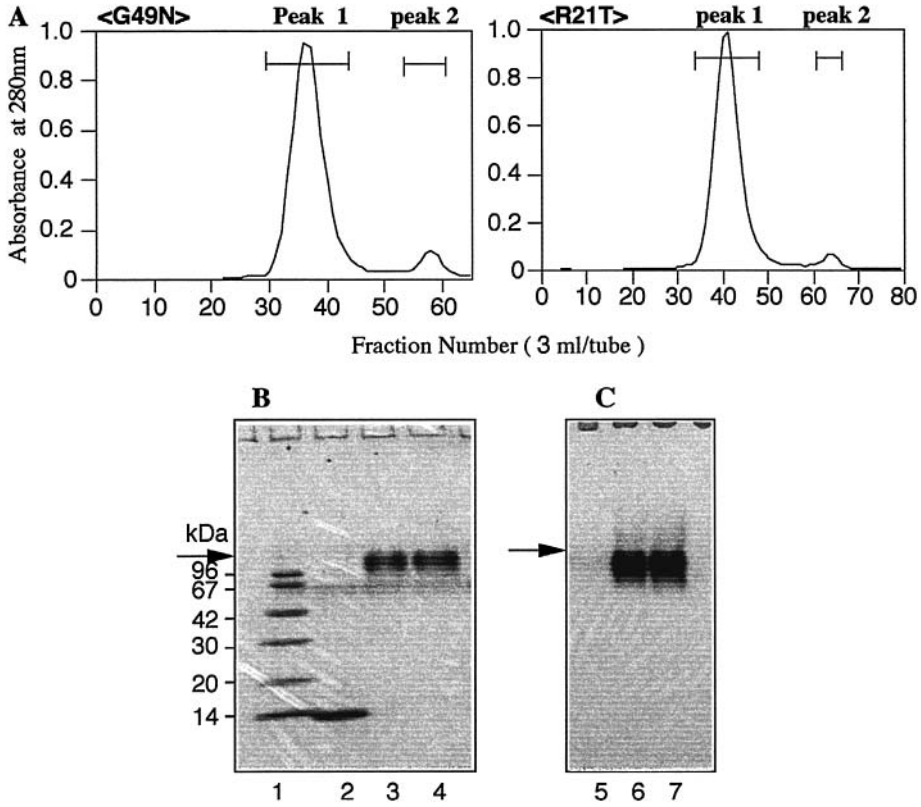

Figure 2. (A) Isolation of glycosylated lysozyme secreted from *S. cerevisiae* carrying G49N or R21T lysozyme cDNA with Sephacryl S-300 HR column. (B, C) SDS-PAGE pattern of G49N and R21T mutant lysozyme [(B) protein staining; (C) carbohydrate staining]: (lane 1) molecular weight makers; (lanes 2 and 5) native lysozyme; (lanes 3 and 6) G49N (peak 1); (lanes 4 and 7) R21T (peak 1).

PAGE pattern (Figure 1B). SDS-PAGE analysis showed that the mutant polymannosyl lysozymes (G49N and R21T) had higher molecular weight bands compared to the native lysozyme. On the other hand, the SDS-PAGE pattern stained with carbohydrate-specific reagent showed a strongly stained broad band near the boundary between the stacking and separating gels for both mutants (Figure 1C). The result suggests that the mutants are glycosylated in the novel N-link form with a large polysaccharide chain. As shown in Figure 1A, the lysozyme activity of the glycosylated lysozymes was greatly decreased for G49N and R21T, indicating that the steric hindrance with a large polysaccharide attached occurs around the substrate binding site when the insoluble cell wall of *M. lysodeikticus* was used as a substrate for the hyperglycosylated lysozyme. Further purification of the glycosylated lysozymes was done to remove nonglycosylated and oligomannosyl lysozymes. Figure 2 shows the elution profiles of mutant lysozyme (G49N and R21T) using Sephacryl S-300 HR column (panel A) and the SDS-PAGE pattern of the purified proteins (panel B). After purification with a Sephacryl S-300 HR column, one main peak was obtained for each mutant (Figure 2A). The results obtained by SDS-PAGE analysis of the main peaks are similar to those obtained after the proteins were purified by a CM-Toyopearl column. The carbohydrate composition of the mutant lysozymes was determined using the phenol−sulfuric acid reaction employing mannose as a standard. The lengths of polymannose chains of mutant lysozymes were found to be 310 and 340 residues/mol of lysozyme for G49N and R21T, respectively. The results indicate that the mutant lysozymes may be N-linked polymannosylation (hyperglycosylation) which occurs in *S. cerevisiae*.

Purification of Protein—Galactomannan Conjugates Prepared by the Maillard Reaction. The protein−galactmannan conjugates are also purified by gel filteration and ion-exchange chromatography to remove the native proteins (data not shown). The purified protein−galactomannan conjugates showed high molecular mass bands in the tops of the separating and stacking gels compared to the native proteins in both protein (lane 3, Figure 3A) and carbohydrate (lane 6, Figure 3B) staining gels. The results indicated that lysozyme and soybean P34 protein were covalently attached to galactomannan through the Maillard reaction between the ϵ-amino groups in the proteins and the reducing-end carbonyl groups in galactomannan during dry heating at 60 °C and 65% relative humidity. Similar results were reported for an ovalbumin−dextran mixture (*10*), a Pronase-treated gluten−dextran mixture (*11*), and a lysozyme−dextran conjugate (*12*). To further elucidate the attachment of galactmannan to lysozyme and soybean P34 protein, the free amino group of lysozyme and soybean P34 protein after polysaccharide attachment was estimated as a percentage relative to the absorbance (A_{340}) of the native lysozyme or soybean P34 protein and was found to be ~30% less than that of native lysozyme and soybean P34 (data not shown). Results revealed that the protein molecules of each lysozyme and soybean P34 protein were covalently conjugated to galactmannan.

Effect of Polysaccharide Attachment to Proteins on IgG and IgE Production. The effect of polysaccharide attachment using genetic and Maillard-type modifications on lysozyme-specific IgG production is shown in Figure 4. The antigen-specific IgG level of polysaccharide-attached lysozyme was determined by ELISA as a relative binding capacity to that of the

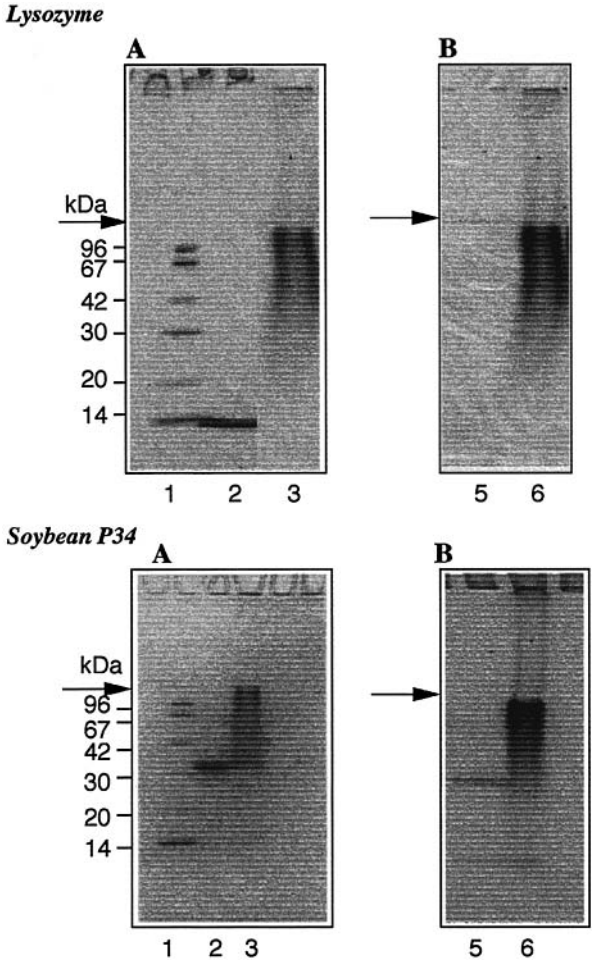

Figure 3. SDS-PAGE patterns of lysozyme (top) and soybean P34 protein (bottom) and galactomannan conjugate [(A) protein staining; (B) carbohydrate staining]: (lane 1) molecular marker; (lanes 2 and 5) native lysozyme (top) or P34 protein (bottom); (lanes 3 and 6) galactomannan conjugates of lysozyme or P34 protein.

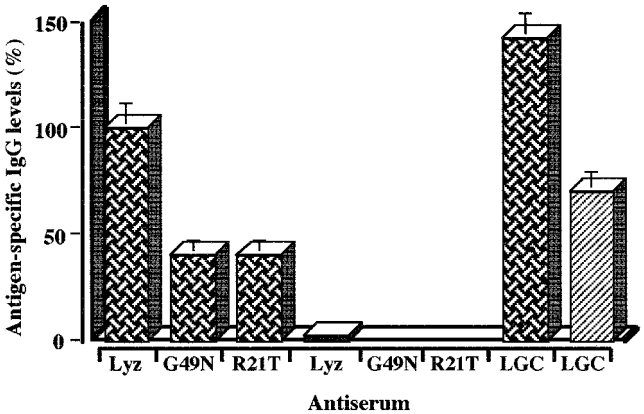

Figure 4. Effect of polysaccharide attachment to lysozyme using the yeast expression system and Maillard-type reaction on the protein-specific IgG production. Each well of a 96-well ELISA was coated with lysozyme (Lyz), G49N or R21T, or LGC as solid phase antigen. Values are means of nine replicates (±SD).

native lysozyme. When lysozyme was used as a solid phase antigen and cross-reacted with the sera obtained from mice immunized with G49N, R21T, or galactomannan conjugate, the production levels of IgG were found to be 40.00, 40.00, and 142.5%, respectively (Figure 4). When G49N and R21T were used separately

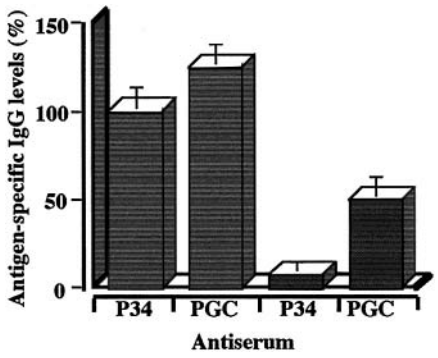

Figure 5. Effect of polysaccharide attachment using the Maillard-type reaction on the protein-specific IgG production of soybean P34 protein (P34). Each well of a 96-well ELISA was coated with P34 protein or PGC. Values are means of nine replicates (±SD).

as a solid phase antigen and cross-reacted with the sera obtained from mice immunized with lysozyme, G49N, or R21T, the IgG production level of lysozyme was greatly reduced (2.5%), whereas G49N and R21T did not produce IgG (Figure 4). The attachment of polysaccharide to lysozyme using the Maillard reaction also significantly decreased the IgG level to <70% when lysozyme−galactomannan conjugate (LGC) was used as a solid phase antigen and cross-reacted with the sera obtained from mice immunized with LGC (Figure 4). The attachment of polysaccharide to lysozyme using the yeast expression system was found to be very effective in reducing the IgG level compared to LGC. On the other hand, the attachment of polysaccharide to soybean P34 using the Maillard-type reaction significantly increased the IgG level to 125% (Figure 5) when soybean P34 protein was used as a solid phase antigen and cross-reacted with the sera obtained from mice immunized with P34 protein−galactmannan conjugate (PGC). When PGC was used as a solid phase antigen and cross-reacted with sera obtained from mice immunized with P34 or PGC, the IgG level was greatly reduced and was found to be 7.5 and 50% for P34 and PGC, respectively (Figure 5). For both lysozyme and soybean P34 protein, when the native proteins were used as solid phase antigens and cross-reacted with sera obtained from mice immunized with the Maillard-type protein conjugates, the IgG production level was greatly elevated and exceeded that of the native proteins. However, when the conjugated proteins were used as solid phase antigens and cross-reacted with the sera obtained from mice immunized with the native proteins or the conjugates, the IgG level was greatly reduced. On the other hand, the attachment of polysaccharides to lysozyme using the yeast expression system was found to be effective in reducing the IgG level even when native lysozyme was used as a solid phase antigen and cross-reacted with the sera obtained from mice immunized with G49N or R21T. The explanation for this difference may lie in the structural differences between the native protein and its conjugate as well as the difference between the Maillard-type and genetic attachment of polysaccharide to the proteins. Because the linked polysaccharides in the yeast expression system are large and branched polymannosyl chains, they might mask the protein epitope and result in the reduction of the allergenicity of the protein. Results indicate that the attachment of polysaccharide using the yeast expression system was very efficient in decreasing IgG levels compared to the Maillard-type polysaccharide attachment.

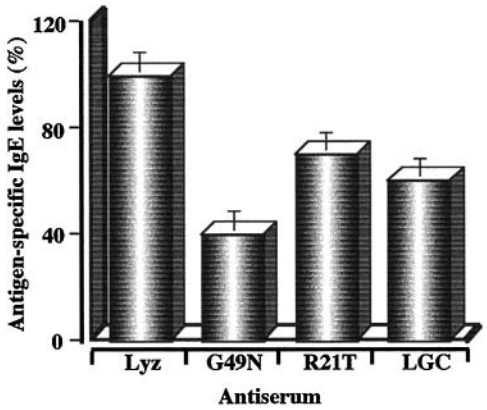

Figure 6. Levels of IgE production of lysozyme (Lyz), G49N, R21T, and LGC using biotinylated lysozyme as a solid phase antigen. Values are means of nine replicates (±SD).

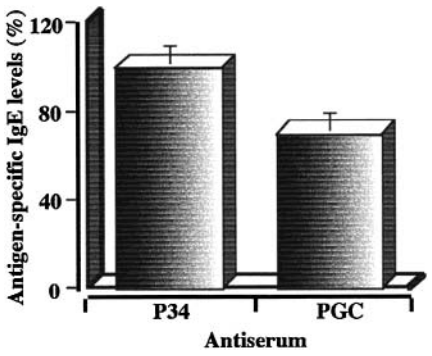

Figure 7. Levels of IgE production of soybean P34 protein (P34) and PGC using biotinylated P34 protein as a solid phase antigen. Values are means of nine replicates (±SD).

The effect of polysaccharide attachment to lysozyme on IgE production level is shown in Figure 6. When lysozyme was used as a solid phase antigen and cross-reacted with the sera obtained from mice immunized with G49N, R21T, or LGC, the IgE levels were decreased to 40, 70, and 60%, respectively (Figure 6). Results suggest that the attachment of polysaccharide at position 49 was very effective in reducing IgE levels compared to that at position 19 and galactomannan conjugate. Although the production of IgG is elevated by the conjugation with galactomannan, the production of IgE is significantly decreased. In a similar manner, the attachment of polysaccharide to soybean P34 using the Maillard-type reaction was found to reduce the IgE level of P34 to 70% when P34 was used as a solid phase antigen and cross-reacted with serum obtained from mice immunized with PGC (Figure 7). In contrast to the IgG production, the IgE production was decreased even when the native proteins were used as solid phase antigens and cross-reacted with the sera obtained from mice immunized with the protein−galactomannan conjugates. This is most likely due to the fact that galactomannan and mannose chains may activate the macrophage and induce Th1-specific immune response, which stimulates the production of IgG, and reduce Th2-specific immune response, which stimulates the production of IgE; therefore, the IgE production level significantly decreased. It has long been recognized that immunization with polysaccharide or infection with polysaccharide-encapsulated bacteria induces a murine antibody response in which the IgM and IgG isotypes predominate (*24*). Perimutter et al. (*24*) also reported that the polysaccharide-induced stimulation of IgG production occurs independently of endotoxin. Immunological properties of allergen chemically modified with synthetic copolymer of *N*-vinylpyrrolidone and maleic anhydride showed that when the protein conjugates were injected intraperitoneally into mice, they induced IgG and did not induce significant quantities of IgE (*8*). Conjugation of immunostimulatory DNA to the short ragweed allergen amb a 1 enhanced its immunogenicity and reduced its allergenicity (*9*). The attachment of polysaccharide using the yeast expression system was found to be very efficient in reducing both IgG and IgE production levels compared to the Maillard-type reaction. Because food allergy is mediated by the allergen-specific IgE antibody, both polysaccharide attachment methods to the allergenic proteins are effective in reducing the allergenicity of the proteins.

In conclusion, the attachment of polysaccharide to proteins using the yeast expression system is an ideal method to mask the epitope structure of the allergenic protein. The Maillard-type polysaccharide conjugation method is also found to be effective in reducing the allergenicity of the proteins. The present study supports the fact that the stimulation of IgG antibody responses is a reflection of protein immunogenicity, whereas protein allergenicity is associated with the induction of strong IgE responses.

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