

Reduction of Antigenicity of Cry j I, Major Allergen of Japanese Cedar Pollen, by the Attachment of Polysaccharides

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An attempt was made to mask the allergenic structure of a major allergen protein, Cry j I (CJI), in Japanese cedar pollen using the Maillard-type polysaccharide conjugation. The SDS-PAGE pattern of the CJI-galactomannan conjugate prepared by the Maillard reaction showed broad bands widely distributed from 50 kDa to more than 100 kDa, suggesting the attachment of galactomannan. The competitive enzyme-linked immunosorbent assay showed that the IgE antibody in the sera of cedar pollen-sensitive patients reacted strongly with CJI, while it did not react with the CJI-galactomannan conjugate. This result suggests that the antigenicity of CJI is greatly reduced by the conjugation with galactomannan.

Key words: Cry j I; CJI-galactomannan conjugate; antigenicity; IgG epitope; IgE epitope

Pollen from Japanese cedar (*Cryptomeria japonica*) is a potent seasonal aeroallergen in Japan and more than 10% of the population suffers from cedar pollinosis. Two major allergens in Japanese cedar pollen have been identified as Cry j I and Cry j II.^{1–3)} CJI is a basic glycoprotein with the molecular mass of 41–45 kDa. The molecular heterogeneity may originate from the presence or absence of N-linked carbohydrate chains. It has been reported that a major allergen of Japanese cedar pollen is CJI, designated according to WHO allergen nomenclature.²⁾

Although most patients depend on symptomatic treatments, it is desirable to develop a fundamental therapy without any side effects. Among many therapies, oral immunotherapy for the treatment of allergic diseases is a promising method as one of the therapies for modulating immune response, although the immunologic molecular mechanism has not been well elucidated. The principle ground of oral immunotherapy is based on the oral tolerance induced by orally administered antigens. A possible molecular mechanism for oral tolerance is the suppression of T-cell reactivity due to the constant exposure to the immunodominant T-cell deter-

minant. Therefore, provided that the epitope of IgE can be masked in the presence the T-cell determinant, the ideal tolerogen can be obtained.

We reported that a major soy allergen, P34, was masked by the attachment of galactomannan using the Maillard reaction in the dry state between the ϵ -amino group in protein and the reducing-end carbonyl group in polysaccharide.⁴⁾ In addition, we reported that the production of IgE against P34 was reduced by the intraperitoneal administration of P-34-galactomannan conjugate,⁵⁾ while the production of IgG was not suppressed. Therefore, protein-polysaccharide conjugation is a promising approach to reduce the antigenicity of allergens.

Thus, this paper describes the effects of masking the allergenic structure of a major allergen protein, CJI, in Japanese cedar pollen, using the Maillard-type polysaccharide conjugation.

Materials and Methods

Materials. Japanese cedar pollen was supplied by Shinkyo Sangyo Co. (Yamaguchi, Japan). Sugi pollinosis patient antisera from three volunteers were provided by Dr. M. Muto, Medical School of Yamaguchi University. CM-Toyopearl 650M and DEAE Toyopearl were purchased from Tosoh (Tokyo, Japan). Biotin-conjugated goat anti-human IgG was purchased from ICN Pharmaceuticals, Inc (Ohio, USA). Goat anti-human IgE polyclonal antibody was purchased from Chemicon International Inc (CA, USA). HRP-streptavidin was purchased from Vector Laboratories, Inc (CA, USA). Unless otherwise stated, all reagents used in this study were of reagent grade.

Preparation of CJI. CJI was prepared according to the method of Yasueda *et al.*¹⁾ To remove the fat in cedar pollen, 100 g of pollen were soaked and shaken hard in 250 ml of diethyl ether using a separatory funnel. Finally, diethyl ether was removed on the glass filter. Defatted pollen was extracted in 2,000 ml

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of 0.125 M ammonium bicarbonate (pH 8.0) at room temperature for 48 h. After extraction, the pollen was separated from the supernatant by centrifugation ($15,000 \times g$, 15 min). The supernatants were used as crude extracts (CE). Solid ammonium sulfate was added to CE at 4°C with stirring to ensure 80% saturation (561 g/l). After it was stirred overnight, the precipitate was collected by centrifugation ($18,500 \times g$, 40 min). The precipitate was dissolved in 200 ml of 0.05 M Tris-HCl (pH 7.8) and dialyzed against the same buffer. The dialyzed CE was centrifuged ($10,000 \times g$, 15 min) to remove the residue, and then diluted to twice by 0.05 M Tris-HCl buffer (pH 7.8). The diluted solution was put on a column of DEAE Toyopearl. The elution passed through DEAE Toyopearl was collected and dialyzed against 0.01 M acetate buffer (pH 5.0). The solution diluted five times with the above acetate buffer was put on CM-Toyopearl equilibrated with 0.01 M acetate buffer (pH 5.0). After the column was washed with the acetate buffer, the adsorbed proteins were eluted with a linear gradient with 0–0.4 M NaCl in 0.01 M acetate buffer (pH 5.0). The peak detected by absorbance at 280 nm was collected, dialyzed against distilled water, and lyophilized. The lyophilized sample was dissolved in 4 ml of distilled water and put on a Sephadex G-100 column (2.6×100 cm, Pharmacia Uppsala, Sweden) equilibrated with 0.05 M ammonium bicarbonate (pH 8.0) at room temperature. Proteins were eluted with 0.05 M ammonium bicarbonate solution at a rate of 20 ml/h. The peak detected by absorbance at 280 nm was collected, dialyzed against distilled water, and lyophilized.

CJI-Galactomannan conjugation. The attachment of galactomannan to CJI done out using the Maillard reaction in the dry state according to the method of Kato *et al.*⁶⁾ The CJI-galactomannan powder mixture in the weight ratio of 1:4 were dissolved in water at 10% (w/v) and lyophilized. CJI-galactomannan mixtures were dry-heated at 60°C under 65% relative humidity (RH) in a desiccator containing saturated KI solution in the bottom for 2 weeks. The Maillard reaction between the ϵ -amino groups in protein and the reducing-end carbonyl groups in polysaccharide is accelerated in the low water activity described above. The conjugate thus obtained was purified as described below.

Purification of CJI-galactomannan Conjugates. To remove galactomannan-free CJI, the CJI-galactomannan conjugate was purified by cation-exchange chromatography using a CM-Toyopearl 650 column. Elution was done with a linear gradient with NaCl (0–0.5 M) in 0.01 M acetate buffer (pH 5.0). The peak of CJI-galactomannan conjugate was collected and dialyzed against distilled water and then lyophilized.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE). SDS-PAGE was done using the method of Laemmli⁷⁾ with a 15% acrylamide separating gel and a 5% acrylamide stacking gel containing 0.1% SDS. A 0.2% sample (20 μ l) was prepared in 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 with 0.2% Coomassie brilliant blue R250 and destained with 10% acetic acid containing 20% methanol.

Measurement of free amino groups. Measurement of free amino groups was done as follows: A 50- μ l protein of CJI solution (0.2%) was added to 1 ml of 0.2 N sodium borate buffer, pH 9.0, and then 1 ml of TNBS reagent, consisting of equal volumes of 0.1% trinitrobenzenesulfonic acid and 0.126% sodium sulfide, was added. This mixed solution was left at 37°C for 2 h and subsequently cooled to room temperature before the absorbance was measured at 420 nm.

Biotinylation of the antibodies. Goat anti-human IgE polyclonal antibody was biotinylated according to the protocol using a Biotin Labeling Kit (Roche Diagnostics, Germany). One mg of anti-human IgE antibodies were dissolved in 1 ml of PBS and added to 7.6 μ l biotin-7-NHS solution (20 mg/ml). The mixture was incubated for 2 h at room temperature with gentle stirring. After biotinylation, non-reacted biotin-7-NHS was removed by gel filtration on a Sephadex G-25 column. The purified biotinylated antibodies were eluted in PBS (pH 7.5) to the final volume of 2.5 ml, and stored at 4°C.

IgG binding of CJI-galactomannan conjugate. The binding of IgG antibody with CJI-galactomannan conjugate was measured by competitive ELISA according to the Huang and Chu method⁸⁾ with a slight modification. Fifty μ l of CJI or CJI-galactomannan conjugate solution (0.8 μ g/ml, on the basis of protein concentration) as a competitor was mixed with 50 μ l of a 1:5 dilution of each patient sera, and it incubated at 37°C for 1 hour. A hundred μ l of CJI (40 μ g/ml) in PBS (0.01 M phosphate buffer saline, pH 7.4) was added to each well of a 96-well ELISA microtiter plate. The plate was kept at 37°C for 1 hour. After the solution had been removed, the wells were washed 4 times with PBS. After washing, the wells were incubated with 200 μ l of blocking solution (5.0% BSA in PBS) at 37°C for 1 h, and then washed 4 times with PBS to remove the excess BSA. To each well was added 0.1 ml of sera solution containing each competitor. The plate was gently mixed and incubated at 37°C for 2 h. After the plate was washed 3 times with PBS, 0.1 ml of biotin-conju-

gated goat anti-human IgG was added to each well, and then the mixtures were incubated at 37°C for 1 h. After the plate was washed with PBS, 0.1 ml of a 1:5000 dilution of HRP-streptavidin in PBS was added. The wells were incubated at 37°C for 1 h and washed with PBS. The remaining IgG was detected by adding 0.1 ml of freshly prepared OPD (*o*-phenylene diamine dihydrochloride) substrate solution [13 mg of OPD plus 4 μ l of 30% hydrogen peroxide in 12 ml of 0.05 M citrate-phosphate buffer (pH 5.0)]. After incubation at room temperature for 5 min, the reaction was stopped by adding 100 μ l of 4 N H₂SO₄. The absorbance at 492 nm was measured using an automatic microplate reader (Bio-Rad Laboratories, model 450, Hercules, CA).

IgE binding of CJI-galactomannan conjugate. The binding of patient IgE antibody with CJI-galactomannan conjugate was measured by competitive ELISA. Competitive ELISA was done using the biotin-avidin method. Fifty μ l of CJI or CJI-galactomannan conjugate solution (0.8 μ g/ml, on the basis of protein concentration) was mixed with 50 μ l of each patient sera. A 100 μ l of CJI (40 μ g/ml) in PBS (0.01 M phosphate buffer saline, pH 7.4) was added to each well of a 96-well ELISA microtiter plate. The plate was kept at 37°C for 1 hour. After the solution had been removed, the wells were washed with PBS. Subsequently washing, the wells were incubated with 200 μ l of blocking solution (5.0% BSA in PBS) at 37°C for 1 h, and then were washed 3 times with PBS to remove the excess BSA. To each well was added 0.1 ml of the patient sera solution containing each competitor. The plate was gently mixed and incubated at 37°C for 2 h. After the plate was washed 3 times with PBS, 0.1 ml of biotinylated anti-human IgE antibody (0.3 μ g/ml) was added to each well, and then the mixtures were incubated at 37°C for 1 h. After the plate was washed with PBS, a 0.1 ml of HRP-streptavidin (5 μ g/ml) in PBS was added. The wells were washed with PBS, and after incubation at room temperature for 5 min. The remaining IgE was detected in the same way as the standard ELISA described above.

Results

Isolation and Purification of CJI from Japanese Cedar Pollen

Since CJI is a basic protein, CM-Toyopearl was used to purify the protein after the contaminant protein were removed by DEAE-toyopearl. The elution pattern of CJI on a column of CM-toyopearl is shown in Fig. 1A. The peak (fraction No. 35–58) was further purified on a column of Sephadex G-100 (Fig. 1B). The peak (fraction No. 100–130) was collected and analyzed by SDS-PAGE (Fig. 1C). The purified CJI showed bands with the molecular masses

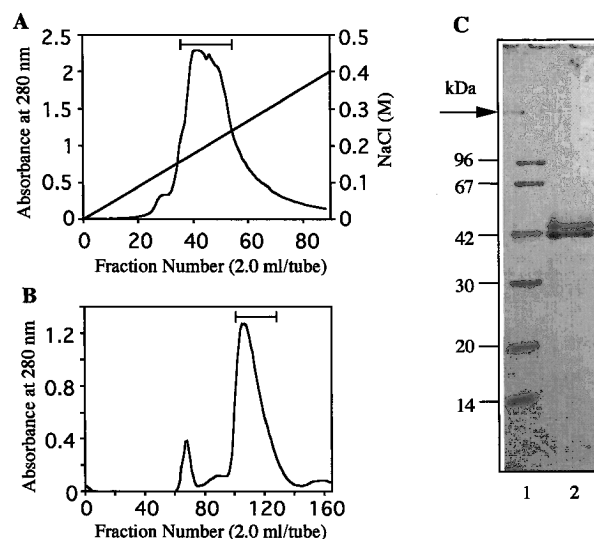


Fig. 1. Isolation and Purification of CJI from Japanese Cedar Pollen.

(A) Elution pattern on CM-Toyopearl column. (B) Elution pattern on Sephadex G-100 column. (C) SDS-PAGE pattern of purified CJI. Lane 1, Molecular marker; Lane 2, Purified CJI.

of about 41 and 45 kDa. These bands were strongly cross-reacted with CJI antibody by immunoblotting (Data not shown). The N-terminal amino acid sequence of both bands was Asp-Asn-Pro-Ile, consistent with the sequence of CJI. These results suggest that the upper and lower bands are CJI and that the difference of MW is attributed to the degree of N-linked glycosylation.

Purification of CJI-Galactomannan Conjugates Prepared by Maillard Reaction

The CJI-galactomannan conjugate was purified by cation-exchange chromatography to remove the free CJI and galactomannan. The SDS-PAGE pattern of purified CJI-galactomannan conjugate showed high molecular mass broad bands widely distributed from 50 kDa to more than 100 kDa. The broad band is attributed to the widely distributed molecular size and the binding number of galactomannan (Fig. 2). The attachment of galactomannan to CJI increased with dry-heating time and reached a maximum after 2 weeks. This binding behavior is almost the same as that of ovalbumin and lysozyme, rigid globular proteins. These results indicated that CJI was 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. Similar results were reported for an ovalbumin-dextran conjugate,⁶ a lysozyme-dextran conjugate,⁹ and a soybean allergen P34-galactomannan conjugate.^{4,5} To confirm the attachment of galactomannan to CJI, the free amino groups of CJI after galactomannan attachment were measured. The result suggests that

3–4 lysyl residues in CJI were masked with galactomannan on the average, while 13–14 lysyl residues remained free.

IgG binding of CJI-galactomannan conjugate

The effects of galactomannan attachment to CJI on the human-IgG antibody recognition were detected by competitive ELISA. Competitive ELISA was done using three human patient sera. The IgG antibody recognition of CJI-galactomannan conjugate was compared with that of native CJI as a contrast of antigenicity. As shown in Fig. 3, the cross-reactivity of CJI-galactomannan conjugate decreased in the range of 15–35%. This result suggested that a part of IgG epitopes of CJI might be covered by galactomannan, but most of the IgG epitopes were not covered. It seems likely that the CJI has various polyclonal epitope structures.

IgE binding of CJI-galactomannan conjugate

The effect of galactomannan attachment to CJI on the IgE antibody recognition was detected by competitive ELISA. Competitive ELISA was done using three human patient sera. For all three sera, IgE antibody recognition was greatly decreased in the range of 95–98%. That is, the cross-reactivity of IgE with CJI-galactomannan conjugate greatly reduced (Fig. 4). These results suggested that IgE epitopes of CJI were masked by galactomannan, and

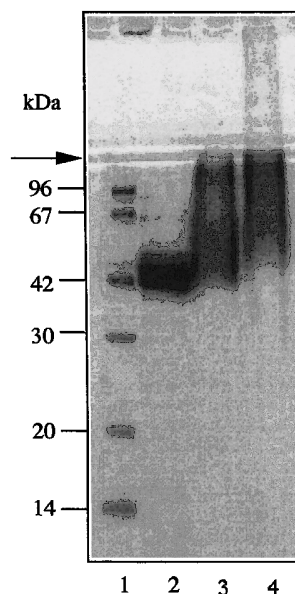


Fig. 2. SDS-PAGE Patterns of CJI and CJI-Galactomannan Conjugate(GM).

Lane 1, molecular marker; Lane 2, native CJI; Lane 3, CJI-GM (weight ratio of 1:2); Lane 4, CJI-GM (weight ratio of 1:4). All CJI-GM were dry-heated at 60°C, in 65% RH in a desiccator containing saturated KI solution in the bottom, for 2 weeks.

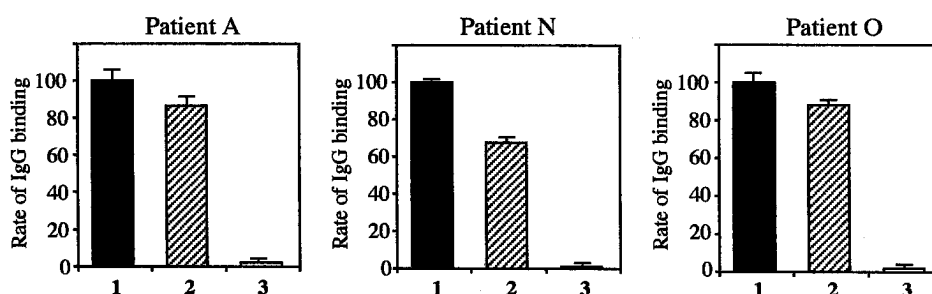


Fig. 3. Human-IgG Binding to CJI and CJI-Galactomannan Conjugate.

The IgG binding was indicated as a percentage of that for CJI. Lane 1, CJI; Lane 2, CJI-GM; Lane 3, control. Control means no competitor in existence. Values are indicated as means of six replicas ($\pm$ SD).

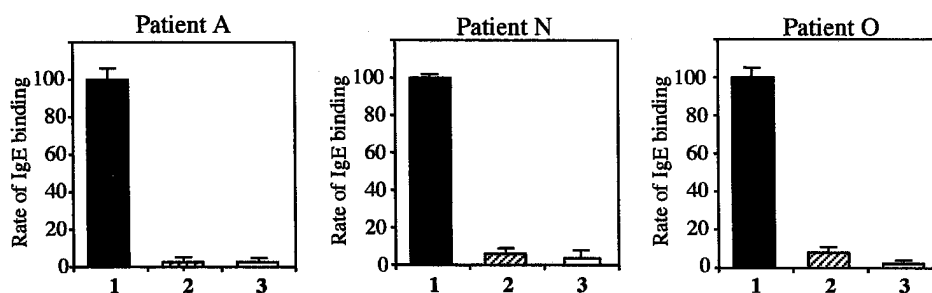


Fig. 4. Human-IgE Binding to CJI and CJI-Galactomannan Conjugate.

The IgE binding was indicated as a percentage of that for CJI. Lane 1, CJI; Lane 2, CJI-GM; Lane 3, control. Control means no competitor in existence. Values are indicated as means of six replicas ($\pm$ SD).

that the antigenicity of CJI was reduced by conjugation with galactomannan.

Discussion

The binding of galactomannan to CJI is specific to the surface ϵ -amino groups and a limited number of galactomannans are attached. Calculating from the analysis of ϵ -amino groups, only three to four ϵ -amino groups were reacted with galactomannan after 2 weeks. This specific attachment of polysaccharide to protein through the Maillard reaction is supported from our previous report on the lysozyme-polysaccharide conjugate prepared by Maillard reaction in dry-heating.¹⁰⁾ Only the lysyl residue at position 97 of lysozyme specifically reacted with polysaccharide after 2 week's dry-heating.

The attachment of galactomannan with CJI was very effective to reduce the binding with IgE antibody in patient sera, although it was not as effective in reducing the binding with IgG antibody. Dry-heating of CJI in the absence of galactomannan was not effective for either antibody binding. Therefore, the attachment of galactomannan is important to reduce the binding with antibodies. Sakaguchi *et al.* reported that IgE epitope-specific mAb binding was significantly decreased after heat treatment.¹¹⁾ This observation suggests that the epitope regions of IgE for CJI are specific to the tertiary structure. Tamura *et al.* reported that CJI-immunized mouse IgE antibodies reacted strongly to CJI peptide 145–152 (VHPQDGDA) as a sequential epitope.¹²⁾ This epitope region may be sterically masked by three to four galactomannans attached to lysine residues in lysine-rich peptides 78–83, 275–279, and 321–326. It is probable that longer galactomannan is more effective to reduce the binding. These results suggest that the IgE epitope of CJI is masked three-dimensionally by steric hindrance of the attached galactomannan. IgG epitopes were also masked, but it not so effectively. This result suggests that most of IgG epitopes may be specific to an amino acid sequence or far from conjugated galactomannan.

We previously reported that the attachment of polysaccharide to the soy protein allergen P34 induced the IgG production and reduced the IgE production by the mice injected intraperitoneally with the allergen.⁵⁾ Immunological properties of allergens chemically modified with synthetic copolymer of N-vinylpyrrolidone and maleic anhydride seems to occur in similar change. When the protein-copolymer conjugates were injected intraperitoneally into mice, they induced IgG and did not induce significant quantities of IgE.¹³⁾ From these data, it is probable that CJI-galactomannan conjugate also may induce IgG, and suppress the production of IgE. Further study to elucidate the production of antibodies is now progress.

In clinical application for oral immunotherapy to induce the immuno-tolerance, the injection or administration of allergen have a possibility to cause anaphylactic shock. A mild tolerogen is desirable to induce the immuno-tolerance. CJI-galactomannan conjugate can be useful as an immuno-tolerogen, because it reduced binding with IgE antibody. Furthermore, CJI-galactomannan conjugate is constructed by a naturally occurring reaction without any synthetic chemicals. Therefore, CJI-galactomannan conjugate may be a safe material for the design of medicine for pollinosis.

In conclusion, the attachment of polysaccharide to protein is a useful technique for reduction of antigenicity. It is new approach to protein engineering for immunology.

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