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Antigenicity of the Oligosaccharide Moiety of the Japanese Cedar (*Cryptomeria japonica*) Pollen Allergen, *Cry j* I

Key Words

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Abstract

Cry j I, a major allergenic glycoprotein of *Cryptomeria japonica* (Japanese cedar, Sugi), is the most common pollen allergen in Japan. Carbohydrate analysis and lectin staining indicated that *Cry j* I possesses the fucose xylose-containing *N*-linked oligosaccharide which previously has been found in some plant glycoproteins. Rabbit polyclonal anti-*Cry j* I IgG antibodies were found to be highly cross-reactive with two other plant glycoproteins which have the same type of oligosaccharides, and the cross-reactivities were completely abolished on chemical deglycosylation of the glycoproteins. Enzyme-linked immunosorbent assay inhibition showed that the majority (up to 70%) of the anti-*Cry j* I rabbit IgG antibodies recognized the oligosaccharide moiety of *Cry j* I. The carbohydrate epitopes were found to be only partially involved in the binding of specific IgE antibodies from a pool of human patient sera.

Cry j I is a nonprofilin allergen of the Japanese cedar (*Cryptomeria japonica*) and was first isolated and characterized by Yasueda et al. [1]. The IgE-binding sites on *Cry j* I were considered to be conformational, and efforts at mapping of antigenic epitopes using monoclonal antibodies suggested the presence of several peptide epitopes and a glycopeptide, the glycosylation of which might be required for the IgE binding of the peptide [2–4]. The importance of the carbohydrate moiety to the allergenicity/cross-reactivity has been proposed for many allergens [5–8, see ref. 13]. However, whether the carbohydrate moieties are directly involved in the B cell and/or T cell tropic epitopes remains unclear for pollen allergens. In this study, we focused on the oligosaccharide chains of *Cry j* I and studied their antigenicities as to IgG and IgE and those of two other plant glycoproteins/glycopeptides with the oligosaccharide structure already determined.

Cry j I was purified from Japanese cedar pollen collected in Nikko, Japan, according to the method of Yasueda et al. [1], with a slight modification. Subfractions of *Cry j* I were separated by polyacrylamide gel electrophoresis in the presence of 0.1% sodium dodecyl sulfate [9], using 9.5% acrylamide separation gels, and then blotted onto nitrocellulose membranes.

The lectin reactivities of *Cry j* I were studied using horseradish peroxidase (HRP) labeled lectins (Honen, Japan) according to the method of Kitagaki-Ogawa et al. [10]. *Solanum tuberosum* agglutinin (EY Laboratories, USA), a glycoprotein which contains 44% (w/w) carbohydrate, 89% of which is arabinose [11], was used as a control for all HRP lectins. The preservation of the activity of all the HRP lectins was confirmed by the reactivity toward fetuin, porcine submaxillary mucin, or ovalbumin. As shown in figure 1, the doublet bands of *Cry j* I were equally stained with concana-

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valin A, *Lens culinaris* agglutinin, and *Ulex europaeus* agglutinin I, which are known to recognize D-mannose or L-fucose on N-linked oligosaccharides, suggesting that *Cryj* I possesses N-linked oligosaccharides with a trimannosyl core substituted with L-fucose at the innermost N-acetylglucosamine, as was revealed for *Clerodendron trichotomum* agglutinin (CTA) [10]. The positive staining with *Gri-fonia simplicifolia* agglutinin II and *Phaseolus vulgaris* agglutinin E suggested the presence of branching N-acetylglucosamine at the trimannosyl core. *S. tuberosum* agglutinin was not stained at all by these lectins, suggesting that the specific carbohydrate structure was essential for the binding with HRP lectins, and the staining was not by non-specific binding. Though *S. tuberosum* agglutinin is a N-acetylglucosamine-specific lectin; it was no longer active after blotting onto a membrane under reducing condition, as shown by negative staining with *P. vulgaris* agglutinin and *U. europaeus* agglutinin I (fig. 1D, F), both of which contained N-acetylglucosamine as a component sugar. *Cryj* I was not stained with *Ricinus communis* agglutinin, soybean agglutinin, or *Dolichos biflorus* agglutinin (data not shown).

The carbohydrate composition of *Cryj* I was analyzed by employing the method of Kitagaki-Ogawa et al. [10]. *Cryj* I contained hexosamine:mannose:fucose:galactose:xylose at 4.5:3.0:2.8:1.6:0.8; the value of D-mannose being taken as 3.0 for the molar ratio calculated. This composition together with the lectin reactivities indicated that *Cryj* I possesses N-linked oligosaccharides with a trimannosyl chitobiose core substituted with fucose and xylose which has been reported for various plant glycoproteins. The presence of galactose and excess hexosamine and fucose may suggest a fucosylated N-acetylglucosamine branch.

The antigenicity of the oligosaccharide moiety of *Cryj* I as to IgG and IgE was studied in comparison with those of two plant glycoproteins: a galactose/N-acetylglucosamine-specific lectin (CTA) and a mannose/glucose-specific *Sophora japonica* bark agglutinin II (B-SJA-II). The two lectins were purified as previously described [10, 12] and deglycosylated with trifluoromethanesulfonic acid [13]. Rabbit normal or anti-*Cryj* I sera and human allergic sera (radioallergosorbent test class 4) were kindly supplied by Torii Yakuhin Co., (Japan). The rabbit sera were further purified to IgG using an Ampure PA kit (Amersham, UK).

Immunoblotting of *Cryj* I and the two plant glycoproteins was performed, using rabbit anti-*Cryj* I IgG. *Cryj* I was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis in parallel with CTA and B-SJA-II, and then the glycoproteins were blotted onto PVDF membranes. After blocking with 2% bovine serum albumin in 0.01 M

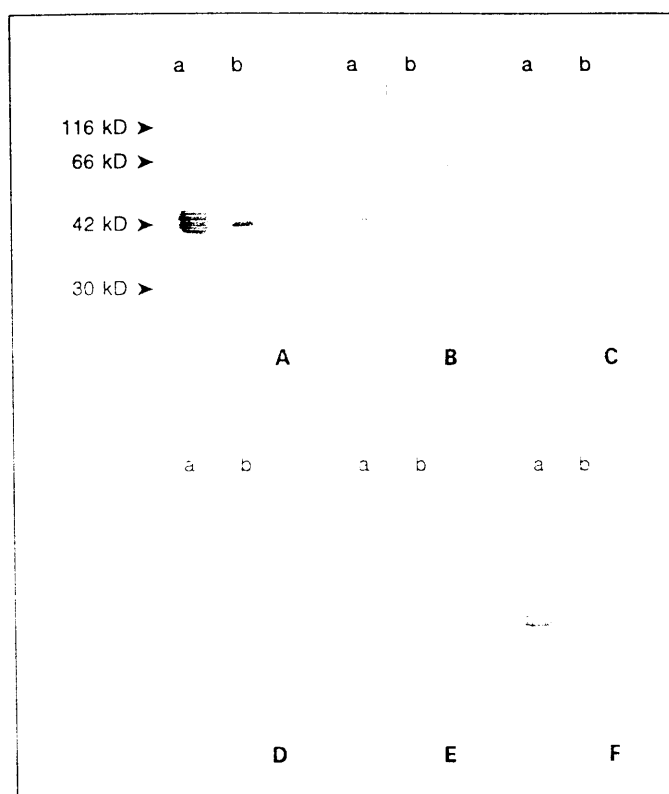
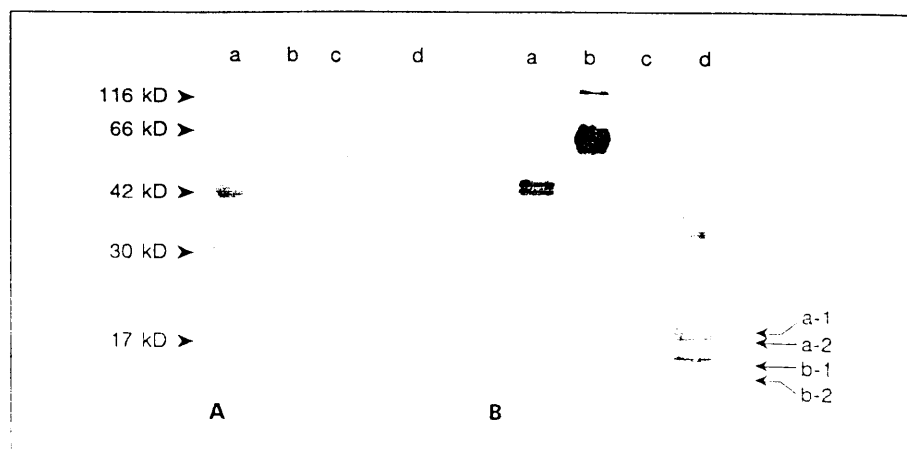


Fig. 1. Lectin reactivity of *Cryj* I. **A** Coomassie brilliant blue staining; lane a: *Cryj* I (4 µg/lane), lane b: STA (4 µg/lane). **B** HRP-concanavalin A staining; lane a: *Cryj* I (0.6 µg/lane), lane b: STA (0.6 µg/lane) in all panels **B-F**. **C** HRP-*L. culinaris* agglutinin staining. **D** HRP-*U. europaeus* agglutinin I staining. **E** HRP-*G. simplicifolia* agglutinin II. **F** HRP-*P. vulgaris* agglutinin E staining. The position of molecular weight markers are indicated on the left.

Tris-buffered saline (pH 7.5), the membranes were incubated with rabbit normal or anti-*Cryj* I IgG prediluted 1:500 for 1 h at room temperature. After further washing and blocking with 1% bovine serum albumin, the bound IgG was detected with HRP-labeled goat antirabbit IgG (1:1,000, 1 µg/ml; KPL, USA). The color was developed with 4-chloro-1-naphthol/H₂O₂. All three subunits of CTA were glycosylated and the structure of the predominant oligosaccharide of CTA was previously determined to be Xylβ1→2 (Manα1→6) (Manα1→3) Manβ1→4GlcNAcβ1→4 (Fucα1→3)GlcNAc [10]. The carbohydrate contents of the B-SJA-II subunits are in the order of a-1>a-2>b-1, but b-2 was not glycosylated, while all four subunits of B-SJA-II exhibit carbohydrate-binding activity [12]. Eighty percent of the total oligosaccharides of B-SJA-II had the same structure as those of CTA, and the rest of the oligosaccharides had the same structure trimmed [unpubl. data]. As shown in fig-

Fig. 2. Immunostaining of CTA and B-SJA-II with rabbit anti-*Cry j* I IgG before and after deglycosylation. **A** Coomassie brilliant blue staining; lane a: *Cry j* I (5 µg/lane), lane b: CTA (8 µg/lane), lane c: deglycosylated CTA (6 µg/lane), lane d: B-SJA-II (5 µg/lane). **B** Immunostaining with rabbit anti-*Cry j* I IgG; lanes a-d: same as in **A**, but the amount loaded in each lane was 1/40 of that in **A**. The positions of molecular weight markers are indicated on the left and B-SJA-II subunits on the right.



ure 2b, only glycosylated forms of the lectin subunits were stained with rabbit anti-*Cry j* I IgG, and the intensity of the staining was dependent on the carbohydrate contents, but not on the carbohydrate-binding activities of the lectins. These results suggested that oligosaccharides of CTA and B-SJA-II contribute to the cross-reactivity with rabbit anti-*Cry j* I IgG.

An enzyme-linked immunosorbent assay was performed to study the cross-reactivity of *Cry j* I-specific human IgE to these glycoproteins. Microtiter plates (Immulon 200, Greiner) were coated with *Cry j* I or CTA by incubation at 4°C overnight, followed by washing and blocking with 2% bovine serum albumin in tris-buffered saline. 100 µl of rabbit anti-*Cry j* I IgG (1:500) or 50 µl of human allergic sera (1:4) was added to the wells, followed by incubation for 1 h at room temperature. After further washing and blocking with 1% bovine serum albumin, the bound antibodies were detected by incubation with HRP-labeled goat antirabbit IgG (1:1,000) or alkaline-phosphatase-labeled horse anti-human IgE (1:1,000, EAST kit; Shionogi Seiyaku, Japan), respectively. The plates were washed again and developed with 150 µl of *o*-phenylenediamine/H₂O₂, and then 50 µl of 4 *N* sulfuric acid was added to stop the reaction, followed by reading at 490 nm for the rabbit IgG system or according to the manufacturer's instructions for the human IgE system.

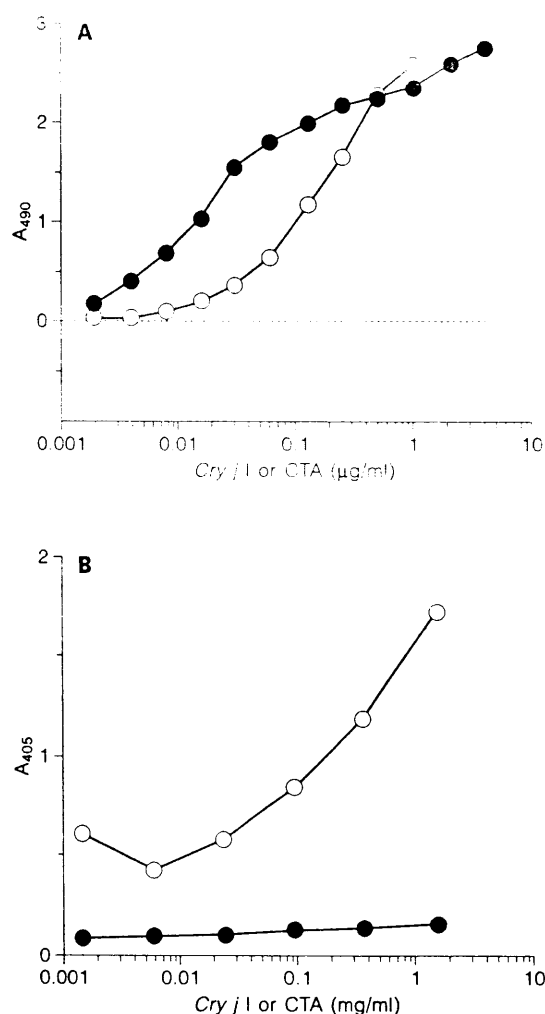


Fig. 3. Cross-reactivities of rabbit anti-*Cry j* I IgG antibodies (**A**) and human allergic sera (**B**) to CTA on enzyme-linked immunosorbent assay. CTA (●) or *Cry j* I (○) was coated onto a well and then subjected to the assay as described in text. The dashed line in **A** indicates the reactivities with normal rabbit IgG.

As shown in figure 3, rabbit anti-*Cryj* I IgG recognized CTA as well as *Cryj* I; in contrast, human IgE bound very little CTA as compared with *Cryj* I (fig. 3b).

Enzyme-linked immunosorbent assay inhibition was performed to evaluate the content of oligosaccharide-specific antibodies in rabbit anti-*Cryj* I IgG. The inhibitors used were intact CTA and CTA glycopeptides/peptides produced by extensive digestion of intact or trifluoromethanesulfonic acid treated CTA with actinase E (Kaken Seiyaku, Japan), followed by heat denaturation at 100°C for 5 min. The extensive actinase E digestion of CTA destroyed its lectin activity and most of the peptide epitopes, if any, cross-reactive to the anti-*Cryj* I antibody. The diluted rabbit IgG antibodies were preincubated for 2 h at room temperature with four-fold serial dilutions of the inhibitors. After preincubation, the IgG-inhibitor mixtures were transferred to plates precoated with *Cryj* I and then subjected to enzyme-linked immunosorbent assay as described above. As shown in figure 4, intact CTA and CTA glycopeptides equally inhibited the binding of rabbit anti-*Cryj* I IgG to *Cryj* I, while the CTA peptides obtained from deglycosylated CTA did not show any inhibitory activity, showing that the peptide portions do not contribute to the cross-reactivity. The results suggested that up to 70% of rabbit anti-*Cryj* I IgG antibodies recognize carbohydrate epitopes which accounts for the cross-reactivities toward CTA and B-SJA-II. In contrast, CTA exhibited low cross-reactivities toward human anti-*Cryj* I IgE (fig. 3). The results suggest that the oligosaccharides of *Cryj* I contribute little to the direct reactivity with IgE, and the oligosaccharides may only participate in the modification of conformational epitopes in peptide portions.

These results show that the majority of rabbit anti-*Cryj* I IgG antibodies recognize oligosaccharides typical of plant glycoproteins. Since this type of oligosaccharides has been reported to be highly allergenic [14] and immunogenic [14–17] to several glycoproteins, antibodies against these gly-

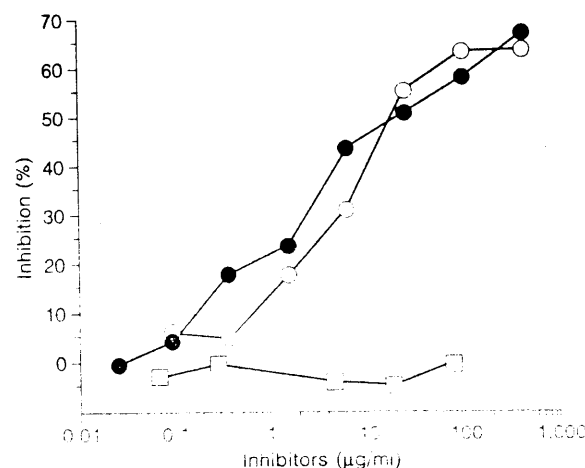


Fig. 4. Enzyme-linked immunosorbent assay inhibition. The binding of rabbit anti-*Cryj* I IgG antibodies to *Cryj* I was inhibited by intact CTA glycoproteins (□), glycopeptides (●), or peptides (○).

coproteins probably cross-react with *Cryj* I. Since IgG is considered to be a potential blocking antibody as to desensitization, various plant glycoproteins might be effectively used as desensitizers as to pollen allergies, substituting for crude pollen extracts. The determination of oligosaccharide structures and identification of IgG epitopes of *Cryj* I are now in progress in our laboratory.

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References

- 1 Yasueda H, Yui Y, Shimizu T, Shida T: Isolation and partial characterization of the major allergen from Japanese cedar pollen. *J Allergy Clin Immunol* 1983;71:77-86.
- 2 Kawashima T, Taniai M, Taniguchi Y, Usui M, Ando S, Kurimoto M, Matuhasi T: Antigenic analyses of Sugi basic protein by monoclonal antibodies. I. Distribution and characterization of B-cell-tropic epitopes of *Cry j* I molecules. *Int Arch Allergy Immunol* 1992;98:110-117.
- 3 Kawashima T, Taniai M, Taniguchi Y, Usui M, Ando S, Kurimoto M, Matuhasi T: Antigenic analyses of Sugi basic protein by monoclonal antibodies. II. Detection of immunoreactive fragments in enzyme-cleaved *Cry j* I. *Int Arch Allergy Immunol* 1992;98:118-126.
- 4 Taniai M, Kawano T, Takakura R, Yamamoto S, Usui M, Ando S, Kurimoto M, Panzani R, Matuhasi T: Epitopes on *Cry j* I and *Cry j* II for the human IgE antibodies cross-reactive between *Cyperus sempervirens* and *Cryptomeria japonica* pollen. *Mol Immunol* 1993;30:183-189.
- 5 Oka S, Shigeta S, Ono K, Jyo T: An epitope residing in carbohydrate chains of a sea squirt antigen termed Gi-ep. *J Allergy Clin Immunol* 1987;80:57-63.
- 6 Haavik S, Paulsen BS, Wold JK: Glycoprotein allergens in pollen of timothy. V. Significance of the carbohydrate moiety for the immunological activity of a basic glycoprotein allergen. *Int Arch Allergy Appl Immunol* 1987;83:231-237.
- 7 Mucci N, Liberatore P, Federico R, Forlani F, Di Felice G, Afferni C, Tinghino R, De Cesare F, Pini C: Role of carbohydrate moieties in cross-reactivity between different components of *Parietaria judaica* pollen extract. *Allergy* 1992;47:424-430.
- 8 van Ree R, Aalberse RC: Pollen-vegetable food crossreactivity: Serological and clinical relevance of crossreactive IgE. *J Clin Immunoassay* 1993;16:124-130.
- 9 Lämmler UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature* 1970;227:680-685.
- 10 Kitagaki-Ogawa H, Matsumoto I, Seno N, Takahashi N, Endo S, Arata Y: Characterization of the carbohydrate moiety of *Clerodendron trichotomum* lectins. Its structure and reactivity toward plant lectins. *Eur J Biochem* 1986;151:779-785.
- 11 Matsumoto I, Jimbo A, Mizuno Y, Seno N, Jeanloz RW: Purification and characterization of potato lectin. *J Biol Chem* 1983;258:2886-2891.
- 12 Ueno M, Ogawa H, Matsumoto I, Seno N: A novel mannose-specific and sugar specifically aggregatable lectin from the bark of the Japanese pagoda tree (*Sophora japonica*). *J Biol Chem* 1991;266:3146-3153.
- 13 Edge ASB, Faltynek CR, Hof L, Reichert LE, Weber P: Deglycosylation of glycoproteins by trifluoromethanesulfonic acid. *Anal Biochem* 1981;118:131-137.
- 14 Prenner C, Mach L, Glossl J, Marz L: The antigenicity of the carbohydrate moiety of an insect glycoprotein, honey-bee (*Apis mellifera*) venom phospholipase A₂. *Biochem J* 1992;284:377-380.
- 15 Kurosaka A, Yano A, Itoh N, Kuroda Y, Nakagawa T, Kawasaki T: The structure of a neural specific carbohydrate epitope of horseradish peroxidase recognized by anti-horseradish peroxidase antiserum. *J Biol Chem* 1991;266:4168-4172.
- 16 Ramirez-Soto D, Poretz R: The (1-3)-linked α -L-fucosyl group of the N-glycans of the *Wistaria floribunda* lectins is recognized by a rabbit antiserum. *Carbohydr Res* 1991;213:27-36.
- 17 Lauriere M, Lauriere C, Chrispeels MJ, Johnson KD, Sturm A: Characterization of a xylose-specific antiserum that reacts with the complex asparagine-linked glycans of extracellular and vascular glycoproteins. *Plant Physiol* 1989;90:1182-1188.