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Structures and contribution to the antigenicity of oligosaccharides of Japanese cedar (*Cryptomeria japonica*) pollen allergen *Cry j* I: relationship between the structures and antigenic epitopes of plant *N*-linked complex-type glycans

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The oligosaccharide structures of *Cry j* I, a major allergenic glycoprotein of *Cryptomeria japonica* (Japanese cedar, sugi), were analysed by 400 MHz ^{1}H-NMR and two-dimensional sugar mapping analyses. The four major fractions comprised a series of biantennary complex type N-linked oligosaccharides that share a fucose/xylose-containing core and glucosamine branches including a novel structure with a nongalactosylated fucosylglucosamine branch.

Rabbit polyclonal anti-*Cry j* I IgG antibodies cross-reacted with three different plant glycoproteins having the same or shorter N-linked oligosaccharides as *Cry j* I. ELISA and ELISA inhibition studies with intact glycoproteins, glycopeptides and peptides indicated that both anti-*Cry j* I IgGs and anti-*Sophora japonica* bark lectin II (B-SJA-II) IgGs included oligosaccharide-specific antibodies with different specificities, and that the epitopic structures against anti-*Cry j* I IgGs include a branch containing α1-6 linked fucose and a core containing fucose/xylose, while those against anti-B-SJA-II IgGs include nonreducing terminal mannose residues. The cross-reactivities of human allergic sera to miraculin and *Clerodendron Trichotomum* lectin (CTA) were low, and inhibition studies suggested that the oligosaccharides on *Cry j* I contribute little or only conformationally to the reactivity of specific IgE antibodies.

Keywords: *Cry j* I, pollen allergen, carbohydrate epitope, *N*-linked oligosaccharide.

Abbreviations: *Cry j* I, a major allergenic glycoprotein of *Cryptomeria japonica*; B-SJA-II, *Sophora japonica* bark lectin II; CTA, *Clerodendron trichotomum* lectin; TFMS, trifluoromethanesulfonic acid; HRP, horseradish peroxidase.

Introduction

The importance of the carbohydrate moiety for allergenicity/cross-reactivity has been proposed for many allergens [1–6]. *N*-Linked oligosaccharides have been reported to be included in the IgE-determinant on honey bee venom phospholipase A2 [1, 2] as well as the *O*-glycans on a sea squirt allergen [3]. Moreover, *N*-glycans on a number of plant glycoproteins are immunogenic, leading to highly cross-reactive antisera [4–9]. To understand the functions of carbohydrates in various allergic or immune reactions, the relationship between the carbohydrate structures and antigenicity needs to be clarified. Preliminarily, we focused on the oligosaccharide chains of *Cry j* I [10], a major allergenic glycoprotein of *C. japonica* which is the

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most common pollen allergen in Japan, and is one of the two major allergens for Japanese cedar pollinosis [11, 12]. *Cry j* I is a glycoprotein with a 6% (weight/weight) carbohydrate content [11]. *Cry j* II is considered to be a protein with an N-terminal amino acid sequence distinct from that of *Cry j* I [12]. Previous characterization by means of a lectin binding assay and composition analyses indicated that *N*-linked oligosaccharides with xylose and fucose were present on *Cry j* I [10]. Immunoassaying suggested that the oligosaccharides contributed to the epitopes of anti-*Cry j* I rabbit IgGs. Since IgG is considered to be a potential blocking antibody as to desensitization, the characterization of the IgG epitope is as biologically important as that of an allergic epitope.

In this study, we first determined the oligosaccharide structures of *Cry j* I and found a discrepancy with those reported recently by others [13]. The contribution of the oligosaccharides of *Cry j* I to antigenicity with respect to IgG and IgE was elucidated in comparison with other plant glycoproteins having different types of oligosaccharides. The structural motifs responsible for the antigenicity of *N*-linked complex oligosaccharides on plant glycoproteins are discussed.

Materials and methods

Materials

Cry j I was purified from Japanese cedar pollen collected at Nikko, Japan, according to Yasueda *et al.* [11], with a slight modification. Partial purification of the crude pollen extract was performed by batch-wise adsorption of DEAE-cellulose and CM-cellulose column chromatography. Further purification by gel filtration chromatography was performed on a Sephacryl S-200HR column. *Cry j* II was purified according to Sakaguchi *et al.* [12]. Miraculin, *Sophora japonica* bark lectin II (B-SJA-II), and *Clerodendron trichotomum* lectin (CTA) were purified as previously described [14–16], respectively, and deglycosylated with trifluoromethanesulfonic acid (TFMS) [15]. Deglycosylation was confirmed by the negative lectin staining after blotting onto a membrane [17], and the decrease in molecular mass observed on SDS-PAGE. *N*-Oligosaccharide glycoamidase A was purchased from Seikagaku Kogyo Co., Japan. Pepsin and exoglycosidases were from Sigma Chemical Co., USA. Standard pyridylamino-glucose oligomers were purchased from Takara Shuzo Co., Japan. Reference pyridylamino-oligosaccharide X010.1 (Scheme 1) was prepared from CTA [16]. A TSK-GEL DEAE-5PW column (0.75 × 7.5 cm) and an Amide-80 column (0.46 × 25 cm) were purchased from Tosoh Corp., Japan, and a Shim-pack CLC-ODS column (0.6 × 15 cm) from Shimadzu Co., Japan. Rabbit anti-*Cry j* I sera and human allergic sera (RAST class 4: totally 66 batches from 21 patients) were supplied by Torii Yakuhin Co., Japan. Rabbit anti-B-SJA-II and anti-TFMS-treated B-SJA-II antisera were prepared by a standard immunization protocol [18]. The rabbit sera were further purified into IgGs with an Ampure PA Kit (Amersham, UK). Horseradish peroxidase (HRP)-labelled goat anti-rabbit IgG was purchased from Kirkegaard & Perry Lab, Inc., USA, and an EAST kit containing alkaline phosphatase-labelled horse anti-human IgE was from Shionogi Seiyaku Co., Ltd., Japan.

Preparation of pyridylamino-oligosaccharides

Oligosaccharides were prepared from *Cry j* I according to the previously described method [16] with modifications. *Cry j* I (8 mg) was extensively digested with pepsin and oligosaccharides were released from the resultant glycopeptides by exhaustive digestion with *N*-oligosaccharide glycoamidase A (2 mU) at 37 °C overnight. The mixture was boiled and digested with actinase E (120 μg) at 37 °C for 3 days, boiled for 10 min, and then digested with N-oligosaccharide glycoamidase A (1 mU). The reaction mixture was evaporated to dryness. The reducing ends of the oligosaccharides were pyridylaminated and purified as described by Kondo *et al.* [19].

Isolation and identification of pyridylamino-oligosaccharides

PA-oligosaccharides were separated by size-fractionation HPLC on a TSK-GEL Amide-80 column and reversed-phase HPLC on a Shimpack CLC-ODS column according to the method described by Tomiya *et al.* [20]. PA-oligosaccharides were detected by fluorescence, using excitation and emission wave-lengths of 320 and 400 nm, respectively. The structures of PA-oligosaccharides were analysed by the two-dimensional sugar-mapping method [20, 21]. The elution positions (expressed as glucose oligomers) of PA-oligosaccharides were compared with the data for the respective reference oligosaccharides [22]. The structures of the reference pyridylamino-oligosaccharides are shown in Scheme 1. HPLC on a DEAE-5PW column was performed under the conditions described previously [23]. α-L-Fucosidase digestion was performed under the extensive digestion condition of a 5 M excess of the enzyme, while other exoglycosidase digestions were performed as described previously [20], and acid defucosylation according to Ishihara *et al.* [24].

^{1}H-NMR measurements

^{1}H-NMR spectra of deuterium exchanged PA-oligosaccharides were acquired at 400 MHz with a GX-400 spectrometer (Japan Electron Optical Laboratory, Tokyo) as described previously [23]. One-dimensional spectra were obtained using a pulse sequence containing a composite refocusing pulse [25] to reduce the water hump, and the transmitter frequency was located at the

same position as the water resonance. Lorenzian-Gaussian resolution enhancement was applied to Fourier transformation and chemical shifts were related to acetone as an internal standard, with δ of 2.216 or 2.213 ppm, respectively, for 30 °C or 65 °C.

Carbohydrate analyses of pyridylamino-oligosaccharides

The carbohydrate composition of each pyridylamino-oligosaccharide was analysed as described by Suzuki *et al.* [26].

ELISA and ELISA inhibition

ELISA was performed according to the method described previously [10]. For the rabbit IgG system, the results were corrected by subtraction of the value for normal rabbit IgG as a control. The inhibitors used were intact miraculin, CTA and *Cry j* I, and their glycopeptides and peptides. The glycopeptides or peptides were produced by extensive digestion of intact or TFMS-treated glycoproteins with actinase E, followed by heat denaturation at 100 °C for 5 min. ELISA inhibition was performed by the same procedure as described previously [10].

Immunoblotting

Immunoblotting of glycoproteins was performed according to the method previously described [10].

Results

Previous lectin binding studies on blotted *Cry j* I showed that the two bands of *Cry j* I (45 kDa and 50 kDa) obtained on SDS-PAGE were equally stained with all the HRP-labelled lectins tested that were specific for N-linked oligosaccharides [10]. Lectin staining of *Cry j* II was always negative (unpublished results), suggesting that it did not contain *N*-linked oligosaccharides. Glycoforms in the subfractions of *Cry j* I were suggested to be very similar to each other, therefore the oligosaccharides were released from the whole *Cry j* I without its separation into subfractions in this study.

Isolation of oligosaccharides

As Fig. 1A shows, the pyridylamino-oligosaccharides derived from *Cry j* I were separated into four major fractions, **a–d**, on size-fractionation HPLC (Amide-80 column), and each fraction gave a single peak, respectively, on reversed-phase HPLC (CLC-ODS column; data not shown), the elution positions being plotted in Fig. 1B. Each fraction of pyridylamino-oligosaccharides, **a–d**, was eluted at the position of neutral pyridylamino-oligosaccharides on a DEAE-5PW column (data not shown), suggesting the absence of negative charges such as those of phosphate and sulfate groups, or uronic acid. The molar

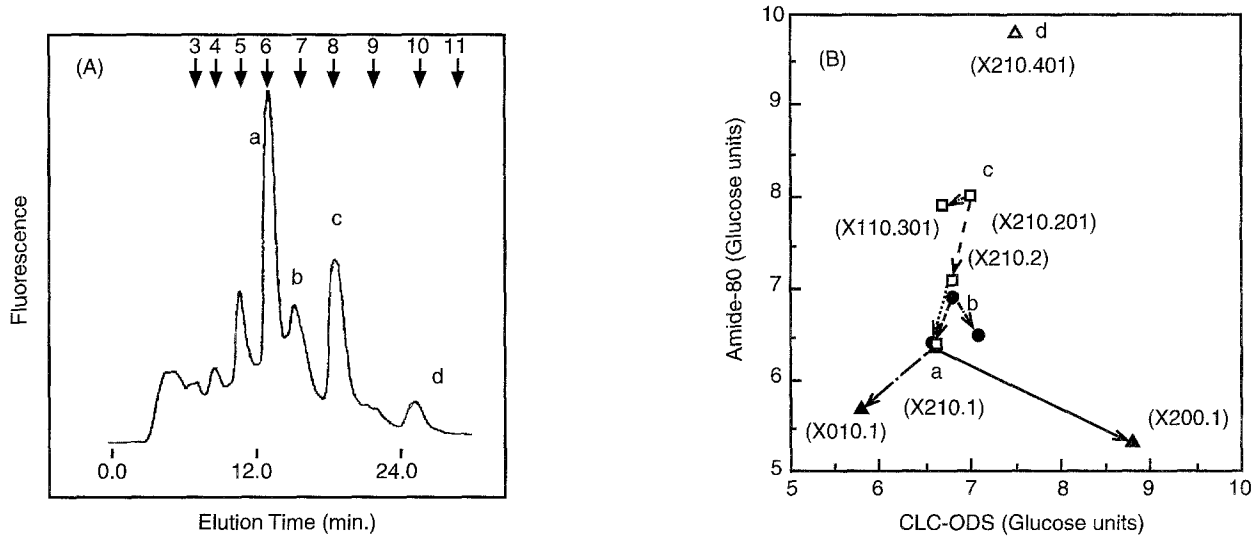


Figure 1. Separation and analysis of pyridylamino-oligosaccharides of *Cry j* I by two-dimensional HPLC. Pyridylamino-oligosaccharides were fractionated by size-fractionation HPLC on an Amide-80 column (A). HPLC was performed as described in the text. The arrows indicate the elution positions of glucose oligomers expressed as the numbers of glucose units. Fractions **a–d** eluted from the Amide-80 column were separately collected and further analyzed by the two-dimensional mapping method, as described in the text.

Two-dimensional sugar map of intact and exoglycosidase-digested pyridylamino-oligosaccharides of *Cry j* I (B). The scales for the two-dimensional sugar map are the numbers of glucose units obtained on CLC-ODS and Amide-80 columns for the *X* and *Y* axes, respectively. Arrows indicate the directions of the changes in the positions on digestion with glycosidases: α-L-fucosidase (---->), β-D-galactosidase (···>), β-*N*-acetylglucosaminidase (-·-·->), and defucosylation with trifluoroacetic acid (——>). The abbreviation of the reference pyridylamino-asialooligosaccharide corresponding to the elution position of each fraction from *Cry j* I is shown in the figure, and its structure is listed in Scheme 1. The abbreviations for the fractions correspond to those in (A).

ratio of fractions **a**, **b**, **c** and **d** was 50:17:28:5 (Table 2). The recoveries of pyridylamino-oligosaccharides were estimated from the sum of the fluorescence of the purified fractions in comparison with that of a known amount of pyridylamino-GlcNAc. The recovery of the oligosaccharides after size-fractionation HPLC was estimated to be 70% of the carbohydrate content of *Cry j* I (6% weight/weight [11]).

Characterization of the oligosaccharides by two-dimensional sugar mapping analysis

The elution positions expressed as glucose oligomers were plotted on a two-dimensional sugar map, as shown in Fig. 1B, and compared with those of the respective reference oligosaccharides. The reference oligosaccharides (except X010.1) were unavailable and the assignments were done by comparison with two-dimensional sugar map data reported previously [22]. The fractions obtained on sequential exoglycosidase digestion or acid defucosylation were analysed again, the results being plotted in Fig. 1B. The elution positions of oligosaccharides **a**, **c** and **d** coincided, within experimental error, with those of reference oligosaccharides, X210.1 (laccase compound c), X210.201 (laccase compound e), and X210.401 (laccase compound f) [27], respectively. The structures of the reference oligosaccharides are shown in Scheme 1. The elution position of oligosaccharide **b** did not correspond to that of any known oligosaccharides.

Oligosaccharide **a**. The decrease in size on β-*N*-acetylglucosaminidase digestion of oligosaccharide **a** was determined to be 0.7–0.8 glucose units on size-fractionation HPLC. It has been shown that each *N*-acetylglucosamine residue behaves like 0.4 glucose units, and each galactose residue like 1.0 glucose units on an Amide-80 column [20]. The results suggested that oligosaccharide **a** has two non-reducing terminal GlcNAc residues. When the digestion product was co-injected with the major oligosaccharide, X010.1, from CTA, a single peak was observed on both CLC-ODS and Amide-80 columns. Furthermore, the acid treatment of oligosaccharide **a** resulted in a compound with the same elution position as X200.1. The chromatographic behaviour of oligosaccharide **a** agreed with the structure initially indicated by its elution position, i.e. X210.1.

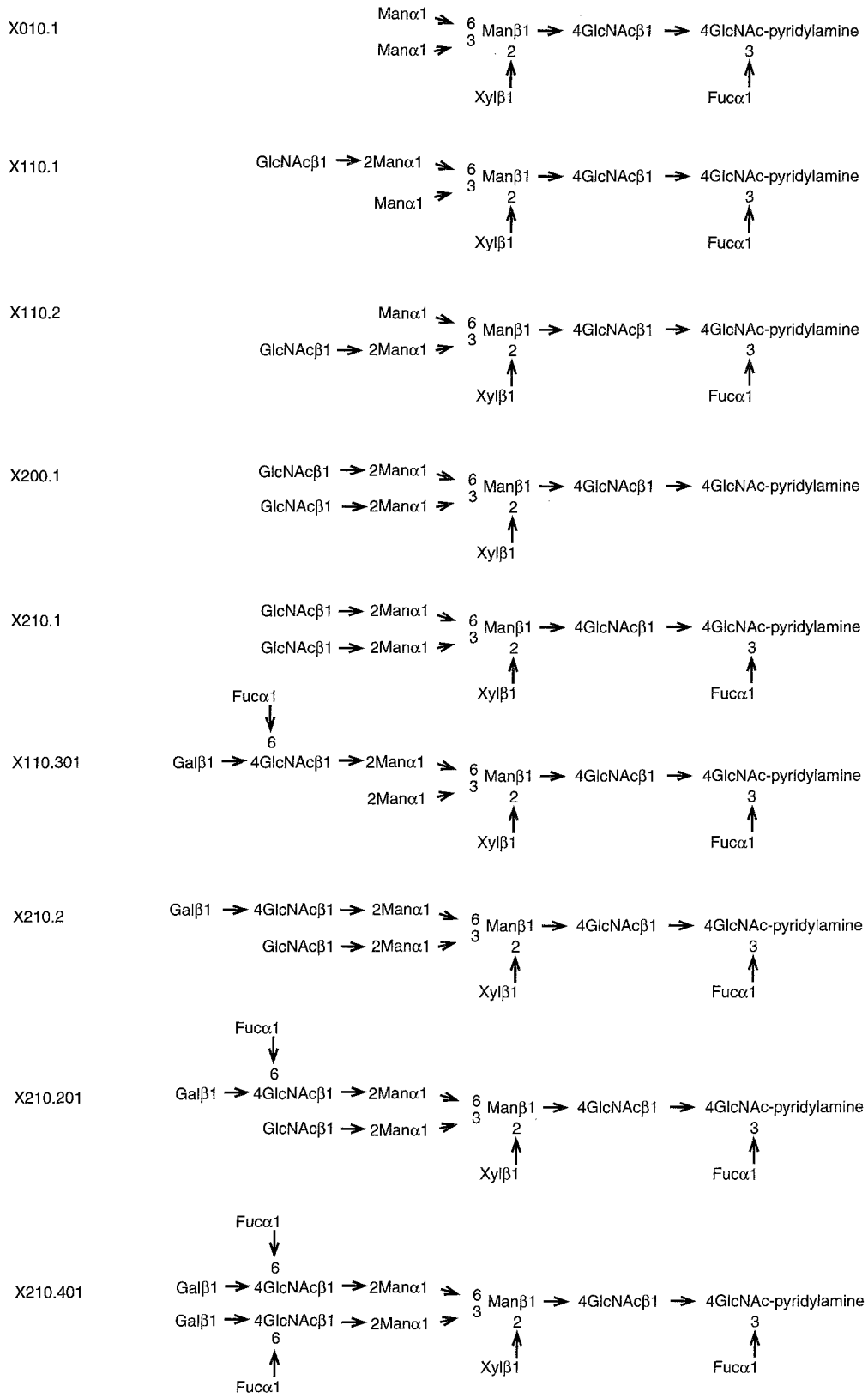
Oligosaccharide **b**. The elution position of oligiosaccharide **b** shifted to that of oligosaccharide **a** on extensive α-L-fucosidase digestion, and when the digestion product was co-injected with oligosaccharide **a** onto the two HPLC columns, they co-eluted as a single peak on both HPLC columns. When the fucose residue is linked by an α1-6 linkage to a GlcNAc-5 or 5′ residue, it is liberated only on extensive digestion and, furthermore, it protects the attached β-GlcNAc residue from β-*N*-acetylglucosaminidase digestion [28]. The position of the fucose residue on oligosaccharide **b** can be predicted by comparing the elution position of the β–acetylglucosaminidase-digested product with those calculated using the elution parameter for L-fucose and the known elution positions of X110.1 and X110.2. The contribution of the α1-6 linked fucose residue to the elution position on a CLC-ODS column has been determined to be +0.2 ~ +0.3 glucose units [22]. After β-*N*-acetylglucosaminidase digestion of oligosaccharide **b**, the decrease in size was assessed to be 0.4 glucose unit on size fractionation HPLC and the elution position on CLC-ODS column shifted to the position of 7.2 glucose units, which corresponded to the predicted elution position of X110.1 with an α1-6 linked fucose on GlcNAc-5′ (7.3–7.4 glucose units), but not to that of X110.2 with an α1-6 linked fucose on GlcNAc-5 (6.0–6.1 glucose units). It was therefore suggested that the fucose residue was linked to GlcNAc-5′ not to GlcNAc-5. The elution position did not change on β-galactosidase digestion. From these results, oligosaccharide **b** was proposed to have a novel structure hitherto unreported, having a fucosylated *N*-acetylglucosamine on the mannose α1-6 arm.

Oligosaccharide **c**. The elution position of oligosaccharide **c** shifted to that of X210.2 on α-L-fucosidase digestion and to that of oligosaccharide **a** on subsequent β-galactosidase digestion, whereas the elution position of oligosaccharide **c** did not change on direct β-galactosidase treatment, even on extensive digestion. It was suggested that the α-L-fucosyl residue was linked to the branching *N*-acetylglucosamine, resulting in the protection of the galactosyl residue from β-galactosidase digestion, as was reported for laccase compound f [27]. The chromatographic behaviour of oligosaccharide **c** agreed with the structure initially indicated by its elution position, i.e. X210.201.

Oligosaccharide **d**: The elution position of fraction **d** shifted to that of fraction **a** on α-L-fucosidase and subsequent β-galactosidase treatment. The chromatographic behaviour of oligosaccharide **d** agreed with the structure initially indicated by its elution position, i.e. X210.401.

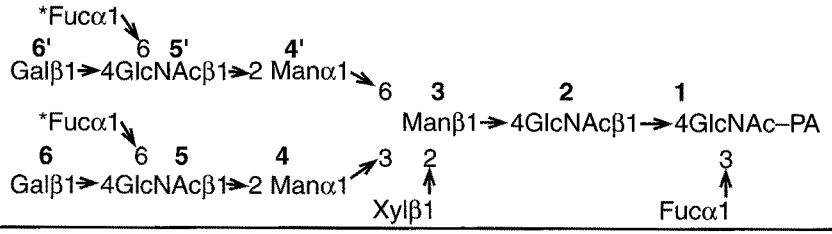
^{1}H-NMR spectroscopy and carbohydrate analyses

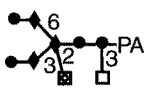
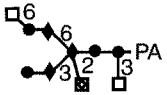
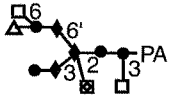
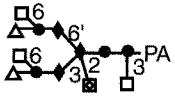
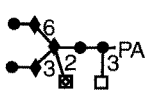
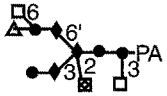
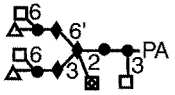
The structures of PA-oligosaccharides of *Cry j* I were further confirmed by 400 MHz ^{1}H-NMR spectroscopy, as listed in Table 1, in comparison with the chemical data for oligosaccharides of X210.1, X210.201 and X210.401 [27] (all are listed in Scheme 1). From these results, the structures of *Cry j* I oligosaccharides **a**, **c** and **d** were determined to be the same as those of the reference pyridylamino-oligosaccharides X210.1, X210.201 and X210.401, respectively. As shown in Table 1, the structure-reporter groups of oligosaccharide **b** indicated the existence of two kinds of α-L-fucosyl residues and the absence of galactose-6′. As listed in Table 1, oligo-



Scheme 1. Structures of reference pyridylamino-oligosaccharides. The abbreviations are according to Lee *et al.* [22].

Table 1. Chemical shifts (ppm) of structural reporter signals of pyridylamino-oligosaccharides from *Cry j* I.



Reporter group	Residue	*Chemical shifts*[a] *in Cry j I oligosaccharides*				*Reference compounds*		
		Fraction a 	*Fraction b* 	*Fraction c* 	*Fraction d* 	*210.1*[b] 	*210.201*[b] 	*210.401*[b] 
H-1 of	2	4.591 [4.611]	4.594 [4.610]	4.595 [4.792]	4.595 [4.792]	4.592	4.591	4.592
	3	4.848 [4.830]	4.852 [4.832]	4.849 [4.830]	[4.833]	4.857	4.855	4.856
	4	5.139 [5.146]	5.139 [5.143]	5.140 [5.143]	5.137 [5.143]	5.140	5.139	5.139
	4′	4.897 [4.882]	4.898 [4.882]	4.901 [4.878]	4.887 [4.872]	4.899	4.900	4.901
	5	4.513 [4.536]	4.514 [4.535]	4.514 [4.535]	[4.580]	4.511	4.511	4.533
	5′	4.544 [4.555]	4.531 [4.555]	4.577 [4.609]	4.575 [4.608]	4.546	4.576	4.576
	6	–	–	–	4.491	–	–	4.490
	6′	–	–	4.500 [4.494]	4.499	–	4.499	4.499
	Fuc	5.042 [5.037]	5.042 [5.037]	5.042 [5.037]	5.042 [5.038]	5.048	5.047	5.048
	*Fuc[c]	–	5.003 [5.004]	5.002 [5.002]	4.999 [5.004]	–	5.000	4.999
	Xyl	4.427 [4.465]	4.426	4.426 [4.460]	4.425	4.425	4.424	4.423
H-2 of	3	4.237 [4.242]	4.244 [4.243]	4.240 [4.242]	4.241 [4.245]			
	4	4.147 [4.150]	4.153 [4.150]	4.152 [4.148]	4.151 [4.151]			
	4′	4.102 [4.086]	4.099 [4.084]	4.098 [4.083]	4.057 [4.088]			
NAc of	1	1.929 [1.934]	1.934 [1.938]	1.927 [1.933]	[1.934]	1.956	1.950	1.954
	2	2.043 [2.041]	2.041 [2.038]	2.044 [2.040]	2.040 [2.038]	2.043	2.042	2.042
	5	2.043 [2.041]	2.041 [2.038]	2.044 [2.047]	2.040 [2.038]	2.043	2.042	2.042
	5′	2.043 [2.041]	2.041 [2.038]	2.044 [2.042]	2.040 [2.038]	2.043	2.042	2.042
	Fuc	1.192 [1.194]	1.192 [1.194]	1.191 [1.192]	1.191 [1.192]	1.197	1.195	1.197
	*Fuc[c]	–	1.170 [1.173]	1.172 [1.174]	1.170 [1.174]	–	1.170	1.170

The symbols used are: ●; GlcNAc, ◆; Man, □; Fuc, ⊠; Xyl, △; Gal.
[a]Chemical shifts were actually measured with internal acetone ($\delta = 2.216$ ppm in D_2O at 30 °C). Values in brackets were obtained at 60 °C with internal acetone ($\delta = 2.213$ ppm in D_2O).
[b]Data from [27]. Values were obtained with internal acetone ($\delta = 2.216$ ppm in D_2O at 30 °C).
[c]*Fuc denotes an α-L-Fuc residue linked to branching GlcNAc-5 or 5′.

saccharide **b** gave a different chemical shift for the H-1 proton of GlcNAc-5′ from that of oligosaccharide **a**, the difference being 0.013 ppm, indicating that a fucose residue was bound to GlcNAc-5′ in oligosaccharide **b**.

The carbohydrate compositions of oligosaccharides **a**–**d** were found to differ in L-fucose and D-galactose contents, the molar ratios of Fuc:Gal (mol/mol) being: oligosaccharide **a**, 1:0; oligosaccharide **b**, 2:0; oligosaccharide **c**, 2:1; and oligosaccharide **d**, 3:2; but to be the same in the molar ratio of GlcNAc:Man:Xyl = 4:3:1. The data in Table 1 and the carbohydrate compositions supported the structures proposed on the basis of the results of two-dimensional sugar mapping analyses. The proposed structures are listed in Table 2.

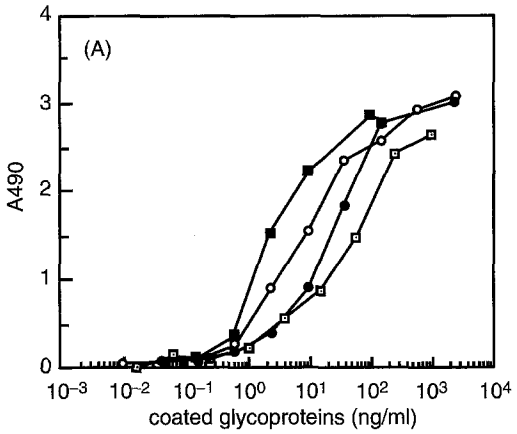
Contribution of oligosaccharides to the antigenicity of Cry j I

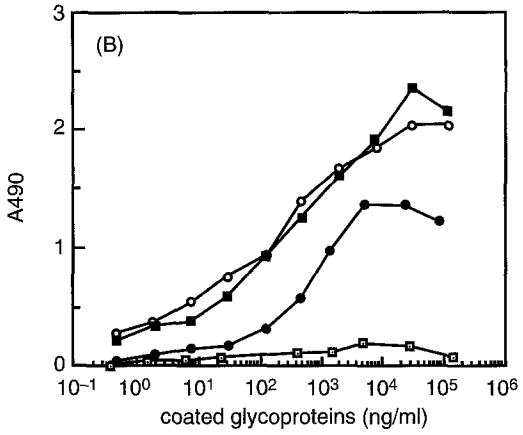
The antigenicity of the oligosaccharide moiety of *Cry j* I as to IgG and IgE was studied in comparison with those of miraculin, B-SJA-II and CTA, for which oligosaccharide structures have been elucidated. Miraculin, a taste-modifying glycoprotein from miracle fruit with a carbohydrate content of 13.9% (weight/weight), has been determined to contain bi- and mono-antennary complex-type oligosaccharides (molar proportions: 4.1% and 19.1%, respectively), which are the same as the agluco-samino structures of oligosaccharides d and c of *Cry j* I, respectively [28]. X010.1 and its trimmed structure lacking the α1-3 linked mannose were also involved (molar proportions: 58.0% and 18.8%, respectively). A galactose/*N*-acetylgalactosamine-specific non-legume lectin, CTA, and a mannose/glucose-specific legume lectin, B-SJA-II, have carbohydrate contents of 20% and 24% (weight/weight), respectively. The major oligosaccharides of both CTA and B-SJA-II (molar proportions: 70% and 80%, respectively) were determined to be the same as X010.1 [16], and the minor oligosaccharide fractions were commonly two kinds of trimmed structures of X010.1, lacking fucose or α1-3 linked mannose (unpublished data). CTA and B-SJA-II are thus considered to have almost the same glycan moieties having the same structures which are shorter than heptasaccharide with slight differences in their compositions. Since B-SJA-II has self-aggregating properties in the absence of specific sugars [15], CTA was used in inhibition assays for B-SJA-II to evaluate the contribution of the glycan moieties.

As shown in Fig. 2(A), rabbit anti-*Cry j* I IgGs cross-reacted with miraculin, B-SJA-II and CTA, as well as *Cry j* I, with similar maximum absorbance. In contrast, rabbit anti-B-SJA-II IgGs bound to CTA and miraculin as well as B-SJA-II, but very little to *Cry j* I (Fig. 2B). Western blotting analysis supported the different cross-reactivities of these IgG antibodies toward the glycoproteins (Fig. 3). As shown in Fig. 3B, anti-*Cry j* I IgGs

Table 2. Proposed structures of pyridylamino-oligosaccharides in *Cry j* I. The given molar ratios are the averages of duplicate determinations.

Oligosaccharide	*Structure*	*Molar ratio*
		%
a	GlcNAcβ1→2Manα1↘6 GlcNAcβ1→2Manα1↗3 Manβ1→4GlcNAcβ1→4GlcNAc–pyridylamine 2 3 Fucα1 ↑ ↑ ↓ Xylβ1 Fucα1 6	50
b	GlcNAcβ1→2Manα1↘6 GlcNAcβ1→2Manα1↗3 Manβ1→4GlcNAcβ1→4GlcNAc–pyridylamine 2 3 Fucα1 ↑ ↑ ↓ Xylβ1 Fucα1 6	17
c	Galβ1→4GlcNAcβ1→2Manα1↘6 GlcNAcβ1→2Manα1↗3 Manβ1→4GlcNAcβ1→4GlcNAc–pyridylamine 2 3 Fucα1 ↑ ↑ ↓ Xylβ1 Fucα1 6	28
d	Galβ1→4GlcNAcβ1→2Manα1↘6 Galβ1→4GlcNAcβ1→2Manα1↗3 Manβ1→4GlcNAcβ1→4GlcNAc–pyridylamine 2 3 6 ↑ ↑ ↑ Xylβ1 Fucα1 Fucα1	5





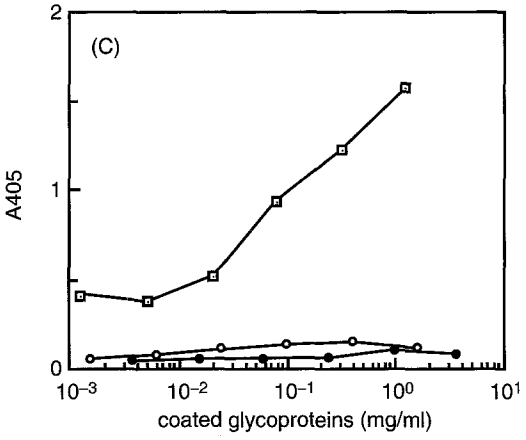


Figure 2. Cross-reactivities of rabbit anti-*Cry j* I IgG antibodies (A), rabbit anti-B-SJA-II IgG antibodies (B), and human allergic sera (C) to glycoproteins on ELISA. Miraculin (—●—), B-SJA-II (--■--), CTA (—○—) or *Cry j* I (—⊡—), was coated onto a well and then subjected to ELISA. After blocking with 1% BSA, 100 μl of rabbit anti-*Cry j* I IgG (1:500) or 50 μl of human allergic sera (1:4) were added to the wells, followed by incubation for 1 h at r.t. After further washing and blocking, the bound antibodies were detected by incubation with HRP-labelled goat anti-rabbit IgG (1:1000) or alkaline phosphatase-labelled horse anti-human IgE (1:1000; EAST kit, Shionogi Seiyaku Co., Ltd., Japan), respectively. The plates were washed again and developed with 150 μl of *o*-phenylendiamine/H_2O_2, and then 50 μl of 4 N sulfuric acid was added to stop the reaction, followed by reading absorbance at 490 nm for the rabbit IgG system or according to the manufacturer's instructions for the human IgE system. The results were corrected by subtracting the value for normal rabbit IgG as a control.

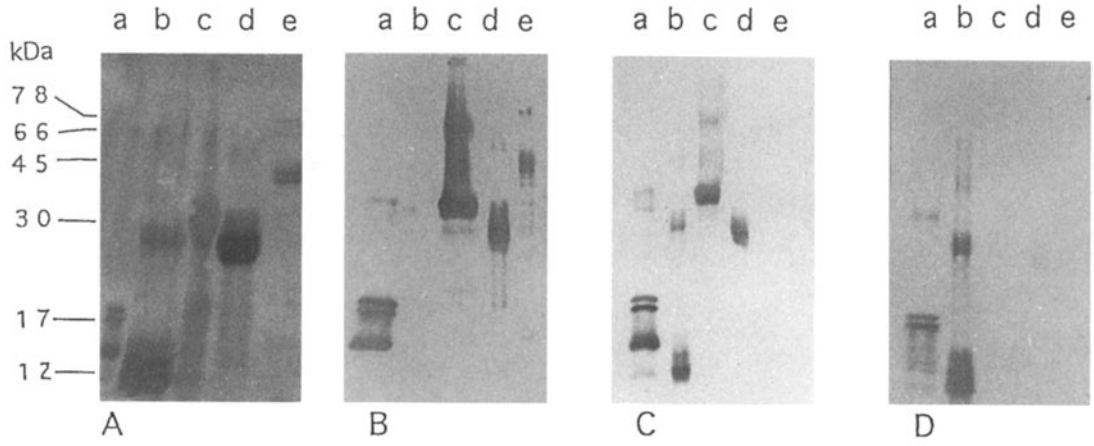


Figure 3. Immunostaining of glycoproteins with rabbit anti-*Cry j* I IgG, anti-B-SJA-II IgG and anti-deglycosylated B-SJA-II IgG. Coomassie brilliant blue staining (A); lane a: B-SJA-II (8 μg per lane), lane b: deglycosylated B-SJA-II (10 μg per lane), lane c: CTA (8 μg per lane), lane d: miraculin (4 μg per lane), lane e: *Cry j* I (5 μg per lane). Immunostaining with rabbit anti-*Cry j* I IgG (B); anti-B-SJA-II IgG (C); and anti-deglycosylated B-SJA-II IgG (D). (B), (C), (D); lanes a–e are the same as in A, but the amount loaded in each lane was 1/10 of that in (A). The positions of molecular weight markers are indicated on the left. Dimer bands appeared around 30 kDa for intact and TFMS-treated B-SJA-II.

cross-reacted with all the glycoproteins except deglycosylated B-SJA-II, suggesting that the oligosaccharides of B-SJA-II contributed to the cross-reactivity. Anti-B-SJA-II IgGs bound to all the glycoproteins but *Cry j* I (Fig. 3C). The antibodies raised against deglycosylated B-SJA-II did not cross-react with miraculin, CTA or *Cry j* I (Fig. 3D). Since B-SJA-II retained its carbohydrate-binding activity after deglycosylation with TFMS and was considered to have an active peptide conformation like the native form (unpublished results), the different cross-reactivities of these antibodies shown in Fig. 3C and D can be attributed to the oligosaccharide-specific antibodies. Anti-B-SJA-II IgGs are therefore suggested to include antibodies recognizing the oligosaccharide epitope common to these glycoproteins (except *Cry j* I) that must contain exposed mannosyl residues, while a part of anti-*Cry j* I IgGs recognized another oligosaccharide epitope common to all these glycoproteins. In contrast to IgGs, allergic human IgEs bound very little miraculin and CTA compared to *Cry j* I (Fig. 2C). Since the tested sera were obtained from 21 patients, it can be regarded that IgEs from most patients had little cross-reactivities against these glycoproteins.

ELISA inhibition was performed to determine the contents of oligosaccharide-specific antibodies in rabbit anti-*Cry j* I IgGs and IgEs of human allergic sera (Fig. 4A and B). Glycopeptides and peptides prepared from the glycoproteins and deglycosylated proteins, respectively, were used for inhibition assays and the contribution of the glycan moieties was evaluated from the difference of their inhibitory potency. The extensive actinase E digestion of miraculin and CTA destroyed most of the peptide epitopes, if any, cross-reactive to the anti-*Cry j* I antibodies and lectin activity. As shown in Fig. 4A, miraculin glycopeptides inhibited the binding of rabbit anti-*Cry j* I IgG to *Cry j* I in a concentration-dependent manner up to the same inhibition level as intact *Cry j* I, while the miraculin peptides obtained from deglycosylated miraculin did not show any significant inhibitory activity, showing that the peptide portions do not contribute to the cross-reactivity. These results suggested that rabbit anti-*Cry j* I IgG antibodies predominantly recognized carbohydrate epitopes. Intact CTA and CTA glycopeptides equally inhibited the binding of rabbit anti-*Cry j* I IgGs to *Cry j* I to a moderate level, but to a lesser extent than miraculin glycopeptides, while CTA peptides did not. These results suggested that the heptasaccharide core commonly present in CTA and miraculin, and the branch portion specifically present in miraculin, separately participated in the epitopic structures. Intact miraculin showed lower inhibitory activity than miraculin glycopeptides, suggesting that the cross-reactive oligosaccharide epitopes were mostly cryptic in intact miraculin and exposed on proteolysis or adsorption onto a plastic surface (Fig. 2A).

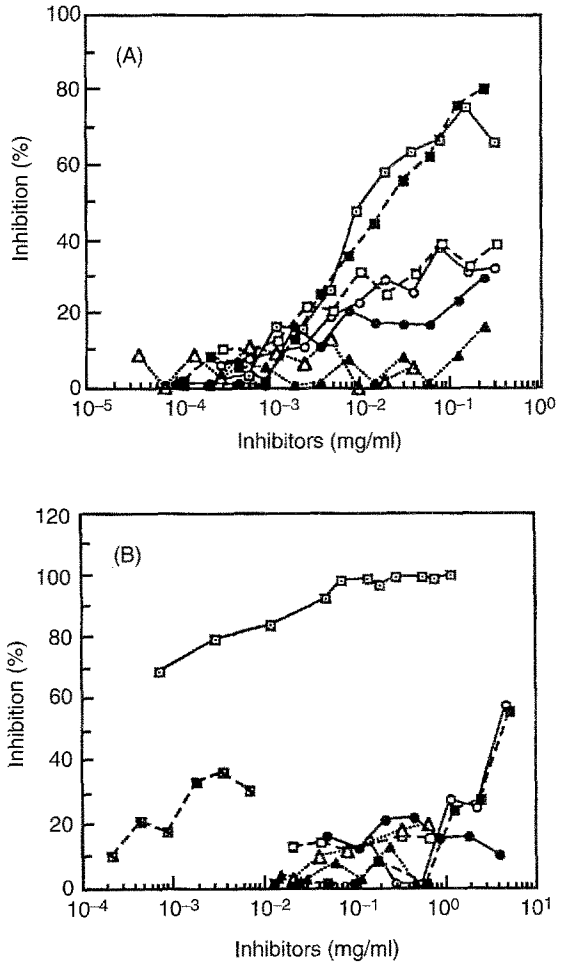


Figure 4. ELISA inhibition for rabbit anti-*Cry j* I IgG (A) and human allergic IgE (B). The binding of antibodies to *Cry j* I coated onto a well was inhibited by intact glycoproteins, glycopeptides or peptides. The symbols used in (A) and (B) are; intact miraculin (—●—), miraculin glycopeptides (--■--); miraculin peptides (···▲···), intact CTA (—○—), CTA glycopeptides (--□--), CTA peptides (···△···), and intact *Cry j* I (-⊡-). *Cry j* I glycopeptides (--⧅--) are shown in (B).

In contrast, the contribution of oligosaccharide moieties was found to be very little as to IgE-binding of *Cry j* I (Fig. 4B). *Cry j* I glycopeptides showed low inhibitory activity compared to intact *Cry j* I, indicating that most IgE epitopes were cleaved by proteolysis. None of the tested inhibitors showed comparable inhibition, although miraculin glycopeptides and intact CTA exhibited some inhibitory activity at very high concentrations, which were three magnitudes higher than the concentrations of *Cry j* I glycopeptides.

Discussion

The major oligosaccharides of *Cry j* I were revealed to be a series of compounds carrying biantennary glucosamine branches linked to α-mannosyl residues of the

common core unit, Manα1-6(Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc. Variations were found only in fucosylation and galactosylation of the glucosamine branches. The structures of oligosaccharides **a**, **b** and **c** accounted for the characteristic reactivity of *Cry j* I to *Grifonia simplicifolia* lectin II [10], which is specific to α or β-linked *N*-acetylglucosamine residues [29].

The same structures as those of oligosaccharides **a**, **c** and **d** have been reported for laccase produced by sycamore cells [27], whereas the structure of oligosaccharide **b** has not yet been reported for other glycoproteins. The oligosaccharide variations present in laccase have been reported to be caused by degradation enzymes after excretion into the culture medium, the largest oligosaccharide synthesized in the Golgi apparatus, like **d** in *Cry j* I, being sequentially trimmed to $Man_2FucXylGlcNAc_2$ [30]. In similar ways, the oligosaccharides, $Man_3(\pm Fuc)XylGlcNAc_2$ and $Man_2XylGlcNAc_2$, which are widely present in plant glycoproteins, have been shown to be degradation products that appear outside the Golgi apparatus [31]. *Cry j* I does not contain these short oligosaccharides, however, as revealed in this study; it has been shown to be localized in the exine of the cell wall, orbicles attached to the cell wall, and Golgi bodies in the mature pollen grains of *C. japonica* by immunocytochemical studies involving monoclonal and polyclonal antibodies [32]. Since cell wall components and orbicles are considered to be synthesized and transported in two ways, i.e. cell wall targeting via Golgi vesicles after synthesis in Golgi bodies, and extracellular accumulation of tapetal substances synthesized and excreted from tapetal cells of the anther, we cannot conclude whether variations in *Cry j* I oligosaccharides are generated in Golgi bodies or not. If oligosaccharide **b** is a processing intermediate in Golgi bodies, the presence of a fucosylated *N*-acetylglucosamine branch on *Cry j* I would suggest that a fucose residue must be transferred onto an *N*-acetylglucosamine-5′ residue preceding the transfer of a galactose-6′ residue in the processing of oligosaccharides.

Recently, Hino *et al.* reported four kinds of carbohydrate structures, A–D, present on *Cry j* I [13], the structure of oligosaccharide B being identified as Galβ1–4(Fucα1-6)GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)(Xylβ1-2)Manβ1-4GlcNAcβ1-4GlcNAc-pyridylamine, and those of oligosaccharides A, C and D coinciding with those of **a**, **c** and **d** in this report. The reason for the discrepancy is not known, but it can be attributed to individual differences in the pollen material. Otherwise, oligosaccharide B might be an artifact derived from oligosaccharide C. Oligosaccharide **b** in this report cannot be an artifact derived from oligosaccharide **c** since galactose-6′ residues are very resistant to galactosidase digestion as long as fucose is linked by an α1-6 linkage to *N*-acetylglucosamine-5′ [27], in addition to the chemical stability of the galactoside linkage compared to the α1-3 linkage of fucose to *N*-acetylglucosamine-1.

This work showed that rabbit anti-*Cry j* I IgG antibodies predominantly recognized the oligosaccharide moiety of *Cry j* I, and two kinds of epitopic motifs were found for this antibody, i.e. branches containing α1-6 linked fucose, and the heptasaccharide core containing α1-3 linked fucose and β-xylose. Anti-B-SJA-II IgGs were shown to include oligosaccharide-specific antibodies that critically required the nonreducing terminal mannose residue(s) of the core for binding. None of these structural motifs has been found yet in mammalian glycoproteins and thus they could be immunogenic epitopes. In fact, fetuin and asialofetuin did not cross-react with either of these antibodies (unpublished results). The model epitopic residues proposed for each antibody are summarized in Fig. 5. As shown in Fig. 5A, the branch portion and the Fuc/Xyl-containing core could be recognized as separate motifs, as shown by ELISA inhibition; i.e. miraculin glycopeptides completely inhibited the bindings of polyclonal anti-*Cry j* I IgGs while CTA glycopeptides incompletely inhibited them. However, a part of the antibodies may be directed against the complexes of these residues, since α1-3 linked and α1-6 linked fucoses can be in a spatially close position, as shown in Fig. 5A. The heptasaccharide structure shown in Fig. 5B has been reported to be a ubiquitous cross-reactive carbohydrate determinant on a number of plant glycoproteins [2, 3, 7–9], α-mannosyl residues and α1-3 linked fucose being shown to be epitopic residues on HRP [7], and the antigenicity of β-xylosyl residue being suggested in another report [9]. In this work, *Cry j* I did not cross-react with anti-B-SJA-II IgGs even when it contained Fuc/Xyl, suggesting that the exposed mannosyl residues and Fuc/Xyl residues in the core are recognized as a single epitope by anti-B-SJA-II IgGs, as shown in Fig. 5B. As judged from the results, attention must be paid to oligosaccharide antigenicity and the direction of cross-reactivity when antibodies against plant glycoproteins are used for immunochemistries, and when producing a recombinant allergen with *N*-glycosylation sites in a plant expression system.

The contribution of the oligosaccharide moiety of *Cry j* I was found to be very little with respect to allergic human IgE-binding to *Cry j* I although the possibility cannot be denied that *Cry j* I oligosaccharides are involved in the IgE epitope in some of the patients. As shown in Fig. 4B, some IgE epitopes still remained after the proteolysis of *Cry j* I. Since glycosylation often protects the neighbouring peptide portion from extensive proteolysis [33], it is suggested that peptides containing glycosylation sites are epitopes against anti *Cry j* I-IgE, and that the oligosaccharides may contribute to the formation of active conformational epitopes of the peptide portions [34].

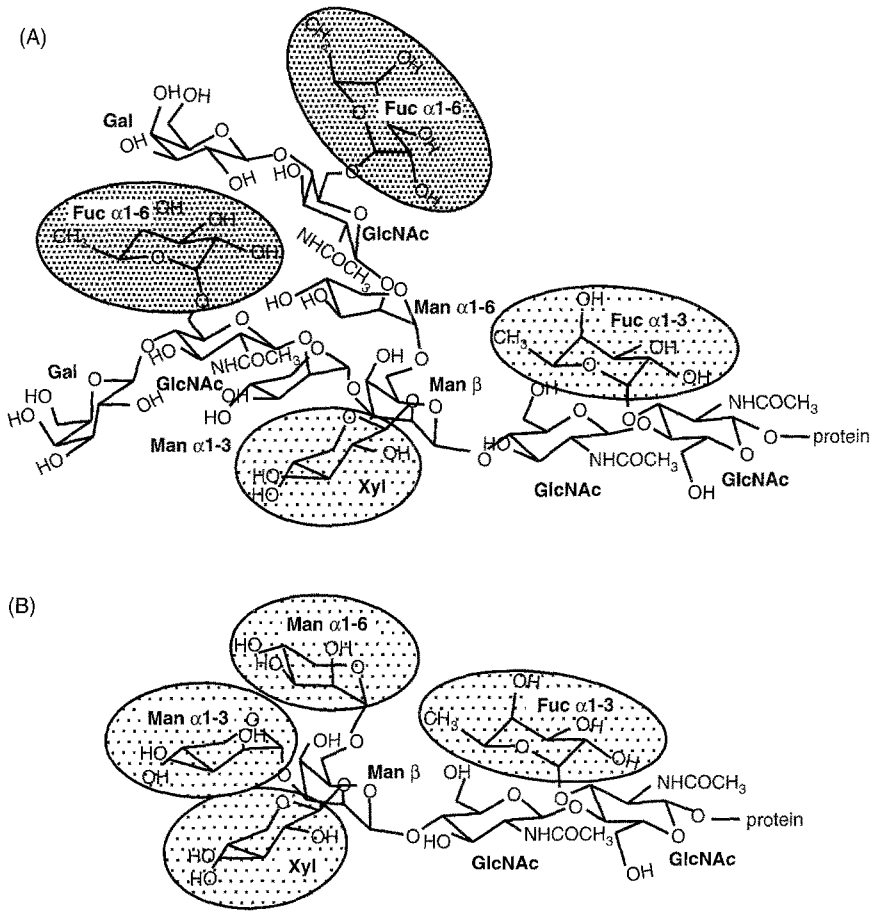


Figure 5. Molecular models of the epitopic structures for the antibodies of anti-*Cry j* I IgG (A) and anti-B-SJA-II IgG (B). The epitopic sugar residues are shown by shaded ovals. The oligosaccharides of *Cry j* I are represented by oligosaccharide **d** in (A), and those of B-SJA-II are represented by X010.1 in (B). The spatial arrangement of the Manα-1-6(Xylβ1-2)Manβ1-4GlcNAcβ1-4(Fucα1-3)GlcNAc moiety is cited from Fig. 8 in [35].

In this study, *N*-glycans were revealed to be immunogenic but not allergenic in *Cry j* I. Specific IgG antibodies against *Cry j* I oligosaccharides may play a role as blocking antibodies in desensitization of pollinosis. Conversely, the fact that *Cry j* I exhibited low cross-reactivity with the antibodies against a ubiquitous plant carbohydrate determinant may make it a special antigen than cannot be recognized by the immune network in the sensitization process for pollinosis. Further studies are required on the biological importance of the oligosaccharide-recognizing antibodies to these aspects.

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