

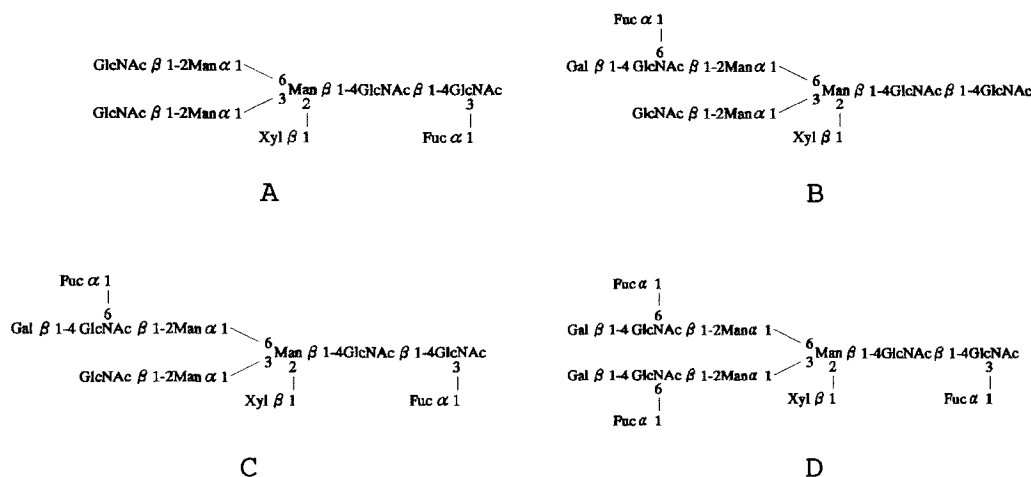
Carbohydrate Structures of the Glycoprotein Allergen *Cry j* I from Japanese Cedar (*Cryptomeria japonica*) Pollen

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The glycoprotein allergen *Cry j* I from Japanese cedar (*Cryptomeria japonica*) pollen was treated with pepsin and glycopeptidase A to release asparagine-linked oligosaccharides. The reducing ends of the oligosaccharides were aminated with the fluorescent reagent 2-aminopyridine. The oligosaccharide derivatives were purified by gel permeation chromatography and reversed-phase HPLC. Their structures were determined by sequential exoglycosidase digestion and 500 MHz ¹H-NMR spectroscopy. Four oligosaccharide structures, A, B, C, and D, were identified as the xylose-containing complex-type. They were present at a molar ratio of 8 : 1 : 6 : 1. By amino acid sequence analyses of the tryptic peptides, Asn-170 and Asn-333 of *Cry j* I were found to carry asparagine-linked oligosaccharides.



Key words: allergen, carbohydrate structure, *Cry j* I, glycoprotein, ¹H-NMR.

Japanese cedar (*Cryptomeria japonica*) pollen is one of the main causes of seasonal allergic reactions. *Cry j* I and *Cry j* II were isolated as pollen allergens and characterized by Yasueda *et al.* (1) and Sakaguchi *et al.* (2), respectively. *Cry j* I is quite different from *Cry j* II in terms of molecular weight, antigenicity, and N-terminal amino acid sequence (3). In SDS-PAGE analysis, *Cry j* I showed multiple bands, as is generally observed with glycoproteins. Taniai *et al.* reported an important role of the carbohydrate moiety of *Cry j* I in the binding of the allergen to human IgE antibodies (4). From pepsin- or V8 protease-digested *Cry*

j I, they obtained two peptide fragments which shared the same amino acid sequence, one being glycosylated and the other not. They also found that some human IgE antibodies could bind to the glycosylated peptide but not to the unglycosylated one. Important roles of carbohydrate moieties in other allergens for binding to human IgE antibodies have also been pointed out by several investigators (4-9). However, the complete structures of the oligosaccharide chains of plant allergens, including *Cry j* I, have not been reported, except for those of *Art v* II (an allergen of mugwort pollen) by Nilsen *et al.* (6).

Here, we report the structural analysis of the asparagine-linked oligosaccharides and glycosylation sites of the glycoprotein allergen *Cry j* I.

Abbreviations: Fuc, fucose; Gal, galactose; GlcNAc, N-acetylglucosamine; TPCK-, N-tosyl-L-phenylalanyl chloromethyl ketone; Man, mannose; M3FX, Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA; M3X, Man α 1-6(Man α 1-3)(Xyl β 1-2)Man β 1-4GlcNAc β 1-4GlcNAc-PA; PA-, pyridylamino; Xyl, xylose.

MATERIALS AND METHODS

Materials—*Cry j I* was purified from Japanese cedar pollen as described previously (3). Glycopeptidase A (almond) and β -*N*-acetylhexosaminidase (jack bean) were purchased from Seikagaku Kogyo, α -L-fucosidase (bovine kidney) from Boehringer Mannheim, β -galactosidase (*Aspergillus oryzae*) from Toyobo, and TPCK-treated trypsin from Sigma Chemical. Bio-gel P-4 (200–400 mesh) and Bio-gel P-4 super fine (under 400 mesh) were purchased from Bio-Rad Laboratories, TSKgel HW-40F, a TSKgel Amide-80 column (0.46 $\times$ 25 cm), and a TSKgel Sugar AX-I column (0.46 $\times$ 15 cm) from Tosoh, and a Cosmosil 5C18-P column (1.0 $\times$ 25 cm) from Nacalai Tesque. A Shim-pack CLC-ODS column (0.6 $\times$ 15 cm) was purchased from Shimadzu, and a 218TP54 column (0.46 $\times$ 25 cm) from Vydac. Deuterated water, D₂O; 99.8% atom %D and 99.96% atom %D, was purchased from Nacalai Tesque and MSD Isotopes, respectively.

Preparation of Oligosaccharides from *Cry j I*—Oligosaccharides were released from 150 mg of *Cry j I* by sequential digestion with pepsin and glycopeptidase as reported by Hotta *et al.* (10). The oligosaccharides were separated by gel permeation chromatography on a Bio-gel P-4 column (2.5 $\times$ 47 cm) equilibrated with distilled water, and the oligosaccharides were then eluted with distilled water. Each fraction was assayed for neutral sugar using resorcinol-sulfuric acid (11), and the positive fractions were pooled.

Pyridylation of Oligosaccharides—Oligosaccharides were reductively aminated using 2-aminopyridine with sodium cyanoborohydride (12). The pyridylamino derivatives of the oligosaccharides (PA-oligosaccharides) were loaded on a column (1.6 $\times$ 40 cm) of TSKgel HW-40F to remove excess reagents.

Isolation and Identification of PA-Oligosaccharides by HPLC—The PA-oligosaccharides were loaded on a gel permeation column of Bio-gel P-4 super fine (2.6 $\times$ 94 cm) equilibrated with 10 mM ammonium acetate buffer (pH 6.2) and eluted at a flow rate of 0.4 ml/min at 62°C with the same buffer. The eluate was monitored by measuring UV absorbance at 280 nm, and three PA-oligosaccharide fractions were collected. Each fraction was further purified by reversed-phase HPLC with a Cosmosil 5C18-P column. The elution was performed at a flow rate of 5.0 ml/min at 40°C with an eluent system composed of solvent A (100 mM ammonium acetate buffer, pH 4.0) and solvent B (0.5% 1-butanol in solvent A) using a stepwise gradient of 20–50% solvent B in 25 min followed by 50–100% solvent B in 10 min. The eluate was monitored by measuring fluorescence intensity using excitation and emission wavelengths of 320 and 400 nm, respectively.

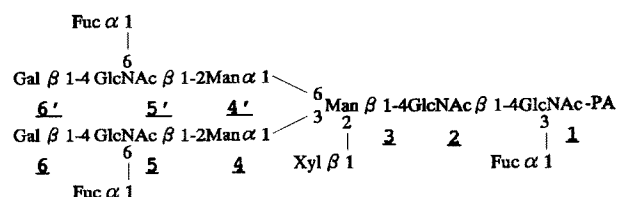
Sugar Composition Analysis—Each PA-oligosaccharide obtained from *Cry j I* was hydrolyzed with 4 N trifluoroacetic acid at 100°C for 3 h, and re-*N*-acetylated and pyridylaminated (13). The pyridylaminated monosaccharides were quantified by ion-exchange HPLC on a TSKgel Sugar AX-I column (14).

Exoglycosidase Digestion—Each PA-oligosaccharide (100–200 pmol) was sequentially digested for 20 h at 37°C with 0.1–0.5 unit of α -L-fucosidase in 50 mM citrate-phosphate buffer (pH 6.0), β -galactosidase in 0.1 M ammo-

nium acetate buffer (pH 3.6), and β -*N*-acetylhexosaminidase in 50 mM citrate-phosphate buffer (pH 5.0), for 20 h at 37°C.

Identification of PA-Oligosaccharides by HPLC “Two-Dimensional Sugar Mapping Technique”—Sample PA-oligosaccharides were analyzed by two different modes of HPLC on a reversed-phase Shim-pack CLC-ODS column and a size-fractionation TSKgel Amide-80 column as described by Tomiya *et al.* (15). The values of the elution position of the PA-oligosaccharides (expressed as PA-glucose unit numbers) on each column were compared with those of reference PA-oligosaccharides.

¹H-NMR Spectroscopic Analysis—The PA-oligosaccharide samples were repeatedly dissolved in D₂O (99.8% atom %D) at room temperature and lyophilized for exchange of labile protons with deuterium. Prior to ¹H-NMR spectroscopic analysis, the sample was redissolved in D₂O (99.96% atom %D) and transferred into a 5 mm NMR microtube (Shigemi). Spectroscopic analyses were performed on an α -500 spectrometer (JEOL) operating at 500 MHz, and data processing was carried out using NMR1 software on a DEC computer. The probe was kept at 30°C. Chemical shifts (δ) were expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane 1-sulfonate (DSS) and measured by reference to internal acetone (δ = 2.216 ppm in D₂O at 30°C). The coding of the sugar residues used is as follows:



Trypsin Digestion of *Cry j I*—*Cry j I* was pyridylethylated using 4-vinylpyridine and tri-*n*-butylphosphine as described by Friedman *et al.* (16). Pyridylethylated *Cry j I* was then dissolved in 300 μ l of 0.2 M ammonium acetate buffer (pH 8.6), and 15 μ g of TPCK-treated trypsin was added. The mixture was incubated for 17 h at 37°C, and boiled for 5 min to stop the enzymatic reaction. The resulting fragments were separated by reversed-phase HPLC on a 218TP54 column equilibrated with solvent C (0.1% trifluoroacetic acid in distilled water). The elution was performed at a flow rate of 0.5 ml/min at 40°C with an eluent system composed of solvent C and solvent D (90% acetonitrile in solvent C), using a linear gradient of 0–100% solvent D in 100 min. Peptide fragments were detected by measurement of UV absorbance at 214 nm.

NH₂-Terminal Amino Acid Sequence Analysis—The NH₂-terminal amino acid sequences of the peptide fragments from the trypsin-digested *Cry j I* were determined on an Applied Biosystems 473A protein sequencer.

RESULTS

Separation of PA-Oligosaccharides—Figure 1 shows an HPLC profile on a TSKgel Amide-80 column of *Cry j I* derived PA-oligosaccharides. Four peaks, A, B, C, and D, were observed. To obtain enough of each PA-oligosaccharide for structural analyses, a gel permeation column of Bio-gel P-4 super fine was used. As shown in Fig. 2, three

fractions (Fr. 1, Fr. 2, and Fr. 3) were obtained. Each was further purified on a reversed-phase HPLC 5C18-P column. Both Fr. 1 and Fr. 2 gave a single peak, and Fr. 3 gave two peaks (data not shown). Analyses of the four peaks obtained on the Amide-80 column revealed that the peaks from Fr. 1 and Fr. 2 corresponded with D and C, respectively, and that the two peaks from Fr. 3 corresponded with B and A.

Sugar Compositions of the PA-Oligosaccharides—Sugar compositions of the four PA-oligosaccharides are shown in Table I. The relative proportions of mannose, xylose, and *N*-acetylglucosamine were nearly equal among all four PA-oligosaccharides, but the relative proportions of galactose and fucose were different among these PA-oligosaccharides. All four oligosaccharides were considered to have a xylose-containing complex-type structure.

Characterization of PA-Oligosaccharides A-D using Two-Dimensional Mapping Technique—PA-Oligosaccharides purified using a Cosmosil 5C18-P column were

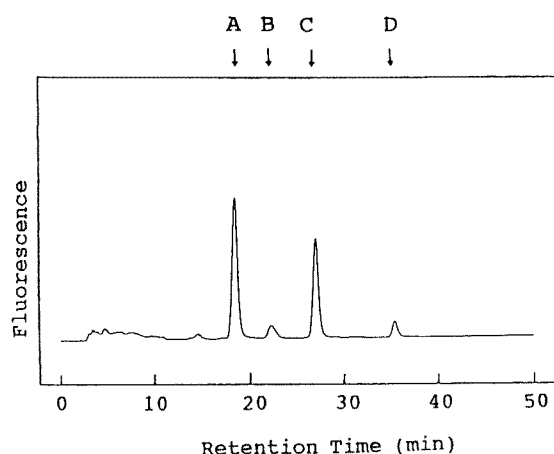


Fig. 1. Chromatogram of PA-oligosaccharides obtained from Cry j I on an Amide-80 column. Four peaks, A, B, C, and D, were observed.

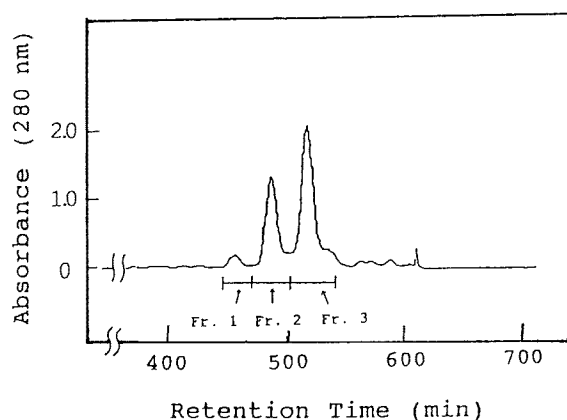


Fig. 2. Chromatogram of PA-oligosaccharides obtained from Cry j I on a Bio-gel P-4 super fine column. The column (2.6 × 94 cm) was equilibrated with 10 mM ammonium acetate buffer, pH 6.2, and elution was performed with the same buffer. PA-Oligosaccharides were detected by measurement of UV absorbance at 280 nm. Three fractions, demarcated with a horizontal line, were collected.

analyzed by a two-dimensional mapping technique using TSKgel Amide-80 and Shim-pack CLC-ODS columns. The results are shown in Table II and Table III. The elution positions of PA-oligosaccharide A resembled that of the laccase-derived xylose-containing complex-type PA-oligosaccharide X3.4 reported by Tomiya *et al.* (15), the structure of which was $\text{GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-6}(\text{GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-3})(\text{Xyl}\beta 1\text{-2})\text{Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4}(\text{Fuc}\alpha 1\text{-3})\text{-GlcNAc-PA}$ (Table II). After digestion of PA-oligosaccharide A with β -*N*-acetylhexosaminidase, a new peak was observed on the two columns. The new position resembled that of the reported $\text{Man}\alpha 1\text{-6}(\text{Man}\alpha 1\text{-3})(\text{Xyl}\beta 1\text{-2})\text{Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4}(\text{Fuc}\alpha 1\text{-3})\text{GlcNAc-PA}$ (M3FX) (15). These results suggest that the structure of PA-oligosaccharide A was the same as that of PA-oligosaccharide X3.4. It has been reported that on an Amide-80 column, a galactose residue behaves like a 1.0 glucose unit, an *N*-acetylglucosamine residue behaves like a 0.5 glucose unit and a fucose residue bound to the outermost GlcNAc residue through the α 1-6 linkage behaves like a 1.0 glucose unit (15). On this basis, the number of sugar residues released was estimated from the values of the subtraction-of-glucose units. After sequential digestion of PA-oligosaccharide B with α -L-fucosidase, β -galactosidase, and β -*N*-acetylhexosaminidase, new peaks were observed on an Amide-80 column at positions which showed deletion of one fucose residue, one galactose residue and two *N*-acetylglucosamine residues, respectively (Table III). The elution positions of the final digest were in good agreement with those of the reported PA-oligosaccharide $\text{Man}\alpha 1\text{-6}(\text{Man}\alpha 1\text{-3})(\text{Xyl}\beta 1\text{-2})\text{Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4GlcNAc-PA}$ (M3X) (17). The elution positions of PA-oligosaccharides C and D resembled those of the laccase-derived xylose-containing complex-type oligosaccharides X3.5 and X3.6, respectively, as reported by Tomiya *et al.* (15). The structures were $\text{Gal}\beta 1\text{-4}(\text{Fuc}\alpha 1\text{-6})\text{GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-6}(\text{GlcNAc}\beta 1\text{-2Man}\alpha 1\text{-3})(\text{Xyl}\beta 1\text{-2})\text{Man}\beta 1\text{-4GlcNAc}\beta 1\text{-4}(\text{Fuc}\alpha 1\text{-3})\text{-}$

TABLE I. Sugar composition of PA-oligosaccharides A-D.

PA-Oligosaccharide	Molar ratio ^a				
	Gal	Fuc	Man	Xyl	GlcNAc
A	0.0	1.0	3.0	1.2	3.8
B	1.0	1.0	3.0	1.1	3.5
C	1.1	2.1	3.0	0.8	4.1
D	2.1	3.1	3.0	0.8	3.8

^aMolar ratios were calculated by taking the value of mannose as 3.0.

TABLE II. Elution positions of PA-oligosaccharides from Cry j I and reference PA-oligosaccharides on Shim-pack CLC-ODS and TSKgel Amide-80 columns.

PA-Oligosaccharide	Glucose unit ^a	
	Shim-pack CLC-ODS	TSKgel Amide-80
A	6.8	6.4
X3.4 ^b	7.0	6.5
B	8.8	7.6
C	7.3	8.1
X3.5 ^b	7.2	8.3
D	7.7	10.2
X3.6 ^b	7.7	10.3
M3FX ^b	5.6	5.7
M3X ^c	7.2	4.8

^aThe values show PA-glucose unit numbers which express the elution positions of PA-glucose oligomers. ^bData from Ref. 15. ^cData from Ref. 17.

TABLE III. Shifts in elution positions of PA-oligosaccharides A-D after sequential exoglycosidase digestion.

PA-Oligosaccharide	Sequential digestion with	Shim-pack CLC-ODS	TSKgel Amide-80		Released ^c
		Glucose unit ^a	Glucose unit ^a	Δ Glucose unit ^b	
A	No treatment	6.8	6.4		
	α -L-Fucosidase	6.8	6.4	0.0	
	β -Galactosidase	6.8	6.4	0.0	
	β -N-Acetylhexosaminidase	5.6	5.5	0.9	2 GlcNAc
B	No treatment	8.8	7.6		
	α -L-Fucosidase	8.6	6.5	1.1	1 Fuc
	β -Galactosidase	8.7	5.6	0.9	1 Gal
	β -N-Acetylhexosaminidase	7.2	4.8	0.8	2 GlcNAc
C	No treatment	7.3	8.1		
	α -L-Fucosidase	7.0	7.2	0.9	1 Fuc
	β -Galactosidase	6.8	6.4	0.8	1 Gal
	β -N-Acetylhexosaminidase	5.6	5.5	0.9	2 GlcNAc
D	No treatment	7.7	10.2		
	α -L-Fucosidase	6.9	8.1	2.1	2 Fuc
	β -Galactosidase	6.7	6.4	1.7	2 Gal
	β -N-Acetylhexosaminidase	5.6	5.5	0.9	2 GlcNAc

^aThe values show PA-glucose unit numbers which express the elution positions of PA-glucose oligomers. ^bThese values showed subtraction-of-glucose units on an Amide-80 column. ^cThe numbers and types of sugar residues released were presumed from the values of the Δ glucose units.

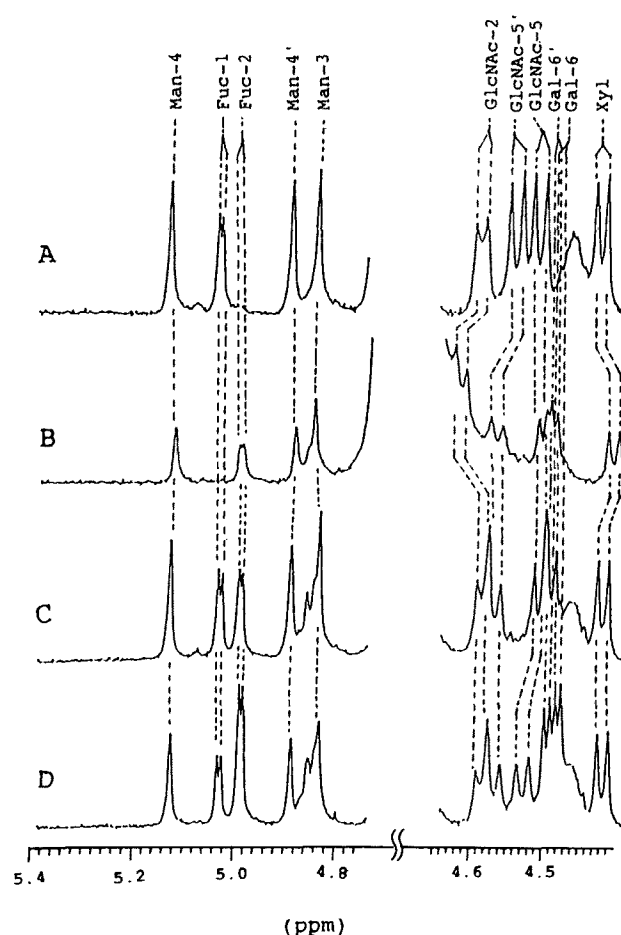


Fig. 3. Structural reporter-group regions of the 500 MHz ¹H-NMR spectra of PA-oligosaccharides A-D. The probe was kept at 30°C. The residue numbers (Man-4, Fuc-1, Fuc-2, and etc.) were used as described in "MATERIALS AND METHODS."

GlcNAc-PA and Gal β 1-4(Fuc α 1-6) GlcNAc β 1-2Man α 1-6[Gal β 1-4(Fuc α 1-6)GlcNAc β 1-2Man α 1-3](Xyl β 1-2)-

Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA, respectively (Table II). After sequential digestion of PA-oligosaccharide C with α -L-fucosidase and β -galactosidase, new peaks were observed on an Amide-80 column at positions which showed deletion of one fucose residue and one galactose residue, respectively (Table III). PA-Oligosaccharide D was digested sequentially with exoglycosidase as described for PA-oligosaccharide C. The digested PA-oligosaccharide was eluted on an Amide-80 column at a position corresponding to two fucose residues and two galactose residues less than before digestion (Table III). The elution positions of the galactosidase digest of both PA-oligosaccharides (C and D) on an Amide-80 column were shifted to positions similar to those of PA-oligosaccharide A. Further treatment of the digests with β -N-acetylhexosaminidase gave the same results as obtained for PA-oligosaccharide A. From these results, the structures of PA-oligosaccharides C and D were estimated to be the same as those of PA-oligosaccharides X3.5 and X3.6, respectively.

¹H-NMR Analyses of PA-Oligosaccharides—For NMR analysis, 20–100 nmol of each PA-oligosaccharide (A–D) was used. The structural reporter-group regions of the 500 MHz ¹H-NMR spectra of PA-oligosaccharides A–D are given in Fig. 3. Chemical shift values for the anomeric protons and the methyl protons of all four PA-oligosaccharides were compared with those of three reference PA-oligosaccharides, X3.4, X3.5, and X3.6 (18). As shown in Table IV, the chemical shifts of the anomeric and methyl groups of PA-oligosaccharides A, C, and D were all in good agreement with those from PA-oligosaccharides X3.4, X3.5, and X3.6, respectively. Comparison of the chemical shift data for PA-oligosaccharide B with those of X3.5 showed that the anomeric and methyl proton signals belonging to Fuc-1 were absent, and that the anomeric proton signal for GlcNAc-2 (4.630 ppm) agreed with that of another reference PA-oligosaccharide, the structure of which was GlcNAc β 1-4GlcNAc-PA as reported by Koyama *et al.* (19). This indicated the lack of a fucose residue bound to the innermost GlcNAc-1 through the α 1-3 linkage. From these results, the structures of PA-oligosaccharides A, B, C, and D were proposed to be as shown in Scheme 1.

Glycosylation Sites of Cry j I Protein—To determine the

PA-Oligosaccharide	Chemical shifts of anomeric protons ^a										
	GlcNAc-2	Man-3	Man-4	Man-4'	GlcNAc-5	GlcNAc-5'	Gal-6	Gal-6'	Fuc-1 ^b	Fuc-2 ^b	Xyl
X3.4 ^c	4.592	4.857	5.140	4.899	4.511	4.536	— ^d	—	5.048	—	4.425
A	4.596	4.849	5.139	4.897	4.512	4.544	—	—	5.042	—	4.427
B	4.630	4.860	5.137	4.898	4.513	4.579	—	4.503	—	5.005	4.415
X3.5 ^c	4.591	4.855	5.139	4.900	4.511	4.576	—	4.499	5.047	5.000	4.424
C	4.592	4.849	5.139	4.901	4.514	4.578	—	4.499	5.042	5.001	4.427
X3.6 ^c	4.592	4.856	5.139	4.901	4.533	4.576	4.490	4.499	5.048	4.999	4.423
D	4.593	4.853	5.139	4.900	4.534	4.578	4.491	4.498	5.045	5.001	4.424

PA-Oligosaccharide	Chemical shifts of methyl protons ^a				
	GlcNAc-2	GlcNAc-5	GlcNAc-5'	Fuc-1 ^b	Fuc-2 ^b
X3.4 ^c	2.043	2.043	2.043	1.197	—
A	2.044	2.044	2.044	1.192	—
B	2.041	2.041	2.041	—	e
X3.5 ^c	2.042	2.042	2.042	1.195	1.170
C	2.043	2.043	2.043	1.191	1.171
X3.6 ^c	2.042	2.042	2.042	1.197	1.170
D	2.041	2.041	2.041	1.195	1.170

^aChemical shifts are expressed in ppm from internal DSS but were actually measured with internal acetone ($\delta = 2.216$ ppm in D₂O at 30°C). ^bFuc-1 represents a fucose residue bound to the innermost GlcNAc-1. Fuc-2 is a residue bound to outer chain(s) GlcNAc-5 and/or GlcNAc-5'. ^cData from Ref. 18. ^dSignals were not detected. ^eSignal was not detected due to the limited amount of sample.

PA-	Oligosaccharide structure	Relative amount (mol%)
A	GlcNAc β 1-2Man α 1 GlcNAc β 1-2Man α 1 Xyl β 1 Man β 1-4GlcNAc β 1-4GlcNAc Fuc α 1	50
B	Fuc α 1 Gal β 1-4GlcNAc β 1-2Man α 1 GlcNAc β 1-2Man α 1 Xyl β 1 Man β 1-4GlcNAc β 1-4GlcNAc	6
C	Fuc α 1 Gal β 1-4GlcNAc β 1-2Man α 1 GlcNAc β 1-2Man α 1 Xyl β 1 Man β 1-4GlcNAc β 1-4GlcNAc Fuc α 1	37
D	Fuc α 1 Gal β 1-4GlcNAc β 1-2Man α 1 Gal β 1-4GlcNAc β 1-2Man α 1 Xyl β 1 Man β 1-4GlcNAc β 1-4GlcNAc Fuc α 1	6

Scheme 1. Proposed structures of the asparagine-linked oligosaccharides obtained from Cry *i* I.

ment T-1 and the 14th residue of fragment T-2 (X) were unidentifiable; that is, the corresponding phenylthiohydantoin amino acid could not be extracted. In comparison with the whole amino acid sequence of the *Cry j* I protein (20), the 9th residue of fragment T-1 was Asn-333 and the 14th residue of fragment T-2 was Asn-170. The amino acid sequences of ³³³Asn-Ala-Thr- and ¹⁷⁰Asn-Ser-Ser- were consistent with that of the Asn-X-Ser/Thr motif which represents a possible *N*-glycosylation site. By sugar com-

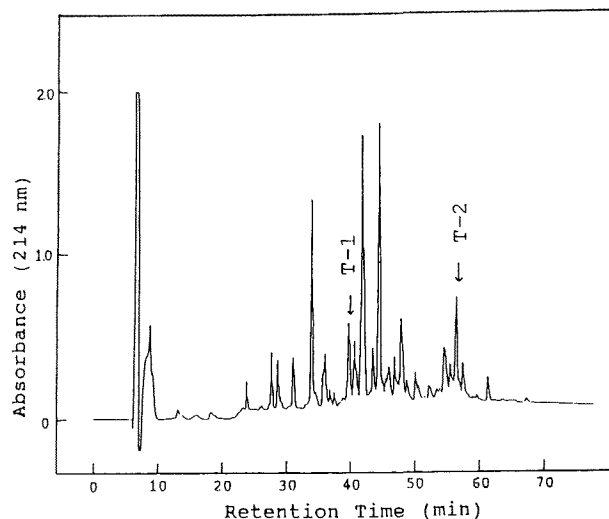


Fig. 4. HPLC profile of trypsin-digested *Cry j* I on a reversed-phase HPLC. Two fragments, T-1 and T-2, containing neutral sugar were obtained.

position analysis, fragments T-1 and T-2 were both found to contain galactose, fucose, mannose, xylose, and *N*-acetylglucosamine (data not shown). These results indicate that Asn-170 and Asn-333 were both *N*-glycosylated. To analyze the glycoforms of the two glycosylated peptides, fragments T-1 and T-2 were digested with glycopeptidase and the oligosaccharides released were pyridylaminated. The PA-oligosaccharides obtained from T-1, T-2, and *Cry j* I were analyzed on an Amide-80 column. The elution patterns of the PA-oligosaccharides obtained from the two fragments were similar with those obtained from *Cry j* I (Fig. 5). In addition, the molar ratio of PA-oligosaccharides A-D was 8 : 1 : 6 : 1 in each elution pattern. This indicates that the glycoforms of T-1 and T-2 were the same as those of *Cry j* I.

DISCUSSION

Analyses using a combination of the two-dimensional sugar mapping technique and $^1\text{H-NMR}$ spectroscopy established that the PA-oligosaccharides released from the glycoprotein allergen *Cry j* I have xylose-containing complex-type structures (Scheme 1). Analysis of the PA-oligosaccharides by HPLC using a TSKgel Amide-80 column showed four oligosaccharides, A, B, C, and D, to exist at a ratio of 8 : 1 : 6 : 1. Several allergens containing carbohydrate moieties have been isolated from other plants (5), but a complete structural characterization of the oligosaccharide has been reported only for the glycoprotein allergen *Art v* II from the pollen of mugwort (6). Oligosaccharides from *Art v* II contain a mixture of high-mannose oligosaccharides ($\text{Man}_{5-9}\text{GlcNAc}_2$); however, in *Cry j* I, oligosaccharides having these structures were not found. Xylose-containing complex-type oligosaccharides linked to asparagine residues have been reported in numerous glycoproteins of plant origin (18, 21-23). These glycoproteins contain the oligosaccharides M3X and/or M3FX. However, neither the oligosaccharide M3X nor M3FX itself was found in *Cry j* I. Moreover, to our knowledge, our laboratory was the first to identify the structure of oligosaccharide B (shown in

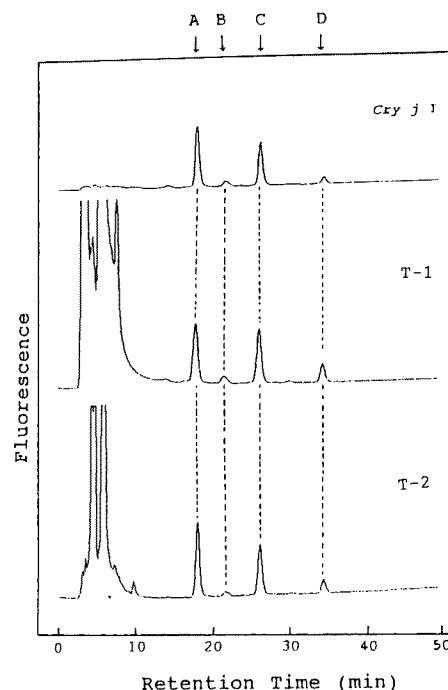


Fig. 5. HPLC profiles of PA-oligosaccharides obtained from *Cry j* I, T-1, and T-2 on an Amide-80 column. Arrows indicate the elution positions of PA-oligosaccharides A-D. The peaks eluted between 2 and 12 min are due to contamination.

Scheme 1) as $\text{Gal}\beta 1-4(\text{Fuc}\alpha 1-6)\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-6(\text{GlcNAc}\beta 1-2\text{Man}\alpha 1-3)(\text{Xyl}\beta 1-2)\text{Man}\beta 1-4\text{GlcNAc}\beta 1-4\text{GlcNAc-PA}$.

No difference in glycoforms was observed between the oligosaccharides bound to Asn-170 and Asn-333. The cDNA analyses by Sone *et al.* (20) and Namba *et al.* (manuscript in preparation) indicate that *Cry j* I has at least four possible *N*-glycosylation sites. In this study, Asn-170 and Asn-333 were confirmed to be similarly glycosylated.

Concerning the relationship between the oligosaccharides linked to glycoproteins and their antigenicity, Kurosaka *et al.* (24) demonstrated in inhibition experiments that a fucose residue bound to the innermost GlcNAc-1 through an $\alpha 1-3$ linkage greatly contributed to the antigenicity of horseradish peroxidase in rabbits. Three oligosaccharides derived from *Cry j* I, A, C, and D, contain the $\alpha 1-3$ linked fucose residues. Therefore, it is reasonable to speculate that these oligosaccharides may have antigenicity, although experiments to examine this possibility were not carried out in this study.

Nilsen *et al.* (6) reported that native *Art v* II contains at least two IgE-binding epitopes, the major one responsible for binding to about 70% of *Art v* II-specific human IgE antibodies and the other epitope(s) responsible for about 30% of the IgE antibodies. Furthermore, they showed that the former is destroyed when the allergen is denatured with SDS and 2-mercaptoethanol, and that the latter is resistant to denaturation and deglycosylation. IgE-binding epitopes on pollen allergens are reported to be generally conformational (7, 8). The importance of the carbohydrate moieties for IgE-binding has been pointed out (4-9). Tanai *et al.* demonstrated that IgE antibodies in the sera of

French patients allergic to *Cupressus sempervirens* bound to the glycosylated, but not to the unglycosylated, peptide Asn-328-Arg-353 containing Asn-333 derived from enzyme-digested Cry j I (4), although the binding strength was reduced to about 10% that of native Cry j I. These results suggest that the cross-reactive IgE antibodies from the French patients recognize a minor IgE-binding epitope which is partially resistant to peptic digestion but completely dependent on the carbohydrate linked to Asn-333. Further studies to clarify the biological roles of the carbohydrate moieties of this allergen are in progress in our laboratory.

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REFERENCES

1. Yasueda, H., Yui, Y., Shimizu, T., and Shida, T. (1983) Isolation and characterization of the major allergen from Japanese cedar (*Cryptomeria japonica*) pollen. *J. Allergy Clin. Immunol.* **71**, 77-86
2. Sakaguchi, M., Inouye, S., Taniai, M., Ando, S., Usui, M., and Matuhasi, T. (1990) Identification of the second major allergen of Japanese cedar pollen. *Allergy* **45**, 309-312
3. Taniai, M., Ando, S., Usui, M., Kurimoto, M., Sakaguchi, M., Inouye, S., and Matuhasi, T. (1988) N-terminal amino acid sequence of major allergen of Japanese cedar pollen (Cry j I). *FEBS Lett.* **239**, 329-332
4. Taniai, M., Kayano, T., Takakura, R., Yamamoto, S., Usui, M., Ando, S., Kurimoto, M., Panzani, R., and Matuhasi, T. (1993) Epitopes on Cry j I and Cry j II for the human IgE antibodies cross-reactive between *Cupressus sempervirens* and *Cryptomeria japonica* pollen. *Mol. Immunol.* **30**, 183-189
5. Haavik, S., Paulsen B.S., and Wold, J.K. (1985) Glycoprotein allergens in pollen of timothy. I. Investigation of carbohydrates extracted from pollen of timothy (*Phleum pratense*) and purification of a carbohydrate-containing allergen by affinity chromatography on concanavalin A-Sepharose. *Int. Arch. Allergy Appl. Immunol.* **78**, 197-205
6. Nilsen, B.M., Sletten, K., Paulsen, B.S., O'Neill, M., and van Halbeek, H. (1991) Structural analysis of the glycoprotein allergen Art v II from the pollen of mugwort (*Artemisia vulgaris* L.). *J. Biol. Chem.* **266**, 2660-2668
7. Duffort, O.A., Carreira, J., Nitti, G., Polo, F., and Lombardero, M. (1991) Studies on the biochemical structure of the major cat allergen *Felis domesticus* I. *Mol. Immunol.* **28**, 301-309
8. Nilsen, B.M. and Paulsen, B.S. (1986) Allergens in pollen from Mugwort (*Artemisia vulgaris* L.) I. Characterization of a crude and a partly purified extract of Mugwort pollen with particular emphasis on the glycoprotein allergen Ag7. *Int. Arch. Allergy Appl. Immunol.* **80**, 113-121
9. Sward-Nordmo, M., Paulsen, B.S., and Wold, J.K. (1988) The glycoprotein Ag-54 (Cla h II) from *Cladosporium herbarum*. Further biochemical characterization. *Int. Arch. Allergy Appl. Immunol.* **85**, 295-301
10. Hotta, T., Ishii, I., Ishihara, H., Tejima, S., Tarutani, O., and Takahashi, N. (1985) Comparative study of the oligosaccharides of human thyroglobulins obtained from normal subjects and patients with various diseases. *J. Appl. Biochem.* **7**, 98-103
11. Monsigny, M., Petit, C., and Roche, A.C. (1988) Colorimetric determination of neutral sugars by a resorcinol sulfuric acid micromethod. *Anal. Biochem.* **175**, 525-530
12. Hase, S., Ibuki, T., and Ikenaka, T. (1984) Reexamination of the pyridylation used for fluorescence labeling of oligosaccharides and its application to glycoproteins. *J. Biochem.* **95**, 197-203
13. Takemoto, H., Hase, S., and Ikenaka, T. (1985) Microquantitative analysis of neutral and amino sugars as fluorescent pyridyl-amino derivatives by high-performance liquid chromatography. *Anal. Biochem.* **145**, 245-250
14. Suzuki, J., Kondo, A., Kato, I., Hase, S., and Ikenaka, T. (1991) Analysis by high-performance anion-exchange chromatography of component sugars as their fluorescent pyridylamino derivatives. *Agric. Biol. Chem.* **55**, 283-284
15. Tomiya, N., Awaya, J., Kurono, M., Endo, S., Arata, Y., and Takahashi, N. (1988) Analysis of N-linked oligosaccharides using a two-dimensional mapping technique. *Anal. Biochem.* **171**, 73-90
16. Friedman, M., Krull, L.H., and Cavins, J.F. (1970) The chromatographic determination of cystine and cysteine residues in proteins as s-β-(4-pyridylethyl)cysteine. *J. Biol. Chem.* **245**, 3868-3871
17. Takahashi, N. and Muramatsu, T. (1992) *Handbook of Endoglycosidases and Glycoamidases* pp. 199-322, CRC Press, Florida
18. Takahashi, N., Hotta, T., Ishihara, H., Mori, M., Tejima, S., Bligny, R., Akazawa, T., Endo, S., and Arata, Y. (1986) Xylose-containing common structure unit in N-linked oligosaccharides of laccase from Sycamore cells. *Biochemistry* **25**, 388-395
19. Koyama, S., Daiyasu, H., Hase, S., Kobayashi, Y., Kyogoku, Y., and Ikenaka, T. (1986) ¹H-NMR analysis of the sugar structures of glycoproteins as their pyridylamino derivatives. *FEBS Lett.* **209**, 265-268
20. Sone, T., Komiya, N., Shimizu, K., Kusakabe, T., Morikubo, K., and Kino, K. (1994) Cloning and sequencing of cDNA coding for Cry j I, a major allergen of Japanese cedar pollen. *Biochem. Biophys. Res. Commun.* **199**, 619-625
21. Ishihara, H., Takahashi, N., Oguri, S., and Tejima, S. (1979) Complete structure of the carbohydrate moiety of stem bromelain. *J. Biol. Chem.* **254**, 10715-10719
22. Hase, S., Koyama, S., Daiyasu, H., Takemoto, H., Hara, S., Kobayashi, Y., Kyogoku, Y., and Ikenaka, T. (1986) Structure of a sugar chain of a protease inhibitor isolated from Barbados pride (*Caesalpinia pulcherrima* Sw.). *J. Biochem.* **100**, 1-10
23. Kimura, Y., Hase, S., Kobayashi, Y., Kyogoku, Y., Ikenaka, T., and Funatsu, G. (1988) Structures of sugar chains of Ricin D. *J. Biochem.* **103**, 944-949
24. Kurosaka, A., Yano, A., Itoh, N., Kuroda, Y., Nakagawa, T., and Kawasaki, T. (1991) The structure of a neural specific carbohydrate epitope of horseradish peroxidase recognized by anti-horseradish peroxidase antiserum. *J. Biol. Chem.* **266**, 4168-4172