

Glycoform Analysis of Japanese Cypress Pollen Allergen, Cha o 1: A Comparison of the Glycoforms of Cedar and Cypress Pollen Allergens

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A Japanese cypress (*Chamaecyparis obtusa*) pollen allergen, Cha o 1, is one of the major allergens that cause allergic pollinosis in Japan. Although it has been found that Cha o 1 is glycosylated and that the amino acid sequence is highly homologous with that of Japanese cedar pollen allergen (Cry j 1), the structure of *N*-glycans linked to Cha o 1 remains to be determined. In this study, therefore, we analyzed the structures of the *N*-glycans of Cha o1. The *N*-glycans were liberated by hydrazinolysis from purified Cha o 1, and the resulting sugar chains were *N*-acetylated and pyridylaminated. The structures of pyridylaminated *N*-glycans were analyzed by a combination of exoglycosidase digestion, two dimensional (2D-) sugar chain mapping, and electrospray ionization mass spectrometry analysis. Structural analysis indicated that the major *N*-glycan structure of Cha o1 is GlcNAc₂Man₃Xyl₁-Fuc₁GlcNAc₂ (89%), and that high-mannose type structures (Man₉GlcNAc₂, Man₇GlcNAc₂) occur as minor components (11%).

Key words: *N*-glycan structure; antigenic oligosaccharide; Japanese cypress pollen allergen; Cha o 1; *Chamaecyparis obtusa*

In previous studies,^{1,2)} we determined the chemical structures of *N*-glycans linked to two cedar pollen

allergens, Cry j 1 (Japanese cedar pollen allergen) and Jun a 1 (mountain cedar pollen allergen), and revealed that biantennary plant complex type *N*-glycans harboring Lewis a epitope occur in their *N*-glycan moieties. This finding suggests that the Lewis a epitope may play a critical role in pollinosis, but the occurrence of this epitope in plant glycoallergens has been reported from only three pollen allergens, Cup a 1 (pollen allergen from Arizona cypress),³⁾ Cry j 1, and Jun a 1. Japanese cypress pollen is another major allergic pollen in Japan, and Cha o 1 is a major allergen contained in the cypress pollens. Although it is known that Cha o 1 is *N*-glycosylated,⁴⁾ the detailed chemical structures of the *N*-glycan moiety remain to be determined. Hence, in this study, we analyzed the structures of *N*-glycans linked to the Japanese cypress pollen allergen Cha o 1 to determine whether plant specific antigenic oligosaccharide or Lewis a epitope occurs in the allergen. First, we purified Cha o 1 from an extract of the cypress pollens by a combination of ion-exchange chromatography and gel-filtration, using an antiserum against Cha o 1 to monitor the elution position of the allergen. We found that Cha o 1 occurred in two forms, with the same *N*-terminal amino acid sequence but different molecular weights, suggesting that the numbers of the *N*-glycosylation sites might be different from each other. Furthermore, structural analysis of *N*-glycans revealed

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Abbreviations: PA-, pyridylamino; RP-HPLC, reverse-phase HPLC; SF-HPLC, size-fractionation HPLC; ESI-MS, electrospray ionization mass spectrometry; CBB, Coomassie Brilliant Blue; Hex, hexose; HexNAc, *N*-acetylhexosamine; Deoxyhex, deoxyhexose; Pen, pentose; 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; MFX, Xyl β 1-2Man β 1-4GlcNAc β 1-4(Fuc α 1-3)GlcNAc-PA; GN2M3FX, GlcNAc β 1-2Man 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; M9A, Man α 1-2Man α 1-6(Man α 1-2Man α 1-3)-Man α 1-6(Man α 1-2Man α 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA; M7A, Man α 1-2Man α 1-6(Man α 1-3)Man α 1-6(Man α 1-2Man α 1-3)Man β 1-4GlcNAc β 1-4GlcNAc-PA

that Cha o 1 bears both a biantennary complex type and a high-mannose type oligosaccharide. The predominant occurrence of the antigenic plant complex type structure, GlcNAc₂Man₃Xyl₁Fuc₁GlcNAc₂ (GN2M3FX), is a characteristic common to allergenic pollen glycoproteins.¹⁻³⁾ But the Lewis a unit was not found in the *N*-glycans of Cha o 1, in contrast with Cup a 1, Cry j 1, and Jun a 1, indicating that the Lewis a epitope is not involved in the symptoms of pollinosis.

Materials and Methods

Materials. An Asahipak NH2P-50 4E column (0.46 × 25 cm) was purchased from Showa Denko (Tokyo, Japan), and a Cosmosil 5C18-AR column (0.6 × 25 cm) from Nacalai Tesque (Kyoto, Japan). GN2M3FX were prepared from glycoproteins from rice culture cells.⁵⁾ M7A and M9A were prepared from royal jelly glycoproteins.⁶⁾ Antiserum against Cha o 1 was a generous gift of Meiji Co. Antiserum against β 1-2 xylose-containing *N*-glycans was a generous gift of Dr. Arnd Strum (Friedrich-Miescher Institute, Basel, Switzerland).⁷⁾

Purification of Cha o 1. Purification of Cha o 1 was basically done as described in a previous paper.⁴⁾ During the purification steps, the elution of Cha o 1 was monitored by immunoblotting assay using antiserum against Cha o 1. Japanese cypress pollen (100 g) was defatted in acetone (500 ml), and the resulting defatted pollen was suspended in 125 mM (NH₄)₂CO₃ and sonicated in an ice bath for 5 min. After incubation for 16 h at 4 °C, the extract was centrifuged at 8,000 rpm for 20 min, and the resulting supernatant was 80% saturated with ammonium sulfate. The protein precipitate was dissolved in a small amount of 10 mM Tris-HCl buffer,

pH 7.8, and dialyzed against the same buffer (5-liter) for 2 d. After centrifugation, the resulting supernatant (total OD_{280 nm}, A_{280 nm} × sample volume = 717.60) was applied to a DEAE cellulose column (4 × 40 cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.8. The run-through fraction was pooled and dialyzed against 10-liter (5-liter × 2 times) of 10 mM Na-acetate buffer, pH 5.0, containing 0.1 M NaCl for 2 d. The dialyze was applied to a SP-Toyopearl (3 × 22 cm) column equilibrated with the same buffer. After the column was washed with the same buffer (about 600 ml), bound proteins were eluted by 10 mM Na-acetate buffer, pH 5.0, containing 0.5 M NaCl. The bound fraction (the Cha o 1 fraction) was dialyzed against 10-liter (5-liter × 2 times) of 10 mM Na-acetate buffer, pH 5.0, containing 0.1 M NaCl for 2 d, and the resulting dialysate (total OD_{280 nm} = 29.68) was applied to the SP-Toyopearl (3 × 22 cm) column equilibrated with the same buffer. In a second cation-exchange chromatography, the bound proteins were eluted by a linear gradient of NaCl from 0.1 M to 0.5 M in 10 mM Na-acetate buffer, pH 5.0. The Cha o 1 fraction, indicated by a horizontal bar in Fig. 1-A, was pooled and concentrated to about 7 ml by Amicon Centriprep-30. The total OD_{280 nm} of the concentrated sample was 5.26. Cha o 1 was further purified by gel-filtration with a Superdex 200 column (GE-Healthcare, 1.6 × 120 cm) in 25 mM Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl. As shown in Fig. 1-B, Cha o 1 was separated from some other contaminative proteins, but two protein bands (around 47 kDa) were detected on the SDS-gel by CBB staining (Fig. 2-I) and on the PVDF membrane by immunoblotting using the antiserum against Cha o 1 (Fig. 2-II), suggesting that Cha o 1 occurs in several isoforms, as reported by Suzuki *et al.*⁴⁾ Furthermore, as shown in Fig. 2-III, the purified Cha o 1 was recognized by the

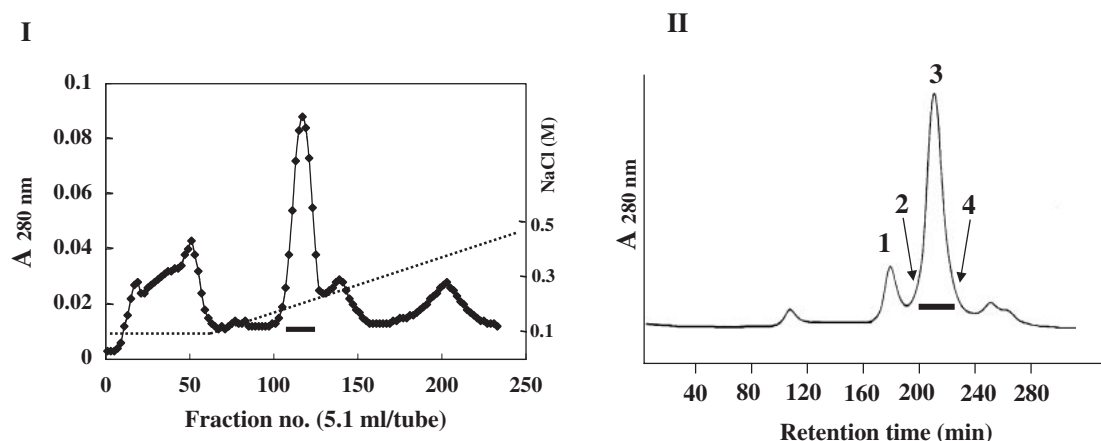


Fig. 1. Purification of Cha o 1.

I, SP-Toyopearl chromatography of partially purified Cha o 1. Partially purified Cha o 1 was loaded on a SP-Toyopearl column in 10 mM Na-acetate buffer, pH 5.0. The allergen was eluted by a linear gradient of NaCl from 0.1 M to 0.5 M in 10 mM Na-acetate buffer, pH 5.0. The Cha o 1-containing fraction, detected by immunoblotting, was pooled, as indicated by a horizontal bar. II, Gel-filtration of the Cha o 1 fraction obtained in I. The concentrated Cha o 1 fraction obtained by SP-Toyopearl chromatography was loaded on a Superdex 200 column (1.6 × 120 cm). The column was developed with 25 mM Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl at a flow rate of 0.8 ml/min.

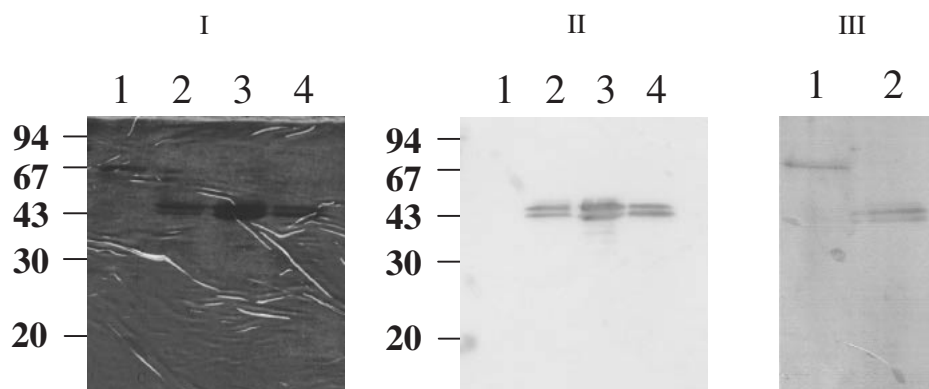


Fig. 2. SDS-PAGE and Immunoblotting.

I, CBB staining of 20% polyacrylamide gel. Lane 1, peak 1 in Fig. 1-II; lane 2, position 2 in Fig. 1-II; lane 3, peak 3 in Fig. 1-II; lane 4, position 4 in Fig. 1-II. II, Immunoblotting using anti-Cha o 1 antibody. Lane 1, peak 1 in Fig. 1-II; lane 2, position 2 in Fig. 1-II; lane 3, peak 3 in Fig. 1-II; lane 4, position 4 in Fig. 1-II. III, Immunoblotting using anti- β 1-2xylose antibody. Lane 1, peak 1 in Fig. 1-II; lane 2, peak 3 in Fig. 1-II. Each immunoblot was done as described in previous papers.^{4,8)}

anti- β 1-2 xylose antibody,^{7,8)} indicating that Cha o 1 bears plant-complex type N-glycans. After two protein bands were cut on PVDF membrane separately, each N-terminal amino acid sequence was analyzed, and it was found that the two proteins had the same N-terminal sequence (D-N-P-I-D-), indicating that they were Cha o 1. Based on these results, we used the Cha o 1 fraction, indicated by a horizontal bar in Fig. 1-II, in glycoform analysis without further purification.

Preparation of pyridylaminated N-glycans from Cha o 1. N-Glycans were released by hydrazinolysis (100°C, 12 h, in 200 μ l of anhydrous hydrazine) from the lyophilized Cha o 1 (2.6 mg). After N-acetylation of the hydrazinolysate with saturated ammonium bicarbonate (400 μ l) and acetic anhydride (20 μ l), the acetylated hydrazinolysate was desalted using Dowex 50 $\times$ 2 resins. Pyridylation of the sugar chains was done by the method of Natsuka and Hase.⁹⁾ Separation of PA-sugar chains was done by HPLC on a Jasco 880-PU HPLC apparatus with a Jasco 821-FP Intelligent Spectrofluorometer, using the Shodex Asahipak NH2P-50 column (0.46 $\times$ 25 cm) and the Cosmosil 5C18-AR column (0.6 $\times$ 25 cm). On the Cosmosil 5C18-AR column, the PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.05% TFA linearly from 0 to 10% at a flow rate 1.2 ml/min. In the case of size-fractionation HPLC using the Asahipak NH2P-50 column, the PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 36% to 62% linearly for 60 min at a flow rate of 0.7 ml/min.

Electrospray ionization (ESI) mass spectrometry. ESI-MS analysis of PA-sugar chains was done as described in our previous reports,^{5,6)} using a Perkin Elmer Sciex API-III triple-quadrupole mass spectrometer with an atmospheric-pressure ionization ion source.

Glycosidase digestion of PA-sugar chains. Digestion with jack bean α -mannosidase, diplococcal β -N-acetylglucosaminidase, and *Aspergillus* α -1,2-mannosidase was done using about 200 pmol of the PA-sugar chains under the conditions described in our previous reports.^{5,10)} The resulting glycosidase-digests were analyzed by SF-HPLC using the Asahipak NH2P-50 column (0.46 $\times$ 25 cm).

Results and Discussion

Purification of PA-sugar chains

First, the PA-sugar chains from Cha o 1 were partially purified by RP-HPLC, as shown in Fig. 3. Three peaks

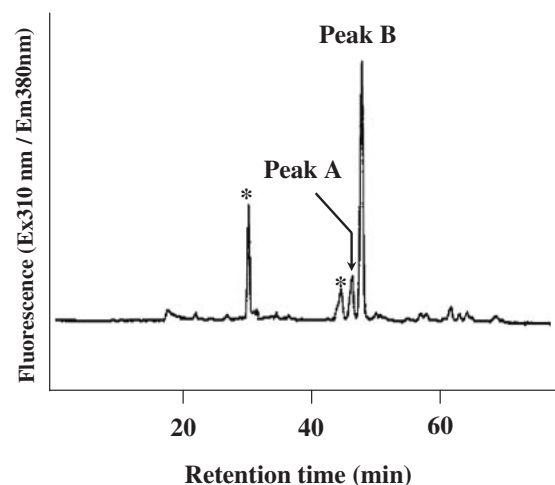


Fig. 3. RP-HPLC Profiles of PA-Sugar Chains from Cha o 1.

Peak A and peak B were confirmed to be relevant N-glycans by ESI-MS, and were used for structural analysis. PA-derivatives were loaded on the Cosmosil 5C 18-AR column (6.0 $\times$ 250 mm) equilibrated with 0.05% TFA. The PA-sugar chains were eluted by an increase in acetonitrile concentration. Star mark (*) shows contaminative peaks.

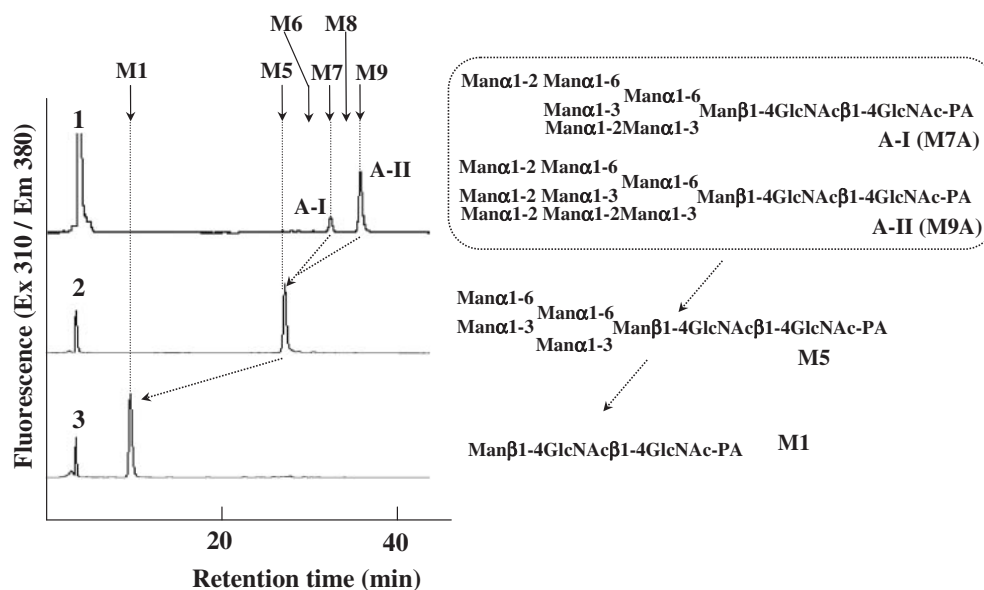


Fig. 4. SF-HPLC of Exoglycosidase Digests of Peak A.

1, PA-sugar chains in peak A in Fig. 3; 2, the *Aspergillus* α -1,2-mannosidase digest of 1; 3, the jack bean α -mannosidase digest of 2. Arrows (M1, M5, M6, M7, M8, and M9) indicate the elution positions of authentic PA-sugar chains: Man1GlcNAc2-PA, Man5GlcNAc2-PA, Man6GlcNAc2-PA, Man7GlcNAc2-PA, Man8GlcNAc2-PA, and Man9GlcNAc2-PA.

were observed in the region where *N*-glycans are expected to be eluted (40–70 min), but it was determined by exoglycosidase digestion that peaks A and B were relevant pyridylaminated *N*-glycans. A small peak eluted just before peak A was not digested by some exoglycosidases, suggesting that this peak might not be *N*-glycan, although MS/MS analysis of it to confirm the presence of GlcNAc-PA (m/z 300) could not be done due to the small amount of the sample.

Structures of PA-sugar chains peak A

When peak A in Fig. 3 was analyzed by SF-HPLC, two PA-sugar chains were detected, as shown in Fig. 4-1. The elution positions of these two PA-sugar chains corresponded to those of authentic PA-sugar chains: A-I to Man7GlcNAc2 and A-II to Man9GlcNAc2-PA. Furthermore, the elution positions of A-I and A-II on RP-HPLC corresponded to those of M7A and M9A respectively. When these PA-sugar chains were treated with *Aspergillus* α -1,2-mannosidase, a new product was eluted at the elution position of M5A (Man5GlcNAc2-PA), suggesting that A-I and A-II contained two and four α 1-2 mannose residues respectively (Fig. 4-2). The product was further converted to M1 (Man1GlcNAc2-PA) by jack bean α -mannosidase, as shown in Fig. 4-3.

From these results, the structures of A-I and A-II were proposed to be M7A and M9A, respectively, as shown in Fig. 4.

Structural analysis of *N*-glycans in peak B

As shown in Fig. 5-I, only one PA-sugar chain was detected in peak B in Fig. 3. The elution position

corresponded to that of GN2M3FX. The elution position of this PA-sugar chain on RP-HPLC also corresponded to that of GN2M3FX (data not shown), suggesting that the structure of this *N*-glycan is the biantennary plant complex type structure, GlcNAc2Man3Xyl1Fuc1GlcNAc2. ESI-MS analysis of peak B showed a single signal at m/z 1674.5 [(M + H)⁺] (Fig. 5-II), suggesting that this PA-sugar chain consisted of (HexNAc)3 (Hex)3(Deoxyhex)1(Pen)1(HexNAc-PA) or GlcNAc2-Man3Xyl1Fuc1GlcNAc2-PA. The deduced structure was further confirmed by exoglycosidase digestion. This PA-sugar chain was converted to M3FX with diplococcal β -*N*-acetylglucosaminidase, suggesting that two GlcNAc residues were bound by a β 1-2 linkage (Fig. 5-I-2). The product was further converted to MFX by jack bean α -mannosidase digestion (Fig. 5-I-3). The data of 2-D sugar chain mapping, exoglycosidase digestion, and ESI-MS analysis suggested that the structure of peak B was GN2M3FX, as shown in Fig. 5.

Comparison of structural feature of *N*-glycans linked to cedar pollen allergen (*Jun a 1* and *Cry j 1*) and cypress pollen allergen (*Cha o 1*)

As shown in Table 1, we have found that the cedar pollen allergens (*Jun a 1* and *Cry j 1*) carry plant-complex type *N*-glycans containing the Lewis a antigen (Gal β 1-3(Fuc α 1-4)GlcNAc β 1-) unit.^{1,2)} In addition to these two cedar pollen allergens, it has been reported that Arizona cypress pollen allergen, *Cup a 1*, also bears the Lewis a unit in the *N*-glycan moiety.³⁾ Although the physiological function of the Lewis a unit in plant *N*-glycans is still obscure, there is a possibility that the antigenic unit is involved in the symptoms of pollinosis.

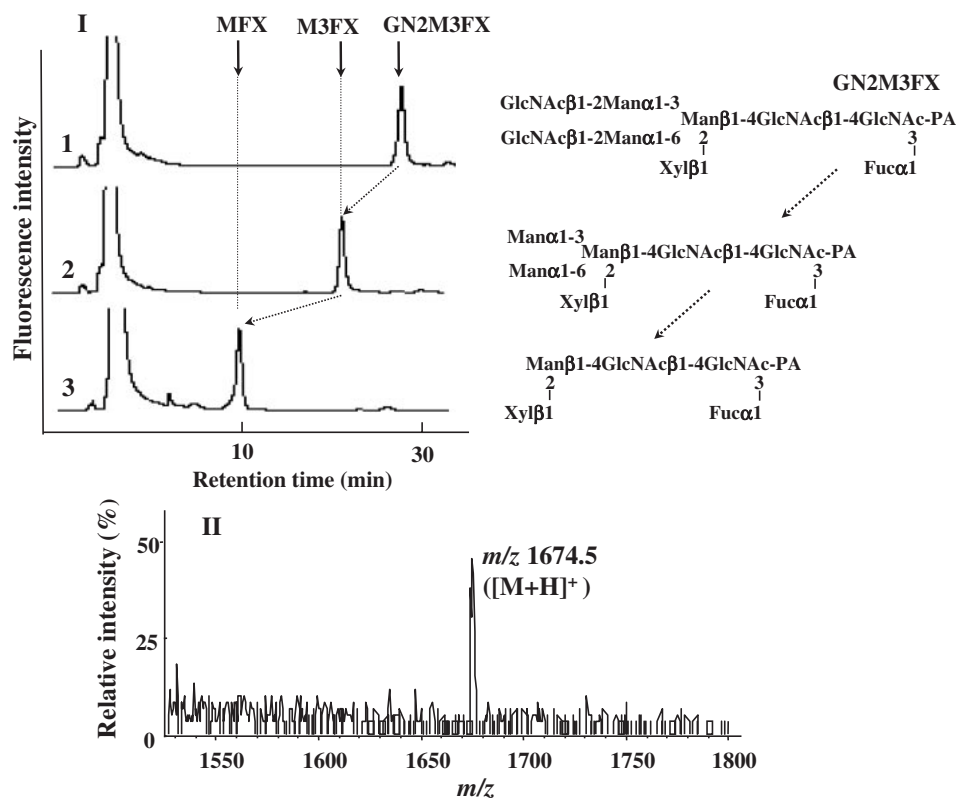


Fig. 5. SF-HPLC of Exoglycosidase Digests of Peak B and ESI-MS Spectrum.

I, SF-HPLC of exoglycosidase digests of peak B. 1, PA-Sugar chain in peak B in Fig. 3; 2, the diplococcal β -GlcNAc-ase digest of I; 3, the jack bean α -mannosidase digest of 2. II, ESI-MS spectrum of Peak B. The PA-sugar chain was observed as a single charged ion, $[M + H]^+$.

Table 1. Comparison of N-Glycan Structures of Two Cedar Pollen Allergens (Jun a 1, Cry j 1) and Cha o 1

Structures of N-Glycans	Jun a 1-A ^a	Jun a 1-B ^a	Cry j 1 ^b	Cha o 1
GlcNAc β 1-2 $\left\{ \begin{array}{l} \text{Man}\alpha 1-6 \\ \text{Man}\alpha 1-3 \end{array} \right.$ Man β 1-4GlcNAc β 1-4GlcNAc Xyl β 1 $\left\{ \begin{array}{l} 2 \\ 1 \end{array} \right.$ Fuc α 1 $\left\{ \begin{array}{l} 3 \\ 1 \end{array} \right.$	ND	3%	ND	ND
GlcNAc β 1-2Man α 1-6 GlcNAc β 1-2Man α 1-3 Xyl β 1 Fuc α 1 Gal β 1-3GlcNAc β 1-2 $\left\{ \begin{array}{l} \text{Man}\alpha 1-6 \\ \text{Man}\alpha 1-3 \end{array} \right.$ Man β 1-4GlcNAc β 1-4GlcNAc Xyl β 1 $\left\{ \begin{array}{l} 2 \\ 1 \end{array} \right.$ Fuc α 1 $\left\{ \begin{array}{l} 3 \\ 1 \end{array} \right.$	75%	76%	47%	89%
Gal β 1-3GlcNAc β 1-2 $\left\{ \begin{array}{l} \text{Man}\alpha 1-6 \\ \text{Man}\alpha 1-3 \end{array} \right.$ Man β 1-4GlcNAc β 1-4GlcNAc Xyl β 1 $\left\{ \begin{array}{l} 2 \\ 1 \end{array} \right.$ Fuc α 1 $\left\{ \begin{array}{l} 3 \\ 1 \end{array} \right.$	23%	21%	38%	ND
Gal β 1-3GlcNAc β 1-2 $\left\{ \begin{array}{l} \text{Man}\alpha 1-6 \\ \text{Man}\alpha 1-3 \end{array} \right.$ Man β 1-4GlcNAc β 1-4GlcNAc Xyl β 1 $\left\{ \begin{array}{l} 2 \\ 1 \end{array} \right.$ Fuc α 1 $\left\{ \begin{array}{l} 3 \\ 1 \end{array} \right.$	2%	ND	15%	ND
Man α 1-2Man α 1-6 Man α 1-2Man α 1-3 Man α 1-2Man α 1-2Man α 1-3 Man α 1-2Man α 1-6 Man α 1-3 Man α 1-2Man α 1-3 $\left\{ \begin{array}{l} \text{Man}\alpha 1-6 \\ \text{Man}\alpha 1-3 \end{array} \right.$ Man β 1-4GlcNAc β 1-4GlcNAc	ND	ND	ND	9%
Man α 1-2Man α 1-6 Man α 1-3 Man α 1-2Man α 1-3 $\left\{ \begin{array}{l} \text{Man}\alpha 1-6 \\ \text{Man}\alpha 1-3 \end{array} \right.$ Man β 1-4GlcNAc β 1-4GlcNAc	ND	ND	ND	2%

a, reference 1; b, reference 2

Hence, in this study, we analyzed the glycoform of *N*-glycans of Japanese cypress pollen allergen, Cha o 1, one of the major pollen glycoallergens in Japan. Molecular cloning of Cha o 1 revealed that this Japanese cypress pollen allergen is highly homologous with the two cedar pollen allergens (Cry j 1 and Jun a 1), and that there are six Asn-X-Thr/Ser sequences.⁴⁾ Although two (Asn(259)-Pro-Thr(261) and Asn(272)-Asp-Thr(274)) of them might not be glycosylated, it is not known whether the remaining four *N*-glycosylation sites are actually glycosylated. As described in a previous report,⁴⁾ Cha o 1 was purified in two isoforms with the same *N*-terminal amino acid sequence (D-N-D-I-P) but different molecular weights (Fig. 2-I), and these two isoforms were recognized by the antiserum against plant complex type *N*-glycans (Fig. 2-III). The difference in molecular weight of the two Cha o 1 molecules might reflect the difference in the numbers of actually glycosylated sites in the four *N*-glycosylation consensus sequences.

Structural analysis of *N*-glycans revealed that the complex type *N*-glycan harboring the Lewis a epitope did not occur in Cha o 1 and that major structure was the biantennary plant complex type structure: GlcNAc β 1-2Man 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 (GN2M3FX) (Fig. 5 and Table 1). This result suggest that the Lewis a epitope in the *N*-glycan moiety is not involved in the symptoms of pollinosis. It is noteworthy, however, that a high content of the GM2M3FX structure is a common feature of the *N*-glycans of allergenic pollen glycoproteins (Jun a 1, Cry j 1, Cup a 1, olive pollen allergen Ole e 1, *Ginkgo biloba* pollen glycoprotein, and palm pollen glycoprotein).^{1-3,11-13)} Comparing the cedar pollen allergens (Jun a 1 and Cry j 1) and Arizona cypress pollen allergens (Cup a 1), the occurrence of high-mannose type *N*-glycans appears to be specific to Japanese cypress pollen allergen (Cha o 1), although the content is very low. Among plant glycoallergens, it has been reported that a peanut allergen, Ara h 1, carries the high-mannose type *N*-glycans (Man6-5GlcNAc2) in addition to xylosylated *N*-glycans.¹¹⁾ And recently it has been proposed that the *N*-glycan of Ara h 1 is a ligand of dendritic cell-specific ICAM-grabbing nonintegrin (DC-SIGN) and that it acts as a Th2 adjuvant.¹⁴⁾ Since DC-SIGN is a C-type lectin specific to mannose residues in glycans, it is possible to assume that the *N*-glycans linked to Cha o 1 are ligands of DC-SIGN, and that Cha o 1 can prime Th2-skewed T-cell responses. Detail analysis of the immunological activity of the *N*-glycans of Cha o 1 to dendritic cells is necessary to determine the involvement of plant *N*-glycans in the symptoms of pollinosis.

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