

Structural Analysis of *N*-Glycans of Storage Glycoproteins in Soybean (*Glycine max*. L) Seed[†]

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The structures of *N*-linked sugar chains (*N*-glycans) of storage glycoproteins in soybean seeds have been identified. Eight pyridylaminated (PA-) *N*-linked sugar chains were derived and purified from hydrazinolysates of the storage glycoproteins by reverse-phase HPLC and size-fractionation HPLC. The structures of the PA-sugar chains purified were first identified by two-dimensional PA-sugar chain mapping and ion-spray mass analysis, considering the results of sugar composition analysis or sequential exoglycosidase digestion. The deduced structures were further analyzed by ion-spray tandem mass spectrometry and 500 MHz ¹H-NMR spectrometry. The eight structures fell into two categories; the major class (96.6%) was a typical high mannose-type, the minor class was a xylose containing-type (Man₃Xyl₁GlcNAc₂, Man₃Fuc₁Xyl₁GlcNAc₂; 3.4%).

Key words: *N*-glycan; plant glycoprotein; allergenic oligosaccharide; ion-spray mass spectrometry; *Glycine max*

In plant cells, some physiological functions of free *N*-glycans, which would be liberated by some *N*-glycan releasing enzymes (endo- β -GlcNAc-ase, PNGase), have been postulated.^{1,2)} However, only a few *N*-glycan releasing enzymes have been purified and characterized from plant cells.^{3–7)} As a first step to discover the physiological function(s) of free *N*-glycans and some *N*-glycan releasing enzymes during seed germination or development of seedlings, we have started to purify and characterize some plant endo- β -GlcNAc-ases^{8,9)} and analyze structures of *N*-glycans of storage glycoproteins in some seeds^{9–11)} or free *N*-glycans in the seedlings.¹²⁾ On the way to survey the activities of *N*-glycan releasing enzymes in several kinds of plant cells, we have found such activities in soybean seeds, and purified and characterized two *N*-glycan releasing enzymes.⁹⁾ In this report, we describe the structural analysis of *N*-glycans linked to the storage glycoproteins in soybean seeds to show the occurrence of endogenous substrates for these two *N*-glycan releasing enzymes.

From the viewpoint of physiological activity of plant origin glycoprotein against mammalian cells, *N*-glycan moiety with x1 $\rightarrow$ 2 fucose and β 1 $\rightarrow$ 3 xylose residues of allergenic

plant glycoproteins in foods and pollen has been reported to be a candidate as the epitope in some atopic allergies.^{13–15)} Recently Gomez *et al.* showed that fucose and/or xylose-containing *N*-glycans prepared from α -amylase inhibitors from wheat and barley flours could be recognized by IgE prepared from baker's asthma patients.¹⁶⁾ And they also showed the IgE could bind to some storage glycoproteins in the seed of pea (*Pisum sativum*), soybean (*Glycine max*), and common bean (*Phaseolus vulgaris*).¹⁶⁾ Considering these facts and our results from structural analysis of *N*-glycans of storage glycoproteins in pea seeds,¹⁰⁾ M3FX, M3X, and M4X structures may be some epitopes for such allergic patient's IgE. On the other hand, the structural analyses of *N*-glycans of some soybean glycoproteins, such as soybean agglutinin¹⁷⁾ and 7S soybean globulin,¹⁸⁾ showed that these glycoproteins bear only high mannose-type *N*-glycans. And the detailed structures of xylose (and fucose) containing *N*-glycans have not been established from the glycoproteins in soybean seeds thus far. In this report, we demonstrate the occurrence of xylose (and fucose) containing *N*-glycans, which should be responsible for the allergenic epitopes,¹⁶⁾ among the storage glycoproteins of soybean

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Abbreviations: endo- β -GlcNAc-ase, endo- β -*N*-acetylglucosaminidase; PNGase, peptide: *N*-glycanase; PA-, pyridylamino; RP-HPLC, reverse-phase HPLC; SF-HPLC, size-fractionation HPLC; IS-MS, ion-spray mass spectrometry; MS/MS, tandem mass; Man, D-mannose; Xyl, D-xylose; Fuc, L-fucose; GlcNAc, *N*-acetyl-D-glucosamine; GN2, chitobiose-PA; M1, Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M5A, Man β 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M6A, Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M6B, Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M6C, Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3)Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M7A, Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M7B, Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M7C, Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3)Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M8A, Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M8B, Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3)Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M8C, Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3)Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M9A, Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3)Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 2Man α 1 $\rightarrow$ 3)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M3X, Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)(Xyl β 1 $\rightarrow$ 2)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA; M3FX, Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)(Xyl β 1 $\rightarrow$ 2)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4(Fuc α 1 $\rightarrow$ 3)GlcNAc-PA.

seeds.

Materials and Methods

Materials. *Glycine max* L. cv. Tamahomare produced in 1995 in Okayama prefecture was used. A Cosmosil 5C18-AR column (0.60 × 25 cm) was purchased from Nacalai Tesque, Inc., and an Asahipak NH2P-50 column (0.46 × 25 cm) from Showa Denko Co. α -Mannosidase (Jack bean) was purchased from Sigma. β -mannosidase (*Achatina fulica*) from Seikagaku Kogyo Co. Hydrazine anhydrous was purchased from Pierce. M9A was prepared from 350-kDa Royal Jelly Glycoprotein.¹⁹⁾ M8A and M7A were from *Abrus precatorius* agglutinin.²⁰⁾ M7B, M7D, M6B, and M5A were from the B-chain of ricin D.²¹⁾ and M3FX, M3X, M4X, and M4C were from *Ricinus communis* agglutinin.²²⁾ M6A, M6C, M7C, M8B, and M8C were prepared from M9A by partial hydrolysis as described in our previous report.²³⁾ Man α 1→6(Man α 1→3)Man β 1→4GlcNAc β 1→4GlcNAc-PA (M3) was from the storage glycoproteins in the pea seeds.¹¹⁾ Man β 1→4GlcNAc β 1→4GlcNAc-PA (M1) was derived from M6B by α -mannosidase digestion, and GlcNAc β 1→4GlcNAc-PA (GN2) was from M1 by β -mannosidase digestion.

Preparation of the storage glycoproteins from *Glycine max* seeds. Defatted soybean seed powder (58 g) was suspended in 50 mM Tris-HCl buffer (pH 8.0, 1000 ml) containing 0.5 M NaCl and the slurry was stirred for 2 h at 4°C. The homogenate was squeezed through four layers of Miracloth (Calbiochem Co.) and centrifuged. After centrifugation (8000 × *g* for 20 min.), the supernatant was 100% saturated with ammonium sulfate. The resulting precipitate was dissolved in deionized water and dialyzed exhaustively against deionized water. Finally the dialyzate including the resulting precipitate was lyophilized and the lyophilizate was used as the storage glycoproteins in the following experiments.

Pyridylamination of the sugar chains. N-Glycans were released by hydrazinolysis (100°C, 12 h, in 20 ml of hydrazine anhydrous) from the storage glycoproteins (3.5 g). After N-acetylation of the hydrazinolysate with the saturated ammonium bicarbonate (20 ml) and acetic anhydride (0.8 ml), the acetylated hydrazinolysate was desalted by Dowex 50 × 2 resin. Pyridylamination of the sugar chains was done by the method of Kondo *et al.*²⁴⁾ Separation of PA-sugar chains was done by HPLC on a JASCO 880-PU HPLC apparatus equipped with a JASCO 821-FP Intelligent Spectrofluorometer, using a Cosmosil 5C18-AR column (0.6 × 25 cm) or an Asahipak NH2P-50 column (0.46 × 25 cm). On the Cosmosil 5C18-AR column, the PA-sugar chains were eluted by increasing the acetonitrile concentration in 0.02% TFA linearly from 0 to 15% for 40 min at flow rate 1.2 ml/min. In the case of size-fractionation HPLC using the Asahipak NH2P-50 column, the PA-oligosaccharide was eluted by increasing the water content in the water-acetonitrile mixture from 30% to 50% linearly for 25 min at a flow rate of 0.8 ml/min.

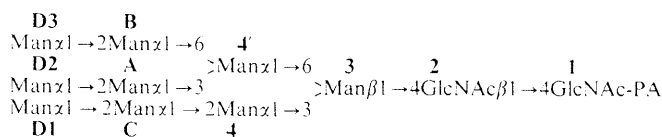
Carbohydrate analysis. Carbohydrate composition was analyzed by gas-liquid chromatography (GLC) as trimethylsilyl derivatives after methanolysis (1.5 N methanolic HCl) by the method of Mega *et al.*²⁵⁾ using myo-inositol as an internal standard. For GLC analysis, a Hitachi G-3000 gas chromatograph with a DB-1 capillary column (30 m × 0.25 mm, J & W Scientific) was used.

Glycosidase digestions. All digestions with α -mannosidase or β -mannosidase were done using about 100 pmol of the PA-sugar chains under the conditions described in our previous report.²¹⁾ The reactions were stopped by boiling the mixtures for 3 min and a part of the digest was analyzed by SF-HPLC. In the case of analyses of the glycosidase digests, the PA-sugar chains were eluted by increasing the water content in the water-acetonitrile mixture from 18% to 40% linearly at a flow rate of 0.8 ml/min.

Ion-spray mass spectrometry. The mass spectrometer used was a Perkin Elmer Sciex API-III, triple-quadrupole mass spectrometer with an atmospheric-pressure ionization ion source. The mass spectrometer was operated in the positive mode; the ion spray voltage was 4200 V. Samples were typically dissolved in 50% acetonitrile/water (containing 0.05% formic acid) and at a concentration of approximately 10 pmol/ μ l and introduced into the electrospray needle by mechanical infusion through a micro syringe at a flow rate of 5 μ l/min. The collisionally activated dissociation (CAD) spectrum was measured with argon as the collision gas. The collision energy was 60–100 eV. The scanning was done with a

step size of 0.5 Da and the CAD daughter ion spectrum was recorded from m/z 200.

500 MHz ^{1} H-NMR spectrometry. ^{1} H-NMR measurements at 500 MHz were done on a Varian VXR 500 Instrument at 27°C. A sample was dissolved in 99.96% deuterated water (Aldrich) and acetone was used as the internal standard. Coding of mannosyl and N-acetylglucosaminyl residues used in this paper is as follows:



Results and Discussion

Purification of pyridylaminated (PA-) sugar chains from the storage glycoproteins of soybean seeds

PA-sugar chains from the storage glycoproteins of soybean seeds were purified by a combination of reversed-phase (RP-) HPLC and size-fractionation (SF-) HPLC. Five PA-sugar chain fractions (I–V) were obtained from PA-derivatives of a hydrazinolysate by RP-HPLC (Fig. 1A). In this report, we used a 0.02% TFA/acetonitrile solvent system instead of the 0.1 M ammonium acetate/1-butanol solvent system²⁶⁾ or 0.1 M phosphate/1-butanol solvent system.²⁷⁾ Since this solvent system contains no salts, the PA-sugar chains fractionated could be easily and directly analyzed by the ion-spray mass spectrometry or ^{1} H-NMR spectrometry without a desalting process. The run-through fraction (between 5–10 min) seems to contain an excess of free 2-aminopyridine and some PA-sugar chains having only one GlcNAc residue at the reducing-end side, since IS-MS/MS analyses of some PA-sugar chains in the run-through fraction showed the fragment ions m/z 300 (GlcNAc-PA) and m/z 462 (ManGlcNAc-PA) but not at m/z 503 (GlcNAc₂-PA) (data not shown). Hase *et al.* have shown that such sugar chains occur as the degradation products of the released sugar chains during hydrazinolysis and pyridylamination.²⁶⁾ More recently we also found high mannose-type free N-glycans bearing only one GlcNAc residue in pea seedlings run through a C18 column.¹²⁾ The small peaks found between 10–18 min are not likely to be N-glycans, since MS/MS analyses of these peaks showed no signal at m/z 300 (GlcNAc-PA). The PA-sugar chain fractions (I–V) were further purified by SF-HPLC using an Asahipak NH2P-50 column. As shown in Fig. 1B, eight PA-sugar chains (A–H) were purified: A and B from Fraction I, C and D from Fraction II, E from Fraction III, F from Fraction IV, and G and H from Fraction V. Structural features of these purified PA-sugar chains are summarized in Table I. Eight N-glycan structures deduced by the elution positions on a 2D sugar chain map^{28,29)} were further supported by IS-MS analysis. The observed molecular masses for the purified PA-sugar chains agreed well with the expected molecular weights for each of the deduced structures. The parent ions [(M+H)⁺] for all eight PA-sugar chains purified by SF-HPLC using the water/acetonitrile system were mainly observed as single-charged ions on IS-MS spectra. PA-Sugar chains A, and G, of which the elution positions did not correspond to those of high mannose-type sugar chains (Man_{9–4}GlcNAc₂-PA) on SF-HPLC, were found to be xylose-containing type structures by sugar component analysis (Table I).

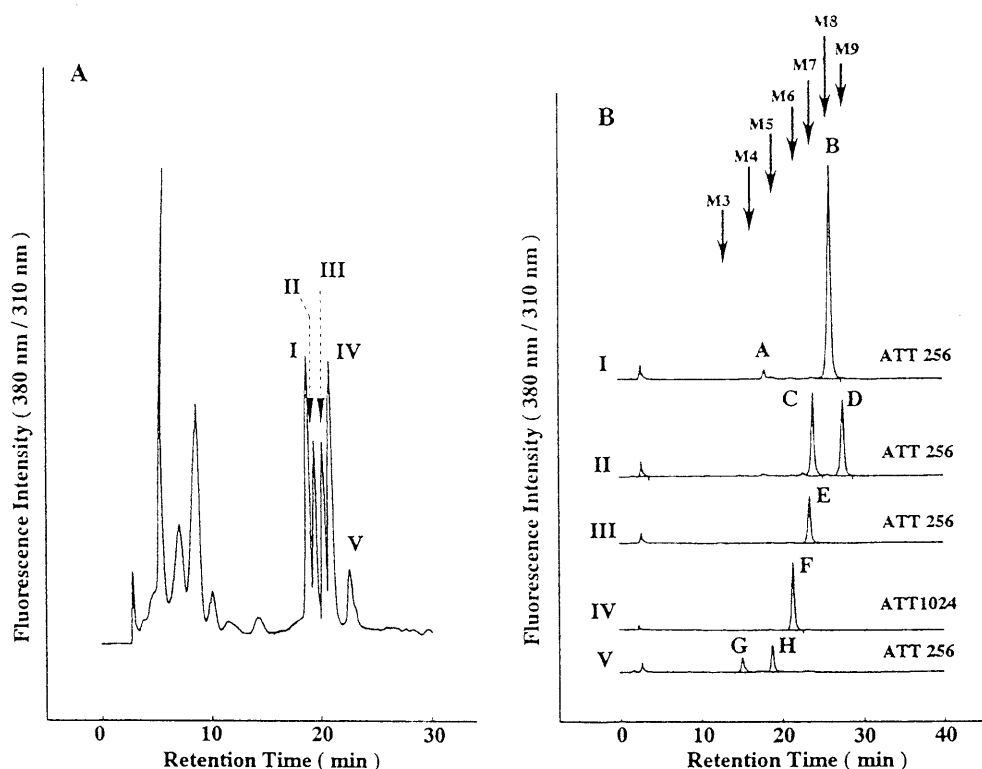


Fig. 1. HPLC of PA-Sugar Chains Prepared from the Storage Glycoproteins in Soybean Seeds.

A: RP-HPLC of PA-derivatives from soybean storage glycoproteins. PA-derivatives were put on a Cosmosil 5C18-AR column (0.6 × 25 cm) and eluted as described in the text.

B: Size-fractionation HPLC of the partially purified PA-sugar chains by RP-HPLC in A.

PA-Sugar chain fractions obtained in A were put on a Asahipak NH2-P50 column (0.46 × 25 cm) and eluted as described in the text. Numbers I–V indicate the PA-sugar chain fractions obtained in A. M3; M4, M5, M6, M7, M8, and M9 in B are the elution positions of M3, M4C, M5A, M6B, M7B, M8A, M9A, respectively. ATT means the integrator attenuation.

Table I. Characterization of PA-Sugar Chains from Storage Glycoproteins in Soybean Seeds

Peak name	A	B	C	D	E	F	G	H
Molecular Size ^a	M3FX	M8	M7	M9	M7	M6	M3X	M5
RP-HPLC Position	M3FX	M8A	M7A	M9A	M7B	M6B	M3X	M5A
Man	3.0						3.0	
Xyl	0.9						1.0	
Fuc	0.9							
GlcNAc/GlcNAc-PA	1.7						1.6	
IS-MS								
[M+H] ⁺	1268	1801	1638	1963	1638	1476	1122	1314
Expected	1267	1800	1637	1962	1637	1475	1121	1313
	(M3FX)	(M8A)	(M7A)	(M9A)	(M7B)	(M6B)	(M3X)	(M5A)

^a Molecular size was measured by size-fractionation HPLC. Mn = (Man)_nGlcNAc₂-PA. M3FX = Man₃Fuc₁Xyl₁GlcNAc₂-PA. M3X = Man₃-Xyl₁GlcNAc₂-PA.

MS/MS analysis and ¹H-NMR analysis of PA-sugar chains

To confirm the deduced structures for eight PA-sugar chains released and purified from the soybean glycoproteins, the structures of these PA-sugar chains were further analyzed in detail by IS-MS/MS spectrometry and ¹H-NMR.

(I) Analysis of xylose-containing sugar chains (PA-sugar chains A and G). The elution positions of PA-sugar chain A exactly corresponded to those of authentic M3FX on both RP- and SF-HPLC (Table I), and the sugar component analysis showed that this PA-sugar chain consists of Man (3.0), Fuc (0.9), Xyl (0.9), GlcNAc (1.0), and PA-GlcNAc (0.7). The molecular mass [M+H]⁺ was found

to be 1268, which agreed with the calculated molecular mass (1267) for M3FX. As shown in Table II, all relevant signals could be assigned as fragment ions derived from the deduced M3FX structure. The fragment ion at *m/z* 446.0 corresponding to Fuc-GlcNAc-PA indicated that a fucosyl residue is bound to the innermost GlcNAc residue. Moreover, all reporter signals observed on ¹H-NMR spectrum agreed well with those of M3FX from one of the pokeweed mitogens (PL-B)³⁰⁾ as shown in Table III. The chemical shifts of the Xyl H-1 (δ = 4.458 ppm) and Fuc H-1 (δ = 5.064 ppm), in combination with coupling constant *J*_{1,2} (7.5 Hz) and (3.7 Hz), are indicative of β- and α-configura-

Table II. MS/MS Analysis of PA-Sugar Chains from Storage Glycoproteins in Soybean Seeds

Fragment ions $[M+H]^+$ (m/z)	A	B	C	D	E	F	G	H
GlcNAc-PA (300.0)	++++	++	+++	++	++++	++++	++++	++++
FucGlcNAc-PA (446.0)	++	ND	ND	ND	ND	ND	ND	ND
GlcNAc ₂ -PA (503.0)	++	++	++	++	++	++	++	++
Man ₁ GlcNAc ₂ -PA (665.5)	+	+	+	+	+	+	+	+
Man ₁ Xyl ₁ GlcNAc ₂ -PA (797.5)	+	ND	ND	ND	ND	ND	+	ND
Man ₂ GlcNAc ₂ -PA (827.5)	+	+	+	+	+	+	+	+
Man ₂ Xyl ₁ GlcNAc ₂ -PA (959.5)	+	ND	ND	ND	ND	ND	+	ND
Man ₃ GlcNAc ₂ -PA (989.5)	+	+	+	+	+	+	+	+
Man ₃ Xyl ₁ GlcNAc ₂ -PA (1121.5)	++	ND	ND	ND	ND	ND	++++	ND
Man ₄ GlcNAc ₂ -PA (1152.0)	ND	+	+	+	+	+	ND	++
Man ₃ Fuc ₁ Xyl ₁ GlcNAc ₂ -PA (1268.0)	+++	ND	ND	ND	ND	ND	ND	ND
Man ₅ GlcNAc ₂ -PA (1314.0)	ND	+	+	+	+	+	ND	++++
Man ₆ GlcNAc ₂ -PA (1476.0)	ND	+	++	+	++	++++	ND	ND
Man ₇ GlcNAc ₂ -PA (1638.0)	ND	+	++++	+	++++	ND	ND	ND
Man ₈ GlcNAc ₂ -PA (1801.0)	ND	++++	ND	+	ND	ND	ND	ND
Man ₉ GlcNAc ₂ -PA (1962.5)	ND	ND	ND	++++	ND	ND	ND	ND

+, 10–25% relative intensity; ++, 26–50% relative intensity; +++, 51–75% relative intensity; +++, 76–100% relative intensity. ND, not detected.

Table III. Chemical Shifts (ppm) of Structural Reporter Group Signals of PA-Sugar Chains Observed on 500 MHz ¹H-NMR Spectrometry

Reporter groups	Residue	A	M3FX ^a	B	M8A ^b	C	M7A ^b	D	M9A ^b	E	M7B ^c	F	M6B ^c	G	M3X ^c	H	M5A ^c
H1 of	4	5.137	5.134	5.345	5.340	5.340	5.349	5.335	5.332	5.345	5.351	5.353	5.359	5.125	5.128	5.102	5.100
	4'	4.930	4.927	4.875	4.868	4.876	4.869	4.867	4.868	4.875	4.878	4.876	4.880	4.917	4.921	4.875	4.880
	A	ND	ND	5.088	5.093	5.095	5.082	5.400	5.402	5.098	5.098	5.102	5.100	ND	ND	5.102	5.100
	B	ND	ND	5.150	5.145	5.151	5.145	5.145	5.145	4.910	4.914	4.913	4.916	ND	ND	4.910	4.914
	C	ND	ND	5.311	5.305	5.058	5.053	5.311	5.308	5.309	5.316	5.058	5.064	ND	ND	ND	ND
	D1	ND	ND	5.049	5.045	ND	ND	5.053	5.058	5.049	5.051	ND	ND	ND	ND	ND	ND
	D2	ND	ND	ND	ND	ND	ND	5.053	5.058	ND	ND	ND	ND	ND	ND	ND	ND
	D3	ND	ND	5.049	5.045	5.046	5.043	5.053	5.058	ND	ND	ND	ND	ND	ND	ND	ND
	Xyl	4.458	4.452	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	Fuc	5.064	5.063	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	4.436	4.438	ND	ND
	H2 of	3	4.267	4.273	4.218	4.216	4.223	4.220	4.214	4.212	4.220	4.227	4.219	4.227	4.249	4.257	4.237
	4	4.048	4.046	4.096	4.095	4.121	4.114	4.102	4.098	4.098	4.097	4.119	4.121	4.037	4.040	4.080	4.081
	4'	3.999	3.996	4.152	4.143	4.155	4.149	4.152	4.152	4.149	4.154	4.154	4.157	3.978	3.995	4.151	4.154
	A	ND	ND	4.072	4.070	4.074	4.068	4.102	4.108	4.067	4.069	4.071	4.076	ND	ND	4.074	4.067
	B	ND	ND	4.028	4.026	4.020	4.016	4.013	4.012	3.984	3.995	3.991	3.998	ND	ND	3.982	3.992
	C	ND	ND	4.111	4.108	4.074	4.068	4.102	4.108	4.114	4.112	4.071	4.076	ND	ND	ND	ND
	D1	ND	ND	4.072	4.070	ND	ND	4.074	4.071	4.067	4.073	ND	ND	ND	ND	ND	ND
	D2	ND	ND	ND	ND	ND	ND	4.074	4.071	ND	ND	ND	ND	ND	ND	ND	ND
	D3	ND	ND	4.072	4.070	4.074	4.068	4.074	4.071	ND	ND	ND	ND	ND	ND	ND	ND
NAc of	1	1.936	1.937	1.944	1.941	1.941	1.941	1.943	1.942	1.943	1.945	1.943	1.942	1.946	1.949	1.943	1.946
	2	2.057	2.059	2.061	2.058	2.061	2.058	2.062	2.061	2.060	2.063	2.060	2.064	2.067	2.075	2.061	2.070
CH3 of	Fuc	1.206	1.209	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

^a Reference 30. ^b Reference 19. ^c Reference 21.

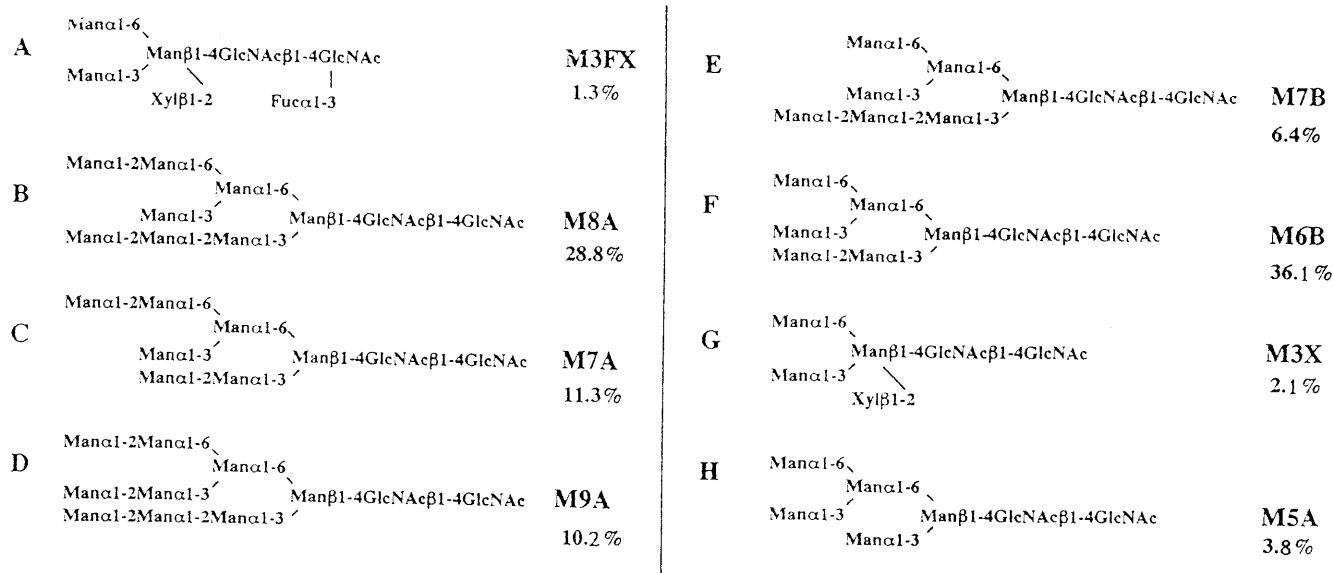
tion of the linkages to Man-3 and GlcNAc-1, respectively. These results showed that the structure of PA-sugar chain A should be M3FX [Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)(Xyl β 1 $\rightarrow$ 2)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4(Fuc α 1 $\rightarrow$ 3)GlcNAc-PA] as shown in the Scheme.

The elution positions of PA-sugar chain G exactly corresponded to those of authentic M3X on both RP- and SF-HPLC (Table I), and the sugar component analysis showed that this PA-sugar chain consists of Man (3.0), Xyl (1.0), GlcNAc (0.9), and PA-GlcNAc (0.7). The molecular mass $[M+H]^+$ was found to be 1122, which agreed with the expected molecular mass (1121) for M3X. As shown in Table II, all relevant signals observed on MS/MS spectrum could be reasonably assigned to the fragment ions

derived from the M3X structure. The structure of this PA-sugar chains was further analyzed by ¹H-NMR spectrometry (Table III). The relevant chemical shifts of structural reporter signals of the PA-sugar chain were also consistent with those of M3X from the A-chain of ricin D.²⁰⁾ Considering these results together, the structure of PA-sugar chain G was M3X [Man α 1 $\rightarrow$ 6(Man α 1 $\rightarrow$ 3)(Xyl β 1 $\rightarrow$ 2)Man β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc-PA] as shown in the Scheme.

These β 1 $\rightarrow$ 2 xylose-containing type N-glycans (M3FX and M3X) are known to be widely distributed in various plant glycoproteins, however, this is the first report showing the detailed structures of these antigenic N-glycans of storage glycoproteins in soybean seeds.

(2) High mannose-type structures (PA-sugar chain B, C,



Scheme Proposed Structures of *N*-Glycans Linked to the Storage Glycoproteins in Soybean Seeds.

A-H correspond to PA sugar chains A-H in Fig. 1B. The pyridylamino residues are omitted in this scheme.

D, E, F, and H). Since the elution positions of the PA-sugar chain B, C, D, E, F, and H exactly corresponded to each elution position of authentic high mannose-type PA-sugar chains on the 2D sugar chain map, the structures could be deduced as shown in Table I. Actually, sequential exoglycosidase digestion of these PA-sugar chains showed that these sugar chains consist of several α -mannosyl residues (4–8 residues) and one β -mannosyl residue besides PA-chitobiose, indicating these sugar chains are typical high mannose-type structures (Fig. 2). The observed molecular masses ($[M+H]^+$) of PA-sugar chains B, C, D, E, F, and H exactly corresponded to M8A, M7A, M9A, M7B, M6B, and M5A, respectively (Table I). Moreover, as shown in Table II, all relevant fragment ions derived by the tandem mass spectrometry from each parent molecule were well assigned, indicating the serial loss of each sugar residue. The deduced structures of these six PA-sugar chains were further analyzed by 500 MHz $^1\text{H-NMR}$. As shown in Table III, every structural reporter group signal of these six PA-sugar chains is in good agreement with those of authentic PA-sugar chains already reported; PA-sugar chain B, C, and D for M8A, M7A, and M9A, respectively, PA-sugar chain E, F, and H for M7B, M6B, and M5A, respectively. Considering these results described above together, the structures of these six high mannose-type sugar chains were as shown in the Scheme. Among these structures, M9A was the same structure found in soybean agglutinin,¹⁷⁾ M8A, M7A, M7B, and M6B were the same structures found in 7S soybean globulin.¹⁸⁾

Structural features of *N*-glycan of the storage glycoproteins in soybean seeds

The estimated structures of eight *N*-glycans prepared from the storage glycoproteins in soybean seeds fell into two categories: the major class was a typical high mannose-type, the minor class was a xylose containing-type ($\text{Man}_3\text{Xyl}_1\text{GlcNAc}_2$, $\text{Man}_3\text{Fuc}_1\text{Xyl}_1\text{GlcNAc}_2$). Among these structures, $\text{Man}_8\text{GlcNAc}_2$ (28.8%), $\text{Man}_6\text{GlcNAc}_2$ (36.1%) were the dominant structures, and it has been revealed that the *N*-glycans linked to the storage glyco-

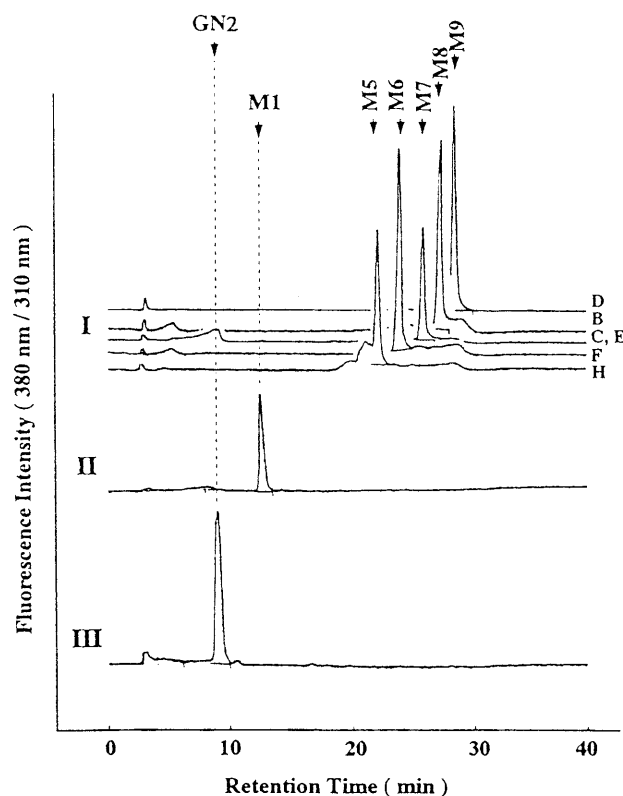


Fig. 2. Size-fractionation HPLC of α - and β -Mannosidase Digests of PA-Sugar Chain B, C, D, E, F, and H.

I, native PA-sugar chain B, C, D, E, F, and H; II, α -mannosidase digest of I; III, β -mannosidase digest of II.

proteins are almost high-mannose type structures (96.6%). We have recently found and purified two *N*-glycan releasing enzymes from soybean extracts: one is an endo- β -*N*-acetylglucosaminidase (endo- β -GlcNAc-ase) which could hydrolyze the chitobiose linkage of high mannose-type sugar chains, and the other is a peptide: *N*-glycanase (PNGase) which could release both high mannose-type and xylose-containing sugar chains.⁹⁾ Therefore, the result obtained in this study demonstrates that the soybean seeds contain

the various endogenous substrates for these two *N*-glycan releasing enzymes; several high-mannose type *N*-glycans which can be substrates for endo- β -GlcNAc-ase and xylose-containing type *N*-glycans which can be substrates for PNGase.

From the viewpoint of physiological activity of plant origin *N*-glycans, it has been recently reported that the *N*-glycans have some physiological effects against the mammalian cells. Gomez *et al.*¹⁶⁾ have shown that IgE prepared from the allergic patients (baker's asthma patients) could bind to fucose an/or xylose containing *N*-glycan moieties of the highly allergenic α -amylase inhibitors from the wheat and barley flours. And they have also found that such IgE could bind to several storage glycoproteins having β 1 $\rightarrow$ 2 xylosyl and α 1 $\rightarrow$ 3 fucosyl residues in seeds of pea (*Pisum sativum*), soybean (*Glycine max*), and common bean (*Phaseolus vulgaris*).¹⁶⁾ Concerning *N*-glycans of storage glycoproteins in pea seeds, the structures have already been identified, and we demonstrated that some xylose-containing *N*-glycans (M3FX, M4X, and M3X), which would be responsible for the allergenic epitopes described in the previous report,¹⁶⁾ occur in the storage glycoproteins.¹⁰⁾ On the other hand, concerning *N*-glycans of soybean glycoproteins, only several high mannose-type structures linked to soybean agglutinin¹⁷⁾ and 7S globulin¹⁸⁾ have been identified. Gomez *et al.*¹⁶⁾ reported the occurrence of xylose-containing *N*-glycans that could be recognized by IgE from baker's asthma patients in soybean seeds, however, the detailed structures of such *N*-glycans have remained to be analyzed. In this report, we have first found *N*-glycan structures having a β 1 $\rightarrow$ 2 xylose residue (M3FX and M3X) in the soybean storage glycoproteins, which would be responsible for one of the allergenic epitopes.¹⁶⁾ Since Tsuji *et al.*³¹⁾ have recently purified and characterized a soybean allergen (Gly m Bd 28K), which is a 26-kDa glycoprotein bearing xylosyl and fucosyl residues and can bind to IgE from soybean-sensitive patients, the M3FX structure described in this report should be linked to the soybean allergen.

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