

The Carbohydrate Moiety of the Bermuda Grass Antigen BG60

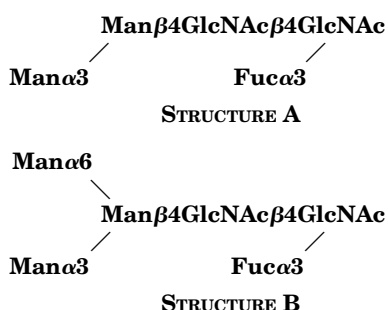
NEW OLIGOSACCHARIDES OF PLANT ORIGIN*

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BG60 is an important allergen of Bermuda grass (*Cynodon dactylon*) pollen, which causes allergic responses in human. It was suggested that its carbohydrate moiety may be relevant to allergic reaction (Su, S. N., Lau, G. X., Shu, P., Yang, S. Y., Huang, S. W., and Lee, Y. C. (1996) *J. Allergy Clin. Immunol.*, in press). Therefore, the structure of the carbohydrate moiety in BG60 was investigated. The N-linked oligosaccharides were released from the glycopeptides of BG60 by digesting with a glycoamidase from sweet almond and reductively aminated with a fluorescent reagent, 2-aminopyridine. The mixture of pyridylaminated oligosaccharides were separated by high-performance liquid chromatography (HPLC) using an octadecylsilyl (ODS) column. Five oligosaccharide fractions were isolated, and each fraction was found to be homogeneous by HPLC on an amide-silica column. The structure of each of the oligosaccharides was analyzed by the two-dimensional mapping technique (Tomiya, N., Awaya, J., Kurono, M., Endo, S., Arata, Y., and Takahashi, N. (1988) *Anal. Biochem.* 171, 73–90), in tandem with sequential exoglycosidase digestion. The two most abundant oligosaccharides, A and B, have an unusual structural feature, i.e. the presence of an L-Fuc α -(1,3)-linked to Asn-linked GlcNAc without a Xyl β -(1,2)-linked to the branching Man (see below). To the best of our knowledge, these are the first such oligosaccharides found in plant glycoproteins.



Bermuda grass (*Cynodon dactylon*) pollen is one of the most common causes for airway allergic disease in hot and humid climate (1–3). It contains at least 53 antigenic proteins that can induce immune response in rabbit (4), of which more than 12 have been shown to possess IgE binding activity (4–7). One of the major allergens with an apparent molecular weight of 56–60 kDa, designated BG60, was shown to be a basic protein containing three to four components with the pI values of 9.8–10.5. Two of the components, which share immunological identities, have been purified and partially characterized (8, 9). Recently, we reported that BG60 contains 4.6% carbohydrate consisting of mannose (Man), N-acetylglucosamine (GlcNAc), fucose (Fuc) in an approximate ratio of 3:2:1 and a minute amount of xylose (10).

The role of carbohydrate in allergens has been controversial. The best known allergens, AgE and AgK of ragweed pollen, contain no carbohydrate (11, 12). However, the involvement of the carbohydrate in antigenicity has been repeatedly demonstrated (13–16). Our recent study showed that BG60 lost its ability to bind monoclonal antibody upon treatment with periodate (10). We now report the results of our structural investigation on the carbohydrate moiety of BG60 and found its major constituents are oligosaccharides hitherto unreported in the plant glycoproteins.

EXPERIMENTAL PROCEDURES

Materials

Bermuda grass pollen was purchased from International Biologicals Inc., Piedmont, OK. Homogeneous BG60 was prepared as described (10). Glycoamidase A (glycopeptidase A, EC 3.5.1.52) from sweet almond, β -N-acetylhexosaminidase, and α -mannosidase (jack beans) were from Seikagaku Kogyo Co., Tokyo, Japan. Pepsin was from Sigma. The pyridylamino (PA)¹ derivatives of isomalto-oligosaccharides (4–20 glucose residues) and of reference oligosaccharides (code numbers² 000.1, 000.1FX, M2.1FX, and M1.1FX) were from Nakano Vinegar Co. (Handa City, Japan). The following oligosaccharides were prepared by the methods described previously: M1.1F by β -xylosidase digestion of M1.1FX (17), 000.1X from rice α -amylase (18), and 100.1FX from lac-case of sycamore cells (19).

Methods

Determination of Proteins and Carbohydrates—Protein was determined by the method of Bradford (20) using bovine serum albumin as

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¹ The abbreviations used are: PA, pyridylamino; BG, Bermuda grass; GlcNAc or GN, N-acetyl-D-glucosamine; Man or M, D-mannose; Xyl or X, D-xylose; Fuc or F, L-fucose; HPLC, high-performance liquid chromatography; RP, reverse phase; ODS, octadecylsilyl; HOHAHA, homonuclear Hartmann-Harn spectroscopy; PLA, phospholipase A₂.

² Code numbers assigned to the oligosaccharides are cited from Ref. 22. The detailed structures of these and other oligosaccharides are shown in the legend to Fig. 2.

standard. Carbohydrates were analyzed by the orcinol- H_2SO_4 reagent (21).

Preparation, Derivatization, and Characterization of the BG60 Oligosaccharides—The oligosaccharides were released from 18 mg of BG60 by sequential digestion with pepsin and glycoamidase A and fractionated by gel filtration (22) on a Bio-Gel P-4 (1×38 cm, in water) and was reductively aminated with 2-aminopyridine using sodium cyanoborohydride (23), and the resultant PA-oligosaccharides were purified by gel filtration on a Sephadex G-15 column (1×38 cm, in 10 mM ammonium bicarbonate). The PA-oligosaccharide mixture was separated and characterized by HPLC using the two-dimensional sugar mapping technique (24). First, the PA-oligosaccharides were separated on a reverse phase column (Shim-pak HRC-ODS, 6×150 mm, Shimadzu, Kyoto, Japan) under the conditions described previously (24). Each oligosaccharide separated on the ODS column was collected and applied to an amide-silica column (Amide-80, 4.6×250 mm, Tosoh, Tokyo, Japan) for adsorptive chromatography, elution being performed as described previously (24). In both HPLC systems, PA-oligosaccharides were detected by fluorescence using excitation and emission wavelengths of 320 and 400 nm, respectively. The elution positions of the PA-oligosaccharides were expressed in glucose unit, as defined by the elution positions of the PA-isomalto-oligosaccharides (tetraose-dodecaose) (24). The coordinates of each oligosaccharide on the ODS- and amide-silica columns were plotted on the two-dimensional sugar map (24) and compared with the coordinates of known *N*-linked oligosaccharides on the map.

Exoglycosidase Digestion—Each oligosaccharide isolated on the ODS and amide columns was sequentially digested with exoglycosidases (β -*N*-acetylhexosaminidase, α -mannosidase, and β -xylosidase) under the condition described previously (17, 22). After each step of exoglycosidase digestion, the elution coordinates of the trimmed oligosaccharides were located on the two-dimensional map to verify the structural identity.

NMR Measurement—Prior to NMR measurement, the PA-oligosaccharide (about 20 nmol as neutral oligosaccharide) isolated by HPLC was purified by gel filtration on a Sephadex G-15 column eluted in 10 mM ammonium bicarbonate. All NMR spectra were recorded on a Bruker AMX-400 spectrometer. ^1H NMR spectra were recorded with 32,000 data points and a spectral width of 6,000 Hz. An exponential window function with a broadening factor of 0.5 Hz was used for sensitivity enhancement. Measurement of two-dimensional HOHAHA (25) was in the phase-sensitive mode (19) with spectra widths of 3,000 Hz. The mixing time of 120 ms was used for the two-dimensional HOHAHA experiment, and the probe temperature was set to 26 °C throughout the two-dimensional experiments. Two-thousand data points were used in the t_2 dimension, and 64 transients were acquired for each of 512 t_1 blocks. Prior to two-dimensional Fourier transformation, the acquired data were multiplied by Gauss function in t_2 and by a shifted sine-square function in t_1 , and zero-filled to yield a matrix $1024 (F_1) \times 1024 (F_2)$ of the real data points. The solvent resonance was suppressed by selective irradiation during the preparation period of 1.2 s.

RESULTS

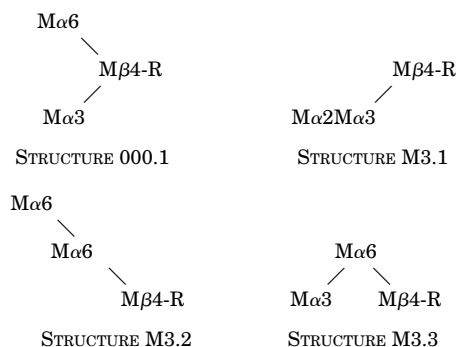
The Oligosaccharides from BG60—The sequential digestion of BG60 with pepsin and glycoamidase A released more than 90% of the total carbohydrate in BG60 as reducing oligosaccharides. The PA-oligosaccharides derived from BG60 were separated into five fractions by reverse phase HPLC on the ODS-silica column (Fig. 1A). Each of the fractions, labeled A–E, showed a single peak upon further chromatography on the amide-silica column (Fig. 1B). The elution coordinates of the oligosaccharides A and B did not coincide with any known oligosaccharides on the existing two-dimensional sugar map³ (Fig. 2A), indicating that these oligosaccharides have novel structures. The elution position of the oligosaccharides C, D, and E coincided with those of the known standard oligosaccharides designated as 000.1X, M2.1FX, and 100.1FX, respectively (Fig. 2B).

NMR Analysis of Oligosaccharide B—Oligosaccharide B, the major oligosaccharide from BG60 (Fig. 1A), was analyzed by ^1H NMR. The chemical shift values for the structural reporter groups of oligosaccharide B are summarized in Table I. Also

shown are two reference compounds: laccase a is an *N*-acetylglucosamine-type oligosaccharide from laccase of sycamore cells, containing a β -(1,2)-linked xylose and α -(1,3)-linked L-fucose (19), and PLA-9 is an oligosaccharide from honeybee venom phospholipase A_2 , having two α -(1–3)-linked fucose residues (FUC and FUC')⁴ linked to two different GlcNAc residues (14).

The chemical shifts of the anomeric and methyl protons of fucose of oligosaccharide B (5.057 and 1.20 ppm) are essentially the same as those for laccase a (5.053 and 1.201 ppm) and those for one of the fucoses (Fuc) in PLA-9 (5.052 and 1.20 ppm). If the fucose residue is α -(1,6)-linked to the PA-modified *N*-acetylglucosamine, the chemical shifts of its anomeric and methyl protons would be 5.127–5.132 and 1.26–1.27 ppm, respectively, clearly distinguishable from those found in oligosaccharide B. The chemical shifts of the H-5 of the fucose in oligosaccharide B (4.22 ppm) and the fucose (Fuc) linked to the PA-modified GlcNAc in PLA-9 (4.25 ppm) are very close, but the corresponding chemical shift of the H-5 of the other fucose (Fuc') in PLA-9 (4.862 ppm), α -(1,3)-linked to the outer *N*-acetylglucosamine, is significantly different. These results clearly indicate that L-Fuc residue in oligosaccharide B is linked to the PA-modified GlcNAc through an α -(1,3)-linkage. All other chemical shift data are consistent with the structure of oligosaccharide B shown in Table II. For example, the H-1 signals of 4.857, 5.122, and 4.910 agree well with those of Man3, Man4, and Man4' of laccase a oligosaccharide from sycamore cells.

Structural Characterization of PA-oligosaccharides Using the Two-dimensional Mapping Technique—The result of the monosaccharide analysis revealed that the oligosaccharide B can be represented as $\text{Fuc}_1\text{GlcNAc}_2\text{Man}_3$. Based on the known *N*-linked oligosaccharides, there can be four possible structures for the arrangement of the three mannosyl residues as shown below (with code numbers), where M is Man and R is GlcNAc β 4GlcNAc.



These four structures are completely discernible in the two-dimensional map.

In order to determine which arrangement of mannoses corresponds to the structure of oligosaccharide B, oligosaccharide B was defucosylated by a controlled hydrolysis with trifluoroacetic acid (26). The coordinates of the resultant oligosaccharide matched with those of the core structure (000.1) on the two-dimensional map (Fig. 2A). The identity was further confirmed by co-chromatography with authentic 000.1. As mentioned above, the NMR data also support this conclusion. The fucose residue in oligosaccharide B could not be removed by the α -fucosidase from bovine kidney. This further supports that the Fuc residue in oligosaccharide B is not α -(1,6)-linked to the PA-modified GlcNAc residue. This is also in complete agreement with the conclusion drawn from the NMR data. Thus we

³ There are more than 250 neutral oligosaccharides (*N*-glycoside type) from glycoproteins recorded by this method (22, 24, 42) at the time of investigation.

⁴ "FUC" and "FUC'" are used in this paper to denote the specific fucosyl residues in PLA-9 to avoid confusion with the generic "Fuc" which designates fucose.

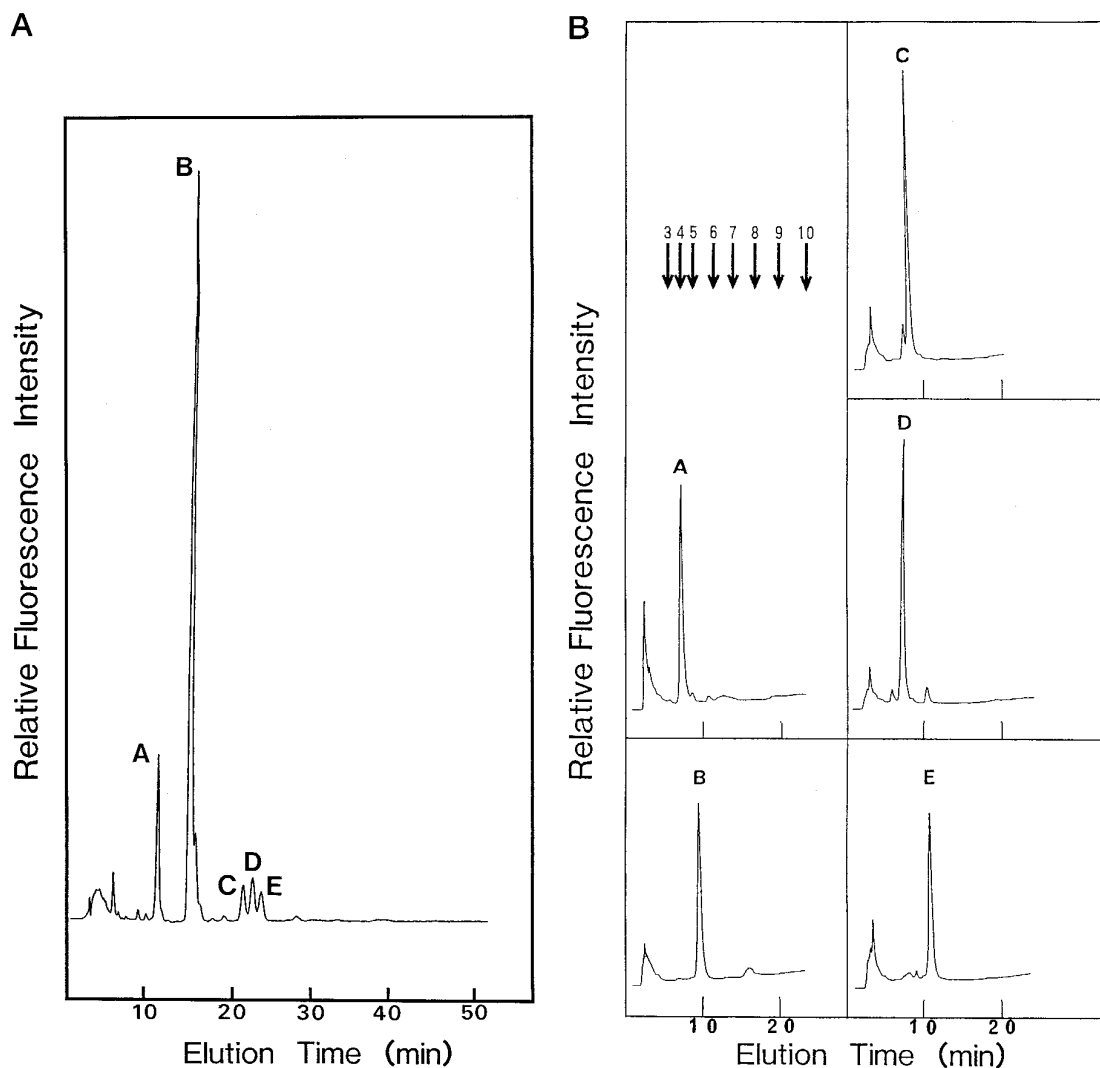
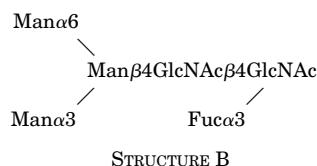
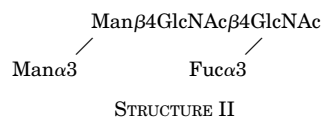
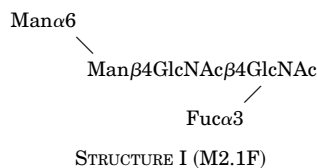


FIG. 1. HPLC of PA-oligosaccharides of BG60 on ODS- and amide-silica columns. A, the profile on the ODS-silica column. Chromatographic conditions: isocratic elution with 10 mM sodium phosphate, pH 3.8, at 1 ml/min. Structural assignment of each peak is shown in Table II. B, the profiles on the amide-silica column eluted under the conditions described under "Experimental Procedures." The arrows indicate the elution positions of reference isomalto-oligosaccharides of the degree of polymerization marked.

conclude that oligosaccharide B has a hitherto unreported structure as shown below.



Digestion of the oligosaccharide A with α -mannosidase changed the elution coordinates from 4.9 (ODS) and 4.2 (amide-silica) to 5.3 (ODS) and 3.0 (amide-silica) and corresponds to the oligosaccharide M1.1F (Fig. 2A). The decrease in the amide column coordinate corresponds to a loss of a single mannosyl residue. Therefore, the structure of the oligosaccharide A is either I or II (shown below).



The structure I is a known oligosaccharide (code number M2.1F) with the coordinates of 6.1 and 4.2⁵ on the two-dimensional map. There is no authentic compound of the structure II, and thus the experimental coordinates are not available for comparison. However, the coordinates for an oligosaccharide can be computed from the "unit contribution" values with a reasonable accuracy (27, 28). The computed coordinates for the structure II are 5.0 and 4.3, which are very close to the observed values for the oligosaccharide A (4.9, 4.2) and considerably different from those from the known structure I. On the other hand, the computed coordinate values for the structure I are 5.7, 4.3. Thus, the structure II can be justly assigned to the oligosaccharide A.

The deduction presented above is based on a hypothesis that

⁵ When the coordinates for a given oligosaccharide are listed, they are in the order of ODS-amide silica.

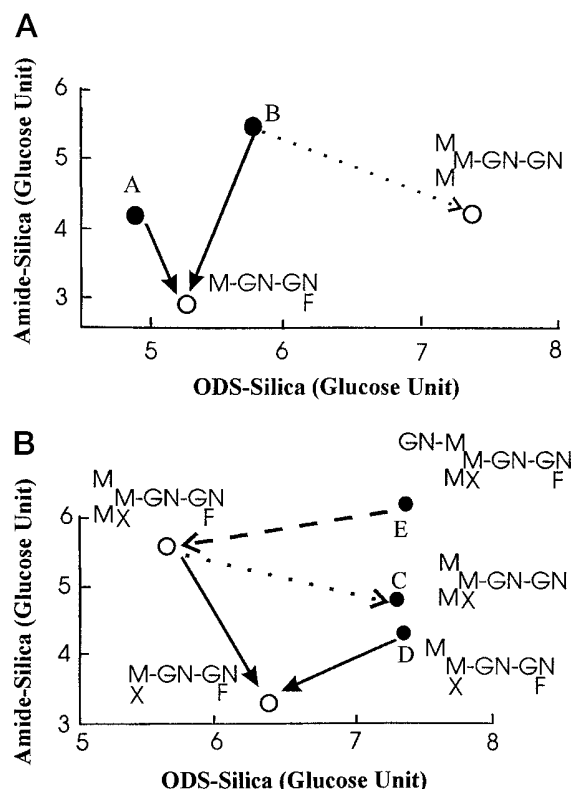
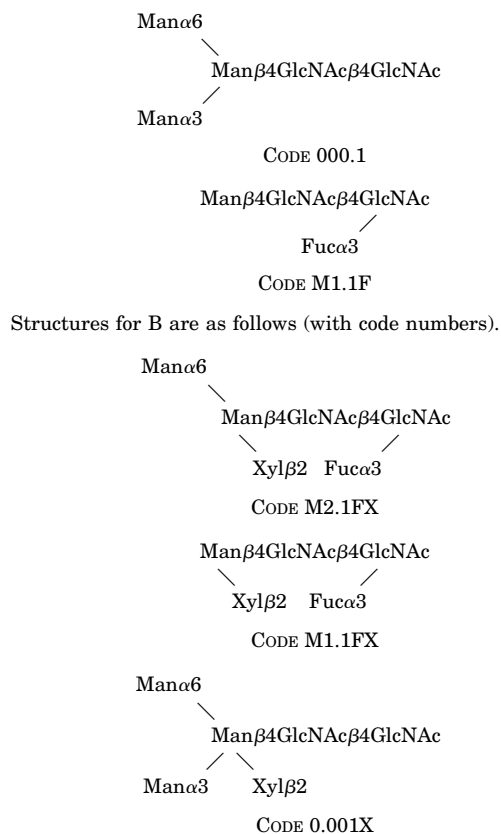


FIG. 2. Identification of oligosaccharides A-E by sequential digestion with exoglycosidases and two-dimensional mapping. The arrows indicate the direction of the changes in the elution positions after digestion with exoglycosidases: solid right arrow, α -mannosidase; dashed right arrow, β -N-acetylhexosaminidase. The α -(1,3)-linked Fuc was chemically removed (dotted right arrow) as described under "Experimental Procedures." The detailed structures of the oligosaccharides shown in this figure (●, samples; ○, reference compounds) are as follows for A (with code numbers).



oligosaccharide A is related to oligosaccharide B or derived from the trimannosyl core. This is a fair hypothesis, since other oligosaccharides from BG60 (Table II) are all related to the trimannosyl core.

The minor oligosaccharides, C, D, and E, all turned out to be of known structure (Table I). Coordinates of the PA derivatives of C, D, and E matched those of 000.1X, M2.1FX, and 100.1FX, respectively, and co-chromatography of each PA derivative with the respective reference PA-oligosaccharide on two different HPLC columns gave a single peak in all cases.

Trimming of the PA-oligosaccharides C, D, and E with exoglycosidases, carried out in parallel with their corresponding reference PA-oligosaccharides, also confirmed their structural identity at each stage of trimming. The changes in the coordinates in the two-dimensional map are shown in Fig. 2B. The trimming was terminated when the common core trisaccharide, $\text{Man}\beta 4\text{GlcNAc}\beta 4\text{GlcNAc}$, was obtained. The elution coordinates and the proposed structures of the oligosaccharides from BG60 are summarized in Table II.

DISCUSSION

Currently available glycoamidases failed to release oligosaccharides from intact BG60. However, once the glycopeptides were prepared from BG60, the glycoamidase from sweet almond worked effectively to release nearly all of the carbohydrates as reducing oligosaccharides. This establishes that the carbohydrates contained in BG60 were nearly totally asparagine-linked. It should be pointed out that glycoamidase from *Flavobacterium* (peptide N-glycosidase F) was ineffective in releasing oligosaccharides from the BG60 (data not shown). This is now understandable since the majority of the oligosaccharides in BG60 contains a Fuc residue α -(1,3)-linked to the GlcNAc residue connected to Asn (29, 30).

Of the five oligosaccharides isolated from BG60, the oligosaccharide B (68.3% of total) is the predominant, followed by the oligosaccharide A (11.9% of total). These oligosaccharides (~80% combined) are devoid of xylose and will be referred to as Type I oligosaccharides of BG60. The other three oligosaccharides (C, D, and E) make up only 9% of the total and are characterized by the presence of xylose β -(1,2)-linked to the branching Man, typically found in plant glycoproteins. These would be referred to as Type II.

To the best of our knowledge, the Type I oligosaccharides of BG60 are the first oligosaccharide chains from plant glycoproteins that contain α -(1,3)-linked Fuc without any xylose. Oligosaccharides frequently found in plant glycoproteins contain both Fuc and Xyl, e.g. oligosaccharide 000.1FX (Fig. 2B) (19, 31-35) or contain only Xyl, e.g. 000.1X (Fig. 2B) (18, 36-38). Although the oligosaccharides 5-A found among the 14 oligosaccharides of honey bee venom PLA₂ (14) is identical to oligosaccharide B in BG60, oligosaccharide 5-A is a minor (only 3.9%) component among the PLA₂ oligosaccharides. In con-

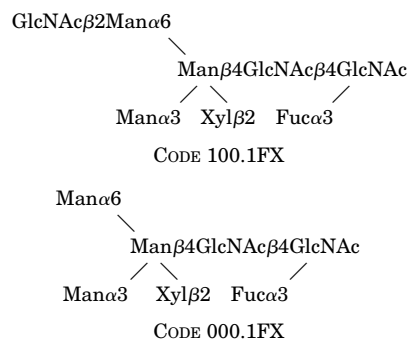


TABLE I

Reporter group	Residue ^a	Chemical shift ^b		
		Peak B	Laccase a ^c	PLA-9
H-1	GlcNAc-2	4.596		4.604
	Man-3	4.857	4.857	
	Man-4	5.122	5.120	5.105
	Man-4'	4.910	4.907	4.922
	Fuc	5.057	5.053	5.052
	Fuc'			5.132
H-2	Man-3	4.256		4.246
	Man-4	4.034		4.167
	Man-4'	3.974		3.983
H-5	Fuc	4.22 ^d		4.25
	Fuc'			4.862
NAc	GlcNAc-2	2.041	2.041	2.050
CH ₃	Fuc	1.20	1.201	1.20
	Fuc'			1.27

[illegible]

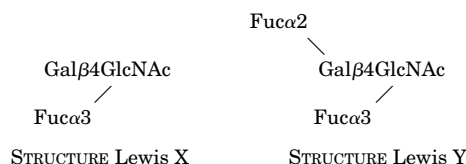
PEAK B

LACCASE A

PLA-9 (14)

^d By two-dimensional HOHAHA.

A structural feature common to all the oligosaccharides of BG60 (excepting the oligosaccharide C) is the fucose α -(1,3)-linked to the PA-modified GlcNAc. The fucose at this position was claimed to be very important in the IgE and IgG binding of the honey bee venom PLA₂ (14). Since this disaccharide segment also exists in the Lewis X and Lewis Y antigens (shown below) in human glycoproteins (39), complete epitopes for the immunological reactions involving honeybee PLA₂ and BG60 must involve a more extended structure than the mere Fuca3GlcNAc segment. Whether fucose-less oligosaccharide C of BG60 can play a part in the immunochemical reaction remains to be elucidated.



Formation of the Type I oligosaccharides in BG60 merits some comments. According to Tezuka *et al.* (40), neither xylose nor fucose can be directly transferred to the trimannosyl core structure (code number 000.1) during the biosynthesis of the oligosaccharides of the sycamore cell laccase. Both xylosyl and fucosyl transferases require the presence of *N*-acetylglucosamine β -(1,2)-linked to the mannose α -(1,3)-linked to the β -mannosyl residue. Thus, the minimum structure required by these two glycosyltransferases is shown below.

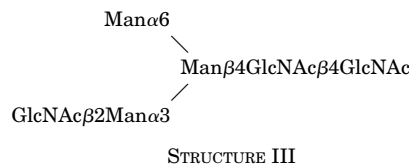


TABLE II
PA-oligosaccharides from BG60: elution positions, structures, and molar %

Oligosaccharides (code no. ^a)	Elution positions observed (reported)	Structures	Mol %
A	4.9, 4.2		11.9
B	5.8, 5.5		68.3
C (000.1X)	7.3, 4.7 (7.2, 4.8)		3.27
D (M2.1FX)	7.3, 4.3 (7.3, 4.4)		3.55
E (100.1FX)	7.3, 6.0 (7.1, 6.2)		2.16

^b Expressed in Glc units, in the orders of the value from the ODS column followed by the value from amide-silica column.

was caused by extracellular degradation in subcellular organelles such as protein bodies or vacuole of plant secretory glycoproteins. The existence of several exoglycosidases in the sycamore cell culture medium was also demonstrated (41). Formation of Type I oligosaccharides in BG60 and the heterogeneity of the BG60 oligosaccharides may also be the result of degradative reactions rather than of imperfect biosyntheses.

Acknowledgment—We thank Dr. Reiko T. Lee for careful reading the manuscript and offering valuable comments.

Note Added in Proof—While this manuscript was in preparation, reports of the structure indicative of the oligosaccharide B in this report, as very minor constituents in soybean peroxidase (0.9%) (Gray, S. S., Yang, B. Y., Hull, S. R., Venzke, D. P., and Montgomery, R. (1996) *Glycobiology* **6**, 23–32) and in horseradish peroxidase (4%) (Yang, B. Y., and Montgomery, R. (1996) *Carbohydr. Res.* **287**, 203–212) appeared.

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