the results reported by Cardinale and Abeles that the observed minimum value of optical rotation of proline is consistent with the observed deuterium isotope effect at the α-position. The overshoot produced by α-amino-ε-caprolactam racemase is relatively low, but the isotope effects at the α-position are higher than those observed in the proline racemase reaction. This could be related to the fact that both D and L enantiomers are produced from either of the two enantiomers of α-amino-ε-caprolactam and that the proton abstracted from the substrate is mostly exchanged with solvent while the substrate is present on the enzyme.

Registry No. H_2, 1333-74-0; D_2, 7782-39-0; α-amino-ε-caprolactam racemase, 52652-64-9; D-α-amino-ε-caprolactam, 28957-33-7; L-α-amino-ε-caprolactam, 21568-87-6; [α-^{2}H]-D-α-amino-ε-caprolactam hydrochloride, 99560-23-3; [U-^{14}C]-L-α-amino-ε-caprolactam hydrochloride, 99560-25-5; [α-^{2}H]-L-α-amino-ε-caprolactam hydrochloride, 99560-24-4.

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Xylose-Containing Common Structural Unit in N-Linked Oligosaccharides of Laccase from Sycamore Cells†

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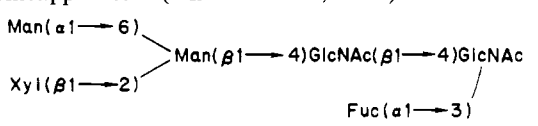
ABSTRACT: The structures of asparagine-linked oligosaccharides of laccase excreted by sycamore (*Acer pseudoplatanus* L.) cells are reported. Peptic glycopeptides obtained from the laccase were treated with *N*-oligosaccharide glycopeptidase (EC 3.5.1.52) to release the oligosaccharide moieties. The oligosaccharides thus obtained were fractionated into six components by gel filtration, thin-layer chromatography, and high-performance liquid chromatography. The structures of the isolated oligosaccharides were determined by sugar analysis, exoglycosidase digestion, and methylation analysis in combination with high-resolution proton nuclear magnetic resonance spectroscopy. It was found that (1) the six oligosaccharides are a series of compounds of xylose-containing biantennary complex types that share as the core a common structural unit, i.e., Xylβ1→2(Manα1→6)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc, and (2) mannose, *N*-acetylglucosamine, galactose, and fucose residues are additionally linked to the core as the outer chain moieties.

We have previously reported the complete structure of the asparagine-linked oligosaccharide of bromelain isolated from the pineapple stem (Ishihara et al., 1979):



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Compositional analyses and partial structural determinations reported so far indicate that not only mannose and *N*-acetylglucosamine but also fucose, galactose, and xylose are present in plant glycoproteins. The presence of complex-type oligosaccharides in plant glycoproteins has recently been suggested in *Phaseolus vulgaris* phytohemagglutinin by Vitale et al. (1984) or in α-amylase by Mitsui et al. (1985). Complete structural studies of these oligosaccharides, however, have not yet been accomplished.

In the present paper, we report a structural study of oligosaccharides of laccase isolated from the suspension-cultured cells of sycamore (*Acer pseudoplatanus* L.). *N*-Oligosaccharide glycopeptidase cleaves quantitatively β-aspartylglucosylamine linkages in glycopeptides, releasing a carbohydrate chain with a chitobiose unit at the reducing end and leaving behind a carbohydrate-free peptide (Takahashi, 1977; Takahashi & Nishibe, 1981). This enzyme has a broad specificity with respect to the carbohydrate moiety; nonsialylated complex-type oligosaccharides as well as high-mannose-type and hybrid-type oligosaccharides are released from a variety of glycopeptides (Ishihara et al., 1983; Hotta et al., 1985; Tarentino & Plummer, 1982). Peptic glycopeptides derived from laccase were digested with the glycopeptidase, and the mixture of oligosaccharides thus obtained was completely separated by techniques including high-performance liquid chromatography (HPLC).[1]

High-resolution proton nuclear magnetic resonance (NMR) was used along with a variety of standard techniques to propose the structure of a series of oligosaccharides possessing as the common core the hexasaccharide that has been shown to exist in bromelain. The significance of the existence of this type of oligosaccharides will also be discussed briefly.

Materials and Methods

Enzymes and Standard Oligosaccharides. *N*-Oligosaccharide glycopeptidase from almond (obtainable as glycopeptidase A), α-L-fucosidase from *Charonia lampas*, and α-mannosidase, β-galactosidase, and β-*N*-acetylhexosaminidase from jack bean were purchased from Seikagaku Kogyo. Pepsin was purchased from Sigma. β-Xylosidase was prepared from almond emulsion by the method of Yoshima et al. (1979). Isomalto oligosaccharides of 4–20 glucose units were prepared from the partial acid hydrolysate of dextran (Nishigaki et al., 1978). Stem bromelain oligosaccharide $Man_2Xyl_1Fuc_1GlcNAc_2$ was prepared from purified stem bromelain that was purchased from Jintan-Dorf Co. (Yasuda et al., 1970).

Other Chemicals. The following materials were purchased from the sources indicated: Sephadexes G-15 and G-200 (superfine) were from Pharmacia; Bio-Gel P-4 (200–400 mesh) was from Bio-Rad; sodium cyanoborohydride and sodium borodeuteride were from Aldrich; 2-aminopyridine and dextran (M_r 200 000–300 000) were from Wako Pure Chemical Industries.

Preparation of Laccase from Sycamore Cells. Laccase excreted by sycamore (*Acer pseudoplatanus* L.) cells was isolated from the culture medium by the method of Bligny & Douce (1983). It was confirmed that the laccase preparation obtained is homogeneous and contains 45% carbohydrate. The monosaccharide composition of the laccase preparation was determined by the method described below, and the results obtained are listed in Table I.

Preparation of Oligosaccharides from the Sycamore Cell Laccase by N-Oligosaccharide Glycopeptidase Digestion. A total of 12.8 mg of the sycamore-cell laccase was used for the isolation of oligosaccharides. Laccase glycopeptides obtained by pepsin digestion were treated with *N*-oligosaccharide glycopeptidase as described previously (Hotta et al., 1985). The oligosaccharide fraction was collected by gel filtration on a Bio-Gel P-4 column and further purified by passing it through columns of the ion-exchange resins Dowex 50W-X8 (H^+) and Amberlite IRA-400 (CO_3^{2-}) (Nishibe & Takahashi, 1981).

Separation of Oligosaccharides by Thin-Layer Chromatography (TLC). The mixture of oligosaccharides was separated by TLC on a silica gel plate (Merck Catalog No. 5721) according to the method of Holmes & O'Brien (1979). Each fraction was eluted with water.

Isolation of Pyridylamino Oligosaccharides by HPLC. Oligosaccharides (200 μg each as neutral sugar) separated by TLC were reductively aminated with 2-aminopyridine by the use of sodium cyanoborohydride (Hase et al., 1984). The pyridylamino derivatives of oligosaccharides thus prepared were purified by gel filtration on a Sephadex G-15 column and were isolated and identified by HPLC on a Shim-Pack CL-C-ODS column (6 × 150 mm, Shimadzu) according to the method of Hase et al. (1984).

Exoglycosidase Digestion of Oligosaccharides. Each fraction of oligosaccharides (about 20 μg each as neutral sugar) separated by TLC was digested with 0.1–0.5 unit of β-galactosidase, β-xylosidase, β-*N*-acetylhexosaminidase, α-mannosidase, or α-L-fucosidase at 37 °C for 20 h. For a limited α-mannosidase digestion, the incubation time was reduced to 2 h. The reaction mixtures were heated at 100 °C for 5 min to terminate the digestion. Resulting oligosaccharides were desalted by ion-exchange resins as described previously (Nishibe & Takahaski, 1981). For digestion using β-galactosidase and β-xylosidase, 0.2 M citrate–phosphate buffer (pH 3.5) was used. Digestion with β-*N*-acetylhexosaminidase, α-mannosidase, and α-L-fucosidase was performed in 0.2 M citrate–phosphate buffer (pH 5.0), 0.05 M acetate buffer (pH 4.5), and 0.1 M acetate buffer (pH 4.0) containing 0.5 M NaCl, respectively.

Methylation Analysis. Oligosaccharide (about 50–100 μg each as neutral sugar) separated by TLC was reduced and methylated by the method of Hakomori (1964). The permethylated products were analyzed as described by Takahashi et al., (1985) on a JEOL-DX300 gas chromatograph–mass spectrometer.

Other Analytical Procedures. Total neutral sugar was determined by the phenol–H_2SO_4 reaction (Dubois et al., 1956). The laccase preparations or the oligosaccharides separated by TLC were hydrolyzed by 2.5 M trifluoroacetic acid at 100 °C for 6 h in an evacuated sealed tube (Arakawa et al., 1976). The monosaccharides obtained were analyzed by HPLC on a ISA-07/S2504 column (4 × 250 mm) was described by Mikami & Ishida (1983).

^1H NMR Measurements. Prior to NMR measurements, pyridylamino derivatives of each oligosaccharide (about 200 μg each as neutral sugar) isolated by HPLC were purified by gel filtration on a Sephadex G-15 column. Samples were dissolved in 99.8% D_2O, lyophilized, and dissolved again in 99.8% D_2O at concentrations of 150–700 μM. NMR measurements were made on a Bruker WM-400 spectrometer operating at 400 MHz in the Fourier-transform mode. All

[1] Abbreviations: Fuc, L-fucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; GlcN, D-glucosamine; Man, D-mannose; Xyl, D-xylose; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid sodium salt; ppm, parts per million.

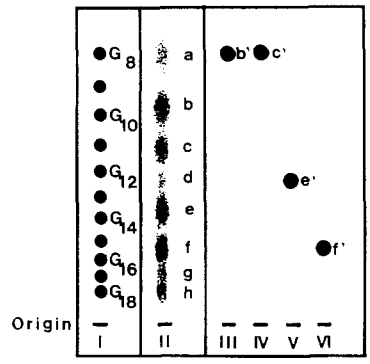


FIGURE 1: TLC profiles of laccase oligosaccharides. Oligosaccharide fractions obtained by *N*-oligosaccharide glycopeptidase digestion of laccase were separated by Bio-Gel P-4 column chromatography and applied on a silica gel plate as described in the text. 1-Propanol/acetic acid/water (3:3:2 by volume) was used as a solvent. Oligosaccharides were visualized with orcinol–H_2SO_4 reagent: (lane I) mixture of standard glucose oligomers (numbers indicate glucose units); (lane II) laccase oligosaccharide fraction; (lanes III–VI) reaction products by *N*-acetylhexosaminidase (jack bean) digestion of compounds b (lane III), c (lane IV), e (lane V), and f (lane VI).

measurements were made at 30 °C. Typically, 2000 transients were accumulated for each measurement. Chemical shifts are expressed from internal DSS but were actually measured with internal acetone. The chemical shift of acetone in reference to internal DSS was determined to be 2.216 ppm in D_2O at 30 °C. Chemical shift values reported are accurate to 0.002 ppm.

Results and Discussion

N-Oligosaccharide Glycopeptidase Digestion of Laccase Glycopeptides. Oligosaccharides linked to the peptic glycopeptides of laccase (12.8 mg) were liberated from the peptide moieties by *N*-oligosaccharide glycopeptidase digestion. About 90% of the total carbohydrates in the original glycopeptides was recovered as asparagine-linked oligosaccharides by the enzyme digestion.

Relative Abundance and Molar Carbohydrate Composition of the Oligosaccharides. The oligosaccharide fractions obtained by glycopeptidase digestion were separated by Bio-Gel P-4 column chromatography and were desalted by passing them through ion-exchange resins. A portion of the sample (about 1 mg) was chromatographed on a silica-gel plate for oligosaccharide separation. For comparison, a series of isomalto oligosaccharides were run on the same plate. Figure 1 shows a typical oligosaccharide profile. Eight fractions corresponding to isomaltooctaose–isomaltooctadecaose were designated a–h in the order of mobility. Each of these fractions was eluted from the plate with water. The carbohydrate composition of fractions a–h was determined after hydrolysis with trifluoroacetic acid, and the results are summarized in Table I. It was confirmed that each of the fractions contains a sufficiently homogeneous oligosaccharide, because, as Table I shows, integral numbers were obtained for each constituent. All of the compounds a–h are characterized by the presence of one xylose and three mannose residues. The contents of fucose, galactose, and *N*-acetylglucosamine are different in compounds a–h. This suggests that these glycans possess structures related closely to that of the carbohydrate moiety of pineapple stem bromelain, which we have reported previously (Ishihara et al., 1979). The amount of oligosaccharides recovered from fractions a–c, e, and f was 45, 84, 196, 193, and 200 μg, respectively. Compounds d, g, and h were obtained in an amount too small for any further structural analyses.[2]

Table I: Carbohydrate Composition of the Whole Laccase Molecule, Compounds a–h, and Stem Bromelain

	molar ratio[a]				
	GlcN	Man[b]	Fuc	Gal	Xyl
laccase	4.4	3.0	2.3	1.2	1.0
compd a	2.0	3.0	1.1	0	1.0
compd b (b1 + b2)	3.1	3.0	0.9	0	1.0
compd c	4.1	3.0	1.1	0	1.0
compd d	4.1	3.0	1.2	0.9	1.0
compd e	4.0	3.0	1.9	1.0	1.0
compd f	4.0	3.0	3.0	2.1	1.0
compd g	4.9	3.0	2.8	1.8	1.0
compd h	5.0	3.0	4.2	2.8	0.8
stem bromelain[c]	2.0	2.0	1.0	0	1.0

[a] The amount of each sugar was determined by an HPLC procedure for monosaccharides described under Materials and Methods. [b] In the laccase and bromelain compounds, molar ratios were calculated by taking the value of mannose as 3.0 and 2.0, respectively. [c] Xylβ1→2-(Manα1→6)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc (Ishihara et al., 1979).

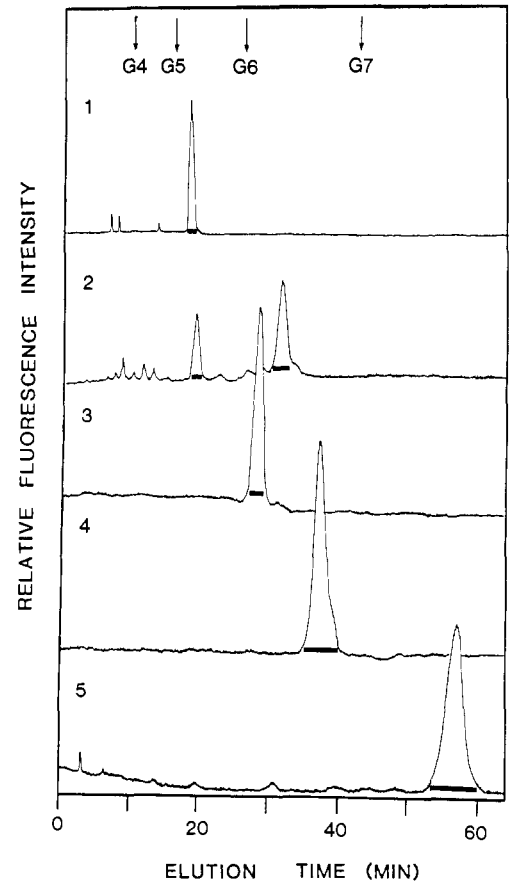


FIGURE 2: HPLC profiles of compounds a–c, e, and f from laccase. Each compound separated by TLC (Figure 1) was extracted with water, aminated, and applied on HPLC as described in the text. Elution was carried out at 50 °C with 0.1 M phosphate buffer (pH 3.8) at a flow rate of 1 mL/min, and fluorescence was detected with an excitation and emission wavelength of 320 and 400 nm, respectively. (1) Compound a; (2) compound b; (3) compound c; (4) compound e; (5) compound f. Arrows indicate the eluting positions of standard glucose oligomers (numbers indicate glucose units). The oligosaccharide fractions indicated by bars were pooled.

In order to check the purity of compounds a–c, e, and f, the pyridylamino derivative of the individual oligosaccharide was

[2] Glycopeptidase-undigested fraction (about 10% of the total carbohydrates in the original glycopeptides) separated by a Bio-Gel column was further treated by hydrazinolysis. No other oligosaccharides were detected on the plate.

separately subjected to HPLC. As Figure 2 shows, compound b was separated into two peaks (designated b1 and b2) on the elution profile. The ratio of yield of b1 and b2 was 1:1.7. By contrast, compounds a, c, e, and f all gave a single peak. Hereafter, compounds contained in each of these fractions will also be referred to as a, b1, b2, c, e, and f.

Exoglycosidase Digestion. It was observed that on incubation with jack bean *N*-acetylhexosaminidase compounds b (b1 + b2) and c are converted to smaller oligosaccharides (Figure 1, b′ and c′, respectively), which gave the same mobility as compound a. It was also shown that compound e is converted to e′. By contrast, on treatment with the enzyme, no change in mobility was observed for compound f (Figure 1, f′). The digestion products b′, c′ and e′ were eluted from the TLC plate, and the composition of monosaccharides of each oligosaccharide was determined. It was confirmed that 1 mol of *N*-acetylglucosamine is removed from b and e. This indicates that 1 mol of *N*-acetylglucosamine is linked to the nonreducing end of b and e. It was demonstrated in a similar manner that 2 mol of *N*-acetylglucosamine exists at the nonreducing end of c.

Limited digestion by jack bean α-mannosidase, which cleaves the Manα1→3Man linkage in preference to the α1→6 linkage (Berman & Allerhand, 1981), was performed as in the case of the *N*-acetylhexosaminidase digestion described above. The result of this experiment shows that 1 mol of Manα1→3 linkage is present at the nonreducing end of a. By contrast, less than $^2/_3$ mol of Manα1→3 linkage was detectable at the nonreducing end of b. As clearly demonstrated by the HPLC experiment (Figure 2), b is actually a mixture of two oligosaccharides, b1 and b2. In view of the above result, we suggest that b1 and b2 have different susceptibility to α-mannosidase.

Methylation Analysis. Table II summarizes the results of the methylation analysis of the laccase oligosaccharides a, b1 + b2, c, e, and f that were separated by TLC. These results show that compound a has a structure that is identical with that of bromelain except for the external Manα1→3 residue (Ishihara et al., 1979). It was also demonstrated that compounds b and c have the same core structure as compound a, with an additional 1 mol and 2 mol of GlcNAcβ1→2 residue at the nonreducing terminals, respectively. The results of the methylation analysis also show that compounds e and f have the same structural unit as compound c with one set and two sets of one galactose and one fucose residue at their nonreducing terminals, respectively.

After compound c (80 μg) was incubated with *Charonia lampas* α-L-fucosidase, the resulting oligosaccharide was analyzed by methylation analysis. It was observed that after defucosylation 1,5,6-tri-*O*-methyl-2-(*N*-methylacetamido)-2-deoxyglucitol, which was detected in methylation analysis of intact compound c, disappeared completely and the 1,3,5,6-tetra-*O*-methyl derivative appeared instead (see Table II). This result established that in compound c a fucose residue is linked to position 3 of the reducing *N*-acetylglucosamine.

Compound f (80 μg) was sequentially analyzed by almond β-xylosidase digestion and methylation analysis. We observed that after dexylosylation 4-mono-*O*-methylmannose, which was detected in intact compound f, disappeared completely with concomitant appearance of 2,4-di-*O*-methylmannose. This result indicates that a xylose residue is linked to position 2 of the innermost mannose residue. Comparisons of the above data with those for bromelain suggest that the laccase oligosaccharide series have the common core structure Xylβ1→2-(Manα1→6)Manβ1→4GlcNAcβ1→4(Fucα1→3)GlcNAc

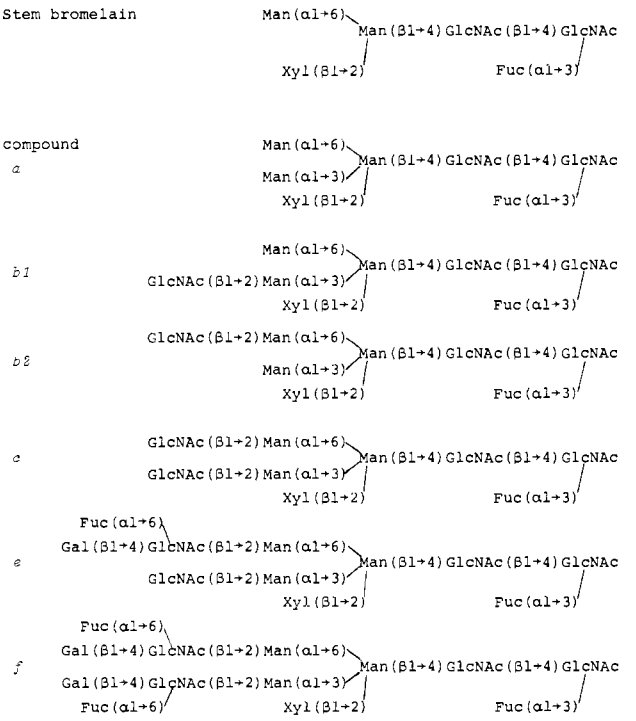


FIGURE 3: Proposed structures of the oligosaccharides obtained from laccase excreted by sycamore cells.

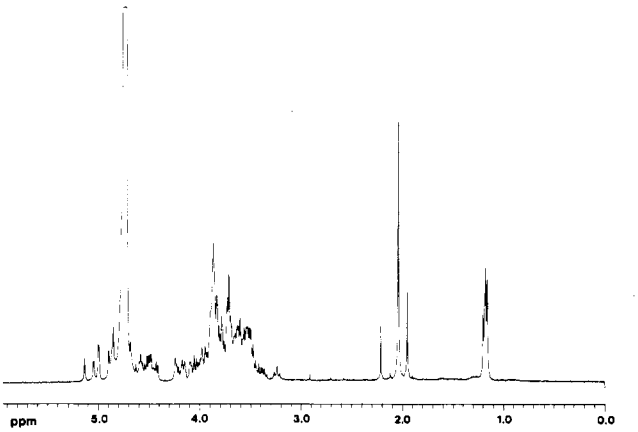


FIGURE 4: The 400-MHz ^{1}H NMR spectrum of compound f. Chemical shifts are in ppm from DSS. The signal of acetone is at 2.216 ppm. The probe temperature was 30 °C.

that exists in bromelain. On the basis of the results of the glycosidase digestion and the methylation analysis described above, we propose in Figure 3 the structures of oligosaccharides a, b1, b2, c, e, and f.

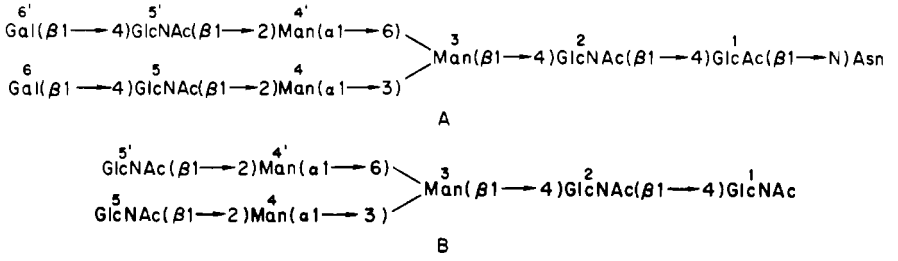
Structural Analyses by ^{1}H NMR. In the experiments of the methylation analyses described above, the positioning of the fucose and xylose residues was actually confirmed only in the cases of compounds c and f, respectively. In order to reach a more decisive conclusion about the structure of the oligosaccharides, ^{1}H NMR spectral data were collected and analyzed. For NMR measurements, about 50–200 μg each of pyridylamino derivatives of the oligosaccharides a, b1, b2, c, e, and f were purified by means of HPLC (see Figure 2). As an example, the ^{1}H NMR spectrum of compound f is given in Figure 4. In Figure 5, the spectra of the CH_3 and H-1 protons of compounds a, b1, b2, c, e, and f are compared with that of bromelain. Table III summarizes the observed chemical shift data. It should be noted that the pyridylamino derivatives of oligosaccharides were used in the present NMR study. Chemical shift data have been compiled for a series

Table II: Molar Ratios of Methylated Alditol Acetates in the Hydrolysates of the Permethylated Compounds a–c, e, f, c″ (Fucosidase Digested), and f″ (Xylosidase Digested) and Bromelain

methylated alditol acetates	molar ratio							
	a	b (b1 + b2)	c	e	f	c″	f″	bromelain
2,3,4-Me_3Fuc	0.8	0.9	0.7	1.8	2.6	0	2.5	0.9
2,3,4-Me_3Xyl	0.8	0.7	0.9	0.9	0.8	0.7	0.1	0.8
2,3,4.6-Me_4Gal	0	0	0	0.9	1.8	0	1.9	0
2,3,4,6-Me_4Man	2.0[a]	1.0[a]	0	0	0	0	0	1.0[a]
3,4,6-Me_3Man	0	1.0	2.0[b]	2.0[b]	2.0[b]	2.0[b]	2.0[b]	0
3,4-Me_2Man	0	0	0	0	0	0	0	0.7
2,4-Me_2Man	0	0	0	0	0	0	0.6	0
4-MeMan	0.7	0.8	0.7	0.9	0.9	0.8	0.1	0
3,4,6-Me_3GlcNAc(Me)	0	0.8	1.6	0.9	0	1.7	0	0
3,6-Me_2GlcNAc(Me)	0.7	0.8	0.7	0.9	0.8	0.7	0.7	0.8
1,3,5,6-Me_4GlcNAc(Me)	0	0	0	0	0	0.6	0	0
1,5,6-Me_3GlcNAc(Me)	0.6	0.6	0.5	0.5	0.6	0	0.5	0.6
3-MeGlcNAc(Me)	0	0	0	nd[c]	nd[c]	0	nd[c]	0

[a] Molar ratios were calculated by taking the value of 2,3,4,6-Me_4Man (2,3,4,6-tetra-*O*-methylmannose) as either 1.0 or 2.0. [b] Molar ratios were calculated by taking the value of 3,4,6-Me_3Man (3,4,6-tri-*O*-methylmannose) as 2.0. [c] In compounds e, f, and f″, 3-MeGlcNAc(Me) [3-mono-*O*-methyl-2-deoxy-2-(*N*-methylacetamido)glucitol] was not detectable under the experimental conditions.

Chart I



of glycopeptides (Vliegenthart et al., 1983). The pyridylamino derivative of an oligosaccharide from fibrinogen, which is of biantennary complex type, Galβ1→4GlcNAcβ1→2Manα1→6(Galβ1→4GlcNAcβ1→2Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc, was used to check the effect of the modification of GlcNAc-1 upon the observed chemical shifts. A significant difference in chemical shift was observed for GlcNAc-2 and Man-3 resonances as a consequence of pyridylamination. However, it was confirmed that Man-4, Man-4′, and other residues that are further apart from GlcNAc-1 do not show any significant shift upon modification of GlcNAc-1. On the basis of these results, spectral assignments for bromelain, which has the known structure, can be made unambiguously with the help of spin-decoupling experiments.

Comparison of the chemical shift data listed in Table III with those compiled previously for a number of oligosaccharides (Vliegenthart et al., 1983) demonstrates that a particularly large shift is observed for the H-1 proton of Man-3. As Figure 5 and Table III show, bromelain and compounds a, b1, b2, c, e, and f give quite similar shifts for the H-1 proton of Man-3. It has been demonstrated that bromelain has a xylose residue that is linked to Man-3 in the β1→2 linkage. It has also been shown in the present work that a xylose is linked to Man-3 in the β1→2 linkage in compound f. On the basis of these results, we conclude that in compounds a, b1, b2, c, e, and f the xylose residue is always bonded to Man-3 in the β1→2 linkage as in the case of bromelain.

Compound a gives peak 4, which is not present in the spectrum of bromelain. On the basis of the result of methylation analysis described above, we are able to assign peak 4 to the H-1 proton of Man-4. Peak 4 is also observed for compounds b1, b2, c, e, and f. Peak 4 observed for compound b2 gives a chemical shift that is identical with that for compound a. By contrast, chemical shifts observed for peak 4 for compounds b1, c, e, and f are all identical but are significantly different from those observed for compounds a and b2. These results indicate that (1) in compounds a and b2 Man-4 is at a nonreducing terminal and (2) an *N*-acetylglucosamine residue is bonded to Man-4 in compounds b1, c, e, and f (see Figure 3). Chemical shifts of peak 4′, which originates from the H-1 proton of Man-4′, are quite similar for bromelain, compound a, and compound b1. This means that compounds a and b1 possess Man-4′ at a nonreducing terminal as in the case of bromelain. Peak 4′ observed for compounds b2, c, e, and f gives identical chemical shifts, which are identical but significantly different from those for compounds a and b1. This indicates that an *N*-acetylglucosamine residue is bonded to Man-4′ in compounds b2, c, e, and f.

Compound c gives two pairs of doublets for the anomeric protons of GlcNAc-5 (4.511 ppm) and GlcNAc-5′ (4.546 ppm). The chemical shift observed for each pair of the doublets coincides with that observed for GlcNAc-5 (4.513 ppm) of compound b1 or that for GlcNAc-5′ (4.544 ppm) of compound b2. This result is consistent with the above conclusion derived on the basis of the chemical shift data observed for Man-4 and Man-4′. It has been reported that the chemical shifts of the H-1 proton of GlcNAc-5 and GlcNAc-5′ are identical in the case of oligosaccharides A and B (Chart I). The reported chemical shift values are 4.582 and 4.558 ppm for oligosaccharides A and B, respectively [Tables IV and V of Vliegenthart et al. (1983)]. The present results show that the chemical shifts observed for the H-1 protons of GlcNAc-5 and GlcNAc-5′ of compound c are both significantly different from those observed for compound B. The chemical shift difference observed for GlcNAc-5 is much larger than that for GlcNAc-5′. These results indicate that the presence of the xylose residue bonded to Man-3 at position 2 affects the chemical shifts for GlcNAc-5 and GlcNAc-5′ in a different way; a much larger shift is observed for GlcNAc-5, which is

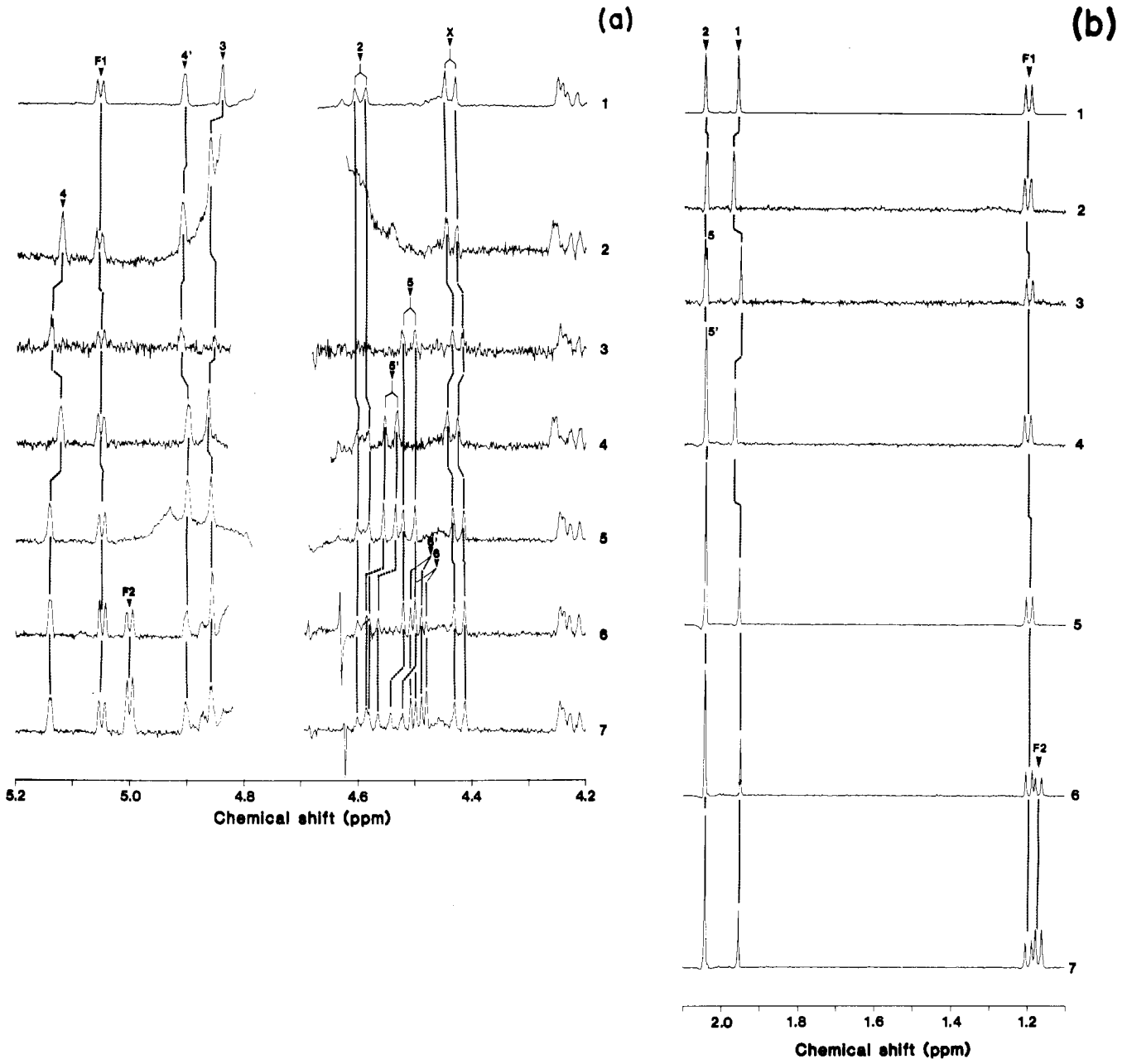


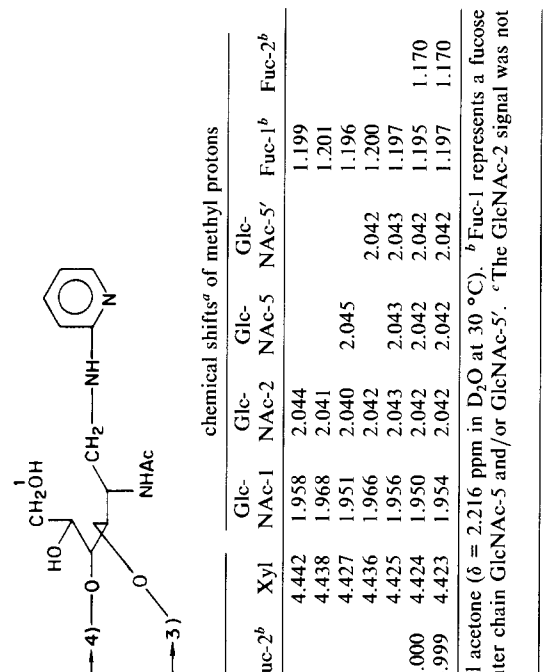
FIGURE 5: The 400-MHz ^1H NMR spectra of the anomeric (a) and methyl (b) protons of oligosaccharides of (1) bromelain, (2) compound a, (3) compound b1, (4) compound b2, (5) compound c, (6) compound e, and (7) compound f. X, Xyl; F1, fucose linked to GlcNAc-1; F2, fucose linked to GlcNAc-5 and/or GlcNAc-5′; 1, GlcNAc-1; 2, GlcNAc-2; 5, GlcNAc-5; 5′, GlcNAc-5′; 3, Man-3; 4, Man-4; 4′, Man-4′; 6, Gal-6; 6′, Gal-6′. Chemical shifts are in ppm from DSS. The probe temperature was 30 °C.

bonded to the Man-4(α1→3)Man-3 branch, as compared to GlcNAc-5′, which is linked to Man-4′(α1→6)Man-3 branch.

In compound e, where one galactose and one fucose residue are added to compound c, a large shift is observed for the H-1 proton of GlcNAc-5′; no change in chemical shift is observed for the H-1 proton of GlcNAc-5. These results indicate that in compound e the galactose and the fucose residues are both linked to GlcNAc-5′.

In compound f, one galactose and one fucose residue are further added to compound e. As listed in Table III, compounds e and f give identical chemical shifts for the H-1 proton of GlcNAc-5′. By contrast, a significant shift of 0.022 ppm is observed for the H-1 proton of GlcNAc-5. This indicates that in compound f one galactose and one fucose residue are both bonded to GlcNAc-5 of compound e. This observation is consistent with the above conclusion that the galactose and the fucose residues are both linked to GlcNAc-5′ in compound e. Chemical shift data observed for the H-1 proton of Gal-6 and Gal-6′ suggest that the galactose residues are bonded to GlcNAc-5 and GlcNAc-5′ in the β1→4 linkage [Table V of Vliegenthart et al. (1983)]. Chemical shifts observed for the CH_3 and H-1 protons of Fuc-1 of compounds a, b1, b2, c, e, and f are all very close to those observed for bromelain. This clearly indicates that Fuc-1 is bonded to GlcNAc-1 in the α1→3 linkage in all the compounds examined in the present work. The CH_3 and H-1 proton resonances due to Fuc-2, which are observed for compounds e and f, give chemical shifts that are significantly different from those for Fuc-1 (see Figure 5 and Table III). It should be noted, however, that in compounds e and f the chemical shifts observed for Fuc-2 residues are all identical. This suggests that Fuc-2 residues bonded to GlcNAc-5 and GlcNAc-5′ are in the same environments. With Fuc-2 bonded to GlcNAc-5 or GlcNAc-5′ in the α1→3 linkage, the chemical shifts of the CH_3 and H-1 protons of

Table III: Chemical Shifts of Anomeric Protons and Methyl Protons for the Pyridylamino Derivatives of Oligosaccharides of Laccase (Compounds a, b1, b2, c, e, and f) and Bromelain



compd	chemical shifts[a] of anomeric protons									chemical shifts[a] of methyl protons					
	Glc-NAc-2	Man-3	Man-4	Man-4′	Glc-NAc-5	Glc-NAc-5′	Gal-6	Gal-6′	Fuc-1[b]	Fuc-2[b]	Xyl	Glc-NAc-1	Glc-NAc-2	Glc-NAc-5	Glc-NAc-5′	Fuc-1[b]	Fuc-2[b]
bromelain	4.599	4.839		4.906					5.053		4.442	1.958	2.044			1.199	
compd a	[c]	4.857	5.120	4.907					5.053		4.438	1.968	2.041			1.201	
compd b1	4.593	4.851	5.138	4.910	4.513				5.051		4.427	1.951	2.040	2.045		1.196	
compd b2	4.594	4.862	5.121	4.898		4.544			5.051		4.436	1.966	2.042		2.042	1.200	
compd c	4.592	4.857	5.140	4.899	4.511	4.546			5.048		4.425	1.956	2.043	2.043	2.043	1.197	
compd e	4.591	4.855	5.139	4.900	4.511	4.576		4.499	5.047	5.000	4.424	1.950	2.042	2.042	2.042	1.195	1.170
compd f	4.592	4.856	5.139	4.901	4.533	4.576	4.490	4.499	5.048	4.999	4.423	1.954	2.042	2.042	2.042	1.197	1.170

[a] Chemical shifts are expressed in ppm from internal DSS but were actually measured with internal acetone (δ = 2.216 ppm in D_2O at 30 °C). [b] Fuc-1 represents a fucose residue that is bonded to the innermost GlcNAc-1. Fuc-2 is a fucose residue that is bonded to the outer chain GlcNAc-5 and/or GlcNAc-5′. [c] The GlcNAc-2 signal was not detectable due to the limited amount of the sample.

Fuc-2 would be quite different from those observed for compounds e and f [Tables XV–XXI of Vliegenthart et al. (1983)]. As described above, Gal-6 and Gal-6′ are bonded to GlcNAc-5 and GlcNAc-5′ in the β1→4 linkage. Therefore, we conclude that Fuc-2 is bonded to GlcNAc-5 and GlcNAc-5′ in the α1→6 linkage. All of these results are quite consistent with the structure of compounds a, b1, b2, c, e, and f proposed in Figure 3.

The structures of the laccase oligosaccharides established in the present study have three interesting features. First, the laccase oligosaccharides are all of the biantennary complex type, which has not yet been proved to exist in the plant glycoproteins. Second, the oligosaccharides contain a xylose residue linked to a β-mannosyl residue by β1→2 linkage. Xylose in asparagine-linked oligosaccharides is known to exist only in the case of plant origin. Third, in all the laccase oligosaccharides, fucose is attached by the α1→3 bond to the *N*-acetylglucosamine that is involved in protein–carbohydrate linkage. In compounds e and f, fucose is linked by the α1→6 bond to the outer *N*-acetylglucosamines. In marked contrast, in number of cases reported so far, an α-fucosyl residue is linked to the C-6 position of the proximal *N*-acetylglucosamine of the core portion.

At present, very little information is available about the biosynthesis and processing of asparagine-linked oligosaccharides present in plant glycoproteins. This is because little is known concerning the exact structures of these oligosaccharides. The presence of the xylose-containing common core of the oligosaccharides in pineapple and sycamore suggests that in the higher plant there exists a pathway regulated in a similar way for the biosynthesis of this type of oligosaccharides.

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Registry No. Compound a, 99299-10-2; compound b1, 99309-00-9; compound b2, 99309-01-0; compound c, 99299-11-3; compound e, 99309-02-1; compound f, 99309-03-2; laccase, 80498-15-3.

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Stopped-Flow Fluorescence Studies on Binding Kinetics of Neurotoxins with Acetylcholine Receptor

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ABSTRACT: Acetylcholine receptor from *Narke japonica* electroplax exhibits a fluorescence change upon binding of snake neurotoxins. This fluorescence change primarily arises from the conformational change of the acetylcholine receptor and reflects the binding process of the toxin with the receptor. The time dependence of the fluorescence change has been monitored for 28 short neurotoxins and 8 long neurotoxins by using a stopped-flow technique. The steady-state fluorescence change is of the same order of magnitude for the short neurotoxins but varies among the long neurotoxins. Nha 10, a short neurotoxin with weak neurotoxicity, causes no fluorescence change in the receptor but can still bind to the receptor with sufficiently high affinity. The substitution of the conserved residue Asp-31 to Gly-31 in Nha 10 is probably responsible for the reduced neurotoxicity. The rate constants for the binding of the neurotoxins to the receptor have been obtained by analyzing the transient fluorescence change. The rate constants show surprisingly a wide range of distribution: $(1.0\text{–}20.5) \times 10^6$ M^{-1} s^{-1} for short neurotoxins and $(0.26\text{–}1.9) \times 10^6$ M^{-1} s^{-1} for long neurotoxins. Examination of the relationship between the rate constants of fluorescence change of the short neurotoxins and their amino acid sequences, thermal stability, hydrogen–deuterium exchange behavior, overall net charge, etc. reveals the following. Positive charges on the side chains of residues 27 and 30 and overall net charge of the neurotoxin govern the magnitude of the binding rate of the neurotoxin with the receptor.

The nicotinic acetylcholine receptor (AChR)[1] from fish electric organ and from mammalian skeletal muscle is a complex of four homologous membrane proteins, in the mole ratio of $\alpha_2\beta\gamma\delta$. All of the five subunits are required to elicit

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