

## The Structure of a Neural Specific Carbohydrate Epitope of Horseradish Peroxidase Recognized by Anti-horseradish Peroxidase Antiserum\*

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**Antiserum raised against horseradish peroxidase (HRP) recognizes a neural specific carbohydrate antigen in *Drosophila* and other insects. The epitopic activity of the carbohydrate moiety of HRP recognized by anti-HRP antiserum was measured by a newly developed enzyme-linked immunosorbent assay, in which HRP glycopeptides conjugated with bovine serum albumin were coated onto the wells and then reacted with goat anti-HRP antiserum. HRP sugar moieties released by almond glycopeptidase A digestion of HRP pepsin digests were subjected to pyridylation. Pyridylamino oligosaccharides were separated into seven fractions by reverse-phase high performance liquid chromatography. The major fraction, which comprised about 80% of the total sugars, reacted strongly with anti-HRP antiserum. The carbohydrate structure of this fraction was determined by sugar composition analysis and 600-MHz <sup>1</sup>H NMR spectroscopy as follows: Man $\alpha$ 1 $\rightarrow$ 6(Man $\alpha$ 1 $\rightarrow$ 3)(Xyl $\beta$ 1 $\rightarrow$ 2)Man $\beta$ 1 $\rightarrow$ 4GlcNAc $\beta$ 1 $\rightarrow$ 4(Fuc $\alpha$ 1 $\rightarrow$ 3)GlcNAc. Analyses of reactivity with anti-HRP antiserum of various oligosaccharide derivatives obtained from the major fraction by exoglycosidase digestion and partial acid hydrolysis indicated that  $\alpha$ 1 $\rightarrow$ 6-linked mannose and  $\alpha$ 1 $\rightarrow$ 3-linked fucose are predominantly involved in the epitopic structure.**

As the nervous system develops, it makes complex networks by forming synapses between specific neurons (1). It is natural to assume that cell surface molecules are involved in the recognition between neural cells. Antibodies that specifically react with neural cells have been powerful tools in studying the nature of the molecules that mediate cell-cell interactions. Antiserum raised against horseradish peroxidase (HRP)<sup>1</sup> specifically recognizes the nervous system of *Drosophila* and other insects and has been used as a neural specific probe in

studies on neural development (2).

Recently, antibodies against HRP have been shown to recognize carbohydrate moieties that are expressed on the surface of neurons (3, 4). These carbohydrate antigens are expressed on some neural proteins, especially on fasciclin I and II, only when they are present on neural cells (3). Fasciclin is considered to mediate cell-cell recognition (5). Thus, the characterization of the nature of the carbohydrate moiety recognized by the antiserum against HRP is of great importance.

In this paper, we reveal the structure of the major HRP carbohydrate unit, which is thought to share the antigenic determinant on neural cells as defined by the anti-HRP antiserum. The epitopic structure necessary for anti-HRP antiserum recognition was also deduced by comparing the reactivity with anti-HRP antiserum of various pyridylamino oligosaccharide derivatives obtained from the major carbohydrate unit of HRP.

### EXPERIMENTAL PROCEDURES

**Materials**—HRP (type IV), pepsin, and proteinase K were purchased from Sigma. Pronase P was from Kaken Kagaku Co., Tokyo. Goat anti-peroxidase antiserum was from Cappel Laboratories, West Chester, PA. Swine anti-goat IgG was from Tago Inc., Burlingame, CA. Glycopeptidase A (almond) and  $\alpha$ -mannosidase (jack bean) were from Seikagaku Kogyo, Tokyo.  $\beta$ -Xylosidase (*Charonia lampas*) was a gift from Seikagaku Kogyo, Tokyo. YMC-gel ODS S-5 columns (4.6  $\times$  300 and 10  $\times$  300 mm) were purchased from Yamamura Chemical Laboratories, Kyoto, and TSK-gel amide 80 (4.6  $\times$  250 mm) was from Tosoh Ltd., Tokyo. Sephadex G-15 and G-25 were purchased from Pharmacia LKB Biotechnology AB, Uppsala. Man<sub>6</sub>GlcNAc<sub>2</sub> was prepared from ovalbumin. Pyridylamino *N*-acetylglucosamine (PA-GlcNAc) and pyridylamino *N*-acetylmannosamine were gifts from Dr. S. Hase, Osaka University. M3FX (Man<sub>3</sub>Xyl<sub>1</sub>Fuc<sub>1</sub>GlcNAc<sub>1</sub>PA-GlcNAc<sub>1</sub>) from ricin D was a gift from Dr. Y. Kimura, Okayama University (6).

**Isolation of Pyridylamino Oligosaccharides from HRP**—HRP glycopeptides obtained by pepsin digestion were digested with almond glycopeptidase A according to the procedure of Hotta *et al.* (7). The digest was applied onto a column of Sephadex G-25 (2  $\times$  93 cm), which was equilibrated and eluted with 0.05 M pyridine/acetic acid, pH 5.0. The fractions containing oligosaccharides, determined by the orcinol method (8), were collected and reductively aminated with 2-aminopyridine by sodium cyanoborohydride, according to the method of Hase *et al.* (9). The PA derivatives of oligosaccharides thus obtained were purified by gel filtration on a Sephadex G-15 column (1  $\times$  42 cm) that was equilibrated and eluted with 10 mM ammonium acetate buffer, pH 6.0, and further fractionated by reverse-phase high performance liquid chromatography (HPLC), obtaining one major and several minor PA oligosaccharides (Fig. 2).

**Preparation of Modified Derivatives from the Major Fraction (Fraction 3)**—Fraction 3, equivalent to 100  $\mu$ g of mannose as determined by the orcinol method, was derivatized as follows. Defucosylated

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<sup>1</sup> The abbreviations used are: HRP, horseradish peroxidase; BSA, bovine serum albumin; Fuc, fucose; Xyl, xylose; PA, pyridylamino; HPLC, high performance liquid chromatography; ODS, octadecylsilane; M3FX, Man<sub>3</sub>Xyl<sub>1</sub>Fuc<sub>1</sub>GlcNAc<sub>1</sub>PA-GlcNAc<sub>1</sub>.

Fraction 3 was prepared according to the method of Yasuda *et al.* (10). Ten  $\mu$ l of Fraction 3 was added to 1 ml of trifluoroacetic acid and kept at 20 °C for 20 h. Dextrolylated Fraction 3 was prepared by digestion with 0.1 unit of  $\beta$ -xylosidase in 60  $\mu$ l of 0.1 M citrate/phosphate buffer, pH 4.0, containing 0.5 M NaCl and 100 mM mannono-1,4-lactone at 37 °C for 24 h. Derivatives devoid of 1 or 2 mannose residues were obtained by digesting Fraction 3 with 1 unit of jack bean  $\alpha$ -mannosidase in 30  $\mu$ l of 0.05 M sodium citrate buffer, pH 4.5, at 37 °C for 2 and 26 h, respectively. The mild  $\alpha$ -mannosidase digestion cleaves the Man $\alpha$ 1 $\rightarrow$ 3Man linkage in preference to the  $\alpha$ 1 $\rightarrow$ 6-linkage (11, 12). Extensive digestion cleaves both linkages. The enzymatic reaction was terminated by boiling the reaction mixtures in water for 2 min. Digests were centrifuged to remove insoluble materials and fractionated by HPLC.

**Fractionation of PA Oligosaccharides by HPLC**—PA oligosaccharides from HRP were fractionated by HPLC with a Shimadzu model LC-6A system on a YMC-gel ODS S-5 column (10  $\times$  300 mm). Elution was performed at a flow rate of 1 ml/min using two solvents, A and B. Solvent A was 0.1 M ammonium acetate buffer, pH 4.0, and solvent B was 0.1 M ammonium acetate buffer, pH 4.0, containing 1% butanol. The column was equilibrated with solvent A. After sample injection, the column was eluted by a linear gradient from solvent A to solvent B in 100 min. PA derivatives prepared from Fraction 3 were fractionated by successive HPLC on TSK-gel amide 80 and YMC-gel ODS S-5 columns (4.6  $\times$  250 mm). The amide 80 column was eluted according to Tomiya *et al.* (13). The ODS column was eluted as described above. PA sugars were detected fluorometrically at an excitation wavelength of 320 nm and an emission wavelength of 400 nm.

**Enzyme-linked Immunosorbent Assay**—Reactivity of HRP oligosaccharides with the antiserum was determined in 96-well microtiter plates coated with HRP glycopeptides. These glycopeptides, thoroughly digested with Pronase P and proteinase K, were prepared according to the procedure of Snow *et al.* (3). Glycopeptides equivalent to 1 mg of HRP were conjugated with 2 mg of bovine serum albumin (BSA) by overnight incubation at room temperature in the presence of 30 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/HCl in 1 ml of phosphate-buffered saline. The reaction mixture was extensively dialyzed against distilled water and lyophilized. BSA-conjugated glycopeptides were dissolved in phosphate-buffered saline. One hundred and fifty  $\mu$ l of the above solution containing an appropriate quantity of conjugates was added to each well and incubated overnight at 4 °C. Wells were washed twice with phosphate-buffered saline containing 1% BSA (solution A) and incubated with solution A at room temperature for 1 h to prevent nonspecific binding of antibodies. The solution was aspirated, and 150  $\mu$ l of goat anti-HRP antiserum appropriately diluted with phosphate-buffered saline containing 0.1% BSA (solution B) was added to each well, then allowed to stand at room temperature for 2 h. Wells were washed three times with solution A, then 150  $\mu$ l of swine anti-goat IgG conjugated with alkaline phosphatase diluted 1:8000 with solution B were added to each well. After a 2-h incubation at room temperature, each well was washed three times with solution A and incubated for 30 min after the addition of 150  $\mu$ l of 1 mg/ml *p*-nitrophenyl phosphate solution in 9.7% diethanolamine, pH 9.8. The reaction was terminated by the addition of 50  $\mu$ l of 3 M sodium hydroxide, and the absorbance of each well at 405 nm was determined using a microplate reader (model 3550, Bio-Rad). Samples were added to the wells prior to the addition of the first antibody for the inhibition assay.

**Sugar Composition Analyses**—Hexosamines were determined with a Hitachi L-8500 amino acid analyzer after hydrolysis in 4 M HCl at 100 °C for 6 h. To determine PA hexosamines, samples and standard PA monosaccharides (PA-GlcNAc and PA-ManNAc) were hydrolyzed in 6 M HCl at 100 °C for 8 h and then applied to a HPLC YMC-gel ODS S-5 column (4.6  $\times$  250 mm) isocratically eluted with 0.25 M citrate buffer, pH 4.0, containing 1% acetonitrile at a flow rate of 1 ml/min. For the determination of neutral sugars, samples were hydrolyzed in 4 M trifluoroacetic acid at 100 °C for 5 h, and the neutral sugars were determined by high performance anion-exchange chromatography on a Dionex Bio-LC 4500i system with a pulsed amperometry detector II. Monosaccharides were separated on a Dionex CarboPac PA-1 column (4  $\times$  250 mm). The column was initially equilibrated with 7% eluant 1 (50 mM sodium hydroxide containing 1.5 mM acetic acid) and 93% eluant 2 (water) at a flow rate of 0.8 ml/min. The column was isocratically eluted for 15 min, followed by elution with 100% eluant 1 for 10 min, and then the column was returned to the initial conditions for 15 min prior to the next injection.

Postcolumn addition of 300 mM sodium hydroxide was carried out at 0.8 ml/min.

**$^1\text{H}$  NMR Measurements at 600 MHz**—NMR spectra of oligosaccharides were measured on a Bruker AM-600 (600-MHz) spectrometer. Ambient probe temperature was 30 °C. The free induction decay signals obtained from the first increment of a two-dimensional NOESY experiment with presaturation during relaxation (4.5-s) and mixing (0.15-s) periods were Fourier transformed to obtain the solvent-suppressed NMR spectra. Acetone was used as an internal standard for chemical shifts ( $\delta$  = 2.216 ppm).

## RESULTS

**Isolation of the PA Oligosaccharides from HRP that React with Anti-HRP Antiserum**—In order to estimate the antigenic reactivity of the PA oligosaccharides from HRP, we developed an enzyme-linked immunosorbent assay in which each well was coated with glycopeptides by a new method. In general, glycopeptides are not very adsorbent. Therefore, either the glycopeptides or the well itself must be treated in order to coat the well. Special procedures include acylation of glycopeptides with palmitic anhydride or the use of a plate whose surface is chemically modified to covalently bind to the amino groups of glycopeptides. We attempted both methods but did not obtain satisfactory results, especially in inhibition assays using oligosaccharides as inhibitors. We thus developed a new well-coating method in which glycopeptides were first conjugated with BSA and then added to the well. These conjugates stably adsorbed to the well, enabling us to test the reactivity of carbohydrates with antibody in an inhibition assay. Fig. 1a shows the reactivity of a constant dilution of anti-HRP antiserum with various amounts of glycopeptide-BSA conjugates added to each well. The antibodies reacted with glycopeptides proportionally to the concentration of coated glycopeptides over 4 ng of mannose/well.

Antiserum activity against glycopeptides, oligosaccharides, and PA oligosaccharides prepared from HRP were compared with respect to the ability to inhibit the reaction between the antibodies and the HRP glycopeptides coated on the wells. Fig. 1b shows the effects of these materials on the reactivity of a constant antiserum dilution with a fixed concentration of HRP glycopeptides equivalent to 30 ng of mannose in each well. They all inhibited the reaction dose-dependently almost to the same extent, indicating that antibodies reacted with the carbohydrate moieties of HRP. Attachment of a peptide portion or the pyridylation of the reducing end of the oligosaccharides had little influence on reactivity. PA oligosaccharides were fractionated by reverse-phase HPLC into seven fractions (Fractions 1–7) (Fig. 2). Fraction 3 comprised about 80% of the total PA oligosaccharides. This fraction was also eluted as a single peak on a HPLC column of TSK-gel amide 80. There was no significant difference in the reactivity with the anti-HRP antiserum between Fraction 3 and the total PA oligosaccharides from HRP as estimated by the inhibitory activity (data not shown).

**Elucidation of Carbohydrate Structure of Fraction 3**—The carbohydrate composition of Fraction 3 was analyzed on an amino acid analyzer (for amino sugars) and two types of HPLC system, one with a pulsed amperometry detector (for neutral sugars) and the other with a fluorometric detector (for PA-GlcNAc) as described under "Experimental Procedures." The results indicated that Fraction 3 was composed of Man, Xyl, Fuc, GlcNAc, and PA-GlcNAc with a molar ratio of 3.2:1.0:1.3:0.9:1.0. The PA oligosaccharide was then analyzed by  $^1\text{H}$  NMR spectroscopy. Fig. 3 shows the 600-MHz  $^1\text{H}$  NMR spectrum of Fraction 3. The spectrum was almost superimposable with that of M3FX from ricin D (6) or Fraction B1 from Barbados pride protease inhibitor (14). The chemical shifts of Fraction 3 were in good agreement with those of

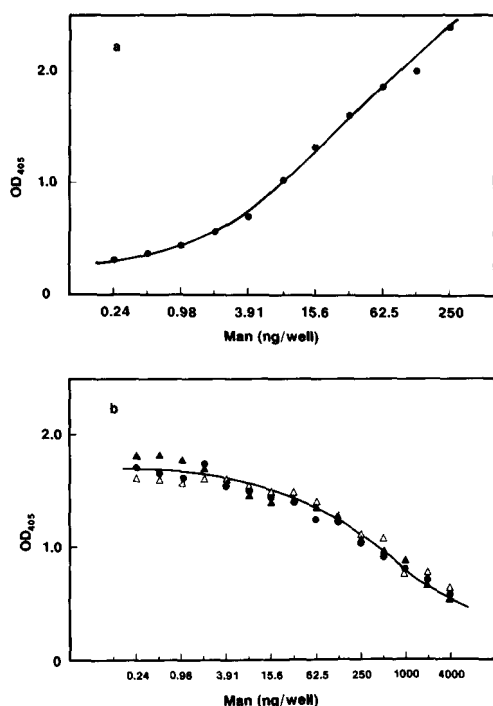


FIG. 1. Reactivity of the antiserum with sugar chains from HRP. *a*, binding of the antibody to coated glycopeptides. Antiserum diluted 1:1600 with solution B was added to wells coated with various amounts of glycopeptide-BSA conjugates. The amount of antibody bound to the well was determined using an enzyme-linked immunosorbent assay with alkaline phosphatase as described under "Experimental Procedures." The *abscissa* indicates the amounts of coated glycopeptides in terms of mannose equivalents as determined by the orcinol method (8). *b*, comparison of the reactivity of glycopeptides, oligosaccharides, and PA oligosaccharides from HRP. The activity of these materials in inhibiting the binding of the antibodies to the coated well was determined as described under "Experimental Procedures." The antiserum was diluted 1:1600, and each well was coated with glycopeptides equivalent to 30 ng of mannose. The *abscissa* indicates the amounts of the added materials in terms of mannose equivalent as determined by the orcinol method.  $\blacktriangle$ , glycopeptides;  $\triangle$ , oligosaccharides;  $\bullet$ , pyridylamino oligosaccharides.

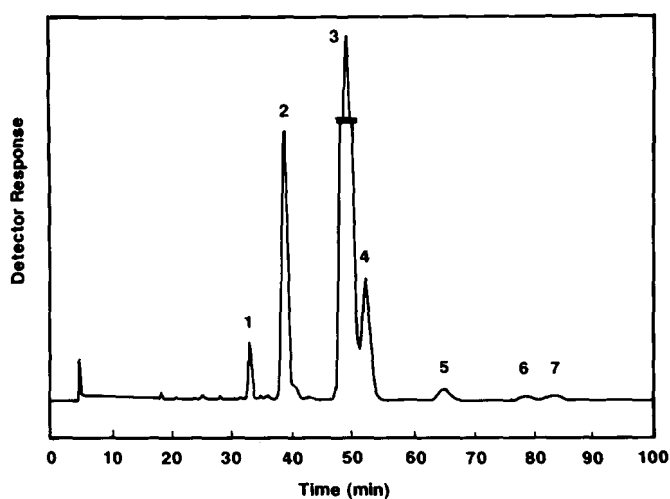


FIG. 2. High performance liquid chromatography of PA oligosaccharides. HPLC was carried out as described under "Experimental Procedures."

previously described PA oligosaccharides and were assigned as shown in Fig. 3. Furthermore, the sugar composition of Fraction 3 was almost the same as that of M3FX and Fraction

B1. On the basis of these lines of evidence, the carbohydrate structure of this fraction was determined as described in Fig. 4 and found to be identical with that of M3FX from ricin D or Fraction B1 from the protease inhibitor.

**Epitopic Structure Defined by Anti-HRP Antiserum**—Reactivity of the antibody with the modified derivatives of Fraction 3 was analyzed to investigate the minimum structure required to react with the antibody. Defucosylated, dextrolylated, and demannosylated derivatives from Fraction 3 were prepared as described under "Experimental Procedures." All derivatives were purified by successive HPLC on amide 80 and ODS columns and subjected to neutral sugar determination to confirm the quantitative and selective removal of particular sugars (see Table I). As shown in Fig. 5, upon removal of the xylose residue, the binding activity of Fraction 3 decreased only slightly. Removal of  $\alpha 1 \rightarrow 3$ -linked mannose resulted in a further reduction in the binding activity even when the xylose residue remained intact. However, it should be noted that this derivative still retained significant reactivity with the antibody. Removal of a fucose residue or 2  $\alpha$ -linked mannose residues, on the other hand, completely destroyed the binding activity of the oligosaccharides.  $\text{Man}_6\text{GlcNAc}_2$  prepared from ovalbumin did not show any inhibitory activity. All these results indicated that terminal mannose residues,  $\alpha 1 \rightarrow 6$ -linked mannose in particular, and  $\alpha 1 \rightarrow 3$ -linked fucose are essential for recognition by the anti-HRP antiserum; that is, they represent the epitopic structure for the antibody.

#### DISCUSSION

Antiserum against HRP is used as a neural specific probe that stains the surface of all axon pathways in the central and peripheral nervous system of the *Drosophila* or grasshopper embryos (2). The antiserum reportedly recognizes a carbohydrate moiety which is specifically expressed on the neural cell surface. Such a carbohydrate moiety is carried by at least 17 glycoproteins, including fasciclin I and II (3). Fasciclin I and II are expressed on different subsets of axon fascicles during development and are thought to be adhesion molecules specific to the nerve cell network (5). It is speculated that sugar chains of these molecules play an important role in the recognition between cells or molecules. Similarly, carbohydrate chains of the neural cell adhesion molecule are also considered to be involved in the aggregation of neural cells expressing L1 and neural cell adhesion molecule (15, 16).

Although antiserum against HRP is widely used, the biochemical nature of the epitope it recognizes is still unknown. The epitope of nerve cells must share structural features with that of HRP. Therefore, we decided to investigate the structure of the epitope on the HRP molecule. The sugar chains of HRP were released, pyridylaminated, and fractionated. The structure of the major fraction, which reacted with the antibody to the same extent as the total PA oligosaccharides, was determined on the basis of the results of the carbohydrate composition analyses and 600-MHz  $^1\text{H}$  NMR spectrum. It was revealed to be the same oligosaccharide unit as has been isolated from several plant glycoproteins, as shown in Fig. 4 (9, 11, 14, 17, 18). Sugar chains of plant glycoproteins are highly processed and have a common structure (19). Several plant lectins also have the same sugar chains as Fraction 3. Some lectins such as *Phaseolus vulgaris* leucoagglutinin (20) or wheat germ agglutinin (21), as well as HRP, are used as retrograde transport tracers. Their carbohydrates may be recognized by membrane proteins on the nerve cells, which work as receptor molecules. Carbohydrates on nerve cell surfaces, which share an epitope with those of HRP, might

FIG. 3. 600-MHz  $^1\text{H}$  NMR spectrum of Fraction 3. About 600  $\mu\text{g}$  of Fraction 3 was dissolved in 600  $\mu\text{l}$  of  $\text{D}_2\text{O}$ . Owing to the water suppression by homogenized decoupling, the resonances around HDO were suppressed. H-1, NAc, and  $\text{CH}_3$  represent the anomeric, N-acetyl, and methyl protons, respectively. Coding of sugar residues is described in the legend to Fig. 4.

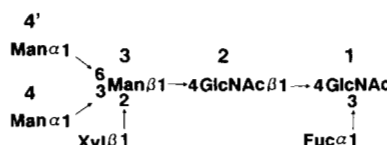
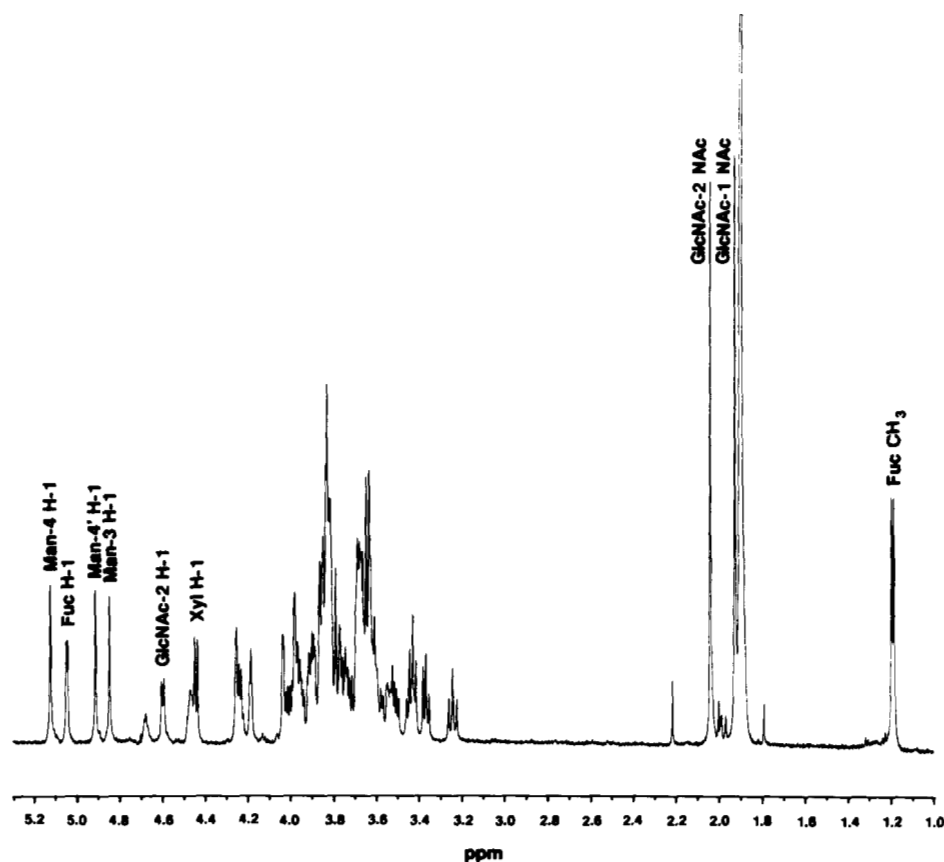


FIG. 4. Proposed structure of the major oligosaccharide from HRP. Numbers above the sugar residues represent the coding of each monosaccharide.

TABLE I

Molar ratios of neutral sugars of modified derivatives from Fraction 3

The molar ratios were determined on the basis of 1 residue of fucose or xylose.

| Derivatives                | Mannose | Xylose | Fucose          |
|----------------------------|---------|--------|-----------------|
| Demannosylated derivatives |         |        |                 |
| Lacking 2 mannose residues | 0.99    | 1.00   | 0.92            |
| Lacking 1 mannose residue  | 1.84    | 1.00   | 0.99            |
| Defucosylated derivative   | 2.95    | 1.00   | ND <sup>a</sup> |
| Dexylosylated derivative   | 3.27    | ND     | 1.00            |

<sup>a</sup> ND, not detected.

interact with such a receptor, resulting in the formation of nerve cell networks.

Utilizing our inhibition assay, we demonstrated that  $\alpha 1 \rightarrow 6$ -linked mannose and  $\alpha 1 \rightarrow 3$ -linked fucose are predominantly responsible for the formation of the epitope structure. The antibody does not strongly require  $\alpha 1 \rightarrow 3$ -linked mannose and xylose for recognition since derivatives devoid of the sugar residues retained significant reactivity with the antibody. When we removed  $\alpha 1 \rightarrow 6$ -linked mannose residue by  $\alpha$ -mannosidase digestion,  $\alpha 1 \rightarrow 3$ -linked mannose was also removed. It is therefore possible that the complete loss of the binding activity of the carbohydrate chain caused by this treatment

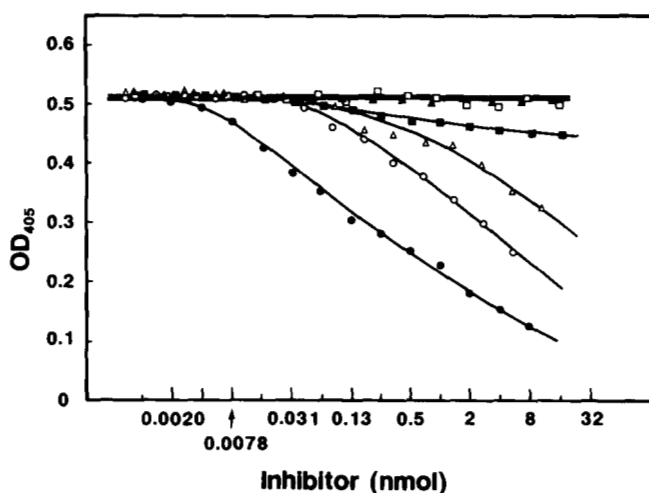


FIG. 5. Reactivity of antiserum with PA oligosaccharide derivatives prepared from Fraction 3. Inhibitory activity was determined as described in the legend to Fig. 1. The antiserum was diluted to 1:6400, and glycopeptides equivalent to 8 ng of mannose were coated to each well.  $\square$ ,  $\text{Man}_6\text{GlcNAc}_2$  prepared from ovalbumin;  $\bullet$ , Fraction 3;  $\blacksquare$ , defucosylated derivative;  $\circ$ , dexylosylated derivative;  $\triangle$ ,  $\blacktriangle$ , derivatives lacking 1 and 2 mannose residues, respectively.

might represent the combined effect by removing both  $\alpha$ -mannosyl residues rather than removing  $\alpha 1 \rightarrow 6$ -linked mannose alone. However, this is not likely because of the fact that anti-HRP antiserum reacts with pineapple stem bromelain, in which the carbohydrate structure lacks  $\alpha 1 \rightarrow 3$ -linked mannose (4, 22, 23). The molecular model of the carbohydrate chain of bromelain has been proposed using  $^1\text{H}$  and  $^{13}\text{C}$  NMR by Bouwstra *et al.* (24) as shown in Fig. 6. According to this model,  $\alpha 1 \rightarrow 6$ -linked mannose and  $\alpha 1 \rightarrow 3$ -linked fucose locate

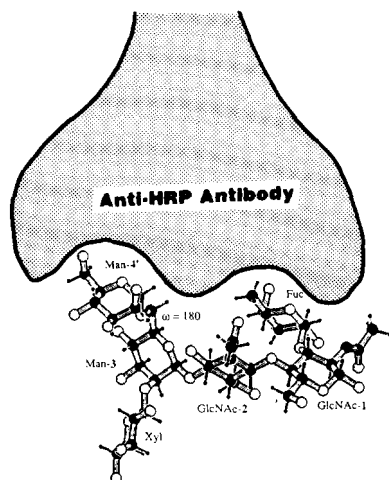


FIG. 6. Molecular model of the epitopic structure for the anti-HRP antibody. The spatial arrangement of the carbohydrate chain from bromelain was adopted from Fig. 8 in Ref 24.

spatially on the same upper side of the sugar chain owing to the restricted rotamer population between the C-5 and C-6 bonds of  $\beta$ -mannose ( $\omega = 180^\circ$ ). This spatial positioning of these 2 sugar residues seems to be quite advantageous for the antibody to recognize them. The conformation of two kinds of similar oligosaccharides has also been compared (25). One is identical with Fraction 3, as is the other except that fucose is attached to the reducing terminal *N*-acetylglucosamine via an  $\alpha 1 \rightarrow 6$ -linkage instead of an  $\alpha 1 \rightarrow 3$ -linkage. This difference in the attachment of fucose brings about the conformational changes in the carbohydrate chains. The sugar chain with the  $\alpha 1 \rightarrow 3$ -linked fucose has the same conformation as that of bromelain, which should also be recognized by the anti-HRP antibody. However, in the sugar chain with the  $\alpha 1 \rightarrow 6$ -linked fucose,  $\alpha 1 \rightarrow 6$ -linked mannose and  $\alpha 1 \rightarrow 6$ -linked fucose locate spatially on the opposite sides of the sugar chain backbone, which may not be recognizable by the antibody used in the study.

Kaladas *et al.* (26) determined the epitopic structure recognized by a rabbit anti-carbohydrate antibody raised against mitogenic lectin from *Wistaria floribunda* seeds. The antibody reacted best with the carbohydrate  $\text{Man}\alpha 1 \rightarrow 6\text{Man}\alpha 1 \rightarrow 6\text{Man}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 4\text{GlcNAc}$ . This structure is similar to the epitope for anti-HRP antiserum but differs in several respects: (a) substitution of the  $\beta$ -mannosyl residue at position 3 with  $\alpha$ -mannose abolishes its reactivity, (b) additional  $\alpha 1 \rightarrow 6$ -linked mannose is involved, and (c) fucose  $\alpha 1 \rightarrow 3$ -linked to *N*-acetylglucosamine at the reducing terminal is not involved. At present, it is unknown whether this sugar moiety is expressed on nerve cells or not.

In this paper, we proposed the structure of a carbohydrate chain that is recognized by anti-HRP antibody and deduced the sugar residues involved in the binding to the antibody. Although the structure of the carbohydrates expressed on nerve cells remains to be elucidated, it should be shared with

the antigenic determinant elucidated here. Isolation of glycoproteins from grasshoppers that react with anti-HRP antibody and elucidation of the structure of the carbohydrate chains of such glycoproteins may help clarify the role of the carbohydrates in the nervous system. These studies are now in progress in our laboratory.

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