Psathyrella velutina Mushroom Lectin Exhibits High Affinity toward Sialoglycoproteins Possessing Terminal N-Acetylneuraminic Acid α2,3-Linked to Penultimate Galactose Residues of Trisialyl N-Glycans

COMPARISON WITH OTHER SIALIC ACID-SPECIFIC LECTINS

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A lectin from the fruiting body of the Psathyrella velutina mushroom (PVL) was found to bind specifically to N-acetylneuraminic acid, as well as to GlcNAc (Ueda, H., Kojima, K., Saitoh, T., and Ogawa, H. (1999) FEBS Lett. 448, 75–80). In this study, the glycan sequences that PVL recognizes with high affinity on sialoglycoproteins were revealed. Among sialic acid-specific lectins only PVL could reveal the sialylated N-acetyllactosamine structure of glycoproteins in blotting studies, based on the dual specificity. The affinity of PVL to fetuin was measured by surface plasmon resonance to be 10^7 M^{-1}, which is an order of magnitude higher than those of Sambucus nigra agglutinin and Maackia amurensis mitogen, whereas affinity to asialofetuin was ~0 and to asialoagalactofetuin was 10^8 M^{-1}, suggesting that PVL exhibits remarkably high affinities toward glycoproteins possessing trisialo- or GlcNAc-exposed glycans. Transferrin was separated into fractions that correspond to the sialylation states on an immobilized PVL column. Transferrin possessing trisialylglycans containing α2,3-linked N-acetylneuraminic acid on the β1,4-linked GlcNAc branch bound to the PVL column and eluted with GlcNAc; those containing only α2,6-linked sialic acids were retarded, whereas other transferrin fractions passed through the column. These results indicate that PVL is a lectin with potential for separation and detection of sialoglycoproteins because of its dual specificity toward sialylglycans and GlcNAc exposed glycans.

Sialic acids play an important role as ligands in cell biology. Sialylation of glycoproteins changes under pathological conditions as well as during developmental stages, and altered sialylation often has significant implications in the physiological role of glycoproteins (1–3). Lectins that recognize the linkages or modifications of sialic acid are therefore indispensable as reagents in biochemical research and diagnostic analyses. Among sialic acid-specific lectins purified from plants and other sources, several lectins have been employed as tools for the detection and separation of sialic acid-containing glycoconjugates (3, 4). Lectins that distinguish various linkages of sialic acid are elderberry bark lectin (SNA; Sambucus nigra agglutinin) (5), Japanese elderberry lectin (SSA; S. sieboldiana agglutinin) (6), and Polyoporus squamosus lectin (7) that are specific for NeuAcα2,6Gal/GalNAc sequences; Maackia amurensis mitogen (MAL) that reacts with greatest affinity to the trisaccharide sequence NeuAcα2,3Galβ1,4GlcNAc/Glc, which is usually present in N-linked oligosaccharides (8), and the hemagglutinin from the same source (MAH) that displays higher affinity for a disialylated tetrasaccharide found in the O-linked oligosaccharide NeuAcα2,3Galβ1,3( NeuAcα2,6)GalNAc (9). In contrast to these sequence-specific lectins, wheat germ lectin (WGA) and Limax flavus lectin have been used as general tools to bind sialylated glycoconjugates (10, 11). A mushroom lectin from Hericium erinaceum recognizes N-glycolyneuraminic acid (12), but further specificity toward linkage or glycan-type is unknown. To harness these lectins in biological research, the binding specificities toward glycoconjugates over monosaccharide or oligosaccharide sequences have to be characterized because the most frequent, and irreplaceable function of the lectins, is to detect specific glycotopes in glycoconjugates in situ, and to analyze or isolate certain glycoconjugates possessing the glycotopes from mixtures.

A lectin from the fruiting body of Psathyrella velutina (PVL) has been known as a GlcNAc-specific lectin (13–15). In contrast to other GlcNAc-specific lectins from higher plants that recognize β1,4-linked GlcNAc preferentially in the oligosaccharide sequence (16), PVL exhibits a strong preference for the GlcNAc

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1 The abbreviations used are: SNA, Sambucus nigra agglutinin; PVL, Psathyrella velutina lectin; GlcNAc, N-acetylglucosamine; NeuAc, N-acetylneuraminic acid; HRP, horseradish peroxidase; TBS, Tris-buffered saline; MAH, Maackia amurensis hemagglutinin (Maackia amurensis lectin II); MAL, Maackia amurensis leukoagglutinin; SSA, Sambucus sieboldiana agglutinin; WGA, wheat germ agglutinin; LPHA, Phaseolus vulgaris leucoagglutinin; GS II, Griffonia simplicifolia lectin II; SPR, surface plasmon resonance; \( k_{	ext{ass}} \), association rate constant; \( k_{	ext{diss}} \), dissociation rate constant; PA, pyridylamine; P-transferrin, pass-through transferrin on the PVL column; R-transferrin, retarded transferrin on the PVL column; B-transferrin, bound transferrin on the PVL column; HPLC, high performance liquid chromatography.
monomer over the chitooligosaccharides and binds nonreducing terminal GlcNAc of other linkages better than that of the β1,4-linkage (13, 14). Because glycosylation with exposed GlcNAc residues lacking terminal galactosylation, or sialylation, has been reported for several pathological markers, such as IGs from rheumatoid arthritis patients (17) and rat hepatoma (18), the remarkable specificity toward nonreducing terminal GlcNAc prompted PVL use as a GlcNAc-detection reagent in diagnostic analyses (19–22). Recently, we observed that PVL binds to several sialoglycoproteins with no GlcNAc residues exposed. We also found that PVL recognizes N-acetyleneuraminic acid (NeuAc) at the same site as the GlcNAc-binding site, and displays differential binding to oligosaccharides depending on the affinity for nonreducing terminal NeuAc residues (23). In this study, the detailed binding specificity of PVL toward sialoglycoproteins was studied in comparison with other sialic acid-specific lectins. An immobilized PVL column was shown to have potential to distinguish trisialoglycans in glycoproteins, especially those containing α2,3-linkages. The precise specificity of PVL expands its utility for the detection of pathological changes in sialylation on one hand, but on the other hand, it raises the need for reconsideration of the results of diagnostic and biological assays hitherto performed that regard PVL as a GlcNAc-specific lectin.

**EXPERIMENTAL PROCEDURES**

**Materials**—PVL was purified from the fruiting bodies of *P. velutina* mushrooms collected in Japan, and PVL-Sepharose and fetuin-Sepharose were prepared by reacting amino groups of PVL (5 mg) or fetuin (40 mg) with formyl-Sepharose (2 g) by reductive amination as reported previously (23). SNA, human α2,6-sialyl glycoprotein (orosomucoid), fetuin from fetal calf serum, and human transferrin were purchased from Sigma. Asialoglycoproteins were prepared by desialylation of intact glycoproteins by either treatment with 0.01 M HCl at 80 °C for 1 h or digestion with sialidase (EC 3.2.1.18, from *Vibrio cholerae*). Roche Molecular Biochemicals) with 0.1 unit/mg glycoprotein in 0.1 M citrate buffer (pH 5.5) containing 1 mM CaCl2 and 1 mM phenylmethylsulfonyl fluoride at 37 °C overnight. MAL, SSA, concanavalin A, and biotin-WGA were purchased from Seikagaku Kogyo (Tokyo, Japan), and biotin-MAH was from VectaStreptavidin peroxidase and HRP-conjugated anti-rabbit IgG antibody were purchased from Zymed Laboratories Inc. (South San Francisco, CA) and ImmPRESS alkaline phosphatase conjugate (Vector Laboratories, Inc., Burlingame, CA). The enzyme-linked immunosorbent assay was performed according to the method of Nakane et al. (24).

**Interaction between Lectins and Glycoproteins by Surface Plasmon Resonance (SPR)**—All procedures were performed at 25 °C using a BIAcore 2000 SPR apparatus, CM5 sensor chip, and an amine coupling kit (BIACORE AB, Uppsala, Sweden). After equilibration with HEPES-buffered saline, the surface of the sensor chip was activated with a 1:1 mixture of N-hydroxysuccinimide and N-ethyl-N′-dimethylaminopropylcarbodiimide, ligands (40 μg/ml) in 10 mM sodium acetate buffer (pH 4.3) were injected onto the activated surface, and then the remaining N-hydroxysuccinimide and carbodiimide esters were blocked by the addition of 10 mM ethanalamine hydrochloride (pH 8.5). Each step was performed for 21 min at a constant flow rate of 5 μl/min. The reference flow cell was prepared without the ligand.

The lectin-glycoprotein interaction was analyzed in 10 mM Tris-buffered saline (TBS) (pH 7.5) at a flow rate of 5 μl/min. Suitable conditions to suppress the aggregation tendency were determined for PVL. kinetic parameters were calculated by the BIAevaluation software version 3.0.

**Affinity Chromatography of PVL on Fetuin-Sepharose**—PVL (50 μg) in 1 ml of TBS was mixed with 2 ml of fetuin-Sepharose gel and poured into the column (0.9 × 3.8 cm, V0 = 2.4 ml) at 4 °C. After incubation for 3 h, the column was washed with TBS, and then PVL was eluted with 0.2 M GlcNAc in TBS and then 0.1 M acetic acid. Thirty drops of each fraction was collected, and the elution of PVL was monitored by enzyme-linked immunosorbent assay; a 100-μl aliquot of each fraction was added to the wells of an Immulon 1 plate (Dynatech Laboratories, Chantilly, VA) and immobilized overnight at 4 °C. The wells were washed with TBS, blocked with 5% bovine serum albumin, TBS for 2 h, and then incubated for 3 h with 100 μl of anti-PVL serum diluted to 1:1,000 with TBS. After washing with TBS, 100 μl of HRP-conjugated anti-rabbit IgG antibody diluted to 1:1,000 with 1% bovine serum albumin, TBS was added. After incubation for 3 h, the color was developed and read at 490 nm using a microplate reader as described previously (23).

**Affinity Chromatography of Sialoglycoproteins on PVL-Sepharose**—A mixture of sialoglycoproteins, α2,3-acetylglycoprotein (50 μg), fetuin (225 μg), and transferrin (40 μg) in 200 μl of 10 mM ammonium acetate (pH 6.5) was applied to a PVL-Sepharose column (0.3 × 13.5 cm, V0 = 1.49 ml) at 4 °C. The glycoproteins were successively eluted with 10 mM ammonium acetate (pH 6.5) and then the same solution containing 10 mM GlcNAc. Twenty drops of each fraction was collected, measured for absorbance at 280 nm, and subjected to SDS-PAGE under reducing conditions. The resolved protein bands were detected with Coomassie Brilliant Blue and examined for lectin reactivity.

**Isolation and Characterization of Transferrin Oligosaccharides**—The procedures, including chromatographic conditions, have been described previously (27, 28). Briefly, native human transferrin (9 mg) was proteolyzed, and oligosaccharides were released from the resultant glycopeptides with glycoamidase A. The oligosaccharides were isolated and labeled with 2-amino pyridine (29). The mixture of pyridylamino (PA)-oligosaccharides was separated by HPLC on a TSK gel DEAE-5PW column according to the sialic acid number. The neutral, mono-, di-, tri-, and tetrasialylated and retarded fractions were evaporated and applied to an ODS silica gel column, and each separated PA-oligosaccharide was then applied to an anion silica column to separate it on the basis of size. Each elution time for the peak on these two columns was recorded in glucose units, and compared with those of authentic reference compounds (two-dimensional map). Identification of a sample PA-oligosaccharide was confirmed by co-chromatography with a suitable reference N-glycan on the two columns. Further confirmation of each PA-oligosaccharide was carried out by digestion of sialidases (β-galactosidase, β-N-acetylgalactosaminidase, and α-L-fucosidase) under conditions as described previously (27, 30) to analyze the asialo-structure on the sugar map. The α2,3-linkage of NeuAc was determined as previously described (30, 31).

Three fractions of transferrin were separated on a PVL-column: pass-through (P-transferrin), retarded (R-transferrin), and bound (B-transferrin) fractions were dialyzed, and dried. The oligosaccharides were released from each transferrin by hydrazinolysis at 100 °C for 2 h using a HCN solution in 20% C206 according to the manufacturer’s instructions. The oligosaccharides were N-acetylated and pyridylamidated (32). PA-oligosaccharides from each transferrin were analyzed on a TSK gel DEAE-5PW column, and then an ODS silica column in the same manner as described above.

**Chemical Analyses**—Quantitation of sialic acid of sialoglycoproteins was performed by procedures reported previously (33) after hydrolysis of sialoglycoproteins (1.8–3.3 μg) in 200 μl of 0.25% HCl at 80 °C for 1 h. The protein concentration was determined using the protein assay reagent (Bio-Rad).

**Interaction between Lectins and Glycoproteins**—All procedures were performed at 25 °C using a BIAcore 2000 SPR apparatus, CM5 sensor chip, and an amine coupling kit (BIACORE AB, Uppsala, Sweden). After equilibration with HEPES-buffered saline, the surface of the sensor chip was activated with a 1:1 mixture of N-hydroxysuccinimide and N-ethyl-N′-dimehtylaminopropylcarbodiimide, ligands (40 μg/ml) in 10 mM sodium acetate buffer (pH 4.3) were injected onto the activated surface, and then the remaining N-hydroxysuccinimide and carbodiimide esters were blocked by the addition of 10 mM ethanalamine hydrochloride (pH 8.5). Each step was performed for 21 min at a constant flow rate of 5 μl/min. The reference flow cell was prepared without the ligand.

The lectin-glycoprotein interaction was analyzed in 10 mM Tris-buffered saline (TBS) (pH 7.5) at a flow rate of 5 μl/min. Suitable conditions to suppress the aggregation tendency were determined for PVL. kinetic parameters were calculated by the BIAevaluation software version 3.0.
recognized only sialyl residues of the glycoproteins. The dominant binding of MAL and MAH to fetuin, but not to transferrin, was consistent with the reported specificity toward α2,3-linked NeuAc residues of these lectins (9, 38). On the other hand, the predominant binding of SNA to transferrin is consistent with the reported specificity of SNA to α2,6-linked NeuAc residues (5).

SSA has been reported to behave basically as a Gal-binding lectin, but the inhibition test for glycoprotein precipitation indicated that SSA exhibits an affinity to α2,6-sialylated Gal several thousand times that to free Gal (6). As shown in Fig. 1F, lanes 1 and 2, binding of HRP-SSA to fetuin was enhanced by desialylation, whereas binding to transferrin was slightly attenuated (lane 4 and 5). These results indicate that the glycoprotein reactivity of SSA is ambiguously changed by desialylation, although a weak preference seems to exist for the α2,6-linked NeuAc abundant in transferrin rather than β-Gal, but not α2,3-linked NeuAc. WGA has been reported to recognize both nonreducing terminal and bisecting GlcNAc and clusters of NeuAc residues (39–41). However, it bound better to asialo- and asialo-agalactotransferrin than to transferrin (Fig. 1G, lanes 4–6), although WGA bound to fetuin but not to asialo- and asialo-agalactotransferrin (Fig. 1G, lanes 1–3). These results indicate that SSA and WGA have severe limitations as NeuAc-detecting reagents that can judge the presence of sialyl residues in an unknown sample from the reactivity change caused by desialylation. In this point, PVL is a useful probe to the penultimate-sialylated N-acetyllactosamine structure when used in combination with desialylation and degalactosylation.

**Kinetic Parameters of PVL Toward Glycoproteins Measured by SPR**—The interactions between PVL and glycoproteins were analyzed by SPR in comparison with SNA and MAL. Fetuin (647 resonance units), asialofetuin (911 resonance units), asialo-agalactofetuin (797 resonance units), or transferrin (4247 resonance units) was captured on the sensor chip, and lectin solutions were introduced for 2 min for PVL, and introduced 3 min for SNA and MAL. When PVL was injected in TBS at a flow rate of 5 μl/min, it gave fluctuating, irregularly shaped binding curves, and fitting of the sensorgrams could not be achieved. Because PVL sometimes clogged the tube and stopped the flow, the irregularity would be caused by the heterogeneous aggregation state of PVL. A suitable condition to analyze the interaction of PVL was introducing a diluted PVL solution of less than 100 nM in 10% glycerol, TBS, and increasing the flow rate to 20 l/min at 25 °C. As shown in Figs. 2, A and B, the binding response of fetuin and asialo-agalactofetuin was dependent on the concentration of PVL. Detectable binding of PVL to asialofetuin was not observed on SPR (data not shown). Whereas the binding of PVL to transferrin had not reached saturation even at 120 s, it dissociated at the rate of 0.5 s−1 after the injection was stopped. This did not fit any binding equation model and could not be reproduced with BIA-simulation software so that the binding parameters could not be calculated. The binding may include heterogeneous interactions, such as aggregation of PVL or nonspecific interaction on the sensor chip that interfere with the specific interaction between PVL and transferrin, because the addition of 0.05% SDS in the buffer suppressed it, and then made the binding curves saturable on SPR. SNA and MAL exhibited concentration-dependent binding curves to fetuin (Fig. 2, C and D) and transferrin (data not shown), but they did not bind to asialofetuin and asialo-agalactofetuin (data not shown).

Kinetic parameters of the interactions are summarized in Table I. The association constant (Ka) of PVL for fetuin was 14 and 166 times those of SNA and MAL, respectively. The Ka of
A. Transferrin (34)

- two N-linked glycans per molecule

B. Fetuin (35-37)

- three N-linked glycans
- and
- three O-linked glycans per molecule

C. α1-acid glycoprotein (43)

- five N-linked glycans per molecule

Scheme 1. The major oligosaccharide structures, total number, and types of glycans of glycoproteins used in this study. References for major oligosaccharide structures are indicated in parentheses.

SNA and MAL for fetuin had been reported to be $8.82 \times 10^5 \text{ M}^{-1}$ and $2.78 \times 10^5 \text{ M}^{-1}$, respectively, when SNA and MAL were captured on the sensor chip and fetuin was injected as ligand (42); the $K_a$ values of PVL are 44 and 140 times these values. The results indicate that PVL binds to fetuin with remarkably high affinity compared with SNA and MAL, exhibiting a high association rate constant ($k_{ass}$) that may be related to oligomerization of PVL to enhance the avidity. On the other hand, PVL did not exhibit detectable binding to asialofetuin. The $K_a$ of PVL to fetuin was increased by 4.9 times after the exposure of GlcNAc.

Affinity Chromatography of PVL on the Fetuin-Sepharose Column—The interaction between PVL and fetuin was studied by affinity chromatography, as shown in Fig. 3A. Detection of the eluted PVL with rabbit anti-PVL antibody indicated that PVL bound to a fetuin column and eluted sugar-specifically. Binding of PVL to the fetuin-Sepharose gel, and its elution with GlcNAc, demonstrates its specificity for terminal sialyl residues, consistent with the observation that PVL binding to fetuin was inhibited with GlcNAc on an enzyme-linked immunosorbent assay, and PVL interacts with NeuAc at the same binding site as GlcNAc (23).

Affinity Chromatography of Sialoglycoproteins on a PVL-Sepharose Column—The resolution efficiency of the PVL-Sepharose column was examined by applying a mixture of three sialoglycoproteins and eluting with 10 mM GlcNAc. As shown in Fig. 3B and C, the effluent contained transferrin and trace amounts of fetuin (Fig. 3C, fractions 3–7). The major component of the eluted fractions changed successively from transferrin (fractions 22–24) to fetuin (fractions 23–30) and subsequently to α1-acid glycoprotein (fractions 24–34). The elution order of the glycoproteins was correlated with their apparent sialic acid concentrations: 3.3, 14, and 17 mol/mol for transferrin, fetuin, and α1-acid glycoprotein, respectively. Human α1-acid glycoprotein has a higher amount of NeuAc residues on five N-glycans of mainly tri- and tetrasialo-types with
Interaction of *P. velutina* Lectin with Sialoglycoproteins

**A. PVL - fetuin**

![Graph A](image)

**B. PVL - asialo-agalactofetuin**

![Graph B](image)

**C. SNA - fetuin**

![Graph C](image)

**D. MAL - fetuin**

![Graph D](image)

**Fig. 2. Sensorgrams of the interactions of PVL with immobilized glycoproteins by SPR.** The surface of the CM5 sensor chip was activated with N-hydroxysuccinimide/N-ethyl-N’-(dimethylaminopropyl)carbodiimide at a flow rate of 5 μl/min for 21 min. Forty μg/ml ligand in 10 mM sodium acetate buffer (pH 4.3) was immobilized onto the chip, and the blocking was performed with 1.0 M ethanolamine hydrochloride (pH 8.5) for 21 min. The reference flow cells were prepared in an analogous manner without a ligand. Various concentrations (100, 50, 25, 12.5, and 6.25 nM) of PVL solution were introduced onto the glycoprotein-immobilized sensor chip for 2 min for PVL and 3 min for SNA and MAL. The flow rate was 20 μl/min for PVL and 5 μl/min for SNA and MAL running buffer was TBS, 10% glycerol for PVL and TBS for SNA and MAL, and the temperature was 25 °C.

**Table I**

Kinetic parameters of PVL, SNA, and MAL for immobilized fetuin, asialo-agalactofetuin, or transferrin by SPR

<table>
<thead>
<tr>
<th>Gliyoprotein</th>
<th>Fetuin</th>
<th>Asialo-agalactofetuin</th>
<th>Transferrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVL</td>
<td>$k_{\text{on}}$ (M$^{-1}$s$^{-1}$)</td>
<td>$1.35 \times 10^7$</td>
<td>$4.60 \times 10^5$</td>
</tr>
<tr>
<td></td>
<td>$k_{\text{off}}$ (s$^{-1}$)</td>
<td>$3.45 \times 10^{-3}$</td>
<td>$2.39 \times 10^{-3}$</td>
</tr>
<tr>
<td></td>
<td>$K_a$ (M$^{-1}$)</td>
<td>$3.91 \times 10^7$</td>
<td>$1.92 \times 10^8$</td>
</tr>
<tr>
<td>SNA</td>
<td>$k_{\text{on}}$ (M$^{-1}$s$^{-1}$)</td>
<td>$9.66 \times 10^7$</td>
<td>$9.54 \times 10^3$</td>
</tr>
<tr>
<td></td>
<td>$k_{\text{off}}$ (s$^{-1}$)</td>
<td>$3.48 \times 10^{-3}$</td>
<td>Not bound</td>
</tr>
<tr>
<td></td>
<td>$K_a$ (M$^{-1}$)</td>
<td>$2.78 \times 10^6$</td>
<td>$1.97 \times 10^{-3}$</td>
</tr>
<tr>
<td>MAL</td>
<td>$k_{\text{on}}$ (M$^{-1}$s$^{-1}$)</td>
<td>$6.36 \times 10^7$</td>
<td>$4.84 \times 10^9$</td>
</tr>
<tr>
<td></td>
<td>$k_{\text{off}}$ (s$^{-1}$)</td>
<td>$2.70 \times 10^{-3}$</td>
<td>$4.35 \times 10^2$</td>
</tr>
<tr>
<td></td>
<td>$K_a$ (M$^{-1}$)</td>
<td>$2.35 \times 10^8$</td>
<td>$7.18 \times 10^{-3}$</td>
</tr>
</tbody>
</table>

both α2,3- and α2,6-linkages (Scheme 1C) (43) than human transferrin (Scheme 1A) and bovine fetuin (Scheme 1B).

The effluents from the PVL column (Fig. 3B) were divided into pass-through fractions of non-interactive glycoproteins (elution volume 1.2–2.3 ml, fractions 3–4), and glycoproteins that were retarded by weak interactions (2.4–4.0 ml, fractions 5–7), according to the differential elution positions of PA-oligosaccharides on the same column observed in our previous study (23). The index elution positions are shown by arrows X, Y, and Z in Fig. 3B; X indicates the position of a non-interactive oligosaccharide, PA-xyllose, at 2.3 ml; Y indicates a fraction significantly retarded in the column (PA-trisialooligosaccharide, Neu5Aca2–6Galβ1–4GlcNAcβ1–2Manα1–6 Neu5Aca2–6Galβ1–4GlcNAcβ1–4GlcNAcβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc–PA) eluted at 3.7 ml. The two PA-distialooligosaccharides, Neu5Aca2–6Galβ1–4GlcNAcβ1–2Manα1–6 Neu5Aca2–6Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc–PA and Neu5Aca2–3Galβ1–4GlcNAcβ1–2Manα1–6 Neu5Aca2–3Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc–PA, eluted at the intermedial position between X and Y (2.6–3.1 ml). When applied alone, transferrin was reproducibly separated into pass-through, retarded, and bound and eluted fractions, which were designated P-transferrin (fractions 3–4), R-transferrin (fractions 5–7), and B-transferrin (fractions 22–
Fig. 3. Affinity chromatography of PVL on a fetuin-Sepharose column (A) and sialoglycoproteins on a PVL-Sepharose column (B and C). A, PVL (50 µg) in 1 ml of TBS was mixed with 2 g of fetuin-Sepharose, and the mixture was incubated in the column (0.9 × 3.8 cm) for 3 h at 4 °C. After washing with TBS, PVL was eluted with 0.2 M GlcNAc in the same buffer, and then 0.1 N acetic acid at the positions indicated by the arrows. To monitor the elution of PVL, a 100-µl aliquot of each eluted fraction was immobilized on a microtiter plate and allowed to react with anti-PVL polyclonal antibody as described in the text. B and C, the mixture of sialoglycoproteins, 50 µg of α1-acid glycoprotein, 225 µg of fetuin, and 40 µg of transferrin in 200 µl of 10 mM ammonium acetate (pH 6.5) was applied to the PVL-Sepharose column (0.3 × 13.5 cm), eluted with the same solution, and then with 10 mM GlcNAc in the same solution at the position indicated by an arrow. Proteins were detected by the absorbance at 280 nm (B), and each fraction was loaded onto SDS-PAGE using 8% polyacrylamide gel (C) as described in the text. On affinity chromatography of sialoglycoproteins on a PVL-Sepharose column and sialoglycoproteins on a PVL-fetuin-Sepharose column (A), and sialoglycoproteins on a PVL-Sepharose column (B and C), the migration distances were analyzed by lectin blotting studies and three-dimensional mapping oligosaccharide analyses.

Lectin Blotting Studies of Native, P-, and B-Transferrins Separated on a PVL Column—Sialic acid analysis indicated that B-transferrin contained 1.4 times more NeuAc residues than P + R-transferrin (5.0 and 3.5 mol of NeuAc per 1 mol of transferrin, respectively), coinciding with the migration of B-transferrin to a slightly higher position than P + R-transferrin on SDS-PAGE (Fig. 4A). As shown in Fig. 4, B and C, the binding of biotin-PVL was completely prevented by acid desialylation, and biotin-GS II, which is a GlcNAc-specific lectin, did not bind to any fraction (Fig. 4D), demonstrating that PVL bound to NeuAc residues of transferrin but not to the nonreducing terminal GlcNAc or protein portions. The difference between P + R-transferrin and B-transferrin was remarkable for MAL reactivity (Fig. 4E), indicating that the α2,3-linked NeuAc residues are contained in only B-transferrin. In contrast, the weaker binding with biotin-SNA of B-transferrin, than P + R-transferrin, suggests that the amount of α2,6-linked NeuAc was smaller in B-transferrin than in P + R-transferrin (Fig. 4F). Biotin-WGA bound with P + R- and B-transferrin irrespectively (Fig. 4G). Branch-dependent lectins, biotin-L-PHA (Fig. 24), respectively. Because PVL may differentiate the microheterogeneity of glycans in each transferrin fraction, the glycan structures were analyzed by lectin blotting studies and three-dimensional mapping oligosaccharide analyses.
and biotin-Con A (Fig. 4I), inversely reacted with these fractions, suggesting that the triantennary glycans recognizable by L-PHA are abundant in B-transferrin, whereas biantennary glycans recognizable by concanavalin A are abundant in P-/H11001R-transferrin. The observation is consistent with the affinity of PA-sialooligosaccharides observed on a PVL column (23), that the tri- (Y) and tetra-sialooligosaccharides-PA (Z) were retarded or bound on the column, but disialobiantennary glycans eluted at the intermediate position between the pass-through fraction (X) and trisialooligosaccharides, as shown by arrows in Fig. 3. Therefore, the trisialooligosaccharides that may distinguish clearly the P-, R-, and B-transferrins were focused on.

PA-Trisialooligosaccharide Structures of Native, P-, R-, and B-transferrins by Three-dimensional HPLC—Oligosaccharides released from P-, R-, and B-transferrins were subjected to oligosaccharide analysis, and monosialo-, disialo-, and trisialooligosaccharide fractions of each transferrin that were eluted by DEAE-HPLC were separately applied to ODS-HPLC. The monosialo-PA- and disialo-PA-oligosaccharide fractions did not show significant differences among the transferrins on ODS-HPLC (data not shown). As shown in Fig. 5, a remarkable difference was found in the ODS-HPLC chromatogram of trisialo-PA-oligosaccharide fractions. All structures of the trisialooligosaccharides of native transferrin were therefore analyzed to assign each PA-oligosaccharide peak of P-, R-, and B-transferrins in Fig. 5.

The PA-trisialooligosaccharides of native transferrin were further analyzed by two-dimensional mapping after desialylation with neuraminidase. The structures of the PA-asialooligosaccharides were assigned from the elution position on a two-dimensional mapping.
dimensional data map, and compared with the structures of originating PA-oligosaccharides predicted from the elution positions on the three-dimensional data map (30). When an elution position of a PA-trisialooligosaccharide could not be assigned to the known PA-oligosaccharides, it was analyzed for the presence and position of the \( \alpha_2,3 \)-linked NeuAc. The structures of PA-trisialooligosaccharides of the native transferrin are summarized in Table II.

From the elution positions on ODS-HPLC as shown in Fig. 5, peaks “e” and “f” detected in B-transferrin coincided with the elution positions of PA-trisialooligosaccharides T-4 and T-5 in native transferrin, which contain one \( \alpha_2,3 \)-linked NeuAc on the GlcNAc\( \beta_1,4 \) branch of the Man\( \alpha_1,3 \) arm and two \( \alpha_2,6 \)-linked NeuAc residues on other branches (Table II). R-transferrin specifically contained peaks “a” and “d,” the elution positions of which correspond to T-1 and T-6, indicating that oligosaccharides containing three \( \alpha_2,6 \)-linked NeuAc residues are retarded on the PVL column irrespective of the branching pattern. Peak “b” in R-transferrin corresponded to T-3 (and T-2), indicating that the 2,2,6 branch does not result in tight binding even when it contains \( \alpha_2,3 \)-linked NeuAc residues. The broad peak “c” in R-transferrin, which may correspond to merged peaks e and f, might have arisen from an overload of transferrin on a PVL column. The PA-trisialooligosaccharide fraction obtained from P-transferrin did not contain a PA-oligosaccharide component. These results indicate that the presence of \( \alpha_2,3 \)-linked NeuAc residues on the GlcNAc\( \beta_1,4 \) branch of the Man\( \alpha_1,3 \) arm in the trisialoglycan is essential for the high affinity of glycoproteins with PVL, and the presence of trisialoglycan containing three \( \alpha_2,6 \)-linked NeuAc residues or \( \alpha_2,3 \)-linked NeuAc residues on other branches only retards the elution of the glycoprotein on a PVL column.

**DISCUSSION**

Kochibe and Matta (13) originally observed that PVL primarily recognizes GlcNAc residues and agglutinates human erythrocytes, although ambiguity about the nature of the glycan receptor on erythrocytes remained. The subsequent conclusion that the carbohydrate-binding specificity of PVL was the non-reducing terminal \( \beta \)-GlcNAc residue, but not GalNAc or NeuAc, was based on the behavior of various complex-type and human milk oligosaccharides on a PVL column (14). When we used PVL to characterize plasma glycoproteins, however, a number of glycoproteins in normal plasma reacted positively with PVL on the membrane, which re-opened the question about the reported specificity. With this as a start, we found the PVL activity of recognizing NeuAc at the same binding site as GlcNAc (23).

The interactions between PVL and fetuin or transferrin in this study indicate that the terminal sialic acid residues contribute to the PVL binding, which is detectable by various biochemical binding assays on agarose gel (Fig. 3), membrane (Fig. 1), and microtiter plates (23), although PVL exhibits an affinity to asialo-agalactofetuin an order of magnitude higher than to fetuin by SPR. This study revealed that PVL bound with high affinity toward glycoproteins possessing trisialoglycans with \( \alpha_2,3 \)-linked NeuAc in the GlcNAc\( \beta_1,4 \) branch, whereas those possessing trisialoglycan with only three \( \alpha_2,6 \)-linked NeuAc residues were retarded in the PVL column. It explains the higher reactivity of fetuin than transferrin with PVL, because fetuin contains those glycans as a major N-glycan (Scheme 1B). On the contrary, the presence of \( \alpha_2,3 \)-linked NeuAc in disialoglycan showed little effect on the elution position of the glycoprotein on a PVL column, i.e. P- and B-transferrins showed essentially the same composition of PA-disialooligosaccharides on ODS-HPLC (data not shown). In support, disialobiantennary glycan with two \( \alpha_2,3 \)-linked NeuAc residues, as well as that with two \( \alpha_2,6 \)-linked NeuAc residues from unsialylated glycans, was only slightly retarded on a PVL column (23). The very weak affinity of PVL toward disialoglycans irrespective of their sialyl linkages would be the reason the reactivity of PVL toward NeuAc-containing oligosaccharides was previously overlooked (14).

MAL has been utilized as the best lectin specific to \( \alpha_2,3 \)-linked NeuAc in N-glycans (38). One of the major differences between PVL and MAL is that PVL exhibits the remarkable affinity toward trisialoglycan with \( \alpha_2,3 \)-linkage, whereas MAL recognizes both disialo- and trisialoglycans with \( \alpha_2,3 \)-linkage (42). Another difference is that PVL exhibits branch dependence, that is, it shows the highest affinity for \( \alpha_2,3 \)-linked NeuAc in the branch linked at C-4 of the mannose \( \alpha_1,3 \) arm (T-4 and T-5 in Table II), and to a lesser extent for \( \alpha_2,3 \)-linked NeuAc in the branch linked at C-2 of mannoses of the \( \alpha_1,3 \) arm and the \( \alpha_1,6 \) arm (T-3 in Table II), but MAL binds independently of the branching position of GlcNAc at the mannos residue in the complex chains. Furthermore, PVL has high affinity to O-glycans possessing \( \alpha_2,3 \)-linked NeuAc, whereas MAL binds with low affinity to the O-glycans possessing sialic acid \( \alpha_2,3 \)-linked to galactose. Coincidently, PVL agglutinates erythrocytes well, whereas MAL is very weak in hemagglutinating activity because much of the \( \alpha_2,3 \)-linked sialic acids on erythrocytes are in O-glycans rather than in N-glycans (38). In support, PVL bound to bovine submaxillary mucin by enzyme-linked immunosorbent assay (23), and PA-oligosaccharides derived from \( \alpha_1,3 \)-Gal and \( \alpha_1,2 \)-glicopilids bound to a PVL column and eluted with 0.1 M GlcNAc (23), suggesting that the O-glycans of glycoorins and glycolipids on erythrocytes may be receptors for hemagglutination. The advantageous properties of PVL in detecting or separating glycoconjugates are its ability to recognize those possessing GlcNAc-exposed glycans, those possessing sialylation isoforms, and others without these terminal residues. The specific elution of bound sialoglycoproteins on a PVL column is performed with GlcNAc, as shown in Fig. 3, whereas glycoproteins possessing GlcNAc-exposed glycans are eluted with 0.1 M AcOH (23). Furthermore, PVL is easily purified by a one-step procedure (23), and is stable for more than 1 year without a detectable decrease in carbohydrate binding activity at \(-20^\circ\text{C}\) in a solution containing 10% glycerol or at \(4^\circ\text{C}\) as an immobilized adsorbent, in contrast to the multipurification steps and the short shelf-life of MAL.

The glycoform of transferrin has been conventionally classified into tetrasialo-, pentasialo-, and hexasialo-forms according to the number of sialic acids on one molecule. A carbohydrate-deficient transferrin is utilized as a diagnostic plasma marker for carbohydrate-deficient glycoprotein syndrome and hepatic disorders induced by alcohol abuse (44–46). Other pathological changes in glycosylation have been reported for plasma haptoglobin during ovarian carcinoma. The increase of \( \alpha_2,3 \)-sialic acid residues could be accompanied by an increased branching from biantennary to triantennary oligosaccharides. The increase of \( \alpha_2,3 \)-sialic acid residues in the PVL column was 2.5 times, and decreased SNA binding (47). In the same pathological states, glycosylation of the plasma \( \alpha_1 \)-proteinase inhibitor was reported to undergo opposite changes in sialylation and branching, and the reactivities of MAL and SNA changed inversely (47). The increase of \( \alpha_2,3 \)-sialic acid residues with increased branching of glycans is also considered to accompany hepatocarcinoma (34). All these glycoproteins are produced in hepatocytes, and haptoglobin possesses a similar glycosylation to that of transferrin in a normal state. Therefore, PVL might become a useful diagnostic reagent to detect glycosylation changes in these cases.

On the other hand, diagnostic measurement using PVL of GlcNAc-exposed IgG in plasma of rheumatoid arthritis pa-
### TABLE II
Structure of trisialylated PA-oligosaccharides on human transferrin

<table>
<thead>
<tr>
<th>Number (mol %)</th>
<th>Structure</th>
<th>Glc units (ODS-column)</th>
<th>Peak Assignment of Figure 5</th>
</tr>
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<tr>
<td>T-1 (7.6%)</td>
<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}6Manα1\textsubscript{2}Manβ1→4GlcNAcβ1→4GlcNAe→PA</td>
<td>8.2</td>
<td>a (R-transferrin)</td>
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<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}6Manα1\textsubscript{2}Manβ1→4GlcNAcβ1→2Manα1</td>
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<td></td>
</tr>
<tr>
<td>T-2 (2.4%)</td>
<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}6Manα1\textsubscript{2}Manβ1→4GlcNAcβ1→4GlcNAe→PA</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}6Manα1\textsubscript{2}Manβ1→4GlcNAcβ1→2Manα1</td>
<td></td>
<td></td>
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<tr>
<td>T-3 (13.9%)</td>
<td>NeuAcα2→3Galβ1→4GlcNAcβ1\textsubscript{1}6Manα1\textsubscript{2}Manβ1→4GlcNAcβ1→4GlcNAe→PA</td>
<td>9.1</td>
<td>b (R-transferrin)</td>
</tr>
<tr>
<td></td>
<td>NeuAcα2→3Galβ1→4GlcNAcβ1\textsubscript{1}6Manα1\textsubscript{2}Manβ1→4GlcNAcβ1→2Manα1</td>
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<td></td>
</tr>
<tr>
<td>T-4 (28.4%)</td>
<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}4GlcNAe→PA</td>
<td>13.0</td>
<td>c (R-transferrin)</td>
</tr>
<tr>
<td></td>
<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}3Manα1\textsubscript{2}Manβ1→4GlcNAcβ1→4GlcNAe→PA</td>
<td></td>
<td>e (B-transferrin)</td>
</tr>
<tr>
<td>T-5 (29.0%)</td>
<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}4GlcNAe→PA</td>
<td>13.1</td>
<td>c (R-transferrin)</td>
</tr>
<tr>
<td></td>
<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}4GlcNAe→PA</td>
<td></td>
<td>f (B-transferrin)</td>
</tr>
<tr>
<td>T-6 (9.5%)</td>
<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}4GlcNAe→PA</td>
<td>15.3</td>
<td>d (R-transferrin)</td>
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<td></td>
<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}4GlcNAe→PA</td>
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<tr>
<td>T-7 (1.5%)</td>
<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}4GlcNAe→PA</td>
<td>16.7</td>
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<td>T-8 (0.5%)</td>
<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}4GlcNAe→PA</td>
<td>19.6</td>
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<td>NeuAcα2→6Galβ1→4GlcNAcβ1\textsubscript{1}4GlcNAe→PA</td>
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tients requires attention in interpreting the results obtained by the most current methods (19–22) because these methods are based on the previously reported specificity of PVL (14). Based on this study, it would be effectively carried out by measuring the reactivity of PVL of samples before and after desialylation treatment to distinguish the binding of PVL to the GlcNAc-exposed IgGs and the sialylated IgGs.

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