Interaction of a lectin from *Psathyrella velutina* mushroom with *N*-acetylneuraminic acid

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Received 18 January 1999; received in revised form 22 February 1999

Abstract A lectin from the fruiting body of *Psathyrella velutina* has been used as a specific probe for non-reducing terminal *N*-acetylglucosamine residues. We reveal in this report that *P. velutina* lectin recognizes a non-reducing terminal *N*-acetylneuraminic acid residue in glycoproteins and oligosaccharides. Binding of biotinyl *P. velutina* lectin to *N*-acetylneuraminic acid residues was prevented by desialylation of glycoconjugates and was distinguished from the binding to *N*-acetylglucosamine. Sialooligosaccharides were retarded or bound and eluted with *N*-acetylglucosamine on a *P. velutina* lectin column, being differentiated from each other and also from the oligosaccharides with non-reducing terminal *N*-acetylglucosamine which bound more strongly to the column.

Key words: *N*-acetylneuraminic acid-specific lectin; Sialoglycoprotein; Pyridylamino-oligosaccharide; Biotinylated polymeric sugar-probe; Mushroom; *Psathyrella velutina*

1. Introduction

A number of fungal lectins have been reported [1,2] and several lectins from *Basidiomycetes* (mushroom) have proved to be useful probes for the detection and fractionation of the specific carbohydrate structures of glycoconjugates [3–5]. A lectin from the fruiting body of *Psathyrella velutina* (PVL) is known as an *N*-acetylglucosamine (GlcNAc)-specific lectin [6,7]. In contrast to other GlcNAc-specific lectins from higher plants, which recognize β1,4-linked GlcNAc preferentially in the oligosaccharide sequence [8], PVL exhibits a strong preference for the GlcNAc monomer over the chitooligosaccharides and binds non-reducing terminal GlcNAc of other linkages better than that of the β1,4-linkage [7,9]. The remarkable specificity towards the non-reducing terminal GlcNAc residue leads to the expectation that PVL would be a useful reagent for glycoconjugate separation and histochemical detection of specific markers because glycosylation lacking terminal galactosylation or sialylation has been reported in several indicators of pathological states, such as IgGs from rheumatoid arthritis patients [10] and rat hepatoma [11]. In fact, the use of PVL as a GlcNAc detection reagent in diagnostic and biochemical analyses is increasing [12–15].

In this study, we demonstrated the ability of PVL to recognize NeuAc and GalNAc and the utility of the immobilized lectin in the fractionation of sialylated oligosaccharides.

2. Materials and methods

2.1. Materials

Fruiting bodies of *P. velutina* were collected in Gunma Prefecture, Japan and stored at −20°C until use. B-SJA-I, a galactose-specific lectin from bark of *Sophora japonica*, was purified as described previously [16]. Fetuin (fetal calf serum), human transferrin, bovine submaxillary mucin (BSM) and *N*-hydroxysuccinimide biotin were purchased from Sigma Chemicals (St Louis, MO, USA). Asialoglycoproteins were prepared by desialylation of intact glycoproteins by two methods, i.e. mild acid treatment with 0.01 M HCl at 80°C for 1 h or digestion with neuraminidase (from *Vibrio cholerae*, Boehringer Mannheim GmbH, Mannheim, Germany) with 0.1 U/mg glycoprotein in acetate-buffered saline (pH 5.5) containing 1 mM $CaCl_2$ and 1 mM PMSF at 37°C for 40 h. Asialo-agalactofetuin and asialo-agalactotransferrin were prepared by digestion of asialoglycoproteins with β-galactosidase (from jack bean, Boehringer Mannheim) with 0.04 U for 1 mg of glycoprotein in citrate buffer (pH 3.5) at 37°C, overnight. Biotinylated polymeric sugar-probes (BP-probe) and *N*-acetylchitooligosaccharides (a mixture of pentamer and hexamer: $GlcNAc_{5-6}$) were from Seikagaku Kogyo (Tokyo, Japan). Streptavidin-biotinylated HRP complex was purchased from Amersham (Buckinghamshire, UK) and *N*-acetylneuraminic acid (NeuAc) was from Nacalai Tesque (Kyoto, Japan). Pyridylamino (PA)-oligosaccharides containing α2,6-linked or α2,8-linked NeuAc were purchased from Takara Shuzo (Kyoto, Japan) and one containing α2,3-linked NeuAc (2A4-200.4) [17] and another containing terminal GlcNAc (200.1) [18] were prepared as described previously. The structures of these oligosaccharides are shown in Scheme 1.

2.2. Preparation of affinity adsorbent

$GlcNAc_{5-6}$ was immobilized to amino-Sepharose by reductive amination. Briefly, amino-Sepharose (10 g) was suspended in 8 ml of 0.2 M K_2HPO_4 containing 800 mg of $GlcNAc_{5-6}$ and 125 mg of $NaCNBH_3$ and incubated at 37°C for 4 days with shaking. The unreacted amino groups were *N*-acetylated as described previously [19]. Acetamido-Sepharose was prepared by *N*-acetylation of amino-Sepharose without coupling of sugars. PVL-Sepharose was prepared by reacting amino groups of PVL (5 mg) with formyl-Sepharose 6B (2 g) by reductive amination as reported previously [20] in the presence of 50 mM GlcNAc as a protecting sugar.

2.3. Purification of PVL

All procedures were carried out at 4°C. Extraction and subsequent acid treatment were performed as described previously [7]. The extract was neutralized with concentrated NaOH and centrifuged at $700 \times g$ for 20 min. The supernatant was mixed with 1.5 g of $GlcNAc_{5-6}$-Sepharose 6B gel in a nitrocellulose tube followed by dialysis against several changes of phosphate-buffered saline, pH 7.2, containing 5 mM EDTA and 10% glycerol. The contents of the dialyzer were packed into the column (1×2.2 cm) and extensively washed with the same

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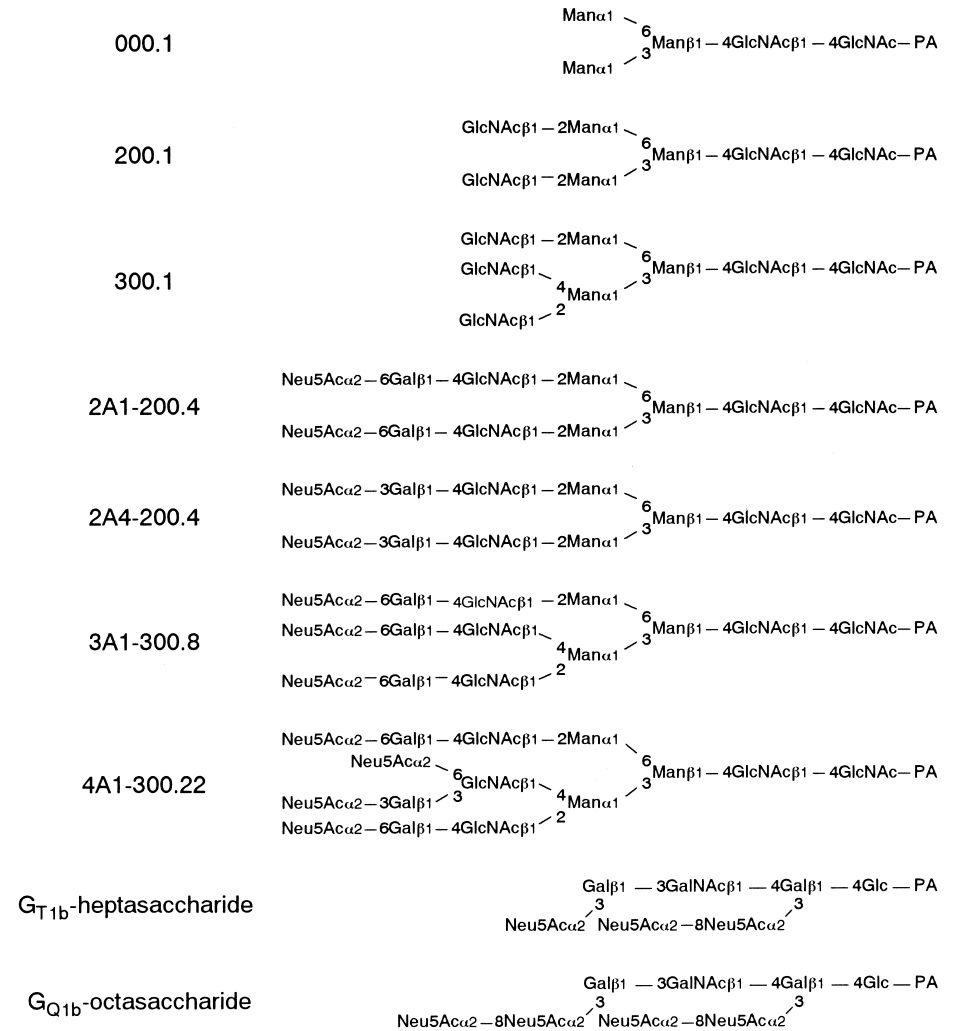
Abbreviations: PVL, *Psathyrella velutina* lectin; PBS, 10 mM phosphate buffer (pH 7.0)-0.13 M NaCl; $GlcNAc_{5-6}$, *N*-acetylchitooligosaccharides (a mixture of pentamer and hexamer); BSM, bovine submaxillary mucin; NeuAc, *N*-acetylneuraminic acid; LacNAc, *N*-acetyllactosamine; BP-probe, biotinylated polymeric sugar-probe; PA-oligosaccharides, pyridylamino-oligosaccharides; B-SJA-I, a galactose-specific lectin from *Sophora japonica*

PII: S0014-5793(99)00334-8



Scheme 1. Structures of PA-oligosaccharides. The abbreviations for PA derivatives of *N*-linked oligosaccharides are according to Tomiya et al. [23].

buffer. PVL was eluted with 0.2 M GlcNAc in the same buffer. The protein concentration was determined by using Protein Assay Reagent (Bio-Rad, Hercules, CA, USA). SDS-PAGE was carried out using 11% polyacrylamide separation gels under reducing conditions with 5% mercaptoethanol [21].

2.4. Preparation of biotin-labelled PVL or B-SJA-I

Biotinylation of PVL was performed using *N*-hydroxysuccinimide biotin [7]. To 1.5 mg PVL in 1.2 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.13 M NaCl (PBS) and 10% glycerol, 150 μl of 0.1% *N*-hydroxysuccinimide biotin was added and the mixture was allowed to stand for 4 h at room temperature in the presence of 10 mM GlcNAc in the reaction buffer. The reaction was stopped by adding 0.1 M Tris followed by dialysis against 10% glycerol. Biotinyl B-SJA-I was prepared in the same manner as described above except the absence of GlcNAc in the reaction buffer.

2.5. Solid phase assay

100 μl of the glycoprotein or PVL solutions were added to the wells of an Immulon 1 plate (Dynatech Laboratories, Chantilly, VA, USA) and immobilized overnight at 4°C. The subsequent procedures were performed at room temperature. After wells were washed with PBS and blocked with 3% BSA-PBS for 2 h, 100 μl of biotinyl lectin or BP-probes was added to immobilized glycoproteins or PVL, respectively. After incubation for 1 h, the wells were washed three times with PBS. 100 μl of streptavidin-biotinylated HRP complex diluted to 1/1000 with PBS was added and incubated for 1 h, followed by washing with PBS. Then, the color was developed with 0.04% *o*-phenylenediamine and 0.01% H_2O_2 in 0.1 M citrate-phosphate buffer (pH 5.0). The absorbance of each well at 490 nm was read using a microplate reader and the averages of duplicate determinations were plotted. For inhibition assays, various concentrations of inhibitors were pre-incubated with immobilized PVL or biotinyl PVL and then solid phase assays were performed as described above.

2.6. Affinity chromatography of PVL on acetamido-Sepharose

Purified PVL (500 μg) in PBS was incubated with 2 g of acetamido-Sepharose gel overnight under the same conditions as $GlcNAc_{5-6}$-Sepharose gel in the purification procedure. After being packed into the column (0.6×6 cm), the gel was washed with PBS and eluted with 0.2 M GlcNAc.

2.7. Affinity chromatography of PA-oligosaccharides on PVL-Sepharose

100 pmol of PA-oligosaccharide in 10 mM ammonium acetate (pH 4.5) was applied to a PVL-Sepharose column (0.3×13.5 cm, $V_T = 1.49$ ml). After washing with 10 mM ammonium acetate (pH 4.5), the column was eluted with 0.1 M GlcNAc in the same buffer or 0.1 M acetic acid. The fractions were collected in three drops and the fluorescence of each fraction was measured by a flow injection in the same buffer using a spectrofluorometer connected to an HPLC pump.

3. Results and discussion

3.1. Purification of PVL

Originally, PVL was purified by a three step procedure: chitin column affinity chromatography, ion exchange chroma-

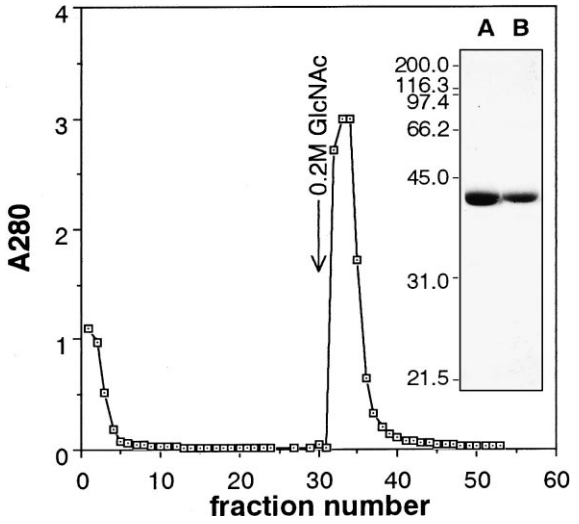


Fig. 1. Purification of PVL by one step affinity chromatography. The acid-treated extract of the fruiting body of *P. velutina* was mixed with 1.5 g of $GlcNAc_{5-6}$-Sepharose gel, followed by dialysis against phosphate-buffered saline (pH 7.2), containing 5 mM EDTA and 10% glycerol, and then packed into the column (1×2.2 cm). After the gel was washed, PVL was eluted with the same buffer containing 0.2 M GlcNAc (arrow). PVL, purified in this study (A) or by the previous procedure (B), was loaded on each lane of the 11% acrylamide gel and run for SDS-PAGE and stained with Coomassie brilliant blue (inset).

tography, followed by re-chromatography on the chitin column [7]. As shown in Fig. 1, PVL was adsorbed onto the column of $GlcNAc_{5-6}$-Sepharose 6B and specifically eluted with 0.2 M GlcNAc. The yield of 20 mg of PVL from 20 g of *P. velutina* tissue was three times higher than the original method [7]. The maximum adsorption capacity was 13 mg/g gel. PVL gave a single band corresponding to about 40 kDa, as shown in Fig. 1 (inset A), which was consistent with the reported molecular weight (Fig. 1, inset B) [7]. PVL was thus purified by single step affinity chromatography.

3.2. Interaction of PVL with sialoglycoproteins

As shown in Fig. 2A, the binding of biotinyl PVL was observed for the sialoglycoproteins examined. The fetuin- and transferrin-binding of biotinyl PVL were found to be completely prevented by treatment with neuraminidase, as shown in Fig. 2B and C, or by acid treatment of fetuin or transferrin (data not shown). Biotinyl PVL bound to asialo-agalactotransferrin (Fig. 2B) or asialo-agalactofetuin (Fig. 2C) because GlcNAc was exposed at the non-reducing terminals. In parallel experiments, the binding of a galactose-specific lectin, B-SJA-I, for immobilized glycoproteins was examined (Fig. 2D). Biotinyl B-SJA-I bound well to asialofetuin but not to fetuin. Degalactosylation of asialofetuin considerably lowered the binding to B-SJA-I. The reversed reactivity of PVL and B-SJA-I to fetuin and its derivatives indicates that these glycoproteins were immobilized enough for lectin detection, regardless of its oligosaccharide structures, and these lectins respectively recognized the specific carbohydrates in oligosaccharides of glycoproteins. In addition, HRP-PVL was shown to bind to fetuin and asialo-agalactofetuin but not to asialofetuin, by Western blot analysis (data not shown). These results clearly suggest that PVL bound to intact sialoglycoproteins by recognizing non-reducing terminal NeuAc residues but not by interacting with other parts of the glycoproteins. On the other hand, BSM has been reported to possess *O*-linked oligosaccharides containing a core-type 4 structure having non-reducing terminal β1-3- and β1-6-linked GlcNAc residues [22] and thus BSM and asialo-BSM may interact with PVL at the GlcNAc residues.

As shown in Fig. 2E–G, GlcNAc commonly inhibited the interaction of PVL with the sialoglycoproteins. Interestingly, whereas the PVL-fetuin- or PVL-BSM-binding was not affected, the PVL-transferrin-binding was decreased to 30% in the presence of 16 mM NeuAc (Fig. 2E).

Since these interactions were most effectively inhibited with GlcNAc rather than NeuAc, the relationship of GlcNAc- and NeuAc-binding sites were therefore studied using monosaccharide-linked BP-probes.

3.3. Interaction of PVL with BP-probes and N-acetyl groups

PVL was immobilized on the well and reacted with seven kinds of BP-probe. PVL bound concentration-dependently to BP-α-NeuAc as well as BP-β-GlcNAc and to BP-*N*-acetyllactosamine (LacNAc) and BP-β-GalNAc to a lesser extent, but not to BP-β-Glc, BP-α-GalNAc or BP-sialyl Lewis X (Fig. 3A). As shown in Fig. 3B, the binding of PVL to BP-α-NeuAc was completely inhibited by a lower concentration of GlcNAc (25 mM) than that required to inhibit the binding to BP-β-GlcNAc (200 mM). The binding of PVL to BP-β-GalNAc or BP-LacNAc was also inhibited with 3 mM or 1.5 mM GlcNAc, respectively (data not shown). These results indicate that PVL interacts not only with non-reducing terminal GlcNAc but also with NeuAc and GalNAc and furthermore, internal GlcNAc of the LacNAc structure, at the common binding site.

Affinity chromatography showed the binding of PVL for *N*-acetyl groups. As shown in Fig. 3C, purified PVL was completely adsorbed onto an acetamido-Sepharose column and eluted with 0.2 M GlcNAc, while it passed through a Sepharose column (data not shown). The result suggests that the acetamido group plays an essential role in the binding of PVL and substitution groups linked to carbon atoms of the pyranose ring affect the affinity to PVL. As shown in Fig. 3D, the α-NeuAc, β-GalNAc have identical configurations with β-GlcNAc as to the equatorial C-2 acetamido group, the equatorial C-3 hydroxyl group and the equatorial C-1 substitution of the β-GlcNAc pyranose ring, which are considered to be critical for contact with the binding site of PVL. The specificities found here were not detected previously by a hemagglutination inhibition assay [7]. The heterogeneity of four binding sites existing in a PVL monomer might be responsible for the specificity gap revealed here from that predicted by a hemagglutination inhibition assay which depends on multivalent binding sites.

3.4. Affinity chromatography of PA-oligosaccharides on PVL-Sepharose

Affinity chromatography of various PA-oligosaccharides was performed using a PVL-Sepharose column with 10 mM ammonium acetate buffer (pH 4.5), because the maximum binding of PVL to GlcNAc was observed at around pH 4 (our unpublished results) and a high sensitivity of fluorescence of PA-oligosaccharides was obtained at an acidic pH. As shown in Fig. 4, sialooligosaccharides showed differential chromatograms on the PVL column depending on the number

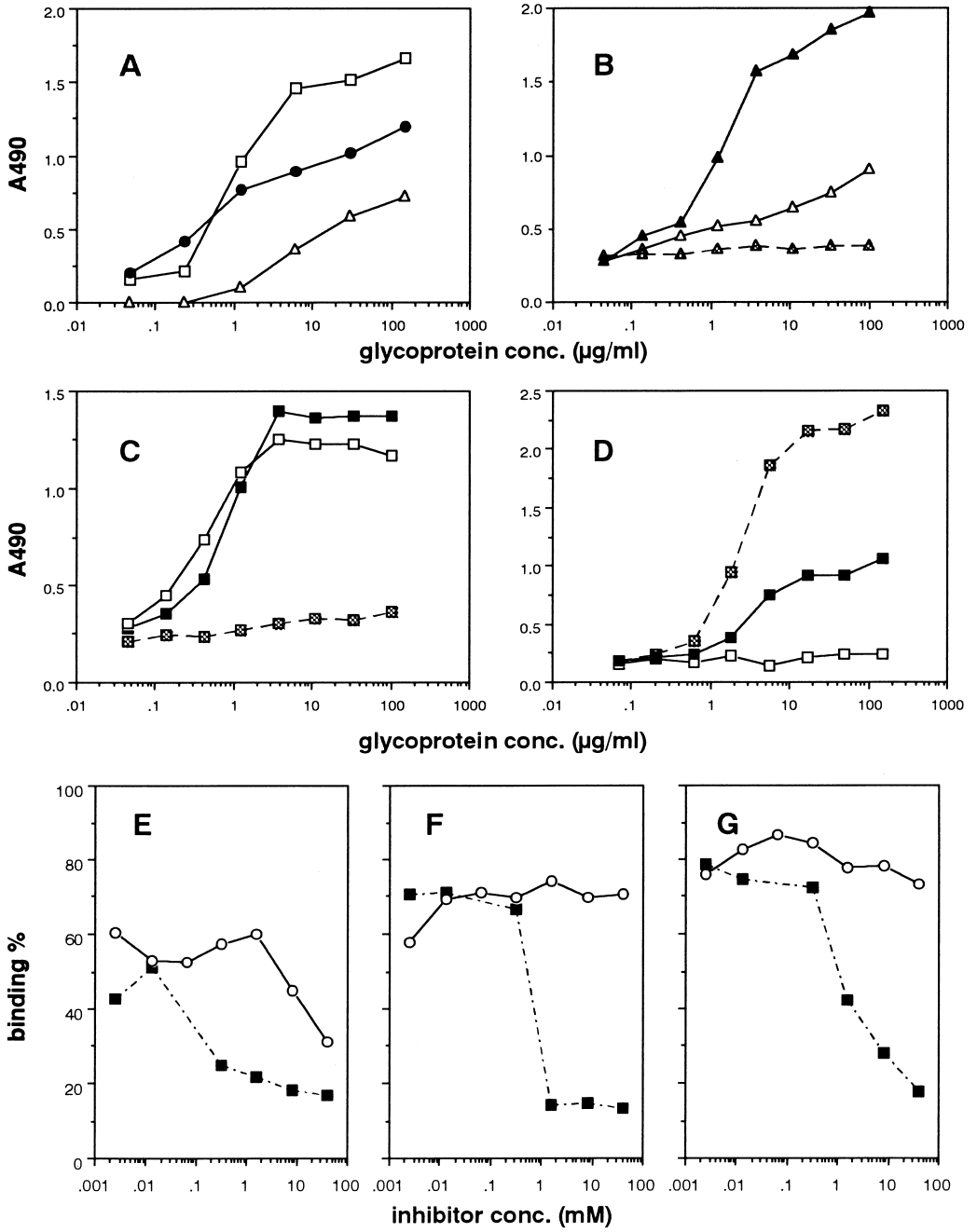


Fig. 2. Reactivities of biotinyl PVL with sialoglycoproteins. Solutions (100 μl) of glycoproteins were serially diluted in PBS and immobilized on microtiter plates. Biotinyl PVL or biotinyl B-SJA-I (10 μg/ml) was added to the wells and a solid phase assay was performed as described in the text. A: reactivity with sialoglycoproteins: fetuin (□), BSM (●) and transferrin (△). B: effects of desialylation and degalactosylation of transferrin on binding of biotinyl PVL. C and D: effects of desialylation and degalactosylation of fetuin on binding of biotinyl PVL (C) and biotinyl B-SJA-I (D). Symbols used are: B, transferrin (open triangel), asialotransferrin (hatched triangel) and asialoagalactotransferrin (filled triangel); C and D, fetuin (□), asialofetuin (hatched square) and asialoagalactofetuin (■). E–G: effects of GlcNAc or NeuAc on the interaction between sialoglycoprotein and biotinyl PVL. After immobilization of 10 μg/ml of transferrin (E), fetuin (F) or BSM (G), biotinyl PVL (10 μg/ml) pre-incubated with various concentrations of GlcNAc (■) or NeuAc (○) for 1 h was added to each well. The binding percentage is represented by the proportion of the absorbance at 490 nm to that in the absence of inhibitors.

of sialic acid residues and the position of its linkage. PA-Xyl or 000.1 were eluted first, while other PA-oligosaccharides were retarded or bound to the column. Disialooligosaccharide with the NeuAcα2-6Gal structure, 2A1-200.4, weakly interacted with the PVL column, but that with the NeuAcα2-3Gal structure, 2A4-200.4, and trisialooligosaccharides, 3A1-300.8, interacted more strongly. Surprisingly, tetrasialooligosaccharides, 4A1-300.22, bound to the PVL column and eluted with 0.1 M GlcNAc. These results indicate that NeuAc residues play an important role in the binding of sialooligosaccharides and sialoglycoproteins to PVL for the first time. When an other trisialooligosaccharide or tetrasialooligosaccharide of glycolipid origin (G_{T1b}-heptasaccharide or G_{Q1b}-octasaccharide, respectively, see Scheme 1) was applied onto

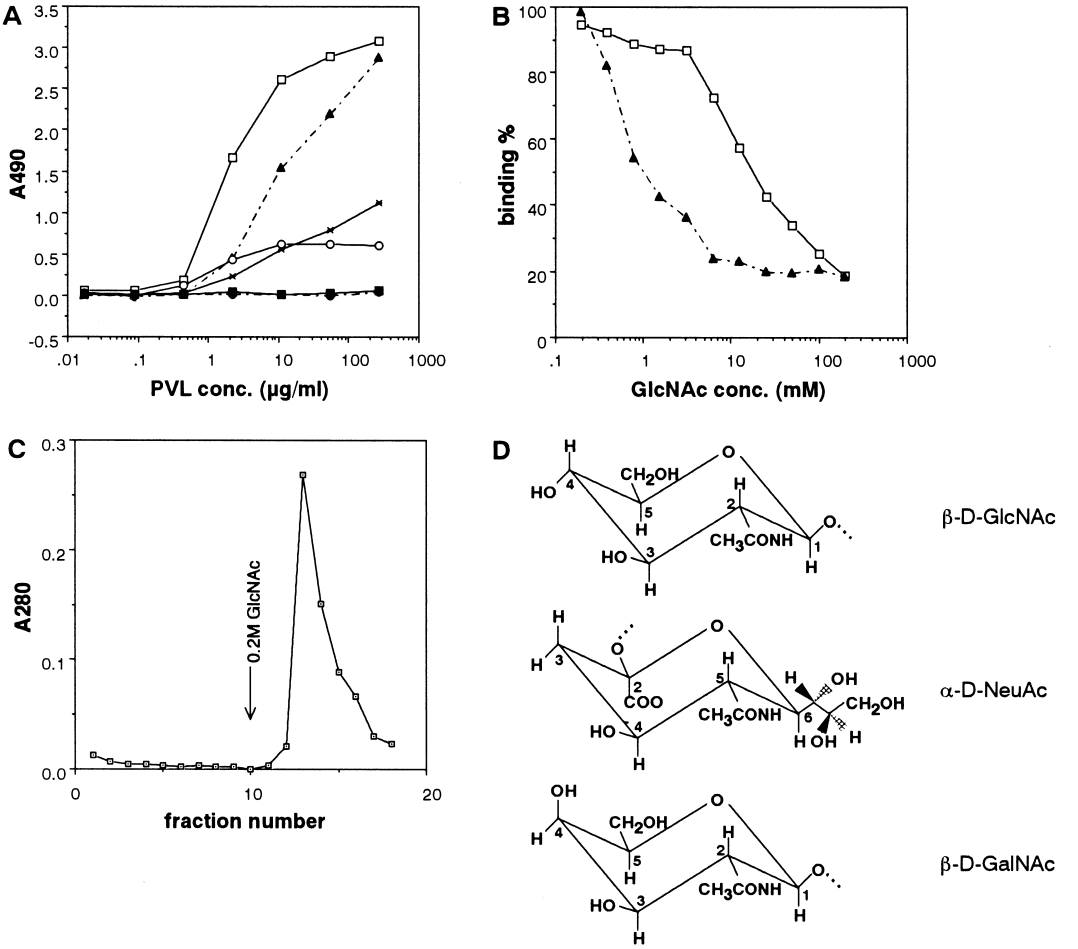


Fig. 3. Interactions of PVL with BP-probes and acetamido-Sepharose. A: a solution (100 μl) of PVL was serially diluted in PBS and immobilized on microtiter plates. BP-probes (5 μg/ml) were added to the wells and a solid phase assay was performed. B: after immobilization of PVL (10 μg/ml), BP-probes (5 μg/ml) were added and co-incubated with various concentrations of GlcNAc as inhibitor. The binding percentage is represented by the proportion of the absorbance at 490 nm to that in the absence of GlcNAc. BP-probes were: β-GlcNAc (□), α-NeuAc (▲), LacNAc (×), β-GalNAc (○), α-GalNAc (●) and β-Glc (■). C: affinity chromatography of PVL on acetamido-Sepharose. Purified PVL (500 μg) in PBS was incubated with acetamido-Sepharose (2 g) and packed into the column (0.6×6 cm). After washing with PBS, PVL was eluted with PBS containing 0.2 M GlcNAc at the arrow. D: common structural features of β-GlcNAc*p*, α-NeuAc*p* and β-GalNAc*p*. The relationships between the acetamido (position 5 of NeuAc and 2 of GlcNAc and GalNAc) and the hydroxyl groups (position 4 of NeuAc and 3 of GlcNAc and GalNAc) and between the acetamido and the substitution groups linked to an adjacent carbon atom (position 6 of NeuAc and 1 of GlcNAc and GalNAc) are all in the *trans* configuration.

a PVL column, they bound to the column and partly eluted with 0.1 M GlcNAc. The oligosaccharides still bound to the column were eluted with 0.1 M acetic acid (data not shown). On the other hand, PA-oligosaccharides with non-reducing terminal GlcNAc residues (200.1, 300.1) bound to the PVL column but hardly eluted with 0.1 M GlcNAc. These oligosaccharides were eluted with 0.1 M acetic acid (Fig. 4).

Differential binding of PVL to oligosaccharides appears to be due to both the number and linkage of non-reducing terminal NeuAc and GlcNAc residues. The interaction with glycoproteins is interpretable by the relative affinity for oligosaccharides found here: (1) PVL bound more strongly to fetuin (containing mainly 3A1-300.8) than transferrin (containing mainly 2A1-200.4) (Fig. 2A). (2) Transferrin-binding of PVL was inhibited by NeuAc (Fig. 2E), but fetuin-binding of PVL was not (Fig. 2F).

These findings indicate that the well known specificity of PVL to non-reducing terminal GlcNAc has to be corrected. PVL-Sepharose is a useful tool for the separation of glycoconjugates containing NeuAc or terminal GlcNAc from others in terms of the number or linkage. Chemically-labelled PVL also could detect sialyl residues and differentiate them from GlcNAc residues when used in combination with glycosidase treatment of glycoconjugates.

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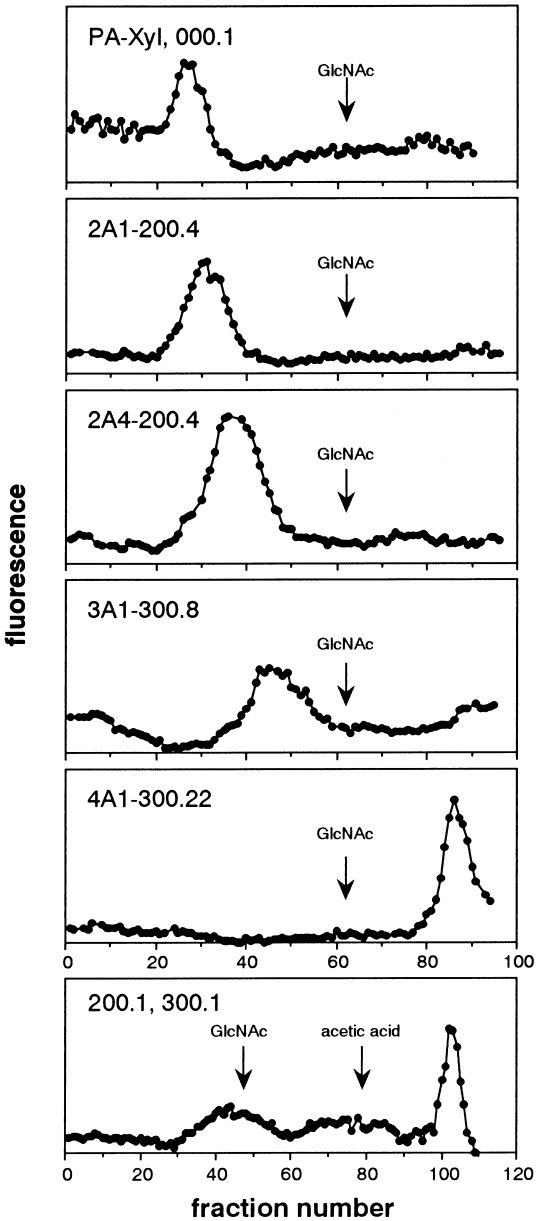

Fig. 4. Affinity chromatography of PA-oligosaccharides on PVL-Sepharose. PA-oligosaccharides (each about 100 pmol) in 10 mM ammonium acetate/acetic acid (pH 4.5) were applied to a PVL column (0.3×13.5 cm) and eluted with the same buffer. The elution buffer was changed to 0.1 M GlcNAc in the same buffer or 0.1 M acetic acid at the positions indicated by arrows. PA-oligosaccharides were detected using a spectrofluorometer as described in the text. The structures of PA-saccharides are shown in Scheme 1.

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