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CARBOHYDRATE-BINDING SPECIFICITIES OF FIVE LECTINS THAT BIND TO *O*-GLYCOSYL-LINKED CARBOHYDRATE CHAINS. QUANTITATIVE ANALYSIS BY FRONTAL-AFFINITY CHROMATOGRAPHY*†

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ABSTRACT

The carbohydrate-binding specificities of lectins purified from *Agaricus bisporus* (ABA-I), *Arachis hypogaea* (PNA), *Bauhinia purpurea* (BPA), *Glycine max* (SBA), and *Vicia villosa* (VVA-B_4) have been studied by affinity chromatography on columns of the immobilized lectins, and quantitatively analyzed by frontal affinity chromatography. These five lectins could be classified into two groups with respect to their reactivities with typical mucin-type glycopeptides, β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→3)-Ser/Thr (**2**) and α-D-Gal*p*NAc-(1→3)-Ser/Thr (**3**). One group, which consists of ABA-I, PNA, and BPA, preferentially binds to **2**, and the other, which consists of SBA and VVA-B_4, shows higher affinity for **3** than for **2**. Among the lectins tested, only ABA-I was found to bind to a sialylated glycopeptide, which was prepared from human erythrocyte glycophorin A and contains three tetrasaccharide chains having the structure of α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-[NeuAc-(2→6)]-α-D-Gal*p*NAc-(1→, with an association constant of 15 μM, whereas the association constants of the other four lectins for this sialylated glycopeptide were <3.5 mM. On the other hand, removal of the β-D-galactopyranosyl group from a glycopeptide containing sequence **2** resulted in decreased association constants for the three lectins of the first group, especially ABA-I and PNA. The two lectins of the second group showed a high affinity for **3**, but SBA preferentially interacted with oligosaccharides containing the α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-D-Glc*p*NAc sequence, prepared from a blood group A-active oligosaccharide.

INTRODUCTION

Lectins are useful tools for the separation of oligosaccharides and glycopeptides, and the study of their structures. The carbohydrate-binding specificities of lectins

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Leu/Ser-Ser(R)-Thr(R)-Thr(R)-Glu/Gly-Val-Ala-Hse

1 R = α-NeuAc-(2→3)-β-D-Gal*p*-(1→3)-[α-NeuAc-(2→6)]-α-D-Gal*p*NAc-

2 R = β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-

3 R = α-D-Gal*p*NAc-

α-D-Gal*p*NAc-(1→3)-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc
(2 on Gal*p*; 4 on Glc*p*NAc)

4 R= H, R′ = α-L-Fuc*p*

5 R = R′ = H

6 R = R′ = α-L-Fuc*p*

Scheme 1. Structures of glycopeptide CB-II (**1**) obtained from human erythrocyte glycophorin A, and a blood group A-active hexasaccharide (**4**) and a blood group A-active heptasaccharide (**6**) isolated from the faeces of breast-fed infants.

that preferentially bind to asparagine-linked carbohydrate chains have been extensively studied through the use of affinity chromatography on immobilized-lectin columns[1–5]. On the other hand, the binding specificities of lectins that specifically bind to so-called mucin-type sugar chains have been studied by hapten-inhibition assays in most cases. The binding specificities of five lectins purified from *Agaricus bisporus, Arachis hypogaea, Bauhinia purpurea, Glycine max*, and *Vicia villosa* were studied by several groups[6–13]; however, the differences in their binding specificities for naturally occurring oligosaccharides and glycopeptides have not yet been clarified. We report herein the carbohydrate-binding specificities of the five lectins, as determined in binding studies using immobilized-lectin columns, and the association constants of the five lectins for various sugar sequences, as determined through frontal analysis.

EXPERIMENTAL

Materials. — Blood group A-active hexa- and hepta-saccharides, isolated from the faeces of breast-fed infants, were obtained from BioCarb Chemicals (Lund, Sweden). Porcine submaxillary mucin was purified from porcine submaxillary glands according to the method of Katzman and Eylar[14]. Sephadex G-25, Sepharose 4B, and DEAE-Sephadex A-25 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden), galactose oxidase from *Dactylium dendroides* from P-L Biochemicals, Inc. (Milwaukee, Wis., U.S.A.), sialidase from *Arthrobacter*

ureafaciens from Nakarai Chemicals Ltd. (Kyoto, Japan), lactose and D-galactose from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), and *N*-acetyl-D-galactosamine and D-galactosamine hydrochloride from Sigma Chemical Co. (St. Louis, MO., U.S.A.).

Preparation of lectins and lectin columns. — *Agaricus bisporus* agglutinin I (ABA-I) was purified by affinity chromatography as reported previously[7]. *Arachis hypogaea* and *Bauhinia purpurea* agglutinins (PNA and BPA) were purified by the methods of Lotan and Sharon[15], and Osawa and Irimura[16], respectively, except for the use of D-galactose–Sepharose 4B as an affinity adsorbent. *Glycine max* agglutinin (SBA) was purified by the method of Lis and Sharon[17], except for the use of D-galactosamine–Sepharose as an affinity adsorbent. *Vicia villosa* agglutinin B_4 (VVA-B_4) was purified according to the method of Tollefsen and Kornfeld[18]. The purified lectins were coupled to cyanogen bromide-activated Sepharose 4B by the method of Cuatrecasas and Anfinsen[19].

*Preparation of Gal-Sepharose and GalN-Sepharose**. — Gal-Sepharose 4B was prepared as follows. Lactose (9.0 g in 15 mL of 0.2M borate buffer, pH 8.0) and sodium cyanoborohydride (2.0 g in 3 mL of the same buffer, pH 8.0) were mixed with hydrazine-Sepharose 4B (70 mL), prepared by the method of Matsumoto *et al.*[20]. The mixture was incubated for 72 h at 37° with constant stirring. The gel was washed on a glass filter with distilled water, and then the remaining free hydrazide groups on the gel were blocked by *N*-acetylation with acetic anhydride (0.2 mL × 3) for 1 h at 37°. GalN-Sepharose 4B was prepared according to the method of Allen and Neuberger[21]. Galactosamine hydrochloride (0.8 g in 20 mL of distilled water) was mixed with CH-Sepharose 4B (Pharmacia, 70 mL), and then 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide (2.3 g) was added to the suspension. The pH of the mixture was maintained at 5.0 with 0.1M NaOH, and the mixture was incubated for 20 h at room temperature.

Preparation of labeled glycopeptides and oligosaccharides. — The structures of the glycopeptides and oligosaccharides **1–6** used in this study were confirmed by analysis of the component sugars and methylation analysis. An N-terminal glycopeptide of human erythrocyte glycophorin A (CB-II; **1**) was prepared from tryptic fragment[22] T1 according to the method of Prohaska *et al.*[23]. Compound **1** (5.0 μmol) was labeled by *N*-acetylation with [1-^{14}C]acetic anhydride (0.73 GBq/mmol; Amersham International) according to Tai *et al.*[24]. A tritiated disaccharide alditol and a tritiated *N*-acetylgalactosaminitol were prepared from porcine submaxillary mucin (PSM) by the β-elimination reaction. PSM-Glycopeptides (200 μg), prepared through repeated Pronase digestions, were incubated with NaB^3H_4 (185 GBq; 12.6 GBq/mmol; New England Nuclear) in 0.1M NaOH (0.2 mL) for 40 h at 40°. The mixture was made neutral by the addition of acetic acid, and then loaded onto a column (0.8 × 35 cm) of Sephadex G-25, equilibrated with distilled water, to

*When not indicated, sugars are in the D configuration, except for L-fucose, and in the pyranose form as glycosides.

recover the labeled sugars. After treatment of the tritiated oligosaccharide alditols with sialidase from *Arthrobacter ureafaciens*, the desialylated oligosaccharide alditols were subjected to gel permeation chromatography on two columns (7.2 × 500 mm) of Bio-Gel P-4 (−400 mesh) in an h.p. liquid chromatograph (Tri-rotar; Japan Spectroscopic Co. Ltd., Tokyo, Japan)[25].

Blood group A-active hexasaccharide **4** (85 nmol) and heptasaccharide **6** (48 nmol) were reduced with NaB^3H_4 (74 MBq; 12.6 GBq/mmol; New England Nuclear) in 10mM NaOH (0.1 mL) for 2 h, and then $NaBH_4$ (5 mg) was added. The reaction was continued for 2 h at room temperature. Each blood group A-active oligosaccharide was desalted on a small column of Dowex 50W-X8 (H^+), equilibrated with distilled water. The labeled oligosaccharide alditols, eluted with distilled water, were freed from boric acid by repeated evaporation with methanol.

Modification of glycopeptides and oligosaccharides. — (a). Removal of sialic acid. Glycopeptide CB-II (**1**; 0.5 μmol) was incubated at 37° with sialidase from *Arthrobacter ureafaciens* (0.5 units) in 0.1M sodium acetate buffer (pH 5.5, 0.5 mL) under a toluene layer for 24 h, followed by heating for 3 min at 100° to stop the reaction. The mixture was passed through a column (1.5 × 20 cm) of DEAE-Sephadex A-25, equilibrated with 50mM ammonium formate buffer, pH 6.4, and the desialylated glycopeptide, CB-II (−SA) (**2**), was recovered in the unbound fraction.

(b). Smith periodate degradation. The galactose residues of **2** were removed by Smith periodate degradation. Compound **2** (0.2 μmol) was oxidized in 50mM sodium acetate buffer (pH 4.0, 0.5 mL) containing 75mM sodium metaperiodate for 48 h, at 4°, in the dark. The reaction was terminated by addition of 1,2-ethanediol (1 drop). After 1 h at room temperature, 0.1M sodium borate buffer, pH 8.0, containing 0.5mM $NaBH_4$ (0.5 mL) was added, and then the mixture was incubated for 3 h at room temperature. The mixture was made neutral with acetic acid and desalted by gel filtration on a column (1.5 × 35 cm) of Sephadex G-25. The periodate-oxidized–borohydride-reduced glycopeptide was hydrolyzed in 50mM HCl for 1 h at 80°, and the mixture was evaporated to dryness to give **3**.

(c). Mild acid hydrolysis. The fucose residue of the blood group A-active hexasaccharide **4** was removed by mild acid hydrolysis. An unlabeled sample (50 nmol) was hydrolyzed in 0.15M HCl for 1.5 h at 80°. After evaporation to dryness, the resulting oligosaccharide was purified by gel permeation chromatography on two columns of Bio-Gel P-4, as described earlier. Removal of the fucose residue was confirmed by compositional analysis. The terminal nonreducing *N*-acetylgalactosamine group of fucose-less hexasaccharide was radioactively labeled by the galactose oxidase–NaB^3H_4 method. The sample (15 nmol) was incubated with galactose oxidase from *Dactylium dendroides* (25 units) in 10mM sodium phosphate buffer (pH 7.3, 0.1 mL) for 2.5 h at 37°, and then reduced by adding NaB^3H_4 (74 MBq, 12.6 GBq/mmol; New England Nuclear) in 0.1M NaOH (10 μL). The incubation was continued for a further 3 h at room temperature with the addition of M $NaBH_4$ after 2 h. The labeled oligosaccharide was applied to small columns of Dowex 50W-X8 and Bio-Rad AG1-X8 ion-exchange resins equilibrated and eluted with distilled water.

Lectin-affinity chromatography. — A radioactively labeled sample was loaded onto a lectin column (0.5 × 12 cm), equilibrated with 10mM sodium phosphate buffer, pH 7.3, containing 0.15M NaCl. The column was eluted with the same buffer (8.4 mL), and then with the same buffer containing an appropriate haptenic sugar (12 mL). Fractions (0.6 mL) were collected at a flow rate of 8 mL/h.

Frontal-affinity chromatography. — Frontal-affinity chromatography was carried out at room temperature according to the method of Kasai and Ishii[26,27]. A radioactively labeled sample in 10mM sodium phosphate buffer, pH 7.3 containing 0.15M NaCl, was continuously applied to a lectin column (0.5 × 12 cm), previously equilibrated with the same buffer. Fractions (0.52 mL) were collected at a flow rate of 5 mL/h, and the radioactivity of each fraction was measured with a liquid-scintillation counter. The association constants were calculated according to the equation given in the Results section.

RESULTS

Lectin-affinity chromatography. — To elucidate the carbohydrate-binding specificities of the five lectins, affinity chromatographies on lectin columns were performed. Of the four isolectins from *Agaricus bisporus*, only the major component, ABA-I, was immobilized on Sepharose 4B, because our previous study did not demonstrate any differences in their binding specificities[7].

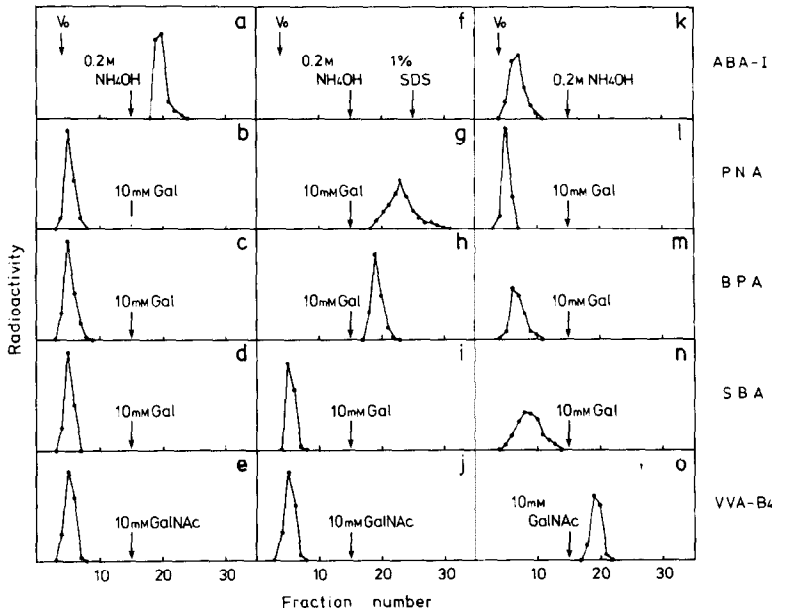

Fig. 1. Elution profiles of intact and modified glycopeptides on lectin columns. Glycopeptides **1** (panels a–e), **2** (panels f–j), and **3** (panels k–o) were applied to a lectin column and eluted as described in the Experimental section. Arrows indicate the void volume (V_0) and where the buffer was changed.

Fig. 1 shows the elution profiles of glycopeptide **1** and the modified glycopeptides on the five lectin columns. Glycopeptide **1**, obtained on cyanogen bromide cleavage of human erythrocyte glycophorin A, constitutes the *N*-terminal end of the glycoprotein and contains three *O*-linked tetrasaccharides having the same structure[22]. Intact **1** was retained on ABA-I-Sepharose and eluted with 0.2M NH_4OH (see Fig. 1a), whereas it was recovered in the void volume fraction with the other four lectin columns without any retardation (see Figs. 1b–e). However, desialylated **1** (**2**), prepared through digestion of **1** with sialidase, was bound by columns of ABA-I-, PNA- and BPA-Sepharose (see Figs. 1f–h), and was eluted with 10mM D-galactose from the PNA- and BPA-Sepharose columns. Glycopeptide **2** was bound to the ABA-I column so tightly that it could not be eluted with any of the eluents tested, such as 0.2M NH_4OH, 0.5M GalNAc, 3M KSCN, and 1% sodium dodecyl sulfate. On the other hand, neither SBA- nor VVA-B_4-Sepharose apparently showed any interaction with **2**, and so it was recovered in the void-volume fraction (see Figs. 1i,j). When the galactosyl group linked β-(1→3) to the 2-acetamido-2-deoxygalactose residue of **2** was removed by Smith periodate degradation, the degradation product (**3**) was no longer bound by columns of ABA-I-, PNA-, and BPA-Sepharose (see Figs.1k,m), indicating that the β-galactosyl group of the glycopeptide was essential for the high-affinity binding to these three immobilized lectins. From the PNA column, **3** was recovered without any retardation, but it was retarded by the ABA-I and BPA columns. This suggested that **3** still had weak ability to bind to the ABA-I and BPA columns, even after removal of the β-galactosyl group. In contrast with intact **1** or desialylated **1** (**2**), which appeared to show no apparent interaction with the SBA column, **3** was shown to interact weakly with the SBA column (see Fig. 1n). Furthermore, **3** was bound by a VVA-B_4 column and eluted with 10mM 2-acetamido-2-deoxygalactose (see Fig. 1o). These results demonstrated that SBA and VVA-B_4 preferably bind to α-GalNAc-(1→3)-Ser/Thr rather than to β-Gal-(1→3)-α-GalNAc-(1→3)-Ser/Thr. However, a disaccharide alditol, β-Gal-(1→3)-GalNAcol, was found to show no apparent interaction with the five immobilized lectins, suggesting that the pyranose form of the *N*-acetylgalactosamine residue was essential for the interaction. As for oligosaccharides containing an *N*-acetylgalactosamine residue at their nonreducing termini, no interaction was observed between the lectin columns tested and the blood group A-active hexa- and hepta-saccharide **4** and **6**. However, removal of the fucosyl group from the hexasaccharide **4** to give **5** increased its affinity for the SBA and VVA-B_4 columns, although it was not completely bound by either column. With the ABA-I, PNA, and BPA columns, however, no interaction was detected.

Frontal analysis by means of lectin-affinity chromatography. — The carbohydrate-binding specificities of the lectins were analyzed quantitatively by means of frontal-affinity chromatography[26,27]. The association constants were calculated according to eq. (*1*), where $[A]_0$ is the concentration of the test sample, *Bt* the total

$$1/[A]_0 \cdot (V - V_0) = (1/Bt \cdot K) \cdot (1/[A]_0 + 1/Bt) \qquad (1)$$

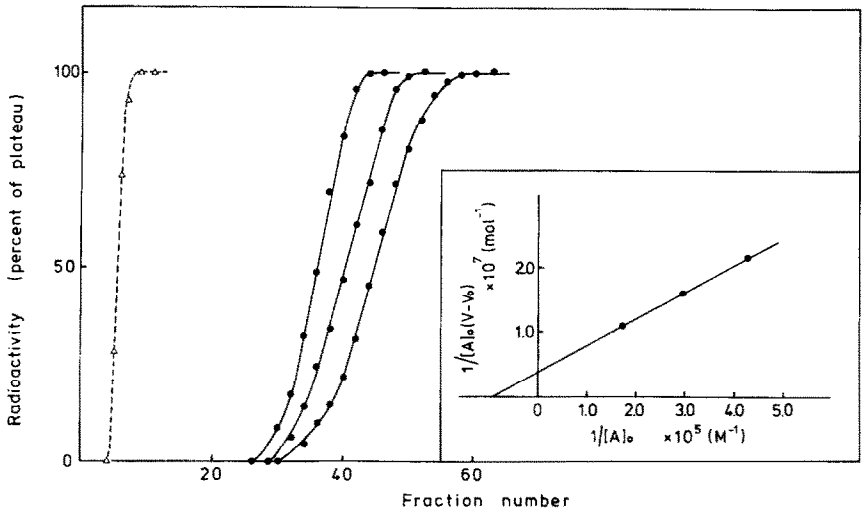

Fig. 2. Frontal affinity chromatography of glycopeptide **2** on a PNA column. Compound **2** was chromatographed on a PNA column as described in the Experimental section at concentrations of 5.7, 3.4, and 2.3μM (●——●) (from left to right). To determine the V value, 5.5μM 2-acetamido-2-deoxy-D-[1-^{3}H]galactitol was loaded onto the column (△-----△) in the presence of 0.1M lactose. The $1/[A]_0(V - V_0)$ *vs.* $1/[A]_0$ plot of the results is shown in the inset.

amount of the active lectin immobilized on a column, V_0 the elution volume of the carbohydrate that does not interact with the lectin, and V the elution volume of the test sample. The void volumes (V_0) for the ABA-I and VVA-B_4 columns (2.8 mL) were determined from the elution volume of tritiated 2-acetamido-2-deoxy-D-glucitol in the presence of 50mM 2-acetamido-2-deoxy-D-glucose, and those of the PNA, BPA, and SBA columns (2.7 mL) from the elution volume of the respective lectins in the presence of 0.1M lactose.

If $1/[A]\cdot(V - V_0)$ is plotted *versus* $1/[A]_0$, a straight line is obtained, and the intercept on the ordinate is $1/Bt$, the intercept on the abscissa $-K_a$, and the slope $1/Bt\cdot K_a$. Once the amount of the active lectin immobilized on a column (Bt) is obtained, the association constant can be determined with eq. (2).

$$K_a = 1/\{(Bt/V - V_0) - [A]_0\} \tag{2}$$

Fig. 2 shows the elution profile on frontal affinity chromatography of **2** on a column of PNA-Sepharose. The V values of **2** for three different concentrations, 5.7, 3.4, and 2.3 μM, were 18.6, 20.8, and 23.0 mL, respectively. From the $1/[A]_0\cdot(V - V_0)$ *vs.* $1/[A]_0$ plot, shown in the inset, the amount of active PNA immobilized was determined to be $2.9\cdot10^{-7}$ mol, and the association constant of PNA for **2** was calculated to be 0.86mM.

Furthermore, based on the $1/[A]_0\cdot(V - V_0)$ *vs.* $1/[A]_0$ plots shown in Fig. 3, the Bt values and association constants (K_a, see Fig. 4) for the other four lectin

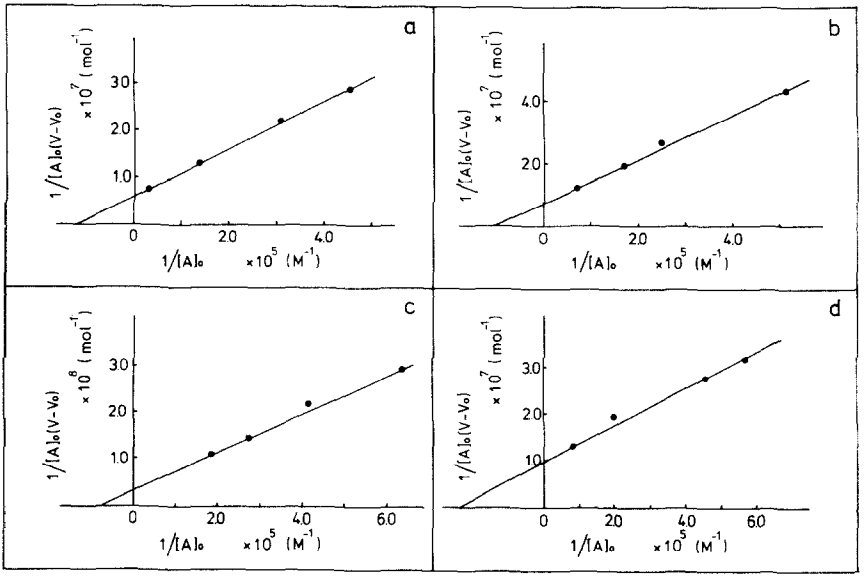

Fig. 3. $1/[A]_0(V - V_0)$ *vs.* $1/[A]_0$ plots: (a) **1** on a ABA-I column; (b) **2** on a BPA column; (c) **3** on a SBA column; and (d) **3** on a VVA-B_4 column.

columns for glycopeptides derived from **1** were calculated: *Bt*, $1.6 \cdot 10^{-7}$ mol for ABA-I; *Bt*, $5.9 \cdot 10^{-8}$ mol for BPA; *Bt*, $2.7 \cdot 10^{-8}$ mol for SBA; and *Bt*, $1.0 \cdot 10^{-7}$ mol for VVA-B_4.

Figure 5 shows the results of frontal chromatographies of various carbohydrates on a column of ABA-I. The elution fronts of glycopeptides **1, 2** and **3**

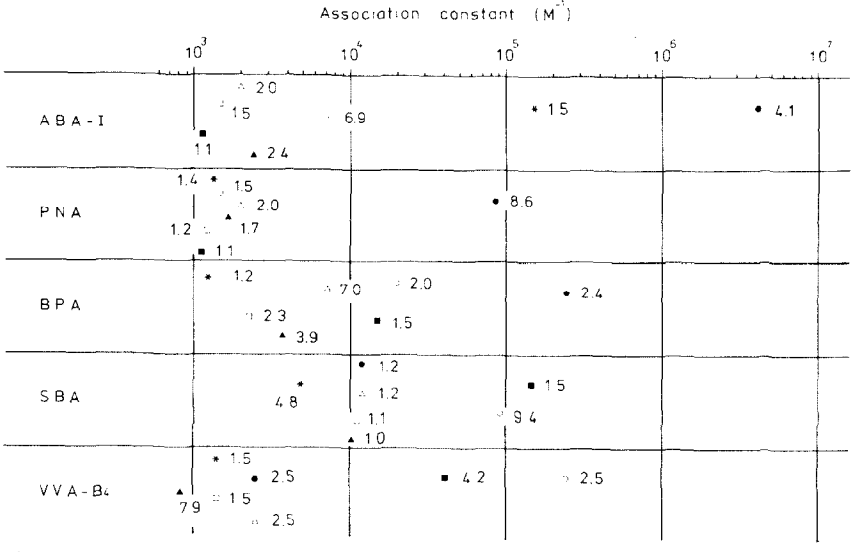

Fig. 4. Association constants of the five lectins for glycopeptides and oligosaccharide alditols. The numbers are association constants: (*) **1**; (●) **2**; (○) **3**; (□) **4**; (■) **5**; (▲) **6**; and (△) β-D-Gal*p*-(1→3)-D-GalNAcol.

were retarded by different degrees. Compound **2** was the slowest and **3** the fastest eluted from the column, indicating that their association constants for ABA-I decrease in the following order: **2, 1**, and **3**. This was in good agreement with the data obtained on conventional affinity chromatography (see Figs. 1a, f, and k).

Similarly, frontal analysis of the other four lectin columns was performed, and their association constants for glycopeptides and oligosaccharides were calculated to be as summarized in Fig. 4. ABA-I showed the largest association constant of 4.1μM for the desialylated glycopeptide **2**, and the presence of sialic acid residues on both the D-galactose and 2-acetamido-2-deoxy-D-galactose residues resulted in a decrease in the association constant to 15μM. Removal of the galactose residues from **2** (to give **3**) markedly reduced the association constant to 6.9mM. The association constants for a disaccharide alditol, β-D-Gal*p*-(1→3)-D-GalNAcol, and A-active oligosaccharides were <3.0mM.

PNA showed quite a low affinity for the sialylated glycopeptide **1** with an association constant of 1.4mM. Removal of the sialic acid residues from **1** (to give **2**) increased the association constant to 0.86mM. However, the subsequent removal of the galactosyl group (to give **3**) caused a decrease in the association constant for the glycopeptide to 1.5mM. These results indicate that PNA strongly interacts with the β-galactosyl group in the β-D-Gal*p*-(1→3)-D-GalNAc sequence, as several investigators reported[8–11], but the pyranose structure of the internal 2-acetamido-2-deoxy-D-glucose residue was also important for the interaction with PNA, as the association constant for β-D-Gal*p*-(1→3)-D-GalNAcol was about one-fortieth of that for **2**.

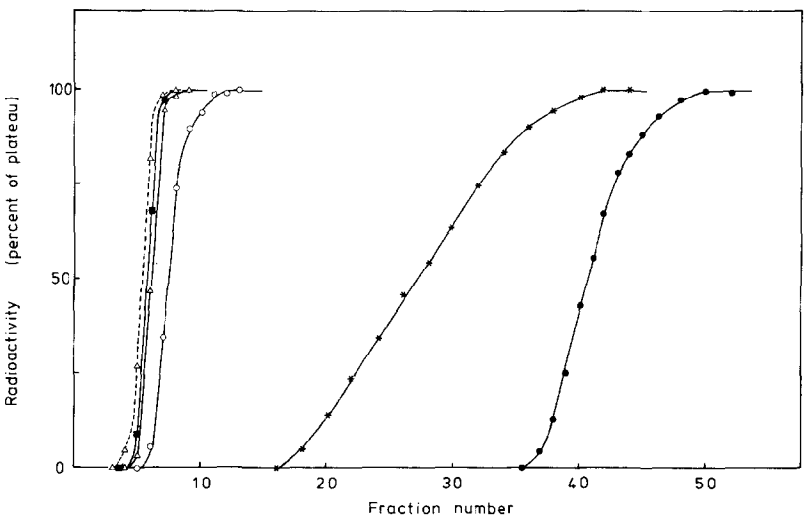

Fig. 5. Frontal-affinity chromatography on a ABA-I column. Glycopeptides and oligosaccharide alditols were chromatographed as described in the Experimental section: **1** (7.1μM, *——*), **2** (8.3μM, ●——●), **3** (1.6μM, ○——○), **5** (0.89μM, ■——■), β-D-Gal*p*-(1→3)-D-GalNAcol (6.7μM, △——△), and 2-acetamido-2-deoxy-D-galactitol in the presence of 50mM 2-acetamido-2-deoxy-D-galactose (△——△).

The association constant of BPA for **2** was estimated to be 24μM, which was about 3-fold greater than that of PNA, and about one-fifth of that of ABA-I. The association constant of these three lectins for **2** were in the following order: ABA-I > BPA > PNA. BPA also recognized both 2-acetamido-2-deoxy-α-D-galactopyranosyl residues linked to the serine or threonine residue (Tn hapten), and the terminal nonreducing 2-acetamido-2-deoxy-α-D-galactopyranosyl groups of a blood group A-active oligosaccharide which is devoid of the L-fucosyl group (**5**). The association constants of BPA for a Tn hapten (**3**) and for **5** were estimated to be 0.2 and 0.15mM, respectively. The presence of an α-L-fucosyl group at the penultimate D-galactosyl residue, however, reduced the association constant to 2.3mM.

SBA and VVA-B_4 were found to show similar binding specificities in that both can interact with terminal 2-acetamido-2-deoxy-D-galactopyranosyl groups, but they showed some differences in their specificities for the internal sugar sequence. The association constant of SBA for blood group **5** (15μM) was about 4-fold larger than that of VVA-B_4 (42mM). On the other hand, the association constant of **3** for SBA (0.94mM) was about one-third of that for VVA-B_4 (25μM). The association constants of SBA, VVA-B_4, and BPA for the Tn hapten (**3**) were in the following order: VVA-B_4 > SBA > BPA, and those for **5**: SBA > VVA-B_4 > BPA.

DISCUSSION

In this study, we investigated the binding specificities of five lectins which preferably bind to serine/threonine-linked sugar chains, rather than asparagine-linked sugar chains, and found that these lectins could be classified into two groups on the basis of their reactivities with typical serine/threonine-linked sugar chains, β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→3)-Ser/Thr (**3**) and α-D-Gal*p*NAc-(1→3)-Ser/Thr (**3**). One group comprises ABA, PNA, and BPA, which show higher affinities for **2** than for **3**. The other group comprises SBA and VVA-B_4, which react preferentially with **3** rather than **2**.

We reported previously that the activities of ABAs were effectively inhibited by a glycopeptide **1**, obtained from human erythrocyte glycophorin A, and a synthetic glycosylamino acid, β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→3)-*N*-tosyl-L-serine[7]. The data obtained in hapten inhibition assays demonstrated that ABAs recognize a disaccharide sequence, β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→, and that the α-D linkage to serine or threonine residues is essential[6,7,11]. In this study, the association constant of ABA-I, the major component of the four isolectins of *A. bisporus*, for glycopeptide **1** was estimated to be 15μM by means of frontal analysis. Removal of the sialyl groups from **1** resulted in an ~27-fold increase in the association constant to 4.1μM, and subsequent removal of the galactosyl groups markedly reduced the association constant to 6.9mM (see Fig. 4). ABA-I showed low affinity for β-D-Gal*p*-(1→3)-D-GalNAcol and oligosaccharides with a 2-acetamido-2-deoxy-D-galactopyranosyl group at their nonreducing termini. These results

demonstrated that sequence **2** was essential for the interaction with ABAs. It is likely that glycophorin A serves as a major receptor for ABA on human erythrocytes. It should be noted that ABA-I–Sepharose showed high affinity for both sialylated and nonsialylated structure **2**, and that it is a useful tool for the isolation of glycoproteins and glycopeptides containing such structures.

The binding specificities of PNA and BPA were found to be similar to that of ABA-I with respect to their preferential binding to glycopeptides containing a β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→ sugar sequence, but were somewhat different in the following points. Only ABA-I has the ability to bind to sialylated glycopeptides. BPA shows rather high affinity for glycopeptides bearing 2-acetamido-2-deoxy-D-galactopyranosyl group at their nonreducing termini, such as the Tn-hapten, but PNA shows only low affinity for the latter. ABA-I shows moderate affinity for the Tn-hapten.

The previous studies involving hemagglutination inhibition assays indicated that the binding activity of BPA was inhibited most by 2-acetamido-2-deoxy-D-galactose among simple sugars, and more effectively by a synthetic glycosylamino acid β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc-(1→3)-*N*-tosyl-L-serine[8,9]. Thus, it was concluded in the previous studies that BPA mainly recognizes a 2-acetamido-2-deoxy-D-galactose residue in the disaccharide, β-D-Gal*p*-(1→3)-D-GalNAc. In the present study, however, the data obtained on lectin affinity chromatography indicated that BPA has a 12-fold higher K_a value for structure **2** than for structure **3**. Nevertheless, BPA showed stronger interaction with oligosaccharides containing a 2-acetamido-2-deoxy-D-galactopyranosyl group at their nonreducing ends, such as **5**, than ABA-I or PNA.

An important common characteristic of the three lectins in the first group is that their affinities for β-D-Gal*p*-(1→3)-D-GalNAcol are quite low. This indicates that the pyranose form of the 2-acetamido-2-deoxy-D-galactose residue in the glycopeptide is essential for the interaction with these lectins.

In the conventional lectin affinity chromatography, the elution of the applied substance from the column depends on the amount of lectin immobilized on the column. For example, **2** was eluted with 10mM D-galactose faster from the BPA column than for the PNA column (see Figs. 1g and h), although the data obtained on frontal affinity chromatography (see Fig. 4) clearly showed that the association constant of BPA for **2** was ~3-fold larger than that of PNA. This can be explained by the observation that the amount of PNA immobilized was 0.29 μmol whereas that of BPA was 59 nmol.

SBA and VVA-B_4, which have similar selective binding specificities to the glycosylamino acid α-D-Gal*p*NAc-(1→3)-Ser/Thr, showed, however, somewhat different reactivities with **3** (Tn-hapten) and **5**. VVA-B_4 showed a much higher affinity for **3** than for **5**. These results are in a good agreement with the data reported by Tollefsen and Kornfeld[13], *i.e.*, that VVA-B_4 shows a high specificity for the Tn antigenic structure. Of the sugar chains tested, SBA, however, interacted most with **5**. The presence of an α-(1→2)-linked L-fucosyl group in **4** seems to

interfere with its interaction with both SBA and VVA-B_4, as the association constants of SBA and VVA-B_4 for **5** were 14- and 28-fold larger, respectively, than those for **4**, as judged from the results obtained by frontal analysis (see Fig. 4). This confirmed the previous observation by Pereira and Kabat[12] that α-D-Gal*p*NAc-(1→3)-[α-L-Fuc*p*-(1→2)]-β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-hexenetriol was a less potent inhibitor of SBA-induced precipitation than its defucosylated derivative.

The data obtained by both conventional and frontal affinity chromatographies (shown in Figs. 1 and 4) demonstrated that glycopeptides having association constants of about 50μM or greater, were bound by lectin columns. This value, however, should be tentative, as it may vary depending on the amount of an active lectin immobilized on the column.

Recently, the carbohydrate-binding specificities of several lectins have been studied by use of lectin-affinity chromatography. This method is advantageous because of the rather simple procedure and the small amount of carbohydrate required. However, its main disadvantage seems to be the difficulty encountered in the quantitative comparison of different immobilized lectins in terms of their interactions with a carbohydrate sample. Therefore, frontal analysis was employed to overcome this and to compare quantitatively the carbohydrate-binding specificities of five lectins that preferentially bind to mucin-type sugar chains.

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