Interaction of asparagine-linked oligosaccharides with an immobilized rice (*Oryza sativa*) lectin column

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The carbohydrate-binding specificity of rice (*Oryza sativa*) lectin was investigated by testing the ability of radioactively labelled glycopeptides and oligosaccharides to bind to a rice lectin–Sephacrose 4B column. Rice lectin binds asparagine-linked oligosaccharides through the core *NN'*-diacetylchitobiose moiety. Whereas β 1-4-mannose enhances the binding strength only to a small extent, α 1-3-linked core mannose increases it considerably. A core α 1-6-linked mannose residue has a slightly inhibitory effect. Binding is not affected when one or both of the α -mannose residues are substituted with mannose at C-2, C-3 and C-6 or with *N*-acetylglucosamine (GlcNAc) at C-2 positions. The presence of an α 1-6-fucose residue attached to the asparagine-linked GlcNAc also does not affect the binding. The binding of complex biantennary glycopeptides is not altered by the presence of one or two galactose residues in the non-reducing terminus, but the presence of one or two sialic acid residues decreases the binding capacity. A bisecting β 1-4-linked GlcNAc attached to β -linked mannose residue enhances the binding of sialo, asialo and asialoagalacto complex biantennary-type glycopeptides. Bisected hybrid-type glycopeptides bind very tightly to a rice lectin–Sephacrose 4B column. Substitution of α 1-3-mannose residue at C-2 and C-4 with GlcNAc completely inhibits the binding of both bisected and non-bisected complex asparagine-linked glycopeptides. *O*-Glycosidically linked oligosaccharides containing GlcNAc bind very weakly to a rice lectin column. The applicability of immobilized rice lectin columns in the fractionation of asparagine-linked oligosaccharides is discussed.

INTRODUCTION

Lectin–Sephacrose columns have proved very useful in the separation and in structural studies of soluble and membrane-bound glycoconjugates [1–10].

Rice (*Oryza sativa*) seeds contain a GlcNAc-binding lectin. It was purified by affinity chromatography on *p*-aminobenzyl 2-acetamido-2-deoxy-1-thio- β -D-glucoside–succinylaminoethylaminyl–Sephacrose 4B. Rice lectin has an *M_r* of 36000 and is composed of four polypeptide chains of two non-identical forms [11]. It has two bound Ca^{2+} ions and lacks covalently bound carbohydrate. However, if the extraction and purification procedures are performed at acidic pH, a low-*M_r* (~12000) glycoprotein containing only arabinose and xylose co-purifies (about 1%) with rice lectin (I. Poola, unpublished work). In haemagglutination-inhibition studies, Poola *et al.* [11] have found that rice lectin binds GlcNAc, its derivatives and polymers, and, to a lesser extent, GalNAc. Though NeuAc was a non-inhibitor, NeuAc α 2-3Lac and NeuAc α 2-6Lac showed weak inhibition. Rice lectin has four carbohydrate-binding sites per molecule of protein, and thermodynamic studies of carbohydrate binding to the lectin showed that it has greater affinity for GlcNAc and its β 1-4-linked oligomers than has WGA [11–15].

Immobilized rice lectin bound Asn-linked glycoproteins only partially. Ovomucoid, which has several GlcNAc-terminated Asn-linked oligosaccharides [16],

was poorly bound to the column [11]. This finding prompted us to investigate in detail the structural determinants of rice-lectin specificity for Asn-linked oligosaccharides. We have studied the elution profiles of several Asn-linked oligosaccharides and found that the immobilized rice lectin binds Asn-linked oligosaccharides through the core Asn-linked *NN'*-diacetylchitobiose moiety, and core β 1-4- and α 1-3-linked mannose residues enhance the binding strength. The binding of Asn-linked oligosaccharides is also influenced by the outer sugar residues and their linkages.

EXPERIMENTAL PROCEDURES

Materials

Sephacrose-4B was from Pharmacia Fine Chemicals (Uppsala, Sweden). CNBr was from Eastman–Kodak (Rochester, NY, U.S.A.). GlcNAc was from Pfanstiehl (Waukegan, IL, U.S.A.). Bio-Gel P-2 (–400 mesh) was from Bio-Rad (Richmond, CA, U.S.A.). Jack-bean (*Canavalia ensiformis*) α -mannosidase was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Endo- β -*N*-acetylglucosaminidase H (from *Streptomyces plicatus*) was from ICN Immunobiologicals (Lisle, IL, U.S.A.). β -Mannosidase from human placenta was a gift from Dr. J. Clarke, Dr. R. Pearce, Dr. D. Mahuran and Dr. J. W. Callahan of the Research Institute, Hospital for Sick Children, Toronto, Canada. It was partially purified by Con A–Sephacrose chromatography, and other exo-

Abbreviations used: WGA, wheat-germ agglutinin; Con A, concanavalin A; E-PHA, erythroagglutinating phytohaemagglutinin; L-PHA, leucoagglutinating phytohaemagglutinin; DSA, *Datura stramonium* (thorn-apple) agglutinin; PBS, phosphate-buffered saline (50 mM-phosphate/0.15 M-NaCl, pH 7.2); Asn, asparagine; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid.

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Table 1. Structures of the glycopeptides (I–XIX) and oligosaccharides (XX and XXI) used in the present work

Glycopeptide	Structure	Glycopeptide/ oligosaccharide	Structure
I		XII	$\text{Man}\alpha 1\text{-}6\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$ $\text{Gal}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}2\text{Man}\alpha 1\text{-}3\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}6\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$
II		XIII	$\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$ $\text{Man}\alpha 1\text{-}3\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$
III		XIV	$\text{Man}\alpha 1\text{-}6\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$ $\text{Man}\alpha 1\text{-}3\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$
IV		XV	$\text{Man}\alpha 1\text{-}6\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$ $\text{Man}\alpha 1\text{-}3\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$
V		XVI	$\text{Man}\alpha 1\text{-}6\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$ $\text{Man}\alpha 1\text{-}3\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$
VI		XVII	$\text{Man}\alpha 1\text{-}6\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$ $\text{Man}\alpha 1\text{-}3\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$
VII		XVIII	$\text{Man}\alpha 1\text{-}6\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$ $\text{Man}\alpha 1\text{-}3\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$
VIII		XIX	$\text{Man}\alpha 1\text{-}6\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$ $\text{Man}\alpha 1\text{-}3\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$
IX		XX	$\text{Man}\alpha 1\text{-}6\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$ $\text{Man}\alpha 1\text{-}3\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$
X		XXI	$\text{Man}\alpha 1\text{-}6\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$ $\text{Man}\alpha 1\text{-}3\text{Man}\beta 1\text{-}4\text{GlcNAc}\beta 1\text{-}4\text{GlcNAc}\beta\text{-}1\text{Asn-N-}[\text{C}^{14}\text{C}]\text{-Ac}$

glycosidases were removed by passage through the affinity columns specific for β -hexosaminidase and β -galactosidase [17,18]. NaB^3H_4 (319 mCi/mmol) was from New England Nuclear (Boston, MA, U.S.A.). [^{14}C]GlcNAc was prepared by hydrolysis of UDP-*N*-acetyl[^{14}C]glucosamine (New England Nuclear) [19]. Rice lectin was prepared as described by Poola *et al.* [11].

Glycoproteins, glycopeptides and oligosaccharides

[*NeuAc*- ^3H]Glycophorin and alkaline-borohydride-treatment-resistant [^{14}C]dimethyl-fetuin, which was labelled by the reductive methylation with [^{14}C]formaldehyde [20], were gifts from Dr. V. P. Bhavanandan, Hershey Medical Center, Hershey, PA, U.S.A. The purified Asn-linked glycopeptides were obtained from various sources. They were *N*-[^{14}C]acetylated in the peptide moiety as described previously [21]. The structures of all the glycopeptides and oligosaccharides used in the present study are given in Table 1. Glycopeptide I was from Sigma Chemical Co. A mixture of glycopeptides II and III, which was prepared by the sequential exoglycosidase treatment of fibrinogen biantennary-complex-type glycopeptide, was provided by Dr. Brad Bendiak from Dr. H. Schachter's laboratory (Hospital for Sick Children, Toronto, Canada). Proton n.m.r. spectroscopy showed that glycopeptides II and III were present in the ratio of 3:7 (w/w). They were *N*-[^{14}C]acetylated and separated on a Bio-Gel P-2 (–400 mesh) column (1.3 cm $\times$ 113 cm) using 0.1 M-pyridine/acetate buffer, pH 5.2. Glycopeptides IV, VIII and XII, containing core fucose, were prepared from human IgG (patient Tem) as described [21]. Glycopeptide X was biosynthetically prepared from glycopeptide VIII by using rat liver Golgi membranes as a source of GlcNAc transferase II as described [22]. Glycopeptides XIII, XV and XVI with core fucose were from human myeloma IgG (Tem) and supplied by Ms. Jean Orr in Dr. H. Schachter's laboratory. Preparation of glycopeptide IX from human fibrinogen was described previously [23]. Bisected glycopeptide XVII with core fucose prepared from human myeloma IgG (patient Hom) was kindly given by Dr. J. P. Carver, Department of Medical Genetics, University of Toronto, Toronto, Canada. Preparation of glycopeptide XIV from glycopeptide XVII was described earlier [23]. Glycopeptide XIX from α_1 -acid glycoprotein was supplied by Dr. K. Schmid of the University of Boston, Boston, MA, U.S.A. [21]. Isolation of glycopeptide XI from ovalbumin has been reported [23]. A bisected triantennary glycopeptide (XVIII), which was isolated from ovalbumin, was supplied by Dr. S. D. Allen from Dr. H. Schachter's laboratory. High-mannose glycopeptide VI and a hybrid-type glycopeptide VII from ovalbumin were available from previous studies [24]. Glycopeptide V isolated from hen ovalbumin was the same preparation as that used previously [25] and was provided by Dr. H. Schachter. All the glycopeptides except I and XIX were characterized by proton n.m.r. spectroscopy. *O*-Glycosidically linked oligosaccharides XX and XXI were kindly given by Dr. Inka Brockhausen from Dr. H. Schachter's laboratory. An *O*-glycosidically linked oligosaccharide was prepared from [*NeuAc*- ^3H]glycophorin by alkaline borohydride treatment [26], isolated on a Bio-Gel P-2 column and used without further analysis. Per-*N*-acetylated chitobiose and chitotriose were isolated from chitin by the method of Rupley [27].

Labelling of oligosaccharides

Oligosaccharides were labelled with ^3H on the reducing terminal residue by reduction with NaB^3H_4 [28].

Glycosidase treatment

Glycopeptides III, IV and VIII were treated with jack-bean α -mannosidase (0.5 unit) in 0.1 ml of 25 mM-citrate buffer, pH 4.6, for 72 h at 37 °C. Glycopeptide V was treated with 5 munits of endo- β -*N*-acetylglucosaminidase H in 0.05 ml of 100 mM-citrate/phosphate buffer, pH 4.9, for 48 h at 37 °C. Glycopeptide II was digested with 1 unit of β -mannosidase (1 unit will hydrolyse 15 μmol of *p*-nitrophenyl β -D-mannoside to *p*-nitrophenol and D-mannose/h at pH 5.0 at 37 °C) for 20 h at 37 °C, in the presence of 0.5 M-GlcNAc to inhibit any residual hexosaminidase. All the enzyme treatments were conducted in a toluene atmosphere. The reactions were terminated by heating in a boiling-water bath for 3 min and reaction mixtures were deionized on Dowex 50 W-X8 (H^+ form) and Bio-Rad AG-1-X8 (OH^- form) columns. Removal of the sugar residues after α - and β -mannosidase treatments was shown by the increased elution volume on Bio-Gel P-2 (–400 mesh) column.

Preparation of a rice lectin–Sephacrose 4B column

A rice lectin–Sephacrose 4B column was prepared as described previously [11] and by the method of March *et al.* [29], except that the Sepharose 4B (4 ml) was activated with CNBr in the cold and the coupling reaction was conducted in the presence of 15 μmol of *NN'*-diacetylchitobiose and 10 μmol of *NN'N''*-triacetylchitotriose in addition to 1 mmol of GlcNAc. The amount of rice lectin bound to Sepharose 4B was estimated to be approx. 5.5 mg/ml of settled gel by subtraction of the amount of unbound protein in the supernatant and the wash after the coupling reaction. Protein was determined by the method of Lowry *et al.* [30]. The coupled gel was packed into a column (0.5 cm $\times$ 20 cm) and washed with 50 ml of 0.5 M-GlcNAc in PBS to elute the *NN'*-diacetylchitobiose and *NN'N''*-triacetylchitotriose, which were used to protect the rice lectin during coupling reaction. Finally the column was tested for binding affinity by applying [*NeuAc*- ^3H]glycophorin [11].

Affinity chromatography

N-[^{14}C]Acetylated Asn-linked oligosaccharides, ^3H -labelled oligosaccharides, and ^{14}C -labelled GlcNAc oligosaccharides [(1–30) $\times 10^3$ c.p.m.] dissolved in 100 μl of PBS were applied to rice lectin–Sephacrose 4B column previously equilibrated with PBS, and allowed to interact for 30–45 min. The column was eluted with the same buffer (12 ml), followed by 0.2 M-GlcNAc (22 ml) in PBS, except where indicated. The column was eluted with 0.5 M-GlcNAc following the 0.2 M-sugar elution wherever necessary. All the binding experiments were conducted at 4 °C. Fractions (1 ml) were collected at a flow rate of 5 ml/h. Radioactivity in each fraction was determined by liquid-scintillation spectrometry using Aqueous Counting Scintillant (Amersham, Arlington Heights, IL, U.S.A.). The entire fraction was counted for radioactivity. Recoveries of glycopeptides from the column based on radioactivity were more than 85%. The column did not show any change in the binding properties, even after repeated use for at least 20 months when stored at 4 °C in PBS containing 0.04% NaN_3 .

Fig. 1. Chromatography of GlcNAc oligomers and glycopeptides

Conditions were as described in the Experimental procedures section. (a) Elution of *NN'*-diacetylchitobiose alditol (total radioactivity applied 29000 c.p.m.); (b) elution of *NN'N''*-triacylchitotriose alditol (total radioactivity applied 10000 c.p.m.); (c) glycopeptide I (total radioactivity applied 2300 c.p.m.); (d) elution of *NN'*-diacetylchitobiosyl-Asn (total radioactivity applied 1400 c.p.m.); (e) elution of glycopeptide II (total radioactivity applied 5100 c.p.m.). The arrow indicates the point of addition of 0.2 M-GlcNAc.

RESULTS

Binding of GlcNAc oligosaccharides and glycopeptides

The binding characteristics of GlcNAc oligomers on the rice-lectin column are shown in Figs. 1(a) and 1(b). *NN'*-Diacetylchitobiose alditol was eluted as a retarded peak with PBS itself (Fig. 1a). *NN'N''*-Triacylchitotriose alditol bound to the column and was eluted as a sharp peak when 0.2 M-GlcNAc was applied (Fig. 1b). This showed that the affinity of chitin oligosaccharide alditols for rice lectin increased with the increasing number of GlcNAc residues. [14 C]GlcNAc was eluted from the column without any retardation (results not shown), but a glycopeptide, GlcNAc- β -Asn, showed a very slight retardation (Fig. 1c). This observation indicates the contribution of *N*-glycosidic linkage to the binding. GlcNAc β 1-4GlcNAc β -Asn, which was isolated by the β -mannosidase digestion of glycopeptide II, was bound to the column and could not be eluted even with 40 ml of PBS. It emerged as a sharp peak when 0.2 M-GlcNAc was applied (Fig. 1d). We obtained a similar result after treating glycopeptide IV with α - and β -mannosidases. These observations show that *NN'*-diacetylchitobiosyl-Asn is the minimum structure required for binding to the rice-lectin column.

Binding of mannose-type glycopeptides

Glycopeptide II, which has only core β 1-4-linked mannose residues, bound to the column and was eluted with 0.2 M-GlcNAc (Fig. 1e). We obtained a similar profile with a glycopeptide prepared by the α -mannosidase digestion of either glycopeptide III or IV. The increased elution volume compared with *NN'*-diacetylchitobiosyl-Asn suggests that β 1-4-linked mannose participates in the binding and increases the binding strength. We examined the contribution of core α 1-3-linked mannose by applying glycopeptide III. It bound to the column and could be eluted only with 0.5 M-GlcNAc (Fig. 2a). We obtained a similar profile with a glycopeptide prepared by the α -mannosidase digestion of glycopeptide VIII. The 'delayed' elution profile shows that the core α 1-3-linked mannose greatly enhances the binding strength and is a very important determinant in the high-affinity binding of Asn-linked oligosaccharides to the rice-lectin column. Glycopeptide IV, which has all the core mannose residues, bound to the column and was eluted with 0.5 M-GlcNAc (Fig. 2b) at an earlier point than glycopeptide III, suggesting that a core α 1-6-mannose residue decreases the binding strength. High-mannose glycopeptides V and VI showed elution profiles similar to those of glycopeptide IV. However, an oligosaccharide isolated by the endo- β -*N*-acetylglucosaminidase H digestion of glycopeptide V and labelled by reducing with NaB 3 H $_4$ did not show any interaction (results not shown), indicating an absolute requirement for *NN'*-diacetylchitobiose moiety for high-affinity binding of Asn-linked oligosaccharides. The above results also show that substitution of α -mannose residues at C-2, C-3 and C-6 with mannose, and an α 1-6-fucose attached to the GlcNAc linked to Asn, do not alter the binding affinity of Asn-linked glycopeptides to the rice-lectin column.

Binding characteristics of complex bisected and non-bisected biantennary-type glycopeptides

The binding of various biantennary-type glycopeptides is shown in Figs. 2(c) and 2(d) and in Figs. 3(a), 3(b) and

Fig. 2. Chromatography of N - $[^{14}\text{C}]$ acetylated mannose-type and complex non-bisected and bisected biantennary-type glycopeptides on a rice lectin-Sepharose 4B column

Conditions were as described in the Experimental procedures section. (a) Elution of glycopeptide III (total radioactivity applied 4000 c.p.m.); (b) elution of glycopeptide IV (3300 c.p.m.); (c) asialo biantennary glycopeptide with core fucose (XIII) (2130 c.p.m.); (d) asialo biantennary bisected glycopeptide (XIV) (total radioactivity applied was 1000 c.p.m.; in this case the total background radioactivity recovered was approx. 2000 c.p.m.). The two arrows labelled 1 and 2 indicate the points of addition of 0.2 M-GlcNAc and 0.5 M-GlcNAc respectively.

3(c). They show four different types of elution profiles. Asialo and asialoagalacto complex biantennary glycopeptides were bound to the column and their elution could be achieved only with 0.5 M-GlcNAc, as in the case

Fig. 3. Chromatography of N - $[^{14}\text{C}]$ acetylated complex biantennary bisected and non-bisected sialoglycopeptides on a rice lectin-Sepharose 4B column

Conditions were as described in the Experimental procedures section. (a) Biantennary glycopeptide having one sialic acid residue in the non-reducing terminal (XV) (the total radioactivity applied was 2300 c.p.m.); (b) biantennary glycopeptide terminating with two sialic acid residues (XVI) (1400 c.p.m.); (c) bisected biantennary-type glycopeptide having two sialic acid residues in the non-reducing end (XVII) (1700 c.p.m.). The arrow indicates the point of addition of 0.2 M-GlcNAc.

of mannose-type glycopeptides. The elution profile of asialo complex biantennary glycopeptide (XIII) is shown in Fig. 2(c). Glycopeptides VIII, IX, X and XII showed similar elution profiles. These results show that substitution of one or both the α -mannose residues at C-2 positions with GlcNAc and the presence of one or two galactose residues in the non-reducing terminal positions do not alter the binding. These observations also reveal that the β 1-2-linked outer GlcNAc residues do not contribute to the binding. To examine the effect of sialic acid residues in the terminal non-reducing positions, we

Fig. 4. Chromatography of *N*-[^{14}C]acetylated complex triantennary-type glycopeptide (XIX) on a rice lectin-Sepharose 4B column

Conditions were as described in the Experimental procedures section. The total radioactivity applied was 2000 c.p.m. The arrow indicates the point of application of 0.2 M-GlcNAc.

applied glycopeptides XV and XVI. The elution profiles of the above are shown in Figs. 3(a) and 3(b). They bound to the column and were readily eluted with 0.2 M-GlcNAc, showing that one or two α 2-6-linked sialic acid residues in the non-reducing end decrease the binding affinity of complex biantennary-type glycopeptides. We also examined the effect of a bisecting GlcNAc β 1-4-linked to β -mannose residue on the binding affinity of complex biantennary-type glycopeptides to the rice-lectin column. The elution profiles of glycopeptides which have a bisecting GlcNAc (XIV and XVII) are shown in Figs. 2(d) and 3(c) respectively. The complex biantennary sialoglycopeptide with a bisecting GlcNAc (XVII) bound to the column and was eluted with 0.2 M-sugar as a broad extended peak (Fig. 3c). This shows that the binding of complex biantennary sialoglycopeptides is stronger in the presence of a bisecting GlcNAc than those which are not bisected. The asialo and asialoagalacto complex biantennary bisected structures (XIV, XI) bound to the column and were eluted with 0.5 M-GlcNAc as broad extended peaks. The elution of glycopeptide XIV is shown in Fig. 2(d). These results also show that the presence of a bisecting GlcNAc residue increases the binding strength of complex biantennary type glycopeptides to rice-lectin column. A hybrid-type glycopeptide (VII) which has a bisecting GlcNAc was also eluted as a broad extended peak with 0.5 M-GlcNAc.

Binding of complex bisected and non-bisected triantennary-type glycopeptides

We examined the characteristics of asialo (XIX) triantennary complex-type glycopeptide binding to the rice-lectin column. The elution profile is shown in Fig. 4. It does not bind to the column and is eluted from the

Fig. 5. Chromatography of *O*-glycosidically linked oligosaccharides on a rice lectin-Sepharose 4B column

Conditions were as described in the Experimental procedures section. (a) Oligosaccharide XX (total radioactivity applied 1800 c.p.m.); (b) oligosaccharide XXI (800 c.p.m.).

column without retardation, showing that the substitution of α 1-3-linked mannose with GlcNAc residues at C-2 and C-4 positions completely prevents the interaction of Asn-linked oligosaccharides. Alkaline-borohydride-resistant [^{14}C]dimethyl-fetuin, which has Asn-linked sialylated triantennary complex-type oligosaccharides, was also eluted from rice lectin-Sepharose 4B column without retardation (results not shown). We found a similar profile with complex bisected triantennary glycopeptide (XVIII) terminating in GlcNAc (results not shown).

Binding of *O*-glycosidically linked oligosaccharides

We tested the *O*-glycosidically linked oligosaccharides which contain GlcNAc in their structure (XX and XXI) for binding to the rice-lectin column. Although both were eluted in a retarded manner by just PBS, oligosaccharide XXI was more retarded than XX. Their elution profiles are shown in Figs. 5(a) and 5(b). These observations show that the rice-lectin carbohydrate-binding site recognizes β 1-3- and β 1-6-linked GlcNAc residues. However, an '*O*'-linked oligosaccharide isolated by the alkaline-borohydride treatment of [*NeuAc*- ^3H]glycophorin did not bind to the column and was eluted without retardation (results not shown),

suggesting that clustered oligosaccharides of the above type may be necessary for binding, as previously postulated [11].

DISCUSSION

The experimental results presented here indicate that the rice-lectin column exhibits high affinity and complexity in binding Asn-linked oligosaccharides. Our data indicate that *NN'*-diacetylchitobiosyl-Asn is the minimum structure required for binding to the rice-lectin column, in contrast with WGA column, which required the presence of a bisecting GlcNAc attached to β 1-4-linked mannose in addition to a core *NN'*-diacetylchitobiose moiety for binding [31]. The binding strength of Asn-linked oligosaccharides is not altered when one or both the core α -mannose residues are substituted with mannose at C-2, C-3 and C-6 or with GlcNAc at C-2 positions or by terminal galactose residues. One or two sialic acid residues in the terminal non-reducing positions decrease the binding strength of Asn-linked oligosaccharides, probably by interfering, by steric or charge-charge interactions, with the 'docking' of the glycopeptide at the binding site. A bisecting GlcNAc β 1-4-linked to a β -mannose residue considerably enhances the binding strength of sialo, asialo, asialoagalacto complex biantennary- and hybrid-type glycopeptides. This may be due to an extended binding site, which accommodates the β 1-4-linked bisecting GlcNAc and permits stronger binding.

Complex triantennary-type Asn-linked oligosaccharides which are substituted with GlcNAc at C-2 and C-4 of the α 1-3-linked mannose do not interact with the rice-lectin column and are eluted without any retardation. In this property rice lectin is similar to Con A [21], pea and lentil-lectin [1] and L-PHA columns [3]. The presence of a bisecting GlcNAc also does not enable the triantennary oligosaccharides with C-2 and C-4 branched outer sugars to interact with the rice-lectin column, suggesting the importance of C-4 unsubstituted α 1-3-mannose for high-affinity binding of Asn-linked oligosaccharides. The WGA column does not seem to be affected by the C-2 and C-4 substituted α 1-3-linked mannose, as it binds bisected hybrids having such linkages [31]. These results explain our previous observations [11] of the poor binding to the rice-lectin column of ovomucoid, which has several GlcNAc-terminated oligosaccharide moieties in which α 1-3-mannose is substituted at C-2 and C-4 positions with GlcNAc residues [16].

It would have been very interesting to examine the triantennary complex-type oligosaccharides, which have α 1-6-linked mannose substituted at C-2 and C-6 positions with GlcNAc, and the tetra-antennary type, for their binding characteristics on the rice-lectin column; unfortunately they were not available to us and were not tested. Our limited studies on *O*-glycosidically linked oligosaccharides have shown that those which have GlcNAc interact weakly with the rice-lectin column. If the binding studies are conducted with various GlcNAc-containing *O*-linked oligosaccharides, it may prove useful to fractionate them.

The complex binding affinity of the rice-lectin column towards Asn-linked oligosaccharides could be exploited for the identification and purification of complex biantennary- and hybrid-type glycopeptides in combination with Con A-Sepharose columns. It has been well

established by several workers that Con A-Sepharose columns fractionate Asn-linked oligosaccharides into three classes [2,21,32]: the unbound, weakly bound (which could be displaced with 0.01 M- α -methyl glucoside) and the tightly bound (eluted with 0.5 M- α -methyl mannoside or with 0.1 M- α -methyl mannoside at 60 °C). The unbound fraction consists of tri- and tetra-antennary and bisected biantennary sialo and asialo types [2,21,33,34], the weakly bound fraction consists of non-bisected biantennary types [35,36] and the tightly bound contains high-mannose and certain hybrid types. The Con A-column-unbound bisected biantennary class could be identified and fractionated on a rice-lectin column into sialo and asialo types (see Fig. 3c and Fig. 2d). An E-PHA column is currently employed for the identification of the Con A-column-unbound biantennary bisected class [2]. However, application of this column is limited, owing to its very narrow carbohydrate binding specificity [37]. Although WGA has been shown to be a specific lectin for identifying Asn-linked oligosaccharides which have a bisecting GlcNAc [31], there are very few reports in the literature which have exploited the use of an immobilized-WGA column to fractionate biantennary Asn-linked glycopeptides on the basis of a bisecting GlcNAc. In our experience two commercial WGA-column preparations (E.Y. Laboratories, San Mateo, CA, U.S.A., and Pharmacia Fine Chemicals, Uppsala, Sweden), and also a WGA column prepared by ourselves, failed to show high-affinity binding for either sialo or asialo bisected biantennary Asn-linked glycopeptides. Hence, as an alternative to E-PHA or WGA, we recommend the rice-lectin column for the identification and fractionation of the Con A-column-unbound bisected biantennary class. The Con A-column-weakly-bound non-bisected biantennary class could be further fractionated on the rice-lectin column into sialo and asialo biantennary types (see Figs. 3b and 2c). The rice-lectin column can also be applied to separate the Con A-tightly-bound fraction into at least four peaks. These peaks correspond to non-bisected sialo hybrids (see Fig. 3b), non-bisected asialo hybrids (e.g. swainsonine-induced hybrids in baby-hamster kidney cells [38]) or high-mannose (Fig. 2c), bisected sialo hybrids (Fig. 3c) and bisected asialo hybrids (see the profile of Fig. 2d).

The high affinity of the rice-lectin column for a bisecting GlcNAc also renders it the best choice of column for the identification and characterization of *N*-acetylglucosaminyltransferase III products. Narasimhan *et al.* [39] have preliminarily characterized the product of rat hepatic nodule *N*-acetylglucosaminyltransferase III activity by using glycopeptide IX (Table 1) as substrate on the rice-lectin column. The product gave an extended profile similar to that of Fig. 2(d), indicating the presence of a bisecting GlcNAc. The 500 MHz proton n.m.r. spectrum has indeed indicated the presence of such a bisecting GlcNAc.

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REFERENCES

1. Kornfeld, K., Reitman, M. L. & Kornfeld, R. (1981) *J. Biol. Chem.* **256**, 6633–6640
2. Cummings, R. D. & Kornfeld, S. (1982) *J. Biol. Chem.* **257**, 11235–11240
3. Cummings, R. D. & Kornfeld, S. (1982) *J. Biol. Chem.* **257**, 11230–11234
4. Blake, D. A. & Goldstein, I. J. (1980) *Anal. Biochem.* **102**, 103–109
5. Cummings, R. D. & Kornfeld, S. (1984) *J. Biol. Chem.* **259**, 6253–6260
6. Eckhardt, A. & Goldstein, I. J. (1983) *Biochemistry* **22**, 5290–5297
7. Yamashita, K., Kochibe, N., Ohkura, T., Ueda, I. & Kobata, A. (1985) *J. Biol. Chem.* **260**, 4688–4693
8. Edge, A. S. B. & Spiro, R. G. (1985) *J. Biol. Chem.* **260**, 15332–15338
9. Iturbe, S., Narasimhan, S., Merrick, J. M., Falk, J. A. & Letarte, M. (1986) *J. Immunol.* **136**, 4588–4595
10. Iturbe, S., Narasimhan, S. & Letarte, M. (1986) *J. Immunol.* **136**, 4596–4603
11. Poola, I., Seshadri, H. S. & Bhavanandan, V. P. (1986) *Carbohydr. Res.* **146**, 205–217
12. Poola, I. & Seshadri, H. S. (1979) *Indian J. Biochem. Biophys.* **16** (Suppl.), Abstr. 18
13. Poola, I. & Seshadri, H. S. (1980) *J. Biosci.* **2**, 29–36
14. Poola, I. & Seshadri, H. S. (1984) *Indian J. Biochem. Biophys.* **21**, 137–138
15. Poola, I. & Kella, N. K. D. (1986) *Biochim. Biophys. Acta* **882**, 12–17
16. Yamashita, K., Kamerling, J. P. & Kobata, A. (1983) *J. Biol. Chem.* **258**, 3099–3106
17. Mahuran, D. & Lowden, J. A. (1980) *Can. J. Biochem.* **58**, 287–294
18. Jones, C. S., Mahuran, D., Lowden, J. A. & Callahan, J. W. (1984) *Can. J. Biochem.* **62**, 529–534
19. Cabib, E., Leloir, L. F., Cardini, C. E. (1953) *J. Biol. Chem.* **203**, 1055–1070
20. Jentoft, N. & Dearborn, D. (1979) *J. Biol. Chem.* **254**, 4359–4365
21. Narasimhan, S., Wilson, J. R., Martin, E. & Schachter, H. (1979) *Can. J. Biochem.* **57**, 83–96
22. Narasimhan, S. (1982) *J. Biol. Chem.* **257**, 10235–10242
23. Narasimhan, S., Freed, J. C. & Schachter, H. (1985) *Biochemistry* **24**, 1694–1700
24. Narasimhan, S., Harpaz, N., Longmore, G., Carver, J. P., Grey, A. A. & Schachter, H. (1980) *J. Biol. Chem.* **255**, 4876–4884
25. Vella, G. J., Paulsen, H. & Schachter, H. (1984) *Can. J. Biochem. Cell Biol.* **62**, 409–417
26. Spiro, R. G. & Bhoyroo, V. D. (1974) *J. Biol. Chem.* **249**, 5704–5717
27. Rupley, J. A. (1964) *Biochim. Biophys. Acta* **83**, 245–255
28. Takasaki, S. & Kobata, A. (1978) *Methods Enzymol.* **50**, 50–54
29. March, S. C., Parikh, I. & Cautrecasas, P. (1974) *Anal. Biochem.* **60**, 149–152
30. Lowry, O. H., Rosebrough, N. H., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
31. Yamamoto, K., Tsuji, T., Matsumoto, I. & Osawa, T. (1981) *Biochemistry* **20**, 5894–5899
32. Krusius, T., Finne, J. & Rauvala, H. (1976) *FEBS Lett.* **71**, 117–120
33. Savvidou, G., Klein, M., Grey, A. A., Dorrington, K. J. & Carver, J. P. (1984) *Biochemistry* **23**, 3736–3740
34. Narasimhan, S., Freed, J. C. & Schachter, H. (1986) *Carbohydr. Res.* **149**, 65–83
35. Baenziger, J. U. & Fiete, D. (1979) *J. Biol. Chem.* **254**, 2400–2407
36. Goldberg, D. E. & Kornfeld, S. (1981) *J. Biol. Chem.* **256**, 13060–13067
37. Yamashita, K., Hitoi, A. & Kobata, A. (1983) *J. Biol. Chem.* **258**, 14753–14755
38. Foddy, L., Feeney, J. & Hughes, R. C. (1986) *Biochem. J.* **233**, 697–706
39. Narasimhan, S., Pascale, R., Schachter, H. & Rajalakshmi, S. (1987) *Int. Symp. Glycoconjugates 9th, Lille, France*, E-36

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