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Review

Fractionation of glycoprotein-derived oligosaccharides by affinity chromatography using immobilized lectin columns

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Abstract

Lectin affinity column chromatography is becoming a method of choice for the fractionation and purification of oligosaccharides, especially N-linked oligosaccharides. Using lectin affinity, it is easy to separate structural isomers and to isolate oligosaccharides based on specific features. Further, serial lectin column chromatography, when various lectin columns are used at the same time, can afford a very sensitive method for the fractionation and characterization of extremely small amounts of oligosaccharides. Thus, when used in conjunction with other separation techniques, lectin affinity chromatography can help to purify rapidly oligosaccharides and provide substantial information about their structural features.

Contents

1. Introduction

Within the last few years, tremendous advances have been made in our understanding of glycobiology. Among the explosive growth of research in the field of carbohydrates, glycobiology is defined as comprehension of the structure and function of the glycoconjugates. A prerequisite for the study of the roles of sugar chains in biological interactions, cell–cell and cell–matrix adhesions, binding and uptake of extracellular glycoproteins and intracellular targeting of macromolecules to organelles is that their structural properties are known [1–3]. The excite-

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ment in this area of research has improved many useful techniques for analysing carbohydrates, as reviewed in this special issue.

Recently, the use of lectin immobilized column chromatography not only for separating and determining structure of glycoprotein-derived oligosaccharides and glycopeptides but also for the purification of glycoproteins and the detection of glycoproteins with pathologically altered sugar chains has been evaluated. Among them, this paper will focus only on recent progress in lectin chromatography as an analytical device for oligosaccharides owing to the purpose of this special issue. In contrast to other fractionation techniques such as gel permeation, ion-exchange, partition, reversed-phase and normal-phase chromatography, lectin column chromatography is basically different because the principle of its fractionation is based on the carbohydrate-binding specificities of lectins. Although the method can be used to fractionate both N- and O-linked oligosaccharides of glycoproteins, it is especially useful for the study of N-linked oligosaccharides because they obey structural rules which are effective in their group fractionation. Further, serial lectin column chromatography can afford a very sensitive method for the fractionation and characterization of extremely small amounts of N-linked oligosaccharides found in glycoproteins [4].

Because so many of immobilized lectins are currently available [5], a complete description of all of them is beyond the scope of this paper, which therefore deals with useful lectins which are commercially available and are effective for the fractionation and structural study of glycoprotein-derived oligosaccharides.

2. Preparation of immobilized lectins

Purified lectins should be immobilized to insoluble absorbents for use in lectin column chromatography. Generally the amino groups of lectins are used for this purpose. The most widely used method is the cyanogen bromide (CNBr) method [6]. Immobilization of lectin is carried out by reaction between the amino groups of lectins and the imidocarbonate residue of CNBr-activated Sepharose. Since the coupling reaction occurs at random between the amino group and the imidocarbonate residue, a haptenic sugar should be added to the reaction mixture to protect the carbohydrate binding site of lectins. Reaction between the activated (N-hydrosuccinimide) esters and the amino groups of lectins can also lead to immobilized lectin gels with chemically stable acid–amino bonds [7]. Finally, Schiff base formation between formyl residues and the amino groups of a lectin followed by reduction with sodium cyanoborohydride to form an alkylamine is used for another coupling method [8].

3. Carbohydrate-binding specificities of various immobilized lectins

3.1. *Aleuria aurantia lectin (AAL)*

AAL was found by Kochibe and Furukawa [9] as a mushroom lectin which agglutinates human erythrocytes irrespective of their ABO and Lewis blood types. Recombinant AAL, produced by *E. coli*, is now commercially available (Seikagaku) and shows the same biochemical characteristics and sugar-binding specificity as natural AAL [10]. Complex-type oligosaccharides can be classified by the presence or absence of an α-fucosyl residue linked at the C-6 position of the proximal N-acetylglucosamine residue of the trimannosyl core. The radioactive fucosylated oligosaccharides can be easily separated from their non-fucosylated counterparts by passing through an AAL-Sepharose column (10 mg lectin/ml gel, 1-ml bed volume, 7.5 mm I.D.) [10–12]. The operating procedure of lectin chromatography will be briefly described here. Radioactive oligosaccharides (10^2–10^6 cpm) dissolved in 100 μl of buffer are mounted on the column. After standing for 30 min, chromatography is performed at room temperature (20°C), unless stated otherwise, at a flow-rate of 12 ml/h. Fractions (1 ml) are collected and the radioactivity in each fraction is determined by a liquid scintillation method. Oligosaccharides

with a non-fucosylated core pass through the column without any interaction and are eluted with 10 ml of 10 m*M* Tris buffer (pH 7.4). In contrast, oligosaccharides with a fucosylated core bind to the column and are eluted with 6 ml of 10 m*M* Tris buffer (pH 7.4) containing 1 m*M* L-fucose (Table 1). Binding was not affected by either the structures of the outer-chain moieties or the presence of a bisecting N-acetylglucosamine residue (GlcNAc residue linked to β-mannose, see Table 3). The Fucα1→2Galβ1→4GlcNAc, the Galβ1→4(Fucα1→3)GlcNAc and the Galβ1→3(Fucα1→4)-GlcNAc groups interact weakly with an AAL column, as will be described later. However, oligosaccharides with more than two of these groups bind more strongly than those with only one of the groups. Therefore, care must be taken in interpreting the results of affinity chromatography with regard to the structural determination of oligosaccharides.

3.2. *Concanavalin A (Con A)*

Study of the behaviour of various oligosaccharides in a Con A-Sepharose column revealed that at least two non-substituted or C-2-substituted α-mannosyl residues must be present in a sugar chain to be retained on the immobilized lectin column [13]. This finding was particularly useful for the structural analysis of N-linked oligosaccharides. Since the biantennary complex-type oligosaccharide has two α-mannosyl residues (see Table 3), it binds to the Con A-Sepharose column (10–16 mg lectin/ml gel, 1 ml; Pharmacia Biotech) and is eluted with 10 ml of 5 m*M* α-methylglucopyranoside in Tris-buffered saline containing 10 m*M* Tris (pH 7.4), 0.15 *M* NaCl and 1 m*M* each $CaCl_2$, $MgCl_2$ and $MnCl_2$ (Table 1). However, oligosaccharides with more than three outer chains totally abolish the interaction with the lectin column and are eluted with 10 ml of the same buffer without α-methylglucopyranoside. On the other hand, high mannose-type and hybrid-type oligosaccharides, which have many Manα1→ and Manα1→2 residues, can bind more strongly to the column than biantennary complex-type oligosaccharides and are eluted with 10 ml of 200 m*M* α-methylmannopyranoside in Tris-buffered saline containing 10 m*M* Tris (pH 7.4), 0.15 *M* NaCl and 1 m*M* each $CaCl_2$, $MgCl_2$ and $MnCl_2$ instead of 5 m*M* α-methylglucopyranoside (Table 1). It should be noted that presence of bisecting GlcNAc markedly changes the conformation of the trimannosyl core and weakens the interaction with the Con A column [14]. Further, the presence of the Fucα1→3 residue on the GlcNAc residue of the Galβ1→4GlcNAc group in the outer chain moieties interferes with the binding of oligosaccharides to the Con A column owing to steric hindrance [15].

3.3. *Phytohaemagglutinin-E_4 (E_4-PHA)*

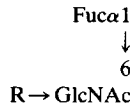
Red kidney bean (*Phaseolus vulgaris*) contains five tetrameric isolectins comprising E and/or L subunits [16]. L_4 (L_4-PHA) binds with high affinity to lymphocytes and induces blastgenesis in these cells. In contrast, E_4 (E_4-PHA) specifically agglutinates erythrocytes, but does not show any blastgenic activity. It is already known that several complex-type oligosaccharides contain a bisecting GlcNAc residue. Irimura et al. [17] reported that bisected biantennary complex-type oligosaccharides with Galβ1→4GlcNAcβ1→ outer chains bind to an E_4-PHA-Sepharose column. Cummings and Kornfeld [18] confirmed this evidence and indicated that none of the non-bisected oligosaccharides can bind to the column. In order to elucidate the precise binding specificity of the lectin column, Yamashita et al. [19] examined the behaviours of various oligosaccharides with bisecting GlcNAc in an E_4-PHA-Sepharose column (5 mg lectin/ml gel, 1 ml; Seikagaku) at room temperature (20°C), and concluded that the minimum structural unit required for binding to the column is as shown in Table 1.

Further, they investigated the behaviours of many oligosaccharides with or without bisecting GlcNAc residue in the lectin column at 4°C. Interestingly, the behaviour of oligosaccharides in the column was different to that at 20°C [20]. Based on these data, it can be concluded that the minimum structural unit required for the binding

Table 1
Structures of the N-linked oligosaccharides which interact with immobilized lectin columns

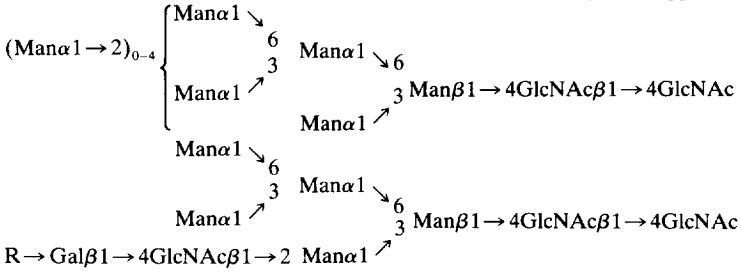
Aleuria aurantia lectin (AAL)

Strong binding oligosaccharides eluted with 1 mM fucose

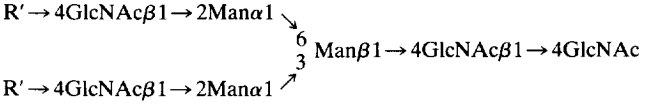


Concanavalin A (Con A)

Strong binding oligosaccharides eluted with 200 m*M* α-methylmannopyranoside

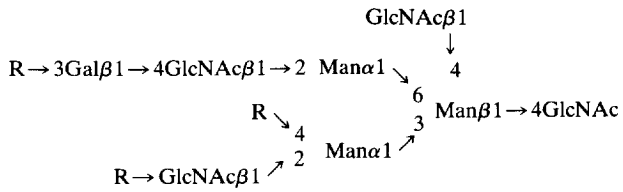


Weak binding oligosaccharides eluted with 5 m*M* α-methylglucopyranoside

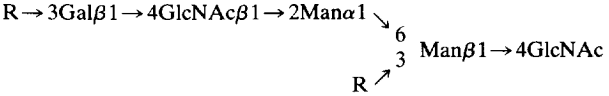


Phytohaemagglutinin E_4 (E_4-PHA)

At 20°C

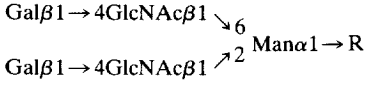


At 4°C

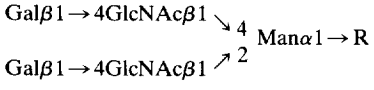


Datura stramonium agglutinin (DSA)

Strong binding oligosaccharides eluted with 1% N-acetylglucosamine oligomers



Retarded oligosaccharides



R = H or sugar; R′ = sugar, in the case of H, oligosaccharides show strong binding and are eluted with 200 m*M* α-methymannopyranoside instead of 5 m*M* α-methyglucopyranoside.

to the column is the pentasaccharide as shown in Table 1. This pentasaccharide passes through at 20°C but is retarded at 4°C in the column [20]. In other words, the requirement for the GlcNAcβ1→2Manα1→3 group, as revealed in the chromatography at 20°C, is lost at 4°C. The results indicated that bisecting GlcNAc changes the steric arrangement of the two outer-chain moieties to fit better in the binding site of an E_4-PHA column. In any case, more effective fractionation of N-linked oligosaccharides can be obtained by performing affinity chromatography at both temperatures.

3.4. *Datura stramonium agglutinin (DSA)*

A DSA-Sepharose column (3 mg lectin/ml gel, 1 ml; Wako) interacts with oligosaccharides containing two N-acetyllactosamine groups with special sequences as shown in Table 1 [21,22]. Oligosaccharides that contain unsubstituted Galβ1→4GlcNAcβ1→6(Galβ1→4GlcNAcβ1→2)Man bind to the lectin column and are eluted with 1% of N-acetylglucosamine oligomers in 10 m*M* Tris-buffered saline (pH 7.4). In contrast, oligosaccharides that contain the Galβ1→4GlcNAcβ1→4(Galβ1→4GlcNAcβ1→2)Man group are retarded at 4°C by the column. This binding characteristic was not affected by either the structure of the inner core portion or the presence of bisecting GlcNAc residue. It should be noted that substitution of the C-3 of the β-galactose residue of the N-acetyllactosamine group with an α-galactose residue (Galα1→3Galβ1→4GlcNAcβ1→) has no effect on the affinity of oligosaccharides to the lectin column, but substitution of the C-3 of the β-N-acetylglucosamine residue of the N-acetyllactosamine group with an α-fucose residue [Galβ1→4(Fucα1→3)GlcNAcβ1→] interferes with the interaction of oligosaccharides with the lectin. These oligosaccharide-binding specificities enable us to separate triantennary oligosaccharides with a C-2,4 branch from tetra- and triantennary oligosaccharides with a C-2,6 branch, as described later.

3.5. *Lectins with specificities directed to the peripheral portions of outer chains*

It is well known that various structures are found in the outer-chain moieties of oligosaccharides derived from glycoproteins, and that they play an important role as the signal of recognition, for example, the clearance of glycoproteins in the bloodstream, intracellular sorting of glycoproteins to lysosomes and leucocyte adhesion to endotherial cells and platelets [1–3]. Accordingly, it is of the utmost importance to obtain structural information on the outer-chain moieties, and affinity chromatography using immobilized lectin columns is useful for this purpose. In Table 2, some lectins are shown and the types of structures with which they interact are listed.

Ricinus communis agglutinin I (RCA-I; Seikagaku) binds basically to terminal β-galactose residues and strongly to oligosaccharides containing a terminal Galβ1→4GlcNAcβ1→ sequence [23,24]. *Allomyrina dichotoma* lectin-II (Allo A-II; EY Laboratories) also interacts with the N-acetyllactosamine group. Interestingly, substitution of the C-6 position of the terminal galactose with acidic residues such as sialic acid or sulfuric acid enhances its affinity [25,26]. *Sambuccus nigra* agglutinin (SNA; EY Laboratories) requires the presence of a sialic acid residue at the C-6 position of the terminal β-galactose or β-N-acetylgalactosamine residue for binding [27]. It should be noted that NeuAcα2→6GalNAc binds to an SNA column but not to an Allo A-II column. On the other hand, *Macckia amurensis* lectin (MAL; Seikagaku) binds to sialylated oligosaccharides containing the terminal sequence NeuAcα2→3Galβ1→4GlcNAc structure but it does not bind to isomers in which sialic acid is linked α2→6 to galactosyl residues [28]. By using the three sialic acid-binding lectin columns with different specificities along with the result of sialidase digestion, the sialic acid linkage of the oligosaccharides can be assigned.

As described above, an AAL-Sepharose column recognizes various fucosyl linkages located

Table 2
Structures of oligosaccharides which interact with immobilized lectin columns

Ricinus communis agglutinin I (RCA-I)
Strong binding oligosaccharides eluted with 10 m*M* lactose
Galβ1→4GlcNAcβ1→R

Retarded oligosaccharides
Galβ1→3GlcNAcβ1→R
Galβ1→3GalNAc
Galβ1→6Galβ1→R

Allomyrina dichotoma lectin-II (Allo A-II)
Strong binding oligosaccharides eluted with 10 m*M* lactose
Siaα2→6Galβ1→4GlcNAcβ1→R
HSO_3^-→6Galβ1→4GlcNAcβ1→R

Retarded oligosaccharides
Galβ1→4GlcNAcβ1→R

Sambuccus nigra agglutinin (SNA)
Strong binding oligosaccharides eluted with 200 m*M* lactose
Siaα2→6Galβ1→4GlcNAcβ1→R
Siaα2→6GalNAc→Ser/Thr

Macckia amurensis lectin (MAL)
Strong binding oligosaccharides eluted with 400 m*M* lactose
$(Siaα2→3Galβ1→4GlcNAcβ1→)_{2-4}→R$

Retarded oligosaccharides
Siaα2→3Galβ1→4GlcNAcβ1→R

Griffonia simplicifolia-I (GS-I)
Strong binding oligosaccharides eluted with 100 m*M* galactose
$(Galα1→3Galβ1→4GlcNAcβ1→)_{2-4}→R$

Retarded oligosaccharides
Galα1→3Galβ1→4GlcNAcβ1→R

Wistaria floribunda agglutinin (WFA)
Strong binding oligosaccharides eluted with 100 m*M* N-acetylgalactosamine
GalNAcβ1→4Galβ1→4GlcNAcβ1→R
GalNAcβ1→4GlcNAcβ1→R

Ulex europaeus I (UEA-I)
Strong binding oligosaccharides eluted with 50 m*M* fucose
Fucα1→2Galβ1→4GlcNAcβ1→R

Aleuria aurantia lectin (AAL)
Retarded oligosaccharides
Fucα1→2Galβ1→4GlcNAcβ1→R
Galβ1→4(Fucα1→3)GlcNAcβ1→R
Galβ1→3(Fucα1→4)GlcNAcβ1→R

Datura stramonium agglutinin (DSA)
Strong binding oligosaccharides eluted with 1% N-acetylglucosamine oligomers
$(Galβ1→4GlcNAcβ1→3)_{1-n}Galβ1→4GlcNAcβ1→R$

Tomato lectin (TL)
Strong binding oligosaccharides eluted with 0.2% N-acetylglucosamine oligomers
$(Galβ1→4GlcNAcβ1→3)_{2-n}Galβ1→4GlcNAcβ1→R$

R = H or sugar. Each lectin gel is packed to make a column of 1 ml (2.6 cm × 0.7 cm I.D.) and chromatography is performed with 10 m*M* Tris buffer (pH 7.4) at a flow-rate of 0.2 ml/min. The amounts of immobilized lectin per 1 ml of gel are as follows: RCA-I, 5 mg; Allo A-II, 3 mg; SNA, 3 mg; MAL, 10 mg; GS-I, 4 mg; WFA, 4 mg; UEA-I, 3 mg; AAL, 10 mg; DSA, 3 mg; TL, 4 mg.

at outer-chain moieties [10–12]. Additionally, *Ulex europaeus* I (UEA-I; EY Laboratories) interacts with the oligosaccharides containing blood group type-2 H antigenic determinant (Fucα1→2Galβ1→4GlcNAc) [29]. Therefore, such binding specificity is useful for separating or identifying oligosaccharides with the determinant. Further, the presence of the Galα1→3 group and the GalNAcβ1→4 group in outer-chain moieties can be detected with *Griffonia simplicifolia* I (GS-I; EY Laboratories) [30,31] and *Wistaria floribunda* agglutinin (WFA; EY Laboratories) [32,33], respectively. A DSA-Sepharose column can be used as an effective tool to separate the multiantennary complex-type oligosaccharides as described in the previous section. In addition, the column can also be applied to determine the peripheral structures of the outer-chain moieties. Complex-type oligosaccharides with at least one Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc group in their outer-chain moieties bind to the column and are eluted with buffer containing N-acetylglucosamine oligomers [22]. On the other hand, tomato lectin (TL; EY Laboratories) shows a high affinity with oligosaccharides which contain three or more linear N-acetyllactosamine sequences [34]. Accordingly, a TL-Sepharose column in combination with a DSA-Sepharose column can separate oligosaccharides containing long poly-N-acetyllactosaminyl repeats from those containing oligosaccharides with the Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc group [34,35].

Combination of the antennary and the various outer-chains will form a large number of different complex-type oligosaccharides. Therefore, one must take care in evaluating the results obtained with these immobilized lectin columns. Some of oligosaccharides interact with different lectin columns at the same time, indicating that such oligosaccharides have different peripheral structures on each different outer-chain moiety. It is noteworthy that these fine specificities described here can be applied to the characterization of not only N-linked but also O-linked oligosaccharides [36,37].

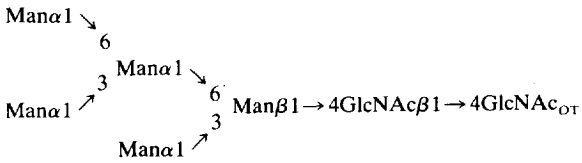
4. Serial lectin column chromatography for the separation of N-linked oligosaccharides

The usefulness of serial lectin columns for the fractionation of complicated N-linked oligosaccharides has been elucidated in many studies [38–50]. Since it is impossible to describe all of them, here a brief explanation of its usefulness as a model case is given.

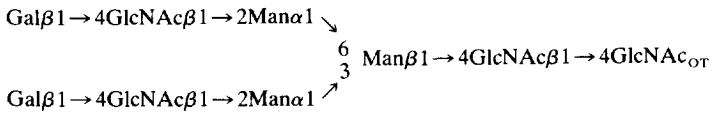
If one obtained a mixture of radioactive N-linked oligosaccharides as listed in Table 3 by hydrazinolysis [51] or N-glycanase digestion [52] of a glycoprotein followed by NaB^3H_4 reduction, one can separate them completely as a single component by serial lectin column chromatography as shown in Fig. 1. First, when the oligosaccharide mixture is subjected to affinity chromatography with the use of a Con A column, it is separated into three fractions. Bi-antennary complex-type sugar chains without a bisecting GlcNAc residue (oligosaccharides II and III in Table 3) and a high mannose-type sugar chain (oligosaccharide I) bind to the column and are eluted sequentially with 5 m*M* α-methylglucopyranoside and 200 m*M* α-methylmannopyranoside, respectively. The remaining oligosaccharides are recovered in the unbound fraction. The fucosylated sugar chain (oligosaccharide III) can be separated from the non-fucosylated sugar chain (oligosaccharide II) by passage through an AAL-Sepharose column. On the other hand, the bisected sugar chain (oligosaccharide IV) in the Con A unbound fraction is fractionated by passage through an E_4-PHA column. Finally, isomeric triantennary complex-type sugar chains mixture can be fractionated by DSA column chromatography based on the binding specificity of this useful lectin: a C-2,4 branched triantennary complex-type sugar chain (oligosaccharide V) is retarded in the column and a C-2,6 branched one (oligosaccharide VI) is bound to the column and eluted with N-acetylglucosamine oligomers. Hence one can obtain individual oligosaccharides shown in Table 3 in this manner. It must be stressed that usually N-linked oligosaccharides of glycoproteins show more complicate patterns. Therefore,

Table 3
N-Linked oligosaccharides used for serial lectin affinity column chromatography

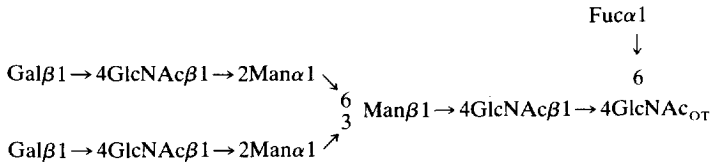
Oligosaccharide I (high-mannose type)



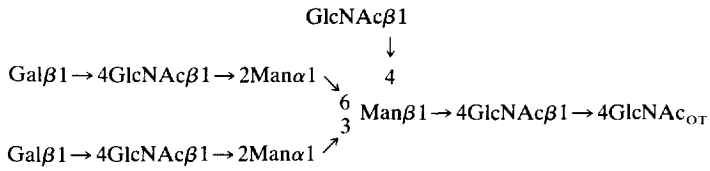
Oligosaccharide II (biantennary type)



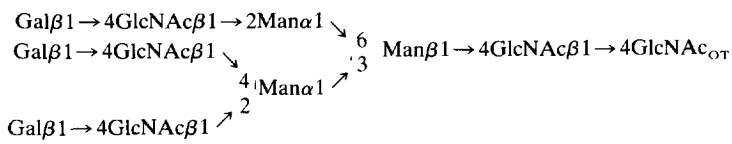
Oligosaccharide III (fucosylated biantennary type)



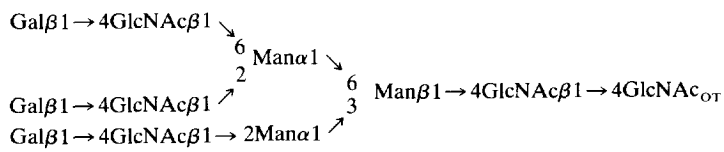
Oligosaccharide IV (bisected biantennary type)



Oligosaccharide V (C-2,4 branched triantennary type)



Oligosaccharide VI (C-2,6 branched triantennary type)



Subscript OT is used to indicate NaB^3H_4-reduced oligosaccharide.

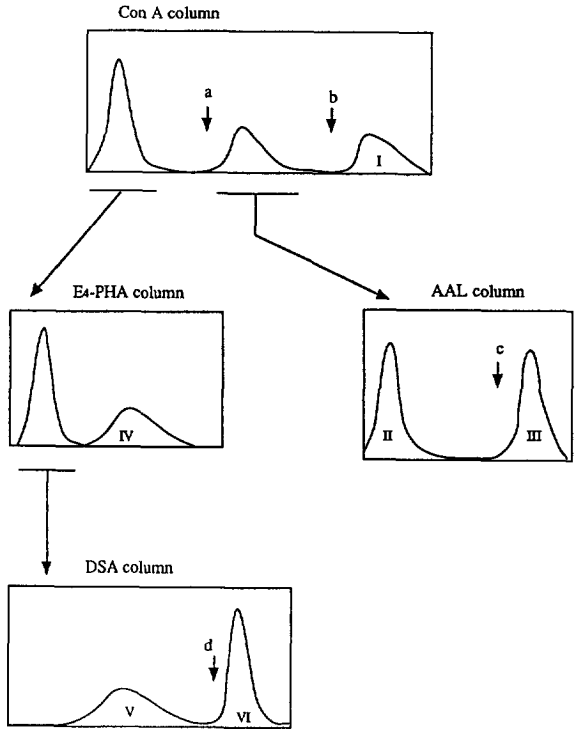


Fig. 1. Serial lectin affinity column chromatography of tritium-labelled oligosaccharide mixture (I–VI in Table 3). Oligosaccharides bound to a Con A column, an AAL column and a DSA column were eluted with 5 m*M* α-methyglucopyranoside (arrow a), with 200 m*M* α-methylmannopyranoside (arrow b), with 1 m*M* fucose (arrow c) and with 1% N-acetylglucosamine oligomers (arrow d). Bars under the elution patterns indicate the fractions pooled, which were then subjected to the next lectin column chromatography. Affinity chromatography on an E_4-PHA column was performed at 20°C. The ordinate and abscissa indicate radioactivity and elution volume, respectively.

lectin affinity column chromatography will become a more effective tool not only for fractionating oligosaccharides but also for elucidating partial structures, in combination with Bio-Gel P-4 column chromatography [53], and digestion with glycosidase with narrow aglycone specificities [54].

As described above, the use of immobilized lectin columns is becoming indispensable for the group separation of N-linked oligosaccharides, and several comments can be added here. As the relative affinity of individual oligosaccharides to the lectin column is fairly constant, one can deduce the structures of an oligosaccharide based on the elution profile from an immobilized lectin column. However, in order to obtain reliable data, one must consider that the elution patterns of oligosaccharides change with the chromatographic conditions, e.g., the density of immobilized lectin, size of column, temperature and flow-rate. Accordingly, columns of immobilized lectins must be standardized before use with oligosaccharides of known structure to ensure the activity. Typical oligosaccharide standards for standardization of chromatography are now commercially available (Oxford GlycoSystems). It is noteworthy that we and another group have developed lectin affinity HPLC columns [50,55], and an RCA HPLC column has been used to analyse the sugar patterns of glycoproteins including IgG samples obtained from healthy individuals and patients with rheumatoid arthritis [50,56,57]. Such an HPLC technique is obviously faster and potentially more reproducible. In the coming years, lectin affinity HPLC will undoubtedly become increasingly popular.

5. Conclusions

Serial lectin column chromatography can be effectively used for fractionation and for structural studies of glycoprotein-derived oligosaccharides as described here. Despite this usefulness, problems of contamination of isolectins with different sugar binding specificities and the limited availability of lectins have sometimes occurred. Recently, in order to overcome such problems, many groups, including ours, have succeeded in producing large amounts of functional recombinant lectins [10,58–60]. Further, recent crystallographic studies and the accumulation of amino acid sequence data on animal and plant lectins have clarified the carbohydrate binding region and the mode of binding [61–64]. Then it was elucidated that substitution of several amino acids in such a region could induce a change in the carbohydrate-binding specificity of an original lectin [65,66]. Progress with such a strategy might lead to the production of a "neolectin" having novel carbohydrate-binding

specificity in future. The addition of further useful lectin and "neolectin" columns to those currently available will surely yield innumerable directions for the development of glycobiology.

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