Analysis of Asparagine-Linked Oligosaccharides by Sequential Lectin Affinity Chromatography

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Abstract

Lectins are proteins that specifically bind to a particular carbohydrate structure. Affinity chromatography with immobilized lectins is a quite effective technique not only for the fractionation of glycoproteins or oligosaccharides but also their structural assessment. In this article, we focus on the separation of glycopeptides and oligosaccharides derived from glycoproteins by affinity chromatography on immobilized lectin columns.

Index Entries: Lectin; oligosaccharide; glycopeptide; glycoprotein; structure.

1. Introduction

Sugar moieties on the cell surface play one of the most important roles in cellular recognition. In order to elucidate the molecular mechanism of these cellular phenomena, assessment of the structure of sugar chains is indispensable. However, it is difficult to elucidate the structures of cell-surface oligosaccharides because of two technical problems. First is the difficulty in fractionating various oligosaccharides heterogenous in the number, type, and substitution patterns of outer sugar branches. The second problem is that very limited amounts of material can be available, which makes it difficult to perform detailed structural studies. Lectins are proteins with sugar binding activity. Each lectin binds specifically to a certain sugar sequence in oligosaccharides and glycopeptides. To overcome the problems described above, lectins are very useful tools. Recently, many attempts have been made to fractionate oligosaccharides and glycopeptides on immobilized lectin columns. The use of a series of immobilized lectin columns, whose sugar binding specificities have been precisely elucidated, enables us to fractionate a very small amount of radioactive oligosaccharides or glycopeptides (ca 10 ng depending on the specific activity) into structurally distinct groups. In this article, we summarize the serial lectin–Sepharose affinity chromatographic technique for rapid, sensitive, and specific fractionation and analysis of asparagine-linked oligosaccharides of glycoproteins.

Structures of asparagine-linked oligosaccharides fall into three main categories termed high-mannose-type, complex-type, and hybrid-type *(1)*. They share the common core structure Manα1–3(Manα1–6)Manβ1–4GlcNAcβ1–4GlcNAc-Asn, but differ in their outer branches (Fig. 1). High-mannose-type oligosaccharides have two to six additional α-mannose residues to the core structure. Typical complex-type oligosaccharides contains two to four outer branches with a sialyllactosamine sequence. Hybrid-type structures have the features of both high-mannose-type and complex-type oligosaccharides, and most of them contain bisecting *N*-acetylglucosamine, which is linked β1–4 to the β-linked mannose residue of the core structure. Recently, a novel type of carbohydrate chain, so-called poly-*N*-acetyllactosamine-type, has been described *(2–5)*.

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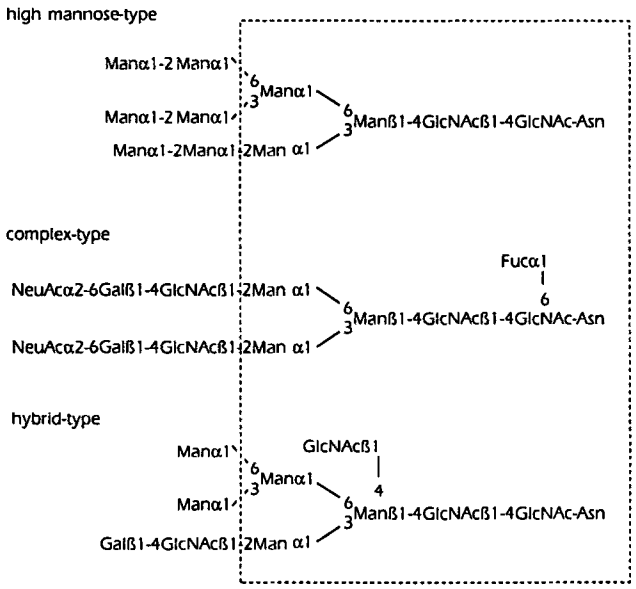


Fig. 1. Structures of major types of asparagine-linked oligosaccharides. The boxed area encloses the core structure common to all asparagine-linked structures.

Its outer branches have a characteristic structure composed of *N*-acetyllactosamine repeating units. It may be classified to be of complex-type. However, it is antigenically and functionally distinct from standard complex-type sugar chains *(4)*. Some of poly-*N*-acetyllactosamine-type oligosaccharides have branched sequences containingGalβ1–4GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6) Gal units *(2,3)*, which is the determinant of the I-antigen.

Glycopeptides or oligosaccharides can be prepared from glycoproteins by enzymatic digestions or chemical methods. The most widely used means for preparing glycopeptides is to digest material completely with pronase. Oligosaccharide can be prepared from glycoproteins or glycopeptides by treating samples with anhydrous hydrazine *(6)* or endoglycosidases. Since the released oligosaccharides retain their reducing termini, they can be radiolabeled by reduction with NaB^3H_4 *(7)*. The primary amino group of peptide backbone of glycopeptides is labeled by acetylation with [^{3}H]- or [^{14}C]-acetic anhydride *(8)*. Before employing columns of immobilized lectins for analyses, oligosaccharides or glycopeptides should be separated on a column of QAE- or DEAE-cellulose based on anionic charge derived from sialic acid, phosphate, or sulfate residues. Acidic oligosaccharides thus separated should be converted to neutral ones for simplifying the following separation. To simplify discussion, the oligosaccharides discussed here do not contain sialic acid, phosphate, or sulfate residues, although these acidic residues, especially sialic acid residues, are found in many oligosaccharides. In most cases, the influence of these residues on the interaction of oligosaccharides with immobilized lectins is weak, but where documentation of the influence of these residues is available, it will be mentioned in the appropriate sections. In this article, we describe the general procedure of serial lectin-affinity chromatography of glycopeptides and oligosaccharides using several well-defined immobilized lectins.

2. Materials

1. Mono Q HR5/5, DEAE-Sephacel, Sephadex G-25 (Pharmacia, Uppsala, Sweden).
2. High-performance liquid chromatograph, two pumps, with detector capable of monitoring UV absorbance at 220 nm.
3. Neuraminidase: 1 U/mL of neuraminidase from *Streptococcus sp.* (Seikagaku Kogyo, Tokyo Japan) in 50 m*M* acetate buffer, pH 6.5.
4. Dowex 50W-X8 (50–100 mesh, H^+ form).
5. Bio-Gel P-4 minus 400 mesh (Bio-Rad, Richmond, CA).
6. HPLC mobile phase for Mono Q: A: 2 m*M* Tris-HCl, pH 7.4; B: 2 m*M* Tris-HCl, pH 7.4, 0.5*M* NaCl.
7. HPLC mobile phase for Bio-Gel P-4: distilled water.
8. HPLC standard for Bio-Gel P-4: partial hydrolysate of chitin, which was prepared according to Rupley *(9)*; 10 µg mixed with 50 µL distilled water. Store frozen.
9. Concanavalin A, *Ricinus communis* lectin wheat germ lectin, *Datura stramonium* lectin, *Maackia amurensis* leukoagglutinin, *Allomyrina dichotoma* lectin, *Amaranthus caudatus* lectin (EY Laboratories, San Mateo, CA), *Phaseorus vul-*

garis erythroagglutinin, and *Phaseorus vulgaris* leukoagglutinin (Seikagaku Kogyo). Immobilized lectins were prepared at a concentration of 1–5 mg lectin/mL of gel (*see* Notes 1 and 2) or obtained commercially (e.g., Pharmacia, EY Laboratories, Bio-Rad, Seikagaku Kogyo): *Galanthus nivalis* lectin, *Lens culinaris* lectin, *Pisum sativum* lectin, *Vicia fava* lectin, pokeweed mitogen, and *Sambucus nigra L* lectin.

10. ^{3}H-$NaBH_4$: 100 mCi of ^{3}H-$NaBH_4$ (sp. 5–15 Ci/mmol; NEN, Boston, MA) mixed with 2 mL 10 m*M* NaOH; store at –80°C.
11. Tris-buffered saline (TBS): 10 m*M* Tris-HCl, pH 7.4, 0.15*M* NaCl.
12. Lectin column buffer: 10 m*M* Tris-HCl, pH 7.4, 0.15*M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$ (*see* Note 3).
13. Methyl-α-mannoside (Sigma, St. Louis, MO): 100 m*M* in TBS; store refrigerated.
14. Methyl-α-glucoside (Sigma): 10 m*M* in TBS; store refrigerated.
15. Lactose (Sigma): 50 m*M* in TBS; store refrigerated.
16. *N*-acetylglucosamine (Sigma): 200 m*M* in TBS; store refrigerated.

3. Methods

3.1. Separation of Acidic Sugar Chains on Mono Q HR5/5 or DEAE-Sephacel and Removal of Sialic Acids

3.1.1. Ion-Exchange Chromatography

1. Equilibrate the Mono Q HR5/5 or DEAE-Sephacel column with 2 m*M* Tris-HCl, pH 7.4, at a flow rate of 1 mL/min at room temperature.
2. Dissolve the oligosaccharides or the glycopeptides in 0.1 mL of 2 m*M* Tris-HCl, pH 7.4, and apply to the column.
3. Elute with 2 m*M* Tris-HCl, pH 7.4 for 10 min, and then with linear gradient (0–20%) of 2 m*M* Tris-HCl, pH 7.4, and 0.5*M* NaCl for 60 min at a flow rate of 1 mL/min.
4. Neutral oligosaccharide are recovered in pass-through fraction. Acidic monosialo-, disialo-, trisialo-, and tetrasialooligosaccharides are eluted out successively by the linear gradient of NaCl.

3.1.2. Removal of Sialic Acid Residues

1. Add 100 µL of neuraminidase buffer and 100 µL of neuraminidase to 10–100 µg oligosaccharides free of buffers or salts. Incubate at 37°C for 18 h.
2. Heat-inactivate the neuraminidase by immersion in a boiling water bath for 3 min.
3. Apply to the column of Dowex 50W-X8 (0.6 cm id × 2.5 cm), wash the column with 1 mL of distilled water, and concentrate the eluates under vacuum.

Alternatively, add 500 µL of 0.1*M* HCl, and heat at 80°C for 30 min. Dry up the sample using an evaporator.

3.2. Separation of Poly-N-Acetyllactosamine-Type Sugar Chains from Other Types of Sugar Chains

Poly-*N*-acetyllactosamine-type sugar chains vary as to the number of *N*-acetyllactosamine repeating units and the branching mode. The structural characterization of poly-*N*-acetyllactosamine-type sugar chains has been quite difficult *(10)*. This type of sugar chain has a higher molecular weight compared to other high-mannose-type, complex-type, or hybrid-type chains. This poly-*N*-acetyllactosamine-type sugar chain is easily separated from others on a column of Bio-Gel P-4 having molecular mass of more than 4000 excluded from the Bio-Gel P-4 column chromatography *(11,12)*.

1. Equilibrate two coupled columns (0.8 cm id × 50 cm) of Bio-Gel P-4 in water at 55°C by use of water jacket.
2. Elute the oligosaccharides at a flow rate of 0.3 mL/min, and collect fractions of 0.5 mL. Monitor absorbance at 220 nm.
3. Collect poly-*N*-acetyllactosamine-type oligosaccharides that are eluted at the void volume of the column. Other types of oligosaccharides included in the column are subjected to the next separation (Sections 3.3.–3.6.) illustrated in Fig. 2. The specificity of the lectins used is summarized in Fig. 3 and Table 1.

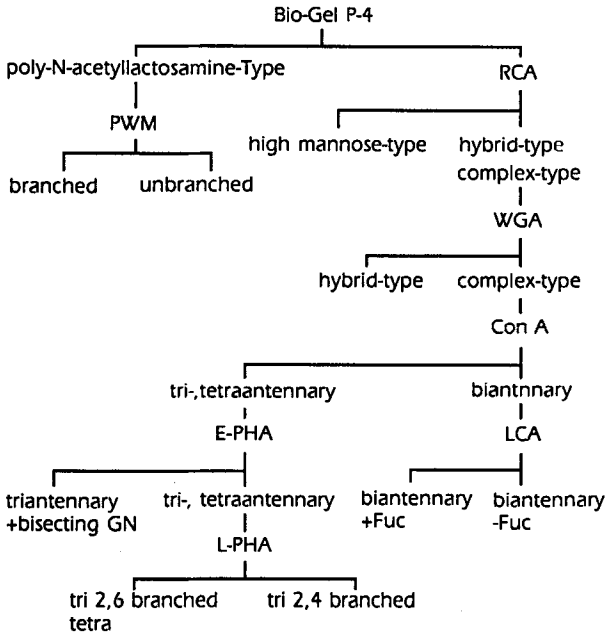


Fig. 2. Scheme of fractionation of asparagine-linked sugar chains by combining affinity chromatography on immobilized lectins.

3.3. Separation of High-Mannose-Type Sugar Chains from Complex-Type and Hybrid-Type Sugar Chains

3.3.1. Affinity Chromatography on Immobilized RCA

After the separation of high-mol-wt poly-*N*-acetyllactosamine-type oligosaccharides, a mixture of the other three types of sugar chains can be separated on a column of *Ricinus communis* lectin (RCA), which recognizes Galβ1–4GlcNAc sequence *(13,14)*.

1. Equilibrate the RCA-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Dissolve oligosaccharides or glycopeptides in 0.5 mL of TBS, and apply to the column.
3. Elute (1.0-mL fractions) successively with three column volumes of TBS, and then with three column volumes of 50 m*M* lactose at a flow rate of 2.5 mL/h at room temperature.
4. Bind both complex-type and hybrid-type sugar chains to the RCA-Sepharose column (*see* Note 4).
5. Collect high-mannose-type oligosaccharides, which pass through the column.
6. Purify the oligosaccharides or glycopeptides from salts and haptenic sugar by gel filtration on Sephadex G-25 column (1.2 cm id × 50 cm) equilibrated with distilled water.

3.3.2. Affinity Chromatography on Immobilized Snowdrop Lectin

High mannose-type glycopeptides, which carry Manα1–3Man units, are specifically retarded on the immobilized snowdrop *(Galanthus nivalis)* lectin (GNA) *(15)*.

1. Equilibrate the GNA-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Dissolve oligosaccharides or glycopeptides in 0.5 mL of TBS, and apply to the column.
3. Elute (0.5-mL fractions) successively with 5 column volumes of TBS, to collect sugar chains lacking Manα1–3Man units or hybrid-type, which are not retarded.
4. Elute with 3 column volumes of 100 m*M* methyl-α-mannoside at flow rate 2.5 mL/h at room temperature to obtain the specifically retarded high-mannose-type glycopeptides that carry Manα1–3Man units.

3.4. Separation of Hybrid-Type Sugar Chains from Complex-Type Sugar Chains

3.4.1. Affinity Chromatography on Immobilized WGA

Wheat germ lectin (WGA)-Sepharose has a high affinity for the hybrid-type sugar chains. It is demonstrated that the sugar sequence GlcNAcβ1–4Manβ1–4GlcNAcβ1–4GlcNAc-Asn structure is essential for tight binding of glycopeptides to WGA-Sepharose column *(16)*.

1. Equilibrate the WGA-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Dissolve glycopeptides in 0.5 mL of TBS and apply to the column.
3. Elute (0.5-mL fractions) successively with 5 column volumes of TBS.
4. Collect hybrid-type glycopeptides with a bisecting *N*-acetylglucosamine residue, which is retarded on WGA column.
5. Collect sugar chains having the typical complex-type (and also high-mannose-type) sugar chains eluted at the void volume of the column with TBS.

Fig. 3. Structures of several complex-type oligosaccharides. The boxed area indicates the characteristic structures recognized by several immobilized lectins.

3.5. Separation of Complex-Type Biantennary Sugar Chains

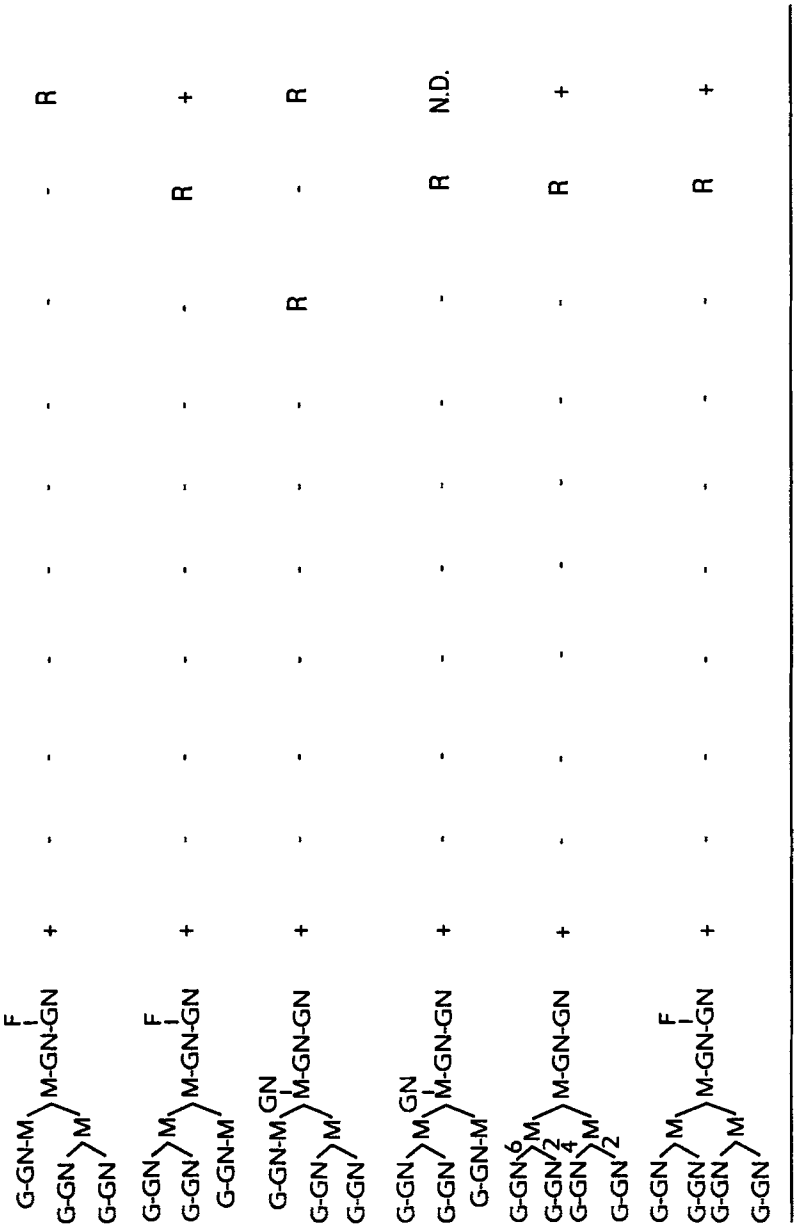
3.5.1. Affinity Chromatography on Immobilized Con A

Oligosaccharides and glycopeptides with tri- and tetraantennary complex-type sugar chains pass through Concanavalin A (Con A)-Sepharose, whereas biantennary complex-type, hybrid-type, and high-mannose-type sugar chains bind to the Con A and can be differentially eluted from the column *(17,18)*.

1. Equilibrate the Con A-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Pass the oligosaccharide mixture of complex-type chain from WGA column through Con A-Sepharose column.

Table 1
Characteristic Structures Recognized by Several Immobilized Lectins

	RCA	GNA	WGA	Con A	LCA	PEA	VFA	E-PHA	L-PHA	DSA
M-M$_{3}^{6}$M-M$_{3}^{6}$M-GN-GN M-M-M$_{3}$	-	R	-	+	-	-	-	-	-	-
M$_{6}^{GN}$M$_{6}^{I4}$M-GN-GN G-GN-M$_{3}$	+	-	R	+	-	-	-	-	-	-
G-GN-M$_{2}$M-GN-GN G-GN-M$_{2}$	+	-	-	+	-	-	-	-	-	-
G-GN-M$_{6}^{F}$M-GN-GN G-GN-M	+	-	-	+	+	+	+	-	-	-
G-GN-M$_{I}^{GN\ F}$M-GN-GN G-GN-M	+	-	-	+	-	-	-	R	-	-
G-GN-M$_{}$M-GN-GN G-GN$_{2}^{4}$M G-GN$_{2}$	+	-	-	-	-	-	-	-	-	R
G-GN$_{2}^{6}$M$_{}$M-GN-GN G-GN-M	+	-	-	-	-	-	-	-	R	+



+, bound; R, retarded; -, not bound; N.D., not determined.

3. Elute (1-mL fractions) successively with 3 column volumes of lectin column buffer.
4. Collect oligosaccharides with tri- and tetraantennary complex-type sugar chains, which pass through a column. Complex-type biantennary glycopeptides or oligosaccharides having bisecting GlcNAc also pass through the column.
5. Elute (1-mL fractions) successively with 3 column volumes of 10 m*M* methyl-α-glucoside and finally with 3 column volumes of 100 m*M* methyl-α-mannoside.
6. Collect complex-type biantennary sugar chains, which are eluted after the addition of methyl-α-glucoside.
7. Collect high-mannose-type and hybrid-type oligosaccharides, or elute glycopeptides after the addition of 100 m*M* methyl-α-mannoside.

3.5.2. Affinity Chromatography on Immobilized LCA, PSA, or VFA

The biantennary complex-type sugar chains bound to Con A-Sepharose column and eluted with 10 m*M* methyl-α-glucoside contains two types of oligosaccharides, which will be separated on a column of lentil lectin (*Lens culinaris* lectin, LCA), pea lectin (*Pisum sativum* lectin, PSA), or fava lectin (*Vicia fava* lectin, VFA) *(19–21)*.

1. Equilibrate the LCA, PSA, or VFA-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Pass the biantennary complex-type sugar chains from the Con A column through LCA, PSA, or VFA-Sepharose column.
3. Elute (1.0-mL fractions) successively with 3 column volumes of lectin column buffer, and then with 3 column volumes of 100 m*M* methyl-α-mannoside at flow rate 2.5 mL/h at room temperature.
4. Collect biantennary complex-type sugar chains without fucose, which pass through the column.
5. Elute bound biantennary complex-type sugar chains having fucose residue attached to the innermost *N*-acetylglucosamine to the column (*see* Note 5).

3.5.3. Affinity Chromatography on Immobilized E-PHA

Complex-type biantennary sugar chains having outer galactose residues and "bisecting" *N*-acetylglucosamine are retarded by *Phaseolus vulgaris* erythroagglutinin (E-PHA)-Sepharose *(14,22)*.

1. Equilibrate the E-PHA-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Apply the pass-through fraction from the Con A column on E-PHA-Sepharose column.
3. Elute (0.5-mL fractions) successively with 5 column volumes of lectin column buffer at a flow rate of 2.5 mL/h at room temperature.
4. Collect biantennary complex-type sugar chains having bisecting *N*-acetylglucosamine residue retarded on the E-PHA column (*see* Note 6). When the elution of the column is performed at 4°C, biantennary complex-type oligosaccharide without bisecting *N*-acetylglucosamine is also retarded by the E-PHA-Sepharose column.

3.6. Separation of Complex-Type Triantennary and Tetraantennary Sugar Chains

3.6.1. Affinity Chromatography on Immobilized E-PHA

E-PHA-Sepharose interacts with high affinity with triantennary (having 2,4 branched mannose) oligosaccharides or glycopeptides containing both outer galactose residues and bisecting *N*-acetylglucosamine residue *(22)*.

1. Equilibrate the E-PHA-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Apply the pass-through fraction from the Con A column on E-PHA-Sepharose column.
3. Elute (0.5-mL fractions) successively with 5 column volumes of lectin column buffer at a flow rate of 2.5 mL/h at room temperature.
4. Collect retarded triantennary (having 2,4 branched mannose) oligosaccharides or glycopeptides containing both outer galactose and bisecting *N*-acetylglucosamine on the E-PHA column. Other tri- and tetraantennary oligosaccharides pass through the column (*see* Note 7).

3.6.2. Affinity Chromatography on Immobilized L-PHA

Phaseolus vulgaris leukoagglutinin (L-PHA), which is isolectin of E-PHA, interacts with triantennary and tetraantennary complex-type glycopeptides having α-linked mannose residue substituted at positions C-2 and C-6 with Galβ1–4GlcNAc *(23)*.

1. Equilibrate the L-PHA-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Apply the pass-through fraction from the Con A column on L-PHA-Sepharose column.
3. Elute (0.5-mL fractions) successively with 5 column volumes of lectin column buffer at a flow rate of 2.5 mL/h at room temperature.
4. Collect retarded triantennary and tetraantennary complex-type glycopeptides having both 2,6-branched α-mannose and outer galactose on the L-PHA column (*see* Note 8). Other tri- and tetraantennary oligosaccharides pass-through the column.

3.6.3. Affinity Chromatography on Immobilized DSA

Datura stramonium lectin (DSA) shows high affinity with tri- and tetraantennary complex-type oligosaccharides. Triantennary complex-type oligosaccharides containing 2,4 substituted α-mannose are retarded by DSA-Sepharose column. Triantennary and tetraantennary complex-type oligosaccharides having α-mannose residue substituted at the C-2,6 positions bind to the column and eluted by GlcNAc oligomer *(24,25)*.

1. Equilibrate the DSA-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Apply the pass-through fraction from the Con A column on DSA-Sepharose column.
3. Elute (0.5-mL fractions) successively with 3 column volumes of TBS at a flow rate of 2.5 mL/h at room temperature to obtain retarded triantennary complex-type sugar chain having 2,4-branched α-mannose on the DSA column.
4. Elute with 3 column volumes of 5 mg/mL *N*-acetylglucosamine oligomer at a flow rate of 2.5 mL/h at room temperature to obtain bound triantennary and tetraantennary complex-type oligosaccharides having α-mannose residue substituted at the C-2,6 positions.

3.7. Separation of Poly-N-Acetyllactosamine-Type Sugar Chains

High-mol-wt poly-*N*-acetyllactosamine-type oligosaccharides are classified into two groups. One is branched poly-*N*-acetyllactoseminoglycan containing Galβ1–4GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)Gal unit, and the other is linear poly-*N*-acetyllactosamine structure, which lacks galactose residues substituted at the C-3,6 positions.

3.7.1. Affinity Chromatography on Immobilized PWM

Branched poly-*N*-acetyllactosamine-type oligosaccharides can be separated by the use of pokeweed mitogen (PWM)-Sepharose column *(26)*. Since the sugar sequence Galβ1–4GlcNAcβ1–6Gal firmly binds to the PWM-Sepharose column, the branched poly-*N*-acetyllactosamine chains can be retained by the column, whereas unbranched ones are recovered without any retardation *(27)* (*see* Note 9).

1. Equilibrate the PWM-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Apply the poly-*N*-acetyllactosamine-type sugar chains separated on Bio-Gel P-4 (*see* Section 3.2.) on PWM-Sepharose column.
3. Elute (1.0-mL fractions) successively with 3 column volumes of TBS and then with three column volumes of 0.1*M* NaOH at a flow rate of 2.5 mL/h at room temperature.
4. Collect unbranched poly-*N*-acetyllactosamine-type sugar chains, which pass through the column.
5. Collect bound branched poly-*N*-acetyllactosamine-type sugar chains.

3.7.2. Affinity Chromatography on Immobilized DSA

Immobilized DSA lectin interacts with high affinity with sugar chains having the linear, unbranched poly-*N*-acetyllactosamine sequence. For the binding to DSA-Sepharose, more than two intact *N*-acetyllactosamine repeating units may be essential *(25)*.

1. Equilibrate the DSA-Sepharose column (0.6 cm id × 5.0 cm) in TBS.

2. Apply the poly-*N*-acetyllactosamine-type sugar chains separated on Bio-Gel P-4 (*see* Section 3.2.) on DSA-Sepharose column.
3. Elute (1.0-mL fractions) successively with 3 column volumes of TBS and then with 3 column volumes of 5 mg/mL GlcNAc oligomer at a flow rate of 2.5 mL/h at room temperature.
4. Collect branched poly-*N*-acetyllactosamine-type sugar chains, which pass through the column, separated from unbranched poly-*N*-acetyllactosamine-type sugar chains, which bind.

3.8. Separation of Sialylated Sugar Chains

The basic Galβ1–4GlcNAc sequence present in complex-type sugar chains may contain sialic acids in α2,6 or α2,3 linkage to outer galactose residues.

3.8.1. Affinity Chromatography on Immobilized MAL

Maackia amurensis leukoagglutinin (MAL) *(28,29)* interacts with high affinity with complex-type tri- and tetraantennary glycopeptides containing outer sialic acid residue linked α2,3 to penultimate galactose. Glycopeptides containing sialic acid linked only α2,6 to galactose do not interacts detectably with the immobilized MAL (*see* Note 10).

1. Equilibrate the MAL-Sepharose column (0.6 cm id × 5.0 cm) in lectin column buffer.
2. Apply the acidic oligosaccharides or glycopeptides separated on Mono Q HR5/5 or DEAE-Sephacel (*see* Section 3.1.1., step 1) on MAL-Sepharose column.
3. Elute (0.5-mL fractions) successively with 5 column volumes of lectin column buffer at flow rate 2.5 mL/h at room temperature.
4. Collect glycopeptides or oligosaccharides containing α2,6-linked sialic acid(s), which pass through the column.
5. Collect retarded glycopeptides or oligosaccharides containing α2,3-linked sialic acid(s).

3.8.2. Affinity Chromatography on Immobilized Allo A

Allomyrina dichotoma lectin (allo A) *(30,31)* recognizes the other isomer of sialyllactosamine compared to MAL. Mono-, di-, and triantennary complex-type oligosaccharides containing terminal sialic acid(s) in α2,6 linkage bound to allo A-Sepharose, whereas complex-type sugar chains having isomeric α2,3-linked sialic acid(s) did not bind to the immobilized allo A.

1. Equilibrate the allo A-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Apply the acidic oligosaccharides or glycopeptides separated on Mono Q HR5/5 or DEAE-Sephacel (*see* Section 3.1.1., step 1) on allo A-Sepharose column.
3. Elute (0.5-mL fractions) successively with 3 column volumes of TBS and then with 3 column volumes of 50 m*M* lactose at a flow rate of 2.5 mL/h at room temperature.
4. Collect glycopeptides or oligosaccharides containing α2,3-linked sialic acid(s), which pass through the column.
5. Elute bound glycopeptides or oligosaccharides having α2,6-linked sialic acid(s) (*see* Note 11).

3.8.3. Affinity Chromatography on Immobilized SNA

Elderberry *(Sambucus nigra L.)* bark lectin (SNA) *(32,33)* shows the same sugar binding specificity as allo A. All types of oligosaccharides that contain at least one NeuAcα2–6Gal unit in the molecule bound firmly to the SNA-Sepharose.

1. Equilibrate the SNA-Sepharose column (0.6 cm id × 5.0 cm) in TBS.
2. Apply the acidic oligosaccharides or glycopeptides separated on Mono Q HR5/5 or DEAE-Sephacel (*see* Section 3.1.1., step 1) on SNA-Sepharose column.
3. Elute (0.5-mL fractions) successively with 3 column volumes of TBS and then with 3 column volumes of 50 m*M* lactose at a flow rate of 2.5 mL/h at room temperature.
4. Collect glycopeptides or oligosaccharides containing α2,3-linked sialic acid(s), which pass through the column.
5. Elute bound glycopeptides or oligosaccharides having α2,6-linked sialic acid(s) in the 50 m*M* lactose eluant.

4. Notes

1. During the coupling reactions, sugar binding sites of lectins must be protected by the addition of the specific haptenic sugars.
2. Immobilized lectin is stored at 4°C. In most cases, immobilized lectin is stable for several years.
3. Some lectins, especially legume lectins, need Ca and Mn ions for carbohydrate binding, so that the buffers used for the affinity chromatography on the lectin column must contain 1 m*M* $CaCl_2$ and $MnCl_2$.
4. Complex-type or hybrid-type oligosaccharides are retarded on a column of RCA-Sepharose rather than tightly bound when their sugar sequences are masked by sialic acids.
5. Intact *N*-acetylglucosamine and asparagine residues at the reducing end are required for tight binding of complex-type oligosaccharides to LCA-, PSA-, or VFA-Sepharose column.
6. High-affinity interaction with E-PHA-Sepharose is prevented if both outer galactose residues on a bisected sugar chain are substituted at position C-6 by sialic acid.
7. Biantennary and triantennary complex-type sugar chains having bisecting GlcNAc can be separated on Bio-Gel P column.
8. L-PHA-Sepharose does not retard the elution of sugar chains lacking outer galactose residues.
9. WGA can be used instead of PWM.
10. *Maakia amurensis* hemagglutinin (MAH), which is an isolectin of MAL, strongly binds to sialylated Ser/Thr-linked Galβ1–3GalNAc, but not to sialylated Asn-linked sugar moieties *(34)*.
11. Oligosaccharides without sialic acid(s) of mono-, di-, tri-, and tetraantennary complex-type are retarded by the allo A lectin column.
12. More detailed reviews on the separation of oligosaccharides and glycopeptides by means of affinity chromatography on immobilized lectin columns have been published *(35,37)*.

5. Summary

Various immobilized lectins can be successfully used for fractionation and for structural studies of asparagine-linked sugar chains of glycoproteins (*see* Note 12). This method needs <10 ng of a radiolabeled oligosaccharide or glycopeptide prepared from a glycoprotein by hydrazinolysis, or by digestion with endo-β-*N*-acetylglucosaminidases or *N*-glycanases. The fractionation and the structural assessment through the use of immobilized lectins make the subsequent structural studies much easier.

References

1. Kornfeld, R. and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Annu. Rev. Biochem.* **54,** 631–664.
2. Tsuji, T., Irimura, T., and Osawa, T. (1981) The carbohydrate moiety of Band 3 glycoprotein of human erythrocyte membrane. *J. Biol. Chem.* **256,** 10,497–10,502.
3. Fukuda, M., Dell, A., Oates, J. E., and Fukuda, M. N. (1984) Structure of branched lactosaminoglycan, the carbohydrate moiety of Band 3 isolated from adult human erythrocytes. *J. Biol. Chem.* **259,** 8260–8273.
4. Merkle, R. K. and Cummings, R. D. (1987) Relationship of the terminal sequences to the length of poly-N-acetyllactosamine chains in asparagine-linked oligosaccharides from the mouse lymphoma cell line BW5147. *J. Biol. Chem.* **282,** 8179–8189.
5. Fukuda, M. (1985) Cell surface glycoconjugates as onco-differentiation markers in hematopoietic cells. *Biochem. Biophys. Acta.* **780,** 119–150.
6. Fukuda, M., Kondo, T., and Osawa, T. (1976) Studies on the hydrazinolysis of glycoproteins. Core structures of oligosaccharides obtained from porcine thyroglobulin and pineapple stem bromelain. *J. Biochem.* **80,** 1223–1232.
7. Takasaki, S. and Kobata, A. (1978) Microdetermination of sugar composition by radioisotope labeling. *Methods Enzymol.* **50,** 554.
8. Tai, T., Yamashita, K., Ogata, M. A., Koide, N., Muramatsu, T., Iwashita, S., Inoue, Y., and Kobata, A. (1975) Structural studies of two ovalbumin glycopeptides in relation to the endo-β-*N*-acetylglucosaminidase specificity. *J. Biol. Chem.* **250,** 8569–8575.
9. Rupley, J. A. (1964) The hydrolysis of chitin by concentrated hydrochloric acid, and the preparation of low-molecular-weight substrates for lysozyme. *Biochem. Biophys. Acta.* **83,** 245–255.
10. Krusius, T., Finne, J., and Rauvala, H. (1978) The poly(glycosyl) chains of glycoproteins . Characterization of a novel type of glycoprotein saccharides from human erythrocyte membrane. *Eur. J. Biochem.* **92,** 289–300.
11. Yamamoto, K., Tsuji, T., Tarutani, O., and Osawa, T. (1984) Structural changes of carbohydrate chains of human thyroglobulin accompanying malignant transformations of thyroid glands. *Eur. J. Biochem.* **143,** 133–144.

12. Tsuji, T., Irimura, T., and Osawa, T. (1980) The carbohydrate moiety of Band-3 glycoprotein of human erythrocyte membranes. *Biochem. J.* **187,** 677–686.
13. Baenziger, J. U. and Fiete, D. (1979) Structural determinants of Ricinus communis agglutinin and toxin specificity for oligosaccharides. *J. Biol. Chem.* **254,** 9795–9799.
14. Irimura, T., Tsuji, T., Yamamoto, K., Tagami, S., and Osawa, T. (1981) Structure of a complex-type sugar chain of human glycophorin A. *Biochemistry* **20,** 560–566.
15. Shibuya N., Goldstein, I. J., Van Damme, E. J. M., and Peumans, W. J. (1988) Binding properties of a mannose-specific lectin from the snowdrop *(Galanthus nivalis)* bulb. *J. Biol. Chem.* **263,** 728–734.
16. Yamamoto, K., Tsuji, T., Matsumoto, I., and Osawa, T. (1981) Structural requirements for the binding of oligosaccharides and glycopeptides to immobilized wheat germ agglutinin. *Biochemistry* **20,** 5894–5899.
17. Ogata, S., Muramatsu, T., and Kobata, A. (1975) Fractionation of glycopeptides by affinity column chromatography on concanavalin A-Sepharose. *J. Biochem.* **78,** 687–696.
18. Krusius, T., Finne, J., and Rauvala, H. (1976) The structural basis of the different affinities of two types of acidic N-glycosidic glycopeptides from concanavalin A-Sepharose. *FEBS Lett.* **71,** 117–120.
19. Kornfeld, K., Reitman, M. L., and Kornfeld, R. (1981) The carbohydrate-binding specificity of pea and lentil lectins. *J. Biol. Chem.* **256,** 6633–6640.
20. Katagiri, Y., Yamamoto, K., Tsuji, T., and Osawa, T. (1984) Structural requirements for the binding of glycopeptides to immobilized *vicia faba* (fava) lectin. *Carbohydr. Res.* **129,** 257–265.
21. Yamamoto, K., Tsuji, T., and Osawa, T. (1982) Requirement of the core structure of a complex-type glycopeptide for the binding to immobilized lentil- and pea-lectins. *Carbohydr. Res.* **110,** 283–289.
22. Yamashita, K., Hitoi, A., and Kobata, A. (1983) Structural determinants of *Phaseolus vulgaris* erythroagglutinating lectin for oligosaccharides. *J. Biol. Chem.* **258,** 14,753–14,755.
23. Cummings, R. D. and Kornfeld, S. (1982) Characterization of the structural determinants required for the high affinity interaction of asparagine-linked oligosaccharides with immobilized *Phaseolus vulgaris* leukoagglutinating and erythroagglutinating lectins. *J. Biol. Chem.* **257,** 11,230–11,234.
24. Cummings, R. D. and Kornfeld, S. (1984) The distribution of repeating [Galβ1,4GlcNAcβ1,3] sequences in asparagine-linked oligosaccharides of the mouse lymphoma cell line BW5147 and PHA^R2.1. *J. Biol. Chem.* **259,** 6253–6260.
25. Yamashita, K., Totani, K., Ohkura, T., Takasaki, S., Goldstein, I. J., and Kobata, A. (1987) Carbohydrate binding properties of complex-type oligosaccharides on immobilized *Datura stramonium* lectin. *J. Biol. Chem.* **262,** 1602–1607.
26. Irimura, T. and Nicolson, G. L. (1983) Interaction of pokeweed mitogen with poly(N-acetyllactosamine)-type carbohydrate chains. *Carbohydr. Res.* **120,** 187–195.
27. Kawashima, H., Sueyoshi, S., Li, H., Yamamoto, K., and Osawa, T. (1990) Carbohydrate binding specificities of several poly-*N*-acetyllactosamine-binding lectins. *Glycoconjugate J.* **7,** 323–334.
28. Wang, W.-C. and Cummings, R. D. (1988) The immobilized leukoagglutinin from the seeds of *Maackia amurensis* binds with high affinity to complex-type Asn-linked oligosaccharides containing terminal sialic acid-linked α-2,3 to penultimate galactose residues. *J. Biol. Chem.* **263,** 4576–4585.
29. Kawaguchi, T., Matsumoto, I., and Osawa, T. (1974) Studies on hemagglutinins from *Maackia amurensis* seeds. *J. Biol. Chem.* **249,** 2786–2792.
30. Sueyoshi, S., Yamamoto, K., and Osawa, T. (1988) Carbohydrate binding specificity of a beetle *(Allomyrina dichotoma)* lectin. *J. Biochem.* **103,** 894–899.
31. Yamashita, K., Umetsu, K., Suzuki, T., Iwaki, Y., Endo, T., and Kobata, A. (1988) Carbohydrate binding specificity of immobilized *Allomyrina dichotoma* lectin II. *J. Biol. Chem.* **263,** 17,482–17,489.
32. Shibuya, N., Goldstein, I. J., Broekaert, W. F., Lubaki, M. N., Peeters, B., and Peumans. W. J. (1987) Fractionation of sialylated oligosaccharides, glycopeptides, and glycoproteins on immobilized elderberry *(Sambucus nigra L.)* bark lectin. *Arch. Biochem. Biophys.* **254,** 1–8.
33. Shibuya, N., Goldstein, I. J., Broekaert, W. F., Lubaki, M. N., Peeters, B., and Peumans, W. J. (1987) The elderberry *(Sambucus nigra L.)* bark lectin recognizes the Neu5Ac(α2–6)Gal/GalNAc sequence. *J. Biol. Chem.* **262,** 1596–1601.
34. Konami, Y., Yamamoto, K., Osawa, T., and Irimura, T. (1994) Strong affinity of *Maackia amurensis* hemagglutinin (MAH) for sialic acid-containing Ser/Thr-linked carbohydrate chains of N-terminal octapeptides from human glycophorin A. *FEBS Lett.* **342,** 334–338.
35. Osawa, T. and Tsuji, T. (1987) Fractionation and structural assessment of oligosaccharides and glycopeptides by use of immobilized lectins. *Ann. Rev. Biochem.* **56,** 21–42.
36. Osawa, T. (1989) Recent progress in the application of plant lectins to glycoprotein chemistry. *Pure & Appl. Chem.* **61,** 1283–1292.
37. Tsuji, T., Yamamoto, K., and Osawa, T. (1993) Affinity chromatography of oligosaccharides and glycopeptides with immobilized lectins, in *Molecular Interactions in Bioseparations* (Ngo, T. T., ed.), Plenum, New York, pp. 113–126.