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REVIEW

Lectins as tools in glycoconjugate research

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Abstract Lectins are ubiquitous proteins of nonimmune origin, present in plants, microorganisms, animals and humans which specifically bind defined monosugars or oligosaccharide structures. Great progress has been made in recent years in understanding crucial roles played by lectins in many biological processes. Elucidation of carbohydrate specificity of human and animal lectins is of great importance for better understanding of these processes. Long before the role of carbohydrate–protein interactions had been explored, many lectins, mostly of plant origin, were identified, characterized and applied as useful tools in studying glycoconjugates. This review focuses on the specificity-based lectin classification and the methods of measuring lectin–carbohydrate interactions, which are used for determination of lectin specificity or for identification and characterization of glycoconjugates with lectins of known specificity. The most frequently used quantitative methods are shortly reviewed and the methods elaborated and used in our laboratories, based on biotinylated lectins, are described. These include the microtiter plate enzyme-linked lectinosorbent assay, lectinoblotting and lectin–glycosphingolipid interaction on thin-layer plates. Some chemical modifications of lectin ligands on the microtiter plates and blots (desialylation, Smith degradation, β-elimination), which extend the applicability of these methods, are also described.

Keywords Enzyme-linked lectinosorbent assay · Glycoconjugate · Lectinoblotting · Lectins · Thin-layer chromatography

Abbreviations

ELLSA	enzyme-linked lectinosorbent assay
GPA	glycophorin A
HOC	human ovarian cyst fluid
TLC	thin-layer chromatography

Introduction

Lectinology can be divided in two major parts, studies on lectins and application of lectins as tools. Studies on lectins are carried out to characterize the structure of lectin molecules, their carbohydrate-binding specificity, conformational/functional properties of their carbohydrate-binding 'pocket', and their biological roles. The second area pertains to the application of lectins with known specificity as powerful tools to study the function of glycoconjugates, both in solution and on cell surfaces [1–6]. Lectins have been widely used for preparative and analytical purposes in biochemistry, cell biology, immunology and related areas [1–3, 7]. They are used for structural characterization of glycoconjugates of unknown structure, identification of lectin-reactive structures in biological materials (body fluids, cells, tissues), fractionation and

Written in bold letters are the mammalian carbohydrate structural units, as shown in Table 1 and Fig. 1.

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purification of glycoconjugates by affinity chromatography, comparative studies *etc*. The choice of proper lectin for such studies and its quality as a tool depends well-defined lectin specificity. Many lectins are apparently specific for a monosaccharide, but they react with various oligosaccharide chains terminating with this sugar with different affinities, because the type of linkage and underlying sugar residues also have significance. Quantitative differences in reactivity pattern exist between various lectins recognizing the same terminal monosaccharide residue [4, 5]. There are also lectins specific for more complex structures which are not inhibited by any monosaccharide. In the present article we describe the specificity of lectins studied by us, and the methods used to determine the lectin–carbohydrate interaction.

Grouping lectins based on recognition of monosaccharides and oligosaccharides

Lectins that can be used as tools to study the glycobiology system are defined as applied lectins. They must be easily obtained, stable, and well characterized in respect of specificity. Before detailed studies for powerful tools, the carbohydrate specificities of applied lectins are classified into six groups according to their specificities to monosaccharides [4, 5]. They are further subgrouped by the affinities to (a) GalNAcα1→*O* to Serine(Threonine) (Ser (Thr)) of the peptide chain; (b) mammalian disaccharide structural units; (c) trisaccharides; (d) the number and location of LFucα1→linked to Galβ1→3/4GlcNAc sequence; and (e) α2→, 3/6 linkages of sialic acid. These structures are frequently found in soluble glycoproteins and as cell surface glycoconjugates in mammals. A scheme of the classification based on monosaccharide specificity and oligosaccharide structures (described in Table 1) showing the highest affinity is shown as follows.

I. GalNAc-specific lectins
   1. **F/A**, GalNAcα1→3GalNAc (Forssman) and GalNAcα1→3Gal (Blood group **A** determinant disaccharide)—*Dolichos biflorus*, *Helix pomatia* and *Wisteria floribunda*.
   2. **A**, GalNAcα1→3Gal—Soybean agglutinin (SBA), Lima bean and *Psophocarpus tetragonolobus*.
   3. **Tn**, GalNAcα1→Ser/Thr—*Vicia villosa* B_4 (VVL-B_4) [8] and *Salvia sclarea* [9].

II. Gal-specific lectins
   1. **T**, Galβ1→3GalNAc ($\mathbf{T}_\alpha$ or $\mathbf{T}_\beta$)—Peanut, *Bauhinia purpurea alba* [10], *Abrus precatorius* [11], *Agaricus bisporus* [12], *Sclerotium rolfsii* [13], *Artocarpus integrifolia* (jacalin) [14], *Artocarpus lakoocha* [15], and *Maclura pomifera* [16], ricin [17] and *Morus nigra* (Morniga G) [18].
   2. **I/II**, Galβ1→3/4GlcNAcβ1→—*Ricinus communis* (RCA_1) [19], *Datura stramonium* (Thorn apple), Wheat germ (WGA) [20], *Erythrina cristagalli* [21], *Erythrina corallodendron* [22] and ricin [17].
   3. **B**, Galα1→3Gal—*Griffonia* (*Bandeiraea*) *simplicifolia* B4 [23].
   4. **E**, Galα1→4Gal—abrin-a, mistletoe toxic lectin-I (ML-I) [24] and *Aplysia* gonad [25, 26].

III. Man and/or Glc-specific lectins recognizing complex N-linked oligosaccharides—*Concanavalin ensiformis* (Jack bean), *Lens culinaris*, *Pisum sativum*, *Hippeastrum* hybrid, *Narcissus pseudonarcissus* and Morniga M [27].

IV. GlcNAc, and/or Galβ1→4GlcNAcβ1→linked specific lectins

Chitin oligosaccharide specific agglutinins—WGA [20], *G*. (*Bandeiraea*) *simplicifolia* II (GSA-II), *Solanum tuberosum*, *Ulex europaeus* (UEA) and *D*. *stramonium*.

V. LFuc-specific lectins (subgroups based on the numbers and location of LFucα linkage)
   1. Monofucosyl-specific agglutinins (blood group **H**, Le^a and/or Le^x)—UEA-I, UEA-II, *Pseudomonas aeruginosa* [28, 29] and *Anguilla anguilla* [30].
   2. Difucosyl-specific agglutinins (Le^b and $Le^{x/y}$) – GSA-IV and *Lotus tetragonolobus*.
   3. Others requiring further characterization—*Salmonella typhimurium* and *Ulva lactuca*.

VI. Sialic acid specific lectins (subgroups based on the recognized linkage of SA)
   1. SAα2→6Gal(NAc)—*Sambucus nigra* [31], *Trichosanthes japonica* and ML-I [24].
   2. SAα2→3Gal—*Agrocybe cylindracea* and *Maackia amurensis* [32].
   3. Others requiring further characterization—*Limax flavus* [33], *Limulus polyphemus* [34] and achatinin-H [35].

Profiles of the binding properties of lectins, based on the affinity of decreasing order of mammalian glycotopes (determinants) is probably one of the easy ways to express carbohydrate specificity and should facilitate the selection of lectins as structural probes for studying mammalian glycobiology.

Eleven mammalian structural units, shown in Fig. 1 and Table 1, have been selected to express the binding properties of Gal and GalNAc specific lectins. They are: (1) **F**, GalNAcα1→3GalNAc; (2) **A**, GalNAcα1→3Gal; (3) **T**, Galβ1→3GalNAc; (4) **I**, Galβ1→3GlcNAc; (5) **II**, Galβ1→4GlcNAc; (6) **B**, Galα1→3Gal; (7) **E**, Galα1→4-

Table 1 Carbohydrate structural units in mammalian glycoproteins and glycosphingolipids [4]

	Codes	Structural units	Sources
1	**F**	GalNAcα1→3GalNAc	Forssman pentasaccharide; animal tissue antigens and human oncofetal glycotopes, mainly in glycosphingolipids
	$\mathbf{F}_{\text{penta-}}$	GalNAcα1→3GalNAcβ1→3Galα1→4Galβ1→4Glc	
	$\mathbf{F}_{\alpha}$	GalNAcα1→3GalNAcα1→Ser/Thr of protein core	In O-linked glycoproteins core
	$\mathbf{F}_{\beta}$	GalNAcα1→3GalNAcβ1→	Glycotope at the nonreducing end of $\mathbf{F}_{\text{penta–}}$
2	**A**	GalNAcα1→3Gal	Human blood group A related **di-**saccharide
	$\mathbf{A}_{\text{h}}$	GalNAcα1→3[Lfucα1→2]Gal	Human blood group A related **tri-**saccharide
3	**Tn**	GalNAcα1→Ser/Thr of protein core	Tn antigen, only in O-linked glycoproteins
4	$\mathbf{T}_{\alpha}$	Galβ1→3GalNAcα1→Ser/Thr of protein core	The mucin-type sugar sequence on the human erythrocyte membrane
	$\mathbf{T}_{\beta}$	Galβ1→3GalNAcβ1→...ceramide	Brain glycoconjugates and gangliosides, GM_1
	I	Galβ1→3GlcNAc	Human blood group precursor type I and II carbohydrate sequences
5	$\mathbf{I}_{\beta}$	Galβ1→3GlcNAcβ1→	
6	**II**	Galβ1→4GlcNAc	Branched or linear repeated **II** sequence is part of blood group I and i epitopes. **I** and **II** are precursors of ABH and Le^a, Le^b, Le^x, Le^y blood group active antigens; most of the lectins reactive with **II** are also reactive with **I**. Lectin Tri-**II** and m**II** determinants are present at the nonreducing end of the carbohydrate chains derived from N- and O-glycans
	$\mathbf{II}_{\beta}$	Galβ1→4GlcNAcβ1→	
	Tri-**II**	Triantennary Galβ1→4GlcNAc	
	m**II**	Multivalent Galβ1→4GlcNAc	
7	**B**	Galα1→3Gal	Human blood group B related **di-**saccharide
	$\mathbf{B}_{\text{h}}$	Galα1→3[Lfucα1→2]Gal	Human blood group B related **tri-**saccharide
8	**E**	Galα1→4Gal	Blood group p^k and P_1 active disaccharide. Sheep hydatid cyst glycoproteins, salivary glycoproteins of the Chinese swiftlet, glycosphingolipids in human erythrocytes, and small intestine
9	**L**	Galβ1→4Glc	Constituent of mammalian milk
	$\mathbf{L}_{\beta}$	Galβ1→4Glcβ1→	Lactosyl ceramides in brain and part of carbohydrate structures in gangliosides
10	**P**	GalNAcβ1→3Gal	Blood group P related disaccharide; glycotope at the nonreducing end of globoside
	$\mathbf{P}_{\alpha}$	GalNAcβ1→3Galα1→	
11	**S**	GalNAcβ1→4Gal	Brain and asialo-GM_2 disaccharide; human blood group Sd(a+) related disaccharide in most human urine secretions, Tamm–Horsfall glycoprotein
	$\mathbf{S}_{\beta}$	GalNAcβ1→4Galβ1→	

α, β Anomer of sugars, *m* multivalent, *Tri* tri-antennary.

Gal; (8) **L**, Galβ1→4Glc; (9) **P**, GalNAcβ1→3Gal; (10) **S**, GalNAcβ1→4Gal; and (11) **Tn**, GalNAcα1→Ser (Thr) of the peptide chain. Thus, the information on carbohydrate specificity of Gal/GalNAc reactive lectins, divided into classes according to their highest affinity for one (or two) of the above disaccharide monomers and/or **Tn** residue, can be completed with data showing an order of reactivity with other disaccharides (Table 2) [4]. These data demonstrate that lectins reacting most strongly with the same oligosaccharide may show differences in order of reactivity with other structures that reflect subtle differences in lectin fine specificity.

Effect of polyvalence of glycotopes in glycan on lectin–glycoform interaction

During the past two decades, it has been observed that many multi-branched oligosaccharides exhibit a significant increment in lectin binding reactivity as compared to their linear counterparts [36, 37]. Based on the results of previous studies, the concept of glycoside cluster effect can be classified into two groups: (a) the 'multi-antennary or simple glycoside cluster effect' as in reaction of galactosides with hepatic lectin [38, 39] and tri-antennary **II** sequences reactive with a galectin from chicken liver (CG-16) [40], or **Tn** glycopeptides. The molecular sizes of these ligands are usually less than 1.5×10^4. The second group is (b) the 'high-density polyvalent or complex glycoside cluster effect', such as polyvalent **Tn** in asialo ovine submaxillary mucin, which generates an enhancement in affinity with VVL-B_4 by 3.3×10^5 and 4.5×10^3 times over Gal and GalNAc, respectively, and is about 1,000 times more active than monomeric **Tn** [8]. In Table 3, the much stronger inhibition of RCA_1 by a panel of cyst glycoproteins than by disaccharides and galactose is shown. A similar phenomenon was also observed with some animal lectins [41]. However, the polyvalences of glycotopes do not always significantly affect carbohydrate protein binding. For example, the potency in the interaction of *P.*

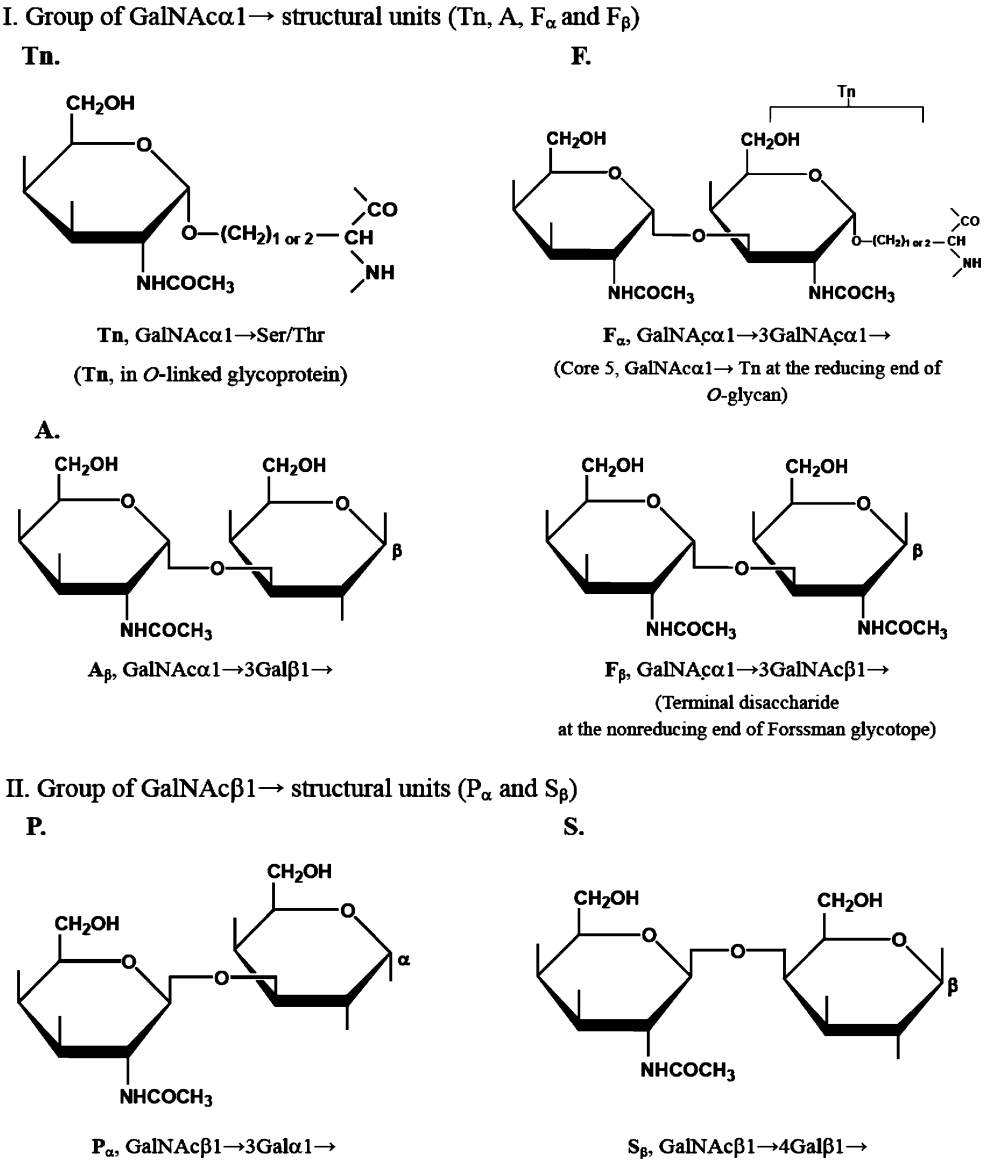


Fig. 1 Mammalian glycoconjugates structural units used to express and classify the carbohydrate specificity of lectins (Adopted and modified from Wang and Wu 2007 [81])

aeruginosa II lectin with LFucα1 polyvalent glycans is about as strong as or weaker than the incremental increase by carbohydrate specificity of monomers [28]. Therefore, to obtain a comprehensive picture of the carbohydrate specificities of a lectin in order to elucidate its functional roles and biomedical applications, the following information should be provided: (1) monosaccharide specificity (Gal, GalNAc, GlcNAc, Man, LFuc, and sialic acid), (2) reactivities to mammalian disaccharides and **Tn** structural units (in decreasing order; Table 2), (3) the most active ligand, (4) simple multivalent or cluster effect of carbohydrate structured units such as **Tn** glycopeptides and multi-antennary glycotopes to inhibit binding, and (5) complex multivalent or cluster effects present in macromolecules with known glycotopes.

The varying reactivities of lectins toward mammalian glycotopes and other sugar units, may reflect the possible existence of different combining sites or subsites in the same molecule, and the differential binding properties of these combining sites (if any) have to be characterized. Furthermore, effects of polyvalence on binding have to be studied. Establishing the relationship among the amino acid sequences of the combining sites of plant lectins and mammalian glycotopes can also be an important direction to be addressed in lectinology.

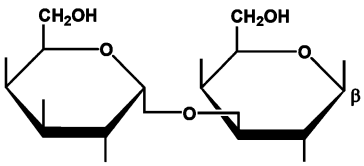
Overview of quantitative methods used to determine lectin–carbohydrate interactions

A similar methodical approach is used for the determination of lectin specificity and application of lectins as tools. It concerns the problem how to measure glycoconjugate–lectin interaction.

Fig. 1 (continued)

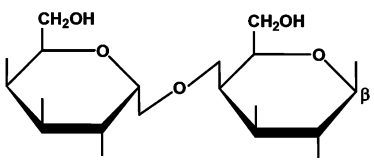
III. Group of Galα1→ structural units (B and E)

B.



B_β, Galα1→3Galβ1→

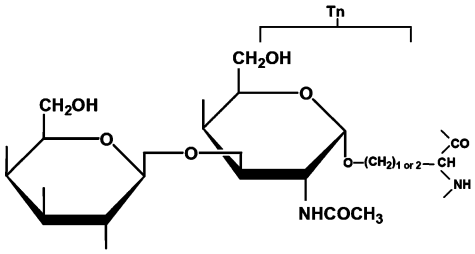
E.



E_β, Galα1→4Galβ1→

IV. Group of Galβ1→ structural units (T_α, T_β, L, I and II)

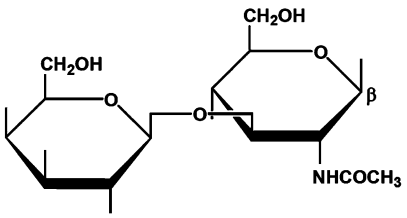
T.



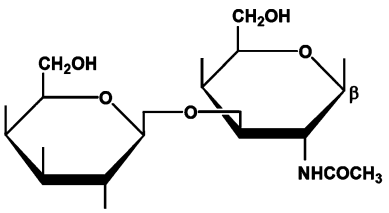
Tα, Galβ1→3GalNAcα1→Ser/Thr

(**Tα**, GalNAcβ1→3Tn at the reducing end of *O*-glycan)

I.

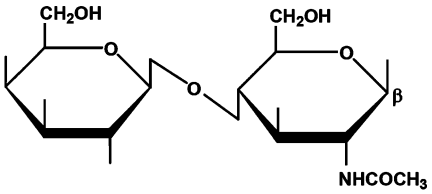


I_β, Galβ1→3GlcNAcβ1→



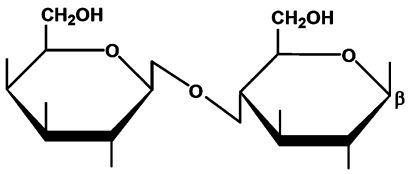
T_β, Galβ1→3GalNAcβ1→

(Terminal disaccharide at the asialo GM1; GSL)



II_β, Galβ1→4GlcNAcβ1→

L.



L_β, Galβ1→4Glcβ1→ (GSL)

In early research, when most lectins were detected by agglutination of erythrocytes (and for this reason were commonly called agglutinins), determination of lectin specificity by semiquantitative hemagglutination-inhibition assay was most frequently used. A more accurate approach was binding of radiolabelled lectins to erythrocytes or other cells [42].

A historical method, which did not require cells, was the quantitative precipitation assay (originally developed for antigen–antibody complexes) introduced in Kabat's laboratory [43]. Lectins precipitate serially diluted high molecular weight glycoconjugates and precipitation is inhibited by various mono- and oligosaccharides. Application of quantitative precipitin/precipitin-inhibition assays (QPA/QPIA) to study the binding properties of lectin glycotopes has several limitations, especially the fact that relatively large amounts of reagents (lectins, glycoproteins and inhibitors) are required. However, due to the accuracy of these methods, various versions have been used for many years in several laboratories. QPA and QPIA have also been

Table 2 Expression of binding properties of Gal/GalNAc reactive lectins by carbohydrate structural units

Codes	Lectins [36, 37, 74, 80-84]	Carbohydrate specificity
F/A	*Dolichos biflorus* (DBA)	$\mathbf{F}_{penta-} > \mathbf{A}_h^a > \mathbf{A} > \mathbf{Tn} >> \mathbf{P}$
	Helix pomatia (HPA)	$\mathbf{F}_{penta-} > \mathbf{A}(> \mathbf{A}_h^b) \geq \mathbf{Tn}, \mathbf{T} >> \mathbf{P}$
	Hog peanut (ABA, *Amphicarpaea bracteata*)	$\mathbf{F}_\beta > \mathbf{A} >> \mathbf{L}$
	Wistaria floribunda (WFA)	$\mathbf{A}(> \mathbf{A}_h^b)$, $\mathbf{F}_{penta-} > \mathbf{F/P} > \mathbf{Tn}$, **I/II** > **L**
	Geodia cydonium (GCA)	$\mathbf{F}_{penta-}$, $\mathbf{A}_h >$**L**> **II**, **T** >**I** >> **E**
	Griffonia (*Bandeiraea*) *simplicifolia*-A_4 (GSI-A_4)	$\mathbf{F}_{penta-} > \mathbf{A}_h >$ GalNAc > **E** > **B** > **I, T** >> **L, II**
F/II	*Caragana arborescens* (CAA)	$\mathbf{F}_{penta-} >$ **II** > sialyl **Tn**
	Wistaria sinensis (WSA)	$\mathbf{F}_{penta-} >$ **P** > **II**, **Tn**, **I** and $\mathbf{A}_h \geq$ **L**/**E**
A	Lima bean (LBA)	Hexa$-\mathbf{A}_h^a > \mathbf{A}_h^b >> \mathbf{B}$
	Soybean (SBA, *Glycine max*)	$\mathbf{A}(> \mathbf{A}_h^b)$, **Tn** and **I/II**
	Vicia villosa (VVA, a mixture of A_4, A_2B_2 and B_4)	$\mathbf{A}(> \mathbf{A}_h^b)$ and **Tn** mainly
Tn	*Vicia villosa* B_4 (VVA-B_4)	Two **Tn** >> one **Tn** >> one or two **T**
	Salvia sclarea (SSA)	Two **Tn** > single or three sequential **Tn** structures
	Glechoma hederacea (GHA)	**Tn** cluster > **Tn** > **A** > $\mathbf{A}_h$ > **F** >> **T**
T	Peanut (PNA, *Arachis hypogaea,*)	**T** >> **I/II** >> **Tn**
	Amaranthus caudatus (ACL)	**T** >> **Tn** >> **I/II**
T/Tn	*Codium fragile* subspecies *tomentosoides* (CFA)	$\mathbf{F}_{penta-}$ and $\mathbf{T}_\alpha >$ **Tn** cluster > $\mathbf{A}_h >$ **T**
	Agaricus bisporus (ABA)	$\mathbf{T}_\alpha$ and **Tn** > **I** >> GalNAc >> **II**, **L**
	Maclura pomifera (MPA)	**T** > **Tn** >> **I/II** and **L**
	Artocarpus integrifolia (jacalin, AIA)	$\mathbf{T}_\alpha > \mathbf{P}_\alpha >$ **T**, **Tn**, **II** > **I** >> $\mathbf{T}_\beta$
	Artocarpus lakoocha (ALA)	$\mathbf{T}_\alpha$, **Tn** cluster >> **T**, **Tn** >> **I/II**
	Bauhinia purpurea alba (BPA)	$\mathbf{T}_\alpha$, **Tn** cluster > **T**, **Tn** > **I/II**
	Morus nigra galactose-specific lectin (Morniga G)	$\mathbf{T}_\alpha >$ **Tn** cluster >> **T** > **Tn**, **P**, Tri-**II**[c]
T/II	*Ricinus communis* toxin (ricin, RCA_2)	**T** > **I/II** and **Tn**
	Abrus precatorius (APA)	**T** > **I/II** > **E** > **B** > **Tn**
	Sophora japonica (SJA)	**T** > **I** ≥ **II** > **L**
	Ricinus communis (RCA_1)	Tri-**II**[c] > **II** ≥ **I** > **E**, **B** > **T**
	Datura stramonium (TAA, thorn apple)	Biantennary **I/II** (penta-2,6) >> **C**[d]
I/II	*Erythrina cristagalli* (coral tree, ECA)	Tri-**II** > **II** > **L**, **I**
	Erythrina corallodendron (ECorL)	Tri-**II** > **II** > **L** > **I**
	Geodia cydonium (GCL)	Tri-**II** > **L** > **II**, **T** > **I**
	Phaseolus vulgaris-L	Tri-**II** > Penta-2,6 > Tri-2,6 > Hepta-3,6 > $\mathbf{II}_\beta$ > GlcNAcβ1,2Man
B	*Griffonia* (*Bandeiraea*) *simplicifolia* -B_4 (GSI-B_4)	**B** > **E** > **A**
	Abrus precatorius toxin-a (Abrin-a)	**E**, **B** > **T**, **L**, **I/II**
E	Mistletoe lectin-I (ML-I)	**E** > **II**, **L** > **T** and **I**

[a] Substitution of Fucα1→2 to subterminal Gal is important for binding.
[b] Substitution of Fucα1→2 to subterminal Gal blocks binding.
[c] Tri-**II**, triantennary **II** glycopeptides.
[d] **C**, chitin disaccharide.

successfully used by us to characterize the binding properties of lectins between the early 1970s and 1990s [44–46], until they were replaced by solid phase assays.

The enzyme-linked immunosorbent assay (ELISA), performed on 96-well microtiter plates, was introduced for determination of antibody activity. In the frequently used version, the antibody bound to the antigen-coated surface of the well was detected using a proper secondary enzyme-linked anti-immunoglobulin antibody. Direct adaptation of this method to lectins was initially based on binding the lectin followed by binding an anti-lectin antibody [47]. However, when several lectins are studied, obtaining an antibody for each lectin is troublesome and this technique was replaced by using labeled lectins, which could be detected by a universal anti-label reagent. One of the reagents for labeling lectins is digoxigenin (DIG) that can be detected with enzyme-linked anti-DIG antibody [48]. This method is relatively popular, because anti-DIG antibodies and DIG-labeled lectins are commercially available. An alternative and very convenient method, in which no antibody is used, is based on the biotin/avidin system. Lectins are biotinylated and detected with enzyme-conjugated avidin (alkaline phosphatase or horseradish peroxidase are most frequently used enzymes). This method was introduced in our laboratories [49] and is described in further part of this article.

In recent years, the microtiter plate methods have lead to micro-array techniques which have been described in

Table 3 Inhibitory potential of human ovarian cyst glycoproteins estimated by amount of cyst glycoproteins giving 50% inhibition of binding of RCA_1 (2.5 ng/50 μl) to asialo human α_1-acid gp (25 ng/50 μl)[a] [19]

Cyst glycoprotein/glycan	Quantity giving 50% inhibition (nanograms)	Mass (weight) relative potency[b]
Cyst OG 10% 2× ppt (**I/II**)	0.2	4.5×10^4
Asialo HOC 350 (**II**, **Le**[a])	4.0	2.3×10^3
HOC 350 (sialyl **II**, **Le**[a])	70.0	1.3×10^2
Cyst MSS 10% 2x ($\mathbf{A_h}$)	90.0	1.0×10^2
Cyst JS phenol insoluble (**H**)	90.0	1.0×10^2
Galβ1→4GlcNAcβ1→2Man ↓ α1-6 Galβ1→4GlcNAcβ1→2Manα1→3Manβ1→4GlcNAcβ1→4GlcNAcβ1-N-Asn ↑ β1-4 Galβ1→4GlcNAc Tri-antennary Galβ1→4GlcNAc (Tri-**II**)	2.1×10^3	4.3
Galβ1→4GlcNAc (**II**)	2.3×10^3	3.9
Gal	9.0×10^3	**1.0**
Galβ1→3GlcNAc (**I**)	1.5×10^4	0.6
GalNAc	8.8×10^5	**0.01**

[a] The inhibitory activity is expressed as the amount of inhibitor giving 50% inhibition; Total volume was 50 μl
[b] Relative potency=quantity of Gal required for 50% inhibition is taken as 1.0/quantity of sample required for 50% inhibition.

rapidly increasing number of publications [50–55]. Microarrays are solid surfaces which can carry a great number of glycans bound (covalently or noncovalently) as small spots. Binding of lectins to immobilized glycans allows rapid and exhaustive analysis of their specificity and is useful for detecting even weak interactions due to multivalent display of ligands on the surface. The various methods described in the literature differ in the ways of derivatization and immobilization of glycans and of determination of lectin binding (frequently fluorescence-based methods were used).

Interaction of lectins with glycoconjugates is also measured by affinity chromatography, using columns of immobilized lectins. The greater the affinity of oligosaccharide or glycopeptide to the lectin, the slower its elution from the column [56]. This method is useful not only for characterization of oligosaccharides, but also for their effective fractionation by using sequential chromatography on various lectin columns [57, 58]. Another approach is frontal affinity chromatography (FAC) [59], in which an excess volume of diluted fluorescently labeled glycan is applied to a lectin column. When the column is saturated with the glycan, it starts to leak and concentration of the glycan in the eluate increases, reaching the concentration equal to that of the applied solution. Comparison of the elution volumes of a ligand not reactive with the lectin and the glycan studied, allows detection of low-affinity interactions and calculation of dissociation constants. Recently, an automated FAC system has been developed, which allows fast analysis of multiple lectins and glycan samples [60].

An excellent method for observation of lectin–carbohydrate interaction 'in real time' is surface plasmon resonance (SPR) [61]. In this method one partner of the reaction is immobilized on a sensor chip channel and solution of the second partner flows over the sensor chip (association stage) that is followed by passing a buffer (dissociation stage). This method allows to follow the interaction and to determine the kinetic and thermodynamic parameters of the reaction. The SPR method was initially designed for measuring interaction between macromolecules, but recently techniques allowing to measure interactions of proteins with small molecules (*e.g.* lectins with glycans) have been developed. However, the SPR method requires an access to rather expensive highly computerized BIAcore or other instrument.

Biotin/avidin based microtiter plate enzyme-linked lectinosorbent assay

Efficient noncovalent binding of glycoproteins or other high-molecular weight glycoconjugates to MaxiSorp plates allows determination of binding of biotinylated lectins to the coated wells by using enzyme-conjugated ExtrAvidin (modified avidin from Sigma) and a proper enzyme substrate [49]. Biotinylation of lectins *via* amino groups is a simple procedure and does not alter their binding capacity [62]. Comparison of lectin binding to different immobilized glycoconjugates (glycoproteins or conjugates of an oligosaccharide with a carrier) or binding of various lectins to one glycoprotein (an example of the latter version is shown in Table 4) can be compared. However, binding assay may

Table 4 Characterization of binding properties of native and asialo-cyst glycoproteins (HOC 350) with established lectins by ELLSA [64]

Lectin	Determinants[a] (carbohydrate specificities)	Amount of lectin (ng)	1.5 (A_{405}) unit (ng)		Maximum A_{405} absorbance[b]		Binding intensity[b]	
			HOC 350		HOC 350		HOC 350	
			Native	Asialo-	Native	Asialo-	Native	Asialo-
DBA	F>A_h>A>Tn	20	–	–	0.01	0.0	–	–
VVL-B_4	mTn>>T	5	–	120.0	0.07	2.5	–	5+
Jacalin	mT, mTn>>I/II	10	–	4.0	0.7	2.6	1+	5+
BPL	T>I/II, Tn	5	–	5.0	0.8	2.6	1+	5+
WGA	mGlcNAcβ1→> mTn	5	200.0	22.0	3.7	4.3	5+	5+
RCA_1	II>I>B>T>>Tn	5	–	30.0	0.3	3.7	±	5+
ECL	I/II>B>Tn	10	–	400.0	0.02	3.3	–	5+
GSI-B_4	B>E>A	25	–	–	0.0	0.01	–	–
M-II	Mβ1→4C	20	–	–	0.1	0.1	–	–
PSA	Mβ1→4C	50	–	–	0.01	0.01	–	–
Lentil	Mβ1→4C	50	–	–	0.01	0.01	–	–

m Multivalent, ***M*** the trimannosidic core structure in N-linked glycoprotein, ***C*** GlcNAcβ1→4GlcNAc (chitin disaccharide)
[a] Carbohydrate specificity of lectins as expressed by lectin determinants shown in Table 1;
[b] Results were interpreted according to the measured A_{405} after 4 h of incubation as follows: 5+ (O.D.≥2.5), 4+ (2.5>O.D.≥2.0), 3+ (2.0>O.D.≥1.5), 2+ (1.5>O.D.≥1.0), 1+ (1.0>O.D.≥0.5), ±(0.5>O.D.≥0.2), and −(O.D.<0.2).

give rise to some uncertainties due to possible differences in coating efficiency or orientation of immobilized ligands. This problem can be overcome by binding of a lectin to one selected ligand and inhibition of the binding not only with oligosaccharides and monosugars, but also with high molecular weight glycoconjugates. This allows comparison of interaction of lectins with various monomeric and multivalent glycoforms in solution [8, 12, 18, 49, 63–65].

This method is accurate, sensitive and allows testing of multiple glycoconjugates [64, 65]. In this system, the amount of reagents required is greatly reduced (about 1,000-times) as compared to that needed for precipitation assays. When starting experiments with a new lectin, it is important to select a proper coating concentration of the target glycoform, because frequently with increasing coating concentration binding of the lectin increases to some point, and then strongly decreases [10, 49, 66]. Optimal binding usually occurred when solution used for coating contained the ligand at 0.2–2μg/ml concentration (10–100ng/50μl/well).

The biotin/avidin-based enzyme-linked lectinosorbent assay may be combined with partial deglycosylation of the glycoprotein by Smith degradation, which can be performed in solution or directly on the plate. For example, when Gal/GalNAc-specific lectins were studied, the plate was coated with asialoglycophorin A (asialo-GPA) containing Galβ1→3GalNAcα→ chains (**T** antigen), which was efficiently transformed on the plate into asialo-agalacto-GPA (**Tn** antigen) [49]. An 'artificial' **Tn** antigen obtained from GPA has several applications [67], it can replace the natural **Tn** antigen from rare **Tn** erythrocytes in studying **Tn**-specific lectins and antibodies. Smith degradation combined with lectin studies can also be applied to other glycoproteins to obtain information on the structure of glycans.

The biotin/avidin-based plate assay facilitated the study of fine specificity of multiple plant and bacterial lectins, where interaction of each lectin with several tens of high- and low-molecular mass glycoforms was examined [12, 18, 19, 21, 28, 68]. Similar studies were performed on galectins, rat galectin-4 (G4-N) and galectin-5, and chicken liver galectin CG-16 [40, 41, 63, 69, 70]. Generally, galectins recognize glycoforms with a terminal β-galactose residue. However, determination of their fine specificity showed quantitative differences in interaction with various oligosaccharide structures. For example, CG-16 showed preference in reactivity with oligosaccharides terminated with Galβ1→4GlcNAc unit, while G4-N reacted better with Galβ1→3GlcNAc-terminated ones. These subtle differences may have significance for functions of lectins *in vivo* and the knowledge of lectin specificity has potential for medical applications.

Methods

Biotinylation of lectins

Lectins were biotinylated *via* amino groups with biotinamidocaproate-*N*-hydoxysuccinimide ester (Sigma), which will be henceforth termed a biotin ester. A 0.025% solution

was obtained by dissolving 500μg of biotin ester in 50μl methanol and mixing this sample with 1.95ml phosphate-buffered saline (PBS; 0.02M phosphate buffer/0.15M NaCl, pH 7.4). A 0.1% lectin solution in PBS was mixed with double volume of biotin ester solution (weight ratio of lectin to the ester should be 2:1) and after 30min at room temperature the sample was dialyzed for several hours against water and overnight against Tris-buffered saline (TBS; 0.05 M Tris–HCl buffer/0.15M NaCl, pH 7.4). The sample was diluted with TBS and 20% sodium azide was added to a final concentration of 0.02% lectin and 0.1% NaN_3. This solution can be stored at 4°C for several months without loss of activity.

The microtiter plate lectin assay

The volumes of all reagents applied to the plate (Nunc, MaxiSorp) were 50μl/well, and all incubations, if not stated otherwise, were performed at room temperature using reagents diluted with TBS containing 0.05% Tween 20 (TBS-T). The plates were coated with a target ligand in 0.05M carbonate buffer, pH 9.6 overnight at 4°C. After washing the plate, biotinylated lectins were incubated in the wells for 30–60min. The plates were washed and the ExtrAvidin–alkaline phosphatase solution (Sigma, diluted 1:10,000) was added. After 1h the plates were washed at least four times and incubated with *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate 5-mg tablets) in 0.05M carbonate buffer, pH 9.6, for 30min, or longer, and the absorbance at 405nm was read in a microtiter plate reader (Fig. 2).

For inhibition studies, the serially diluted inhibitor samples were mixed with an equal volume and constant concentration of lectin solution (can be done in a separate microtiter plate). The control lectin sample was diluted twofold with TBS-T. The lectin concentration used for inhibition should be selected from the binding curve of serially diluted lectin to the plate coated with constant ligand concentration, and should be located below the saturating concentration, usually in the upper part of the decreasing arm of the curve. After 30min incubation at room temperature, the samples were tested in the binding assay. The inhibitory activity is expressed as the concentration of inhibitor giving 50% inhibition of the control lectin binding. All experiments were done at least in duplicate and the standard deviation was usually less than 5% of the mean value. The control wells, where addition of biotinylated lectin or coating were omitted, served as blank (absorbance below 0.1).

Smith degradation on ELISA plates

Wells coated with asialoglycoprotein were filled with 50μl of 0.05M sodium periodate in 0.05M acetate buffer, pH 4.5, and left overnight at 4°C (oxidation of sugars having two vicinal free hydroxyl groups). The plates were washed with TBS, and incubated with 50μl of 0.15M $NaBH_4$ in 0.1M borate buffer, pH 8.0, for 2–3h (reduction of aldehyde groups formed during oxidation). After washing, the plates were filled with 50μl of 0.025M sulfuric acid, tightly covered with parafilm and plastic cover, and incubated overnight at 37°C (selective release of oxidized/reduced sugars). After washing with TBS the plates were ready for the binding assay.

Lectinoblotting and modifications lectin ligands on the blots

This method allows identification of lectin-binding components in cell extracts or other biological material, which is fractionated by electrophoresis and blotted to nitrocellulose or polyvinylidene fluoride (PVDF) membranes. It is a qualitative method which is used to detect the lectin-binding glycoproteins in mixtures, *e.g.* to compare the lectin-binding patterns to glycoproteins of different cells, or cells at different stages of differentiation. The use of biotinylated lectins allows detection of reactive glycoproteins with high sensitivity. This method can also be combined with desialylation and Smith degradation of glycoproteins on the blot, thus allowing detection of cryptic lectin-reactive structures. Degradation on the blot is convenient, because modified glycoproteins are detected at their original positions that may further facilitate their identification. We first used this method to detect glycophorin Mi.III hybrid variant, which is relatively frequent in Asian populations [71]. Glycophorins are unique densely O-glycosylated red cell membrane glycoproteins which, after desialylation or desialylation/Smith degradation, can be specifically detected on the blots of erythrocyte membrane lysates with anti-**T** and anti-**Tn** lectins, respectively. Later, the method was used to characterize glycans of chicken glycophorins with lectins [72].

Some lectin-reactive glycotopes (*e.g.* ABH/Lewis) are present on O-glycans and N-glycans. To identify the characteristic of glycans to which the lectin is bound, the β-elimination of O-glycans by mild alkaline treatment of the blots was performed [73]. Nitrocellulose sheets are mechanically destroyed by alkaline treatment, and Immobilon P (PVDF) transfer membranes, which are not affected, were used. Best results were obtained by treating the blots with 0.055 MNaOH for 16h at 40°C. Sodium borohydride used in the β-elimination procedure was omitted because in these experiments the reduction of the released O-glycans was not necessary. The results obtained with erythrocyte membrane glycoproteins (used as a model) showed that peanut lectin detected specifically all glyco-

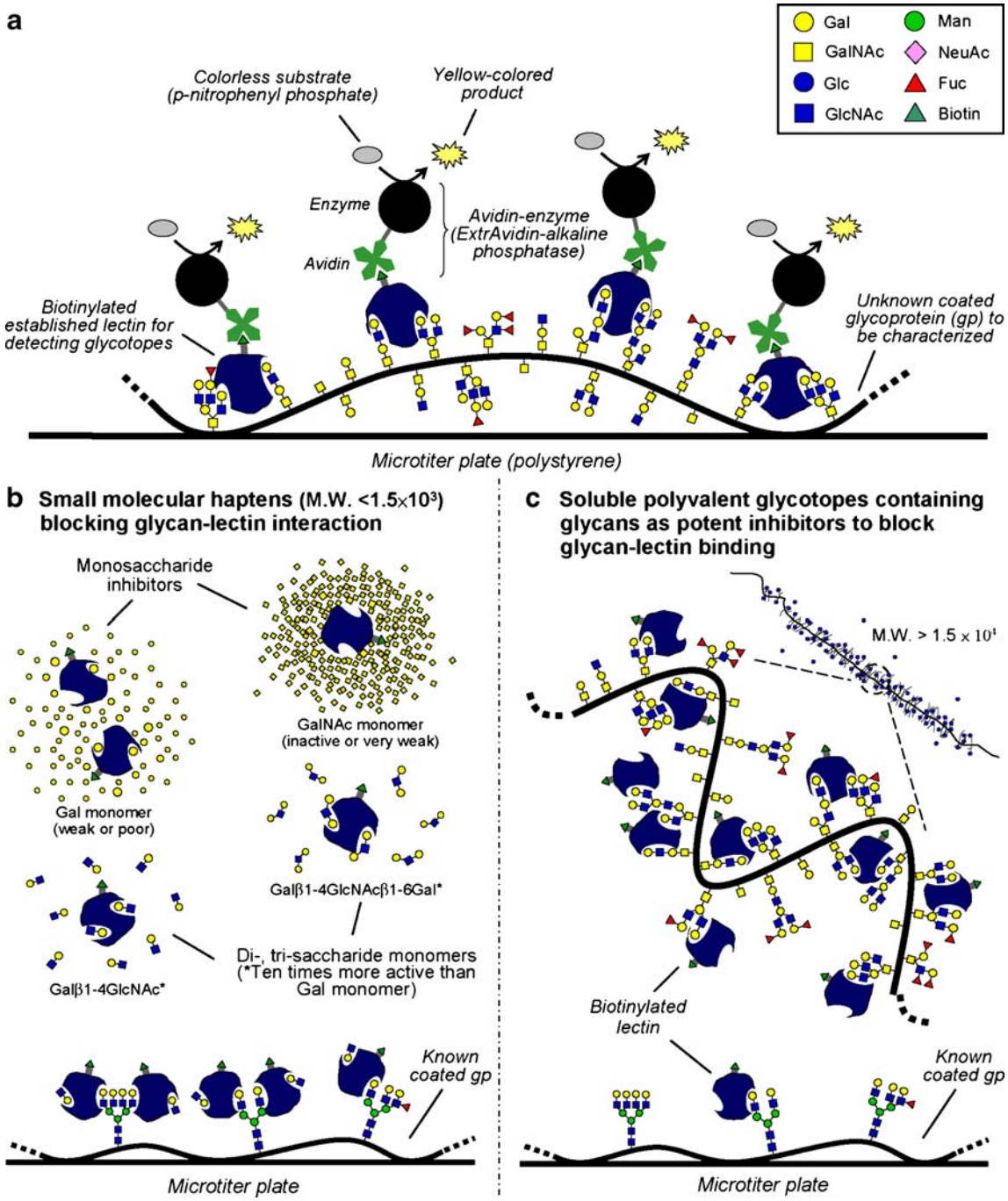


Fig. 2 Principle and an example (RCA_1) of (a) biotin/avidin based microtiter plate enzyme-linked lectinosorbent (ELLSA) and (b, c) inhibition assays as tools for characterizing binding properties of soluble glycoproteins, their inhibitory potential and/or determining carbohydrate specificities of lectins [19, 64]. **a** The uncharacterized glycoprotein immobilized on a microtiter plate can bind an established biotinylated lectin, for *e.g. R. communis* agglutinin-1 (RCA_1), which can recognize glycotopes (Galβ1→4GlcNAcβ) of unknown glycans. The overall activity of ExtrAvidin–alkaline phosphatase complexed to the biotinylated lectin can be estimated. **b** The biotinylated (known) lectin reacting with small haptens (M.W.$<1.5\times10^3$) or clusters (M.W. 1.5×10^3 to 1.5×10^4) in solution can compete with a known coated glycoprotein for binding. Similarly, **c** if a series of well-characterized glycans are precoated in the wells of a microtiter plate, an uncharacterized glycan (M.W.$>1.5\times10^4$), containing polyvalent glycotopes, can be used as an inhibitor to block the binding and its inhibitory potential toward known lectins can also be determined

phorin bands on desialylated blots, and no bands were detected on desialylated and then alkali-treated blots. On the other hand, the reactions of red cell membrane glycoproteins with *Phaseolus vulgaris* lectin (recognizing N-glycans) and anti-peptide antibodies were not affected. This method was used to show that sialyl-Lewis[a] receptors in human colon carcinoma cell line CX-1.1 were carried mainly by O-glycans of mucin-type glycoproteins.

The chemical modifications are cheaper and frequently more efficient than enzymatic deglycosylation with the use of glycosidases. However, Smith-degradation may be less conclusive than sequential glycosidase digestion, when applied to a glycoprotein with glycans of unknown structure.

Methods

Modifications of glycoproteins on blots

To desialylate glycoproteins, blots (nitrocellulose or Immobilon P) were incubated in 0.025M sulfuric acid for 1h at 80°C and washed with water. Smith degradation was performed by successive incubations of the desialylated blots in (1) 0.05M $NaIO_4$ in 0.05M acetate buffer, pH 4.5, overnight at 4°C; (2) 0.15M $NaBH_4$ in 0.1M sodium borate buffer, pH 8.0, for 2–3h at room temperature; (3) 0.025M sulfuric acid for 1h at 80°C. The blots were washed with water between the incubations. For β-elimination, the untreated or desialylated Immobilon P blots were overlaid with 0.055M NaOH, left for 16h at 40°C, and washed with water.

Detection of glycoprotein bands on blots with biotinylated lectins

The blots were blocked with 5% bovine serum albumin (BSA) for 1h at 37°C, or overnight at 4°C, and were overlaid with biotinylated lectin solution (usually 5–10μg/ml) in TBS-T for 1h at room temperature. The blots were washed 5-times for 10min each with 0,15M NaCl and overlaid with ExtrAvidin–horseradish peroxidase conjugate (Sigma; diluted 1:1,000 with TBS-T) for 1h at room temperature. After five to six washes, an enzymatic reaction was developed at room temperature with 4-chloro-1-naphthol (Sigma; 6mg in 2ml methanol, diluted with 10ml TBS and treated with 6μl H_2O_2). Alternatively, ExtrAvidin–alkaline phosphatase conjugate and its substrate, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (both from Sigma) can be used.

Interactions of lectins with glycosphingolipids

Glycosphingolipids have unique oligosaccharide sequences, but also share several glycotopes with glycoproteins [74]. Insolubility of glycolipids in water requires a different experimental approach to study their interaction with lectins and antibodies [75]. Glycolipids can be tested in a microtiter plate assay, but are applied in organic solvent and solvent-resistant plates must be used. Vinyl Assay Plates (COSTAR) are suitable for this purpose. Glycolipids are dissolved in a minimal volume of chloroform/methanol (2:1) and diluted with methanol (concentration of chloroform should not be higher than 5%). These samples are applied to the plates (20μl/well) and after organic solvents evaporate, the plates are ready to use. A commonly used method for fractionation of glycolipids is thin-layer chromatography (TLC) and detection of glycolipids with lectins on the TLC-plates. This method was very helpful in our studies on unique glycosphingolipids identified in rare polyagglutinable NOR erythrocytes.

Preliminary results suggested that the antigen responsible for polyagglutination of NOR erythrocytes is a neutral glycolipid. Therefore, the pool of neutral glycolipids from NOR erythrocytes was fractionated on a TLC-plate and probed with biotinylated lectins (Fig. 3). Three unusual glycolipid bands, absent in control erythrocytes, were detected, two strongly stained by *G. simplicifolia* isolectin B4 (GSL-IB_4, specific for Galα), and one by SBA (specific for GalNAcα/β). It greatly facilitated the isolation of these glycolipids and their structural studies, which showed them to be a globoside elongated with Galα1-4 (NOR1), Galα1-4GalNAcβ1-3Galα1-4 (NOR2) and GalNAcβ1-3Galα1-4 (NOR_{int}), respectively [76, 77].

During these studies the following observations were made: GSL-IB_4 did not react on the plate with P^k glycolipid (Galα1-4Galβ1-4Galβ1-Cer), and SBA did not or very weakly react with globoside (GalNAcβ1-3Galα1-4Galβ1-4Glcβ1-Cer; Fig. 3). These glycolipids contained proper terminal sugars and were present in much greater amount than NOR-related longer glycolipids mentioned above, were, strongly reactive with these lectins. A possible reason is the short oligosaccharide chain in P^k or globoside, and the inhibitory effect of closely located hydrophobic ceramide residue. However, it may not apply to all lectins. For example, *Moluccella laevis* lectin (anti-**Tn**) surprisingly bound to the P^k glycolipid on the TLC plate, but not in the microtiter plate assay [78]. Comparison of the activity of lectins recognizing an α or α/β form of the same sugar gave surprising results. *R. communis* agglutinin 1 (specific for Galα/β related ligands) reacted on the TLC plate with the NOR1 glycolipid only, while GSL-IB_4 (recognizing Galα residue) reacted strongly with NOR1 and NOR2 glycolipids (Fig. 3) [79]. Similarly, SBA recognized only the shortest blood group A glycolipid (migrating in the pentaglycosidic glycolipid region), while *H. pomatia* agglutinin (specific for GalNAcα1-3Gal(NAc)β) reacted strongly not only with the same blood group A glycolipid, but also with its elongated homologs (Fig. 3) [77]. These results indicate that binding of lectins to glycosphingolipids is influenced (in both directions?) by the lengths of their oligosaccharide chains. This binding may also depend on the method used, presumably due to different

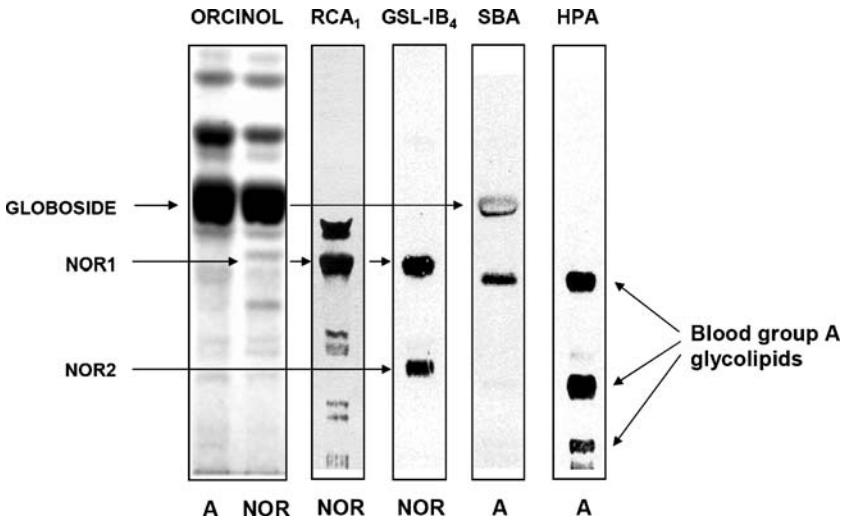


Fig. 3 Detection of human erythrocyte glycosphingolipids with lectins on TLC-plates. The plates containing fractionated glycolipids from blood group A or NOR erythrocytes were stained with orcinol or overlaid with lectins: RCA_1, *R. communis* agglutinin-1 recognizing Galα/β-related ligands; $GSL\text{-}IB_4$, *G. simplicifolia* isolectin B4 specific for Galα-terminating oligosaccharides; SBA, soybean agglutinin specific for GalNAcα/β; HPA, *H. pomatia* agglutinin specific for GalNAcα. NOR1 and NOR2, NOR-related glycolipids, both terminating with Galα1-4GalNAcβ1-3Galα-

conformation or orientation of glycosphingolipids on various surfaces. Therefore, the results of lectin binding to glycosphingolipids should be interpreted with caution, and influence of various factors on this binding is currently unknown.

Method

Binding of lectins to glycosphingolipids on thin-layer plates

Glycolipid samples solubilized in chloroform/methanol (2:1, *v*/*v*) were applied to high-performance TLC plates (Kieselgel 60, Merck) and developed with a solvent system selected for glycolipids studied. Erythrocytes neutral glycolipids were fractionated in chloroform/methanol/water (55:45:10, *v*/*v*/*v*). The dried plates were immersed in 0.05% polyisobutylmethacrylate (Aldrich) in hexane for 1 min, dried again, sprayed with TBS and immersed in 5% BSA for 1 h. The plates were overlaid successively with (1) biotinylated lectin solution (5 μg/ml) in TBS-T containing 1% BSA for 1 h; (2) ExtrAvidin–alkaline phosphatase conjugate (Sigma) diluted with TBS-T/BSA for 1 h; (3) a substrate solution (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, Sigma) until development of color.

Conclusions

Lectins with well defined specificity are excellent tools for several analytical and preparative purposes. They are used to identify and characterize the structure of glycans, when the amount of biological material is not sufficient for instrumental techniques, such as mass spectrometry or nuclear magnetic resonance, or lectins may be supplementary to instrumental research. The methods for determination of lectin–carbohydrate interaction described here do not cover the full spectrum of assays used. They are limited to the most frequently used techniques measuring interaction of lectins with soluble (or solubilized) lectin ligands, with special attention to the methods applied in our laboratories. There are several techniques for detection or determination of lectin-reactive glycoconjugates in cells or tissues, such as fluorescence-activated cell sorter, histochemistry, and others. The choice of the method depends on the purpose and material studied. Use of biotinylated lectins is convenient in many assays. Based on practical and economic considerations, the speed and the accuracy of the assay, the range of binding vision, and the amounts of glycans, lectins and ligands required, the highly sensitive microtiter plate assay by using biotinylated lectins should be one of the best approaches to analyze the specificity of combining sites of lectins and/or to characterize the binding properties (glycotopes) of complex carbohydrates and carbohydrate haptens. This system has been adopted in our labs for over a dozen years and successfully used for determination of specificity of several tens of lectins. As all methods have their own limitations, other methods can be used for advanced purposes, like crystallography for characterization of lectin-binding site, SPR for kinetic studies and other techniques.

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