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Fine sugar specificity of the mistletoe (*Viscum album*) lectin I†

HENRI DEBRAY[1]*, JEAN MONTREUIL[1] and HARTMUT FRANZ[2]

[1] *Laboratoire de Chimie Biologique, Unité Mixte de Recherche du CNRS No. 111, Université des Sciences et Technologies de Lille, F-59655 Villeneuve d'Ascq, France*

[2] *Universität Witten Herdecke, Institute of Phytochemistry, Berlin, Germany*

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The behaviour of *N*-acetyllactosamine-type oligosaccharides and glycopeptides on a column of mistletoe lectin I (MLI) immobilized on Sepharose 4B was examined. The immobilized lectin does not show any affinity for asialo-*N*-glycosylpeptides and related oligosaccharides, which possess one to four unmasked *N*-acetyllactosamine sequences. However, substitution of at least one of the *N*-acetyllactosamine sequences by sialic acid residues, either at O-3 or O-6 of galactose, slightly enhances the affinity of the lectin. Such sialylated *N*-glycosylpeptides or oligosaccharides are eluted from the lectin column by the starting buffer as retarded fractions. Surprisingly, the affinity of the immobilized MLI is higher for P1 antigen-containing glycopeptide isolated from turtle-dove ovomucoid and for glycopeptides from bovine thyroglobulin containing terminal non-reducing Galα1-3Gal sequences. These structures are strongly bound on the lectin column and their elution is obtained with 0.15 M galactose in the starting buffer.

Keywords: mistletoe (*Viscum album*) lectin I; sugar specificity; *N*-acetyllactosamine-type oligosaccharides; P1 antigen

Introduction

Three different lectins have been isolated from mistletoe (*Viscum album*) grown on the locust tree (*Robinia pseudo-acacia*) by affinity chromatography [1]. The three lectins present different molecular weights and monosaccharide specificities. The major mistletoe lectin I (MLI), composed of two different subunits, has a molecular weight of 115 000 and belongs to the D-galactose-specificity group whereas lectin II ($M_r = 60\,000$) and lectin III ($M_r = 50\,000$) are respectively D-galactose/*N*-acetyl-D-galactosamine- and *N*-acetyl-D-galactosamine-specific lectins [2].

Recently, the specificity of MLI was examined by haemagglutination-inhibition assay [3] and by inhibition of binding of the iodinated lectin to a solid-phase cluster ligand by different mono- or oligosaccharides [4, 5]. In this study, the fine sugar specificity of the MLI was examined by analysis of the behaviour of well-defined *N*-acetyllactosamine-type oligosaccharides and glycopeptides on an immobilized MLI-Sepharose 4B column. Preliminary results of this study have been previously reported [6].

* To whom correspondence should be addressed.

† In memory of Hartmut Franz.

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Materials and methods

Isolation and coupling of mistletoe lectin I

Mistletoe lectin I was isolated from mistletoe (*Viscum album*) grown on the locust tree (*Robina pseudoacacia*) by affinity chromatography on acid-treated Sepharose as described [1]. The lectin was coupled to Sepharose 4B that had been CNBr-activated according to the procedure of March *et al.* [7]. The amount of lectin bound to Sepharose was estimated to be 3 mg per ml of gel by subtracting the amount of unbound protein found in the supernatant and washing solutions after coupling. Protein was determined by the method of Lowry *et al.* [8].

Origin of glycopeptides and oligosaccharides

Oligosaccharides 1–3, shown in Table 1, with ^{14}C-labelled terminal non-reducing D-galactosyl groups, were gifts from Dr F Piller (Centre de Biophysique Moléculaire, Orléans). Oligosaccharides 4–12, 17–20 of Table 1, were isolated by Dr G. Strecker from the urine of patients with various lysosomal diseases [9], from mucins of swallows' nest (oligosaccharide 28) [10] and from the jelly coat of *Axolotl* eggs (oligosaccharide 27) [11]. Glycopeptides isolated from human serotransferrin (structure 13), from human lacto-

Table 1. Behaviour of *N*-acetyllactosamine-type oligosaccharides and glycopeptides on an immobilized mistletoe lectin I-Sepharose column.

Compound	*Glycan structure*	*Elution profile*		
		FNR	*FR*	*FE*
1	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcOMe	+		
2	Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcOMe	+		
3	Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)GlcOMe Gal(β1-4)GlcNAc(β1-3)	+		
4	Gal(β1-4)GlcNAc(β1-2)Man(α1-3)Man(β1-4)GlcNAc	+		
5	NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)Man(β1-4)GlcNAc		+	
6	Man(α1-6) Man(β1-4)GlcNAc Gal(β1-4)GlcNAc(β1-2)Man(α1-3)	+		
7	Man(α1-6) Man(β1-4)GlcNAc NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)		+	
8	Gal(β1-4)GlcNAc(β1-2)Man(α1-6) Man(β1-4)GlcNAc Gal(β1-4)GlcNAc(β1-2)Man(α1-3)	+		
9	Gal(β1-4)GlcNAc(β1-2)Man(α1-6) Man(β1-4)GlcNAc NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)		+	
10	NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-6) Man(β1-4)GlcNAc NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)		+	
11	NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6) Man(β1-4)GlcNAc NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)		+	
12	NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6) Man(β1-4)GlcNAc NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)		+	
13	NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6) Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3)		+	
14	Gal(β1-4)GlcNAc(β1-2)Man(α1-6) Fuc(α1-6) Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn Gal(β1-4)GlcNAc(β1-2)Man(α1-3)	+		

Table 1. *(continued)*

Compound	*Glycan structure*	*Elution profile*		
		FNR	*FR*	*FE*
15	NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Fuc(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) /		+	
16	Fuc(α1-3) \ Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Fuc(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) /		+	
17	Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Man(β1-4)GlcNAc Gal(β1-4)GlcNAc(β1-2)Man(α1-3) / Gal(β1-4)GlcNAc(β1-4) /	+		
18	Gal(β1-4)GlcNAc(β1-6) \ Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Man(β1-4)GlcNAc Gal(β1-4)GlcNAc(β1-2)Man(α1-3) /	+		
19	Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ GlcNAc(β1-4)—Man(β1-4)GlcNAc Gal(β1-4)GlcNAc(β1-2)Man(α1-3) /	+		
20	Gal(β1-4)GlcNAc(β1-6) \ Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Man(β1-4)GlcNAc Gal(β1-4)GlcNAc(β1-2)Man(α1-3) / Gal(β1-4)GlcNAc(β1-4) /	+		
21	Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn Gal(β1-4)GlcNAc(β1-2)Man(α1-3) / Gal(β1-4)GlcNAc(β1-4) /	+		
22	NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-6) \ Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) / NeuAc(α2-3)Gal(β1-4)GlcNAc(β1-4) /		+	

(continued)

Table 1. (*continued*).

Compound	*Glycan structure*	*Elution profile*		
		FNR	*FR*	*FE*
23	Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)GlcNAc(β1-2)Man(α1-6) Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn Gal(β1-4)GlcNAc(β1-2)Man(α1-3) Gal(β1-4)GlcNAc(β1-4)	+		
24	Man(α1-6) Man(α1-6) Man(α1-3) GlcNAc(β1-4)——Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn GlcNAc(β1-2)Man(α1-3) Gal(β1-4)GlcNAc(β1-4)	+		
25	Gal(α1-3)Gal(β1-4)GlcNAc(β1-6) [NeuAc(α2-6)]40% or Gal(β1-4)GlcNAc(β1-2)Man(α1-6) Fuc(α1-6) [Gal(α1-3)]60% Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn NeuAc(α2-6)Gal(β1-4)GlcNAc(β1-2)Man(α1-3) Gal(α1-3)Gal(β1-4)GlcNAc(β1-4)			+
26[a]	Gal(α1-4)$_3$ [Gal(β1-4)GlcNAc(β1-6) Gal(β1-4)GlcNAc(β1-4)Man(α1-6) Gal(β1-4)GlcNAc(β1-2) Man(β1-4)GlcNAc(β1-4)GlcNAc(β1-N)Asn NeuAc(α2-6)$_1$ Gal(β1-4)GlcNAc(β1-2)Man(α1-3) Gal(β1-4)GlcNAc(β1-4)]			+
27[b]	Fuc(α1-4)dNloA(α2-6) GalNAc-ol Gal(α1-4)Gal(β1-4)GlcNAc(β1-3) Fuc(α1-2)	+		
28	Gal(α1-4)Gal(β1-4)Gal(β1-4)GlcNAc(β1-6) GalNAc-ol NeuAc(α2-3)Gal(β1-3)	+		

[a] For oligosaccharide 26, brackets around the antennae mean that the exact position of the monosaccharide residues substituting the basic structure is not known.
[b] dNloA, 3-deoxy-D-glycero-D-galacto-nonulosonic acid.

transferrin (structures 14–16), from turtle-dove ovomucoid (structure 26) and from Cohn's fraction IV (structure 22) were gifts from Professor G. Spik. Glycopeptides 21 and 23 were isolated from human α1-acid glycoprotein after extensive pronase digestion as described [12]. Hybrid-type glycopeptide (structure 24) isolated from hen ovalbumin was a gift of Dr A. Kobata (Department of Biochemistry, University of Tokyo, Tokyo, Japan). Glycopeptides from

bovine thyroglobulin (structure 25) were isolated as described [13].

N-glycosylpeptide (structure 14) and several oligosaccharides (structures 4, 6, 8) were chemically desialylated with 0.05 M trifluoroacetic acid for 1 h at 80°C. The purity of all the oligosaccharides and glycopeptides was checked by ^{1}H-NMR spectroscopy.

Labelling of glycopeptides and oligosaccharides

Glycopeptides were labelled with [1-^{14}C]acetic anhydride (0.37–1.1 GBQ mmol^{-1}, The Radiochemical Centre, Amersham, UK) as described [14] and purified by gel filtration on a Bio-Gel P2 column (3 × 75 cm) equilibrated in water. Oligosaccharides were labelled at the reducing terminal monosaccharide residues by reduction with sodium boro[^{3}H]hydride (185–370 GBQ mmol^{-1}, The Radiochemical Centre, Amersham, UK) as described [15] and also purified by gel filtration on a column of Bio-Gel P2 equilibrated with water.

Affinity chromatography of oligosaccharides or glycopeptides on the the immobilized MLI-Sepharose 4B column

Labelled glycopeptides or oligosaccharides (2–30 × 10^{3} disintegrations min^{-1}, 0.1–10 nmol) were applied to the column of MLI-Sepharose 4B (1 × 10 cm) equilibrated at room tempereraturе in 10 mM sodium phosphate buffer (pH 7.2) containing 0.15 M NaCl (PBS). Elution was performed first with PBS at a flow rate of 9 ml h^{-1} and then with PBS containing 0.15 M galactose. Fractions (1.5 ml) were collected and aliquots counted in a Beckman LS-1800 scintillation counter. Recovery of radioactivity from the MLI-column was always >95%.

Results and discussion

The three elution profiles obtained when *N*-glycosylpeptides and related oligosaccharides were applied to an immobilized MLI-Sepharose column are shown in Fig. 1. Oligosaccharides or glycopeptides were eluted at the void volume of the column (fraction FNR; panel A) because of a lack of interaction between the immobilized lectin and the saccharides. Under the experimental conditions used, FNR was eluted between fractions 7 and 8. Other glycans, weakly interacting with the lectin, were eluted from the column by the starting buffer as retarded fractions (fraction FR, panel B). Under the experimental conditions used, FR were eluted between fractions 10 and 18.

The sharp elution profile obtained with 0.15 M galactose in starting buffer (peak FE, panel C) indicates a strong, specific interaction between the lectin and the bound saccharides. Table 1 describes the behaviour of 28 glycopeptides and oligosaccharides on the immobilized MLI. Although MLI belongs to the D-galactose-specificity group, the immobilized lectin presents no affinity for glycopeptides

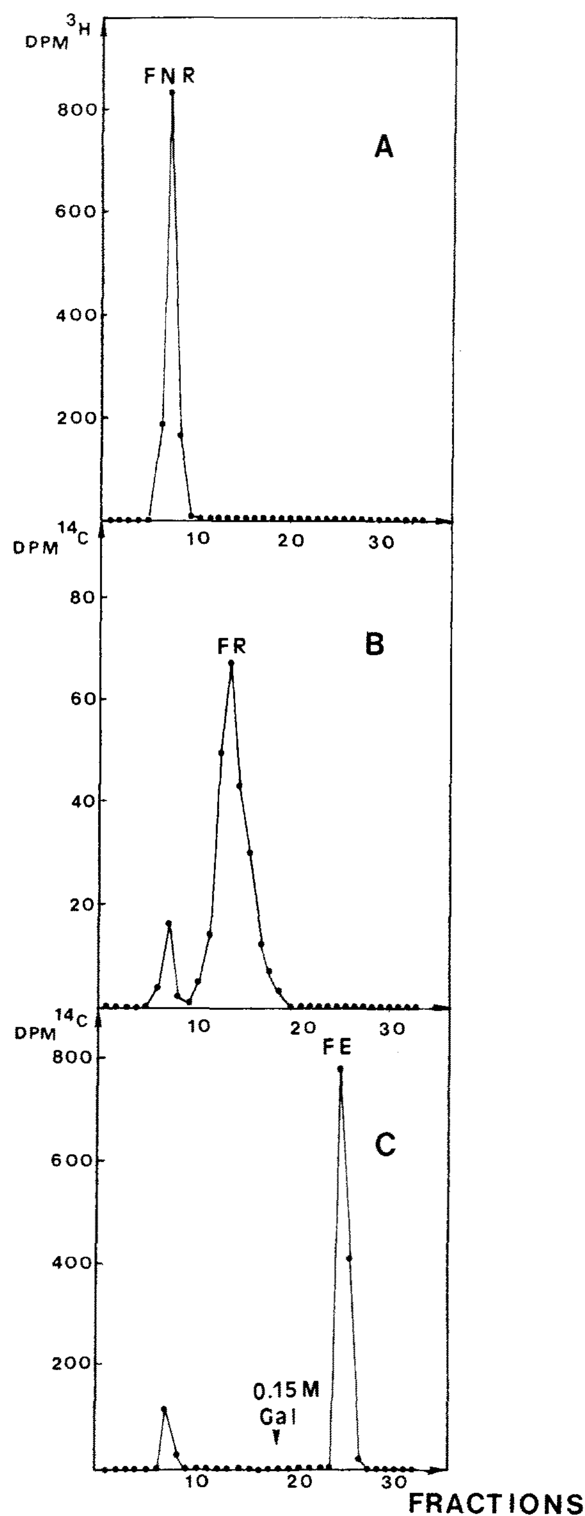

Figure 1. Elution profiles of *N*-glycosylpeptides or related oligosaccharides on an immobilized MLI-Sepharose column. Labelled glycopeptides or oligosaccharides (2–30 × 10^{3} disintegrations min^{-1}, 0.1–10 nmol) were applied to the lectin-Sepharose column (1 × 10 cm) equilibrated at room temperature in a 10 mM phosphate buffer pH 7.2 containing 0.15 M sodium chloride (PBS). Elution was first performed with this buffer at a flow rate of

(continued)

or oligosaccharides with one to four unmasked terminal non-reducing *N*-acetyllactosamine sequences, for example structures 6, 8, 17, 20, 23.

The lectin does not interact with the branched structure 3 with I activity and in which the two Galβ1-4GlcNAc sequences are β1-3- and β1-6-linked to a galactose residue. Oligosaccharide 2 with I activity and which also possesses two *N*-acetyllactosamine determinants but in a linear structure does not interact with the immobilized lectin. Immobilized MLI presents no affinity towards a hybrid-type glycopeptide from hen ovalbumin (structure 24) also possessing an unmasked Galβ1-4GlcNAc residue. Surprisingly, substitution of at least one of the *N*-acetyllactosamine sequences by a sialic acid residue either at O-3 (structures 5 and 9) or O-6 of galactose (structures 7 and 16) enhances the affinity of MLI. These sialylated structures are eluted from the lectin column by the starting buffer as retarded fractions. However, the strength of the interaction does not increase significantly as the number of such sialylated *N*-acetyllactosamine residues increases. For example, structures 7, 15 and 22 with one, two and three sialylated *N*-acetyllactosamine sequences respectively are retarded to the same extent on the lectin column.

MLI also exhibits the same affinity for a sialylated oligosaccharide (structure 12) as for the corresponding sialylated glycopeptide (structure 13). The presence of a fucosyl residue α1-3-linked to a peripheral *N*-acetylglucosamine residue (structure 16) and/or the presence of an α-L-fucose residue at the O-6 position on the N-acetylglucosamine involved in the *N*-glycosylamine linkage (structures 15 and 16) do not modify the interaction between the glycopeptides and the immobilized lectin. However, tetra-antennary *N*-acetyllactosamine-type glycopeptides, isolated from bovine thyroglobulin with two to three substitutions of the *N*-acetyllactosamine moieties by galactose in α-1-3-linkage (structure 25) [16] are strongly bound on the lectin column and are eluted with 0.15 M galactose in the starting buffer. Desialylation of these tetra-antennary glycopeptides does not alter the affinity of the lectin for these compounds. Moreover, the immobilized MLI also presents a strong affinity for a penta-antennary *N*-acetyllactosamine-type glycopeptide from turtle-dove ovomucoid (structure 26). This glycopeptide, which exhibits P1 serological activity, possesses one *N*-acetyllactosamine sequence substituted at O-6 of galactose by an *N*-acetylneuraminic acid residue together with three other *N*-acetyllactosamine sequences each substituted at O-4 of galactose by an α-D-galactose residue and one unmasked *N*-acetyllactosamine sequence [17]. It is strongly bound on the lectin column and its elution is obtained with 0.15 M galactose in the starting buffer. Desialylation of the penta-antennary glycopeptide does not modify the affinity of the lectin for this glycan suggesting that the Galα1-4Gal disaccharide units may represent the determinants recognized with high affinity by MLI. However, neither the oligosaccharide isolated from the jelly coat of *Axolotl* eggs (structure 27) with this terminal non-reducing Galα1-4Gal sequence but substituted at the O-2 position of the penultimate galactose by an α-L-fucose residue, nor the oligosaccharide isolated from mucins of swallows' nest with one Galα1-4Gal terminal non-reducing sequence but β1-4-linked to a galactose residue (structure 28) are recognized by MLI.

Our results confirm and extend data on the carbohydrate specificity of MLI recently obtained by Wu *et al.* [3] by a haemagglutination-inhibition assay showing that galabiose Galα1-4Gal and, to a lesser extent, Galα1-3Gal were the best disaccharide inhibitors for MLI. In our study, immobilized MLI presents the highest affinity for Galα1-4Gal-containing glycopeptide isolated from turtle-dove ovomucoid and for glycopeptides from bovine thyroglobulin with terminal non-reducing Galα1-3Gal sequences. From this point of view, the immobilized MLI could represent a valuable tool for both the fractionation and structural analysis of oligosaccharides and glycopeptides containing these particular saccharidic determinants.

However, the immobilized lectin does not interact at all with *N*-acetyllactosamine-type oligosaccharides or glycopeptides containing up to four terminal non-reducing *N*-acetyllactosamine residues. This result is at variance with those obtained by Wu *et al.* [3] showing that *N*-acetyllactosamine was only two times less active than galabiose in inhibiting erythrocyte agglutination. The apparent lack of interaction of structures containing unmasked *N*-acetyllactosamine residues with immobilized MLI could be explained by assuming that, similarly to immobilized Concanavalin A, *Ricinus communis* agglutinin and *Erythrina* lectins [18–20], immobilized MLI only binds glycans having association constants in solution greater than a minimal value; for Concanavalin A and the *R. communis* lectin, this value was found to be $4 \times 10^6\ \text{M}^{-1}$ [18, 19].

The fact that asialofetuin represents a good ligand for MLI (4) whereas asialoglycopeptides prepared from it are not recognized by the lectin can be explained, because the asialoglycoprotein exhibits at least 14 terminal non-reducing galactose residues per molecule [21]. This yields an avidity effect between the asialoglycoprotein and the lectin while its asialoglycopeptides with only one to three of such galactose residues are not recognized. Surprisingly, the affinity of the immobilized lectin is higher for *N*-acetyllactosamine-type oligosaccharides or glycopeptides with *N*-acetyllactosamine sequence(s) substituted by sialic acid residue(s) either at O-3 or O-6 of galactose. All these sialyated structures, weakly interacting with the immobilized

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$9\ \text{ml h}^{-1}$ and then with PBS containing 0.15 M D-galactose. Fractions (1.5 ml) were collected and aliquots counted by liquid scintillation in a Beckman LS-1800 scintillation counter. A, B and C correspond respectively to profiles obtained with compounds 19 (non-retained fraction FNR), 15 (retarded fraction FR) and 26 (eluted fraction FE).

MLI, are eluted from the column by the starting buffer as retarded fractions.

In this respect, MLI is very similar to other lectins also presenting a specificity towards either α2-3-sialylated *N*-acetyllactosamine residues such as *Maackia amurensis* leukoagglutinin [22] or α2-6-sialylated *N*-acetyllactosamine sequences such as the *Sambucus nigra* agglutinin [23] or the *Allomyrina dichotoma* lectins [24, 25]. From this point of view, immobilized MLI provides another valuable tool for the fractionation of unmasked *N*-acetyllactosamine-containing oligosaccharides and glycopeptides which do not interact with the lectin and oligosaccharides or glycopeptides with terminal NeuAc α2-6/α2-3Galβ1-4GlcNAc sequences which are retarded on the lectin column.

Mistletoe lectin I [2], as well as the *Ricinus communis* toxin RCA_{II} [26] and agglutinin RCA_{I} [27], are glycoproteins consisting of A and B chains linked by disulfide bonds. A chains are powerful toxins while B chains harbour the sugar-binding sites. However, using the same methodology we used here to study MLI specificity, it was shown that, in contrast to immobilized MLI, both RCA_{I} and RCA_{II} interact strongly with oligosaccharides or glycopeptides containing unmasked *N*-acetyllactosamine residues [28, 29]. Moreover, whereas terminal α-2-6-linked sialic acid partially inhibits the interaction of oligosaccharides with RCA_{I}, terminal α-2-3-linked sialic acid abolishes interaction with the immobilized lectin. In contrast, α-2-3- and α-2-6-linked sialic acid equally inhibit but do not abolish oligosaccharide interaction with RCA_{II} [28, 29].

As already emphasized by Wu *et al.* [3], the digalactoside Galα1-4Gal found in glycosphingolipids of mammalian cell membranes, where it can serve as a specific receptor for P adhesins of uropathogenic *Escherichia coli* [30–32], or in glycoproteins of egg white of pigeons and doves [33, 34], may represent the saccharidic determinant recognized by the B chain of the cytotoxic MLI at the cell surface. However, our results show that the terminal non-reducing Galα1-3Gal sequence, characterized in some glycolipids [35] and in glycoproteins such as murine laminin [36] and bovine thyroglobulin [16], may also represent potential binding sites for the B chain of MLI at the cell surface.

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