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The sugar-combining area of the galactose-specific toxic lectin of mistletoe extends beyond the terminal sugar residue: comparison with a homologous toxic lectin, ricin *

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ABSTRACT

Viscumin (the major lectin of mistletoe extract), also known as ML-1, and ricin (RCA II) belong to a group of heterodimeric toxic lectins composed of an A chain, which inhibits protein synthesis, and a B chain, which mediates entry into the cell in a galactose-specific manner. Although most of the binding force for the association of viscumin with galactose-containing ligands is generated by the nonreducing terminal galactose residue, a particular hydroxyl group on the penultimate sugar also appears to participate in the binding, suggesting that viscumin has an extended combining site. In this paper, we give further examples of affinity enhancement by the hydroxyl group situated on the penultimate sugar next to the glycosidic linkage of the terminal galactose. The structure with highest affinity for viscumin thus far discovered is β-D-Gal-(1 → 2)-β-D-Gal. In contrast to viscumin, ricin does not have this extended binding area, as none of the disaccharides tested exhibited significant affinity enhancement.

INTRODUCTION

The major lectin of mistletoe extract, viscumin or ML-1, is a heterodimeric toxic lectin composed of toxic A chain and galactose-binding B chain[1]. There are a number of toxic lectins with this construction in the plant kingdom, the best studied being ricin (RCA-II) from castor beans.

Recently, we reported the binding specificity of ML-1 investigated by an inhibition assay[2]. In that study, a possibility of viscumin having an extended combining area beyond the terminal Gal residue was explored using many disaccharide structures having Gal at the nonreducing terminus. Out of 14 such

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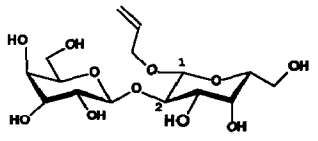
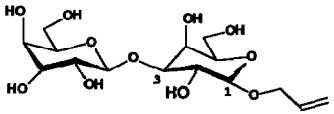


β-D-Gal-(1→2)-β-D-Gal-(1→O)-All



β-D-Gal-(1→3)-β-D-Gal-(1→O)-All

Fig. 1. Structures of β-D-Gal-(1 → 2)-β-D-Gal-(1 → O)-All and β-D-Gal-(1 → 3)-β-D-Gal-(1 → O)-All. The putative interacting OH group on the penultimate Gal is shown in large, boldface type.

disaccharide structures tested, two [allyl (All) β glycosides of β-D-Gal-(1 → 2)-D-Gal and β-D-Gal-(1 → 3)-D-Gal] were found to have binding affinity significantly higher (10–50 fold) than the others. A structural feature on the penultimate sugar that is common to these two, and only these two, disaccharides is the presence of an equatorial OH group close to the intersugar glycosidic linkage, as shown in bold face in Fig. 1. We surmised that this OH group interacts favorably with the sugar-combining area of viscumin.

To obtain further support for this hypothesis, β-D-Gal-(1 → 2)-D-Glc, β-D-Gal-(1 → 3)-D-Glc, and β-D-Gal(1 → 3)-D-Man derivatives were synthesized[3]. Both β-D-Gal-(1 → 2)-D-Glc and β-D-Gal(1 → 3)-D-Glc derivatives have the putative "interacting" equatorial OH group, while β-D-Gal(1 → 3)-D-Man structure has an axial OH group at the corresponding position. Inhibition assays with these and other Gal-terminated disaccharide glycosides showed that the disaccharide structures which possess the OH group next to the intersugar glycosidic linkage (i.e., where R^1 or R^2 = OH in Fig. 2) exhibited affinity enhancement greater than 5-fold, and the disaccharide structure of β-D-Gal-(1 → 2)-β-D-Gal- had the highest affinity to viscumin (33-fold increase in affinity over Gal).

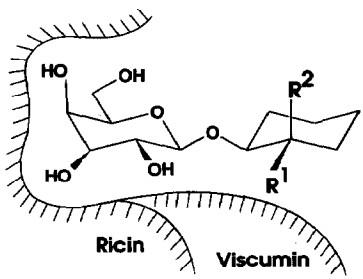


Fig. 2. The combining site of viscumin and ricin for Gal-disaccharides.

In contrast to viscumin, none of the disaccharides and glycosides tested bound to ricin with any significant affinity enhancement over that of galactose.

RESULTS AND DISCUSSION

The results of inhibition assays for viscumin is shown in Table I. The table includes data on newly synthesized disaccharides as well as earlier data[2]. Disaccharides and disaccharide glycosides are divided into four groups based on their binding affinity (Groups 1 to 4 in the order of decreasing affinity). The I_{50} values of galactose and some simple galactosides are in the range 0.5 to 1 mM (ref 2). Thus, disaccharides in Groups 1–3 all have variously enhanced affinity. The structures of all the β-2-, β-3-, and β-4-linked disaccharides are drawn in a "ribbon" configuration, as found for N-acetyl-lactosamine by X-ray crystallographic analysis, and nature of the functional group situated next to the intersugar glycosidic linkage on the front side of the penultimate sugar (R^1 or R^2, see Fig. 2) is compared. All the members in Groups 1 and 2 had an equatorial OH group at this position ($R^1 = OH$), while Group 3 disaccharides had an axial OH group ($R^2 = OH$). The majority of Gal disaccharides belong to Group 4; it includes all the β-(1 → 4)-linked disaccharides ($R^1 = CH_2OH$), all the β-(1 → 3)-linked Gal-

TABLE I

Binding affinity of Gal-terminated disaccharides and disaccharide glycosides to viscumin

	I_{50} [a] (mM)
Group 1	
β-D-Gal-(1 → 2)-β-D-Gal-(1 → O)-All [b]	0.025
β-D-Gal-(1 → 2)-β-D-Gal-(1 → O)-Pr [b]	0.03
Group 2	
β-D-Gal-(1 → 3)-β-D-Gal-(1 → O)-All	0.08
β-D-Gal-(1 → 3)-α-D-Glc-(1 → O)-All	0.08
β-D-Gal-(1 → 2)-D-Gal	0.1
Group 3	
β-D-Gal-(1 → 3)-α-D-Man-(1 → O)-All	0.22
β-D-Gal-(1 → 3)-α-D-Man-(1 → O)-Bn [b]	0.15
β-D-Gal-(1 → 3)-D-Ara	0.18
β-D-Gal-(1 → 2)-D-Glc	0.14
Group 4	
β-D-Gal-(1 → 2)-α-D-Glc-(1 → O)-All	0.48
β-D-Gal-(1 → 4)-D-Glc	0.65
β-D-Gal-(1 → 4)-D-HexNAc	0.36–0.43
β-D-Gal-(1 → 3)-α-D-GlcNAc-(1 → O)-Bn	0.42
β-D-Gal-(1 → 3)-α-D-GalNAc-(1 → O)-All	0.3
β-D-Gal-(1 → 6)-α-D-GlcNAc-(1 → O)-Bn	0.4
α-D-Gal-(1 → 6)-D-Glc	0.65
α-D-Gal-(1 → 3)-β-D-Gal-(1 → O)-Pr	0.5
α-D-Gal-(1 → 2)-β-D-Gal-(1 → O)-Pr	0.45
α-D-Gal-(1 → 2)-α-D-Glc-(1 → O)-All	0.35

[a] Some I_{50} values are from ref 2. [b] Abbreviations are as follows: Al, allyl; Pr, *n*-propyl; Bn, benzyl.

HexNAc disaccharides ($R^1 = NHCOCH_3$), all the (1 → 6)-linked disaccharides, and all the α-linked disaccharides. Thus, disaccharides in Group 4 lack an OH group at R^1 (or R^2). A sole exception is allyl α-glycoside of β-D-Gal-(1 → 2)-D-Glc, which, despite having an OH group as R^1, belongs to Group 4. The strongly enhanced affinity of disaccharides belonging to Group 1 and 2 gives further support to the hypothesis that the equatorial OH next to the intersugar glycosidic bond ($R^1 = OH$) is interacting with the lectin binding site, perhaps via hydrogen bonding[2]. However, a somewhat lower affinity enhancement by a corresponding axial OH (Group 3 disaccharides, $R^2 = OH$) and no enhancement by β-D-Gal-(1 → 2)-α-D-Glc-(1 → O)-All together suggest that the above interpretation is too simplistic.

Interestingly, of all the disaccharide glycosides with $R^1 = OH$, those having the highest [β-D-Gal-(1 → 2)-β-D-Gal-(1 → O)-All/Pro] and the lowest affinity [β-D-Gal-(1 → 2)-α-D-Glc-(1 → O)-All] are both β-D-Gal-2-linked. As shown in Fig. 1, the 2-linked disaccharide glycosides have their aglycon situated immediately next to the intersugar glycosidic linkage. We thought that this proximity of aglycon to the Gal residue may alter the relative orientation of the two sugar rings. For instance, the β-aglycon of a β-D-Gal-(1 → 2)-D-Hex disaccharide projects out towards the Gal residue and crowds into its CH_2OH group. If the disaccharide assumes the conformation of lactose, the allyl aglycon cannot rotate freely. It is conceivable, therefore, that the two rings will rotate against each other to a more suitable orientation (Fig. 3). In the case of an α-aglycon, as in β-D-Gal-(1 → 2)-α-D-Glc-(1 → O)-All, the steric interference is less obvious; still it is possible that the sugar rings may rotate to the opposite direction (Fig. 3). The fact that β-D-Gal-(1 → 2)-β-D-Gal-(1 → O)-All/Pro structures have the highest affinity suggests that the most preferred position of the OH group may be somewhere between the equatorial and axial positions when the two sugar rings assume the lactose-type conformation (Fig. 3).

To find out if the presence of an α- or β-aglycon on β-D-Gal-(1 → 2)-linked disaccharides does affect the binding affinity, the aglycon of β-D-Gal-(1 → 2)β-D-Gal-(1 → O)-All and β-D-Gal-(1 → 2)α-D-Glc-(1 → O)-All was removed to produce two corresponding reducing disaccharides. The removal of the allyl group caused the binding affinity of β-D-Gal-(1 → 2)-β-D-Gal-(1 → O)-All to decrease (the I_{50} rising from 0.03 to 0.1 mM) and that of β-D-Gal-(1 → 2)α-D-Glc-(1 → O)-All to increase (the I_{50} decreasing from 0.5 to 0.14 mM). These results suggest that the aglycon of a 2-linked disaccharide does indeed alter the conformation. It is likely that the structure with the highest affinity for viscumin is represented by β-D-Gal-(1 → 2)-β-D-Hex- structure, where Hex is a pyranose having equatorial 2- and 3-OH groups. The oligosaccharide chains with this structure at the nonreducing terminus, if extant in nature, may be the most potent natural ligands for viscumin.

We wanted to determine whether such an extended binding area also exists in other toxic lectins of the same molecular construction. This group includes ricin, abrin, moddecin, and probably a lectin from *Trichosanthes kirilowii*[4]. Results of

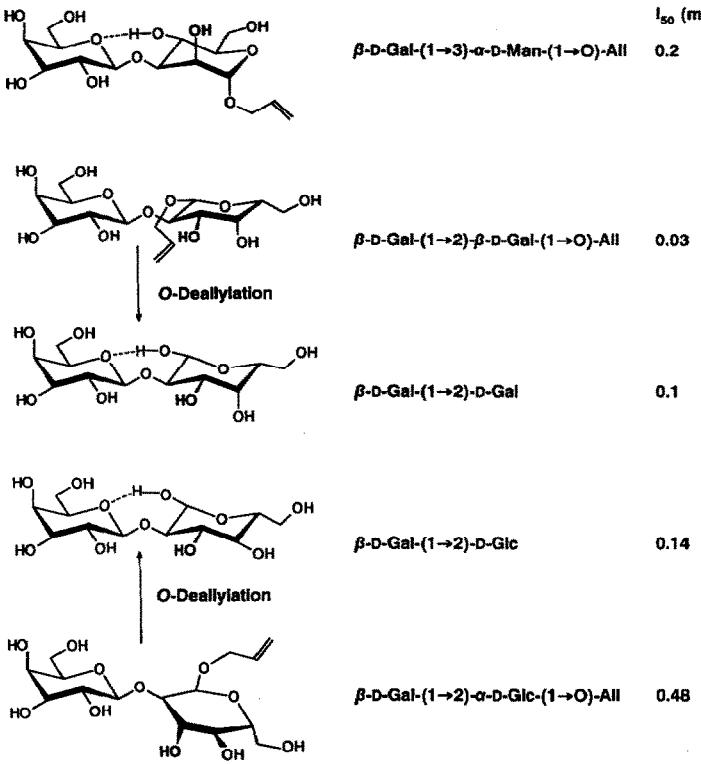


Fig. 3. Probable conformations of β-D-Gal-(1 → 2)- and β-D-Gal-(1 → 3)-linked disaccharides and their allyl glycosides and their corresponding I_{50} values.

inhibition assays using ricin are shown in Table II. For comparison, the corresponding I_{50} values of viscumin are also included. For ricin, the I_{50} values of all the disaccharide glycosides, as well as Gal and galactosides, are without exception in the range 0.3 to 1 mM, suggesting that there is little interaction between the penultimate sugar and the ricin binding site. This result agrees with what was determined from the X-ray crystallographic study, which showed that the Glc residue of lactose appeared to have little contact with the ricin binding area[5]. Rivera-Sagredo et al.[6] synthesized all the possible monodeoxy derivatives of lactose and tested their affinity for ricin. Deoxygenation on the glucose ring in general had only a small effect, the 3-deoxy derivative being 2-fold better and the 6-deoxy derivative, which is a 2-fold worse inhibitor than the parent compound (methyl β-lactoside). In contrast, deoxygenation on the Gal ring (3′-, 4′- and 6′-deoxy) caused decreases in the affinity of 30- to 60-fold, and even the smallest decrease by the 2′-deoxy derivative was 6-fold, indicating again that the interaction between OH groups and the ricin binding site is limited to the Gal group.

Table II also shows that viscumin and ricin, in general, have similar I_{50} values for various Gal-containing ligands. For instance, Gal and lactose have I_{50} values of

TABLE II

Binding of mono- and di-saccharides to ricin and viscumin

	I_{50} (mM)	
	Ricin	Viscumin [a]
D-Gal	1.0	0.9
D-GalNAc	1.7	120
D-Fuc	1.4	3.4
L-Ara	5.0	13
D-Gal6Me	3.3	ND [b]
2d-D-lyxHex [c]	5.8	11.3
α-D-Gal-(1 → O)-Me	0.84	0.48
β-D-Gal-(1 → O)-Me	0.4	0.65
α-D-Gal-(1 → O)-All	0.61	1.0
β-D-Gal-(1 → O)-All	0.38	1.0
β-D-Gal-(1 → O)-SCH_2CN	0.48	0.2
3d-D-xylHex-(1 → O)-All [d]	30	34
4d-D-xylHex-(1 → O)-Pr [e]	NI (20) [f]	~ 70
β-D-Gal-(1 → 2)-β-D-Gal-(1 → O)-All/Pr	0.7	0.03
β-D-Gal-(1 → 2)-α-D-Glc-(1 → O)-All	1.0	0.42
α-D-Gal-(1 → 2)-β-D-Gal-(1 → O)-Pr	0.75	0.45
β-D-Gal-(1 → 3)-β-D-Gal-(1 → O)-All	0.52	0.08
β-D-Gal-(1 → 3)-α-D-Glc-(1 → O)-All	0.4	0.08
α-D-Gal-(1 → 3)-β-D-Gal-(1 → O)-Pr	0.62	0.5
β-D-Gal-(1 → 3)-α-D-GlcNAc-(1 → O)-Bn	0.4	0.42
β-D-Gal-(1 → 3)-α-D-Man-(1 → O)-All	0.74	0.22
β-D-Gal-(1 → 3)-α-D-GalNAc-(1 → O)-All	0.7	0.3
β-D-Gal-(1 → 3)-D-Ara	0.3	0.2
β-D-Gal-(1 → 4)-D-Glc	0.4	0.5
β-D-Gal-(1 → 4)-D-GlcNAc	0.4	0.4
β-D-Gal-(1 → 4)-D-ManNAc	0.3	0.43
α-D-Gal-(1 → 6)-D-Glc	0.88	0.6
β-D-Gal-(1 → 6)-α-GlcNAc-(1 → O)-Bn	0.5	0.45

[a] Some data for viscumin are taken from ref 2. [b] Not determined. [c] "2-Deoxy-D-galactose." [d] "Allyl 3-deoxy-D-galactoside." [e] "Propyl 4-deoxy-D-galactoside." [f] Not inhibiting at the highest concentration tested (20 mM).

0.9 and 0.5 mM, respectively, for viscumin, and 1 and 0.4 mM for ricin. Also similar is the fact that the axial 4-OH group is the most important OH group, followed by the equatorial 3-OH group. There are some differences in the binding characteristics of the two lectins. While simple aglycons (methyl and allyl), either in the α or β orientation, hardly improve the binding affinity to viscumin, a β methyl or β allyl aglycon enhances affinity to ricin by at least 2-fold. The most obvious difference between the two lectins at the monosaccharide level is the effect of the substituent at C-2. Affinity of GalNAc for viscumin is 120-fold lower than Gal, while the difference between Gal and GalNAc for ricin is less than 2-fold. A higher detrimental effect of 2-*N*Ac on binding to viscumin appears to be

mainly due to the steric effect[2]. We speculated that the HO-2 of Gal is closely apposed to the combining area of viscumin, and there is virtually no room to accommodate a group larger than a hydroxyl group. Obviously, for ricin, there is a enough room to accommodate an acetamido group. This effect together with the presence of an interacting OH group at R^1 suggests that the disaccharide ligand is in close contact with the viscumin surface at the front side of the disaccharide as shown in Fig. 2, while the ricin combining site does not make contact in this area.

A considerable difference exists among toxic lectins when their behavior on the binding as well as their toxic effect on various cell types was examined[7–9]. Although all these lectins recognize Gal residues, it is tempting to speculate if subtle differences in the binding characteristics, such as shown here for viscumin and ricin, might modulate the biological behavior of these toxic lectins.

EXPERIMENTAL

Materials.—Preparations of all the Gal-(1 → 2)- and Gal-(1 → 3)-linked disaccharide glycosides have been reported[3]. Viscumin was prepared as described[10]. Ricin (RCA-II) was obtained from E.Y. Labs., Inc. (San Mateo, CA).

General methods.—Iodination of viscumin and ricin was carried out by the Chloramine-T method[11], using 0.5 mCi of $Na^{125}I$, 10 or 20 μg of lectin, and a low concentration of Chloramine-T (0.5 mg/mL). The affinity of various sugars for viscumin and ricin was determined using an inhibition assay as described[2]. Briefly, ^{125}I-labeled viscumin or ricin was incubated at 25°C for 1 h with Sepharose beads modified with 6-aminohexyl lactoside (LacAH-Seph) in the absence or presence of an inhibitor (test sugar) at various concentrations. The amount of ^{125}I-lectin bound to the beads was determined by a γ-counter after the beads had been separated from the incubation mixture by rapid centrifugation through an oil layer (with a density slightly higher than the aqueous layer). A semi-log plot of the degree of inhibition was made against the inhibitor concentration (on the logarithmic axis) scale. The concentration that gave 50% reduction in binding (I_{50}) was determined from each sigmoidal curve.

Binding of ^{125}I-ricin to LacAH-Seph.—Important parameters for binding of ^{125}I-ricin to LacAH-Seph, such as the time required to reach maximum binding and proportionality between the amount of ^{125}I-ricin bound and its concentration in the incubation mixture, were determined as described for viscumin[2]. The time required to reach equilibrium binding at 25°C was 1 h. At a set concentration of ^{125}I-ricin, the total amount of radioactivity bound to an increasing amount of LacAH-Seph showed a typical saturation curve and reached as high as 95% of the input radioactivity, suggesting that the iodinated ricin retained full binding affinity. Under the incubation conditions used for inhibition assays, the amount of ^{125}I-ricin bound was proportional to its concentration in the incubation mixture.

Removal of the allyl aglycon.—The allyl aglycon was hydrolyzed using the method of Boss and Scheffold[12]. To the solutions of β-D-Gal-(1 → 2)-β-D-Gal-(1

→ O)-All, and β-D-Gal-(1 → 2)-α-D-Glc-(1 → O)-All, each 40 mg in 0.6 mL of water, were added 10% palladium-on-charcoal (10 mg) and p-toluenesulfonic acid monohydrate (~ 12 mg), and the suspension was stirred for 6 h at 80°C. Although allyl β-D-galactopyranoside and allyl α-D-glucopyranoside were cleanly removed by overnight treatment under these conditions and produced a single product, Gal and Glc, respectively, allyl glycosides of the disaccharides produced products from hydrolysis of the intersugar glycosidic linkage in addition to the desired reducing disaccharide, β-D-Gal-(1 → 2)-D-Gal or β-D-Gal-(1 → 2)-D-Glc. For this reason, the mixture, after filtration and evaporation, was fractionated by the silica gel chromatography (1.3 × 25 cm) using 3:2:1 EtOAc–glacial acetic acid–water (solvent *A*) as eluant. Fractions containing the products were combined and evaporated. β-D-Gal-(1 → 2)-D-Glc had R_f 0.26, which was identical to that of lactose, while β-D-Gal-(1 → 2)-D-Gal moved slightly slower (R_f 0.24). ^{1}H NMR spectra of the two products showed the expected peaks: for β-D-Gal-(1 → 2)-D-Gal (D_2O): δ 5.49 (d, *J* 3.6 Hz, H-1α), 4.68 (d, *J* 8.2 Hz, H-1β), 4.57 (d, *J* 7.4 Hz, H-1′β); for β-D-Gal-(1 → 2)-D-Glc (in D_2O): δ 5.46 (d, *J* 3.6 Hz, H-1α), 4.73 (d, *J* 8.0 Hz, H-1β), 4.57 (d, *J* 7.4 Hz, H-1′β). The ratio of the H-1α and H-1β peaks was ~ 1:1 for both compounds, and H-1α plus H-1β roughly equaled the H-1′β peak. To further confirm the reducing nature of the products, each was treated with $NaBH_4$, which transformed them into compounds with lower R_f-values than the starting disaccharides. The R_f-values from TLC in solvent *A* were for lactitol, 0.20; β-D-Gal-(1 → 2)-D-Glc-ol, 0.21; and β-D-Gal-(1 → 2)-D-Gal-ol, 0.22. These results suggest that the products were indeed reducing disaccharides, β-D-Gal-(1 → 2)-D-Gal and β-D-Gal-(1 → 2)-D-Glc, respectively. For the inhibition assays, concentrations of the disaccharide solutions were determined by the phenol–H_2SO_4 method[13] using solutions of Gal and a mixture containing equal concentrations of Gal and Glc as standards.

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