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Carbohydrate binding specificity of the T_n-antigen binding lectin from *Vicia villosa* seeds ($VVLB_4$)

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2-Dansylamino-2-deoxy-D-galactose (GalNDns) is a useful fluorescent probe to study the interaction of non-fluorescent sugars with the B_4 lectin from *Vicia villosa* seeds ($VVLB_4$). Binding of the lectin to GalNDns leads to a 5.2-fold increase in Dansyl fluorescence with a concomitant 10 nm blue shift in its emission maximum. The strong binding of GalNDns ($K_a = 7.33 \times 10^4\ M^{-1}$ at 20°C) is due to a favourable entropic contribution to the association process. Among the other sugars studied, GalNAcα1-*O*-Ser followed by MeαGalNAc are the best ligands. 2-Deoxygalactose, galactosamine and galactose are 2013, 469 and 130 times weaker ligands, respectively, as compared to GalNAc, whereas GalNDns is about 2.44 times more potent than GalNAc, indicating that substitutions at the C-2 position of GalNAc have a considerable influence on the binding affinities. Equatorial orientation of the hydroxyl group at C-3 and axial orientation at C-4 as in galactose are important for the interaction with $VVLB_4$. The C-6 hydroxyl group is not indispensable. The binding site of the lectin is directed exclusively towards monosaccharides alone. Interestingly enough, despite its preference for MeαGalNAc over MeβGalNAc, in oligosaccharides, the lectin prefers terminal β-linked GalNAc as compared to the α-linked one.

Vicia villosa lectin; 2-Dansylamino-2-deoxy-D-galactose; Monosaccharide binding; Orientation of hydroxyl group

1. INTRODUCTION

Lectins are carbohydrate binding proteins of non-immune origin, which have been used widely in cell biology, biochemistry and histochemistry to isolate and/or to characterize cell surface carbohydrates and glycoproteins [1–3]. Lectins require configurational and structural complementarity of sugars for interaction to occur. All lectin molecules have two or more carbohydrate binding sites, a property essential for their ability to agglutinate cells or to precipitate complex carbohydrates [1,2,4].

Vicia villosa (Hairy winter vetch) seeds contain at least three lectins, designated B_4, A_2B_2 and A_4, that are composed of two different subunits with M_r 35,900 (subunit B) and 33,600 (subunit A) [5]. The A_4 lectin agglutinates A_1 erythrocytes specifically, but is not the predominant lectin in *Vicia villosa* seeds. B_4, the predominant isolectin agglutinates T_n exposed erythrocytes specifically but does not agglutinate A, B or O-erythrocytes [5]. A_2B_2 isolectins, as expected, display properties characteristic of both A_4 and B_4 isolectins [5].

In 1978, Kimura and Wigzell [6] reported that a surface glycoprotein on mouse T-lymphocytes appeared after immune activation by major histocompatibility complex alloantigens or polyclonal activation with concanavalin A. Expression of this glycoprotein was shown to correlate both in time and extent to the levels of cytotoxicity generated. This glycoprotein (M_r 145,000) which appeared to be selectively expressed on Lyl^-2+ cytotoxic T-lymphocytes was designated T145. *Vicia villosa* agglutinins have attracted much interest with regard to their biological properties since an uncharacterized lectin preparation was reported to bind specifically to this glycoprotein and *Vicia villosa* affinity adsorbant was used to fractionate allogen and mitogen activated blasts into highly cytotoxic and non-cytotoxic cell populations [7]. B_4 lectin was further shown to bind to a L-PHA resistant mouse lymphoma cell line and to erythrocytes having exposed *N*-acetylgalactosamine residues α-linked to serine or threonine (Tn-antigen) [8].

Some information regarding its saccharide specificity is available from hemagglutination inhibition and binding of radio-labeled lectin to immobilized saccharides [8,9]. However, the information obtained by these methods is semiquantitative and hence there is a need for detailed studies of the saccharide binding properties of the lectin. Moreover, an accurate determination of the association constants is necessary for evaluating the thermodynamic parameters associated with the bind-

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Abbreviations: GalNDns, 2-dansylamino-2-deoxy-D-galactose; Dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; $VVLB_4$, *Vicia villosa* B_4 seed lectin; T_n-antigen, GalNAcα1-*O*-Serine/Threonine; GalNAc, *N*-acetyl-galactosamine; Gal, galactose; Meα (or β)Gal, methyl-Meα (or β)-galactopyranoside; Meα (or β)GalNAc, methyl-2-acetamido-2-deoxy-α (or β)galactopyranoside. All sugars are D-sugars.

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ing, which in turn will yield valuable information regarding the forces involved in the interaction as well as information about the nature and dimensions of the combining region, i.e. whether the combining site is extended or not. We report here that *N*-dansylgalactosamine (GalNDns) is an useful fluorescent probe to study the interaction of non-fluorescent sugars to the $VVLB_4$.

Piller et al. [9] have recently reported that $VVLB_4$ could bind to both the terminal as well as internal GalNAc structures, though the evidence for the latter is not apparent from their data. Our data on the other hand suggest that the lectin binds to the terminal GalNAc structure alone.

2. MATERIALS AND METHODS

2.1. *Materials*

$VVLB_4$, GalNAc, MeαGal, MeβGal, 2-deoxygalactose, fucose, galactosamine, Gal and melibiose were products of Sigma Chemical Co., St. Louis, MO, USA. Lactose was obtained from BDH, Poole, England. Gulose, talose, Forssman pentasaccharide, GalNAc(α1–3)Gal, Gal(β1–6)GalNAc and GalNAcα1-*O*-Ser were obtained from Biocarb Chemicals, Lund, Sweden. Gal(α1–3)GalαOMe, GalNAc(β1–3)GalαOMe and Gal(β1–3)GalNAcαOMe were purchased from Carbohydrate International, Arlov, Sweden. GalNDns was synthesized as described earlier [11,12]. Gal(α1–3)Gal and Gal(β1–3)GalNAc were prepared as in [19]. MeαGalNAc and MeβGalNAc were prepared according to the method of Sarkar and Kabat [13]. All other chemicals were of analytical grade.

2.2. *Fluorescence measurements*

The fluorescence spectra were recorded with a Shimadzu RF-5000 Fluorescence Spectrophotometer. The samples were excited at 330 nm and the emission spectra recorded above 450 nm, with slitwidth of 5 nm for both monochromators (Fig. 1).

Titrations of fluorescence for the binding of GalNDns to $VVLB_4$ were performed on a Union Giken FS501A fluorescence polarizer, essentially as described earlier for the binding of GalNDns to other lectins [12,14].

The association constant (K_a) was determined graphically from plots of $\log[P]_f$ vs. $\log\{(F_c-F_o)/(F_\infty-F_c)\}$ (Fig. 2a) according to Eq. 1 [10],

$$\log\{(F_c-F_o)/(F_\infty-F_c)\} = \log K_a + \log [P]_f \quad (1)$$

where F_o, F_c and F_∞ are the fluorescence intensities of GalNDns in the absence, in the presence, and at infinite concentration of $VVLB_4$, and $[P]_f$ is the free protein concentration. Binding of non-fluorescent, inhibitory sugars (L) was studied by monitoring their ability to displace GalNDns (M) from its complex with $VVLB_4$ in substitution titrations. Association constants for the indicator ligand (K_M) and the competing ligand (K_L) were determined from the plots of $[L]_f$ vs. $\{[P]_t/[PM]-1\}\cdot[M]_f$ (Fig. 2b) according to Eq. 2 [17,20].

$$\{[P]_t/[PM] - 1\}\,[M]_f = K_L/K_M\,[L]_f + 1/K_M \quad (2)$$

The enthalpy of interaction was obtained from Van 't Hoff plots of the temperature dependent association constants. Free energy of interaction was obtained from Eq. 3

$$\Delta G = -RT\ln K_a \quad (3)$$

and the entropy changes were obtained from Eq. 4

$$\Delta S = (\Delta H-\Delta G)/T \quad (4)$$

2.3. *Other analytical methods*

Protein concentration was determined by the method of Lowry et al. [15] using bovine serum albumin as a standard. The protein concentration was expressed in terms of a protomer (M_r 35,900). The concentration of GalNDns was determined spectroscopically using a molar absorptivity of 4800 cm^{-1} at 330 nm [16]. Concentration of the other sugars used for titration were determined by weight measurements. All experiments were carried out in PBS.

3. RESULTS

The fluorescence intensity of GalNDns increases about 5.2-fold at infinite protein concentration as determined by a double reciprocal plot of the fluorescence intensity ($1/F$) vs. the protein concentration ($1/[P]$), with a concomitant 10 nm blue shift in the emission maximum. Addition of inhibitory saccharides totally reversed this effect (Fig. 1). The protein concentration-dependent changes in the fluorescence intensities of GalNDns were used to elucidate its K_a for binding to the lectin and the stoichiometries involved therein according to the method of Chipman et al. [10]. A representative plot for GalNDns binding to $VVLB_4$ at 20°C is shown in Fig. 2a. The lectin has 4 binding sites per tetramer for this sugar and an association constant of $7.33 \times 10^4\ M^{-1}$ at 20°C. The binding of non-fluorescent sugars to $VVLB_4$ was studied by following the decrease in the fluorescence of $VVLB_4$–GalNDns complex due to the release of GalNDns bound to $VVLB_4$ upon addition of the inhibitory sugar. A plot for the competitive binding of MeαGalNAc in presence of GalNDns at 20°C is shown in Fig. 2b. The association constant values obtained by this method for various sugars at 20°C are listed in Table II. The binding of some of the sugars to $VVLB_4$ at various temperatures was also studied by this method. The association constant, K_a, for all these sugars decreases with an increase in temperature (Fig. 3).

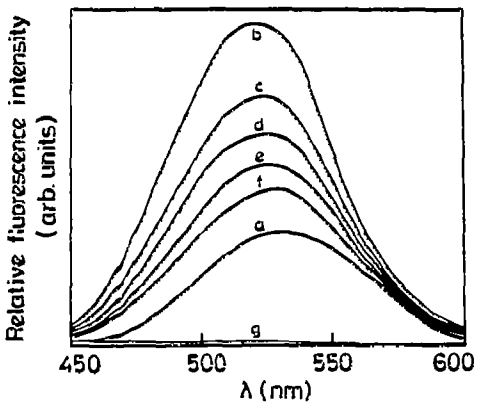

Fig. 1. Binding of GalNDns to $VVLB_4$ and reversal by MeαGalNAc. The fluorescence intensity (curve a) of GalDNns (3.70 μM, 1.8 ml) enhanced significantly in the presence of 15.1 μM $VVLB_4$ (75 μl, 378 μM stock (promoter)) (curve b). The fluorescence intensity decreased (curves c–f) after the addition of various aliquots (c, 10; d, 20; e, 35; f, 45 μl) of MeαGalNAc (2.5 mM). Curve (g) is a buffer control.

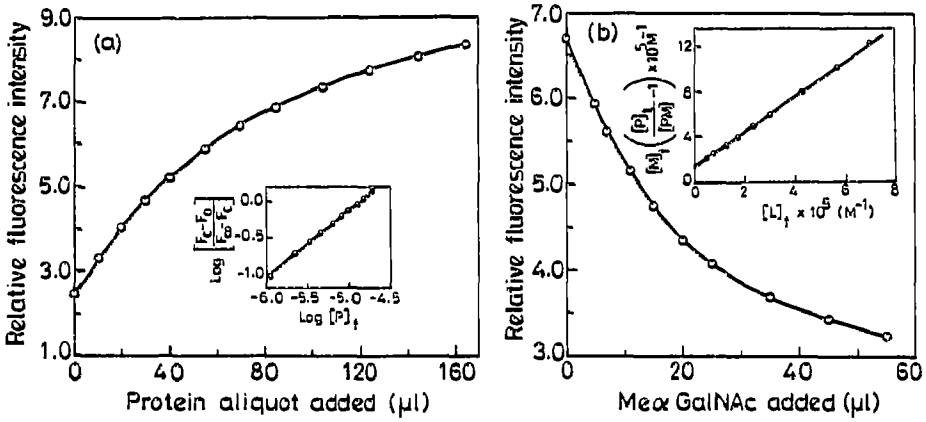

Fig. 2. Titration of GalNDns with $VVLB_4$ at 20°C (a). Competitive binding of MeαGalNAc to $VVLB_4$ in the presence of GalNDns (b). (a) The fluorescence intensity is plotted as a function of the added protein concentration. A 3.70 μM solution of GalNDns (1.8 ml) in PBS was titrated with 256.9 μM $VVLB_4$ (protomer). The inset gives a graphical representation for the determination of the association constant (K_a = 7.33 × 10^5 M^{-1}). (b) To a 3.70 μM solution of GalDNns (1.8 ml) was added 75 μl of $VVLB_4$ (378 μM) at 20°C. The mixture was then titrated with MeαGalNAc (2.5 mM). The decrease in fluorescence intensity was plotted as a function of MeαGalNAc added. From the Y-axis intercept (inset) the K_a for GalNDns was determined to be K_a = 7.34 × 10^5 M^{-1}, and the slope yielded a value of 1.14 × 10^5 M^{-1} for MeαGalNAc at 20°C.

The enthalpy and entropy changes associated with the interaction of these sugars with $VVLB_4$ are listed in Table I.

The strong binding to GalNDns, in contrast to other monosaccharides, is due to a relatively positive entropic contribution (ΔS = −2.29 $JM^{-1}K^{-1}$, ΔH = −27.96 KJM^{-1}) as revealed by thermodynamic parameters (Table I). Binding of this sugar to the lectin also shows that it can accommodate a large hydrophobic substituent on the C-2 carbon of Gal.

As expected GalNAcα1-*O*-Ser is the best ligand (Table II). Among the other sugars studied, MeαGalNAc has the highest value for the association constant whereas its β-linked counterpart is a poor ligand, likewise MeαGal is 11 times better a ligand than Meβ Gal, indicating that the methoxy group in the α-position at C-1 makes a positive contribution to the stabilization of the lectin–sugar complex due to a favourable enthalpic contribution (Table I). Substitution at the C-2 position of Gal has considerable influence on its binding to $VVLB_4$ as is evident from the following: replacement of 2-OH group by H as in 2-deoxygalactose and by NH_2 group in $GalNH_2$ led to a decrease in the affinity by 15.4- and 3.6-fold, respectively. However, substitution at the same locus by an *N*-acetamido group resulted in a 130.4-fold increase in affinity as compared to Gal, probably due to additional van der Waal's interactions or H-bonding between the acetamido group of the sugar and the protein. The introduction of an even bulkier substituent at C-2 as in GalNDns dramatically increases the affinity over Gal by 318.7-fold, probably due to an increase in hydrophobic interactions at this locus.

Non-binding of gulose indicates that the change of the hydroxyl group orientation at C-3 from equatorial to axial abolishes interaction with the lectin. Importance of the axial orientation at C-4 is underlined by the non-reactivity of the lectin with glucose. The C-6 hydroxymethyl group is not critical for the binding as evidenced by the binding affinity of fucose which is comparable to that of Gal.

Binding of oligosaccharides to the lectin did not show any remarkable increase in affinity over that observed for binding to the monosaccharides, leading to the conclusion that the binding sites of this lectin are directed most likely towards monosaccharides alone. An exami-

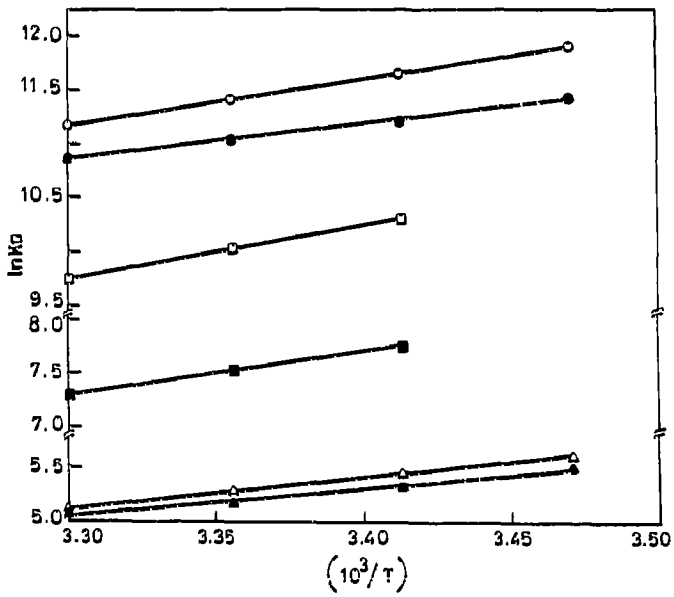

Fig. 3. Van 't Hoff plots for the association of various sugars to $VVLB_4$. The symbols used are: ○, MeαGalNAc; ●, GalNDns; □, GalNac; ■, MeαGal; △, Gal; ▲, Meβ Gal.

Table I

Association constants and the thermodynamic parameters[a] for the binding of sugars to $VVLB_4$

Sugar	K_a (20°C) (10^{-3} M^{-1})	$-\Delta G$ (kJ·mol^{-1})	$-\Delta H$ (kJ·mol^{-1})	$-\Delta S$ (J·mol^{-1}·K^{-1})
1. GalNDns	73.3	27.29	27.96	2.29
2. MeαGalNAc	114.0	28.36	37.75	32.05
3. GalNAc	30.0	25.11	49.04	81.67
4. MeαGal	2.31	18.87	35.73	57.54
5. MeβGal	0.204	12.95	23.75	36.86
6. Gal	0.230	13.25	27.31	47.99

[a] The thermodynamic parameters for MeβGalNAc could not be determined due to the paucity of this sugar.

nation of the binding affinities also shows that the interaction of the lectin with the oligosaccharide takes place through the recognition of the terminal or the non-reducing GalNAc. Thus GalNAc(β1–3)GalαOMe with an association constant of 1.22×10^4 M^{-1} is closer to that of GalNAc ($K_a = 3.0 \times 10^4$ M^{-1} at 20°C) as compared to that of GalαOMe ($K_a = 2.31 \times 10^3$ M^{-1} at 20°C). This indicates that despite the strong preference of $VVLB_4$ for α-linked GalNAc (i.e. MeαGalNAc) over its β-linked counterparts and its stronger binding of MeαGal over MeβGal, in disaccharides such as GalNAc(β1–3)GalαOMe it binds to terminal GalNAc alone irrespective of its linkage. Likewise, the equilibrium constant for binding of Gal(β1–3)GalNAcαOMe, $K_a = 3.09 \times 10^2$ M^{-1} is more readily explained as arising due to recognition of the terminal β-linked galactose than that due to interaction with GalNAcαOMe since it approaches the equilibrium constant of MeβGal ($K_a = 2.04 \times 10^2$ M^{-1}) better than that of GalNAcαOMe ($K_a = 1.14 \times 10^5$ M^{-1}). The explanation given above for the binding of Gal(β1–3)GalNAcOMe is further supported by the value of K_a for lactose (1.5×10^2 M^{-1}) which shows that the affinity of MeβGal for the lectin can be a good measure of the affinity of the terminal β-linked galactose in an oligosaccharide in its interaction with the lectin. The fivefold poorer affinity of melibiose as compared to MeαGal probably arises due to an unfavourable contribution to binding made by the bulkier aglycon in α-linkage to Gal. Similarly Gal(β1–6)GalNAc with K_a of 8.09×10^2 M^{-1} probably interacts with its galactose moiety, as this affinity, while being comparable to that of MeβGal, is nevertheless 37-fold less as compared to the affinity of GalNAc. Binding of subterminal GalNAc is also ruled out by the fact that, had it bound the disaccharides through its subterminal GalNAc, its affinity should have been comparable with that of free GalNAc as by analogy to the similar affinities of Gal and fucose, its substitution at C-6 by galactose is not expected to impair its affinity. The alternative explanation could be that the lectin interacts with the reducing GalNAc; the lower affinity as compared to GalNAc arises due to steric hindrance caused by the bulky substituent present at C-6 position. However, owing to the very large difference between the associa-

Table II

Association constants and free energies of binding for a number of ligands with $VVLB_4$ at 20°C

Ligands	K_a (10^{-3} M^{-1})	$-\Delta G$ (kJ·mol^{-1})	Relative affinity (Gal=1)
1. GalNDns	73.3	27.29	318.7
2. MeαGalNAc	114.0	28.36	495.7
3. MeβGalNAc	33.0	25.34	143.5
4. GalNAc	30.0	25.11	130.4
5. MeαGal	2.31	18.87	10.0
6. MeβGal	0.204	12.95	0.89
7. Gal	0.23	13.25	1.00
8. GalNAc(β1–3)GalαOMe	12.2	22.92	53.04
9. Gal(β1–3)GalNacαOMe	0.309	13.97	1.34
10. Gal(β1–6)GalNac	0.809	16.31	3.52
11. Forssman Penta.	1.99	18.50	8.65
12. Gal(β1–3)GalNAc	1.675	18.08	7.28
13. GalNacα1-*O*-Ser	271.0	30.47	1178.3
14. Gal(α1–3)Gal	0.52	15.23	2.26
15. Gal(α1–3)GalαOMe	0.54	15.33	2.35
16. GalNac(α1–3)Gal	8.42	22.02	36.6
17. Melibiose	0.471	14.99	2.05
18. α-Lactose	0.15	12.20	0.65
19. D-Fucose	0.134	11.93	0.58
20. 2-deoxy-Gal	0.0149	6.58	0.0
21. $GalNH_2 \cdot HCl$	0.064	10.12	0.28
22. Talose	0.14	12.04	0.61
23. Gulose	N.B.*		

*No binding observed

tion constants of Gal(β1–6)GalNAc and GalNAc, and the fact that the β1,6 linkage is expected to be very flexible we feel that the lectin prefers the former mode of binding.

Interestingly enough, Forssman pentasaccharide, which contains α-linked terminal GalNAc, bound to the lectin with a 15-fold poorer affinity as compared to GalNAc, whereas GalNAc(β1–3)GalαOMe proved to be only about a 2.46-fold poorer ligand as compared to GalNAc, which clearly indicates that in oligosaccharides the lectin shows a slight but definite preference for binding to terminal β-linked GalNAc as compared to terminal α-linked GalNAc which would also explain the very poor affinity of the lectin for blood group A-reactive oligosaccharide [5].

4. DISCUSSION

One of the unusual features of the lectin, noted in this study, is that, in spite of its known preference for GalNAc over Gal, the lectin is shown here to interact with the terminal β-linked Gal even if GalNAc is the subterminal sugar. An equally important observation, perhaps, is that despite its strong affinity for MeαGalNAc/MeαGal over MeβGalNAc/MeβGal in oligosaccharides the lectin displays weaker binding to the terminal α-linked GalNAc and Gal when compared with their β-linked counterparts.

GalNAc(α1–3)Gal, Gal(α1–3)Gal and Gal(α1–3)GalαOMe can interact with the lectin through their terminal or subterminal residues. Almost identical affinities of Gal(α1–3)Gal and Gal(α1–3)GalαOMe that are significantly lower than that for GalαOMe together with the fact that GalNAc(α1–3)Gal is a poorer ligand compared to GalNAcαOMe indicates that in the α-linked sugars as well, the lectin binds to the non-reducing hexopyranosyl residues only. This suggestion is also supported by broadly similar ΔG values for the interaction of Gal(α1–6)Glc [note Gal(α1–3)Gal and Gal(α1–3)GalαOMe] which is expected to bind to the lectin through its non-reducing galactose residue only.

The poorer binding of oligosaccharides with terminal α-linked GalNAc or Gal as compared to the disaccharides that contain these sugars in β-linkage can be explained by assuming that the combining site of the lectin is complementary to a single hexopyranosyl residue alone, and is constituted by a moderately deep cleft. This, together with the conformational analyses of these disaccharides, suggests a plausible mechanism of its preference for teminal β-linked GalNAc and Gal structures which is as follows: in GalNAc(α1–3)Gal, Gal(α1–3)Gal the orientation of the sugar rings is such that they form a bent L-shaped structure in which the subterminal Gal is in close proximity to the terminal sugar preventing the entry of the latter in the combining site of the lectin [19]. Similar reasoning would also explain the non-binding of $VVLB_4$ to blood group A-erythrocytes [5]. On the other hand β-linked disaccharides such as GalNAc(β1–3)Gal, Gal(β1–3)GalNAc display linear structures [18] which permit the unhindered access of their terminal residues in the combining site of the lectin. However, the T_n-antigenic structure expectedly is the best ligand as its GalNAc in α-linkage to the hydroxyl group of serine or threonine residues protrudes far enough from the peptide backbone for a sterically favourable interaction with the lectin to take place.

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