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Saccharide Binding to Three Gal/GalNAc Specific Lectins: Fluorescence, Spectroscopic and Stopped-flow Kinetic Studies

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Fluorescence and stopped-flow spectrophotometric studies on three plant lectins from *Psophocarpus tetragonolobus* (winged bean), *Glycine max* (soybean) and *Artocarpus integrifolia* (jack fruit) have been studied using *N*-dansylgalactosamine as a fluorescent ligand. The best monosaccharide for the winged bean agglutinin I (WBA I) and soybean (SBA) is Me-αGalNAc and for jack fruit agglutinin (JFA) is Me-αGal. Examination of the percentage enhancement and association constants (1.51×10^6, 6.56×10^6 and 4.17×10^5 M^{-1} for SBA, WBA I and JFA, respectively) suggests that the combining regions of the lectins SBA and WBA I are apolar whereas that of JFA is polar. Thermodynamic parameters obtained for the binding of several monosaccharides to these lectins are enthalpically favourable. The binding of monosaccharides to these lectins suggests that the -OH groups at C-1, C-2, C-4 and C-6 in the D-galactose configuration are important loci for interaction with these lectins. An important finding is that the JFA binds specifically to Galβ1-3GalNAc with much higher affinity than the other disaccharides which are structurally and topographically similar.

The results of stopped-flow spectrometry on the binding of *N*-dansylgalactosamine to these lectins are consistent with a bimolecular single step mechanism. The association rate constants (2.4×10^5, 1.3×10^4, and 11.7×10^5 $M^{-1}sec^{-1}$ for SBA, WBA I and JFA, respectively) obtained are several orders of magnitude slower than the ones expected for diffusion controlled reactions. The dissociation rate constants (0.2, 3.2×10^{-2}, 83.3 sec^{-1} for SBA, WBA I and JFA, respectively) obtained for the dissociation of *N*-dansylgalactosamine from its lectin complex are slowest for SBA and WBA I when compared with any other lectin-ligand dissociation process.

Abbreviations: SBA, Soybean agglutinin; WBA I, Winged bean agglutinin (Basic); JFA, Jack fruit agglutinin; PNA, Peanut agglutinin; Con A, Concanavalin A; Dansyl (Dns), 5-dimethylaminonaphthalene-l-sulphonyl; 2GalNDns, *N*-dansylgalactosamine; dGal, 2-deoxygalactose; L-Ara, L-arabinose; D-Fuc, D-fucose; L-Rha, L-rhamnose; *N*-acetyllactosamine, Galβ4GlcNAc; melibiose, Galα6Glc.

A large number of lectins have been purified and are in use as tools in glycoconjugate and cell research [1-4]. To understand the mechanism of binding and interaction of lectins with various complex cell surface glycoconjugates, it is essential to know the nature, mechanism and the forces involved in the binding of lectins to simple carbohydrates. Quantitative carbohydrate binding parameters have been determined only for a few lectins. The present investigation was undertaken to measure and compare the carbohydrate binding parameters of three lectins having broadly similar monosaccharide specificity, i.e., galactose/*N*-acetylgalactosamine binding lectins from seeds of soybean (*Glycine max*), winged bean (*Psophocarpus tetragonolobus*) and jack fruit (*Artocarpus integrefolia*).

Materials and Methods

Materials

Winged beans and soybeans were purchased from the University of Agricultural Sciences, Bangalore, India, and the Jack fruit seeds were purchased from the local market. The sugars, reagents for gel electrophoresis and other chemicals used were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Isolation of WBA I, SBA and JFA

Winged bean agglutinin (WBA I), Soybean agglutinin (SBA) and Jack fruit agglutinin (JFA) were isolated and purified to homogeneity following the methods of Khan *et al.* [5], Gordon *et al.* [6] and Suresh Kumar *et al.* [7], respectively. All the three lectins gave single bands on polyacrylamide gel electrophoresis indicating the homogeneity of the preparations. On SDS polyacrylamide gel electrophoresis both SBA and WBA I gave single bands corresponding to the molecular weight of M_r 30 000 and M_r 29 000, respectively. JFA on the other hand gave two bands with M_r of 10 200 and 9 500, respectively.

Preparation of Me-α-GalNAc, Me-β-GalNAc, Galβ1-3GalNAc and Galβ1-3GlcNAc

Me-α-GalNAc and Me-β-GalNAc were prepared by the method of Sarkar and Kabat [8]. Galβ1-3GalNAc and Galβ1-3GlcNAc were synthesized according to the methods of Flowers and Shapiro [9] and Flowers and Jeanloz [10], respectively.

Preparation of GalNDns

GalNDns was synthesized by the method of Lartey and Derechin as described by Swamy *et al.* [11].

Concentration Determination

SBA concentration was determined spectrophotometrically by using an A_{280} (1%, 1 cm) value of 12.8 [12], whereas the concentration of WBA I and JFA was estimated by the method of Lowry *et al.* [13] using bovine serum albumin as standard. SBA and WBA I concentrations were expressed in terms of a monomer of M_r 30 000 and M_r 29 000, respectively. JFA concentrations were expressed in terms of dimer (M_r 20 000) as it was shown

to have two binding sites per molecule of M_r 40 000 [14]. The concentration of GalNDns was determined spectroscopically using a molar absorption of 4 800 cm^{-1} at 330 nm [15] and those of non-chromogenic sugars by weight.

Fluorescence Measurements

Fluorescence spectra of GalNDns in the presence and absence of SBA, WBA I and JFA were recorded on a Perkin-Elmer MPF 44A ratio-recording spectrofluorimeter. The samples were excited at 330 nm and the emission spectra recorded above 450 nm, with slit widths of 5 nm for both the monochromators.

Fluorescence titration measurements were made on a Union Giken FS 501A fluorescence polarizer equipped with photon counting photomultipliers. Samples were excited at 330 nm with a 7 nm slit and emission was monitored by means of a metal interference band pass filter ($\lambda_{0.5}$ = 6.5 nm) centered at 520 nm, together with a 510 nm cut-off filter. Samples in 1 × 1 × 3.5 cm quartz cuvettes were plated in a thermostated copper holder maintained at a constant temperature by means of a temperature bath. The fluorimeter was equipped with a microprocessor which allowed averaging of several readings.

A fixed volume (2 ml) of GalNDns (3.0, 3.75 and 18.0 μM for SBA, WBA I and JFA, respectively) was titrated with addition of defined aliquots of SBA (333 μM), WBA I (169 μM) or JFA (570 μM) in the cuvette. The change in fluorescence intensity was recorded after each addition. The association constants for the interaction of SBA, WBA I and JFA with GalNDns were determined by the method of Chipman *et al.* [16]. Binding of non-fluorescent saccharides to the lectins was studied according to the method of Bessler *et al.* [17] as described by Khan *et al.* [18].

Stopped-flow fluorescence studies were performed on a Union Giken RA 401 stopped-flow spectrometer. Excitation was at 330 nm and emission was monitored above 460 nm by using a cut off filter. The dead time of the instrument was determined to be 0.5 ms. The sample reservoirs and cell compartment were maintained at a constant temperature by means of a temperature bath. In these experiments the concentration of GalNDns was fixed (3.75 μM for SBA, 3.78 μM for WBA I and 12 μM for JFA) and that of the lectins was varied from 10 to 100 times of that of the sugar. The concentrations expressed are those after mixing. The dissociation of GalNDns from the lectin-GalNDns complex was monitored by mixing lectin-GalNDns solution with a 10 mM solution of *N*-acetylgalactosamine or Me-α-Gal.

Results

The fluorescence intensity of GalNDns was enhanced upon binding to SBA, WBA I and JFA (Fig. 1A, B, C). For totally bound sugar 11, 15 and 2-fold enhancement in its fluorescence intensity was observed on binding to SBA, WBA I and JFA, respectively [5, 11, 14]. The enhancement of the fluorescence intensity is accompanied by a 25 nm blue shift in the emission maximum for binding of GalNDns to SBA and WBA I, whereas only a 10 nm blue shift was observed on binding to JFA. Addition of saturating amounts of inhibitory sugars such as *N*-acetylgalactosamine or Me-α-Gal totally reversed these effects.

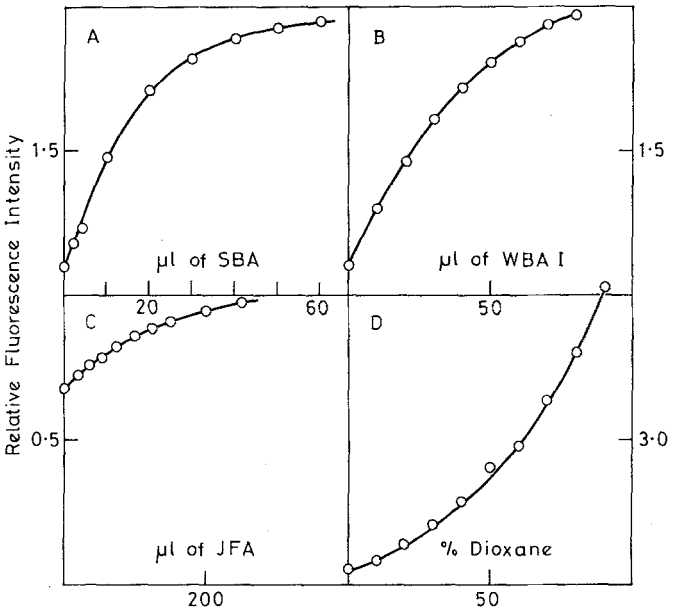

Figure 1. Titration of GalNDns with SBA (A), WBA I (B), JFA (C) and dioxane (D).
A. GalNDns (3.78 μM) was titrated with SBA (333 μM) at 20°C.
B. GalNDns (3.75 μM) was titrated with WBA I (169 μM) at 25°C.
C. GalNDns (12 μM) was titrated with JFA (570 μM) at 20°C.

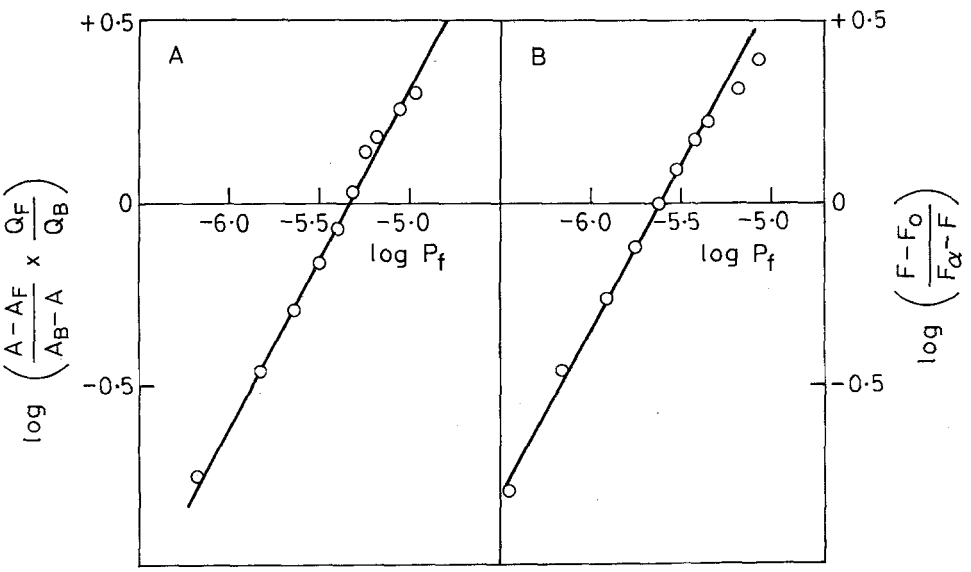

Figure 2. A sample plot for the determination of the association constant of GalNDns for WBA I at 25°C. The association constant, K_a, determined by fluorescence intensity titration is $4.2 \times 10^5\ M^{-1}$.

Table 1. Association constants (K_a) and relative affinity (RA) with galactose = 1, for a number of ligands.

Sugar	SBA		WBA I		JFA	
	10^{-3}x K_a at 20°C	RA	10^{-3}x K_a at 25°C	RA	10^{-3}x K_a at 20°C	RA
GalNDns	1510	6565	417.00	448.00	17.4	13.00
Me-α-GalNAc	130	565	15.70	17.00	N.D.[a]	—
Me-β-GalNAc	N.D.	—	0.48	0.50	N.D.	—
GalNAc	9.2	40	4.55	5.00	3.3	2.50
Me-α-Gal	0.7	3.0	5.52	6.00	40.0	30.00
Me-β-Gal	0.55	2.4	0.60	0.60	0.20	0.15
D-Gal	0.23	1.0	0.93	1.00	1.34	1.00
D-GalN	N.D.	—	0.23	0.20	1.44	1.10
dGal	0.04	0.17	0.21	0.20	0.636	0.47
D-Fuc	0.06	0.26	0.17	0.20	N.B.[b]	—
L-Ara	0.05	0.22	0.044	0.05	N.B.	—
L-Rha	N.D.	—	0.016	0.02	N.B.	—
D-Glc	N.B.	—	0.011	0.01	N.B.	—
Lactose	0.35	1.52	0.04	0.04	0.030	0.002
Melibiose	0.55	2.39	1.95	2.00	6.25	4.66
N-Acetyllactosamine	0.44	1.91	N.D.	—	0.036	0.002
Galβ1-3GlcNAc	N.D.	—	N.D.	—	0.041	0.003
Galβ1-3GalNAc	N.D.	—	N.D.	—	122	91.04

[a] N.D. = not determined.
[b] N.B. = not binding.

A representative plot for GalNDns binding to WBA I at 25°C for fluorescence intensity titration is shown in Fig. 2. The values of the association constant "K_a" for GalNDns binding to SBA, WBA I and JFA were determined according to the method of Chipman *et al.* [16] and are reported in Table 1. The association constants for the binding of GalNDns to SBA, WBA I and JFA were also determined at several temperatures and were found to decrease with increase in temperature for all the three lectins [5, 11, 14]. The corresponding $\Delta H°$ and $\Delta S°$ values are given in Table 2.

The binding of non-fluorescent sugars to SBA, WBA I and JFA was studied by monitoring the decrease in the fluorescence intensity due to the release of GalNDns from its highly fluorescent lectin-complex on addition of the inhibitory sugar. The values of association constants of various sugars for SBA, WBA I and JFA as determined according to the method in [17] are listed in Table 1. The binding of several sugars to these lectins at various temperatures was also studied by this method. The enthalpy and entropy changes associated with the interaction of these sugars with the lectins are listed in Table 2.

The first order approach of the fluorescence to its final value for the reaction of SBA, WBA I and JFA with GalNDns could be interpreted in terms of a single step reaction according to the expression

$$P + D \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} PD$$

Table 2. Thermodynamic parameters for the binding of sugars to SBA, WBA I and JFA.

Sugar	-ΔH° (kJ mol^{-1})			-ΔS° (J mol^{-1}K^{-1})		
	SBA 20°C	WBA I 25°C	JFA 20°C	SBA 20°C	WBA I 25°C	JFA 20°C
GalNDns	37.9	33.6	31.6	10.9	5.2	26.6
Me-α-GalNAc	37.5	30.5	N.D.[a]	49.1	21.9	N.D.
GalNAc	33.5	27.3	55.0	38.6	21.5	120.4
Me-α-Gal	25.6	27.7	55.0	32.8	21.5	99.6
Me-β-Gal	N.D.	22.6	42.0	N.D.	22.6	99.3
D-Gal	N.D.	N.D.	38.0	N.D.	N.D.	70.6
Galβ-1-3GalNAc	N.D.	N.D.	105.0	N.D.	N.D.	261.0

[a] N.D. = not determined.

where P and D refer to the lectin and GalNDns, respectively. Values for k_{+1} and k_{-1} were obtained from the slope and intercept of linear plots of k_{app} vs. [P]. A representative plot of GalNDns-SBA interaction is shown in Fig. 3. The values of the dissociation rate constant k_{-1} was also determined by a direct method by dissociating the lectin (17.5 μM SBA) and GalNDns (15 μM) complex on mixing with excess of *N*-acetylgalactosamine and Me-α-Gal (10 mM solutions). The values of k_{+1} and k_{-1} determined graphically for the GalNDns-SBA interaction (Fig. 3) at 20°C are 2.1×10^5 $M^{-1}sec^{-1}$ and 0.2 sec^{-1}, respectively; and k_{-1} determined by the direct method is 0.2 sec^{-1}. Values of k_{+1} and k_{-1} determined for the interaction of SBA, WBA I and JFA are listed in Table 3. The thermodynamic parameters ΔH* and ΔS* for the association and dissociation reactions obtained by the method of Laidler [19] are listed in Table 4.

Discussion

We have used the fluorescence properties of GalNDns to probe the interaction of various saccharides with SBA, WBA I and JFA. The total reversal of fluorescence enhancement when sufficient amounts of inhibitory sugars are added to a lectin-GalNDns complex proves that the binding of this fluorescent sugar to the lectins is saccharide specific. When the fluorescence intensity of GalNDns was measured as a function of dioxane concentration (Fig. 1D), dramatic enhancement of its fluorescence was observed. Earlier it had been shown by Chen [20] that the fluorescence quantum yield of *N*-dansyltryptophan increased several fold with a concomitant blue shift when the dielectric constant of the solvent was decreased. In other words, the fluorescence quantum yield of the dansyl moiety increases with decrease in the ploarity of its environment. Our observation of 11-, 15- and 1-fold increases in the fluorescence intensity of GalNDns with concomitant blue shift on binding to SBA, WBA I and JFA, respectively, suggests that the "dansyl" moiety is accommodated into an apolar environment when GalNDns binds to these lectins. The difference in the increase in fluorescence intensity

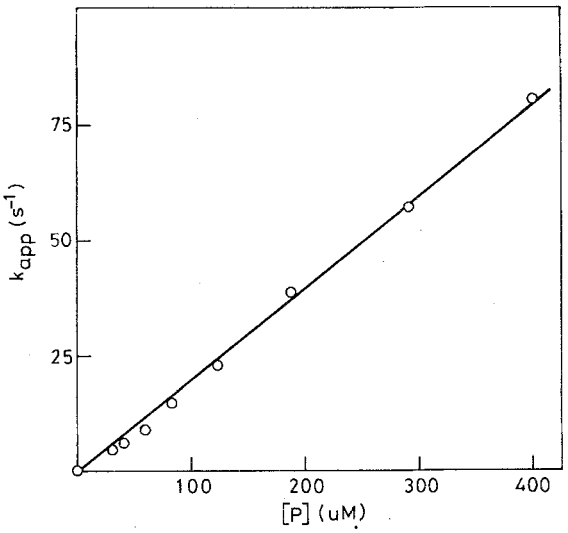

Figure 3. Determination of the rate constant for the association of SBA and GalNDns at 20°C. The concentration of the sugar was fixed at 3.75 μM after mixing. The slope of the line yielded a k_{+1}-value of 2.1 × 10^5 M^{-1} sec^{-1}.

shows that JFA has the least apolar area at its sugar binding site, whereas SBA and WBA I have highly apolar regions at their sugar binding site.

The binding constants of saccharides for SBA, WBA I and JFA indicate certain similarities as well as differences. Galactose is a better ligand for all the three lectins compared to dGal and galactosamine. But all of them exhibited increased affinity on substitution of the amino group at the C-2 position of galactosamine by an acetamido group or the dansyl group as in *N*-acetylgalactosamine or GalNDns. This is apparent from the relative affinities (RA) shown in Table 1, where JFA shows a comparatively lower increase in the binding affinity with substituents like the acetamido group, or a large hydrophobic substituent like the dansyl group, at the C-2 position of galactose than does SBA and WBA I. Interaction of the dansyl group with a hydrophobic area near the binding site is also reflected by a less unfavourable binding entropy (Table 2) when compared with other monosaccharides [14, 21]. Comparison of the binding affinities of SBA, WBA I and JFA for dGal, galactosamine, galactose, *N*-acetylgalactosamine and GalNDns shows that SBA has the highest affinity for hydrophobically-substituted galactosamine, whereas JFA has the least affinity. All the three lectins do not favour alteration of the C-4 hydroxyl group in galactose. The C-5 hydroxymethyl group of galactose seems to provide a favourable binding locus for all the three lectins as indicated by the poorer affinities of D-fucose, L-rhamnose and L-arabinose.

Comparison of the binding affinities of 1-*O*-methyl derivatives of galactose indicates that a methyl group in the α-anomeric position increases the affinity for WBA I and JFA, whereas a β-anomeric derivative constitutes a destabilizing factor. However, SBA does not distinguish appreciably between these two anomeric derivatives of galactose. Of the simple monosaccharides tested, SBA and WBA I bind best to Me-α-GalNAc whereas JFA binds best to Me-α-Gal.

Table 3. Association and dissociation rate constants for the binding of *N*-dansylgalactosamine to SBA, WBA I and JFA.

Lectin	k_{+1} ($M^{-1}s^{-1}$)	k_{-1} (s^{-1})
SBA (20°C)	2.4×10^{5}	0.20
WBA I (25°C)	1.3×10^{4}	3.2×10^{-2}
JFA (24°C)	11.7×10^{5}	83.3

Table 4. Activation parameters for the binding of *N*-dansylgalactosamine to SBA, WBA I and JFA.

Lectin	H_a^* (kJ mol^{-1})	H_d^* (kJ mol^{-1})	S_a^* (J $mol^{-1}K^{-1}$)	S_d^* (J $mol^{-1}K^{-1}$)
SBA	39.2	77.4	- 7.4	5.9
WBA I	41.6	75.0	-26.5	-21.4
JFA	56.1	86.9	59.9	83.7

It is interesting to compare the values of the association constants for mono- and disaccharides for the three lectins. It can be seen from the relative affinities of these lectins towards mono- and disaccharides (Table 1) that SBA and WBA I do not favour binding to disaccharides. WBA I shows a similar anomeric specificity for disaccharides as for monosaccharides; lactose, with a β-linked galactose is a very poor binder compared to melibiose, with the α-linked galactose. But SBA again fails to differentiate between lactose and melibiose, containing the α- and β-linked galactose. JFA alone among these lectins shows a pronounced increase in affinity towards a disaccharide, the "T-antigenic" disaccharide, Galβ1-3GalNAc. The binding of Galβ1-3GalNAc to JFA is accompanied by a large increase in enthalpy, which adequately compensates for the increase in the -ΔS value (Table 2), thus resulting in a 610-fold increase in K_a for the binding of the disaccharide over that for Me-α-Gal. Except for Galβ1-3GalNAc among the β-linked disaccharides, JFA binds better to melibiose, an α-linked disaccharide.

The forward rate constants listed in Table 3 for SBA, WBA I and JFA interaction with GalNDns are several orders of magnitude slower than a diffusion controlled reaction. Bimolecular association rate constants which are slower than a diffusion controlled process have also been reported for the binding of chromogenic ligands to several other lectins [15, 22-26]. The thermodynamic parameters obtained from the temperature dependence of the association and dissociation processes for SBA, WBA I and JFA (Table 4) interaction with GalNDns show significant differences in the activation process for these lectins. SBA and WBA I have enthalpy-controlled association and dissociation processes with very little entropic contribution, whereas JFA has an overall enthalpy-controlled activation process but there is a significant entropy contribution.

The entropy for the association process for SBA and WBA I is very small, indicating that the association process does not involve a highly ordered transition state for these lectins. JFA, however, has a large activation entropy for the association process indicating

that a specific configuration of reactants is required for GalNDns binding to this lectin. SBA and WBA I appear to differ from Con A, PNA and JFA [22, 23] in this respect.

The dissociation rate constants obtained for GalNDns interaction with SBA and WBA I are by far the slowest observed for any lectin-monosaccharide interaction. Several mechanisms have been put forward to account for the slow reaction rates but the one most favoured is a rapid but unobserved formation of lectin-sugar complex (PD_i), followed by a slower observable change of this complex:

$$P+D \underset{k_{-1}}{\overset{k_{+1}}{=}} P.D_i \underset{k_{-2}}{\overset{k_{+2}}{=}} PD$$

$$K_I = \frac{k_{+1}}{k_{-1}} \; ; K_{II} = \frac{k_{+2}}{k_{-2}}$$

The slow dissociation kinetics of lectins could be a favourable property and might lead to an increase in efficiency of the lectins in their interaction with cells [24].

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