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A comparison of the carbohydrate binding properties of two *Dolichos biflorus* lectins

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The carbohydrate binding properties of the *Dolichos biflorus* seed lectin and DB58, a vegetative tissue lectin from this plant, were compared using two types of solid phase assays. Both lectins bind to hog blood group A + H substance covalently coupled to Sepharose 4B and this binding can be inhibited with free blood group A + H substance. However, the binding of the seed lectin is inhibited by D-GalNAc whereas DB58 binding was not inhibited by any monosaccharide tested, thus suggesting that its carbohydrate combining site may be more extensive than that of the seed lectin. The activities of these two lectins also differ from one another in ability to recognize blood group A + H substance adsorbed on to plastic and in the effects of salt and urea on their carbohydrate binding activities. Neither lectin showed glycosidase activity with p-nitrophenyl α-D-GalNAc or p-nitrophenyl β-D-GalNAc.

Keywords: lectins, *Dolichos biflorus*; blood group substance

Introduction

Almost all plant lectins described to date are multivalent and were initially discovered by detecting the ability of their respective plant extracts specifically to agglutinate particular types of erythrocytes or to precipitate complex carbohydrates (for review see [1]). Many of these lectins are found in the seeds of plants, particularly the legumes where they can constitute up to 10% of the soluble protein of the seed extract [2]. In recent years low levels of lectins related to the seed lectins have been found in other legume tissues; some of these lectins are encoded by separate genes and do not agglutinate erythrocytes (for reviews, see [3, 4]). Little has been reported about the carbohydrate binding properties of these vegetative tissue lectins due to difficulties in designing appropriate assays.

The legume, *Dolichos biflorus*, contains a family of lectin genes, some of which are differentially expressed, both spatially and temporally [5, 6]. Two of these lectins, the seed lectin and a lectin from stems, leaves and floral parts, named DB58, are 87% identical in primary structure [7, 8]. These lectins are encoded by separate genes located in the same transcriptional orientation within 3 kb pairs of one another, suggesting that they may have arisen by gene duplication and subsequent divergence [6].

The *Dolichos biflorus* seed lectin is similar to other legume seed lectins. It was first reported by Bird [9, 10] who found that it could specifically agglutinate type A1 erythrocytes and precipitate blood group A substance. Isolation and characterization of this lectin showed that this blood group A specificity is due to its ability to recognize terminal, nonreducing α-linked D-GalNAc residues [11]. Subsequent comparative studies of the specificity of this lectin with other D-GalNAc specific lectins showed that it was distinguished from many of the lectins in this category by its extreme preference for D-GalNAc over D-galactose and its ability to react strongly with the terminal disaccharide [DGalNAc(α1-3)βDGalNAc] of the Forssman antigenic determinant [12–15]. The lectin is a tetramer of 110 000 molecular weight composed of equal amounts of two types of closely related subunits, I and II [16]. Subunit II is thought to arise from subunit I by posttranslational proteolysis of approximately 10 amino acids from the carboxyl terminal end [17]. Carbohydrate binding activity has been detected only with subunit I, and the lectin has two carbohydrate binding sites per tetramer [18].

The DB58 lectin was discovered during a developmental study of the seed lectin when radioimmunoassays employing antibodies against the seed lectin detected low levels of a cross reactive protein in the stems and leaves [19]. Subsequent isolation and characterization of this protein showed that it is a 58 000 molecular weight dimer composed of two closely related subunits [20, 21]. This lectin constitutes only about 0.05% of the total protein of the stems and leaves [22]. DB58 does not agglutinate erythrocytes and was thought to be an inactive form of a lectin [20] until it was found that this protein could bind to immobilized blood group A + H substance in the absence of salt [23]. The present paper reports a comparison of

the carbohydrate binding properties of this lectin with the seed lectin.

Materials and methods

Carbohydrates

The methyl α- and β-glycosides of D-GalNAc were obtained from CalBiochem (La Jolla, CA). All other monosaccharides and methyl glycosides used in this study were purchased from Pfanstiehl (Waukegan, IL) or Sigma (St. Louis, MO). Hog blood group A + H substance was purified by ethanol precipitation [24] from hog gastric mucin (Wilson, Chicago, IL). Ovine submaxillary mucin was purchased from Accurate Chemicals (Westbury, NY) and desialylated by incubation at 80 °C for 1 h in 1 N H_2SO_4.

Lectins

The seed lectin was isolated from *Dolichos biflorus* seeds (F. W. Schumacher & Co., Inc., Sandwich, MA) by affinity chromatography on polyleucyl hog blood group A + H substance [11, 25]. DB58 was extracted from the stems and leaves of 3–4-week-old *Dolichos bifloros* plants and chromatographed on a column of hog blood group A + H substance coupled to Sepharose 4B [23]. This lectin, which was eluted by 0.15 M NaCl from this column, was further purified by Cibacron blue Sepharose and ion exchange chromatography.

The lectins were iodinated using the iodine monochloride procedure [26, 27]. A 20 µl aliquot (1 mCi) of carrier free $Na^{125}I$ (New England Nuclear, Boston, MA) was mixed with 200 µl of 0.1 M HCl containing unlabelled 1 mM ICl and then diluted with 600 µl of 1 M glycine-HCl, pH 8.5. Aliquots of this mixture (350 µl per mg lectin) were then added to the lectin solutions (1 mg ml^{-1} in 0.2 M glycine-HCl, pH 8.5, containing 0.1 M D-GalNAc). After 30 min on ice, the lectin solutions were dialysed extensively against PBS containing 1 mM KI and then against PBS (seed lectin) or 10 mM phosphate buffer, pH 7.2 (DB58). Both the PBS and phosphate buffers contained 0.02% NaN_3. This gentle labelling procedure gave specific activities of 164 cpm ng^{-1} of seed lectin and 393 cpm ng^{-1} of DB58.

Sepharose conjugates

Blood group A + H substance was coupled to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) following the manufacturer's instructions. Before coupling, the blood group substance (30 mg ml^{-1} in 50 mM Tris-HCl, pH 7.5, containing 10 mM $CaCl_2$) was digested with 360 µg ml^{-1} pronase (CalBiochem) for 20 h at 37 °C and repurified by ethanol precipitation. This treatment increases the number of free NH_2 groups available for coupling and was found to improve the capacity of the resin. A control Sepharose conjugate was prepared by coupling ethanolamine (1 M) to CNBr-activated Sepharose 4B. Ethanolamine was chosen for this purpose since it served as the blocking agent in the coupling of the blood group substance to the Sepharose.

ELISA

Immulon 1 polystyrene microtitre plates (Dynatech, Chantilly, VI) were incubated overnight at 4 °C with 100 µl per well of 10 µg ml^{-1} blood group A + H substance in 50 mM $NaHCO_3$, pH 9.0, containing 0.02% NaN_3. Control wells containing only buffer were included on all plates. The plates were washed three times with TBS/Tween (50 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl and 0.05% Tween-20) and incubated at 37 °C for 1 h with 200 µl per well of blocking buffer [TBS/Tween containing 5.0% (w/v) dry milk, 0.03% antifoam A and 0.001% merthiolate]. The plates were then washed three times with TBS/Tween and twice with PBS. Lectin or inhibitor followed by lectin were then added to each well to a final volume of 100 µl and the plates were incubated at 37 °C for 30 min. After washing three times with PBS, 100 µl per well of 2% formalin in PBS was added and the plates were incubated at room temperature for 45 min. The plates were washed three times with TBS and incubated at 37 °C for 30 min with 100 µl per well of a 1:100 dilution in blocking buffer of antiserum against heat denatured seed lectin or DB58. The plates were washed five times with TBS/Tween and then incubated at 37 °C for 30 min with 100 µl per well of goat anti-rabbit IgG (H + L)-horse radish peroxidase conjugate (Cappel Laboratories, Philadelphia, PA) diluted 1:100 in blocking buffer. After washing five times with TBS/Tween, the plates were incubated for 30 minutes at room temperature with 100 µl per well of 50 mM sodium citrate, pH 4.0, containing 600 µM 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) and 0.024% H_2O_2. Optical densities of the wells were read at 405 nm using a V_{max} microtitre plate reader (Molecular Devices, Menlo Park, CA).

Carbohydrate binding assays employing Sepharose-conjugates

The Sepharose conjugates described above were washed three times in graduated centrifuge tubes with 10 mM Na phosphate buffer, pH 7.2, containing 0.02% NaN_3 and 0.05% Tween-20 and resuspended in the same buffer to a 10% stock solution (v/v). Varying amounts of these conjugates were added to Eppendorf tubes containing the iodinated lectins to give a final volume of 200 µl. The tubes were mixed and incubated at room temperature overnight. The tubes were microfuged for 2 min and aliquots of the supernatants were counted in a gamma counter. Inhibition experiments were conducted in a final volume of 100 µl and the samples were processed as above.

Results

Carbohydrate binding properties of lectins as determined by ELISA

Previous attempts to measure DB58 activity by haemagglutination and precipitation assays failed thereby necessitating the development of a solid phase assay for this lectin. An ELISA was initially chosen, and the validity of this assay was tested using the seed lectin. The seed lectin bound to the adsorbed blood group A + H substance, and the extent of binding was dependent on lectin concentration. The inclusion of a final concentration of 0.025% Tween-20 in the assay was found to minimize nonspecific binding in control wells that had not been adsorbed with blood group substance.

Inhibition results for the seed lectin obtained with this ELISA are shown in Fig. 1. As had previously been determined [11], the seed lectin shows a preference for Me α-D-GalNAc over d-GalNAc. The relative inhibitory potency of the glycoside with respect to the free sugar at 50% inhibition measured at two different lectin concentrations (2.1 and 3.5 μg ml^{-1}) was 2.17 and 1.96, respectively. These values are lower but close to the ratios of 2.84–3.25 previously determined by inhibition of precipitation assays [11–13]. The relative inhibitory potency of Me β-D-GalNAc to D-GalNAc determined at the above lectin concentrations was 0.53 and 0.55 compared with the previously determined value of 0.59 [12]. These results again demonstrate the α-anomeric preference of the seed lectin.

Although DB58 had previously been found to bind to blood group A + H substance coupled to Sepharose 4B [23], no binding of this lectin to the blood group substances was detected using the ELISA over a wide range of DB58 concentrations at pH values from 6.0 to 8.2. Reduction of salt concentration did not promote binding. Attempts were made to alter the presentation of the blood group substance in the wells by using homobifunctional and heterobifunctional cross linkers to couple covalently the blood group A + H substance to poly-L-lysine adsorbed to microtitre plates as previously described [28]. Although the seed lectin bound to the blood group substance coupled by such procedures, DB58 did not bind.

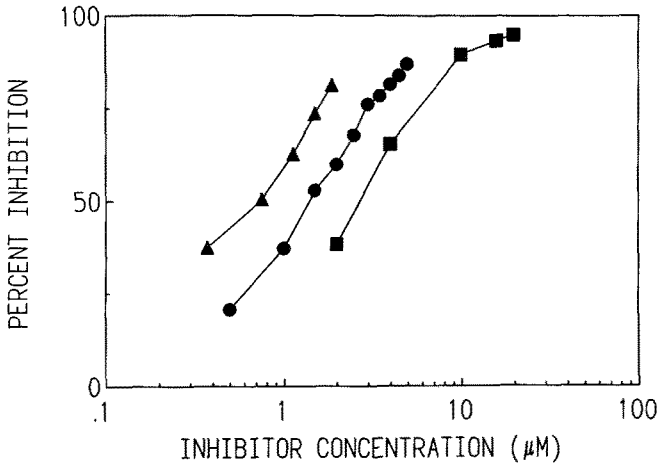

Figure 1. Inhibition of binding of seed lectin to blood group A + H substance adsorbed to wells of microtitre plate. Various concentrations of D-GalNAc (●), Me α-D-GalNAc (▲), and Me β-D-GalNAc (■) were added to wells of the microtitre plate followed by the addition of seed lectin (349 ng) to a final volume of 100 μl per well. The ELISA was conducted as described in the text and the absorbance of the wells at 405 nm was read 30 min after addition of substrate to the wells.

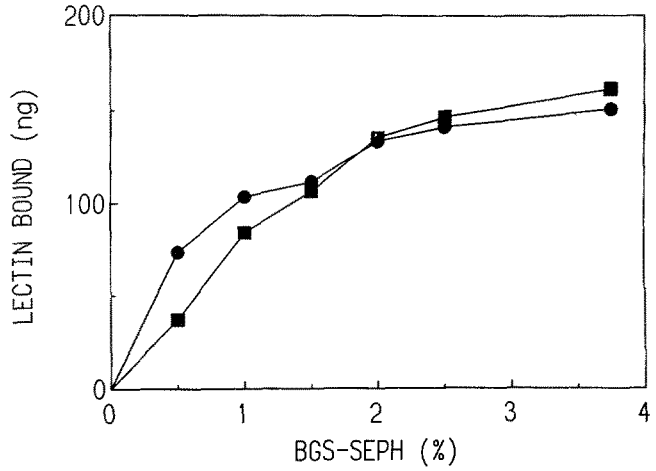

Figure 2. Binding of iodinated lectins to BGS-Seph. ^{125}I-DB58 (291 ng = 5.02 pmol) or ^{125}I-seed lectin (589 ng = 5.35 pmol) were added to increasing amounts of BGS-Seph in a final volume of 200 μl. After incubation at room temperature overnight, binding was measured as described in the text. (●) DB58; (■) Seed lectin.

Glycosidase assay

The inability of DB58 to bind to blood group A + H substance in the above ELISA assay in contrast to its ability to bind to affinity resins with this blood group substance during purification raised the possibility that the pure protein might be enzymatically active. Both the seed lectin and DB58 were assayed for their ability to hydrolyse p-nitrophenyl α-D-GalNAc and p-nitrophenyl β-D-GalNAc. The assays were conducted in 10 mM phosphate buffer, pH 7.2, at substrate concentrations of 2 mM and 1.6 mM, respectively. Neither lectin showed any activity with these substrates.

Carbohydrate binding properties of iodinated lectins determined by binding to blood group A + H-Sepharose (BGS-Seph)

Both the iodinated seed lectin and DB58 bound to BGS-Seph with similar affinities as shown in Fig. 2. The lectins showed no signficant binding to ethanolamine-Sepharose controls that were included in each assay. As had been previously found [23], the presence of NaCl in the buffer prevented DB58 from binding to the BGS-Seph

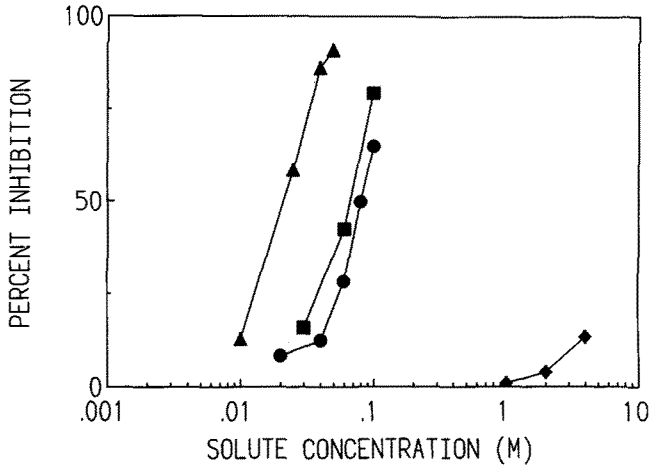

Figure 3. Effect of salts and urea on binding of ^{125}I-DB58 to BGS-Seph. Various amounts of NaCl (●), KCl (■), Na_2SO_4 (▲) and urea (◆) were mixed with ^{125}I-DB58 (72.5 ng) and BGS-Seph (2%) in a final volume of 100 µl. After incubation at room temperature overnight, binding was measured as described in the text. Note that expression of Na_2SO_4 concentration in normality would shift the curve obtained with this salt closer to the curves obtained with the other salts.

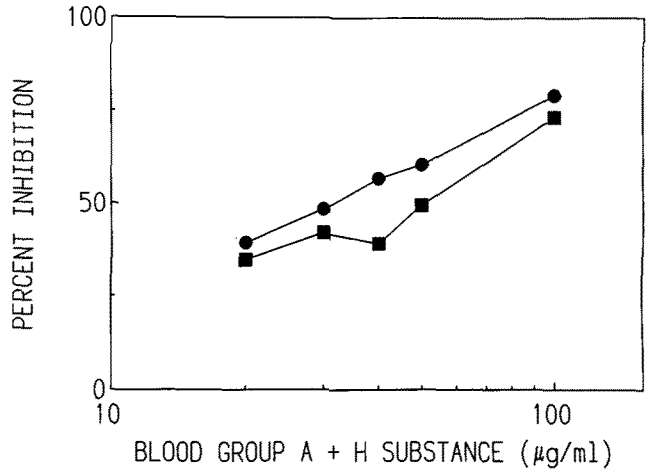

Figure 4. Inhibition of binding of ^{125}I-DB58 and ^{125}I-Seed lectin to BGS-Seph with hog blood group A + H substance. Various concentrations of blood group A + H substance were mixed with (●) ^{125}I-DB58 (72.5 ng = 1.25 pmol) or (■) ^{125}I-Seed lectin (144.9 ng = 1.32 pmol) and BGS-Seph (2%) in a final volume of 100 µl. After incubation at room temperature overnight, binding was measured as described in the text.

(Fig. 3). The inclusion of this salt had no effect on the activity of the seed lectin. DB58 activity was also susceptible to KCl and Na_2SO_4 [Fig. 3]. However, DB58 maintained 96% of its activity in 2 M urea and 87% of its activity in 4 M urea (Fig. 3), whereas the activity of the seed lectin was reduced to less than 20% in these concentrations of urea.

Inhibition experiments with the seed lectin showed that the relative inhibitory potency of Me α-D-GalNAc to D-GalNAc was 2.65. This ratio is in close agreement to the value obtained with the ELISA above and to the previously established values [11–13], thus confirming the validity of this assay for determining lectin specificity.

Hog blood group A + H substance inhibits the binding of DB58 to BGS-Seph as shown in Fig. 4. The amounts of this blood group substance required for inhibition are in the range of amounts required for inhibition of binding of the seed lectin to this resin. No inhibition was obtained with 100 mM concentrations of the following sugars: D-GalNAc, D-GlcNAc, D-ManNAc, D-galactose, D-glucose, D-xylose, L-fucose, Me α-D-GalNAc, Me α-D-Gal, Me β-D-Gal, or Me α-D-Man. Only slight and probably insignificant inhibition was obtained with 100 mM concentrations of D-mannose (8.7%), Me β-D-GalNAc (9.7%), Me α-D-GlcNAc (5.6%) and Me β-D-GlcNAc (15.0%) and with 775 µg ml^{-1} asialofetuin (11.5%). The binding of DB58 was also not inhibited by ovine submaxillary mucin or desialylated ovine submaxillary mucin (156 µg ml^{-1}), thus suggesting that this lectin recognizes a larger entity than a monosaccharide.

Discussion

Previous findings that the DB58 lectin from *Dolichos biflorus* does not agglutinate erythrocytes nor precipitate with blood group A + H substance [20] necessitated the development of a solid phase assay for examining the carbohydrate binding properties of this vegetative tissue lectin. The two types of solid phase assays used in the present study are more sensitive than the hemagglutination and precipitation assays previously used for the seed lectin [11–13] and utilize much less lectin. The validity of both of these assays was established by determining the relative abilities of Me α-D-GalNAc and D-GalNAc to inhibit the seed lectin. The values obtained with both assays were similar to ratios previously obtained using inhibition of precipitation assays [11–13].

Although both the seed lectin and DB58 bind to blood group A + H substance, the lectins differ from one another with respect to the conditions under which they bind. As had previously been found [23], unlike the seed lectin, the carbohydrate binding activity of DB58 is sensitive to salt. The inactivation of this lectin by NaCl, KCl and Na_2SO_4 indicates that this sensitivity is neither cation nor anion specific. In contrast, the inclusion of urea up to 4 M diminished DB58 activity by only 13% whereas it abolished more than 80% of the activity of the seed lectin. Since hydrogen bonds have often been implicated in carbohydrate binding, it is difficult to understand the resistance of DB58 to urea. The *Moluccella laevis* lectin has also been reported to retain its activity in urea at concentrations as high as 8 M [29].

Both the seed lectin and DB58 have similar association constants for blood group A + H substance, however, these

lectins appear to differ from one another in carbohydrate specificity. A difference in monosaccharide specificity was suggested by a previous study employing affinity electrophoresis in which both D-GalNAc and D-GlcNAc were found to give very weak inhibition of DB58 [23]. In the present study although the binding of the seed lectin is inhibited by D-GalNAc, no monosaccharide nor methyl glycoside tested was found to give significant inhibition of DB58 binding. These results suggest that the carbohydrate binding site of DB58 differs from the site of the seed lectin in that it accommodates a more complex, perhaps multiantennary structure. Several plant lectins, such as the seed lectins from the legume, *Phaseolus vulgaris*, have been shown to recognize such complex structures (for review, see [1]). The topography of such a structure might be altered upon adsorption to plastic thus explaining the failure of DB58 to bind to the ligand presented in this fashion. It is also possible that the conformation of the lectin itself may be altered by the environment of the microtitre plate wells, rendering it incapable of recognizing the adsorbed ligand. The inactivation of this lectin at low salt concentrations suggests an extreme sensitivity of this lectin structure to external conditions. Neither DB58 nor the seed lectin exhibited the ability to hydrolyse p-nitrophenyl α- or β-D-GalNAc.

Of the 253 amino acid residues that constitute the primary structures of the mature seed lectin and DB58, differences have been found at 33 positions [6–8]. Although these positions are scattered throughout the sequence, preliminary comparisons of these two lectins by computer modelling showed that all but five of the sequence variations are localized in the vicinity of the carbohydrate binding site [30]. It is thus not surprising that these two similar lectins show differences in carbohydrate binding properties. The different degree of oligomerization of these two lectins [16, 20, 21] may also contribute to these differences in activity.

In addition to their carbohydrate binding sites, a number of plant lectins have also been found to have hydrophobic sites with a high affinity for adenine and adenine derivatives [31]. It is interest that no difference in specificity of these adenine binding sites has been detected between the *Dolichos biflorus* seed lectin and DB58 [32]. The confinement of functional variation between these two lectins to their carbohydrate binding properties makes them valuable tools for the exploration of the relationship of lectin structure to function.

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