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Immunochemical Studies of the Combining Sites of the Two Isolectins, A_4 and B_4, Isolated from *Bandeiraea simplicifolia*[1]

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The specificity of two isolectins, A_4 and B_4, of *Bandeiraea simplicifolia* lectin I (BS-I) was studied by quantitative precipitin, precipitin inhibition, as well as by competitive binding assays using various blood group substances and tritium-labeled human B substance. A_4 precipitated well with A_1, A_2, B, and precursor substances, with A_2 precipitating less strongly than did A_1 substance; H, Le^a and Le^b substances did not react. Precipitin inhibition and competitive binding assays confirmed the precipitin data that A_4 is most specific for terminal nonreducing α-linked 2-acetamido-2-deoxy-D-galactopyranose (DGalNAc) but also reacts with oligosaccharides with terminal nonreducing α-linked DGal, thus accounting for its blood group A and B specificities. Of the oligosaccharides tested, A_4 reacted best with DGalNAcα1 → 3DGal and a trisaccharide DGalNAcα1 → 3DGalβ1 → 3DGlcNAc (A_5II) was equally active, suggesting that the A_4 site is no larger than a disaccharide. B_4 precipitated well with B substances and with a precursor substance to a lesser extent, while A_1, A_2, H, Le^a, and Le^b substances were inactive. Precipitin and competitive binding assays showed that it reacted well with oligosaccharides with terminal α-linked DGal with DGalα1 → 3DGal being most active, while

$$\begin{array}{l} \text{LFuc}\alpha 1 \\ \quad\downarrow \\ \quad 2 \\ \text{DGal}\alpha 1 \rightarrow 3\text{DGal}\beta 1 \rightarrow 4\text{DGlcNAc}\beta 1 \rightarrow 6 - \text{R} \end{array}$$

(BR_L 0.44) was much less active, indicating a substitution at the subterminal residue affects the binding substantially and indicating that the B_4 site involves at least the subterminal α1 → 3 linked DGal. The B_4 site was found to be strictly B specific.

Lectins have been isolated from many different plants, mostly from legumes (1–3), and from certain invertebrates and animals (4). Their main characteristic is their ability to bind sugars specifically and thus many agglutinate A, B, and O erythrocytes and react with certain blood group substances to form precipitates similar to those between antibody and antigen. The precipitation can be inhibited specifically by mono- and oligosaccharides and so their combining sites can be studied by quantitative precipitin and inhibition assays (5).

Two lectins have been extracted from the seeds of *Bandeiraea simplicifolia*. Agglutination of B cells by such seed extracts was first described by Mäkelä and Mäkelä (6). Subsequently, Hayes and Goldstein (7) isolated an α-D-galactosyl binding lectin designated as BS-I. A second lectin (BS-II) was isolated from the same seeds (8); it did not agglutinate A, B, or O cells and was shown to have a combining site most specific for terminal nonreducing DGlcNAc[2] (9).

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[2] Abbreviations used: BS-I, *Bandeiraea simplicifolia* lectin I; DGlc, D-glucopyranose; DGal, D-galactopyra-

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BS-I as initially purified by affinity chromatography on Bio-Gel-melibionate (7), is a glycoprotein of M_r 114,000 consisting of four subunits. Although it showed one band on polyacrylamide gel electrophoresis at pH 4.3 as well as in sodium dodecyl sulfate at pH 7 and gave a single symmetrical peak in ultracentrifugation, multiple bands were seen on isoelectric focusing at pH 9.5. Further studies (10) showed BS-I to be a family of five tetrameric isolectins built like lactic dehydrogenase (11) of two different glycoprotein subunits with different binding specificities. The five isolectins were designated as A_4, A_3B, A_2B_2, AB_3, and B_4 (10). A_4 and A_3B were purified on a Bio-Gel-melibionate column, A_2B_2, AB_3, and B_4 were separated on a column of insolubilized polyleucyl A + H hog mucin blood group substance. The A subunit is most specific for αDGalNAc but also reacts with αDGal, whereas the B subunit is specific only for αDGal.

The purification of the BS-I isolectins and the availability of various blood group substances and oligosaccharides permitted studies of the fine specificity of A_4 and B_4 by quantitative precipitin and inhibition assays as well as by competitive binding assays (12). A_4 was found to precipitate well with A_1, A_2, B, and precursor substances, but not with H or Le^a substances, while B_4 reacted well only with B and very weakly with a precursor substance. Precipitin inhibition and competitive binding assays confirmed that A_4 is most specific for terminal nonreducing α-linked DGalNAc; its site also accommodates α-linked DGal but less well, whereas B_4 is specific only for terminal nonreducing α-linked DGal. Among the oligosaccharides tested A_4 and B_4 were inhibited best by DGalNAcα1 → 3DGal and DGalα1 → 3DGal, respectively, thus accounting for their blood group activities. The trisaccharide DGalNAcα1 → 3DGalβ1 → 3DGlcNAc (A_5II) was as active as DGalNAc-α1 → 3DGal, thus suggesting that the A_4 site is no larger than a disaccharide. Similarly for B_4 DGalα1 → 3DGal was the best inhibitor while

$$\begin{array}{l} \text{LFuc}\alpha 1 \\ \quad\downarrow \\ \quad 2 \\ \text{DGal}\alpha 1 \rightarrow 3\text{DGal}\beta 1 \rightarrow \\ \qquad\qquad 4\text{DGlcNAc}\beta(1 \rightarrow 6) - \text{R} \end{array}$$

(BR_L0.44) with a fucose substitution at the subterminal residue was inactive indicating that the B_4 site involves at least a disaccharide. Their sites differ importantly from anti-A and anti-B sites in that substitution of the subterminal DGal by LFucα1 → 2 decreased activities with A_4 and B_4 while such substitution greatly increased activity with anti-A and anti-B. With this consideration it should be noted that certain bacterial polysaccharides containing terminal nonreducing DGalα1 → 3DGal induce antibodies that agglutinate B erythrocytes specifically (13).

MATERIALS AND METHODS

BS-I A_4 and B_4 were purified from *B. simplicifolia* seeds (10). The insolubilized lectins used in competitive binding assay were prepared by coupling the lectins to cyanogen bromide-activated Sepharose 4B (14–16). The blood group substances used were those isolated from human saliva or cyst fluid and from horse gastric mucosa (17–21). The blood group oligosaccharides used were those isolated and characterized previously (18–22). Monosaccharides were obtained commercially (Nutritional Biochemicals Corp. and Schwarz/Mann Research Laboratories). The ^{3}H-labeled Beach ϕOH insoluble[3] (B substance) was prepared by labeling the free amino group of the polypeptide backbone with [^{3}H]acetic anhydride (23). The labeled products were then isolated by affinity

nose; DGlcNAc, 2-acetamido-2-deoxy-D-glycopyranose; DGalNAc, 2-acetamido-2-deoxy-D-galactopyranose; DManNAc, 2-acetamido-2-deoxy-D-mannopyranose; DGalNH$_2$, 2-amino-2-deoxy-D-galactopyranose; *o,p*-NO$_2$$\phi\alpha$DGalNAc, *o,p*-nitrophenyl-α-2-acetamido-2-deoxy-D-galactopyranose; ϕOH, phenol; Con A, concanavalin A.

[3] These substances were purified by digestion with pepsin and precipitation with ethanol; the dried ethanol precipitates were extracted with 90% phenol and fractionally precipitated by addition of 50% ethanol in 95% phenol to the indicated concentrations. The designation 10 or 20% ppt denotes a fraction precipitated from phenol at an ethanol concentration of 10 or 20%. 2× signifies that a second phenol extraction and ethanol precipitation were carried out; a fraction insoluble in 90% phenol is also obtained (17).

chromatography on a BS-I Sepharose column; and then on a Bio-Gel P-100 column.

Immunochemical methods. Quantitative precipitin and precipitin inhibition assays were by the quantitative microtechniques (24) in final volumes of 200 μl. In each assay 5.25 μg N of A_4 and 5.04 μg N of B_4 lectin were used. In quantitative precipitin assays the tubes were incubated at 37°C for 1 h and then kept at 4°C for 1 week with mixing twice daily. In quantitative inhibition assays, the lectin was incubated with the inhibitors for 30 min at 37°C, antigen was then added and mixed followed by incubation at 37°C for 1 h; the tubes were then left at 4°C for 1 week as for the precipitin assay, centrifuged, washed, and total N was determined by the ninhydrin method (25).

Competitive binding assay. The competitive binding assays were performed as described previously (23, 26). Briefly, a 1:40 dilution of the insolubilized A_4 and B_4 lectins consisting of 2 mg lectin coupled to 1 ml of Sepharose 4B was used; 30 μl of the insoluble diluted A_4 and 50 μl of the diluted B_4 suspension were sufficient to bind 50–60% of approximately 4000 cpm of the labeled Beach φOH insol blood group B substance. A mixture of labeled and unlabeled blood group substances or low molecular weight sugar was added to the lectin in a final volume of 350 μl. The tubes were mixed by constant rotation for 16 h at 4°C. Separation of bound and free label was by repeated centrifugation after which 200 μl of the supernatant was counted for ^3H. All determinations were set up in duplicate.

The data are expressed graphically as percentage inhibition of the binding of labeled antigen against nanomoles of mono- or oligosaccharide or nanograms of blood group substance added. The formula used to compute percentage inhibition is:

$$\left(1 - \frac{\text{total cpm added} - \text{cpm in supernatant with inhibitors}}{\text{total cpm added} - \text{cpm in supernatant without inhibitors}}\right) \times 100.$$

RESULTS

Quantitative Precipitin Assays

The isolectin A_4 reacted well with A_1, A_2, B, and precursor substances but not with H, Le^a, and Le^b substances. Reactions with blood group A substances are shown in Fig. 1A. A_1 substances, Cyst 9 φOH insol, and MSS 10% 2×,[3] reacted equally well with the lectin, 20 μg precipitating all the added lectin with 4 μg giving 50% precipitation. Reaction with A_2 substance, Cyst 14 φOH insol, was weaker, 8 μg being needed for 50% precipitation. Reactions with B substances varied; Beach φOH insol reacted well with 6 μg giving 50% precipitation, while Horse 4 25% reacted less well, 9 μg being needed. Tij φOH insol and Tij 20% 2× with blood group B as well as with some I and i determinants reacted less well. N-1 φOH insol, an Le^a substance,

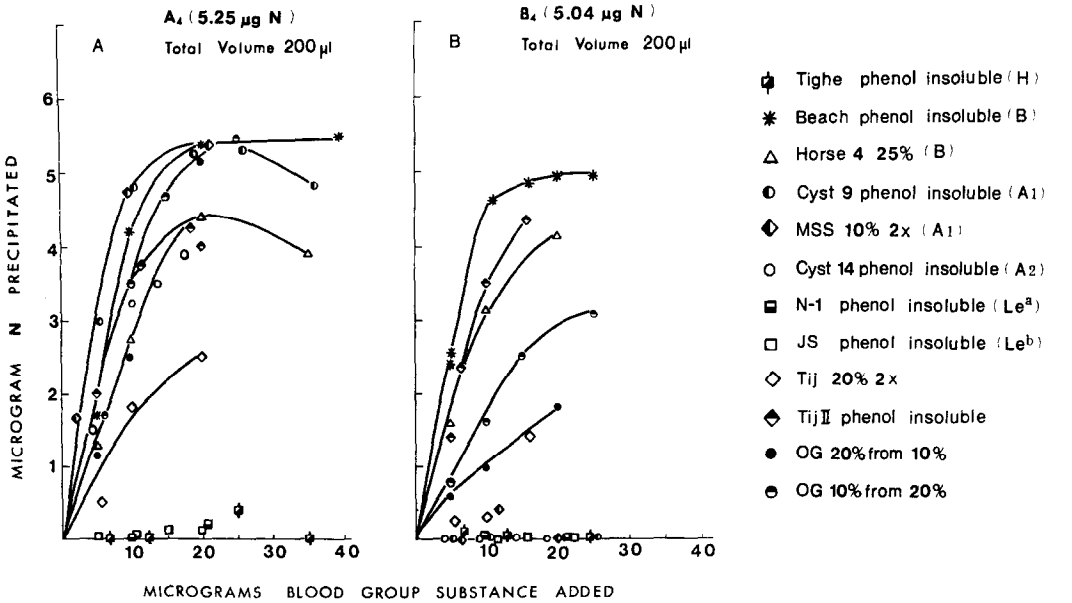


FIG. 1. Quantitative precipitin curves of (A) A_4 and (B) B_4 with various blood group substances.

and JS ϕOH insol, an Le^b substance and Tighe did not react. It also precipitated with a precursor blood group substance OG 10% from 20% and with OG 20% from 10% comparably to Tij 20% 2× thus showing that the lectin can react with the A, DGalNAc and the B, DGal determinants. The reaction with precursor OG substance would suggest some other moiety, most probably terminal DGalNAc linked α to Ser or Thr, known to be present as incomplete chains in blood group substances (27).

The reaction of B_4 with various blood group substances is shown in Fig. 1B. It reacted specifically with B substances but to differing extents; with Beach ϕOH insol 5 μg precipitated 50% of the lectin; with Horse 4 25% and with Tij phenol insoluble 8 μg were needed. Cyst 9 ϕOH insol and MSS 10% 2× (A_1), Cyst 14 ϕOH insol (A_2), N − 1 ϕOH insol (Le^a), and JS ϕOH insol (Le^b) substances did not react. Precursor substances OG 20% from 10%, OG 10% from 20%, and Tij 20% 2× with only about 18% of the B activity of Beach phenol insoluble reacted much less strongly.

Quantitative Precipitin Inhibition Assays (A_4)

These were carried out using various monosaccharides, glycosides, blood groups, and other oligosaccharides as inhibitors. The A_4 inhibition assays were set up to inhibit the precipitin reaction between A_4 and a B substance (Beach ϕOH insol) as shown in Fig. 2A and Table I. The results are in accord with the precipitin data, only oligosaccharides and blood group substance with terminal DGalNAc and DGal were active. The most potent inhibitors were those with terminal nonreducing αDGalNAc while those with αDGal were less active. $pNO_2\phi\alpha$DGalNAc, A oligosaccharides A_5II (DGalNAcα1 → 3DGalβ1 → 3DGlcNAc) (28, 29), and a disaccharide DGalα1 → 3DGal (R_L1.85) (21, 26, 30) were most potent requiring 2, 2.5, and 2.5 nmol for 50% inhibition, respectively. DGalNAcα1 → 6DGal, ethyl αDGalNAc, and methyl αDGalNAc gave 50% inhibition with 3.5 nmol; while free DGalNAc was only one-half as active and $pNO_2\phi\beta$DGalNAc was 4.5-fold less

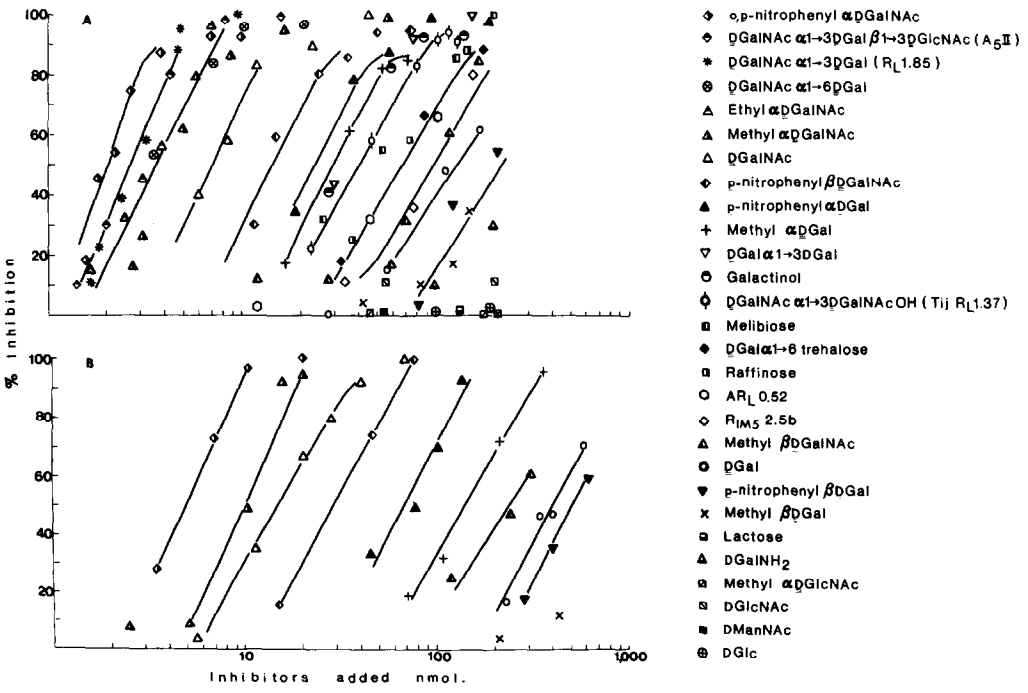


FIG. 2. Inhibition by monosaccharides and various oligosaccharides (A) of precipitation of A_4 (5.25 μg N) by B substance, Beach phenol insoluble (20 μg), (B) of precipitation of A_4 (5.8 μg N) by A substance, Cyst 9 phenol insoluble (15 μg).

TABLE I

ACTIVITIES BY INHIBITION OF PRECIPITIN AND COMPETITIVE BINDING ASSAYS OF VARIOUS OLIGOSACCHARIDES REACTING WITH A_4

Inhibitors	Amount required for 50% inhibition (nmol)		
	A_4 (5.25 μg N) + Beach phenol insoluble (20 μg) (Fig. 2A)	A_4 (5.8 μg N) + Cyst 9 phenol insoluble (15 μg) (Fig. 2B)	Competitive binding assay A_4 insol (30 μl of 1:40) + ^{3}H-labeled Beach phenol insoluble (4000 cpm) (Fig. 4A)
o,*p*$NO_2\phi\alpha$DGalNAc	2.0	5.0	0.39
DGalNAcα1 → 3DGalβ1 → 3DGlcNAc (A_5II)	2.5		
DGalNAcα1 → 3DGal (R_L 1.85)	2.5		
DGalNAcα1 → 6DGal	3.5		
Ethyl αDGalNAc	3.5		
Methyl αDGalNAc	3.5	10	0.8
DGalNAc	7	15	1.9
p$NO_2\phi\beta$DGalNAc	16	29	3.2
p$NO_2\phi\alpha$DGal	24	78	7.8
Methyl αDGal	30	140	7.8
DGalα1 → 3DGal	30		
Galactinol (DGalα1 → 1 myoinositol)	30		
DGalNAcα1 → 3DGalNAcOH (Tij R_L 1.37)	40		
Melibiose (DGalα1 → 6DGlc)	50		
DGalα1 → 6 trehalose	65		
Raffinose (DGalα1 → 6DGlcα1 ⟷ 2βFru)	65		
LFucα1 ↓ 2 DGalNAcα1 → 3DGalβ1 → 4DGlcNAcβ1 → 6-R (AR_L 0.52)	65		
LFucα1 ↓ 2 DGalNAcα1 → 3DGalβ1 → 4DGlcNAcβ1 → 6DGalOH (R_{IM5} 2.5b)	100		
Methyl βDGalNAc	100	240	20
DGal	120	500	70
p$NO_2\phi\beta$DGal	180	600	120
Methyl βDGal	200	—	120
Lactose	>200		
$DGalNH_2$	>200		
Methyl αDGlcNAc	>200		
DGlcNAc	>200		
DManNAc	>200		
DGlc	>200		
LFucα1 ↓ 2 DGalα1 → 3DGalβ1 → 4DGlcNAcβ1 → 6-R (BR_L 0.44)			190

active requiring 7 and 16 nmol for 50% inhibition, respectively. The second group of inhibitors with terminal nonreducing α-linked DGal is less active than those with terminal α-linked DGalNAc; the most potent inhibitor in this group is *p*$NO_2\phi\alpha$DGal, 24 nmol for 50% inhibition, 12-fold poorer than *o*,*p*$NO_2\phi\alpha$DGalNAc. Methyl αDGal, DGalα1 → 3DGal, and galactinol (DGalα1 → 1 myoinositol) were slightly less active,

30 nmol being needed. Other compounds with terminal nonreducing α-linked DGal were melibiose (DGalα1 → 6DGlc), 50 nmol; DGalα1 → 6 trehalose, 65 nmol; raffinose (DGalα1 → 6DGlcα1 ↔ 2βDFru), 65 nmol; for 50% inhibition, respectively. DGal and methyl βDGal were less active, 120 and 180 nmol being needed, respectively. The third group of inhibitors which have terminal nonreducing α-linked DGalNAc had the subterminal residue reduced, DGalNAcα1 → 3DGalNAcOH (Tij R_L1.37) (31), or with a substitution such as the A oligosaccharide

LFucα1
↓
2
DGalNAcα1 → 3DGalβ1 →
4DGlcNAcβ1 → 6R

(AR_L 0.52) and

LFucα1
↓
2
DGalNAcα1 → 3DGalβ1 →
4DGlcNAcβ1 → 6GalOH

(R_{IM5} 2.5b) (22). Reduction of or substitution on the subterminal residue greatly reduced inhibitory power, the reduced compound required 40 nmol and the substituted compounds 65 and 100 nmol, respectively. Lactose, D-galactosamine, methyl βDGalNAc, methyl αDGlcNAc, DGlcNAc, DManNAc, and DGlc were inactive in amounts up to 200 nmol.

Comparable inhibition assays were also set up to inhibit the reaction between A_4 and an A substance (Cyst 9 ϕOH insol) (Fig. 2B and Table I). Results were similar to those with A_4 and the B substance (Beach ϕOH insol) (Fig. 2A) but two- to fourfold more of each inhibitor was needed.

Quantitative Precipitin Inhibition Assays (B_4)

Figure 3 and Table II show the inhibition of precipitation between B_4 and Beach ϕOH insol. DGalα1 → 3DGal was the most active inhibitor, 80 nmol being required for 50% inhibition. In general, compounds that have terminal nonreducing α-linked DGal were active, those tested were methyl αDGal, 85 nmol; pNO$_2\phi\alpha$DGal, 120 nmol; DGalα1 → 6 trehalose, 120 nmol; melibiose, 140 nmol; galactinol, 175 nmol; and raffinose, 225 nmol for 50% inhibition, respectively. It is unusual that pNO$_2\phi\beta$DGal was somewhat better as an inhibitor than pNO$_2\phi\alpha$DGal only 85 nmol as compared to 120 nmol being needed whereas other β-linked DGal compounds such as methyl βDGal and lactose were quite inactive. The identity of the p-nitrophenyl α and βDGal was checked by specific optical rotation and on two different solutions. A B blood group oligosaccharide BR_L 0.44 (20)

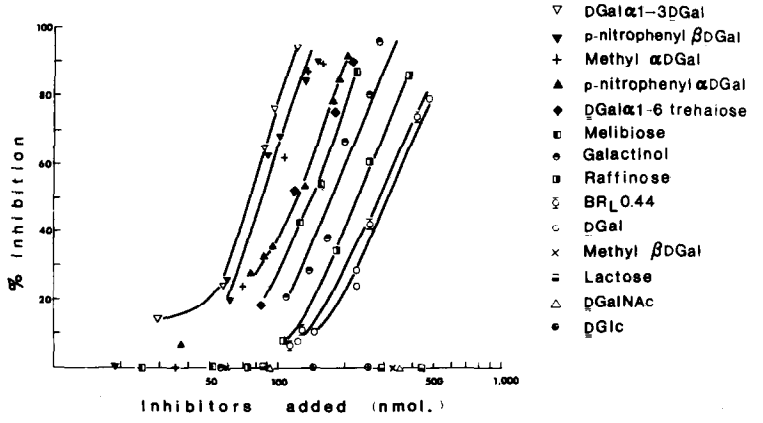


FIG. 3. Inhibition of monosaccharides and various oligosaccharides of precipitation of B_4 (5.04 μg N) with B substance, Beach phenol insoluble (20 μg).

TABLE II

ACTIVITIES BY INHIBITION OF PRECIPITIN AND COMPETITIVE BINDING ASSAYS OF VARIOUS OLIGOSACCHARIDES REACTING WITH B_4

Inhibitors	Amount required for 50% inhibition (nmol)	
	B_4 (5.04 μg N) Beach phenol insoluble (20 μg) (Fig. 3)	Competitive binding assay B_4 insol (50 μl of 1:40) + Beach phenol insol (4000 cpm) (Fig. 4B)
DGalα1 → 3DGal	80	20
$pNO_2\phi\beta$DGal	85	20
Methyl αDGal	85	20
$pNO_2\phi\alpha$DGal	120	45
DGalα1 → 6-trehalose	120	
Melibiose	140	
Galactinol	175	
Raffinose	225	
LFucα1 ↓ 2 DGalα1 → 3DGalβ1 → 4DGlcNAcβ1 → 6-R (BR_L 0.44)	285	
DGal	310	150
Methyl βDGal	>330	340
Lactose	>420	
DGalNAc	>350	>450
DGlc	>250	

LFucα1
↓
2
DGalα1 → 3DGalβ1 →
4DGlcNAcβ1 → 6R

only showed slight activity.

Competitive Binding Assays

Various oligosaccharides and blood group substances were used to inhibit the binding of the insolubilized lectin and ^{3}H-labeled Beach φOH insol. Competitive binding of A_4 and ^{3}H-labeled Beach φOH insol is shown in Fig. 4A and Table I, and that between B_4 and ^{3}H-labeled Beach φOH insol in Fig. 4B and Table II. The results confirmed the precipitin inhibition assays and also required two- to fivefold less inhibitors for 50% inhibition. The most active inhibitors of A_4 are those with terminal α-linked DGalNAc such as $o,pNO_2\phi\alpha$DGalNAc and methyl αDGalNAc while their β-anomers were much less active. Compounds with terminal α-linked DGal also were somewhat less active than those with terminal α-linked DGalNAc. The B_4 competitive binding assays also showed that both DGalα1 → 3DGal, $pNO_2\phi\beta$DGal, and methyl αDGal were equally active and twice as active as $pNO_2\phi\alpha$DGal which required 45 nmol for 50% inhibition. DGal and methyl βDGal were much less active while DGalNAc did not inhibit even at 450 nmol.

Competitive binding of A_4 and ^{3}H-labeled Beach φOH insol by other unlabeled blood group substances is shown in Fig. 5A. The results also support the quantitative precipitin data. A_1, A_2, and B substances competed well but A_2 (Cyst 14) is a weaker inhibitor as compared to A_1 (Cyst 9).

Figure 5B showed the competitive binding of B_4 and ^{3}H-labeled Beach φOH insol by various unlabeled substances. A_1 and A_2 substances showed no activities while B substances competed well. Horse 9 φOH insol (B) reacted well, 1 μg giving 50% inhibition, while Horse 9 10% 2× and Horse 9 20% 2× which required 10 and 20%

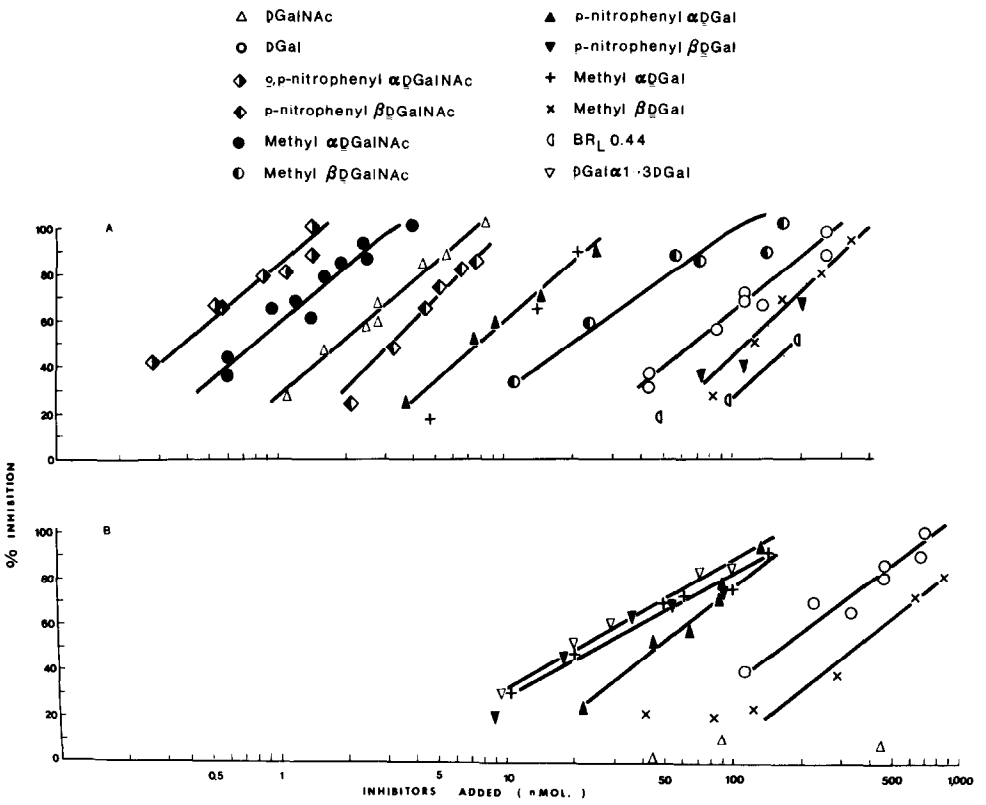


FIG. 4. Competitive binding assays of monosaccharides and oligosaccharides with (A) A_4–Sepharose (30 μl of 1:40) and B substance, ^{3}H-labeled Beach phenol insoluble (4000 cpm), (B) B_4–Sepharose (50 μl of 1:40), and ^{3}H-labeled Beach phenol insoluble (4000 cpm).

ethanol, respectively, for precipitation from phenol were weaker inhibitors, thus showing a decrease in B activities when higher percentages of ethanol were used for precipitation. Such a decrease in activity was not observed with A_4 (Fig. 5A) with Horse 9 10% 2× reacting best; Horse 9 φOH insol and Horse 9 20% 2× were slightly weaker.

DISCUSSION

BS-I consists of two lectins with different specificities (10). Precipitin inhibition studies with A_4 showed DGal and DGalNAc to be good inhibitors, while B_4 was only inhibited by DGal (10). The present findings support and extend this observation in defining further the specificites of A_4 and B_4 by quantitative precipitin, quantitative precipitin inhibition, as well as by competitive binding assays. Inhibition (Fig. 2A) and competitive binding assay (Fig. 4A) with A_4 showed $o,p NO_2\phi\alpha$DGalNAc to be the best inhibitor and 3.5- and 7-fold better than DGalNAc and $pNO_2\phi\beta$DGalNAc and competitive binding assays showed it to be 5- and 8-fold better, respectively. Methyl αDGalNAc is less active than $o,pNO_2\phi\alpha$DGalNAc by both assays; $pNO_2\phi$-glycosides have also been shown to be better inhibitors than the methyl glycosides in Con A (32), *Sophora japonica* (33), peanut agglutinin (34), and BS-II (9). This has also been interpreted as involving a hydrophobic contribution to the binding, thus indicating that there are some hydrophobic interactions between the phenyl ring and the lectin site. The precipitin reaction between A_4 and blood group substance can also be inhibited by compounds with terminal nonreducing α-linked DGal (Fig. 2A); the most active $pNO_2\phi\alpha$DGal is 3.4-fold less active than DGalNAc but 5-fold better than DGal, while methyl αDGal is only 4-fold better than

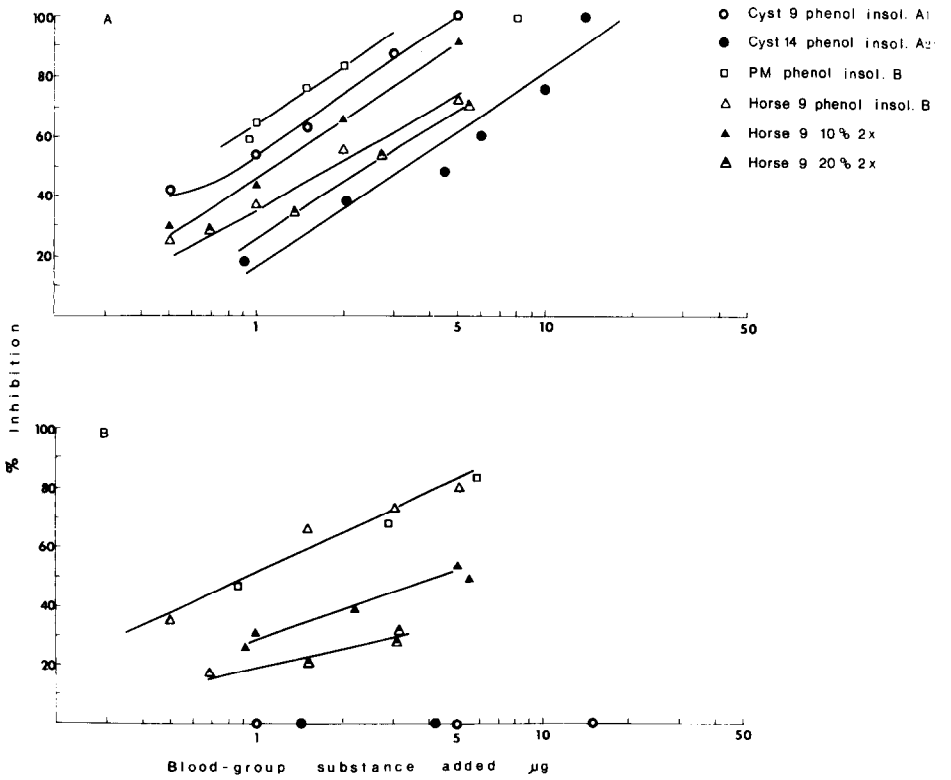


FIG. 5. Competitive binding assays of various blood group substances with (A) A_4–Sepharose (30 µl of 1:40) and B substance, ^{3}H-labeled Beach phenol insoluble (4000 cpm), (B) B_4–Sepharose (50 µl of 1:40), and ^{3}H-labeled Beach phenol insoluble (4000 cpm).

DGal, again showing some hydrophobic interaction of the phenyl ring; DGlc, DGlcNAc, DManNAc, and DGalNH_2 were inactive. Thus the DGal conformation is required for reaction and an equatorial *N*-acetamido group at C2 can enhance activity significantly, but an equatorial NH_2 group diminished it. Studies with di- and higher oligosaccharides with terminal DGalNAc showed the terminal sugar and the α-linkage to play a significant role in binding.

The subterminal sugar is also involved in the binding. Figure 2A shows that the disaccharides DGalNAcα1 → 3DGal and DGalNAcα1 → 6DGal (R_L 1.85) and a trisaccharide DGalNAcα1 → 3DGalβ1 → 3DGlcNAc (A_5II) to have similar activities, while a disaccharide DGalNAcα1 → 3 *N*-acetyl-D-galactosaminitol (Tij R_L 1.37) was 16-fold less active. Two other blood group oligosaccharides

LFucα1
↓
2
DGalNAcα1 → 3DGalβ1 →
4DGlcNAcβ1 → 6DGalactitol

(R_{IM5} 2.5b) and

LFucα1
↓
2
DGalNAcα1 → 3DGalβ1 →
4DGlcNAcβ1 → 6R

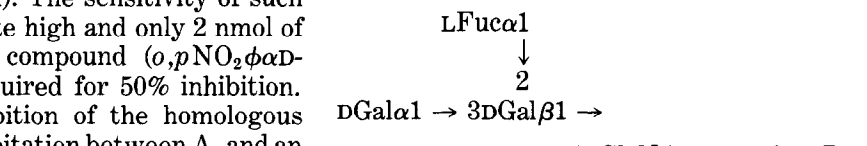
(AR_L 0.52) were tested and were 40- and 26-fold less active than DGalNAcα1 → 3DGal indicating that opening the ring to give the alditol or a substitution with L-fucose at the subterminal residue interferes substantially with the binding.

The inhibition assays with A_4 were done with a heterologous system, that is, using

inhibitors to inhibit the precipitation reaction between A_4 and a blood group B substance (Beach ϕOH insol). The sensitivity of such a system was quite high and only 2 nmol of the most active compound (o,pNO$_2\phi\alpha$DGalNAc) was required for 50% inhibition. Comparable inhibition of the homologous system, the precipitation between A_4 and an A substance (Cyst 9 ϕOH insol), required two- to fourfold more of a given inhibitor (Fig. 2B and Table I).

Competitive binding assays were two- to fivefold more sensitive than precipitin inhibition assay. The most potent inhibitor for binding of ^{3}H-labeled Beach ϕOH insol and A_4 is o,pNO$_2\phi\alpha$DGalNAc, only 0.39 nmol giving 50% inhibition as compared to 2 nmol for the heterologous precipitin inhibition.

The inhibition assays support the precipitin data (Fig. 1A) and explain why A_4 precipitated both A and B substances while H and Le^a substances which lack terminal unsubstituted DGal or DGalNAc did not react. It is interesting to note that the lectin reacted better with A_1 substances (Cyst 9 and MSS) than A_2 (Cyst 14) and it also reacted well with precursor substances (OG 20% from 10% and OG 10% from 20%). This could be in part due to the terminal nonreducing βDGal residues or to the terminal αDGalNAc present as incomplete chains and linked to serine or threonine of the polypeptide backbone. Competitive binding assays also showed A_1 substance (Cyst 9) to be about 3.5-fold more active than A_2 substance (Cyst 14), in accord with quantitative precipitin data. In the inhibition studies of B_4 (Fig. 3) only DGal of the monosaccharides tested showed activity. It is surprising to find methyl αDGal and pNO$_2\phi\beta$DGal to be of similar activity while pNO$_2\phi\alpha$DGal is slightly less active; methyl βDGal is inactive up to 330 nmol, this was found on precipitin inhibition and competitive binding assay. Molecular models did not provide any obvious explanation but perhaps the hydrophobic aglycone of pNO$_2\phi\beta$DGal may make somewhat better contact in the site than it does in pNO$_2\phi\alpha$DGal. Of the disaccharides tested only those with terminal nonreducing αDGal were active. The disaccharide DGalα1 → 3DGal is the most active compound, while a B oligosaccharide BR_L 0.44,



with L-fucose substituted on the subterminal residue has much reduced activity (Fig. 3) as was found for the comparable oligosaccharide in the A_4 system again showing that the subterminal residue is involved in binding.

Figure 5B showed the competitive binding assays of B_4 with various unlabeled blood group substances. A_1 and A_2 substances showed no activity while B substances were active. Horse 9 ϕOH insol (B) reacted well, 1 μg giving 50% inhibition, while Horse 9 10% 2× and Horse 9 20% 2× which required 10 and 20% ethanol for precipitation from phenol were weaker inhibitors showing decreases in B activity as higher percentages of ethanol were used for precipitation. Such a decrease in activities was not observed with A_4 (Fig. 5A).

The above studies show the A_4 site to be most specific for terminal nonreducing α-linked DGalNAc but is able to accomodate αDGal; it involves at least a portion of a subterminal DGal and DGalNAcα1 → 3DGal is somewhat better than DGalNAcα1 → 6DGal as might be expected from its blood group A activity; DGalNAc linked α to other linkages should be tested. The B_4 site is strictly specific for terminal nonreducing DGal, reacting with all compounds with terminal nonreducing α-linked DGal; like A_4 it reacted better with compounds having α1 → 3 than those with α1 → 6 linkages, consistent with its B specificity; oligosaccharides with other linkages should also be tested.

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