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Multivalent II [β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc] and Tα [β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc] specific Moraceae family plant lectin from the seeds of *Ficus bengalensis* fruits

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Abstract—A galactose specific lectin was isolated from the seeds of *Ficus bengalensis* (Moraceae) fruits and designated as *F. bengalensis* agglutinin (FBA). The lectin was purified by affinity repulsion chromatography on fetuin-agarose and was a monomer of molecular mass 33 kDa. Like other Moraceae family lectins, carbohydrate-binding activity of FBA was independent of any divalent cation. FBA did not bind with simple saccharides, however sugar ligands with aromatic aglycons showed pronounced binding. The combining site of FBA recognized preferably Galβ1,4GlcNAcβ1-(II) followed by Galβ1,3GalNAcα1-(Tα) containing glycotopes. Interaction with saccharides revealed that the combining site of FBA could well accommodate a tetrasaccharide, asialo GM1 glycan (Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1-), whereas polyvalent Tn (GalNAcα1-Ser/Thr), one of the well-recognized ligands of Moraceae family lectin, did not interact well with FBA.

Keywords: Moraceae; Lectin; *Ficus bengalensis*; ELLSA; FBA

1. Introduction

The structural domain of carbohydrate-binding site(s) is well conserved in a particular plant lectin family and accordingly these families exhibit an almost similar specificity.[1,2] Galactose/T-specific jacalin related lectins are considered a small homogeneous family of Moraceae lectin family. They have been gaining importance for their exclusively high avidity for human tumor associated Tα (Galβ1,3GalNAcα1-Ser/Thr) and Tn (GalNAcα1-Ser/Thr) antigens on the malignant cells. Simultaneously they exhibit low or weak binding for other possible cross-reactive antigens on the cells, particularly II (Galβ1,4GlcNAcβ1-), Tβ (Galβ1,3GalNAcβ1-) of gangliosides and sialylated T and Tn antigens.[3–7] Lectins with the same mono- and oligosaccharide specificity often demonstrated different specificity for macromolecules and showed changes in specificity from one type of sugar ligand to another type depending on the surface density of carbohydrate on macromolecules.[6] Although polyvalency in the ligands some times broadens the specificity yet clustered Tn and Tα glycotopes are the most interactive for jacalin related Moraceae family lectins.

Jacalin (from *Artocarpus integrifolia*) and ALA (*Artocarpus lakoocha* agglutinin), two important Thomson–Friedenreich antigen (Galβ1,3GalNAc) specific Moraceae plant lectins were purified and characterized with special reference to their specificity to the molecular level, earlier in our laboratory.[4,6,8–10] Jacalin showed specificity for IgA_1 among other immunoglobulins,[11,12] binding for specific regions of HIV,[13] induced differentiation of leukemic cell K 562[14] and potent and selective stimulation of distinct T cell function.[15]

In this article we have described a simple purification procedure, affinity repulsion chromatographic technique of a lectin from the seeds of fruits from *Ficus bengalensis* including its sugar specificity by enzyme linked lectinsorbent assay, ELLSA. Further characterization of the

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lectin with a view to binding toward the mammalian carbohydrate structural units and the corresponding glycotopes at a macromolecular level has been done and compared with other Moraceae lectins.

2. Results and discussion

2.1. Purification and molecular characterization

F. bengalensis agglutinin, FBA was purified by affinity repulsion chromatography on fetuin-agarose column (Fig. 1) and the yield of the lectin was 5.22% (Table 1). FBA on purification exhibited a single band with a molecular weight of 34 kDa in SDS PAGE both under reducing and nonreducing condition (Fig. 2a) and a molecular weight of 33.51 kDa in ES-MS spectra (Fig. 3). Gel filtration on Superose 12 column FBA eluted at 14.7 ml corresponded to 30 kDa (Fig. 2b), which indicated that FBA was a monomeric lectin. FBA was stable at a wide pH range from 5 to 9 and the hemagglutinating activity was maximum from pH 6.5 to 7.5. The activity of the lectin persisted up to 40 °C and was gradually abolished above 40 °C. The

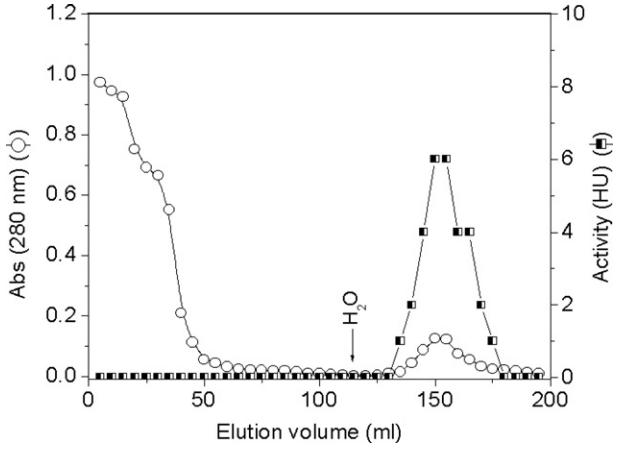

Figure 1. Purification of *Ficus bengalensis* agglutinin (FBA). Affinity repulsion chromatographic profile of *F. bengalensis* seed extract on fetuin-agarose column (10 cm × 1 cm). Protein elution was monitored spectrophotometrically at 280 nm, and activity was observed as hemagglutination of pronase-treated human O erythrocytes.

Table 1. Purification scheme of *Ficus bengalensis* agglutinin[a]

Purification steps	Total protein (mg)	Total activity (HU)[b]	Specific activity (HU/mg)	Recovery of protein (%)
Seed extract	9.88	4160	421.05	100
FBA	0.516	1192	2309.47	5.22

[a] Data shown are mean of three experiments.
[b] Hemagglutination unit (HU) is defined as the minimum amount of protein (μg/mL) showing hemagglutination with pronase-treated human O erythrocytes.

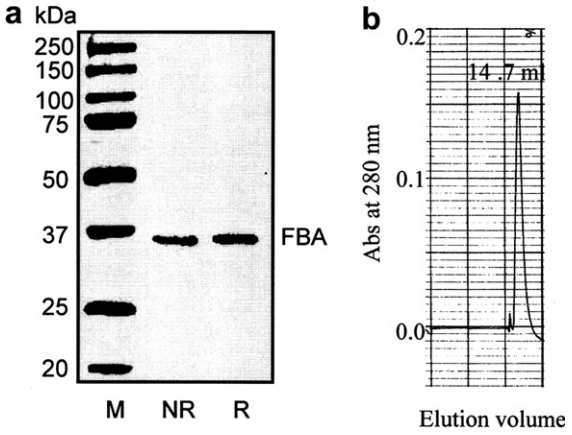

Figure 2. Molecular mass and size of FBA. (a) 11% SDS–PAGE of purified FBA. The gel was stained with Coomassie blue G 250. M—precision plus protein standard from Bio-Rad, NR—denatured but nonreduced FBA, R—denatured and reduced FBA. (b) Gel filtration chromatography of FBA on FPLC by a Superose 12 10/30 column and the elution was monitored at 280 nm.

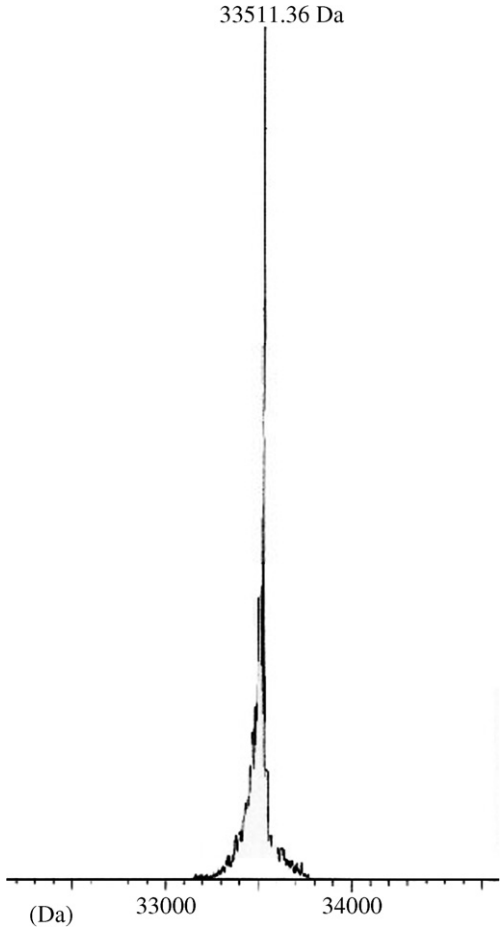

Figure 3. Electron spray ionization mass spectra of FBA.

hemagglutinating activity was totally independent on metal ions. FBA agglutinated human blood groups A, O, and B but the minimum concentration of FBA

required for hemagglutination of A was little more (12.5 μg/mL) than O and B (6.25 μg/mL) (Table 2). However, increase in hemagglutinating activity was observed when the red blood cells were treated with pronase and neuraminidase. The lectin strongly agglutinated rabbit erythrocytes being 32 times more than human A and 16 times more than human O and B erythrocytes. There observed two times increase in hemagglutination than normal rabbit erythrocytes where the cells were treated with either pronase or neuraminidase.

2.2. Carbohydrate specificity

Plant lectins mediate symbiosis between rhizobia and leguminous plants, transport of storage carbohydrates and defense of plants against different kinds of predatory invertebrates and higher animals as well as against phytopathogenic bacteria.[16] Lectins are used recently to study aberrant glycosylation that occur on cell surfaces during pathophysiological processes. To ascertain the biological function and to develop FBA as a potential tool for research in glycobiology, knowledge of the carbohydrate specificity of this lectin becomes imperative. The specificity of FBA was elucidated by the two methods: hemagglutination–inhibition and enzyme-linked solid phase assay. Table 3 shows the results of the hemagglutination inhibition of FBA (2HU) with glycoproteins. The following sugars Gal, $GalNH_2$, GalNAc, Me-αGal, Me-βGal, Glc, GlcNAc, Man, Ara, melibiose, raffinose, Neu5Ac, and lactose even at 200 mM did not inhibit the hemagglutination. However, it was strongly inhibited by N-linked glycans viz., apotransferrin, porcine thyroglobulin (PTG), bovine thyroglobulin (BTG), and fetuin and at high concentration by O-linked glycan namely bovine submaxillary mucin (BSM), porcine submaxillary mucin (PSM), birds' nest glycoprotein (BNG).

The interaction of FBA with immobilized glycoproteins is summarized in Table 4 and the binding profiles with individual glycoconjugates are shown in Figure 4. The binding data was expressed as the amount of glycoproteins (ng) required for binding which corresponded to abs. 1.5 at 492 nm.[6] The results showed that among the glycoproteins tested multiantennary II containing glycoproteins interacted FBA more strongly than polyvalent Tα or Tn containing one. Asialo apotransferrin

Table 2. Hemagglutination profile of FBA hemagglutination ability was represented as the minimum concentration of FBA (μg/mL) required for one visible hemagglutination

Erythrocytes	Normal	Pronase treated	Neuraminidase treated
Human			
A-type	12.5	1.56	1.56
B-type	6.25	0.39	0.78
O-type	6.25	0.39	0.78
Rabbit	0.39	0.18	0.18

Table 3. Hemagglutination–inhibition assay

Inhibitor	MIC[a] (μg/mL)	Inhibitory intensity[b]
Apotransferrin	0.97	+++++
PTG	1.94	++++
BTG	3.88	+++
Fetuin	3.88	+++
BSM	31.2	+
PSM	62.4	+
BNG	62.4	+
Gal	$>3.63\times10^4$	−
$GalNH_2$	$>3.58\times10^4$	−
GalNAc	$>4.42\times10^4$	−
Meα-Gal	$>3.88\times10^4$	−
Meβ-Gal	$>3.88\times10^4$	−
Galα1,6Gal	$>6.84\times10^4$	−
Galα1,6Glcβ1,2Fru	$>1.01\times10^5$	−
Galβ1,4Glc	$>6.84\times10^4$	−
Glc	$>3.63\times10^4$	−
GlcNAc	$>4.42\times10^4$	−
Man	$>3.63\times10^4$	−
Ara	$>3.00\times10^4$	−
Neu5Ac	$>4.89\times10^4$	−

[a] The minimum inhibitory concentration required for inhibiting two HU (pronase-treated human O erythrocytes) of FBA.
[b] Results were interpreted according the relative inhibitory potency. Expression pattern for sugars/glycoproteins were indicated by plus sign (+) and minus sign (−) showing inhibition and noninhibition of hemagglutination, respectively.

Table 4. Binding of biotinylated FBA (15 ng/50 μL) with serially diluted different glycoproteins starting from 1 μg

Curve no. (Fig. 4)	Inhibitor	Quantity[a] (ng) required for $A_{492\,nm}$ 1.5	Maximum binding ($A_{492\,nm}$)
8	Asialo apotransferrin	26.62	2.77
2	Asialo PTG	32.63	2.68
7	Apotransferrin	45.34	2.46
14	Asialo fetuin	53.02	2.64
1	PTG	55.58	2.092
18	Asialo BTG	131.54	2.92
13	Fetuin	179.93	1.9
17	BTG	242	2.84
12	Asialo HCG	251.97	2.108
20	Asialo BNG	464.16	1.558
6	Asialo GM1-BSA	>1000	1.46
19	BNG	>500	1.357
16	Asialo PEM	>1000	0.705
15	PEM	>1000	0.47
10	Asialo BSM	>1000	0.465
9	BSM	>1000	0.437
22	Asialo AGP	>1000	0.35
4	Asialo PSM	>1000	0.315
11	HCG	>1000	0.281
3	PSM	>1000	0.225
21	AGP	>1000	0.215
5	Asialo OSM	>1000	0.167

[a] Amount of glycoproteins required for binding that corresponded to 1.5 unit absorbance at 492 nm after termination of enzymatic reaction at 2 h.

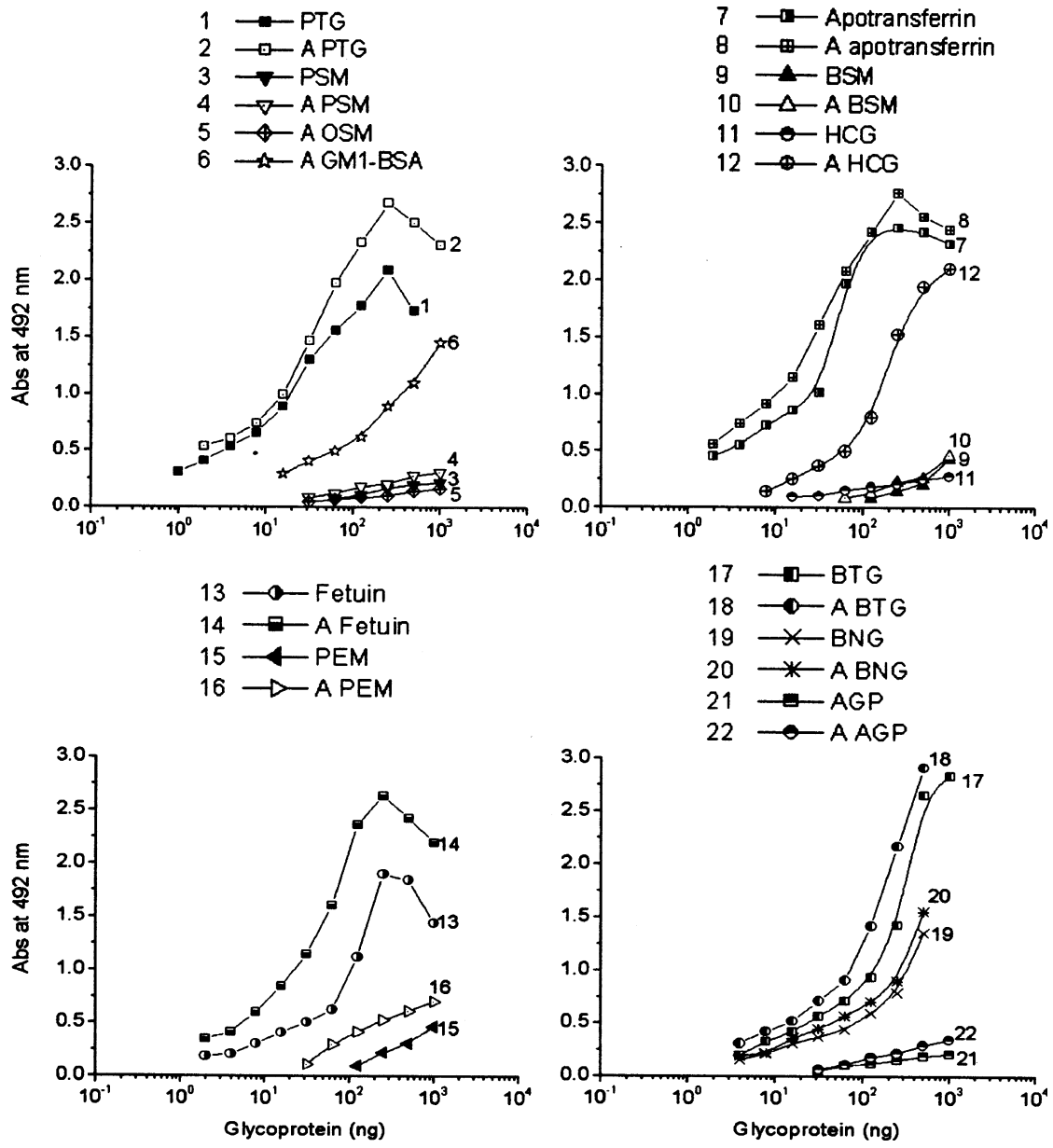

Figure 4. Binding profile of biotinylated FBA with plate coated glycoproteins. FBA (15 ng per well) was used. Wells were coated with serially diluted glycoproteins started from 1 μg (except BNG and asialo BNG, from 500 ng). Binding was measured at 492 nm after 2 h incubation of substrate (OPD) and terminating the reaction by adding 50 μL 6 N H_2SO_4.

that contains exclusively N-linked oligosaccharide (II) interacted best with FBA. Only 26.6 ng was required to reach 1.5 absorbance at 492 nm. In addition, FBA also reacted strongly with other multivalent-II containing N-glycans, that included asialo PTG, apotransferrin, asialo fetuin, PTG; the amount required to reach the value 1.5 was within the range from 32.6 to 55.5 ng. FBA showed less binding activity with asialo BTG, fetuin, BTG, and asialo human chorionic gonadotropin (asialo HCG) since the amount required for binding was appreciably high (131–251.97 ng). A large number of glycoproteins showed very weak binding to FBA even when used at a concentration more than 1 μg (Table 4). Polyvalent Tα containing glycoproteins, such as porcine erythrocyte mucoid (PEM), asialo PEM, and polyvalent Tα and Tn containing glycoproteins such as BSM, PSM and their asialo counterparts were very poor ligands. Asialo ovine submaxillary mucin (asialo OSM), in which Tn is present as major glycan also shared negligible binding. Asialo GM1-BSA (Galβ1,3GalNAcβ1,4Galβ1, 4Glcβ1-O-CH_2-S(CH_2)$_2$CONH–BSA) containing Tβ unit as well as linker chain, interacted with FBA in higher concentration (abs. 1.47 at 1 μg). BNG and asialo BNG bound to FBA but not significantly, whereas human α1 acid glycoprotein (AGP) and its asialo form were weak ligands.

Table 5. Inhibitory percentage of various saccharides to the binding of biotinylated FBA (7.5 ng/50 μL) and plate coated asialo fetuin (250 ng/50 μL)

Curve no. (Fig. 5)	Inhibitor	Maximum amount of sugar inhibitor used (nmol)	Percentage of inhibition[a]
—	Gal	10,000	5.6
—	GalNAc	10,000	3.8
—	Me-αGal	10,000	6.8
—	Me-βGal	10,000	10.54
—	Me-αGalNAc	625	0
—	Me-βGalNAc	625	0
—	Glc	5000	0
—	Man	5000	1.36
—	Neu5Ac	10,000	0
—	*p*-NO_2Ph-αGal	2500	9.18
—	*p*-NO_2Ph-βGal	1250	0
—	*p*-NO_2Ph-αGalNAc	312.5	0
—	*p*-NO_2Ph-βGalNAc	156.25	6.8
2	*p*-NO_2Ph-β-(Galβ1,4Glc)[b]	625	77.88
—	GalNAcα1-Ser/Thr (Tn)	317.36	0
1	Galβ1,4Glc	5000	29.32
3	Galβ1,4GlcNAc (II)	1250	20.5
—	Galβ1,4Gal	10,000	19.28
4	Galα1,6Glc	10,000	21.69
—	Galα1,6Glcβ1,2Fru	5000	2.4
—	GlcNAc	5000	0
—	GalA	5000	0
—	Galα1,3Gal	625	10.44
5	Galβ1,3GalNAc (T)	312.5	16.1
6	Tα-benzyl (Galβ1,3GalNAcα1-benzyl)	312.5	36.66
7	Asialo GM1-OTs (Galβ1,3GalNAcβ1,4Galβ1,4Glcβ-)	625	49.397
8	Asialo GM2-OTs (GalNAcβ1,4Galβ1,4Glcβ-)	1250	21.28

'—' Respective curves are not shown in Figure 5 for the clarity.

[a] The inhibitory activity was estimated and is expressed as the percentage of inhibition by the sugars (nmol).

[b] 185 nmol *p*-NO_2Ph-β-(Galβ1,4Glc) gave 50% inhibition of the binding.

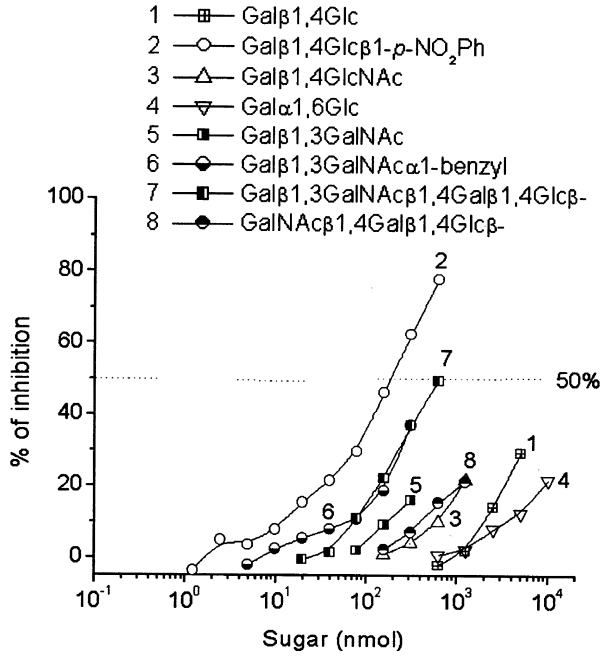

Figure 5. Inhibition of binding between biotinylated FBA and plate coated asialo fetuin by selected saccharides. Wells were coated with 250 ng of asialo fetuin. The lectin was pre-incubated with an equal volume of serially diluted sugars. The final FBA was used 7.5 ng per well.

The effective binding of plate coated glycoproteins with soluble lectin depends on two factors; the nature of protein part that promotes adsorption on the plate and availability of glycan moiety for interaction with lectin. As the percentage of absorbance of the glycoproteins on the microtiter plate assay had not been established, the relative binding ability of different glycoproteins with FBA could not be accurately measured. Therefore, the carbohydrate specificity of the lectin was further confirmed by inhibiting the binding of FBA with asialo fetuin using well-defined glycoproteins. In ELLSA inhibition assay, the percentage of inhibition by the sugars is summarized in Table 5 and the inhibition profiles of interacting sugars are shown in Figure 5. Among the sugars tested *p*-NO_2Ph-β-Lac required 185 nmol for 50% inhibition of the binding. Consistent with the hemagglutination inhibition study all tested monosaccharides were either noninhibitors or very weak inhibitors. Compared to monosaccharides the disaccharides showed better inhibition though all inhibited less than 50% except aromatic aglycon containing ligands.

The inhibition profiles of individual glycoproteins are shown in Figure 6 and the results are summarized in Table 6 as 50% inhibition of FBA–asialo fetuin binding. From the results it was revealed that the polyvalent

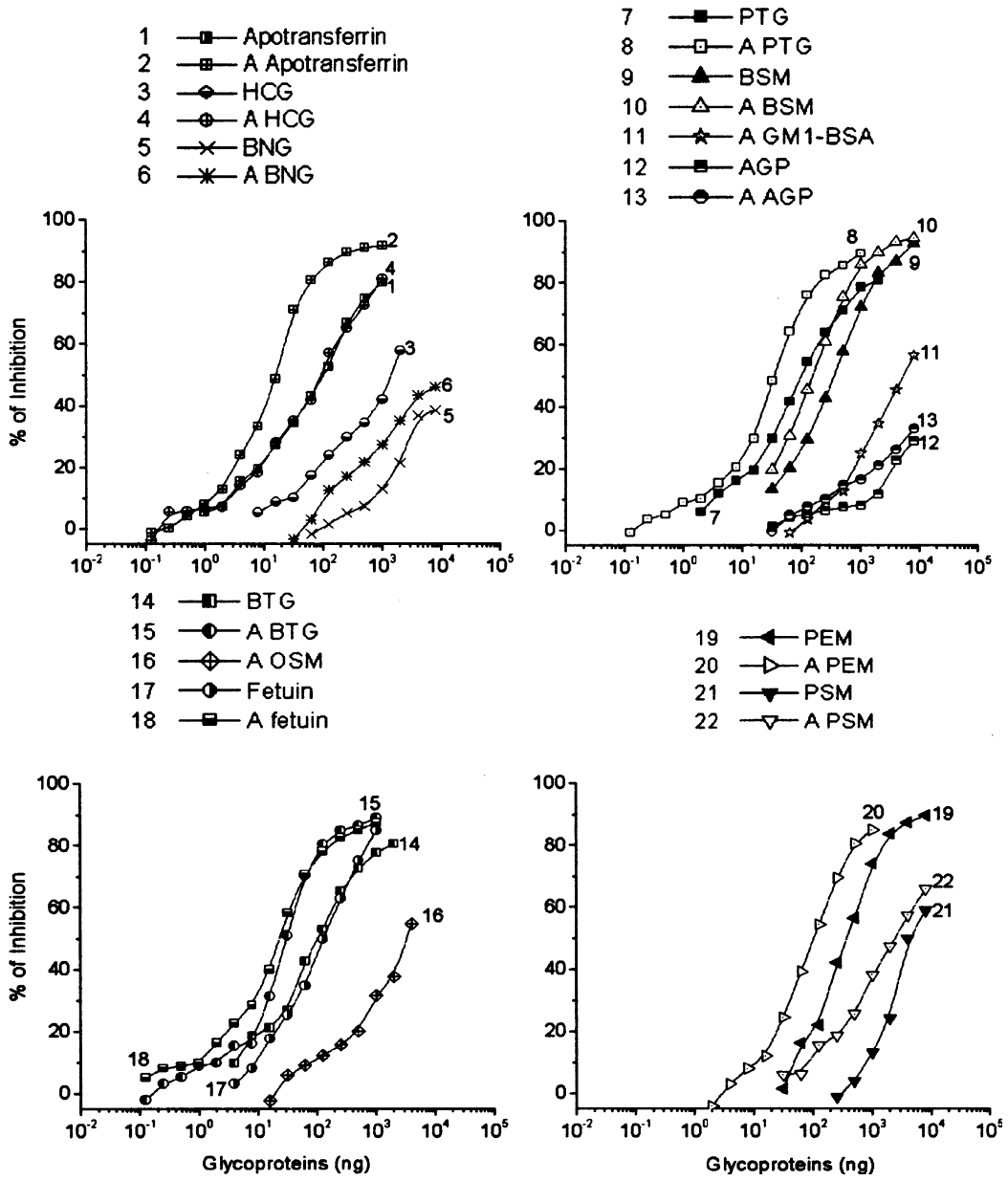

Figure 6. Inhibition of binding between biotinylated FBA and asialo fetuin by different glycoproteins. Wells were coated with 250 ng of asialo fetuin. The lectin was pre-incubated with an equal volume of serially diluted glycoproteins. FBA was added 7.5 ng per well. Amount for 50% inhibition of binding was calculated.

glycotopes present in tested glycans generated a great enhancement of the interaction with the lectin compared to saccharides. Similar results obtained by hemagglutination inhibition assay. Like ELLSA binding assay, multivalent-II containing N-linked glycoproteins were more inhibitory than polyvalent Tα and Tn containing glycoproteins. But as the percentage of specific sugars in individual glycoproteins is different, it cannot be exclusively concluded that the inhibiting potency of multivalent-II containing glycoproteins were more than polyvalent Tα or Tn containing glycoproteins. Asialo apotransferrin was the best inhibitor among the glycoproteins tested and required 15.85 ng for 50% inhibition of FBA–asialo fetuin binding. Asialo fetuin, asialo BTG and asialo PTG were also good inhibitor and requires 23.08, 31.18, and 34.25 ng, respectively, for 50% inhibition of binding. Due to the masking effect of sialic acid the inhibitory potency of these sialoglycoproteins were decreased than their asialo counter part. Asialo HCG (92.76 ng), multivalent-II containing ligand inhibited 50% of the binding. AGP having mostly tri- and tetra-II was poor inhibitor and removal of sialic acid slightly increased the inhibitory activity. These results indicated that the binding affinity of FBA was not only dependent upon the number of II residues but also influenced by its spatial arrangement. Tα and Tn containing asialo PEM

Table 6. Amount of glycoproteins giving 50% inhibition of binding between biotinylated FBA (7.5 ng/50 μL) and plate coated asialo fetuin (250 ng/50 μL)

Curve no. (Fig. 6)	Inhibitor	Quantity giving 50% inhibition[a] (ng)
2	Asialo apotransferrin	15.85
18	Asialo fetuin	23.08
15	Asialo BTG	31.18
8	Asialo PTG	34.25
4	Asialo HCG	92.76
7	PTG	100
1	Apotransferrin	101.9
14	BTG	103.83
20	Asialo PEM	108.85
17	Fetuin	127.68
10	Asialo BSM	154.1
9	BSM	345.76
19	PEM	365.82
3	HCG	1442.92
22	Asialo PSM	2442
16	Asialo OSM	3312.5
21	PSM	4642.21
11	Asialo GM1-BSA	5377.89
6	Asialo BNG	8000 (45.96%)
5	BNG	8000 (38.56%)
13	Asialo AGP	8000 (33.03%)
12	AGP	8000 (29.05%)

[a] The inhibitory activity was estimated from the curves (Fig. 6) and is expressed as the quantity of glycoconjugates (ng) giving 50% inhibition.

and asialo BSM had moderate inhibitory potency and required 108.8 and 154.1 ng for the inhibition a masking effect of sialic acid was also observed with these glycoproteins. Asialo OSM, PSM, and asialo PSM were weak inhibitors and the required amounts for 50% inhibition were 3312.5, 4642.2, and 2442 ng, respectively. BNG and asialo BNG only inhibited 38.56% and 45.96%, respectively, at 8 μg.

It is very interesting and informative to compare the carbohydrate-binding profile of FBA with other well-documented galactose/T-specific jacalin related Moraceae lectins: jacalin,[4,5] ALA,[6] MPA[3] (*Maclura pomifera* agglutinin), and Morniga G[7] (galactose specific lectin from *Moras nigra*). (1) Glycoside-cluster effect in lectin–ligand binding is more prominent in FBA than other members. Because mono- and disaccharides reactivity of jacalin, ALA, MPA, and Moringa G is 10^3–10^4 times less compared to polyvalent ligands but for FBA such reactivity is very poor or not at all seen; (2) jacalin, Morniga G, and FBA have reacted well with multiantennary II containing N-glycans along with polyvalent Tn/Tα, whereas ALA does not show similar trend and only binds polyvalent Tα/Tn glycotopes; (3) polyvalent Tn and Tα are the most potential ligands for jacalin, ALA, MPA, and Morniga G whereas for FBA it is multivalent II; (4) jacalin, MPA, Morniga G, and FBA recognize and react with cryptic form of sugar ligands or sialylated sugar ligands, whereas ALA reacts poorly with them. Thus FBA is unique among the Gal/GalNAc specific Moraceae family plant lectins. No lectin from this group was previously reported to have such multivalent II specificity over Tα/Tn specificity. Due to multivalent II specificity of FBA it may be considered as a recognition molecule for the cancerous tissues where expression of internal multivalent II, besides Tα/Tn tends to occur.[17,18] Thus the binding profile of FBA presents a good demonstration for the requirement of complex glycotopes to explain the lectin-ligand interaction and this may be used as a candidate in glycobiology to study cell surface and to purify glycans.

The polyspecificity of FBA is in good agreement with the host-defending role against microorganisms and insects because the ability to recognize different glycan structure enables the lectin to recognize and interact with a broad range of carbohydrate structures and accordingly widens the spectrum of possible target organisms. This is in good agreement with several previously reported Moraceae family plants.[6,7]

3. Experimental

3.1. Materials

F. bengalensis seeds were obtained from the receptacle of the fruits collected from the garden of our institute. Fetuin-agarose, protease (from *Streptomyces griseus*), neuraminidase (from *Clostridium perfringens*), β-galactosidase (from *Escherichia coli*), biotinamidocaproate-*N*-hydroxy-succinimide ester, antibiotin-HRP, *o*-phenylenediamine were purchased from Sigma, USA. All other chemicals used were of the highest purity available. Me-α/βGal, Me-αGalNAc, asialo GM1, asialo GM2, asialo GM1-BSA were kindly supplied by Professor N. Roy of our department. Meβ-GalNAc, T-disaccharide (Galβ1,3GalNAc) and Tα-benzyl (Galβ1,3GalNAcα1-benzyl) were the kind gift of Professor A. M. Wu (Chang-Gung University, Kwei-san, Taipe, Taiwan). Asialo OSM was generously supplied by Professor E. Lisowska (Institute of Immunology and Experimental Therapy, Wroclaw, Poland). Mild acid hydrolysis of OSM resulted in termination of sialic acid, along with most of the outer L-fucose residues and about 75% of the carbohydrate side chains in asialo OSM were polyvalent Tn.[19,20] PEM was used from our old stock. BNG was obtained from Professor R. Schauer (University of Kiel, Kiel, Germany). HCG was the product of IBSA Institute, Biochimique SA Lugano, Switzerland. All other sugars and glycoproteins were purchased from Sigma, USA. Apotransferrin, PTG, BTG, fetuin, BSM, PSM, HCG, PEM, and BNG were desialylated by 0.01 M HCl at 80 °C for 90 min. Small fragments and HCL were removed by extensive dialysis against H_2O.

Over 53% Tn and 22% GlcNAcβ1,3Tn was present as major carbohydrate side chain in asialo BSM. The next abundant lectin determinant in asialo BSM is Tα and GlcNAcβ1,6Tα.[21,22] PSM has 65% glycan part. Major carbohydrate chains are O-linked through GalNAc to the protein core that are mostly composed of either fucosylated and/or sialylated Tn or Tα, that is, blood group A or H type of glycans.[23] Therefore, asialo PSM has little exposed Tn than Tα. Asialo PEM was a polyvalent Tα containing glycoprotein.[24]

Fetuin from fetal calf serum composed of 22% carbohydrate part. It has six glycosydic chains per molecule; three of them are O-linked sialyl Tα attached to Ser or Thr residues of the protein core and the other three are N-linked with sialyl bi- or tri-antennary glycan of ratio 2:1. Thus asialo fetuin contains two lectin determinants Tα and II.[25]

The carbohydrate side chains of PTG consist of triantennary or biantennary sialyl-II that are part of N-linked glycan, attached to Asn of the protein core.[26] Human apotransferrin also have similar N-linked structure with bi-antennary sialyl II.[27] AGP have a complex type N-linked glycan structure and the carbohydrate units can be grouped by bi-, tri-, and tetra-antennary II of ratio 1:2:2 with or without α1,2Fuc residues.[28,29] HCG is another glycoprotein having exclusively N-linked glycans chains with a α-L-Fuc residue on the Gal.[30] BTG also have multiantennary-II containing N-linked glycan chain.[31,32] Asialo derivative of PTG, BTG, apotransferrin, AGP, and HCG were well examples of multivalent-II.

BNG, the water-soluble mucus glycoprotein was extracted from the salivary gland of Chinese swiftlets and its main constituents are sialic acid rich O-linked glycan. Galβ1,3[GlcNAcβ1,6]GalNAcα1,3GalNAcα-Ser/Thr and Galβ1,3[GlcNAcβ1,6]GalNAcα-Ser/Thr are the most important major structure found in asialo BNG along with Galα1,4Gal.[33]

3.2. Purification of *F. bengalensis* agglutinin (FBA)

The agglutinin was purified from the seed extract by applying affinity repulsion chromatography technique[34] on fetuin-agarose matrix. The seeds (7 g) were extracted with 20 mM phosphate buffer, pH 7.2 containing 150 mM NaCl (PBS) (65 mL). The clarified extract was applied on the affinity column equilibrated with PBS. After washing the unbound material(s) from the column by equilibrating buffer, the bound protein was eluted by deionized water and the fractions (1 mL) were collected in 1 M NaCl (150 μL). The fractions showing hemagglutinating activity were concentrated by ultrafiltration (YM-10) and dialyzed against PBS. Protein content was determined by the Bradford method[35] using BSA as the standard.

3.3. Molecular mass

Gel filtration on Superose 12 10/30 (Amersham Biosciences, Sweden) column by using PBS as eluting buffer at a flow rate 0.4 mL/min and monitored by a 280 nm UV-filter. Blue dextran (2000 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), cytochrome C (12.4 kDa), and aprotinin (6.5 kDa) were used as a molecular mass standard. The purified FBA was subjected to 11% SDS–PAGE for testing homogeneity and molecular weight determination according to the procedure of Laemmli.[36] FBA (5 μg) was treated separately with 1% SDS and 1% SDS with 1% 2-mercaptoethanol at 100 °C. The gel was stained with Coomassie Brilliant Blue G-250 and precision plus protein standards (BioRad) used as molecular weight markers. The molecular weight was further confirmed by electron spray ionization mass spectrometry (Micromass Q-Tof Micro, Waters) by dissolving the salt free FBA in CH_3CN–H_2O (1:1) mixture.

3.4. Hemagglutination and hemagglutination inhibition assay

The hemagglutinating activity of the lectin was determined by incubating a twofold serially diluted FBA solution (25 μL) in PBS with an equal volume of 2% (v/v) normal, pronase and neuraminidase-treated human as well as rabbit erythrocytes suspension in PBS for 30 min at room temperature. The normal and enzyme-treated erythrocytes were prepared as described earlier.[37] Hemagglutination unit is defined as the minimum amount of protein (μg/mL) showing hemagglutination after 30 min at room temperature. The hemagglutination–inhibition assay was performed by preincubating 25 μL of FBA in PBS (2 HU) with known concentration of serially diluted sugars (25 μL) for 2 h in microtiter plate at room temperature. Pronase-treated human erythrocytes (25 μL, 2% v/v) were then added to the wells and the results were noted after 1 h. The inhibitory activity was defined as the minimum concentration (mM) of each sugar-inhibitor required for complete inhibition of hemagglutination.

3.5. Physico-chemical property

Aliquots of FBA in PBS were incubated at different temperatures (20–80 °C) for 30 min and cooled in ice. The activity of the aliquots was tested against pronase-treated human O erythrocytes. The pH stability of FBA was measured by dialyzing the aliquots of FBA against the following buffers for 6 h at 4 °C: 20 mM glycine buffer (pH 4.0), 20 mM acetate buffer (pH 5–5.5), 20 mM PBS (pH 6–9), and 20 mM glycine–NaOH buffer (pH 10). All the aliquots were titrated for hemagglutination

with pronase-treated human O erythrocytes in the respective dialyzing buffers except those at pH 4–5. These were titrated in saline. To examine the requirement of metal ion for the activity of FBA, it was dialyzed against 20 mM PBS, pH 7.2 containing 10 mM EDTA for 6 h followed by exhaustive dialysis against PBS. The demetallized lectin was titrated in PBS against pronase-treated human O erythrocytes.

3.6. Biotinylation of FBA

FBA was biotinylated by biotinamidocaproate-*N*-hydroxy-succinimide ester.[38] 0.2 mg FBA in 1 mL PBS was incubated with 0.1 mg biotin ester in 50 μL methanol for 30 min at room temperature. Then biotinylated FBA was dialyzed extensively against 20 mM PBS, pH 7.0 and was stored at −20 °C.

3.7. Enzyme linked lectin-sorbent assay

The avidity of FBA for various glycoproteins was studied by ELLSA. The binding of fixed amount of FBA to varying amounts of glycoproteins coated on the wells of microtiter plate was checked. In another set of experiments, the amount of FBA was varied keeping the amount of glycoproteins fixed. Based on these studies, 15 ng lectin was considered to be optimal. Enzyme-linked lectin-sorbent assay (ELLSA) was performed with different glycoproteins, according to the procedure of Duk et al.[38] The volume of each reagent added to the 96-well F-bottomed microtiter plates (Maxisorp, NUNC, Denmark) was 50 μL per well. All incubations except coating were performed at 20 °C. The reagents were diluted with 20 mM PBS, pH 7.0 containing 0.05% Tween-20 (PBS-T); this was also used for washing the wells between incubations. The wells were coated with varying amounts of glycoproteins in 50 mM carbonate–bicarbonate buffer, pH 9.6 at 37 °C for 2 h. After washing the wells, different amounts of biotinylated lectin were added to each well, and the plates were left for 1 h, washed and incubated with antibiotin-HRP (diluted 1:5000) in PBS-T for 1 h. Then *o*-phenylenediamine (1 mg/mL) in 50 mM citrate phosphate buffer, pH 5 containing 0.014% H_2O_2 was added to each well and incubated for 2 h. The absorbance was recorded at 492 nm in ELISA reader after addition of 50 μL 6 N H_2SO_4.

Inhibition study was done by mixing serially diluted sugars or glycoproteins with equal volume of 15 ng biotinylated FBA in PBS-T. After 45 min incubation at room temperature the samples were added to the wells of microtiter plate, which was previously coated with 250 ng asialo fetuin and the binding assay was done as described above. The inhibitory activity was estimated from the inhibition curve and expressed as the amount of inhibitor giving 50% inhibition of control FBA bound with coated asialo fetuin. All experiments were done in triplicate and the data presented were mean value of the results. The standard deviation in most experiments was less than 5% of the mean value. The wells devoid of coated glycoproteins gave absorbance less than 0.1 that served as blank.

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