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Catfish (*Clarias batrachus*) serum lectin recognizes polyvalent Tn [α-D-Gal*p*NAc1-Ser/Thr], Tα [β-D-Gal*p*-(1→3)-α-D-Gal*p*NAc1-Ser/Thr], and II [β-D-Gal*p*(1→4)-β-D-Glc*p*NAc1-] mammalian glycotopes

Biswajit Singha [†], Mausumi Adhya [‡], Bishnu P. Chatterjee [*]

Department of Biological Chemistry, Indian Association for the Cultivation of Science, Jadavpur, Kolkata 700 032, India

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ABSTRACT

A new calcium dependent GalNAc/Gal specific lectin was isolated from the serum of Indian catfish, *Clarias batrachus* and designated as *C. batrachus* lectin (CBL). It is a disulfide-linked homodecameric lectin of 74.65 kDa subunits and the oligomeric form is essential for its activity. Binding specificity of CBL was investigated by enzyme-linked lectin-sorbent assay using a series of simple sugars, polysaccharides, and glycoproteins. GalNAc was more potent inhibitor than Gal; and α glycosides of both were more inhibitory than their β counterparts. CBL showed maximum affinity for human tumor-associated Tn-antigens (GalNAcα1-Ser/Thr) at the molecular level and was 3.5 times higher than GalNAc. CBL interacted strongly with polyvalent Tn and Tα (Galβ1,3GalNAcα1-) as well as multivalent-II (Galβ1,4GlcNAcβ1-) antigens containing glycoproteins and intensity of inhibition was 10^3–10^5 times more than monovalent ones. The overall specificity of CBL lies in the order of polyvalent Tn, Tα and II ⋙ monovalent Tn ⩾ Me-αGalNAc > monovalent Tα > Me-βGalNAc > Me-αGal > monovalent T > GalNAc > monovalent F > monovalent II > Me-βGal > Gal.

1. Introduction

Antigenic determinants, Tn (GalNAcα1-Ser/Thr), Tα (Galβ1, 3GalNAcα1-Ser/Thr) which are normally present as cryptic structure, O-glycosidically linked to Ser/Thr on the peptide back bone, are expressed in an unmasked form in ~90% human carcinoma.[1–3] These determinants as well as unmasked N-linked multiantennary-II (Galβ1,4GlcNAcβ1-) containing glycoproteins are expressed as aberrant glycosylation on the tumor cell surface and in circulating glycoproteins in human.[1,4,5] A direct link has been shown to exist between carcinoma progressiveness and the density of Tn/Tα antigens. Thus considering them to be the most specific human carcinoma-associated structures.[6] Although Tn/Tα antigens have been characterized by monoclonal antibodies,[7,8] human macrophage lectin[9] as well as some animal and plant lectins,[10,11] yet new lectins are being discovered that recognize Tα, Tn, and II antigens. One such lectin has been isolated from the serum of Indian catfish *Clarias batrachus*. Very few lectins[12–15] have been isolated from the fish sera and characterized structurally and functionally. However, their detailed carbohydrate specificity has not been elucidated at the molecular level except a Fuc specific lectin from the serum of fresh water eel (*Anguilla anguilla*).[16]

In this communication we have described the purification and molecular properties of a new lectin from *C. batrachus* serum with detailed analysis of its glycan affinity toward mammalian glycotopes by enzyme linked lectin-sorbent assay (ELLSA).

2. Results and discussion

2.1. Purification of *C. batrachus* serum lectin

C. batrachus serum on treatment with 50% ammonium sulfate yielded a protein which showed specific activity 42 with sixfold purification. This by affinity chromatography on melibiose-Sepharose 4B column (Fig. 1A) resulted in purification of 72-fold with specific activity 540. The active fraction was further purified by anion exchange chromatography on Resource Q column (Fig. 1B) that yielded a lectin with specific activity 800 and purification fold 106. Table 1 shows the purification scheme of *C. batrachus* serum lectin (CBL).

* Corresponding author at present address: Department of Biotechnology, West Bengal University of Technology, Salt Lake, Kolkata 700 064, India. Tel.: +91 33 2321 0731/1327; fax: +91 33 2334 1030.
E-mail address: cbishnup@gmail.com (B. P. Chatterjee).
† Glyco-Immunochemistry Research Lab, Institute of Molecular and Cellular Biology, College of Medicine, Chang-Gung University, Kwei-San, Tao-Yuan, 333, Taiwan.
‡ Department of Biochemistry, College of Medicine, Cheju National University, Jeju 690-756, Republic of Korea.

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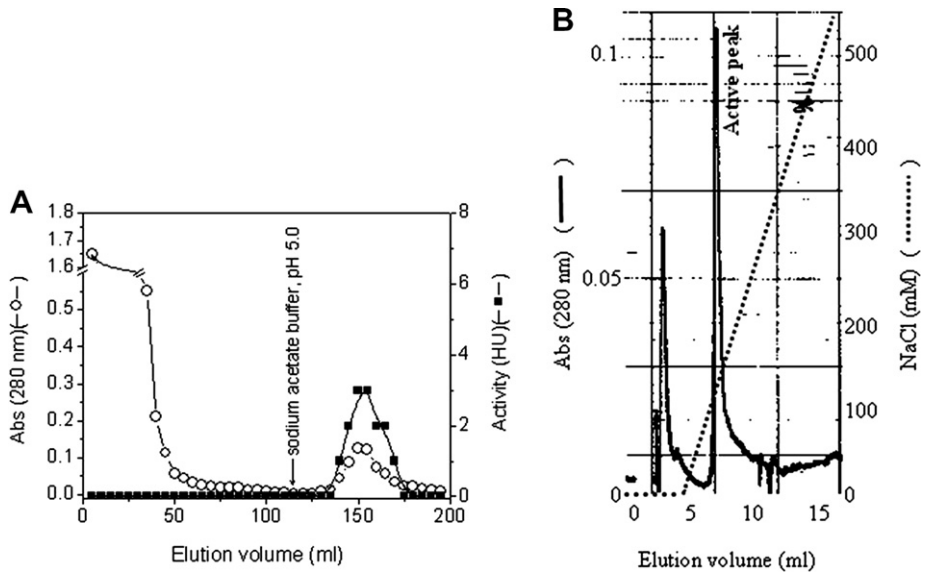

Figure 1. Purification profile of *C. batrachus* lectin. (A) Affinity chromatography of $(NH_4)_2SO_4$ fractionated *C. batrachus* serum on immobilized melibiose column (10 × 1 cm). Elution of lectin was monitored at 280 nm, and the activity was determined by hemagglutination of normal human O erythrocytes. (B) Anion exchange chromatography (Resource Q) of affinity purified lectin. CBL was eluted by a linear NaCl gradient.

Table 1
Purification scheme of *Clarias batrachus* lectin[a]

Purification steps	Total protein (mg)	Total activity (HU[b])	Specific activity (HU/mg)	Purification fold	Recovery of protein (%)
Serum	255	1917.29	7.52	1.0	100
50% Ammonium sulfate precipitated fraction	92.25	3843.75	41.67	5.54	36.18
Affinity purified lectin	0.71	382.7	540.54	71.88	0.28
Resource Q purified lectin	0.40	323.61	800	106.38	0.16

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

2.2. Molecular properties

The molecular mass of native CBL and its subunit(s) was determined by SDS–PAGE, gel filtration chromatography, and ESI-MS. The molecular weight of the lectin could not be determined by 6% SDS–PAGE under non-reducing condition due to unavailability of markers above 250 kDa (Fig. 2A). Under reducing condition only one subunit of molecular mass 72 kDa was obtained. CBL upon gel filtration chromatography on Superose 6 10/300 GL, eluted at 11.5 mL (peak i) that corresponded to molecular mass 780 kDa, whereas on treatment with DTT it eluted at 16.53 mL corresponding to 70 kDa (peak ii) (Fig. 2B). Hemagglutinating activity was detected only with lectin under peak (i) suggesting that the oligomeric nature is an essential requirement for hemagglutinating activity. The oligomeric nature of CBL was further confirmed by gel electrophoresis of proteins collected from both peak fractions at denaturing and reducing conditions (Fig. 2C). The molecular mass of subunit was further confirmed by ESI-MS (Fig. 3), which suggests that CBL is composed of 10 subunits of 74.65 kDa and each is attached by disulfide bond.

The neutral sugar content of CBL was estimated to be 6.88%. The carbohydrate analysis revealed that Glc, Man, Gal, GlcNAc, GalNAc, Rha, and Fuc present as 34.77, 15.86, 30.3, 17.81, 6.15, and 14.59 mole per mole of CBL, respectively. CBL contained high mole percentage of proline as well as isoleucine, glutamic acid/glutamine, serine, and glycin (Table 2).

CBL was stable at a wide range of pH (pH 5–9) and hemagglutinating activity was maximum at pH from 7.2 to 8. The activity of lectin was stable up to 40 °C, persisted 25% up to 50 °C, and was totally lost above that temperature. The activity of the lectin was dependent on Ca^{2+} ion as it abolished after demetalization with EDTA. However, the activity was completely restored after addition of 2.5 mM $CaCl_2$. Other divalent cations, Mg^{2+} and Mn^{2+}, had no effect on hemagglutination.

2.3. Hemagglutination activity

CBL agglutinated human erythrocytes irrespective of blood group O, A, and B with equal potency at a minimum concentration of 1.25 μg/mL and rabbit erythrocytes more strongly (39.5 ng/mL). However, its activity was enhanced eight and twofold when the red blood cells were treated with pronase or neuraminidase.

2.4. Carbohydrate specificity

Hapten inhibition assay (Table 3) showed that among the monosaccharides tested, Me-αGal was the most potent inhibitor (3.08 mM) being four times more in inhibiting the hemagglutination than its parent sugar Gal and two times more than GalNAc. Me-βGal had the same inhibitory potency like Gal. $GalNH_2$ (25 mM) and D-Fuc (50 mM) inhibited at high concentrations. Among oligosaccharides, melibiose (Galα1,6Glc) and raffinose (Galα1,6Glcβ1,2Fru) proved to be the strongest inhibitors (6.25 mM) and lactose (Galβ1,4Glc) was the poorest inhibitor (50 mM).

The binding affinity of CBL for glycoproteins is summarized in Table 4 and the binding patterns are shown in Figure 4. The binding data were expressed as the amount of glycoproteins (ng)

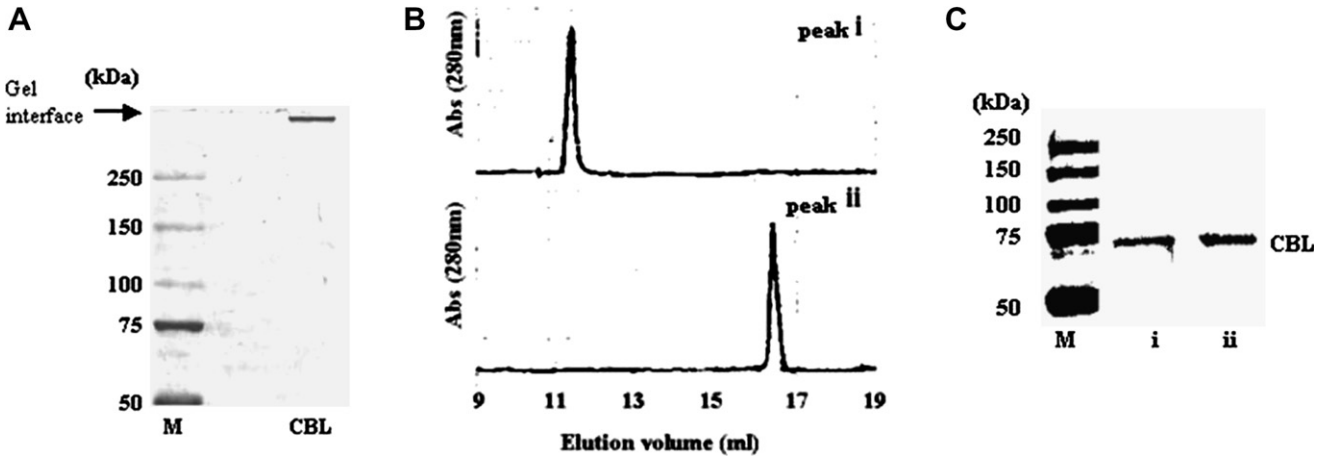

Figure 2. Molecular weight and molecular size of CBL (A) 6% SDS–PAGE of denatured CBL. (B) Gel filtration chromatography of CBL on Superose 6 10/300 GL column. CBL (peak i) and reduced CBL (peak ii) were eluted with 20 mM TBS and monitored at 280 nm. (C) 10% SDS–PAGE; (Lane i) denatured and reduced CBL from peak i and (Lane ii) reduced CBL from peak ii. M Precision plus protein standard (Bio-Rad).

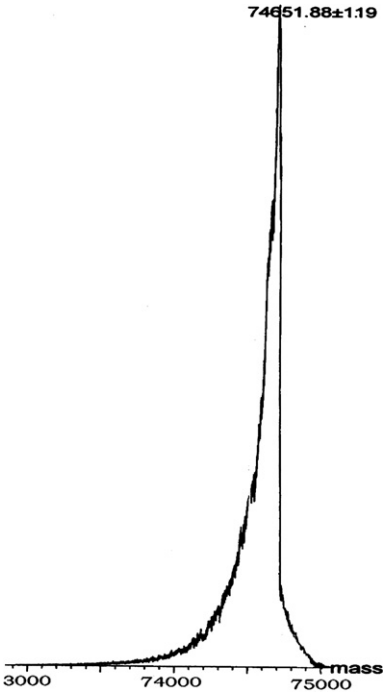

Figure 3. ESI-MS spectrum of CBL subunits, recorded on a Micromass Q-Tof micro (Waters). CBL was injected at 10 nL/min flow rate and 1500 V was used to initiate the ionization.

required for binding which corresponded to Abs. 1.5 at 492 nm.[11] CBL reacted most strongly with polyvalent Tn containing asialo ovine submaxillary mucin (OSM). Moderate binding was observed with asialoagalacto bovine submaxillary mucin (BSM), asialo fetuin, asialoagalacto fetuin, multiantennary II containing asialo porcine thyroglobulin (PTG) and Tn-glycophorin; whereas poor binding was observed with Tn and Tα containing asialo BSM. Other glycoproteins almost did not bind (Table 4).

As the percentage of adsorbance of the glycoproteins on the microtiter plate assay had not been established,[11] the relative binding ability of different glycoproteins with CBL could not be accurately measured. Therefore, the carbohydrate specificity of CBL was further confirmed by inhibiting the binding of CBL with

Table 2
Amino acid composition of CBL

Amino acids	Mol %
Asp	6.3
Glu	8.05
Ser	8.44
Gly	7.8
His	1.22
Arg	2.3
Thr	6.38
Ala	5.77
Pro	12.95
Tyr	2.91
Val	5.4
Met	1.45
Cys	6.06
Ile	11.2
Leu	5.26
Phe	2.6
Lys	4.34
Trp[a]	1.58

Data shown are mean of three experiments.
[a] Trp was measured spectrophotometrically.

Table 3
Hemagglutination-inhibition of CBL by mono- and oligosaccharides

Inhibitor	MIC[a] (mM)	Inhibitory intensity[b]
Me α-Gal	3.12	++++
GalNAc	6.25	+++
Galα1,6Glc	6.25	+++
Galα1,6Glcβ1,2Fru	6.25	+++
Gal	12.5	++
Me-βGal	12.5	++
$GalNH_2$	25	+
Galβ1,4Glc	50	±
D-Fuc	50	±
Glc	>100	–
Man	>100	–
L-Fuc	>100	–
Neu5Ac	>100	–

[a] The minimum inhibitory concentration required for complete inhibition of the binding between two HU of CBL and equal volume of 2% suspension of human erythrocytes.
[b] Results were interpreted according to the relative inhibitory potency. Expression pattern for sugars/glycoproteins is indicated by sign (+), (±) and (–) showing inhibition, moderate inhibition and non-inhibition of hemagglutination, respectively.

Table 4
Binding of biotinylated CBL (25 ng) with serially diluted different glycoproteins

Curve no. (Fig. 4)	Glycoprotein	Quantity[a] (ng) required for A_{492nm}	Maximum binding (A_{492nm})
2	Asialo OSM	6.89	3.0
13	Asialo fetuin	42.45	3.0
5	Asialoagalacto BSM	44.45	3.0
14	Asialoagalacto fetuin	52.63	3.0
16	Asialo PTG	89.16	3.0
11	Tn-glycophorin	89.62	3.0
4	Asialo BSM	272.18	3.0
12	Fetuin	>1000	1.45
10	Asialo glycophorin	>1000	1.3
15	PTG	>1000	1.3
1	OSM	>1000	0.65
3	BSM	>1000	0.44
8	Asialoagalacto BNG	>1000	0.40
9	Glycophorin	>1000	0.39
7	Asialo BNG	>1000	0.36
6	BNG	>1000	0.33

[a] Amount of glycoproteins required for binding that corresponded to 1.5 unit absorbance at 492 nm after termination of enzymatic reaction at 1 h.

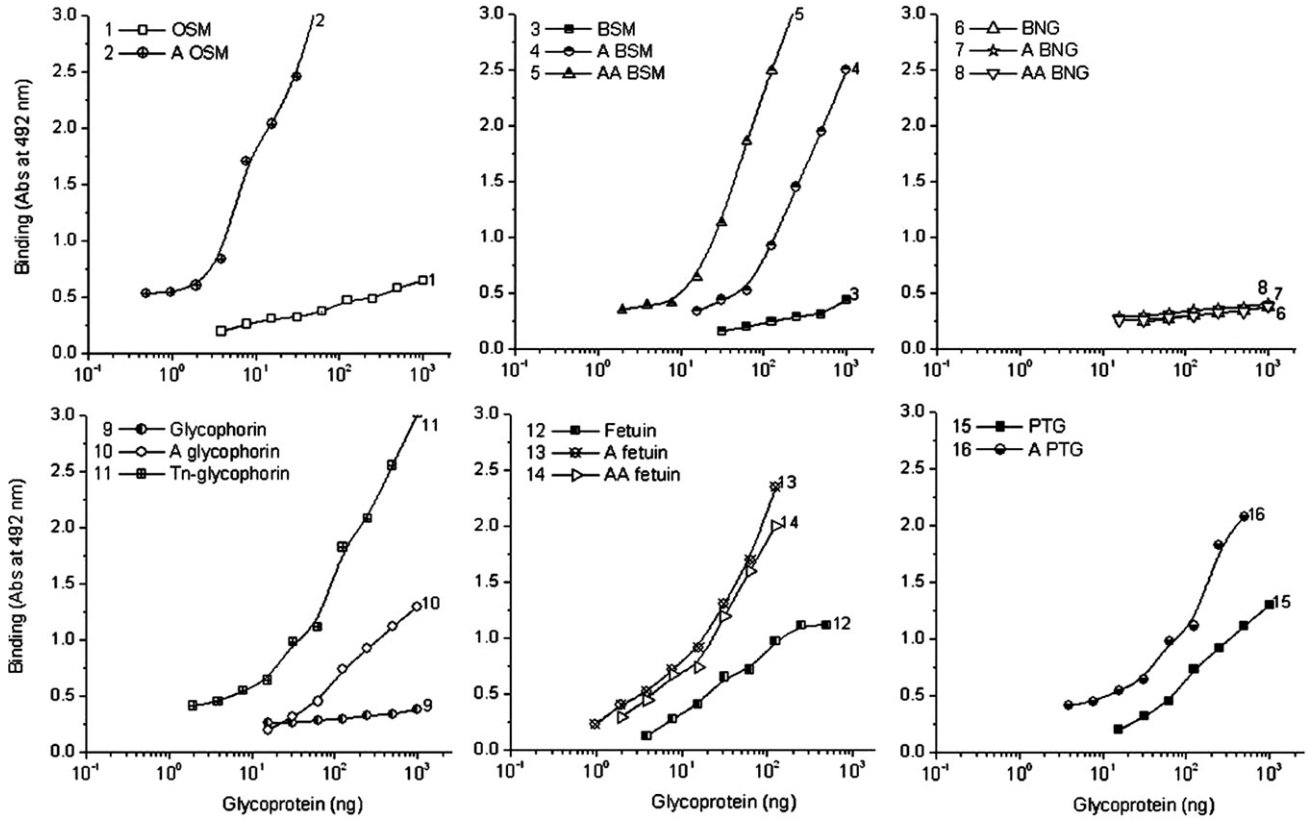

Figure 4. Binding profile of biotinylated CBL (25 ng/50 μL/well) with plate coated glycoproteins (serially diluted from 1 μg). Binding was measured at 492 nm after 1 h incubation with substrate (OPD) followed by termination of the reaction by adding 50 μL of 3 M H_2SO_4. Asialo and asialoagalacto were designated A and AA, respectively.

asialo OSM using sugars, polysaccharides, and glycoproteins. The percentage of inhibition by the sugar inhibitors is summarized in Table 5 and the inhibition patterns are graphically presented in Figure 5. The results showed that the inhibitory potency of α and β glycosides of Gal/GalNAc was more than their parent sugars, and α-glycoside being more inhibitory than β-one. Me-αGalNAc was the best inhibitor among the monosaccharides tested and was 3.33 times more active than GalNAc. The inhibitory activity of Me-βGalNAc and Tn was 1.67- and 3.49-fold higher than GalNAc. D-Fuc and L-Ara showed less inhibitory activity (38.7% and 40.84%, respectively) than Gal, which indicates that OH at C-6 is important for binding. 1,6-Anhydro-Gal was inactive up to 10 μmol, indicating that C-1 OH is important locus for binding. Glc (C-4 epimer of Gal) and GlcNAc showed very little inhibitory activity, which suggests that axial C-4 OH is essential for binding. The pyranose structure of Gal/GalNAc is an essential requirement for binding with CBL since Tag, which has a furanose structure, had a little inhibitory effect. Lyx and Neu5Ac were non-inhibitors even at 10 μmol.

The inhibitory activity of oligosaccharides in CBL-asialo OSM interaction is presented in Table 5. Galα1,6Glc, Galα1,6Glcβ1,2Fru, and stachyose (Galα1,6Galα1,6Glcβ1,2Fru) were effective inhibi-

Table 5
Amount of various saccharides giving 50% inhibition of binding between biotinylated CBL (12.5 ng) and asialo OSM (10 ng)

Curve no. (Fig. 5)	Sugars	Quantity giving 50% inhibition (nmol)[a]	Relative potency[b]
21	GalNAcα-Ser (Tn)	317.36	3.49
7	Me-αGalNAc	332.0[c]	3.33
14	Galα1,6Galα1,6Glcβ1,2Fru	484.39	2.29
23	Galβ1,3GalNAcα-benzyl (Tα-benzyl)	568.85[c]	1.95
4	*p*-NO_2Ph-αGal	610.5	1.81
8	Me-βGalNAc	661	1.67
2	Me-αGal	850.64	1.3
22	Galβ1,3GalNAc (T)	929.88[c]	1.19
6	GalNAc	1107	1
24	GalNAcα1,3GalNAc (F)	1164.8[c]	0.95
6	GalA	1478.16	0.75
13	Galα1,6Glcβ1,2Fru	1506	0.73
12	Galα1,6Glc	1777.82	0.62
16	Galβ1,4GlcNAc (II)	2217.85[c]	0.5
3	Me-βGal	2570.16	0.43
1	Gal	6401.79	0.17
19	Galα1,4Gal	>625 (47.4%)	–
15	Galβ1,4Glc	>5 × 10^3 (44.46%)	–
5	*p*-NO_2Ph-βGal	>1250 (43.36%)	–
17	L-Ara	>10 × 10^3 (40.84%)	–
11	D-Fuc	>10 × 10^3 (38.7%)	–
10	*p*-NO_2Ph-αGalNAc	>312.5 (33.6%)	–
18	Galα1,3Gal	625 (32.04%)	–
–	Tag	>10 × 10^3 (18.7%)	–
20	Galβ1,4Gal	>312.5 (28.26%)	–
–	GalAα1,4GalA	>78.12 (23.11%)	–
9	*p*-NO_2Ph-βGalNAc	>156.25 (26.3%)	–
–	GlcNAc	>10 × 10^3 (13.93%)	–
–	Glc	>10 × 10^3 (12.36%)	–
–	1,6-Anhydro-D-Gal	>10 × 10^3 (0%)	–
–	Lyx	>10 × 10^3 (0%)	–
–	Neu5Ac	>10 × 10^3 (0%)	–

[a] The inhibitory activity was estimated from the curves (Fig. 5) and is expressed as the quantity of sugars (nmol) giving 50% inhibition.
[b] Relative potency of sugars is compared with GalNAc (taken as 1.0).
[c] Extrapolated.

tors. The inhibitory potency of LacNAc (Galβ1,4GlcNAc) (II) was seven times less inhibitory than Me-αGalNAc. T (Galβ1,3GalNAc) and F-disaccharides (GalNAcα1,3GalNAc) were little more inhibitory than II but less potent than Me-αGalNAc. Tα-benzyl (Galβ1,3GalNAcα1-benzyl) exhibited more inhibitory potency than T. The inhibitory potency of T, Tn, Tα, F, and II was in the following order Tn > Tα > T > F > II.

Inhibitory potency of various glycoproteins and polysaccharides to CBL-asialo OSM interaction is summarized in Table 6 and 50% inhibition of binding is shown in Figure 6. Among the glycoproteins tested, asialo OSM was the best inhibitor requiring only 1.16 ng for 50% inhibition and was 2.11 × 10^5 times more potent than GalNAc. Asialoagalacto BSM and Tn-glycophorin, were also stronger inhibitors being 3.6 × 10^4 and 9.9 × 10^3 times more potent than GalNAc, whereas monovalent Tn-glycopeptide (GalNAcα1-Ser/Thr; MW < 3000 Da) and Tn (GalNAcα-1Ser) were much less inhibitory. Asialo BSM and asialo glycophorin had less inhibitory potency compared to asialo OSM. Multivalent II containing glycoproteins, asialo PTG and asialo apotransferrin were among the most potent inhibitors, being 4.9 × $10^4$and 6.93 × 10^3 times more active than GalNAc. Asialoagalacto birds nest glycoprotein (BNG) bearing terminal Galα1,4Gal, and GlcNAcβ1,6GalNAcα1,3GalNAc, and GlcNAcβ1,6Tn was another effective inhibitor that required 10.48 ng for 50% inhibition. Asialo fetuin which contains multivalent II and polyvalent Tα was more potent inhibitor than asialoagalacto fetuin containing only polyvalent Tn. Sialoglycoproteins were weak inhibitors due to masking effect of sialic acid. Fenugreek galactomannan containing Galα1,6-residues in the branching enhanced 96 times inhibitory activity than Me-αGal, whereas larch arabinogalactan containing Galβ1,6-residues in the branching showed no inhibition at all.

Based on the inhibition results of CBL-asialo OSM binding by simple sugars and glycoproteins, it revealed that glycoproteins containing polyvalent glycotopes enhanced the inhibition to a great extent (10^3–10^5 times) in comparison to monovalent Tn, Tα and Tn-glycopeptides and GalNAc. Although binding of monosaccharides and their derivatives with lectins is very specific, yet it is weaker than that with polyvalent glycans. However, when appropriate lectin determinant or sugar ligand when forms cluster with the required geometry, the interaction becomes strong. There are numerous examples where polyvalent carbohydrate could lead to a remarkable increase of binding affinity with lectins.[9,11,16–20] Due to difficulties in synthesizing branched carbohydrate derivatives, natural polyvalent glycoproteins with well-defined glycotopes were used in the present binding study.

It may be concluded from the foregoing results that (1) pyranose structure of hexoses showed better binding than furanose structure, (2) axial C-4 OH of the terminal sugar was important locus for binding, (3) α-anomer of Gal/GalNAc was better inhibitor than β-one, and (4) CBL recognized Tn, T, Tα, and II antigens and polyvalency enhanced the binding. The binding affinity of CBL can be summarized as polyvalent Tn/Tα/II containing glycotopes ⋙ monomeric Tn ⩾ Me-αGalNAc > monomeric Tα > Me-βGalNAc > Me-αGal > monomeric T > GalNAc > monomeric F > monomeric II > Me-βGal > Gal.

Like jacalin,[21,22] *Artocarpus lakoocha* agglutinin (ALA),[11] *Bauhinia purpurea* agglutinin (BPA),[23] *Agaricus bisporus* agglutinin (ABA),[19] and Morniga G,[24] CBL exhibits pronounced specificity for α-GalNAc (GalNAc > Gal). However, CBL exhibits subtle difference in the binding specificity with jacalin and ALA [polyvalent Tn and Tα⋙ polyvalent II (weak)], whereas it shows close specificity with ABA, BPA, and Morniga G [polyvalent Tα, Tn, II (strong)].

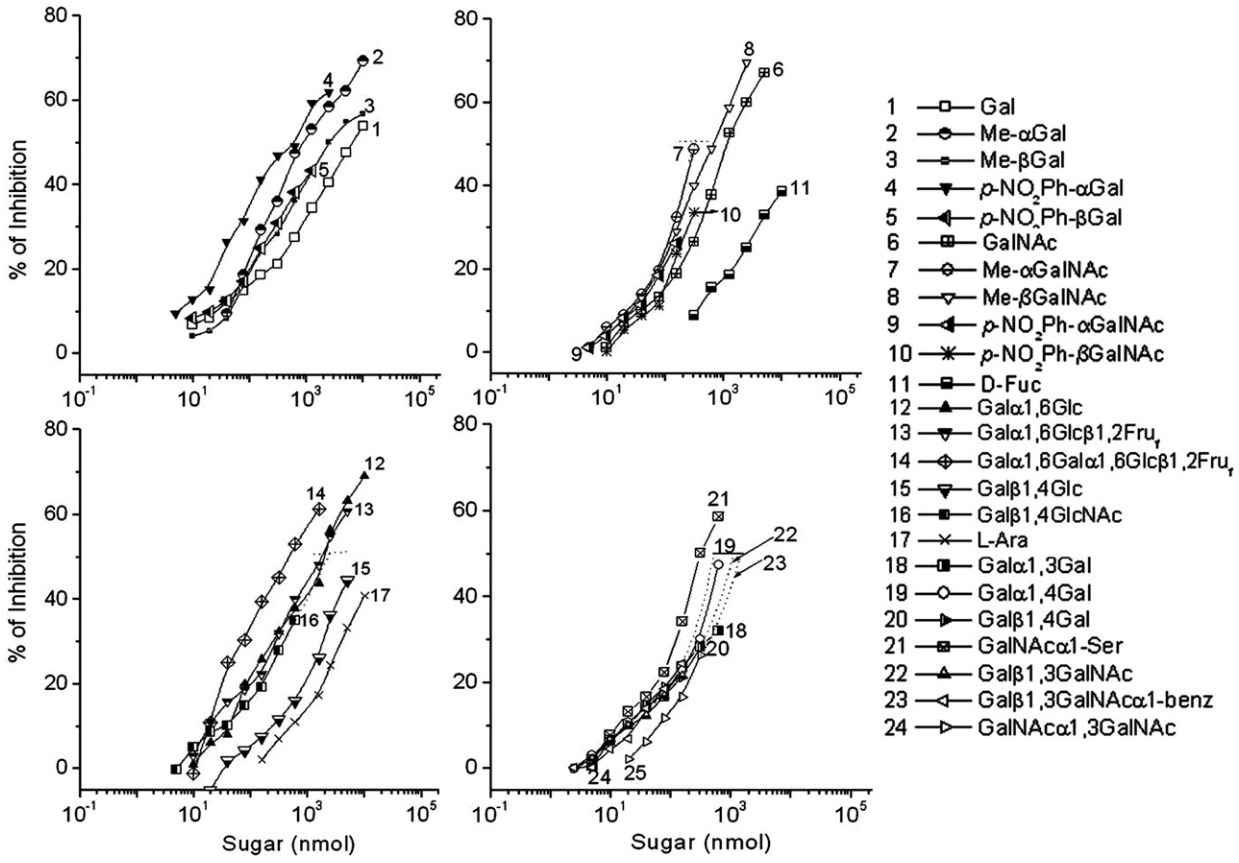

Figure 5. Inhibition of binding between biotinylated CBL (12.5 ng/50 μL/well) and plate coated asialo OSM (10 ng/50 μL/well) by different sugars. 50% inhibition of binding was calculated from the absorbance at 492 nm after terminating the reaction at 1 h. Dotted lines (···) indicate extrapolation of curve. Asialo and asialoagalacto were designated A and AA, respectively.

3. Experimental

3.1. Materials

Blood was drawn from the caudal vessels of healthy catfish, *C. batrachus* and allowed to clot at room temperature for 2 h. Separated serum was centrifuged at 10,000 × g for 10 min and kept at −20 °C till use. Protease (from *Streptomyces griseus*), neuraminidase (from *Clostridium perfringens*), β-galactosidase (from *E. coli*), biotinamidocaproate-*N*-hydroxy-succinimide ester, antibiotin-HRP, *o*-phenylenediamine were purchased from Sigma, USA. Melibiose was immobilized on Sepharose 4B according to the procedure of Porath and Ersson adopted by Teichberg et al.,[25] Meβ-GalNAc, F-disaccharide, T-disaccharide, Tα-benzyl, Tn and Tn-glycopeptide (GalNAcα1-Ser/Thr; MWt <3000 kDa) were the kind gift of Professor A. M. Wu (Chang–Gung University, Kwei-san, Taipe, Taiwan). Tn-glycopeptide was prepared from the asilao OSM according to the method describe by Wu et al.,[26] Meα-Gal, Meβ-Gal, Meα-GalNAc and 1,6-anhydro-Gal[27] were kindly supplied by Professor N. Roy of Indian Association for the Cultivation of Science, Kolkata, India.

OSM, asialo OSM, human glycophorin, asialo, and asialoagalacto glycophorin were generously supplied by Prof. E. Lisowska (Institute of Immunology and Experimental Therapy, Wroclaw, Poland). Desialylation of OSM and glycophorin was done by mild acid hydrolysis using 0.01 M HCl at 80 °C for 90 min and the small fractions were removed by extensive dialysis against water.[11] About 75% of the carbohydrate chains in asialo OSM are composed of polyvalent Tn.[28,29] Asialo glycophorin has 15 O-linked glycan of Tα lectin determinants and one N-linked carbohydrate residues.[30] Tn-glycophorin was obtained from asialo glycophorin by periodate oxidation followed by mild acid hydrolysis.[30] Larch arabinogalactan and fenugreek galactomannan were used from our old stock. Larch arabinogalactan is made up of β-1,3 linked D-Gal backbone carrying β-1,6 linked single or long chain D-Gal branching along with low amount of α-1,6 linked L-Ara_f.[31] Fenugreek galactomannan was isolated form the seeds of *Trigonella foenum-graecum*, and contains D-Man and D-Gal in a 1.25:1 ratio. The main chain of this polysaccharide is composed of β-1,4 linked D-Man backbone carrying α-1,6 linked single D-Gal residue.[32] BNG was obtained from Professor R. Schauer (University of Kiel, Kiel, Germany). The water-soluble mucus glycoprotein (native BNG) was extracted from the salivary gland of Chinese swiftlets. It is primarily constitutes of sialic acid rich O-linked glycan. Galβ1,3[GlcNacβ1,6]GalNAcα1,3GalNAcα-Ser/Thr and Galα1,3[GlcNacβ1,6]GalNAcα-Ser/Thr are the most important major structures present in asialo BNG along with Galα1,4Gal.[33]All other sugar and glycoproteins were purchased from Sigma, USA.

Asialo glycoproteins were prepared from BNG, BSM, porcine submaxillary gland mucin (PSM), α1 acid glycoprotein (AGP), human apotransferrin, PTG, and fetuin by the following procedures. All the glycoproteins (10 mg) were separately dissolved in 50 mM sodium acetate buffer, pH 5.5, and incubated at 37 °C for 24 h with neuraminidase (1 unit).[34] The enzyme was deactivated by heating at 100 °C for 5 min and centrifuged. The supernatant was dialyzed against water and lyophilized. Asialoagalacto glycoproteins were prepared as follows: Asialo fetuin, asialo BSM, asialo BNG, and asialo PSM (5 mg each) were dissolved in 100 mM TBS buffer, pH 7.3, containing 1 mM $MgCl_2$ and 100 mM 2-mercaptoethanol and incubated with β galactosidase (2 units) at 37 °C

Table 6
Amount of various glycoproteins and polysaccharides giving 50% inhibition of binding between biotinylated CBL (12.5 ng) and plate coated asialo OSM (10 ng)

Curve no. (Fig. 6)	Glycoproteins and polysaccharides	Quantity giving 50% inhibition (ng)[a]	Relative potency[b]
2	Asialo OSM	1.16	2.11×10^5
16	Asialo PTG	5.0	4.9×10^4
5	Asialoagalacto BSM	6.78	3.6×10^4
13	Asialoagalacto BNG	10.48	2.3×10^4
22	Asialo fetuin	17.52	1.39×10^4
10	Tn-glycophorin	24.57	9.97×10^3
9	Asialo glycophorin	33.19	7.38×10^3
20	Asialo apotransferrin	35.34	6.93×10^3
23	Asialo fetuin	61.34	3.9×10^3
4	Asialo BSM	81.83	2.99×10^3
8	Glycophorin	151.21	1.62×10^3
21	Fetuin	199.28	1.23×10^3
26	Asialoagalacto PSM	237.42	1.03×10^3
19	Apotransferrin	249.62	981
25	Asialo PSM	354.54	691
15	PTG	478.93	510
18	Asialo AGP	478.93	510
3	BSM	733.34	334
24	PSM	863.08	283
1	OSM	942.21	260
6	Fenugreek galactomannan	1719.39	142
14	Tn-glycopeptide[d]	9.36×10^4	2.6
—	Tn[c]	1.05×10^5	2.33
—	GalNAc[c]	2.45×10^5	1.0
17	AGP	$>10 \times 10^3$ (44.21%)	—
12	Asialo BNG	$>10 \times 10^3$ (43.67%)	—
11	BNG	$>10 \times 10^3$ (40.69%)	—
7	Larch arabinogalactan	$>25 \times 10^3$ (0%)	—

[a] The inhibitory activity was estimated from the curves (Fig. 6) and is expressed as the quantity of inhibitors (ng) giving 50% inhibition.
[b] Relative potency of sugars is compared with GalNAc (taken as 1.0).
[c] Amount required for 50% inhibition (nmol) was calculated as nanogram basis from Figure 5.
[d] Extrapolated.

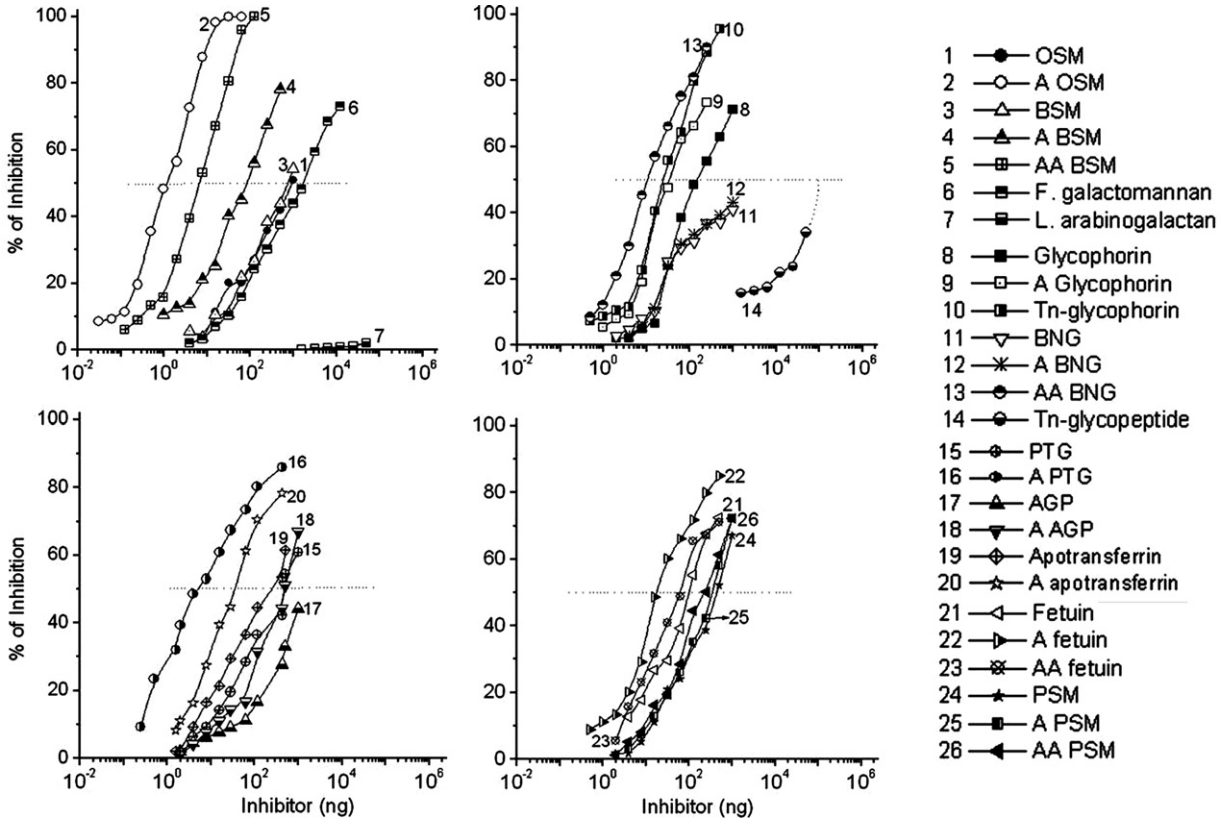

Figure 6. Inhibition of binding between biotinylated CBL (12.5 ng/50 μL/well) and plate coated asialo OSM (10 ng/50 μL/well) by different glycoproteins and polysaccharides. 50% inhibition of binding was calculated from the absorbance at 492 nm after terminating the reaction at 1 h. Dotted lines (···) indicate extrapolation of curve. Asialo and asialoagalacto were designated A and AA, respectively.

for 24 h.[35] The enzyme was deactivated and asialoagalacto glycoproteins were isolated as before.

Over 53% sialyl Tn and 22% GlcNAcβ1,3Tn are present as major carbohydrate side chains in BSM. The next abundant lectin determinant in BSM is sialyl Tα and GlcNAcβ1,6Tα.[36–38] Removal of β-galactose from BSM makes abundance of Tn as compared to asialo BSM. PSM has 65% glycan part. Major carbohydrate chains are O-linked through GalNAc to the protein core that mostly composed of either fucosylated and/or sialylated Tn or Tα, that is, blood group A or H type of glycans. More than 90% of sialic acids in PSM are Neu5Gc.[39] Therefore, asialo PSM and asialoagalacto PSM have very little exposed Tn and Tα. Fetuin from fetal calf serum composed of 22% carbohydrate part. It has six carbohydrate 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 triantennary glycan of ratio 2:1. Thus asialo fetuin contains two lectin determinants Tα and II, but asialoagalacto fetuin has only Tn.[40] The carbohydrate side chains of PTG are biantennary or triantennary sialyl II as N-linked carbohydrate part attached to Asn of the protein core.[41] Human apotransferrin also has similar N-linked structure with bi-antennary sialyl II.[42] AGP has a complex type N-linked glycan structure, and the carbohydrate units of this glycoprotein can be grouped by bi, tri, and tetra antennary II of ratio 1:2:2 with or without α1,2Fuc residues.[43,44] Asialo derivative of PTG, apotransferrin, and AGP are well examples of multivalent II. Other chemicals used were of the highest purity available.

3.2. Purification of CBL

C. batrachus serum was fractionated by 50% $(NH_4)_2SO_4$ precipitation. The pellet was collected and dialyzed in 20 mM TBS, pH 7.2, containing 10 mM $CaCl_2$ (TBS-Ca). The clear dialyzed material was loaded on melibiose-Sepharose 4B column (10 × 1 cm), pre-equilibrated with TBS-Ca. After washing the unbound protein (till Abs. less than 0.002), absorbed protein was eluted with 50 mM citrate buffer, pH 5.0, concentrated by YM-10 and dialyzed against 20 mM Tris–HCl, 10 mM $CaCl_2$, pH 8.0 (TB-Ca). This affinity purified protein was further purified on Resource Q (1 mL) column (Amersham Biosciences), fitted with FPLC system using a salt gradient between 20 mM TB-Ca and 20 mM TB-Ca containing 1 M NaCl. Proteins in different fractions were estimated by the method of Lowry[45] using bovine serum albumin as the standard after removal of Tris.

3.3. Molecular mass

The purified lectin (CBL) was reduced by incubating with 100 mM DTT for 2 h at 37 °C. CBL and the reduced one were subjected to gel filtration on Superose 6 10/300 GL (Amersham Biosciences, Sweden) column which was eluted 20 mM TBS, pH 7.2, at a flow rate of 0.2 mL/min. The molecular mass was determined from the relative elution volume of the following protein standards: blue dextran (2000 kDa), bovine thyroglobulin (669 kDa), apoferritin (449 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). CBL (2 μg) was subjected to gel electrophoresis on 6% SDS–PAGE under denaturing condition by heating the lectin with 1% SDS at 100 °C.[46] Under reducing condition, CBL (2 μg) was heated with 1% SDS and 100 mM DDT at 37 °C for 2 h and subjected to 10% SDS–PAGE.[46] The gels were stained with Coomassie Brilliant Blue G-250. The molecular mass of CBL was determined by comparing the relative mobility of lectin with Precision Plus Protein standards (BioRad). The lectin subunit band was cut from the gel, dialyzed to remove salt, and dried. The molecular mass of CBL was determined by ESI-MS using 10 nL/min flow rate of 100 pmol CBL solution in $CH_3CN–H_2O$ (1:1) mixture on Micromass Q-Tof Micro (Waters) mass spectrometer.

3.4. Hemagglutination and hemagglutination-inhibition assay

The hemagglutinating activity of the lectin with normal, pronase, and neuraminidase-treated human as well as rabbit erythrocytes was determined in TBS-Ca, pH 7.2, as earlier.[20,47] Hemagglutination unit is defined as the minimum amount of protein (μg/mL) showing hemagglutination after 30 min at room temperature. The hapten-inhibition assay was performed with pronase treated human O erythrocytes in TBS-Ca, pH 7.2, as described previously.[20] The inhibitory activity of lectin was defined as the minimum concentration (mM) of each sugar-inhibitor required for complete inhibition of hemagglutination by 2 HU of CBL.

3.5. Physico-chemical property

Aliquots of CBL in TBS-Ca were incubated at different temperatures (20–80 °C) for 30 min and cooled. The activity of the aliquots was tested against human erythrocytes. The pH stability of CBL was measured by dialyzing the aliquots of CBL against the following buffers containing 10 mM $CaCl_2$ for 6 h at 4 °C: 20 mM glycine buffer (pH 4.0), 20 mM acetate buffer (pH 5–6), 20 mM TBS (pH 7–9), and 20 mM glycine–NaOH buffer (pH 10). All the aliquots were tested for hemagglutination with human erythrocytes in the respective dialyzing buffers except those at pH 4–5. These were titrated in the presence of TBS-Ca buffer (pH 7.5). To examine the requirement of metal ion for the activity of CBL, it was dialyzed against 20 mM TBS, pH 7.2, containing 10 mM EDTA for 6 h followed by extensive dialysis against TBS. The demetalized lectin was titrated in TBS containing 0.5–20 mM $CaCl_2$, $MgCl_2$, and $MnCl_2$ against human erythrocytes.

3.6. Sugar and amino acid composition

CBL (300 μg) was hydrolyzed with 2 M TFA in a sealed tube at 120 °C for 2 h.[48] The alditol acetates of the released sugars in chloroform were injected on fused silica HP-5 column (30 m × 0.025 μm × 0.32 mm) in Agilent (model 6890 series GC system) gas chromatograph equipped with a HP 3898 A Chemstation. For amino acid analysis, 50 μg CBL was hydrolyzed in the vapor phase by 6 M HCl containing 1% phenol in PICO-TAG workstation at 110 °C for 22 h. The amino acid was derivatized by phenyl isothiocyanate at 20–25 °C for 20 min. The derivatives were then analyzed in reverse phase HPLC using PICO-TAG C_{18} column (3.9 × 150 mm). Tryptophan was determined spectrophotometrically.[49]

3.7. Biotinylation of CBL

CBL was biotinylated by biotinamidocaproate-*N*-hydroxy-succinimide ester.[50] 0.2 mg CBL in 4 mL TBS containing 2.5 mM $CaCl_2$ was mixed with 0.1 mg biotin ester in 50 μL methanol and left for 30 min at room temperature followed by dialysis against 20 mM TBS, pH 7.2, containing 2.5 mM $CaCl_2$ and was stored at −20 °C.

3.8. Enzyme linked lectin-sorbent assay

ELLSA was performed with different glycoproteins, according to the procedure of Duk et al.,[50] 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 TBS, pH 7.2, containing 2.5 mM $CaCl_2$ and 0.05% Tween-20 (TBS-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, left for 1 h, washed and incubated with antibiotin-HRP (diluted 1:5000) in TBS-T for 1 h. *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 1 h. The absorbance was recorded at 492 nm in ELISA Reader after addition of 50 μL of 3 M H_2SO_4. Inhibition study was done by serially mixing diluted sugars or glycoproteins with equal volume of 25 ng biotinylated CBL solutions. After 45 min incubation at room temperature the samples were added to the wells of microtiter plate, which was previously coated with asialo OSM (10 ng), 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 CBL bound with coated asialo OSM.

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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