Glycobiology Insights

ORIGINAL RESEARCH

Tn/T Specific Agglutinin from Estuarine Crab *Scylla Serrata* with Potent Mitogenic Activity on Mouse Splenocytes and Antiproliferative Effect on Hepatocellular Carcinoma (HepG2) Cell

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Abstract: A lectin was purified from the hemolymph of esturarine crab *Scylla serrata* by successive 40% $(NH_4)_2SO_4$ precipitation, affinity chromatography on asialo fetuin- Sepharose column, Resource Q anion-exchanger in FPLC system and designated as scyllin-2. Scyllin-2 was a homogeneous monomeric protein of molecular mass 75 kD judged by SDS-PAGE and confirmed by ESI-MS-Q-ToF. Its activity was Ca^{+2} dependent being maximum at pH 7.5 and at 20 °C. N-terminal sequence of scyllin-2 showed close resemblance to peanut lectin and histidine kinase A. It agglutinated human O, A, B and AB blood group erythrocytes equally well and showed maximum inhibition with α-Gal by hapten- inhibition study. The detailed carbohydrate specificity of scyllin-2 was determined at the macromolecular level based on the Gal/GalNAc structural units in the mammalian glycoproteins by enzyme-linked lectinosorbent (ELLSA) and inhibition assays. It revealed that scyllin-2 binds specifically to tumor-associated carbohydrate antigens GalNAcα1→Ser/Thr (Tn) and Galβ1→3GalNAcα1→Ser/Thr (Tα). It showed very weak binding with Galβ1→3/4GlcNAc (I/II) glycotopes on glycoproteins and T/Tn covered by sialic acid. Multivalancy of Tn/Tα containing glycoproteins tested resulted in higher binding of 10^2–10^4 order than the respective Gal and GalNAc monomer. Scyllin-2 stimulated proliferation of mouse splenocytes. Analysis of expression of receptors for scyllin-2 on HepG2 by flow cytometry showed the binding of FITC-scyllin-2 to HepG2 was 86.51%, which was nearly comparable to *Artocarpus lakoocha* agglutinin (ALA) (66.41%), another Tn/Tα specific lectin indicating that the glycan structure on HepG2 cell surface shows prevalence of Tn/Tα units. It inhibited proliferation of HepG2 cells (61 μg/ml). The inhibitory effect was comparable to ALA (80 μg/ml). Thus, we have characterized a Tn/T specific invertebrate lectin with biological significance.

Keywords: *Artocarpus lakoocha* agglutinin, ELISA, hemolymph, HepG2, *Scylla serrata,* Scylin-2

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Introduction

Lectins are mono-or multivalent proteins or glycoproteins of non-immune origin that bind stereospecifically and reversibly to diverse sugar structures.[1] They are ubiquitous in the biosphere; have been found to be present in viruses, bacteria, fungi, plants and animals. By virtue of their carbohydrate-binding property they participate in several biological processes such as cell separation,[2] signal transduction,[3,4] opsonization,[5,6] apoptosis,[7] agglutination of erythrocytes and other types of cells including cancer cells. They can be employed for the detection and characterization of glycoconjugates on the surface of cells, to differentiate between malignant and normal cells.[8] This differentiation is observed due to altered glycosylation on cell surface associated with malignancy, its progression and metastasis.[9,10] The detection of glycans in disease states has thus received considerable attention. Particular structural profiles of glycans and their recognition by lectins have been attributed to disease progression which can be monitored by lectin-carbohydrate interaction study thus making it important as a diagnostic tool.[11,12] Many lectins found in the hemolymph of invertebrates which generally bind to sialic acid and/or variety of its derivatives as well as acylhexsomines, act like "natural" antibodies to provide a first line of defense against microbes and foreign substances by binding to and neutralising them, and often promoting phagocytosis by hemocytes. Some of the best examples are horseshoe crab lectins, limulin from the American *Limulus polyphemus*, tachylectins from the Japanese *Tachypleus tridentatus* and carcinoscorpin from the Indian *Carcinoscorpius rotunda cauda.*[13–16] Similarly, C-type lectins from a variety of insect species such as the fleshfly *Sarcophaga peregrina*, the American cockroach *Periplaneta americana*, the silk worm *Bombyx mori*, and the West Indian leaf cockroach *Blaberus discoidalis* involve in pathogen recognition and engulfment.[16] Among invertebrate lectins only a few recognize galactose and its glycosides. Among them hemolymph from swimming crab, *Charybdis japonica*,[17] and cockroach, *Periplaneta Americana,*[18] albumen gland of African land snail, *Achatina fulica,*[19] and *Pomacea urceus* and sponges, *Axinella polypoides,*[20] *Geodia cydonium*[21] contain lectin having specificity toward galactose preferably β-galactosides.

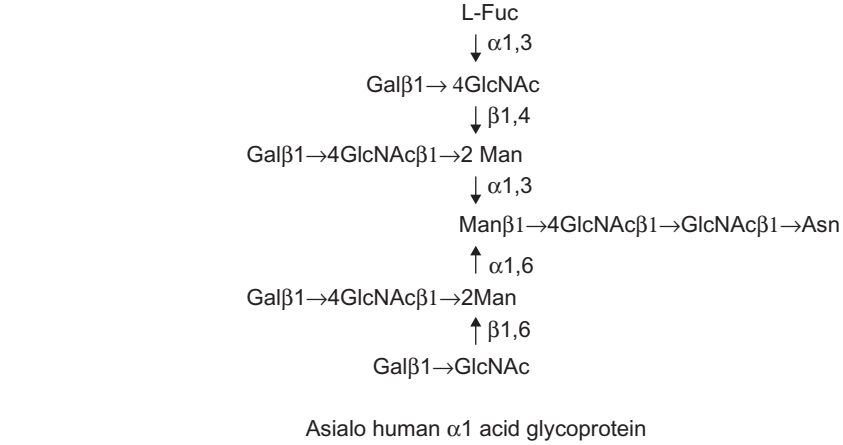
We previously isolated a low molecular weight monomeric lectin, scyllin (~5 kD) from the hemolymph of estuarine crab *Scylla serrata* by affinity chromatography on GalNAc-Sepharon which showed specificity towards Neu5Acα2,3/2,6Galβ1,4GlcNAc, the common anntenaric oligosaccharide chain of complex type *N*-glycan,[22] Scyllin has the property to agglutinate both gram-positive and gram-negative bacteria and inhibited their growth by inhibiting respiration and exogenous glucose oxidation.[23] In the present article we report the isolation of another lectin, scyllin-2, from the hemolymph of *Scylla serrata* and characterization of its carbohydrate binding specificity by enzyme-linked lectinosorbent assay (ELLSA) using mammalian glycoproteins, which contain T (Galβ1→3GalNAc), Tα (Galβ1→3GalNAcα1→Ser/Thr) and Tn (GalNAcα1→Ser/Thr) determinants These are tumor associated carbohydrate antigens, normally cryptic in the peptide core of *O*-glycoproteins. They are expressed in an unmasked form in about 90% of human carcinomas.[24–26] The T/Tn determinants have thus been considered to be the most specific human tumor-associated structures.[27] Many lectins with T/Tn specificity has been characterized[28–33] and novel lectins recognizing T/Tn structures are still being discovered. It revealed that scyllin-2 recognized GalNAcα1→Ser/Thr (Tn) and Galβ1,3GalNAcα1→Ser/Thr (Tα) present as *O*-glycan antigenic structure in mammalian glycoproteins. The mitogenic response of scyllin-2 to mouse splenocytes and comparison of its interaction with hepatocellular carcinoma (HepG2) cells with another T/Tn specific plant lectin, *Artocarpus lakoocha* agglutinin are also described here in.

Materials and Methods

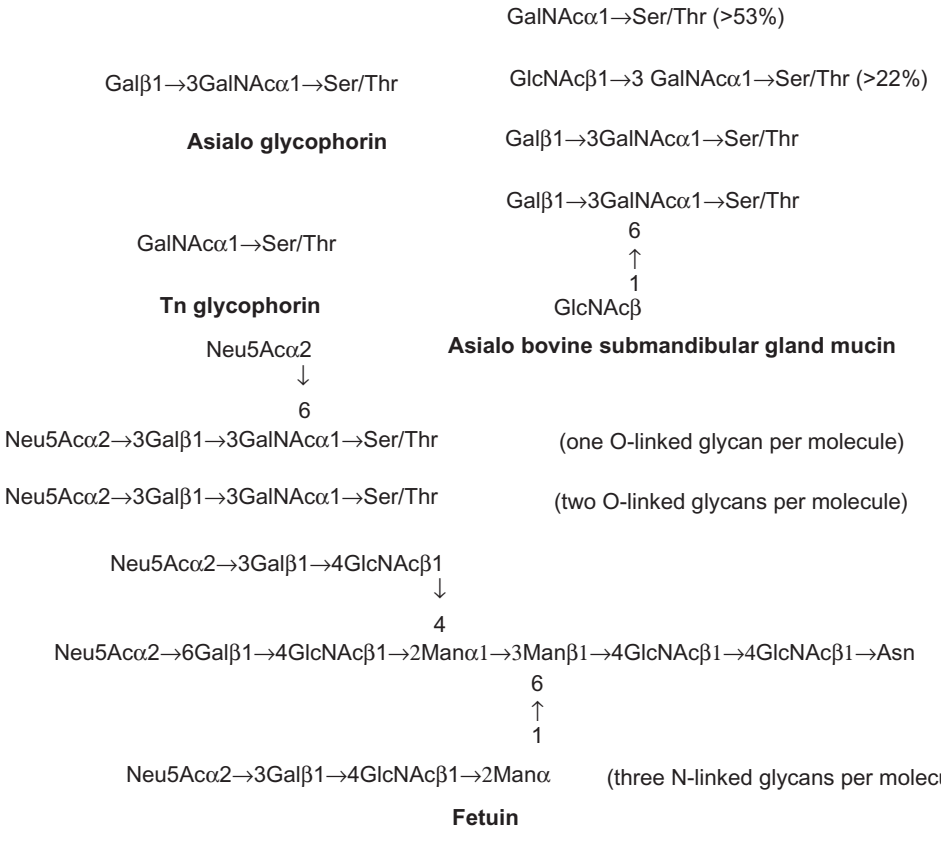
Materials

The estuarine crab Scylla serrata was purchased from local municipal market and identified by Zoological Survey of India, Kolkata. Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 culture medium and fetal bovine serum (FBS) were purchased from GIBCO, USA. Ficoll-Histopaque, 2-mercaptoethanol, penicillin, streptomycin, fungizone, glutamine, biotinamidocaproate-*N*-hydroxy-succinimide ester, antibiotin-HRP, reagents for gel electrophoresis, Bradford reagent and trypan blue were purchased from Sigma, USA. Galactose, mannose, glucose, N-acetyl-galactosamine, N-acetyl-glucosamine, L-fucose, D-fucose, L-arabinose,

D-arabinose,*p*-NO_2-phenyl α-galactoside,*p*-NO_2-phenyl βgalactoside,*p*-NO_2-phenyl N-acetyl α-galactosaminide, *p*-NO_2-phenyl N-acetyl β galactosaminide, lactose, melibiose, raffinose, stachyose and glycoproteins *viz*. fetuin, porcine thyroglobulin (PTG), bovine thyroglobulin (BTG), bovine submandibular gland mucin (BSM), porcine submandibular mucin (PSM), transferrin, human $\alpha_1$acidglycoprotein (AGP) were purchased from Sigma, USA. Ovine submandibular mucin (OSM), asialo OSM, glycophorin, asialo glycophorin, Tn glycophorin were kindly obtained from Prof. E. Lisowska, Institute of Immunology and Experimental Therapy, Wroclaw, Poland. The bird nest glycoprotein (BNG) was the kind gift of Prof. J.F.G Vliegenthart, Utrecht University, Utrecht, The Netherlands. Asialo BSM, Tn containing glycopeptides (MW < 3,000 Da) and T disaccharide (Galβ1→3GalNAc) were the kind gift of Prof. A. M. Wu, Chang-Gung University, Kwei-San, Taipe,Taiwan. Methyl-α-galactose (Me-α-Gal), Methyl-α-N-acetylgalactosamine (Me-α-GalNAc) were kindly



Asialo human α1 acid glycoprotein



obtained from Prof. N. Roy, Department of Biological Chemistry, Indian Association for the Cultivation of Science, Kolkata, India. Asialo glycophorin which has fifteen O-linked glycan of Tα (Galβ1→3GalNAc α1→Ser/Thr) lectin determinants and one N-linked carbohydrate residue was prepared according to Wu and Pigman.[34] Tn-glycophorin was prepared by removing galactose residue from asialo glycophorin by periodateoxidation and mild acid hydrolysis.[35] Fetuin, BSM, PSM, BTG, PTG, AGP, BNG and transferrin 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. Some of the oligosaccharide structures of different glycoproteins are given below.

Tissue culture plates (96 well), petri dishes, culture bottles were procured from Axygen, Sweden. HepG2 cells were obtained from National Centre for Cell Science, Pune. *Artocarpus lakoocha* agglutinin (ALA) was purified and characterized as reported.[28] Asialofetuin was conjugated to CNBr-activated Sepharose 4B following the procedure as described in Pharmacia booklet.

Purification of *S. serrata* hemolymph agglutinin

The hemolymph was collected by cutting spincers and legs of healthy animals of either sex followed by centrifugation at 10,000 rpm at 4 °C for 30 min to remove tissues. The clear supernatant was stored at −20 °C for further use. To the clear supernatant 40% ammonium sulphate was added and stirred at 4 °C for 4 h. The precipitate, separated by centrifugation at 10,000 rpm for 30 min, was dissolved in TBS-Ca (20 mM Tris-HCl, 150 mM NaCl and 20 mM $CaCl_2$, pH 7.5) buffer and dialyzed against the same buffer till free of NH_4^+ ions. The dialyzed sample was loaded on to asialofetuin-Sepharose affinity column (5 cm × 1 cm) pre-equilibrated with TBS-Ca buffer. After elution of the unbound protein by TBS-Ca (pH 7.5), the bound protein was desorbed by 50 mM citrate buffer (pH 5), and immediately neutralized by 1 M Tris-HCl. The active fractions were pooled, mixed, dialyzed against TBS-Ca buffer and concentrated by YM-10 membrane. The semi-purified lectin was added to Resource-Q anion-exchanger in a FPLC system with the starting buffer (20 mM Tris-HCl, 20 mM $CaCl_2$, pH 8) and eluting buffer (20 mM Tris-HCl, 20 mM $CaCl_2$, 1 M NaCl, pH 8) at a flow rate of 2 ml/min. The fractions showing hemagglutinating activity were pooled, dialyzed against TBS-Ca buffer and concentrated by YM10. The protein content of the hemolymph and other fractions was estimated by the method of Bradford using BSA as the standard (Bradford 1976).

Hemagglutination and hemagglutination-inhibition assay

These assays were performed in 96 well polystyrene-U-bottomed microtiter plates.[36] To two-fold serially diluted agglutinin solution (25 µl) in TBS-Ca an equal volume of 2% (w/v, 1×10^6) washed human B erythrocytes suspension in TBS was added. The plate after gentle shaking was incubated for 1 h at room temperature. The above experiment was also performed with pronase and neuraminidase treated human B erythrocytes[36] Hemagglutination titer was defined as the reciprocal of the highest dilution showing visible hemagglutination. This assay was further performed with human O, A, and AB erythrocytes as before.

For hemagglutination-inhibition assay two-fold serially diluted sugar inhibitors (25 µl) in saline was added to an equal volume of agglutinin solution and incubated for 2 h at room temperature. Thereafter, 2% human B erythrocytes suspension in saline (25 µl) was added to each well and incubated for 1 h at room temperature. The degree of hemagglutination was examined and the maximum dilution of the inhibitor solutions showing inhibition was recorded. The controls were set up with equal volumes of saline and erythrocytes, sugar and erythrocytes and agglutinin and erythrocytes.

PAGE, SDS—PAGE and molecular mass

Homogeneity of the agglutinin was performed by electrophoresis in 10% polyacrylamide gel under non-denaturing condition at pH 8.9.[37] The band was visualized by 0.2% Coomassie brilliant blue (G 250) staining followed by destaining in 5% acetic acid containing 20% methanol. SDS-PAGE (10%) was performed under denaturing condition by the method of Laemmli.[38] The sample was treated with 1% SDS in the presence or absence of 2-mercaptoethanol for 5 min at 100 °C. The gel was stained as before. The

molecular mass of the purified agglutinin, scyllin-2 was calculated according to the relative mobility with the Precision Plus Protein™ standards. The molecular mass was also determined by ESI-MS Q ToF mass spectrometry at 5 µl/min flow rate of 90 pmol scyllin-2 in CH_3CN:H_2O:HCOOH (50:50:1) mixture.

pH and thermal stability

The pH stability of scyllin-2 was determined by dialyzing its aliquots against buffers of different pH ranging from pH 3 to 10 for 6 h at 4 °C, *viz*, 50 mM sodium citrate buffer (pH 4–5), 50 mM citrate phosphate buffer (pH 5.2–6.8), 50 mM TBS buffer (pH 7.2–9), 50 mM glycine-NaOH buffer (pH 10). After dialysis hemagglutination activity was observed in the presence of respective buffers against human B erythrocytes. To determine the thermal stability, scyllin-2 in TBS-Ca (pH 7.5) was incubated separately in water bath at different temperatures ranging from 10 to 80 °C for 30 min. The hemagglutinating activity was determined at room temperature as before.

Effect of Ca^{2+} ion

To examine the effect of Ca^{2+} on the activity of scyllin-2, it was chelated out by extensively dialysis against 20 mM TBS containing 50 mM EDTA (pH 7.8). The dialyzed protein was further dialyzed exhaustively against water followed by TBS buffer (pH 7.8). With the dialyzed protein, the hemagglutination assay was performed. To restore the activity of the agglutinin, $CaCl_2$ was added to the dialyzed agglutinin at a concentration ranging from 0–100 mM and the hemagglutination was performed. The above experiment was repeated likewise with Mg^{2+}

N-terminal amino acid sequence

Scyllin-2 was subjected to 10% SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membrane at 100 mA constant current for 18 h using 10 mm 3-cyclohexylamino-1-propane-sulfonic acid (CAPS), pH 11.0 containing 10% methanol as the blotting buffer. After transfer of the protein band to PVDF, the membrane was rinsed several times with Milli Q water and then saturated with 100% MeOH for 2 sec. The blot was next stained with 0.1% ponceus. The membrane was washed with several changes of water, air dried and the protein band excised. The N-terminal amino acid sequence of the electroblotted scyllin-2 was done in a gas-phase protein sequencer (Shimadzu model PPSQ-21 A) consisting of Edman reaction unit followed by HPLC and UV detection.[39]

Circular dichroism study

The circular dichroism (CD) spectra of scyllin-2 and its modified forms were measured at 25 °C in a cell of 1 mm path length of 350 µl capacity in the wave length ranging from 200–250 nm with a Jasco J-815 spectropolarimeter. All samples were scanned at step resolution 0.1 nm, with a scan speed 50 nm per min and 5 scans were taken for each sample. All recordings were done in deionized water, adjusted to pH 7.5. The concentration of the agglutinin in all the experiments was 6 µmol.

Biotinylation of scyllin-2

Scyllin-2 was biotinylated by biotinamido-caproate-*N*-hydroxysuccinimide ester.[35] Briefly, 200 µg scyllin-2 in 1 ml TBS-Ca buffer, pH 7 was mixed with biotin ester (100 µg) in 50 µl methanol and incubated for 30 min at room temperature. The biotinylated scyllin-2 was dialyzed overnight against TBS-Ca buffer and was stored at −20 °C.

Enzyme- linked lectin-sorbant assay

The affinity of scyllin-2 for different glycoproteins was studied by ELLSA.[35] The binding of fixed amount of scyllin-2 to varying amounts of glycoproteins coated on the wells of microtiter plates (Maxisorp, NUNC, Denmark) was checked. In another set of experiment, the amount of scyllin-2 was varied keeping the amount of glycoproteins fixed. Based on these studies 20 ng scyllin-2 was considered to be optimal. The volume of each reagent applied to the plate was 50 µl/well. All incubations except for coating were performed at 20 °C. The reagents, if not otherwise indicated, were diluted with 20 mM TBS, pH 7 containing 0.05% Tween 20 (TBS-T); this was also used for washing the wells between incubations. The wells were coated with 50 µl of varying amounts of glycoproteins (from 0.5 ng to 1.0 µg) in 50 mM sodium carbonate-bicarbonate buffer, pH 9.6 and left overnight at 4 °C. After washing the wells, 50 µl of biotinylated lectin (20 ng) were added to each well and incubated at room temperature for 1 h, washed

and incubated with 50 µl of antibiotin-HRP (1:3000) in TBS-T for 1 h. *O*-phenylenediamine (1 mg/ml) in 50 µl citrate-phosphate buffer (50 mM), pH 5 containing 0.014% H_2O_2 was added to each well and incubated in the dark with gentle shaking for 2 h at room temperature. The absorbance was recorded at 492 nm in ELLSA reader after addition of 50 µl 6 N H_2SO_4 to each well. For inhibition study, the wells were coated with 50 µl of asialo ovine submaxillary mucin (10 ng/well) and incubated overnight at 4 °C. The serially diluted sugars or glycoproteins were mixed with an equal volume of 20 ng biotinylated scyllin-2 in TBS-T and incubated at 20 °C for 45 min. Scyllin-2 diluted to two-fold with TBS-T was used as control. After incubation the samples were tested for binding assay as described above. The inhibitory activity was estimated from the inhibition curve and expressed as the amount of inhibitor (nmol/well) giving 50% inhibition with respect to scyllin-2 bound with asialo OSM as control. All experiments were done in triplicate and the data presented were mean value of the results. The standard deviation in all experiments was less than 5% of the mean value. The wells devoid of glycoproteins recorded absorbance less than 0.1 which served as blank.

Assay for mitogenic activity toward mouse splenocytes

The mitogenic activity of scyllin-2 was determined as described by Mandal and Chowdhury.[40] The splenocytes were aseptically isolated from BALB/c mice by pressing the tissue through a sterilized 100-mesh stainless steel sieve and suspended with RPMI 1640 medium containing 10% FBS, 100 unit penicillin- streptomycin/ml. To cells (10^5 cells/0.1 ml/well) in 96 well cell culture plates (NUNC, Denmark) scyllin-2 was added in various concentrations (0.1, 1, 10, 100 and 200 µg/ml). The wells containing cells cultured in the absence of agglutinin served as negative control and those in the presence of ConA (6 µg/ml) were treated as positive control. Following incubation of the splenocytes at 37 °C in a humidified atmosphere of 5% CO_2 for 72 h, the cells in each well were pulsed with 1 µCi of [^{3}H] thymidine (10 µl) and incubated for another 18 h under the same condition. The cells were then harvested onto a glass fiber filter with an automated cell harvester and the radioactivity was measured using a liquid scintillation counter (Packard). The reported values are the mean of triplicate samples.

Cell culture

HepG2 cell line was cultured and maintained in DMEM medium supplemented with FBS (10%), penicillin and streptomycin (100 IU/ml) at 37 °C in humidified atmosphere of 5% CO_2. The cells attained 70% confluency within 48 h.

FITC labeling of lectins

Flow cytometric analysis

HepG2 cells in culture flask were scraped by cell scraper and washed with 10 mM PBS, pH 7.5. The cells (1×10^6 cells/ml) were dispersed in the same buffer and incubated with 2 µg of FITC-scyllin-2 and FITC-ALA respectively in 100 µl PBS for 1 h at room temperature. The suspension was centrifuged at 2,000 rpm for 10 min. The cells were washed with 10 mM PBS containing

Table 1. Purification scheme of *Scylla serrata* hemolymph agglutinin.[a]

Fraction	Total volume (ml)	Protein concentration (mg/ml)	Total protein (mg)	Total activity (HU)[b]	Specific activity (HU/mg)	Purification fold	Yield (%)
Crude hemolymph	20	17	340	1280	4.0	1.0	100
Ammonium sulphate precipitated fraction	2.0	4.0	8.0	512	64	16	40
Asialo fetuin Sepharose 4B purified agglutinin	1.0	1.0	1.0	256	256	64	20
Resource Q purified agglutinin	0.20	0.08	0.016	205	12800	3200	16

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

5% FBS and dispersed as a homogeneous suspension in 10 mM PBS containing 0.5% paraformaldehyde, 0.1% NaN_3 and 5% FBS (pH 7.5).

Antiproliferative effect of scyllin-2 and ALA on HepG2

The antitumor activity of scyllin-2 and ALA *in vitro* was determined as follows.[41] HepG2 cells were diluted with DMEM supplemented with 10% FBS and seeded (1×10^4 cells/0.2 ml/well) to 96 well cell culture plate (NUNC) and incubated for 3 h. Thereafter scyllin-2 and ALA (10, 20, 40, 80 and 100 µg/ml) were added separately and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h. The cells cultured in the absence of lectin served as a control. 1 µCi [^{3}H] thymidine (10 µl) was added to each well and incubated for 18 h. The cells were harvested onto a GFC membrane by a cell harvester. The incorporated radioactivity was measured using a liquid scintillation counter (Packard).

Results

Purification of *S. serrata* hemolymph agglutinin

Table 1 shows the purification scheme of *S. serrata* hemolymph agglutinin. The crude hemolymph of *S. serrata* on precipitation with 40% ammonium sulphate gave a protein of 16-fold purification with 40% yield. Since the hemagglutinating activity of ammonium sulphate precipitated fraction was effectively inhibited by asialo fetuin, the agglutinin was purified by affinity chromatography on asialo fetuin-Sepharose 4B column following elution with 50 mM citrate buffer (Fig. 1a). The purification achieved was of 64-fold with 20% yield. This was further purified by Resource-Q anion-exchanger (Fig. 1b). The specific activity of scyllin-2 was 12,800 with 3200 fold purification. The recovered activity of scyllin-2 was 16%. The minimum concentration of scyllin-2 required for erythroagglutination was 1.25 µg/ml.

Scyllin-2 was homogeneous as it produced a single band in nondenaturing gel (not shown). Under denaturing condition with or without 2-ME it gave a single band in 10% SDS-PAGE corresponding to molecular mass 75 kD (Fig. 2a). The absolute mass of scyllin-2 obtained by ESI-MS-Q-ToF mass analysis was 74.8 kD (Fig. 2b), indicating that scyllin-2 was a monomeric lectin.

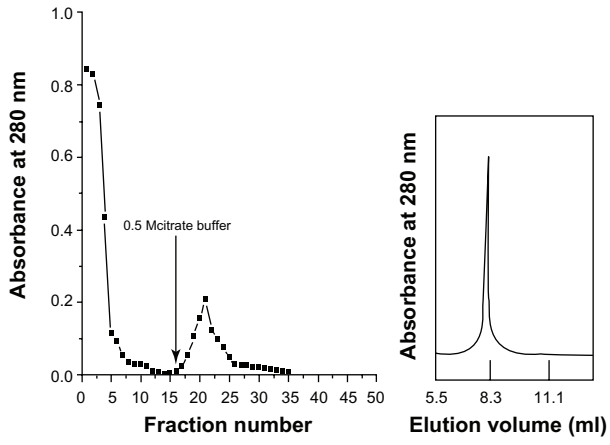


Figure 1. A) Purification of *Scylla serrata* crab hemolymph agglutinin. 0%–40% ammonium sulphate fraction of *S. serrata* crab hemolymph was loaded onto asialo-fetuin Sepharose 4B affinity column (20 cm × 1 cm). After elution of unbound protein with TBS-Ca^{2+} (pH 7.5), the bound protein was desorbed with 50 mM citrate buffer (pH 5.4) and the hemagglutination of the pooled fractions was observed with normal human B erythrocytes. **B**) Elution profile of the asialo-fetuin Sepharose 4B affinity column purified *S. serrata* crab agglutinin on Resource Q anion-exchanger in FPLC system with buffer (20 mM Tris-HCl, 20 mM $CaCl_2$, 1 M NaCl, pH 8) at a flow rate of 2 ml/min.

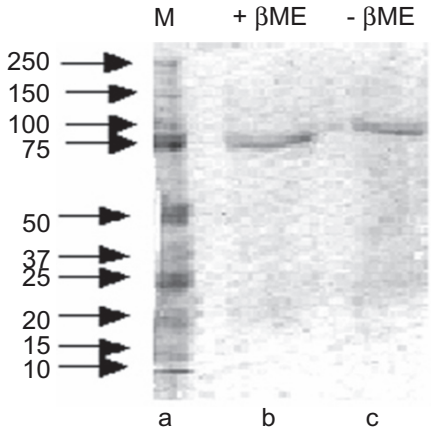


Figure 2a. SDS-PAGE of scyllin-2 on 10% polyacrylamide gel. Scyllin-2 was denatured with 1% SDS in the presence and absence of 2-mercaptoethanol (ME) for 5 min at 100 °C. Lane a, Precision Plus Protein™ Standards from Bio-Rad; lane b, scyllin-2 in the presence of 2-ME; lane c, scyllin-2 in the absence of 2-ME. The protein bands were stained with Coomassie brilliant blue G-250.

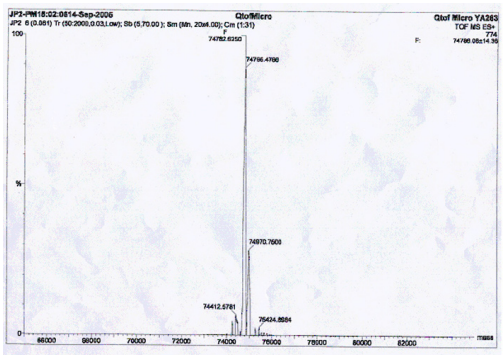


Figure 2b. ESI-MS-Q-ToF mass spectrometry of scyllin-2.

Table 2. Comparison of the N-terminal amino acid sequence of scyllin-2 with other lectins and enzyme protein.

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Scyllin-2	A	E	T	I	S	F	N	F	N	S	Y	S	E	G	N
Peanut	A	E	T	V	S	F	N	F	N	S	F	S	E	G	N
	24														38
	96													107	
Histidine kinase A	A	E	S	I	–	–	N	F	G	S	Y	S	E	G	
Phaseolus vulgaris	A	N	D	I	S	F	N	S	Y	S	E	G			
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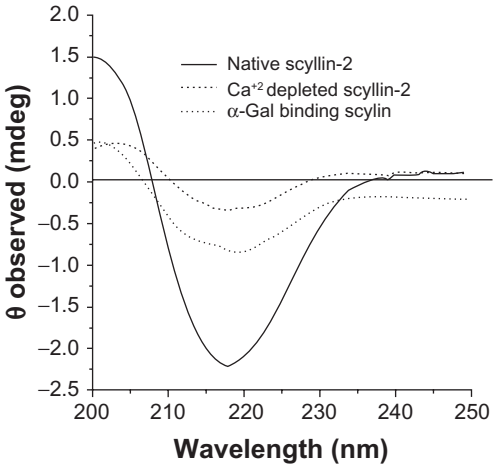

Figure 3. Circular dichroism (CD) spectra of scyllin-2 and modified scyllin-2.

Hemagglutination and hemagglutination-inhibition assay

Scyllin-2 agglutinated human erythrocytes of O, B and AB blood groups strongly (1.25 μg/ml). However, blood group A erythrocytes were agglutinated slightly less (2.5 μg/ml). The pronase and neuraminidase treated erythrocytes were agglutinated more intensely being 8 and 4 times higher than that given by the normal human erythrocytes.

Inhibition study of various saccharides on the hemagglutinating activity of scyllin-2 showed that among the mono- and oligosaccharides tested, Me-α-Gal was the most potent inhibitor requiring 3.12 mM for complete inhibition of 2 HU of lectin. The inhibitory potency of Gal (50 mM) and GalNAc (25 mM) was poor being 16 and 8 times less respectively than the former. Me-β-Gal, Glc, GlcNAc, Man and $ManNH_2$ did not inhibit the hemagglutination even at 200 mM. Among the disaccharides tested, melibiose was the only inhibitor that required 6.25 mM whereas α-lactose, β-lactose, galbiose, sucrose did not inhibit even at 200 mM.

Table 3. Binding of biotinylated scyllin-2 (20 ng/50 μl) with serially diluted different glycoproteins starting from 1 μg by ELISA.

Curve no.	Glycoprotein	Quantity[a] (ng) required for (A_{492} nm) 1.5 unit	Maximum binding (A_{492} nm)
2	Asialo OSM	13.69	3.1
4	Asialo BSM	32.12	2.4
21	Tn glycophorin	141.32	1.7
6	Asialo PSM	232.79	1.7
20	Asialo glycophorin	500	1.4
8	Asialo human α_1 acid glycoprotein	1000	1.6
1	OSM	1000	0.1
3	BSM	1000	0.1
19	Glycophorin	1000	0.1
5	PSM	1000	0.3
12	Asialo BNG	1000	1.3
11	BNG	1000	0.3
7	Human α_1 acid glycoprotein	1000	0.3
13	Fetuin	1000	0.2
14	Asialo fetuin	1000	0.7
15	PTG	1000	0.3
16	Asialo PTG	1000	0.6
17	BTG	1000	0.2
18	Asialo BTG	1000	0.5
9	Transferrin	1000	0.1
10	Asialo transferrin	1000	0.4

[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.

Molecular property

Scyllin-2 was active between pH 6–8 being maximum at pH 7.5, its activity was completely abolished at

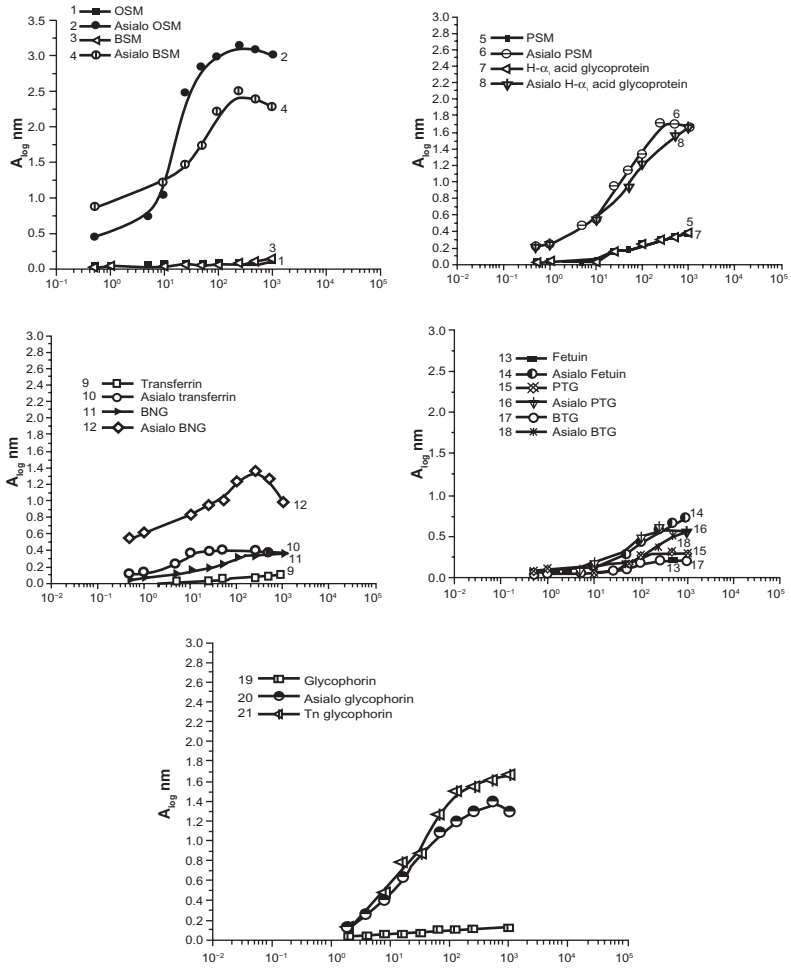

Figure 4. Binding profile of scyllin-2 with serially diluted glycoproteins starting from 1 μg coated in microtiter—plates. The amount of lectin used was 20 ng/well.

pH 9. The activity of scyllin-2 was unaffected up to 25 °C and gradually decreased with the increase in temperature and completely abolished at 80 °C. The hemagglutination activity of scyllin-2 was absolutely dependent on Ca^{2+} ion since on dialysis with EDTA its activity (Titer^{-1} 2^{10}) was abolished. However, after dialysis against TBS, scyllin-2 showed very little activity (Titer^{-1} 2), which was partially restored on addition of Ca^{2+} to the metal free scyllin-2. When the activity of the lectin was checked in the presence of Mg^{2+} and Mn^{2+} the hemagglutinating activity was not altered suggesting that the activity of the lectin was independent of these metal ions.

Amino acid sequencing

The N-terminal amino acid sequence of the 75 kD protein of scyllin-2 and its comparison to that of other lectins and enzyme is presented in Table 2. The N-terminal sequence of 15 amino acid residues of scyllin-2 showed close resemblance to that of peanut lectin at 24–38 residues. Scyllin-2 also showed resemblance to ATP- binding region of kinase A at 96–107 residues and *Phaseolus vulgaris* lectin at 1–12 residues.

Circular dichroism study

Figure 3 shows CD spectra of scyllin-2, Ca^{2+} depleted scyllin-2 and α-Gal bound scyllin-2 at far UV which were expressed as observed ellipticity (θ) in deg.cm^2.dmol^{-1} at a given wave length. High negative ellipticity around 218 nm in native scyllin-2 characterized a structure with very high β-sheet content. There was considerable decrease in ellipticity when CD spectra was measured after Ca^{2+} depletion, and with α-Gal bound scyllin-2, however, the position of the minima of three spectra did not change. This again proved that the lectin's activity was Ca^{2+}

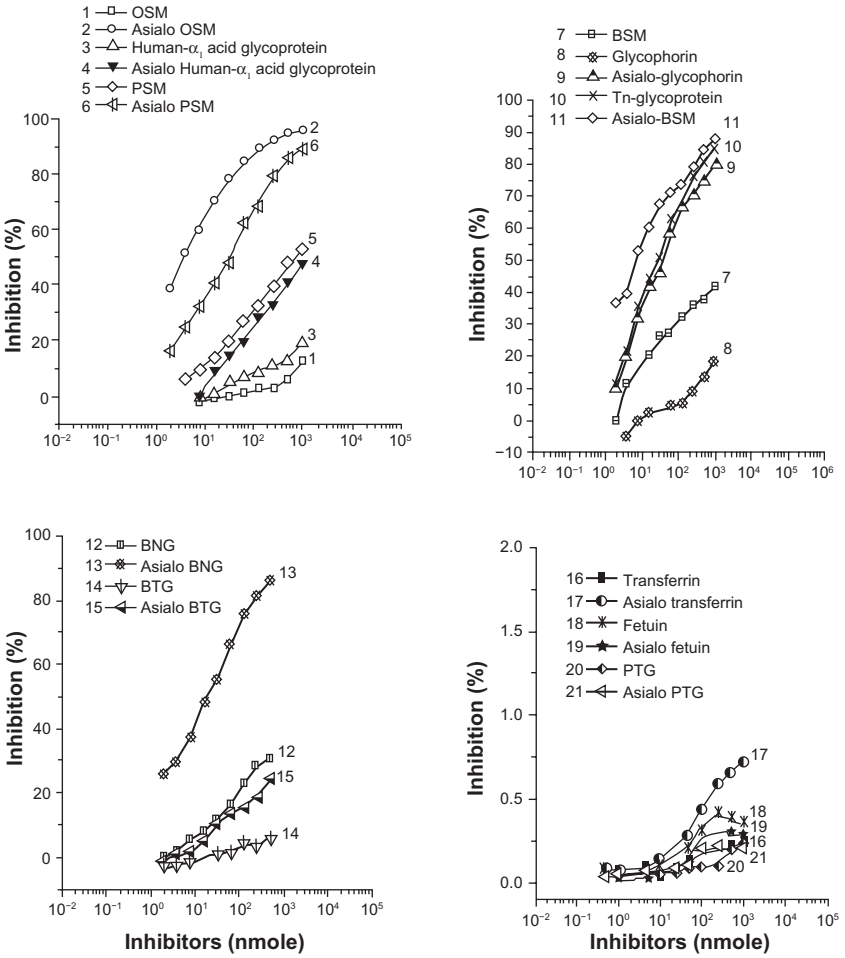

Figure 5. Inhibition of binding between biotinylated scyllin-2 and asialo OSM by different glycoproteins. Wells were coated with 10 ng of asialo OSM. Scyllin-2 (10 ng/well) was preincubated with an equal volume of serially diluted glycoproteins. Amount of glycoproteins for 50% inhibition of binding was calculated.

dependent and it had binding specificity towards α-Gal.

Glycan affinity of scyllin-2

The binding pattern of scyllin-2, with various immobilized glycoproteins and their asialo form is summarized in Table 3 and the binding profiles with the individual glycoproteins are shown in Figure 4. From the binding profile it is clear that scyllin-2 reacted strongly with asialo glycoproteins whereas their sialylated form reacted very poorly. The binding data was expressed as the amount of glycoproteins (ng) required for binding which corresponded to absorbance 1.5 at 492 nm.[28] The results showed that polyvalent Tα and Tn containing asialo glycoproteins such as OSM, BSM, strongly reacted with scyllin-2. However, compared to asialo OSM and BSM, asialo PSM reacted moderately and their sialylated forms showed very weak binding. Among the asialo glycoproteins tested asialo OSM in which Tn, GalNAcα1-Ser/Thr is present as major glycan (>75%)[42] interacted best with the scyllin-2 being 13.69 ng required to reach 1.5 absorbance at 492 nm. In addition, scyllin-2 also reacted strongly with asialo BSM containing 53% Tn[42] requiring 32.12 ng to reach the value 1.5. Scyllin-2 showed less binding activity with asialo PSM since the amount required for binding was appreciably high, 232.79 ng. A large number of glycoproteins showed very weak binding to scyllin-2 even when used at an amount of 1 µg (Table 3). Multivalent-II containing N-glycans such as asialo transferrin, asialo BTG, asialo PTG, asialo fetuin showed very feeble binding with scyllin-2.

Table 4. Amount of glycoproteins giving 50% inhibition of binding between biotinylated scyllin-2 (10 ng/50 μl) and plate coated asialo OSM (10 ng/50 μl).

Curve no.	Glycoproteins inhibitor	Quantity[a] giving 50% inhibition (ng)	Relative potency[b]
2	Asialo OSM	3.7	7.78×10^4
11	Asialo BSM	6.78	4.25×10^4
13	Asialo BNG	18.74	1.5×10^4
10	Tn glycophorin	20.87	1.4×10^4
6	Asialo PSM	34.12	8.4×10^3
9	Asialo glycophorin	38.33	7.5×10^3
5	PSM	659.23	4.4×10^2
1 (Fig. 6)	Gal	2.88×10^5	1
14	BTG	500 (13.81%)	–
15	Asialo BTG	500 (7.65%)	–
12	BNG	500 (30.9%)	–
7	BSM	1000 (41.8%)	–
3	Human α_1 acid glycoprotein	1000 (19.06%)	–
4	Asialo human α_1 acid glycoprotein	1000 (47.56%)	–
8	Glycophorin	1000 (17.99%)	–
1	OSM	1000 (12.18%)	–
21	Asialo PTG	1000 (14.54%)	–
18	Fetuin	1000 (3.9%)	–
19	Asialo fetuin	1000 (15.18%)	–
20	PTG	1000 (13.77%)	–
16	Transferrin	1000 (13.72%)	–
17	Asialo transferrin	1000 (19.26%)	–

[a]The inhibitory activity was estimated from inhibition cruves and is expressed as the amount of glycoproteins giving 50% inhibition.
[b]Relative potency = quantity of Gal (curve no. 1, Fig. 6) required for 50% inhibition is taken as 1.0/quantity of sample required for 50% inhibition.

Inhibition of scyllin-2 and asialo OSM interaction by various glycoproteins

The inhibition profiles of scyllin-2-asialo OSM interaction by various glycoproteins are shown in Figure 5 and the results are summarized in Table 4. It revealed that the polyvalent glycotopes in the tested glycoproteins generated a great enhancement of the interaction with the lectin. Polyvalent Tα and Tn containing glycoproteins were more inhibitory than multivalent –II containing N-linked glycotoproteins. Asialo OSM was the best inhibitor among the glycoproteins tested requiring 3.7 ng for 50% inhibition of scyllin-2-asialo OSM binding and 7.7×10^4 times more potent than Gal whose inhibitory potency was least. Asialo BSM was the next inhibitor that required 6.8 ng for 50% inhibition and was 4.25×10^4 times more potent than Gal. Asialo BNG, Tn glycophorin, asialo PSM and asialo glycophorin were also good inhibitors requiring 18.74, 20.87, 34.12 and 38.33 ng, respectively for 50% inhibition of binding and showed their inhibitory potency to the order of

Table 5. Amount of various saccharides giving 50% inhibition of binding between biotinylated scyllin-2 (10 ng/well) and asialo-OSM (10 ng/50 μl).

Curve no.	Saccharide	Quantity[a] giving 50% inhibition (nmol)	Relative potency[b]
1	Gal	1599.89	1
3	Me-α Gal	80.57	19.86
2	GalNAc	1227.23	1.3
8	*p*-NO_2-Ph-α Gal	61.36	26.07
7	*p*-NO_2-Ph-α GalNAc	195.94	8.17
12	Melibiose	234.39	6.83
18	3α-Galactobiose	380.38	4.21
17	Stachyose	625 (45.36%)	–
20	Digalacturonic acid	78.125 (29.4%)	–
–	4α-Galactobiose	625 (42.5%)	–
22	Galβ1,4Gal	156.25 (30.89%)	–
19	3α, 4β,-Galactotriose	312.5 (5.1%)	–
21	3α, 4β, 3α-Galactotetraose	156.25 (15.19%)	–
16	Raffinose	1250 (31.4%)	–
9	*p*-NO_2-Ph-β GalNAc	312.5 (34.38%)	–
10	*p*-NO_2-Ph-β-Gal	2500 (44.65%)	–
13	Rhamnose	5000 (25%)	–
14	GlcNAc	5000 (14.89%)	–
15	$GalNH_2$	5000 (8.98%)	–
11	Lactose	5×10^3 (32.87%)	–
4	Me-β Gal	2.5×10^4 (47.15%)	–
5	L-Ara	2.5×10^4 (14.237%)	–
–	D-Ara	2.5×10^4 (22.43%)	–
6	L-Fuc	2.5×10^4 (18.87%)	–
–	D-Fuc	2.5×10^4 (28.78%)	–
–	D-Man	2.5×10^4 (28.78%)	–
–	D-Glc	2.5×10^4 (16.26%)	–

[a]The inhibitory activity was estimated from the inhibition cruve and is expressed as the amount of inhibitor giving 50% inhibition.
[b]Relative potency = quantity of Gal (curve no. 1, Fig. 6) required for 50% inhibition is taken as 1.0/quantity of sample required for 50% inhibition.

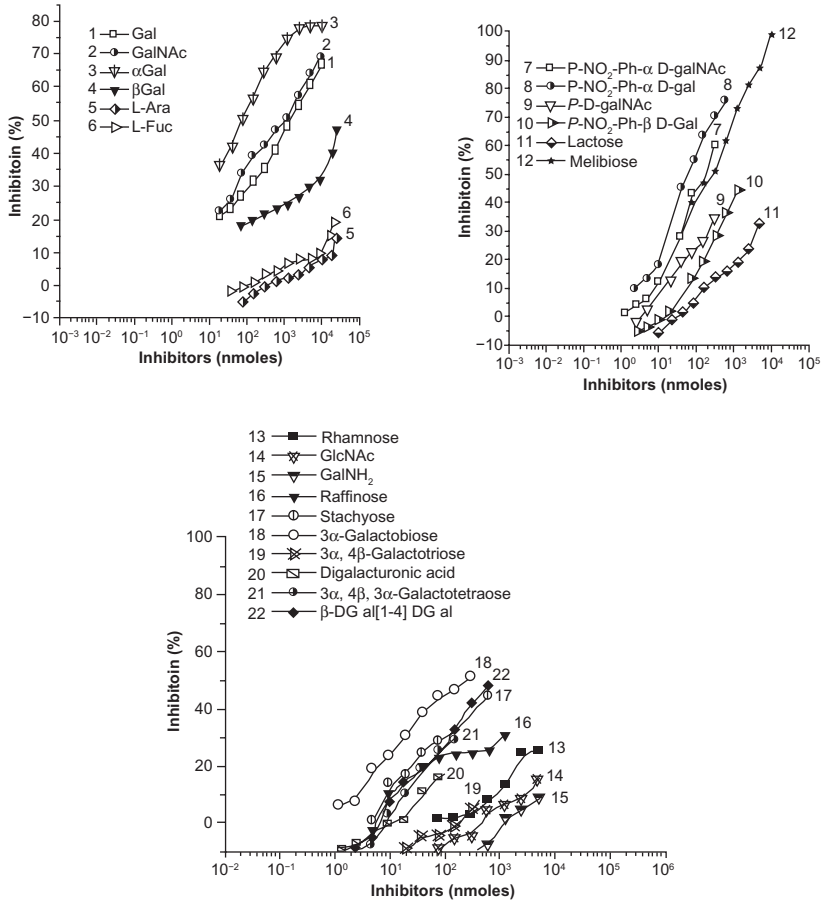

Figure 6. Inhibition of binding between biotinylated scyllin-2 and asialo OSM by various saccharides. Wells were coated with 10 ng of asialo OSM. Scyllin-2 (10 ng/well) was preincubated with an equal volume of serially diluted saccharides. Amount of saccharides for 50% inhibition of binding was calculated.

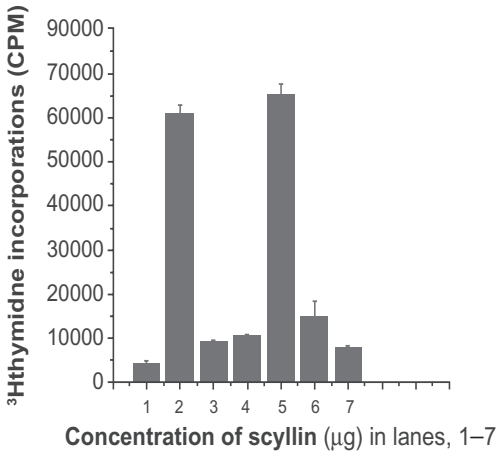

Figure 7. Mitogenic activity of *S. serrata* agglutinin toward mouse splenocytes observed as the count per min (CPM) of [^{3}H] thymidine uptake. Splenocytes without scyllin-2 (-ve control); splenocytes with scyllin-2 (0.1, 1, 10, 100 and 200 μg/ml); splenocytes with ConA (6 μg/ml, +ve control). Data represent mean ± SD of 3 experiments.
**Equals $P < 0.01$ as analyzed by Students t-test.

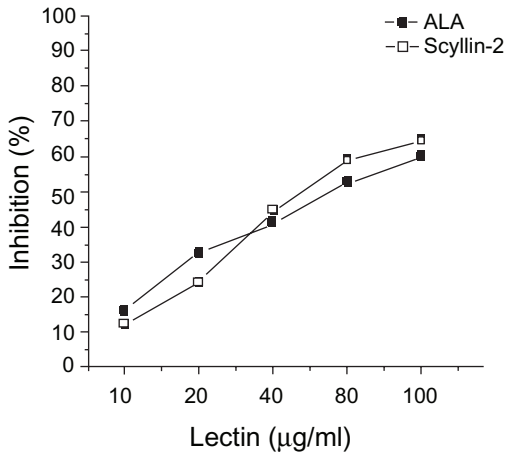

Figure 8. Flow cytometric analysis of expression of scyllin-2 and ALA ligands on HepG2 cells. (a), dot plot of binding with FITC- scyllin-2; (b), histogram of binding with FITC-scyllin-2; (c), dot plot of binding with FITC-ALA; (d), histogram of binding with FITC- ALA; (e), dot plot of binding with FITC-scyllin-2 pre-incubated with α Gal; (f), histogram of binding with FITC-scyllin-2 pre-incubated with α Gal.

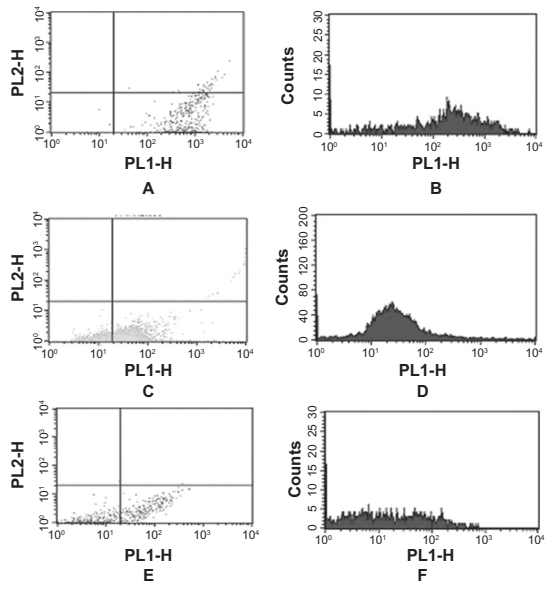

Figure 9. Comparative study of antitproliferative activity of scyllin-2 and ALA on HepG2 (1×10^4 cells/well) cells.

10^3–10^4 times more than Gal (Table 4) whereas sialoglycoproteins due to masking effect of sialic acid showed decreased inhibition than their asialo counter part. PSM gave 50% inhibition with highest amount (659.23 ng) and its inhibitory potency was least being only 4.4×10^2 times than Gal. Asialo human α_1 acid glycoprotein even with 1 µg could not attain 50% inhibition and its sialylated counterpart with the same amount inhibited only 19%. Other glycoproteins and their asialo forms did not inhibit the binding with the amount used (Table 4).

Inhibition of scyllin-2-asialo OSM interaction by mono- and oligosaccharides

Inhibition of scyllin-2-asialo OSM binding by saccharides is summarized in Table 5 and the binding pattern is shown in Figure 6. Among the mono- and oligosaccharides tested *p*-NO_2-Ph-α Gal was the best inhibitor requiring only 61.36 nmol for 50% inhibition of the binding between scyllin-2 and asialo OSM and 26 times more potent than Gal whose inhibitory potency was least. Next potent inhibitor was Me-α Gal (80.57 nmol) which was about 20 times more inhibitory than Gal. GalNAc inhibited the binding almost with the same potency of Gal whereas *p*-NO_2-ph-α GalNAc was 6 and 8 times more potent than GalNAc and Gal respectively. Among oligosaccharides melibiose (234.39 nmol) and 3α-galactobiose (380.38 nmole) were moderate inhibitors 7 and 4 times more potent than Gal. 4α-galactobiose, 3α, 4β-galactotriose, 3α, 4β, 3α-galactotetraose though containing nonreducing α Gal unit did not attain 50% inhibition. *p*-NO_2-Ph-β Gal did not inhibit 50% at 2.5 µmol. Me-β Gal gave 47% inhibition even at 25 µmol. Other sugars tested at 5 to 25 µmol gave poor inhibition.

Mitogenic activity

Lectins are mostly mitogens; they transform resting lymphocytes/splenocytes to a proliferative state which has high implication in immunology. Scyllin-2 showed potent mitogenic response towards BALB/c splenocytes. It showed maximum mitogenic activity at 10 µg/ml concentration. The dose required to show maximum mitogenic activity of scyllin-2 was higher than that required by positive control Con A which showed a comparable activity at 6 µg/ml concentration (Fig. 7). The value obtained by the treatment with scyllin-2 were significantly different from that without treatment ($P < 0.01$).

Flow cytometric analysis of ligand expression of scyllin-2 and ALA on HepG2 cells

The binding of FITC labeled scyllin-2 and ALA with HepG2 was analyzed and compared by flow cytometry. The binding of scyllin-2 with HepG2 was 86.51% (Figs. 8a and 8b) which was considerably higher than that observed with ALA (66.41%) (Figs. 8c and 8d). Scyllin-2 preincubated with α Gal showed low percentage of binding, 44.36% (Figs. 8e and 8f) which was half of the original binding.

Anti-proliferative activity

Both cell proliferation and anti-proliferation are biological processes which are exemplified by lectin binding to cell surface carbohydrate receptors. Lectins exhibit growth inhibitory effect towards many carcinoma cells. Scyllin-2 showed growth inhibitory effect on HepG2 cells. This anti-proliferative activity on HepG2 was compared with another T/Tn specific plant lectin, *A. lakoocha* agglutinin,[28] and was dose dependent. Figure 9 shows that IC_{50} given by scyllin-2

and ALA on HepG2 was comparable being 61 µg/ml and 80 µg/ml respectively.

Discussion

Scylla serrata edible crab exhibits different phenotypes determined by multivariate analysis.[43] Different coastal environment has significant influence on the phenotypic expression of *S. serrata*, and two well discriminated morphs were found in the same location.[43] Environmental temperature is one of the main variables known to cause morphometric features. Such morphological diversity shows variation in their physiological and physico-chemical properties. This is due to regulation of their protein synthesis, carbohydrate synthesis and overall carbohydrate binding property, and biological property. Mercy and Ravindranath[44] reported that a lectin purified from the hemolymph of marine crab *S. serrata* from the Indian Oceanic region recognized NeuGc and bound to bovine and porcine thyroglobulins which contain a NeuGcα2,6 Gal as terminal component of oligosaccharide residues exclusively and the lectin activity was not inhibited by NeuAc and NeuAcα2,3 Gal/GalNAc and Neu5 Acα2,6 Gal. Whereas hemolymph lectin from Thai marine crab *S. serrata* showed binding specificity to sialoglycoconjugates, BSM.[45] Evidence was documented of two other sialic acid specific lectins from the hemolymph of fresh water crab, *Paratelphusa jacquemontii*[46] and of California coastal crab, *Cancer antennarius*.[47] Despite their different habitat, they have almost the same molecular mass 34 kD and 36 kD, respectively. They showed high specificity to BSM which contains 9-*O*-acetyl- and 8, 9-di-*O*-acetyl-N-acetyl neuraminic acid. This was confirmed by abolition of its inhibitory capacity by sialidase treatment and de-*O*-acetylation. Like the occurrence of multiple lectins in sponges, *Axinella polypoides*,[20] *Pelina semitubulosa*,[48] *Halichondria okadii*,[49] and blood clam *Anadara granosa*,[50,51] crustacean hemolymph contains multiple lectins[52,53] whose detailed carbohydrate specificity at the macromolecular level has not been worked out. The hemolymph of the edible crab *Scylla serrata* contains multiple lectins, the phenomenon was ascertained by its cross-adsorption test with erythrocytes (unpublished).We purified earlier a low molecular weight (5 kD) lectin scyllin from *S. serrata* collected from estuary of Gangetic delta that showed binding affinity to complex sialooligosaccharides of mammalian glycoproteins like fetuin, ceruloplasmin, porcine mucin, human glycophorin.[22] Herein, we report a Ca^{2+} dependent second monomeric lectin of molecular mass 75 kD having T and Tn specificity which is close to monomeric lectin of MW 70 kD isolated from Thai *S. serrata* crab.[45] However, lectin from *S. serrata* of Indian Oceanic region has molecular mass of 55 kD and is a heterodimeric lectin of subunits 30 kD and 25 kD respectively.[44]

The N-terminal sequence of 15 amino acid residues of scyllin-2 showed close resemblance to peanut lectin though at different region. Amino acid sequence of scyllin-2 also maintained good homology with histidine kinase A and *Phaseolus vulgaris* lectin as well. Scyllin-2 consists of β-sheet structure. This structure may be suggested to have similarity to peanut (*Arachis hypogea*) agglutinin (PNA), crystallographic structure of which was solved by Banerjee and collaborators.[54,55] This homotetrameric lectin with monomers in a jelly roll fold, associated by non-covalent bonds results in an open structure, which confers a stable behavior to this lectin. A decrease in spectra intensity suggests a propensity of decreasing β sheet structure in Ca^{2+} depleted scyllin-2 and α-Gal bound scyllin-2. Nevertheless, the positions of minima did not change which indicates that β-sheet structure is maintained even in Ca^{2+} depleted scyllin-2 and in α-Gal bound scyllin-2. Also a deconvolution of these spectra (results not shown) agrees with high β- sheet content in these structures. Therefore, these changes in intensity can be related to tertiary or quaternary effects, which can not be measured directly at far-UV. CD in Ca^{2+} depleted scyllin-2 suggests that Ca^{2+} plays a critical role in holding compact β-sheet architecture of scyllin-2. Similar effect was observed in α-Gal bound scyllin-2. Further investigation is necessary.

The results of both binding and inhibition assays revealed a remarkable affinity of scyllin-2 for polyvalent Tn/T containing glycoproteins. Scyllin-2 reacted best with all high-density Tn-containing

glycoproteins (asialo OSM, asialo BSM and asialo PSM), indicating that the high density of polyvalent Tn plays an essential role in binding. It is reasonable to assume that the increase in reactivity of this glycoprotein is due to the exposure of a large number of Tn structures following mild acid hydrolysis.

Like straw mushroom lectin,[56] *Ganoderma capense* lectin,[41] tuber lectin from a wild cobra lily, *Arisaema flavum*,[57] lectin from tubers of Voodoo lily (*Sauromatum venosum*)[58] pinto bean lectin,[59] haricot bean lectin,[60] scyllin-2 is endowed with a potent mitogenic effect towards mouse splenocytes. It also exhibited potent inhibitory effect on the proliferation of HepG2 cell lines. This finding is in concordance with the previous report on many other lectins.[61–63] Lectins have been used for gastrointestinal cell targeting. Tomato lectin and jacalin retained their antitumor activity through the gastrointestinal tract.[64] In the present study flow cytometric data revealed that T/Tn specific lectins scyllin-2 and ALA have their ligand receptor on HepG2.

Studies have shown that individual carbohydrate-protein binding is too weak to affect the specific interaction. Numerous studies have demonstrated that polyvalent display of carbohydrates can lead to remarkable increase of binding affinity.[65] The binding profile of scyllin-2 and the inhibition of binding using polyvalent glycoproteins containing well-defined glycotopes as well as reactive saccharides by ELLSA equivocally established its specificity to Tn/T antigen.

T/Tn antigen occurs on the surface of tumor cells as a mucin-associated antigenic marker and is one of the few chemically well-defined tumor antigens with a proven link to malignancy.[27] They are good marker for several cancerous tissues, including the prognosis of colorectal cancer.[66] From the remarkably strong affinity of scyllin-2 for Tn/T glycoproteins observed in this study without cross-reacting with other glycoproteins, it can be reasonably assumed that scyllin-2 may be an useful tool (1) as a structural probe to detect the aberrant expression of Tn/T epitopes in tissues and cells, (2) in the analysis of *O*-linked glycoproteins, and (3) for the identification and fractionation of glycopeptides and oligosaccharides.

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Disclosures

This manuscript has been approved by all authors. This paper is unique and is not under consideration by any other journal for publication elsewhere. The views expressed in this article do not necessarily reflect those of Libertas Academica. The authors and peer reviewers report no conflicts of interest.

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