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PURIFICATION AND PARTIAL CHARACTERIZATION OF A HEPARIN-BINDING LECTIN FROM THE MARINE CLAM ANADARA GRANOSA*

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Summary : The heparin-binding lectin, Anadarin MS, from the plasma of the marine clam Anadara granosa is purified through affinity chromatography on heparin-Sepharose 4B followed by gel filtration on a Sepharose 6B column. The purified lectin is a pentameric protein of native Mr 300 kDa and is composed of identical subunits of 60 kDa. The pI value of this Ca^{2+}-dependent lectin is 6.2. Anadarin MS agglutinates normal rabbit erythrocytes but not that of human. Aspartic acid, glutamic acid, histidine and glycine are the predominant amino acids. Unlike other reported heparin-binding lectins, Anadarin MS exhibits a unique and strict specificity for iduronic acid containing glycosaminoglycans. This lectin agglutinates infective promastigotes of Leishmania donovani exclusively and can therefore be used as a novel biochemical surface marker for this parasite.

Proteoglycans are remarkable for their highly diversified biological roles. Majority of these roles are regulated through the binding of proteins to the glycosaminoglycan chains of the proteoglycans. Among the proteoglycans, heparin shows the strongest binding ability and several reports of heparin-binding proteins and their proposed functional roles are available (1).

*Dedicated to the memory of Dr. Manju Sarkar.

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Apart from this, another special class of proteins, the endogenous heparin-binding lectins has been discovered (2-7) which are also involved in several biological recognition processes (8,9).

Considering the extensive biological activities of the glycosaminoglycans, especially heparin, the importance of heparin-binding lectins as experimental tools seems to be enormous. Among the numerous purified animal lectins reported so far, few have been demonstrated to be heparin-binding and they are mostly from vertebrate sources. None of these lectins is exclusively specific for iduronic acid containing glycosaminoglycans. In the present communication, we describe the purification and partial characterization of a 'unique heparin-binding lectin from the marine blood clam Anadara granosa. The interaction of this lectin with Leishmania donovani, the causative agent of visceral leishmaniasis, is also notable. As soon as the parasites are transformed from a non-infective phase to an infective population, they are readily agglutinated by this lectin.

Materials and Methods

Sepharose 4B, Sepharose 6B and carrier ampholytes for isoelectric focusing were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Glycosaminoglycans, monosaccharides and other chemicals were obtained from Sigma Chemical Co., USA. The heparin-Sepharose 4B affinity matrix was a gift from Dr. D. Mitra (Indian Institute of Chemical Biology, Calcutta, India). Plasma of Anadara granosa clam was prepared following the procedure described earlier (10). Both Leishmania major (MHOM/IN/71/LRC-L408) and Leishmania donovani (MHOM/IN/83/AG83) were maintained in mammalian tissue culture medium RPMI-1640 (Gibco, USA) (11).

About 10 ml of stored plasma (specific activity : 45.7 units/mg) was passed through a heparin-Sepharose 4B affinity column, previously equilibrated with buffer A (50 mM Tris-HCl buffer, pH 7.3, containing 50 mM $CaCl_2$), at 10°C. The column was then washed thoroughly with the same buffer at the same temperature. Elution of the bound protein was done by 2.0 M NaCl solution at room temperature. Fractions were monitored by measurement of absorbance at 280 nm. The protein peak was dialysed against buffer A, concentrated by a lyophilizer and was subjected to gel filtration on a Sepharose 6B column previously equilibrated with buffer A containing 100 mM D-galactose. The column was eluted with the same buffer. Optical density (at 280 nm) and hemagglutinating activity of the fractions were checked. The peak fractions containing lectin activity were pooled, dialysed against buffer A, concentrated and stored at 4°C.

Protein concentration was determined by the method of Lowry et al.(12) using crystalline bovine serum albumin as a standard. Total carbohydrate was estimated by using D-Gal as standard (13).

Hemagglutination was carried out at room temperature (25°C) using normal rabbit and human erythrocytes (10). Effects of pH, temperature and divalent cations (Ca^{2+}, Mg^{2+} and Mn^{2+}) on lectin activity were demonstrated as described earlier (10). Hemagglutination inhibition test was done by incubating lectin (0.14 µg, containing 10 hemagglutination unit) with serially diluted glycosaminoglycans and other saccharides in microtitre plates. 25 µl of a 2% (v/v) rabbit erythrocyte suspension was then added to the incubated solution and the results were noted after 1h.

Polyacrylamide gel electrophoresis, of the native protein was performed in 6% polyacrylamide gel at pH 8.9 (14). SDS/PAGE in slab was carried out in 10% polyacrylamide gel (15). Dissociation and reduction of the proteins were performed following the procedures mentioned earlier (10). The subunit profile was revealed by Coomassie blue and silver staining. Analytical isoelectric focusing was performed in LKB slab-gel isoelectric focusing apparatus (16).

About 1.9 mg of the purified lectin was applied on a column (90 cm x 1.5 cm) of Sepharose 6B, equilibrated with buffer A containing 100 mM D-galactose. Fractions collected were assayed for activity by the hemagglutination assay technique using normal rabbit erythrocytes and absorbance was measured at 280 nm. The column was calibrated with thyroglobulin, ferritin, catalase and concanavalin A as markers.

Amino acid composition of the purified lectin was analysed with a Pharmacia LKB Alpha Plus amino acid analyser. Samples were prepared as described previously (17). Tryptophan was determined spectrophotometrically (18).

The log(non-infective) and stationary (infective) phase promastigotes of Leishmania donovani and Leishmania major were suspended separately in phosphate-buffered saline (10 mM- sodium phosphate buffer, pH 7.2, containing 150 mM NaCl). 25 µl of this suspension ($1x10^8$ cells/ml) was mixed with the serially diluted lectin. After incubation in a humid chamber at 25°C for 30 min, the samples were examined under a Zeiss microscope. Inhibition studies were carried out using appropriate haptens.

Results

The Ca^{2+} dependent heparin-binding lectin, Anadarin MS, from the plasma of Anadara granosa was purified by affinity chromatography followed by gel filtration. The bound lectin was eluted from the heparin-Sepharose 4B column with 2.0 M NaCl solution. Upon gel filtration on a Sepharose 6B column, the affinity purified material resolved into two distinct peaks, one of which (Peak-II) was active (Fig.1). About 0.09 mg of lectin was purified from 10 ml of crude plasma. The recovery of the purified lectin and the purification fold were 96% and 1493,

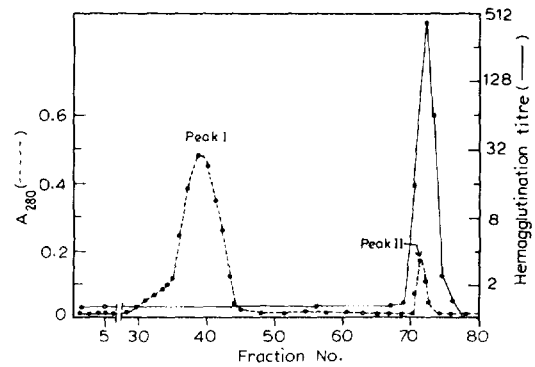


Fig.1. Purification of affinity-isolated protein by gel filtration on a Sepharose 6B column. Fractions collected were assayed for A_{280}(---) and hemagglutination was done by using rabbit erythrocytes (——).

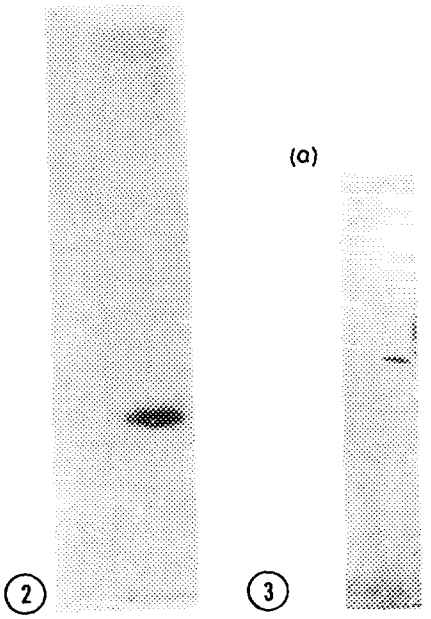
respectively (Table I). The purified lectin produced a single band in 6% alkaline polyacrylamide gel electrophoresis (Fig.2) and isoelectric focusing (Fig.3a). Homogeneity was also confirmed by the single and active protein peak obtained through the gel filtration (Fig.4a).

Anadarin MS strongly agglutinated normal rabbit erythrocytes but not that of human and its optimum range of pH for activity was 7.3 to 7.4. The titre value was found more or less identical over a wide range of temperature. The lectin activity was totally inhibited by Mg^{2+} and Mn^{2+}.

Anadarin MS is a protein having a native Mr of 300 kDa as determined by gel filtration on a calibrated Sepharose 6B column (Fig.4b). On SDS/PAGE, the purified lectin yielded a single band

Table I. PURIFICATION OF ANADARIN MS

Fraction	Total Volume (ml)	Total Protein (mg)	Specific activity (units/ mg)	Recovery (%)	Purification (fold)
Crude Plasma	10	140	45.7	100	1
Heparin-Sepharose 4B affinity column eluate (with 2.OM Nacl)	2.5	1.6	2000	50	43.8
Sepharose 6B eluate	1.2	.09	68266	96	1493



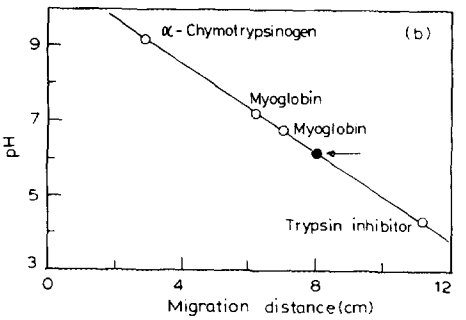


Fig.2. Polyacrylamide gel electrophoresis of Anadarin MS in 6% gel at pH 8.9.

Fig.3. Determination of the pI of Anadarin MS by isoelectric focusing. The purified lectin showed a single band at pH 6.2 (a). In (b), the arrow indicates the position of Anadarin MS.

of Mr 60 kDa (Fig.5). The pI of the purified lectin was found to be 6.2 (Fig.3b). Aspartic acid, glutamic acid, histidine and glycine are the most abundant amino acids of Anadarin MS (Table II).

Hemagglutination inhibition study using normal rabbit erythrocytes revealed that heparin was the most potent inhibitor among the glycosaminoglycans tested. Dermatan sulphate also inhibited the lectin activity but on a molar basis it was less effective an inhibitor than heparin. Chondroitin 4-sulphate, chondroitin 6-sulphate and hyaluronic acid were found to have no effect on binding. All other sugars tested failed to inhibit the lectin activity (Table III).

The lectin agglutinated infective promastigotes of *Leishmania donovani*, but failed to interact with the parasites when they are in the log (non-infective) phase of growth. On the other hand, Anadarin MS agglutinated both the infective and non-infective promastigotes of *L.major* (Table IV).

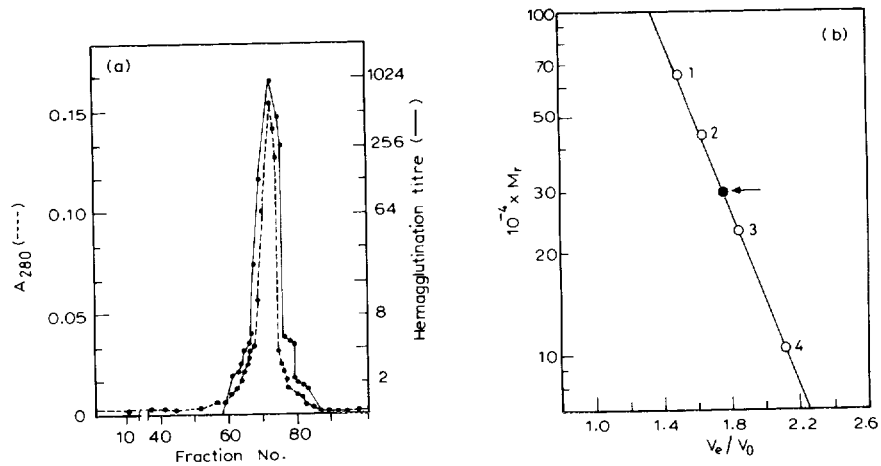


Fig.4. Elution profile of Anadarin MS from gel filtration on a calibrated Sepharose 6B column (a) and determination of its native molecular weight (b).

Discussion

This report describes the purification and partial characterization of a heparin-binding lectin from the plasma of the marine blood clam, Anadara granosa. The native Mr of this

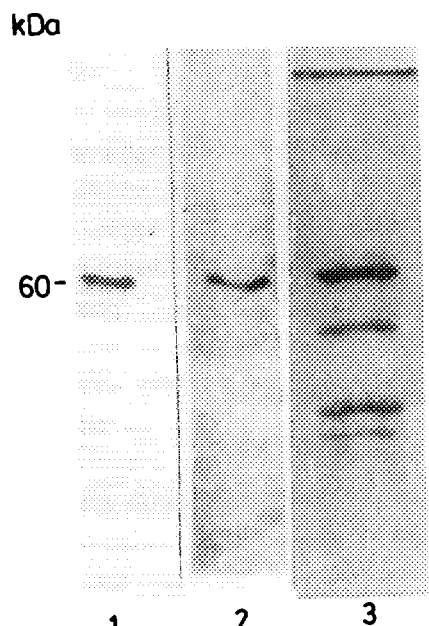


Fig.5. Subunit profile of purified Anadarin MS. 10% SDS/PAGE pattern of the materials eluted from the heparin-Sepharose 4B (Lane 3), purified lectin, stained with Coomassie brilliant blue (Lane 2) and with silver stain (Lane 1).

Table II. AMINO ACID COMPOSITION OF PURIFIED ANADARIN MS

	Composition (no.of residues/mol)
Aspartic acid	41
Threonine	15
Serine	18
Glutamic acid	39
Proline	6
Glycine	27
Alanine	14
Cysteine	2
Valine	16
Methionine	3
Isoleucine	10
Leucine	12
Tyrosine	9
Phenylalanine	20
Histidine	31
Lysine	10
Tryptophan*	11
Arginine	17

*Determined by a spectrophotometric method (18).

lectin is 300 kDa and it is composed of five identical subunits of 60 kDa.

The results of hemagglutination-inhibition study suggest that the topography of the combining sites of Anadarin MS is significantly different from other known heparin-binding lectins (2,7,19,20). Among the glycosaminoglycans tested, the lectin interacts only with heparin and dermatan sulphate, not with hyaluronic acid, chondroitin 4-sulphate and chondroitin 6-sulphate. This selective interaction suggests that the combining sites of the lectin may have strong affinity for iduronie acid and the inhibition is not only due to the effect of polyanions. It is further proved that charge interactions contribute very little to the binding when the charge carrying saccharides such as galacturonic acid, glucuronic acid and sialic acid fail to inhibit the lectin activity. None of the heparin-binding lectins reported so far, exhibits such strict specificity for iduronic acid containing glycosaminoglycans.

Like human placenta, chicken muscles and ascidian hemolymph (21-23), the plasma of Anadara granosa contains a galactose-

Table III. INHIBITION OF ANADARIN MS ACTIVITY BY SIMPLE SUGARS AND GLYCOSAMINOGLYCANS

Inhibitors	Concentration for complete inhibition
Simple sugars	
D-Glucose	-*
D-Galactose	-*
N-Acetyl-D-galactosamine	-*
N-Acetyl-D-glucosamine	-*
Mannose	-*
Lactose	-*
N-Glycolyl neuraminic acid	-*
N-Acetyl neuraminic acid	-*
Glucuronic acid	-*
Galacturonic acid	-*
L-Arabinose	-*
Glycosaminoglycans	mg/ml
Heparin	0.015
Dermatan sulphate	0.62
Chondroitin 4-sulphate	-**
Chondroitin 6-sulphate	-**
Hyaluronic acid	-**

- Noninhibitory up to, *100 mM;
**20 mg/ml concentration

specific lectin (10) along with the heparin-binding one. Although there is an indication that these two lectins may be involved in tissue development (24), it remains to be ascertained whether this concurrent occurrence of two particular types of lectins within the same tissue has any biosynthetic, functional or evolutionary significance.

Table IV. AGGLUTINATION OF LEISHMANIA SPP. BY ANADARIN MS

	L.donovani		L.major	
	non-infective	infective	non-infective	infective
Titre	0	256	512	512
Minimum lectin concentration (µg/ml) required for agglutination	200	3.72	0.95	0.95
Nature of agglutination	-	head to head	head to head	head to head

Specific agglutination of infective promastigotes with Anadarin MS suggests that transition of L.donovani from non-infective to infective phase is accompanied by the expression of glycosaminoglycans on the surface of the parasites. Such modification of surface carbohydrates have been shown to be essential for survival and infectivity of L.major promastigotes (25,26). After further studies, Anadarin MS may be used as a novel biochemical surface marker for the identification and isolation of infective promastigotes of L.donovani. These isolated infective promastigotes may then be used for detailed characterization.

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