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PURIFICATION AND PARTIAL CHARACTERIZATION OF A N-GLYCOLYLNEURAMINIC ACID SPECIFIC LECTIN FROM THE CLAM ANADARA GRANOSA (L)

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Summary : The N-glycolylneuraminic acid-specific lectin (AFL) from the foot muscles of the marine clam Anadara granosa has been purified to homogeneity by affinity chromatography on bovine submaxillary mucin-Sepharose 4B. The Ca^{2+}-dependent lectin agglutinates rabbit erythrocytes. The purified lectin is a tetrameric protein of native M_r 254 kDa having a pI value of 6.65. The M_r of two subunits is 65 kDa each and that of the remaining two is 62 kDa each. The dominant amino acids of the lectin are aspartic acid, glutamic acid, serine and glycine. The lectin activity is inhibited only by N-glycolylneuraminic acid specially when it is present in the macromolecular structure of mucin viz., porcine submaxillary mucin, which is the most potent inhibitor. The binding site does not recognize N-acetylneuraminic acid. Due to this strict specificity, the lectin appears to be unique.

The natural derivatives of neuraminic acid are usually known as sialic acids which are important components of glycoproteins, gangliosides and polysaccharides. Sialic acids exist in these macromolecules as NeuAc or NeuGc and their several mono-, di-, or tri-substituted O-acetyl and/or O-glycolyl derivatives. These multiple forms of sialic acids result in

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Abbreviations : AFL, Anadara granosa foot lectin; PSM, porcine submaxillary mucin; BSM, bovine submaxillary mucin; SSM, sheep submaxillary mucin; NeuAc, N-acetylneuraminic acid; NeuGc, N-glycolylneuraminic acid; 9-OAc-NeuGc, 9-O-acetyl-N-glycolylneuraminic acid; 9-OAc-NeuAc, 9-O-acetyl-N-acetylneuraminic acid; NeuGc α (2 → 6) GalNAc-ol, O-(N-glycolylneuraminyl) α (2 → 6)2-acetamido-2-deoxy-D-galactitol; HCG, human chorionic gonadotropin.

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enormous structural diversities which have some obvious functional significance.

The properly purified sialic acid binding lectins are valuable and potential tools for the detection, quantitation, localization, purification and characterization of sialic acid containing macromolecules. The majority of the sialic acid binding animal lectins have been reported from the invertebrates, quite a few of which are marine. Most of the sialic acid binding lectins bind nonspecifically to NeuAc and its various derivatives (**1-3**) while a few bind more specifically to a particular derivative of NeuAc (**4, 5**). Most of these lectins are also inhibited by NeuGc but the inhibitory potency of this sugar is found to be much less than that of NeuAc. The Pila globosa albumin gland lectin (**6**) shows higher affinity for NeuGc than NeuAc but no report of a lectin which is exclusively specific for NeuGc and NeuGc containing glycoproteins is available. The present communication describes the purification and partial characterization of a unique NeuGc specific lectin from the foot muscles of the marine blood clam Anadara granosa.

Materials and Methods

BSM (**7**), SSM (**7**) and PSM (**8**) were purified from fresh glands. Periodate oxidation of NeuAc was performed according to the methods of Peters et al. (**9**), 9-OAc-NeuGc, 9-OAc-NeuAc, Neuraminic acid and NeuGcα(2 → 6) GalNAc-ol were prepared following the procedures described by Swarnakar et al (**6**). Estimation of bound sialic acid was done through the method of Svennerholm (**10**). All other chemicals, sugars and glycoproteins were obtained from Sigma Chemical Co., U.S.A. Anadara granosa clams were collected from the mangrove dominated Sundarbns, India.

BSM was coupled to CNBr-activated Sepharose 4B (**11**) to produce an affinity matrix. The amount of BSM bound to activated Sepharose 4B was determined by the method of Saifer and Grestenfeld (**12**). The affinity matrix was equilibrated with 50 mM Tris-HCl buffer (pH 7.3) containing 50 mM $CaCl_2$ (buffer A) at 10°C. Foot muscles from 100 live Anadara granosa clams were dissected, homogenized in a blender and extracted with 250 ml of 10 mM ice-cold Tris-HCl buffer (pH 7.3). The extract was then centrifuged at 25000 g for 1 h at 4°C. The clear supernatant was dialysed against buffer A. After estimating the protein and testing the hemagglutinating activity, the dialysed extract was stored at -20°C for the purification of the lectin. About 50 ml of the stored foot extract (Specific activity 34.26 unit/mg) was passed through a Sepharose 4B - BSM affinity column (1.5 x 25 cm), previously equilibrated with buffer A, at 10°C. The column was washed thoroughly with the same buffer at the same temperature. The bound lectin was eluted from the column with 0.05 M Tris-HCl (pH 8.0)/0.15 M NaCl/0.04 M sodium citrate, at room temperature. Fractions of 1.0 ml were collected in test tubes and absorbance was measured at 280 nm. The protein peak was dialysed against buffer A, concentrated and stored for further experiments.

Hemagglutination assay was performed at 25°C in a microtitre system using 2% (v/v) rabbit erythrocyte suspension in saline (**13**). Influence of divalent cations (Ca^{2+}, Mg^{2+}, Mn^{2+}), pH and temperature on hemagglu-

tination were observed following the procedure described earlier (**13**). Inhibition of hemagglutination was performed by incubating lectin (0.84 µg) with serially diluted sugars and glycoprotein in microtitre plate, 2% (v/v) rabbit erythrocytes was then added to the incubated solutions and the minimum amount of sugar and glycoprotein required to inhibit 10 hemagglutination unit was determined.

Protein was estimated by the method of Lowry et al. (**14**) using crystalline BSA (Lot No. 114F-0016 Sigma Chemical Co.) as standard and total carbohydrate was determined (**15**) by using D-Gal and standard. Purified lectin was iodinated with chloramine-T (**16**).

Alkaline polyacrylamide gel electrophoresis of ^{125}I-labelled purified lectin was performed in a 5% gel (**17**). SDS/PAGE was done in a 11% polyacrylamide gel (**18**). Dissociation and reduction of the ^{125}I-labelled purified lectin was carried out through the methods mentioned earlier (**13**). The subunit profile was observed by radioautography. Analytical isoelectric focusing was performed in a LKB slab-gel isoelectric focusing apparatus (**19**).

About 1 ml of ^{125}I-labelled purified lectin was passed through a Sepharose 6B gel filtration column equilibrated with buffer A. Radioactivity of each fraction was checked by a Gamma-ray Spectrometer. The column was calibrated with thyroglobulin, ferritin, catalase and ConA as markers.

Amino acids were analysed with a Pharmacia LKB Alpha Plus amino acid analyser. Samples were hydrolysed by standard acid hydrolysis conditions using 6N HCl at 110°C for 22 h. Protection was done for cysteine, methionine and tyrosine using proper protecting reagents. Tryptophan was determined spectrophotometrically (**20**).

Results

The NeuGc-binding lectin (AFL) from the foot muscle of the bivalve, Anadara granosa was purified in one step through affinity chromatography on a BSM - Sepharose 4B column. The lectin was bound to the affinity column at 10°C in the presence of Ca^{2+}. The Ca^{2+} dependent lectin showed a reduced activity at alkaline pH therefore, citrate buffer (pH 8.0) without Ca^{2+} eluted the bound lectin very effectively. If compared with the crude extract, the specific activity of the purified lectin increased 342 fold with a recovery of 88%. Nearly 1.2 mg of lectin was purified from 540 g of foot muscle (Table I).

Table I. Purification of Anadara granosa foot lectin (AFL)

Fraction	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude foot muscle extract	50	467	16000	34.26	100	1
BSM-Sepharose 4B affinity column eluate	11	1.2	14080	11733	88	342

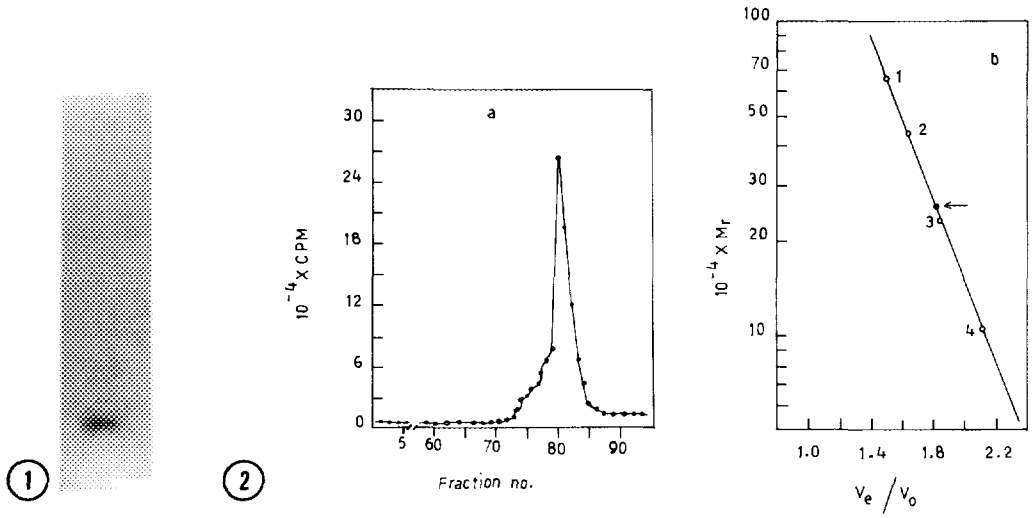

Fig. 1. Polyacrylamide gel electrophoresis of ^{125}I-labelled AFL in 5% gel at pH 8.9.

Fig. 2. Elution profile (a) of ^{125}I-labelled AFL from gel filtration on a calibrated Sepharose 6B column and determination of its native molecular weight (b).

The purified AFL produced a single band on alkaline polyacrylamide gel electrophoresis (Fig. 1). Homogeneity of the purified lectin was confirmed by the single peak obtained from gel filtration (Fig. 2a) and by isoelectric focusing. The lectin was active over a wide range of temperature and its optimum range of pH was 7.1 to 7.4. Unlike Ca^{2+}, Mg^{2+} and Mn^{2+} reduced the titer value drastically.

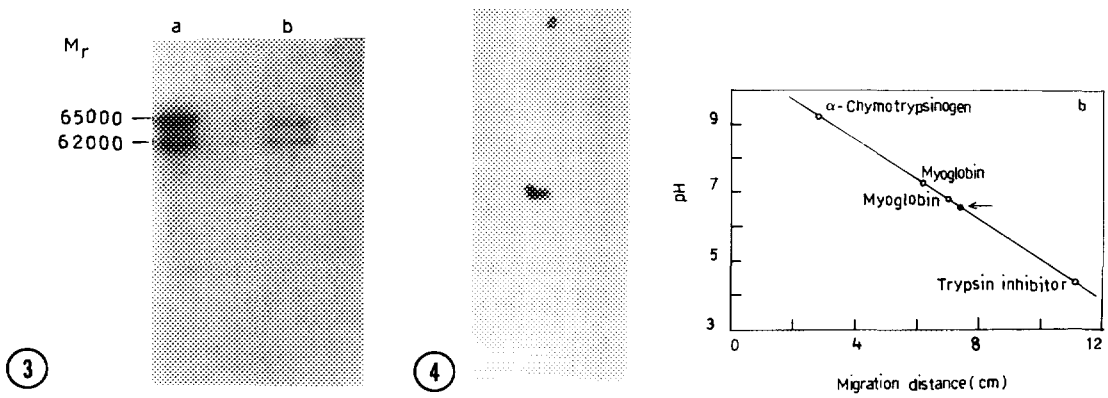

Fig. 3. Subunit profile of AFL. Radioautography of 11% SDS/PAGE of ^{125}I-labelled affinity purified (a) and gel filtrated (b) AFL.

Fig. 4. Determination of the pI of AFL by isoelectric focusing. **(a)** The purified lectin showed a single band at pH 6.65. **(b)** The arrow indicates the position of AFL.

Table II. Amino acid composition of purified AFL

	Composition (no. of residues/mol)
Aspartic acid	25
Threonine	18
Serine	34
Glutamic acid	35
Proline	10
Glycine	23
Alanine	17
Cysteine	3
Valine	10
Methionine	4
Isoleucine	7
Leucine	13
Tyrosine	6
Phenylalanine	12
Histidine	6
Lysine	8
Tryptophan*	14
Arginine	9

*Determined by a spectrophotometric method (20).

The native molecular weight of AFL was found to be 254 kDa (Fig. 2b) from the gel filtration on a calibrated Sepharose 6B column. On SDS/PAGE, the purified lectin resolved into two subunits of 65 kDa and 62 kDa. The gel filtrated lectin revealed similar subunit profile (Fig. 3). On isoelectric focusing the lectin showed a single band at pH 6.65 (Fig. 4). Analysis of amino acid composition of AFL revealed the predominance of aspartic acid, glutamic acid, glycine and serine (Table II).

The results of inhibition studies are presented in the Table III. The most potent inhibitor among the monosaccharides was NeuGc. Neuraminic acid was 11 times less effective than NeuGc. The lectin did not recognize 9-OAc-NeuGc, NeuAc, 9-OAc-NeuAc, periodate treated NeuAc, N-acetyl muraminic acid, colominic acid, glycolic acid and pyruvic acid. *NeuGc α (2 → 6) GalNAc-ol was 3 times more effective than NeuGc but the* best inhibitor was PSM. BSM and fetuin was 22 and 237 times less effective, respectively, than PSM.

Discussion

The NeuGc-specific tissue lectin purified from the foot muscles of Anadara granosa is a tetrameric protein (native M_r 254 kDa). The molecular weight of two subunit is 65 kDa each and that of the

Table III. Inhibition of hemagglutinating activity of AFL by various sugars and glycoprotein

Inhibitors	Concentration (mM) for complete inhibition**
NeuAc	-**
NeuGc	0.38
Neuraminic acid	4.18
Pyruvic acid	-**
Glycolic acid	-**
9-OAc-NeuGc	-**
9-OAc-NeuAc	-**
N-acetyl muraminic acid	-**
Colominic acid	-**
D-Galactose	-*
N-Acetyl-D-galactosamine	-*
D-Glucose	-*
Mannose	-*
Lactose	-*
NeuGcα(2 → 6) GalNAc-ol	0.12
Heparin	-**
PSM	0.0009
BSM	0.02
SSM	-**
Fetuin	0.214
α_1-acid glycoprotein	-**
HCG	-**

**Concentrations are expressed in term of sialic acid content.
'—' non-inhibitory upto *, 100 mM; **, 20 mg/ml.

Acid sugars were neutralized before use.

remaining two is 62 kDa each. Hemagglutination inhibition studies reveal the unique binding property of AFL. The only monosaccharide which is recognized by the combining site of AFL, is NeuGc. The lectin does not interact with glycolic acid indicating that mere presence of a glycolyl group in NeuGc is not enough to make it an inhibitor. Neuraminic acid is a much weaker inhibitor than NeuGc; but when the C-5 of neuraminic acid is substituted with an N-acetyl group, its inhibitory potency is totally lost, even colominic acid is not recognized by the binding site. The few other derivatives of NeuGc and NeuAc tested viz., 9-OAc-NeuGc, periodate-treated NeuAc, 9-OAc-NeuAc, fail to inhibit the activity of AFL. All these indicate a strict specificity of the lectin for NeuGc. This extremely selective affinity is further proved from its interactions with some sialoglycoproteins. The most potent inhibitor of AFL is PSM which contains exclusively NeuGc as its sialic acid (**21, 22**). The sialic acids of BSM and fetuin include both NeuAc and NeuGc (**23, 24**). The inhibitory potency of BSM is much less than PSM but considerably higher

than fetuin. This difference in potency is probably due to the amount of NeuGc present in the said sialoglycoproteins. The disaccharide, NeuGc α (2 → 6) GalNAc-ol, is readily recognized by the combining site and this observation is quite compatible as both PSM and BSM contain this disaccharide as their major carbohydrate chain (**25**, **26**). The binding site of AFL does not interact also with NeuAc present in the macromolecular structure of mucin. SSM, which contains only NeuAc (**27**, **28**), can not inhibit the lectin activity even at a much higher concentration than PSM. Other NeuAc containing sialoglycoproteins viz., HCG, α_1-acid glycoprotein and ovalbumin, show non-inhibitory effect. If taken together, all these observations suggest that AFL is strictly specific for NeuGc, specially when it is present in the macromolecular structure of mucin.

This is probably the first report of a NeuGc-specific lectin which does not recognize either free or bound NeuAc. After further characterization of the binding site by using different derivatives of NeuGc and NeuGc containing oligosaccharides, AFL may be proved as an important tool in the study of sialoglycoconjugates.

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