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N-glycolylneuraminic acid specific lectin from Pila globosa snail

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Summary : A N-glycolylneuraminic acid-specific lectin (PAL) has been purified from an albumin gland extract of the apple snail, Pila globosa. Purification is conducted on a bovine submaxillary mucin-Sepharose 4B affinity matrix followed by gel filtration on a Sepharose 6B column. The lectin agglutinates rabbit erythrocytes. The hemagglutination activity is dependent on Ca^{2+} concentration in a significant manner but with a remarkable behaviour. The lectin is a trimeric glycoprotein of native Mr 440 kDa with 25% carbohydrate and is composed of three nonidentical subunits of molecular weights 190, 145, and 105 kDa. It has a pI of 7.0. The lectin exhibits a unique and strict specificity toward N-glycolylneuraminic acid and this phenomenon discriminates it from other known sialic acid binding lectins. The uniqueness indicates the absolute need for a glycolyl substitution on the amino residue and of a glyceryl side chain on the exocyclic part and an axial -COOH group in neuraminic acid. The presence of an acetyl substitution on the exocyclic part impedes lectin-ligand interaction.

Gastropods are rich sources of lectins which are present primarily in the albumin gland of the snail (**1**). Lectins are multivalent carbohydrate binding proteins or glycoproteins having the ability to bind specifically to cell surface carbohydrates and/or to precipitate glycoconjugates and polysaccharides (**2**). They have numerous applications as specific probes to investigate the role of cell surface carbohydrates during development, differentiation, and malignant transformation. They are also used to study the distribution, localization, and purification of glycoconjugates (**3**).

In spite of the ubiquitous presence of lectins in nature, few have been characterized as specific for sialic acid (**4-10**). However, most of

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Abbreviations : PAL, Pila globosa albumin gland lectin; BSM, bovine sub maxillary mucin; HA, hemagglutination assay; NeuAc, N-acetylneuraminic acid; NeuGc, N-glycolylneuraminic acid; 9-OAc-NeuGc, 9-O-acetyl-N-glycolylneuraminic acid; NeuGc α (2 ⟶ 6) GalNAc-ol, O-(N-glycolylneuraminyl) α (2 ⟶ 6)2-acetamido-2-deoxy-D-galactitol; D-GlcNAc, N-acetyl-D-glucosamine; D-GalNAc, N-acetyl-D-galactosamine; D-ManNAc, N-acetyl-D-mannosamine.

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the sialic acid binding lectins so far reported attach nonspecifically to various derivatives of sialic acid, strictly speaking to derivatives of NeuAc (**5-8**).

Sialic acid describes several derivatives of neuraminic acid. In biological systems the sialic acid in the parent form always exists as NeuAc or NeuGc. In addition, there are usually varieties of the mono-, di-, or tri-substituted O-acetyl and/or O-glycolyl derivatives of the parent molecule. These derivatives exhibit interesting species- and tissue-specific distribution and may change during transformation of cells (**12**). Thus sialic acid-specific lectin has great importance in determining the biological significance of sialoglycoconjugates. The present communication deals with the purification and partial characterization of a NeuGc-specific lectin from the albumin gland of the snail Pila globosa.

Materials and Methods

Pila globosa snails were purchased from a local market. All chemicals were obtained from Sigma Chemical Co., U.S.A. 9-OAc-NeuGc was prepared from NeuGc (unpublished data). Neuraminic acid was prepared through de-N-glycolylation of NeuGc with 0.5 N HCl at room temperature for 2 h. The excess acid was removed and the product was confirmed by NMR spectroscopy. Periodate-treated NeuGc was prepared by treating NeuGc with 10 mM $NaIO_4$ at 25°C for 24 h in the dark (**13**). The methyl ester-2-O-methyl- β-glycoside of NeuGc was prepared by refluxing NeuGc with methanol in the presence of amberlite IR-120 (H^+) (**14**). The compound produced was then identified through IR spectroscopy. The 2-O-methyl-β-glycoside of NeuGc was prepared by treating methylester-2-O-methyl-β-glycoside of NeuGc with 0.1 N NaOH at 25°C for 2 h (**15**). The disaccharide NeuGc α (2→6) GalNAc-ol was prepared from BSM (**11**) followed by thin-layer chromatographic separation on silica-coated plates using ethanol: butanol:pyridine:water:acetic acid, 100:10:10:30:3 (v/v/v/v/v), as solvent (**16**). Normal rabbit erythrocytes were desialylated according to the method of Stockert et al. (**17**). Purified lectin was iodinated with chloramine-T(**18**).

Protein was determined according to the method of Lowry et al. (**19**) using crystalline BSA Lot No.114F-0016 as standard, and total carbohydrate was determined by the phenol-H_2SO_4 method (**20**) using D-Gal as standard.

An affinity matrix was prepared by coupling BSM with CNBr-activated Sepharose 4B according to the method of Cuatrecasas (**21**). The amount of BSM coupled was found to be 1.1 mg BSM/ml Sepharose 4B. The immobilised BSM content was measured by estimating bound sialic acid (**22**). After coupling, the gel was equilibrated with 50 mM Tris-HCl, pH 7.1 (TB), at 10°C. The albumin glands (320 g) from 380 P. globosa snails were dissected, homogenized in a blender, and extracted with 500 ml at 25°C. The extract was centrifuged at 12,000 g for 30 min. Supernatant (450 ml) was centrifuged at 85,000 g for 1.5 h. The clear yellowish-white layer (400 ml) was dialysed against TB. This dialysed sample (400 ml) containing 17 mg/ml protein was tested for agglutinating activity. It was stored at -20°C and later used for purification of the lectin. Before use, this dialysed extract was thawed and passed through a Sepharose 4B-BSM column (1.5 x 30 cm) previously equilibrated with TB at 10°C. After extensive washing (until the A_{280} of effluent came to <0.005), the lectin, bound to the column, was eluted using 75 mM Tris-HCl buffer, pH 8.0, containing 15 mM Ca^{2+} (TBC). Specific fractions eluted from the column

were assayed for absorption at 280 nm. The protein peak obtained by specific elution was dialysed against TB and concentrated. The concentrated eluate was passed through a Sepharose 6B column (1.5 x 90 cm) equili brated with TB for further purification. Fractions (1.1 ml) were collected and monitored by measurement of absorbance at 280 nm and by HA with rabbit erythrocytes.

Hemagglutination was performed with a microtiter plate at 25°C. Samples were serially diluted with saline and mixed with 25 µl of 2% rabbit erythrocyte suspension in saline, incubated for 45 min and scored. The hemagglutination titer was calculated as the reciprocal of the highest dilution of lectin that produced visible agglutination. The hemagglutinating activity of the lectin was also tested with neuraminidase-treated rabbit erythrocytes. Temperature sensitivity of the lectin was evaluated by perfor ming hemagglutination at temperatures ranging from 4 to 37°C. The effect of metal ions on HA was checked at 25°C by adding in each case different concentrations (0.5 - 10 mM range) of $CaCl_2$, $MgCl_2$, and $MnCl_2$. The effect of pH on HA was checked at 25°C by testing the activity of purified lectin in buffers of pH 5.0-10.0. The inhibitory potency of different saccha rides was determined by adding known concentrations of sugar in serial dilution to microtiter plates. The minimum concentration of sugar necessary to inhibit one hemagglutination unit of lectin was recorded.

Alkaline polyacrylamide gel electrophoresis of purified lectin was carried out in a 5% gel (**23**). SDS/PAGE was performed in a 6.5% polyacrylamide gel by the method of Laemmli (**24**). Dissociation and reduc tion of the ^{125}I-labelled purified protein were done by heating for 10 min at 100°C in sample buffers containing 3% SDS, 8 M urea and with or without 2% β-mercaptoethanol. After completion of electrophoresis, the gel was divided into two parts. One part was stained for Coomassie blue and the rest of the gel was used for radioautography. Analytical isoelectric focusing was performed in a LKB slab gel isoelectric-focusing apparatus (**25**). After isoelectric focusing the pI of the purified protein was revealed by radioautography.

About 2 mg of protein samples containing lectin activity was subjec ted to gel filtration on a column (1.5 x 90 cm) of Sepharose 6B equili brated with 50 mM Tris-HCl buffer, pH 7.1, at 25°C. Fractions of 1.1 ml were collected and were assayed for activity by the hemagglutination assay procedure. The column was calibrated with thyroglobulin, ferritin, catalase, and Con A as marker.

Antisera against crude extract were prepared in rabbit as described by Kabat et al. (**26**). The sera were tested by immunodiffusion according to Ouchterlony (**27**) and by immunoelectrophoresis (**28**).

Results

The NeuGc-specific lectin from the albumin gland of the snail P. globosa was purified in two steps : affinity chromatography and gel filtration. The specific activity of purified lectin was increased approxi mately 1600-fold compared to that of the crude extract (Table I), with a recovery of 4% and a yield of 0.34 mg of purified lectin from 320 g of snail albumin gland. The total carbohydrate content of PAL was 25% (Table I). The lectin was bound to the BSM-Sepharose 4B affinity matrix in the cold (10°C) in the absence of Ca^{2+}. It was eluted at room tempera ture with TBC buffer of pH 8.0 containing 15 mM $CaCl_2$ (taking advantage of the reduced activity of crude lectin at alkaline pH and in the presence

Table 1. Purification of NeuGc-specific lectin from the albumin gland of Pila globosa snails

Fractions	Total volume (ml)	Total protein (mg)	Total carbohydrate (mg)	Total activity (units)	Specific activity (units/mg protein)	Purification (fold)
Crude albumin gland extract	40	680	-	32000	47.058	1
BSM-Sepharose 4B affinity column eluate	3.8	1.368	-	19456	14222	302.2
Sepharose 6 B eluate (peak I)*	3.8	0.034	0.008	2560	75294	1600

* The fractions 63 and 64 showing high hemagglutinating activity indicated by the peak 1 bar on fig.1 were pooled, concentrated, and checked for activity and protein content.

of Ca^{2+}). The affinity-isolated active material was further purified by gel filtration on a Sepharose 6B column which yielded two peaks (Fig.1), peak 1 of which was active.

The purified albumin gland lectin showed a single band on poly acrylamide gel electrophoresis at alkaline pH (Fig.2). The lectin was also tested for homogeneity by immunodiffusion and immunoelectrophoresis with rabbit antibody to crude lectin as shown in Fig.3a and Fig.3b, respectively. It gave a single band on the Ouchterlony plate and moved as a single band in immunoelectrophoresis. On gel filtration, with a calibrated column of

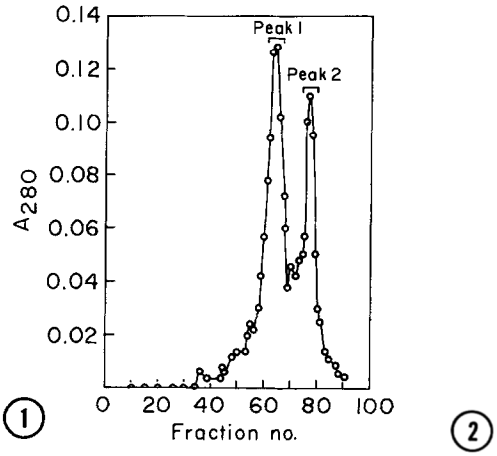


Fig.1. Purification of affinity-isolated lectin by gel filtration on a Sepharose 6B column. Fractions collected were assayed for A_{280} (-O-).

Fig.2. Polyacrylamide gel electrophoresis of PAL at pH 8.3 in 5% gel. Mobility is from top to bottom.

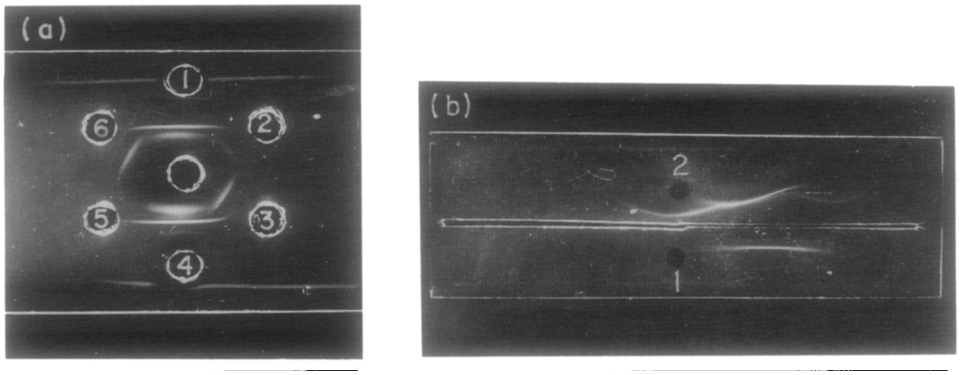


Fig.3. (a) Immunodiffusion and (b) immunoelectrophoretic pattern of PAL.
(a) Immunodiffusion. The centre well contained rabbit antiserum to crude extract of the albumin gland. Wells 1 and 4, Crude extract; Wells 2, 3, and 6, PAL ; Well 5, TB.
(b) Immunoelectrophoresis. Well 1, PAL; Well 2, Crude extract.

Sepharose 6B, the native molecular weight of PAL was found to be 440 kDa (Fig.4). The activity of lectin was maximal near 25°C and in the pH range 6-7. The activity remained unchanged on addition of Mn^{2+} and/or Mg^{2+}, diminished appreciably in the presence of Ca^{2+} (Table II), and attained a saturation limit at 10 mM Ca^{2+} ion. In order to investigate the subunit pattern of native protein, ^{125}I-labelled purified lectin was resolved in SDS/PAGE and autoradiographed. On denaturing with 3% SDS and 8 M urea followed by boiling for 10 min with or without β-mercaptoethanol, the purified lectin dissociated into subunits of mol. wt. 190, 145, and 105 kDa (Fig.5). An autoradiogram from isoelectric focusing on a polyacrylamide gel of ^{125}I-labelled purified lectin also showed a single band at pH 7.0 (Fig.6). All the hemagglutination tests were carried out with rabbit erythro

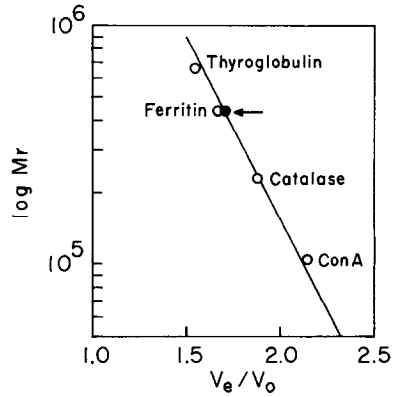


Fig.4. Determination of native molecular weight of PAL by gel filtration on a Sepharose 6B column equilibrated with TB. The arrow indicates the position of PAL.

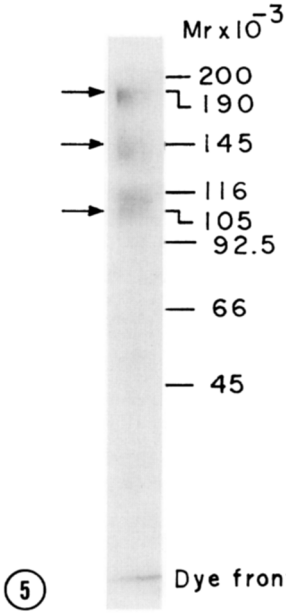
Table II. Effect of Ca^{2+} on the hemagglutinating activity of PAL

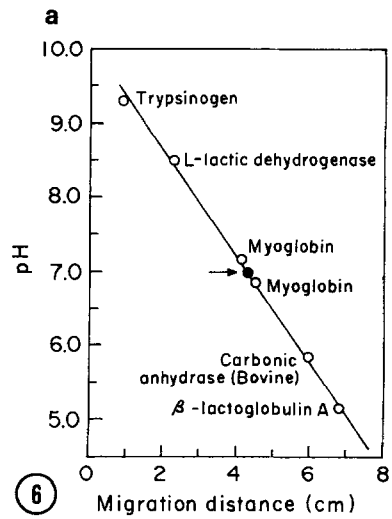
Protein (mg/ml)	Hemagglutination (units/ml)	Concentration of Ca^{2+} (mM)	Specific activity (units/mg)
0.027	320	0	11851
0.027	160	0.5	5926
0.027	36	1.5	1333
0.027	20	3.5	740
0.027	8	6.0	296
0.027	2	8.0	74
0.027	2	10.0	74

The protein concentration was 0.027 mg/ml, pH 7.0, temperature 25°C.

cytes. The hemagglutinating activity of PAL was abolished when assayed by neuraminidase-treated rabbit erythrocytes.

The studies on the inhibition of hemagglutination of purified lectin using various sugars are presented in Table III. Among the monosaccharides, the most effective inhibitor was NeuGc, whereas NeuAc was found to be





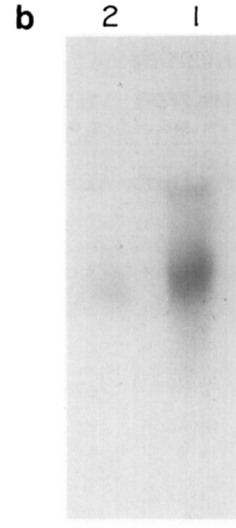


Fig.5. Subunit molecular weight determination of PAL. Radioautography of 6.5% SDS/PAGE of ^{125}I-labelled PAL.

Fig.6. Determination of the pI of PAL by isoelectric focusing in a pH gradient of 3-10. (a) The arrow indicates the position of PAL (pI 7.0). (b) The radioautography of isoelectric focusing.
1. ^{125}I-labelled affinity-isolated lectin.
2. ^{125}I-labelled gel filtration-isolated lectin, i.e., PAL.

Table III. Inhibition by various sugars of hemagglutinating activity of purified lectin using 2% rabbit erythrocytes

Sugars	Minimum concentration required for complete inhibition of one hemagglutination unit (nmoles)*
Neuraminic acid	51.93
NeuAc	202.07
NeuGc	23.9
Glycolic acid	1520
Periodate-treated NeuGc	1040
Pyruvic acid	710.23
NeuGc α (2→6)GalNAc-ol	7.613
2-O-Methyl-β-glycoside of NeuGc	91.83
Methylester-2-O-methyl-β-glycoside of NeuGc	202.86
9-OAc-NeuGc	475.12

*D-Glc, D-Man, D-Gal, D-Fuc, L-Fuc, D-GlcNAc, D-GalNAc, D-ManNAc, D-glucuronic acid, D-galacturonic acid were noninhibitory up to 250 nmoles. Acid sugars were neutralised before use.

almost noninhibitory. The NeuGc was approximately four times more effec tive than the 2-O-methyl-β-glycoside of NeuGc and was three times less effective than α-glycosidically linked NeuGc, i.e., NeuGc α(2→6) GalNAc-ol as revealed by HA inhibition. The inhibitory potency of 2-O-methyl-β-glycoside NeuGc was much greater than that of its esterified derivative. The C7 analogs of NeuGc and 9-OAc-NeuGc were found to be noninhibitory. Neuraminic acid was one half as potent as NeuGc. Purified lectin was not inhibited by glycolic acid and pyruvic acid.

Discussion

This report describes the purification and partial characterization of a NeuGc-specific lectin from the albumin gland of the snail P. globosa. The purified lectin is a trimeric glycoprotein of M_r 440 kDa consisting of three nonidentical subunits of M_r 190, 145, and 105 kDa. The binding site of PAL recognises neuraminic acid as the only potent monosaccharide, and binding occurs with greater specificity only if the C-5 of neuraminic acid is substituted with an N-glycolyl group. But substitution by an N-acetyl group at C-5 of neuraminic acid requires a much higher concentration for inhibition of lectin-induced hemagglutination. Moreover, the mere pre sence of a glycolyl group is neither sufficient nor necessary for lectin ligand interaction as evidenced by inhibition results of glycolic acid. Thus, it may be concluded that the lectin is specific for NeuGc. This novel specificity of the lectin for NeuGc can be confirmed by compairing

it with other lectins which recognise both NeuAc and NeuGc. The inhibitory potencies of NeuGc over NeuAc of CSN (9), $Achatinin_H$ (29), limulin (11), LFA (8), crab lectin (5), and scorpion lectin (4) are reported to be 2.0, 1.3, 1.0, 0.17, <1.0, and 0.5 times, respectively; on the other hand the PAL shows an 8.5 times greater affinity toward NeuGc with respect to NeuAc (Table III). The exocyclic glyceryl side chain of neuraminic acid is indispensable for recognition by lectin because periodate-treated NeuGc is found to be a noninhibitor (Table III). This characteristic of PAL is in contrast to the characteristics of the well-known lectin limulin, in which the glyceryl chain of N-acyl neuraminic acid plays no role in binding (11), and is somewhat similar to those of SNA, which has a strict requirement for the glyceryl side chain of NeuAc (7). The absence of the O-Ac group at the exocyclic part of NeuGc adds a new dimension to our thinking on the structure of the lectin receptor. If there is an O-Ac substitution at the exocyclic part, then the accessibility of sugar faces steric hindrance in binding with lectin (Table III), whereas in the case of $Achatinin_H$ (29) and Cancer antenarrius lectin (5), the O-Ac residue at C-9 facilitates interaction. Thus, the distinguishing feature of PAL is easily perceived. The next questions involved the need for anomeric speci ficity and the requirement for a properly oriented -COOH group at C-1. To evaluate this, we used NeuGc α (2$\rightarrow$6) GalNAc-ol, the methylester-2-O-methyl-β-glycoside of NeuGc, and its deesterified derivative. It must be noted that the methylester-2-O-methyl-β-glycoside of NeuGc is not availa ble to us. The activity of PAL decreases extremely in the presence of NeuGc α (2$\rightarrow$6)GalNAc-ol, whereas the 2-O-methyl-β-glycoside of NeuGc is a poor inhibitor compared to free NeuGc. This result corroborates that the α-anomer of NeuGc is important for binding. The methylester-2-O-methyl β-glycoside of NeuGc is inactive like pyruvic acid, but the deesterified 2-O-methyl-β-glycoside of NeuGc is moderately active, suggesting that the presence of a -COOH group is needed, specifically unlike limulin (11) and carcinoscorpin (9) which also bind D-glucuronic acid because of the mere presence of a -COOH group. It is evident that the presence of a -COOH group at a particular and preferred conformational orientation is required for correct fitting with the binding site of PAL. The contribution of the -OH group at C-4 is yet to be determined.

So far as our knowledge extends, this is the first report of a lectin specific for NeuGc. Affinity binding of the lectin will provide an important tool for easy isolation of free and conjugated NeuGc, e.g., H-D antigen (30), from a mixture of NeuAc and NeuAc containing sialoglycoconju gates. Various sialoglycoconjugates act in diverse ways, e.g., differentiation antigens, determinants of receptor function, determinants of transmembrane signalling, and sometimes acting to mask antigenicity (31). Thus, this

lectin can share in part of the biochemical work done by sialic acid-specific monoclonal antibodies, which are expensive and scarce.

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