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RESEARCH REPORT

Physico-chemical characterization of a natural agglutinin from the hemolymph of a millipede *Thyropygus descriptus*

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Abstract

Natural hemagglutinins with specific affinity for the glycocalyx of rabbit erythrocytes is identified in the hemolymph of the millipedes, *Thyropygus descriptus*, *Xenobolus acuticonus*, *Arthrosphaera disticta* and *A. craspedota*. Of the tested species, maximum hemagglutinability is observed in the hemolymph of the millipede *Thyropygus descriptus.* Further characterization of the hemolymph agglutinin of *Thyropygus descriptus* showed optimum agglutinability at pH 6.5 and temperatures 30 - 35 ºC. Starvation up to 20 days had no influence on the hemagglutinability of the hemolymph. Agglutinability was impervious by change in diet, and inclusion of diverse concentrations of cations or chelators in the buffer. The agglutinin, though agglutinates rabbit, rat and human A erythrocytes, when pre-adsorbed with erythrocytes of a particular species, loses its ability to agglutinate erythrocytes of any species suggesting the presence of a single agglutinin in the hemolymph. In general, the agglutinating activity of the agglutinin is inhibited by the glycoproteins porcine stomach mucin, lactoferrin, bovine submaxillary mucin, transferrin, fetuin and the sugars N-acetyl galactosamine, N-acetyl lactosamine, lactose, galactose and N-acetyl neuraminic acid. The sialic acid specificity of the agglutinin is revealed by the reduction in hemagglutination activity when treated with the desialylated rabbit erythrocytes.

Key words: millipede; *Thyropygus descriptus;* agglutinin; hemolymph; erythrocytes; hemagglutination assay; hemagglutination inhibition assay

Introduction

Lectins, the sugar binding proteins are found in a wide range of organisms including viruses, bacteria, fungi, plants and animals (Sharon, 2008). They react with sugars in glycolipids, glycoproteins, or oligosaccharides and agglutinate erythrocytes via cell surface glycoproteins and glycolipids (Stromberg *et al.,*1991; Sharon, 2008). Their specificity is usually defined in terms of a monosaccharide(s) or simple oligosaccharides that inhibit lectin-induced agglutination (Sharon and Lis, 1972; Goldstein *et al*.,1980). An agglutinin may recognize a part of a sugar (Shimizu *et al*.,1977), a whole sugar (Bretting and Kabat, 1976), their glycosidic linkages (Koch *et al*., 1982) or a sequence of sugars (Kobiler and Mirelman, 1980; Mauchamp, 1982).

Among protostomian invertebrates that are incapable of synthesizing sialic acids, molluscan and arthropodan agglutinins recognize a unique kind of sugar called sialic acids (Mullainadhan *et al*., 1984). The type of sialic acids and the glycosidic linkages with adjacent sugar in an oligosaccharide differ among pathogenic bacteria (Ravindranath and Cooper, 1984) and human cancer (Ravindranath *et al*.,1985b; Higashi *et al*., 1985; Kawai *et al*., 1991). Therefore, lectins specifically recognizing various sialic acids and their carbohydrate binding patterns can be used as tools for identifying various sialyl epitopes on pathogens or in biopsy of malignant tumors. A search for such sialic acid specific lectin is made in a millipede, *Thyropygus descriptus,* a Diplopodan representative of the Super Class Myriapoda, a new group for lectin study. To develop strategies for affinity purification we have studied the physico-chemical properties of the agglutinin.

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Material and Methods

Materials

Millipedes *Thyropygus descriptus*, *Xenobolus acuticonus*, *Arthrosphaera disticta* and *A. craspedota* used in this investigation were collected from the forest region of Kanyakumari (Anayadi and

Table 1 Survey of hemagglutinins in the hemolymph of millipedes

Erythrocytes	HA titer			
	T. descriptus (n = 25)	*X. acuticonus* (n = 25)	*A. disticta* (n = 25)	*A. craspedota* (n = 25)
Rabbit	2048	32	512	1024
Rat	128	0	256	128
Human A	64	0	512	128
Human B	0	0	512	128
Human O	0	0	32	16
Ox	0	0	0	0
Horse	0	0	0	0
Pig	0	0	0	0
Mouse	0	0	0	0

Kodayar) and Tirunelveli (Kalacaud and Thalayanai) Districts, Tamil Nadu, India.

Hemolymph collection

The arthropodial membrane in between the collum and the adjacent segment was punctured after cleaning the area with wet cotton. The exuding hemolymph was collected in 15 ml polypropylene tubes kept on ice and stored in refrigerator.

Erythrocyte collection

Blood from different mammals was collected by venipuncture of the ear (rabbit), fore arm (Human A, B, O and horse), cardiac puncture (mouse and rat) and from the slaughter house (ox and pig) directly in modified Alsevier's medium (pH 6.1) containing sodium citrate (30 mM), sodium chloride (77 mM), glucose (114 mM), neomycin sulphate (100 μg/ml) and chloramphenicol (330 μg/ml) at a ratio of 2:8. Erythrocytes were suspended and washed three times by centrifugation at 4,000*g* for 5 min with ten volumes of Tris-Buffered Saline (TBS) pH 6.5 (Tris-HCl 50 mM, NaCl 100 mM, $CaCl_2$ 10 mM) and resuspended in the same as 1.5 % suspension.

Hemagglutination (HA) Assay

The HA activity of the hemolymph agglutinin was assayed by measuring its ability to agglutinate erythrocytes. HA assays were performed at 30 °C by serial dilution of the hemolymph (25 µl) with TBS (25 µl) and mixing with 25 µl of 1.5 % erythrocyte suspension. HA titer was determined by the visual estimation of erythrocyte agglutination on microtiter plates 60 min after adding the cells. The HA titer (the units of agglutinin activity) is the reciprocal of the highest dilution of the sample that gave agglutination. To develop strategies for affinity purification, HA assay was also performed with high agglutinating rabbit erythrocytes at different pH, temperature and using buffer with different concentrations of cations calcium, magnesium and manganese and chelators, EDTA and EGTA.

Table 2 Effect of moulting on the biochemical factors and HA titer of the hemolymph of the millipede of *Thyropygus descriptus*

Parameters analysed	Premoult (n = 15)	Freshmoult (n = 10)	Postmoult (n = 10)	Intermoult (n = 25)
Volume (ml/animal)	2.568±1.229	2.967±1.09	2.78±1. 922	2. 65±1.27
Water content (mg%)	96.887±1.1	98.35±1.218	97.232±1. 29	97.335±1.209
Calcium (mM)	12.983±0.045	13.101±0.321	12.503±0.245	12.801±0.504
Protein (µg/25µl)	0.430±0.031	0.432±0. 5	0.441±0.053	0.461±0.035
HA Titer	1024	4096	2048	2048

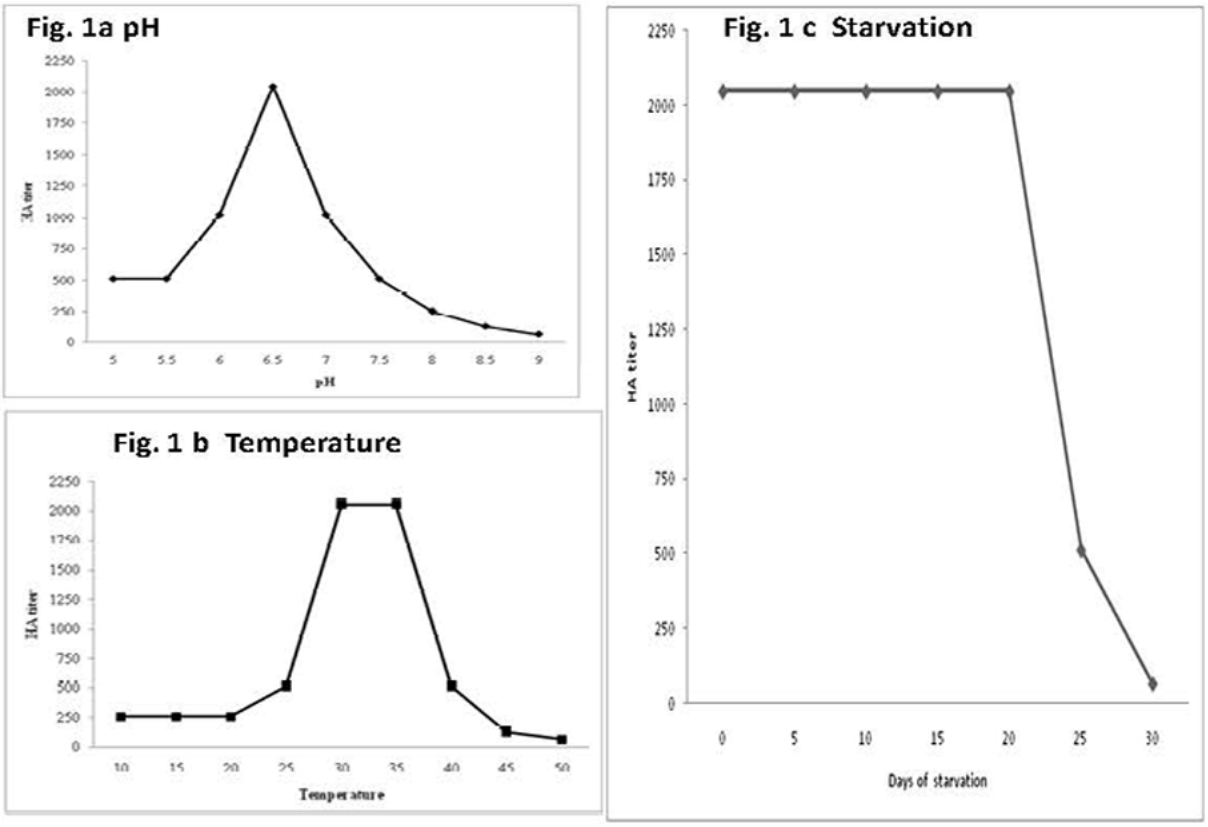

Fig. 1 Effect of pH, temperature and starvation on hemagglutination assay of the hemolymph agglutinin of the millipede *Thyropygus descriptus*

To study the effect of pH on HA titer, the hemolymph sample was mixed with TBS at specific pH (5 - 9) at 1:1 ratio and incubated for 1 h and serially diluted in microtiter plates having TBS of same pH before adding erythrocyte suspension.

To study the effect of temperature on HA titer, the hemolymph sample was incubated for 1 h at specific temperature (10 - 50 °C) and used for HA assay.

To study the effect of cations and chelators on HA titer, the hemolymph sample incubated for 1 h in equal volume of TBS containing specific concentration (0.01,0.1,1.0 and 10 mM) of cations (calcium, magnesium and manganese) and chelators (EDTA and EGTA) was used for HA assay.

In addition, HA was also carried out in the hemolymph obtained from animals fed with different foods, subjected to starvation and various stages of moult cycle after determining the volume, protein, calcium and water content of the hemolymph.

Cross-adsorption test

Packed erythrocytes (rabbit/rat/human A) were prepared by repeated washing of erythrocytes in 0.9 % saline by centrifugation at 4,000*g* for 5 min until we get a clear pellet. Hemolymph was mixed with equal volume of packed rabbit/rat/human A erythrocytes and incubated for 18 h at 4 °C with occasional shaking. After centrifugation, the supernatant was analyzed for HA.

Hemagglutination inhibition (HAI) assay

Hemolymph (25 µl) diluted to sub agglutination concentration (dilution at which hemolymph was able to provide 2 wells HA) was added to each well containing 25 µl of known concentration of serially diluted inhibitors (glycoproteins, mono and oligosaccharides). After incubation for 1 h, 25 µl of 1.5 % rabbit erythrocyte suspension was added. The HAI titer is reported as the reciprocal of the highest dilution of inhibitors giving complete inhibition of agglutination after 60 min.

Protease treatment

Rabbit erythrocytes washed with TBS by centrifugation at 4,000*g* were mixed with equal volumes of mg/ml of trypsin and chymotrypsin and 0.25 mg/ml of pronase and incubated for 4 h at 37 °C. The treated cells were pelleted by low speed centrifugation in TBS-BSA and used for HA assay.

Sialidase treatment

A reaction mixture containing 10 % washed rabbit erythrocytes in TBS-BSA (pH 6.5) and 140 mU sialidase of *C. perfringens* (Sigma-Type X) was incubated at 37 °C for 4 h. Sialidase treated cells were washed with TBS-BSA three times, pelleted by low speed centrifugation and used for HA assay.

Results

HA activity of hemolymph

The hemolymph of all the millipedes *T. descriptus*, *X. acuticonus*, *A. disticta* and *A. craspedota* agglutinated erythrocytes with diverse specificity. All the tested species agglutinated rabbit erythrocytes much higher than the other red blood cells tested (Table 1). Among the species assayed, maximum agglutinability was observed in the hemolymph of the millipede *T. descriptus.* Hence further characterizations were restricted to the hemolymph of the millipede, *T. descriptus.*

Hemolymph had maximum agglutinin activity at pH 6.5 (Fig.1a) and temperature 30 - 35 °C (Fig. 1b).

Calcium, magnesium, manganese, EDTA and EGTA at all the concentrations (0.01, 0.1, 1.0, 10.0 mM) did not have any influence on HA.

Maximum HA was observed in the freshmoult stages than in the pre and post and intermoult stages (Table 2).

Although varieties of food fed to the animals did not alter the HA, prolonged starvation significantly reduced the HA of the hemolymph (Fig. 1c).

HA activity after adsorption with different erythrocytes

When *T. descriptus* hemolymph adsorbed to rabbit, rat and human A erythrocytes was used for HA assay with rabbit, rat and human A erythrocytes, it failed to agglutinate the erythrocytes of any other species except rabbit erythrocytes (Table 3).

Table 3 Cross adsorption assay of the hemolymph agglutinin of the millipede *Thyropygus descriptus*

Erythrocyte adsorbed (n = 10)	HA Titer		
	Rabbit	Rat	Human A
None	2048	128	64
Rabbit	0	0	0
Human A	16 (0)	0	0
Rat	16 (0)	0	0

Values in parenthesis refer to HA titers after second and subsequent adsorptions.

Inhibitors of HA

N-acetyl lactosamine (LacNAc), N-acetyl galactosamine (GalNAc) and lactose effectively inhibited the hemagglutinating activity. Though sialidase treatment reduced the agglutinability, free sialic acid was a weak inhibitor of the hemolymph agglutinin. Among glycoproteins, porcine stomach mucin (PSM) and lactoferrin inhibited the hemolymph agglutinin strongly (Tables 4).

Table 4 Hemagglutination inhibition (HAI) of the hemolymph agglutinin of the millipede *Thyropygus descriptus*

Inhibition by sugars			
Inhibitors (n = 5)	HAI titer	Min. con. req. for HAI (mM)	Inhibitory potency (%)
N-acetyl lactosamine	64	1.062	100
N-acetyl galactosamine	64	1.062	100
Lactose	64	1.062	100
Galactose	16	6.25	25
N-acetyl Neuraminic acid	8	12.5	12.5
Inhibition by glycoproteins			
Inhibitors	HAI titer	Min. con. req. for HAI (µg/ml)	Inhibitory potency (%)
Porcine stomach mucin	2048	4.88	100
Lactoferrin	512	19.53	25
Bovine submaxillary mucin	8	1250	0.39
Transferrin	8	1250	0.39
Fetuin	4	2500	0.195
Thyroglobulin	0	-	-

Table 5 Effect of enzymatic cleavage of rabbit erythrocytes on hemagglutination assay of the hemolymph agglutinin of the millipede *Thyropygus descriptus*

Enzymes used	Site of enzyme action	HA Titer
None	-	2048
Neuraminidase *C. perfringens* Type X (140 mU)	NeuAcα2,3Gal;NeuAc α2,6Gal; NeuAcα2,8Gal;	512
Trypsin (1 mg/ml)	Arg-, Lys-	32768
Chymotrypsin (1 mg/ml)	Tyr-Trp-Phe-Leu-	32768
Pronase (0.25 mg/ml)	All peptide links	65536

Sugars such as N-acetyl glucosamine, glucose, maltose, mannose, xylose, fructose and arabinose and glycoproteins such as bovine and porcine thyroglobulin did not inhibit HA at concentration 50 mM and 2.5 mg/ml respectively.

HA activity of hemolymph after enzymatic alteration of erythrocytes

HA activity got reduced when tested with neuraminidase (sialidase) treated rabbit erythrocytes and increased when tested with protease treated rabbit erythrocytes (Table 5).

Discussion

The hemolymph of all the four millipedes *T. descriptus*, *X. acuticonus*, *A. disticta* and *A. craspedota* recognized rabbit erythrocytes with great specificity. The ability of the millipede agglutinin to agglutinate rabbit erythrocytes argues for the specific recognition of the sugars constituting the glycocalyx of these erythrocytes, which serve as receptors to ligands as in the eukaryotic cells (Hakomori, 1973). It has been found that different animal species have characteristic receptor determinants on their erythrocyte surface (Yamakawa and Suzuki, 1953) and interspecies variations (Yasue *et al*., 1978). Reduction in HA following starvation beyond 20 days and stability of HA after feeding different types of food reveals the presence of a natural agglutinin.

Identification of maximum hemagglutinability in the freshmoult stage of the millipede suggest defense role of this lectin in protecting the animals from foreign invaders and in the development of the millipede. As the exoskeleton of the freshmoult animals is extremely soft it may remain susceptible to the attack of any pathogen. Hence the presence of high amount of agglutinin in the hemolymph could be a defense mechanism to evade microbial attack. Like agglutinins from many other species (Miller *et al*., 1972), the *T. descriptus* agglutinin is also sensitive to pH and temperature. The loss of biological activity of the agglutinin with increased temperature could be related to destabilization of sporadic weak interactions of tertiary structure responsible for native conformation of lectin (Singh and Saxena, 2013). HA assay with different concentrations of cations such as calcium, magnesium and manganese and chelators such as EGTA and EDTA suggests that the lectin is calcium independent.

The removal of HA following adsorption of the hemolymph to rabbit erythrocytes suggest the presence of a single hemagglutinin as reported in *C. antennarius* (Ravindranath *et al*., 1985a), *Scylla serrata* (Mercy and Ravindranath, 1992, 1993) and *Paratelphusa jaquemontii* (Maghil *et al*., 2003). However, the hemolymph when adsorbed to rat and human A erythrocytes continued to agglutinate rabbit erythrocytes suggesting the presence of remnants of agglutinability in the hemolymph capable of recognizing rabbit erythrocytes even after repeated adsorptions. This is supported by the serological studies which show that activity to one type of erythrocyte can be adsorbed by that type of erythrocytes, leaving residual agglutinating activity to other type of erythrocytes (Noguchi, 1903).

The rise in HA activity following treatment of erythrocytes with proteases may be due to the removal of certain proteins which mask the glycocalyx of the rabbit erythrocytes that are specifically recognized by the millipede hemolymph agglutinin.

The potent inhibitor of the agglutinin is PSM, a glycoprotein rich in GalNAc residues. Accordingly, GalNAc also inhibits the agglutinating activity at 1.5 mM concentration, but the ability of the hemolymph to agglutinate rabbit erythrocytes and its reduction following desialylation of erythrocytes argues for the affinity of the agglutinin to sialic acids. Among the sialic acid content of PSM, 90 % exist as N- glycolyl-neuraminic acid and 10 % as N-acetyl neuraminic acid and traces as N-O-acetyl neuraminic acid. High HA with rabbit erythrocytes containing NeuGc (Bhavananthan *et al*., 1964) and inhibition by PSM containing 90 % NeuGc (Schoop and Faillard, 1967) accounts for NeuGc specificity, which can be confirmed only after affinity purification of the lectin.

Conclusion

In spite of various information, the exact specificity of the agglutinin based on sugar specificity can be clearly stated only upon purification. This study provides the physico-

chemical requirements of the hemolymph agglutinin for affinity purification. The presence of sialic acid binding agglutinins in arthropods such as *T. descriptus* that are incapable of synthesizing sialic acids suggest that these agglutinins may be involved in the innate immunity of these organisms.

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