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CHARACTERIZATION OF NATURALLY OCCURING AGGLUTININ FROM THE MIDGUT OF THE RUSTY MILLIPEDE *TRIGONIULUS CORALLINUS*

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ABSTRACT

Natural hemagglutinins with specific affinity for the glycocalyx of rabbit erythrocytes is identified in the extracts of the midgut of the diplopodan millipedes *Thyropygus minusculus* of the family Harpagophoridae, *Jonespeltis splendidis, Chondromorpha severini* of the family Paradoxosomatidae and *Trigoniulus corallinus* of the family Trigoniulidae. Of the various tissues analysed for hemagglutinins, the highest HA titer was observed in the midgut against rabbit erythrocytes. The midgut agglutinin of all the four species also agglutinated rat and pig erythrocytes with various specificities. Further, the physico-chemical characterization was analyzed of the midgut of the rusty millipede *Trigoniulus corallinus*. The midgut agglutinin was sensitive to calcium and EDTA. The maximum hemagglutination observed at pH 6.5 and temperature 35ºC. The hemagglutinability was inhibited by α-lactose, D-galactosamine, dextrose, GlcNAc and lactoferrin. Biochemical parameters like the extract of midgut protein, calcium and water had no significant influence on HA. The sialic acid specificity of the agglutinin is revealed by the reduction in hemagglutination activity when treated with the desialylated rabbit erythrocytes.

INTRODUCTION:

Invertebrates necessitate defend themselves against a variety of pathogens. Invertebrate animals especially arthropods which lack adaptive immune systems, have developed other systems of biological host defense, so called innate immunity, that respond to common antigens on the cell surfaces of potential pathogens [1]. The innate immune system of the arthropods is the first line of inducible host defense against bacterial, fungal and viral pathogens [2]. This defense system is essential for the survival and perpetuation of all multicellular organisms [3]. Lectins/agglutinins are mono/di/polyvalent carbohydrate binding proteins [4, 5] widely distributed within the body fluids and other tissues of some invertebrates [6], play important roles in a wide array of biological processes, including recognition and control of nonself [5]. They can bind to sugar moieties in cell walls or membranes thereby change the physiology of the membrane to cause agglutination, mitosis or other biochemical changes in the cell [7, 8]. Present study we have focused our search on a millipede (Class Myriapoda) because, limited number of millipede lectins have been purified and characterized, including those from

Thyropygus descriptus [9], *Arthrosphaera disticta* [10, 11] and *Gluttonous beauts* [12].

MATERIALS AND METHODS

Materials

Millipedes *Thyropygus minusculus, Jonespeltis splendidis, Chondromorpha severini* and *Trigoniulus corallinus* were used in this investigation.

Animal collection and maintenance

Millipedes *Thyropygus minusculus, Chondromorpha severini, Jonespeltis splendidis and Trigoniulus corallinus* used in this investigation were collected from the swampy areas and coconut groves of Nagercoil, Marthandam, Elavuvilai and Nattalam, Kanniyakumari District, TamilNadu, India. All the species were kept in large cement tanks containing moist bricks, trunk of plantain tree and dried decaying leaves and fed with raw potatoes. Millipedes adapted to the laboratory condition as evidenced by their molting, copulation and deposition of eggs.

Hemolymph collection: The arthrodial membrane in between the column 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.

Preparation of tissue extract: The healthy anaesthetized millipedes were dissected using a pair of clean scissors. The tissues were removed and thoroughly rinsed in cold Tris buffered saline (TBS) to remove the adhering hemolymph. The tissue extracts were prepared by grinding 100 mg each of foregut, midgut and hindgut in 1 ml of cold TBS using a glass tissue grinder. The extracts were centrifuged at 4000 g for 10 min. at 4°C and the supernatant was assessed for hemagglutination activity.

Erythrocytes collection

Blood from different mammals were collected by venipuncture of the ear (rabbit), fore arm (Human A, B, O, dog and horse), cardiac puncture (mouse, squirrel, guinea pig and rat) and from the slaughter house (pig, cow, goat, buffalo, donkey) directly in modified Alsevier's medium (pH 6.1) containing sodium citrate (30 mM), sodium chloride (77 mM), glucose (114 mM), neomycin sulphate (100 g/ml) and chloramphenicol (330 g/ml) at a ratio of 2:8. Erythrocytes were suspended and washed thrice by centrifugation at 4000 g with ten volumes of physiological saline (0.9%) and with Tris-Buffered Saline (TBS) pH 7.5 (Tris-HCl: 50 mM, NaCl: 100 mM; $CaCl_2$: 10 mM) and resuspended in TBS as 1.5% suspension.

Hemagglutination (HA) Assay: The HA activity of the hemolymph/tissue agglutinin was assayed by measuring its ability to agglutinate erythrocytes. HA assays are performed at room temperature (30°C) by serial dilution of the hemolymph/tissue (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 minutes 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.

Effect of pH on HA assay: To assess the effect of pH on the agglutination 25 µl of the extract of the midgut was serially diluted with 25 µl of TBS at varying pH (5 to 11) and incubated for one hour prior to the addition of erythrocytes. Hemagglutination titer was determined after 1 hour of incubation.

Table 1 Taxonomic position of the experimental animals

Phylum - Arthropoda
Superclass - Myriapoda
Class - Diplopoda

Order	Family	Name
Spirostreptida	Herpagophoridae	*Thyropygus minusculus*
Polydesmida	Paradoxosomatidae	*Chondromorpha severini* *Jonespeltis splendidis*
Spirobolida	Trigoniulidae	*Trigoniulus corallinus*

Table 2 Natural hemagglutinins in the millipedes, *Thyropygus minusculus, Trigoniulus corallinus, Chondromorpha severini*, *Jonespeltis splendidis*

Millipedes	**Tissues**	**HA titer**			
		Rabbit	**Rat**	**Pig**	**Human A**
Thyropygus minusculus	Hemolymph	0	0	0	0
	Foregut	8	4	16	0
	Midgut	2048	32	1024	0
	Hindgut	8	2	8	0
Trigoniulus corallinus	Hemolymph	4	2	0	0
	Foregut	8	4		0
	Midgut	2048	1024	1024	16
	Hindgut	8	4	8	0
Chondromorpha severini	Hemolymph	0	0	0	0
	Foregut	4	2	2	0
	Midgut	512	16	8	0
	Hindgut	4	2	2	0
Jonespeltis splendidis	Hemolymph	0	0	0	0
	Foregut	8	2	4	0
	Midgut	1024	32	64	8
	Hindgut	16	8	4	0

Human B, O, Guinea pig, Mice, Cow, Goat, Buffalo, Horse, Squirrel, Donkey & Dog erythrocytes were did not agglutinate the agglutinin of the hemolymph and tissues of the millipedes.

Figure 1 Hemagglutination titer of the midgut of the millipede, *Trigoniulus coralli* in relation to pH.

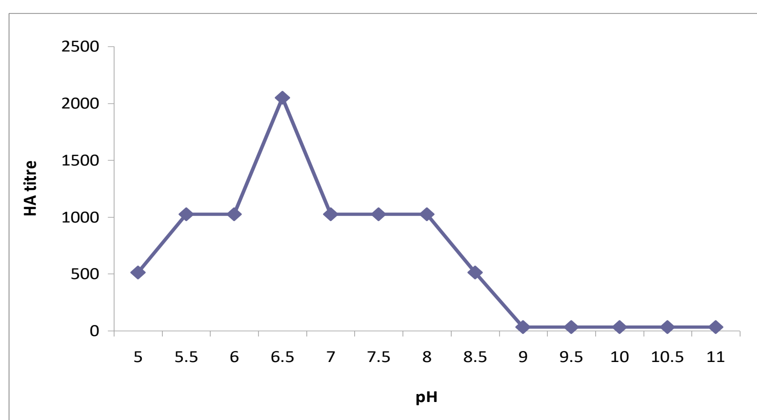

Figure 2: Hemagglutination titer on the midgut extract of the millipede *Trigoniulus corallinus* in relation to temperature

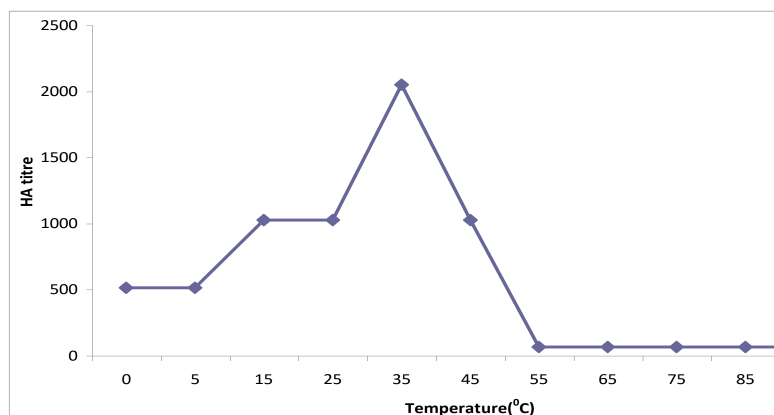

Table 3 Effect of cations and chelators on the hemagglutination titer of the midgut of the millipede, *Trigoniulus corallinus*

Concentration (mM) **(n=10)**	HA Titer					
	$CaCl_2$	$MgCl_2$	$MnCl_2$	EDTA		Trisodium citrate
				Disodium	Tetrasodium	
0	1024	1024	1024	1024	1024	1024
0.01	1024	1024	1024	2048	1024	1024
0.1	1024	1024	1024	2048	1024	1024
1	1024	1024	1024	2048	1024	1024
5	1024	1024	1024	2048	1024	1024
10	2048	1024	1024	256	1024	1024
20	128	512	512	64	512	1024
30	32	32	64	64	512	256
40	32	32	64	0	512	256
50	32	32	64	0	32	256
100	0	0	32	0	0	32

Table 4 Hemagglutination titer of the midgut extract of the millipede, *Trigoniulus corallinus,* after the adsorption with different erythrocytes.

Erythrocytes adsorbed	HA Titer			
	Rabbit	Rat	Pig	Human A
None	2048	1024	1024	16
Rabbit	0	0	0	0
Rat	2(0)	0	0	0
Pig	8(0)	0	0	0
Human A	0	0	0	0

Table 5 HAI of midgut extract of *Trigoniulus corallinus* by various sugars

Sugars (n=5)	HAI titer	Minimum concentration needed for HAI (mM)	Inhibitory potency (%)
α-Lactose	128	0.78	100
D-galactosamine	32	3.125	25
Dextrose	8	12.5	6.25
GlcNAc	4	25	3.125
D-galactose	2	50	1.56

Table 6 HAI of midgut extract of *Trigoniulus corallinus* by various glycoproteins

Glycoprotein (n=10)	HAI titer	Minimum concentration needed for HAI (μg/ml)	Inhibitory potency (%)
Lactoferrin	1024	4.882	100
BSM	4	1250	0.3906
Fetuin	4	1250	0.3906
Apotransferrin	4	1250	0.3906
Thyroglobulin	4	1250	0.3906
PSM	2	2500	0.1953
Transferrin	0	0	0

Table 7 Effect of enzyme treatment of rabbit erythrocytes on HA titer of the midgut agglutinin of the millipede *Trigoniulus corallinus*

Enzymes used (n=25)	HA Titer
None	2048
Trypsin	1024
Neutral Protease	1024
Neuraminidase	4

Table 8 Biochemical analyzed on the midgut extract of the rusty millipede, *Trigoniulus corallinus.*

Characteristics analysed (n=25)	Midgut
Water (%)	69.12±0.2
Calcium (mM)	11.7±0.5
Protein (mg/ml)	34.2±0.4
HA	2048

Effect of temperature on HA assay

To assay the thermal stability of the agglutinin 200 μl of extract of midgut was incubated in aliquots at specific temperatures (0 to 95°C) and was serially diluted with 25 μl of TBS prior to the addition of erythrocytes. Hemagglutination titer was determined.

Effect of cations on HA assay: To assess the effect of cations on agglutinability, the extract of the midgut was serially diluted with 25 μl of TBS with different

concentration of cations (Ca^{2+}, Mg^{2+}, Mn^{2+}) and was incubated at room temperature (30±2ºC) for 1 hour, prior to the addition of erythrocytes. Hemagglutination titer was determined.

Effect of chelators (EDTA and trisodium citrate) of the agglutinin

To assess the effect of chelators of the agglutinin, the extract of the midgut was serially diluted with 25µl of TBS with different concentration of chelators (EDTA and trisodium citrate) and was incubated at room temperature (30±2ºC) for 1 hour prior to the addition of erythrocytes. HA titer was determined.

Cross adsorption assay: Packed erythrocytes (rabbit/rat/pig/human A) were prepared by repeated washing of erythrocytes in 0.9% saline by centrifugation at 4000 g for 5 minutes until we get a clear pellet. Midgut extract was mixed with equal volume of packed rabbit/rat/pig/human A erythrocytes and incubated for 18 h at 4ºC with occasional shaking. After centrifugation, the supernatant was analyzed for HA.

Hemagglutination inhibition assay: HAI of the midgut agglutinin was performed to the test the ability of various sugars (mono and disaccharides) and glycoproteins to inhibit agglutination of rabbit erythrocytes. For this study, 25 µl of inhibitors (sugar/glycoprotein) of known concentration (100 mM sugars / 5 mg/ml glycoproteins) were serially diluted with 25 µl of TBS in microtiter plates. Then to each well, 25 µl extract of midgut diluted to subagglutination concentration in TBS (to give HA of one well) was added, and incubated for 1 hour. After incubation, 25 µl of 1.5% rabbit erythrocytes suspension was added, mixed and incubated. The hemagglutination inhibition titer was reported as the reciprocal of the highest dilution of inhibitor giving complete inhibition of agglutination after 1 hour.

Enzyme treatment of the erythrocytes: Protease treated erythrocytes were prepared following the method of Pereira *et al*., 1981[13] and asialo-erythrocytes were prepared following the method of Ravindranath *et al*., 1988 [14] and Mercy and Ravindranath, 1993 [15].

Estimation of water, calcium and protein content

Water content was estimated following the method of Mullainathan, 1979 [16]. Midgut calcium was measured the procedure of Webster, 1962 [17] and the protein concentration is estimated by Folin-Ciocalteau method of Lowry *et al*., 1951 [18].

RESULTS AND DISCUSSION

Diversity and distribution of millipedes: Four species of millipedes representing three families Herpagophoridae, Paradoxosomatidae, Trigoniulidae of the three orders Spirostreptida, Polydesmida and Spirobolida of the class Diplopoda and super class Myriapoda of the phylum Arthropoda were used for this investigation (Table 1).

HA activity of all the four millipedes: Among the four species assayed, the hemolymph of the millipede *T. corallinus* alone exhibited agglutinability against rabbit and rat erythrocytes. Hemolymph of the other three species of millipedes, *Chondromorpha severini, Thyropygus minusculus*, *Jonespeltis splendidis* failed to agglutinate any of the tested erythrocytes (Table 2). Low agglutinability was observed in the extract of the foregut (HA titer = 2-16), and hindgut (HA titer = 2-16) which recognized rabbit, pig and rat erythrocytes. High HA titer was observed in the extract of the midgut with rabbit (HA titer = 512-2048), rat and pig (HA titer = 16-1024) and feeble HA titer was observed Human A (HA titer = 8-16) erythrocytes (Table 2). The midgut of all the millipedes *Trigoniulus corallinus,*

Chondromorpha severini, Thyropygus minusculus and *Jonespeltis splendidis* recognized rabbit erythrocytes with great specificity. Agglutinin/lectin may recognize a whole sugar or a part of sugar or a sequence of sugar or their glycosidic linkages [19-22]. The high HA titer in the midgut of all the four species of millipedes and low HA titer in the fore and hindgut of all the millipedes with specific affinity for rabbit erythrocytes suggest that agglutinins would have been released from the midgut the site of synthesis or storage of agglutinins in millipedes as reported in the midgut gland of millipedes *Thyropygus descriptus* [9] and *Arthrosphaera disticta* [11]. The weak binding observed in the hemolymph, fore and hindgut could be due to the forward and backward flow of the agglutinin from the midgut, if it is the source of origin of the agglutinin. Ability of the extract of the midgut of all the four species of millipedes to agglutinate rabbit erythrocytes with very high HA titer showed a way of convergence in erythrocyte specificity of the millipedes.

HA activity of the millipede *Trigoniulus corallinus*

Very feeble agglutination was showed in the hemolymph (HA titer = 2-4), foregut (HA titer = 4-8) and hindgut (HA titer = 4-8). The midgut agglutinin agglutinated rabbit (HA titer = 2048), rat and pig (HA titer = 1024) erythrocytes with great potency. In addition the midgut agglutinin also agglutinated human A (HA titer = 16) erythrocytes (Table 3). The midgut agglutinin binds to particular sugar moiety/receptors on the surfaces of the cells that is glycocalyx region of those erythrocytes. Probably the agglutinin may bind to sialic acid of the glycocalyx of these erythrocytes [23]. The erythrocyte specificity of the midgut agglutinin 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 [24].

The HA activity of the agglutinin was sensitive to pH and temperature. The midgut agglutinin activity was maximum at pH 6.5 (Figure 1) and temperature 35ºC (Figure 2). Conformational changes occur due to the change/dissociation of the binding sites of the agglutinin when there is decrease/increase in pH and temperature which may suppress/accelerate the hemagglutination activity. The loss of agglutinating activity of midgut agglutinin with increased temperature may be due to destabilization of sporadic weak interactions of tertiary structure responsible for binding of native agglutinin. Similar activity was also reported in hemolymph of *Rhysida nuda nuda* [25], *Thyropygus descriptus* [26] midgut gland of *Thyropygus descriptus* [9] and *Arthrosphaera disticta* [11]. Maximum HA activity was observed in the presence of 10 mM calcium chloride which got reduced on addition of more than 5 mM Disodium EDTA (Table 3). Cations are involved in stabilizing the primary structure of agglutinins. Probably, the divalent cations may trigger/suppress the hemagglutination activity depending on their concentration. EDTA is known to be a metal-chelating agent. Addition of 0.1 to 5mM disodium EDTA may cleave the excess calcium resulting in an increase in HA titer. Similar activity was also reported in *Paratelphusa jacquemontii* [27], *Emerita emeritus* [28], *Lamella lamellifrons* [29], *Arthrosphaera disticta* [11].

HA activity of the extract of the midgut after adsorption with different erythrocytes

Cross adsorption results showed the presence of single agglutinin in the extract of midgut of the rusty millipede, *Trigoniulus corallinus* as evidenced by the complete disappearance of hemagglutination activity after first or second adsorptions with any of the erythrocyte species that showed agglutination with the midgut gland

agglutinin (Table 4). Removal of agglutinability was also reported in the hemolymph of *Scylla serrata* [30], *Thyropygus descriptus* [26].

Hemagglutination inhibition assay

Among the inhibitors tested for HAI, the agglutinability of the midgut agglutinin with rabbit erythrocytes was inhibited by sugars: α-Lactose, D-galactosamine, Dextrose, GlcNAc and D-galactose (Table 5) and glycoprotein: lactoferrin, a sialoglycoprotein (Table 6). Hydroxyl groups (OH) of carbohydrates may participate in the binding to CRDs of the midgut gland agglutinin [31]. Lactoferrin specificity is also reported in other millipedes [10, 11, 26].

Enzyme treatment: The agglutinability of the midgut agglutinin of the rusty millipede, *Trigoniulus corallinus* got greatly reduced when checked with enzyme treated rabbit erythrocytes (Table 7). The reduction in HA titer could be due to cleave of the glycosidic bonds to the sialic acid residues.

Biochemical factors and HA activity: Studies on the role of biochemical parameters such as water, protein and calcium content of the extract of the midgut showed no influence on hemagglutinating activity (Table 8). Change in biochemical factors also failed to influence the HA activity of the crabs *Episesarma tetragonum* [32], *Varuna litterata* [33], *Lamella lamellifrons* [23], centipede *Rhysida nuda nuda* [25] and millipedes *Thyropygus descriptus* [9] and *Arthrosphaera disticta* [11].

CONCLUSION

The present study described the agglutinability and characterization of the midgut agglutinin of the millipede *Trigoniulus corallinus*. The agglutinin recognized rabbit erythrocytes, exhibiting high HA titer in the presence of Ca^{2+} ions at pH 6.5 and temperature upto 35ºC. The agglutinability was specifically inhibited by the sugars α-lactose, D-galactosamine, dextrose, GlcNAc and glycoprotein lactoferrin. This study provides the physico-chemical characteristics necessary to purify the agglutinin.

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