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Physicochemical characterization of hemagglutinin from the coelomic fluid of the Sea urchin *Tripneustes gratilla* (Linnaeus, 1758)

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Abstract

Lectins are carbohydrate binding proteins with the ability to agglutinate cells. In this study, a naturally occurring hemagglutinin with specific affinity for dog erythrocytes was identified, isolated and purified from the coelomic fluid of the sea urchin *Tripneustes gratilla.* The physicochemical characteristics of the agglutinin showed that its hemagglutinating activity was calcium dependent, optimum at pH 7.5 and temperature between 0 to 30°C and in the presence of 1-10 mM $CaCl_2$ or 5 mM $MgCl_2$. The HA activity was reversibly sensitivity to di and tetra sodium EDTA. Cross adsorption assay of the agglutinin revealed the presence a single lectin. PSM was identified as the potent inhibitor followed by thyroglobulin, BSM, transferrin, and fetuin. The agglutinating activity of the agglutinin is also inhibited by the sugars α-lactose, D-fucose, sucrose, trechalose, L-fucose and melibiose.

Keywords

Tripneustes gratilla, hemagglutination, hemagglutination inhibition assay, cross adsorption assay.

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INTRODUCTION

Lectins are sugar - binding proteins of non immune origin which agglutinate cells or precipitate glycoconjugates. These molecules are capable of specific recognition and reversible binding with carbohydrates without alternating these covalent structures. Lectins are synonymously known as

agglutinins or hemagglutinins. Specificities of lectins are described in terms of the monosaccharide haptens that inhibit their agglutination reactions [1]. In this research echinoderms were studied. The phylum Echinodermata is phylogenitically positioned within the deuterostome lineage of animals that includes the chordata and a few minor invertebrate phyla. There are five extent classes of echinoderms, of which the echinoid class includes sea urchins and sand collars. The cellular mediators of immunity in echinoderms are coelomocytes present in the coelomic fluid which bathes the internal organs and forms the fluid medium, where the coelomocytes (Echinoderm immune cells) are suspended [2-4]. The composition of coelomic fluid is similar to sea water consisting of dissolved salts and other minerals [5]. Since the coelomic fluid is also rich in proteins secreted by the coelomocytes or the surrounding tissues (such as the radial nerve cord), essential for encapsulation of invasive material and clotting reactions, they are involved in cell free (humoral) immune responses. Hilde man and Colleagues demonstrated the ability of several echinoderm species to differentiate self from non self tissues through allograft rejection studies [6-7]. These initial observations were extended in studies of sea urchins was defined [8-10]. These and other studies identified the cells of the open circulatory system in adult echinoderms, the coelomocytes, as the main effectors of defense responses [8], and which initiates response to injury or infection and carries out the clearance of foreign substances [11-13]. In this paper an effort is made to study the physicochemical characterization of natural hemagglutinins in the coelomic fluid of the sea urchin *Tripneustes gratilla*.

MATERIALS AND METHODS

Sample collection

Adult specimens of the sea urchin *Tripneustes gratilla* were collected along the coast of Kanniyakumari District and transferred to the Laboratory in aerated tanks with sea water. The coelomic fluid was obtained from the animals by cutting a slit in to the peristomial membrane.

Hemagglutination assays to determine physicochemical parameters

The physicochemical properties of the sample were determined by hemagglutination assays with coelomic fluid under conditions of varying pH, temperature, divalent cations of diverse concentration and EDTA.

Hemagglutination (HA) assay

HA assay was performed in U-bottomed microtiter plates by two fold serial dilution of 25 µl coelomic fluid with an equal volume of TBS. After dilution, 25 µl of 1.5 % erythrocytes suspension was added to each well mixed and incubated for 60 minutes at room temperature. The HA was recorded as the reciprocal of the highest dilution of the sample giving complete agglutination of erythrocytes [14]. Controls for all assays consisted of the substitution of the sample by TBS.

Effect of physical factors on HA assay

To assess the effect of pH 25 µl of the coelomic fluid was mixed with equal volumes of tris buffered saline (TBS) at different pH (5 -10.5) and was incubated at room temperature (30 ± 2°C) for 1 hour, before adding the erythrocyte suspension.

To study the effect to temperature on HA 25 µl coelomic fluid was incubated at (0-100°C) for 1 hour. After incubation the HA activity of each sample was determined against the respective high agglutinating erythrocytes.

Effect of cations and chelators on HA titer

To study the effect of cations and chelators on HA titer, the coelomic fluid was incubated for 1 hour in equal volume of TBS containing specific concentration (0.01, 0.1, 1.0, 5.0, 10, 20, 30, 40, 50 and 100) of cations (calcium and magnesium) and chelator (EDTA) of 5 mM was used for HA assay.

Cross adsorption assay

Packed erythrocytes (Dog, Rabbit, Buffalo, Cow, Rat, Human, A, B, O and AB) were prepared by repeated washing of erythrocytes in 0.9 % saline by centrifugation at 4, 000 g for 5 minutes until a clear pellet was obtained. Coelomic fluid was mixed with equal volume of packed dog, rabbit, buffalo, cow, rat and human A, B, O and AB erythrocytes and incubated for 18 hr at 4°C with occasional shaking. After centrifugation, the supernatant was analysed for HA.

Hemagglutination inhibition (HAI) assay

25 µl of coelomic fluid diluted to sub agglutination concentration (dilution at which coelomic fluid was able to provide 1 wells HA) was added to each well containing 25 µl of known concentration of serially diluted inhibitors (glycoprotein and sugars). After incubation for 1 hr, 25 µl of 1.5 % dog erythrocytes suspension was added. The HA titer is reported as the reciprocal of the highest dilution of inhibitors giving complete inhibition of agglutination after 60 minutes.

RESULTS

HA profile of *Tripneustes gratilla*

The coelomic fluid of *Tripneustes gratilla* agglutinated dog erythrocytes better than rat, rabbit, pig and human A, B, O and AB erythrocytes (Table 1).

Effect of pH and temperature on HA assay

The coelomic fluid exhibited maximum agglutinin activity at the pH 7.5 and 0 to 30°C (Figure 1a and 1b).

Effect of cations and chelators

Addition of divalent cations (1 -10 mM) (Ca^{2+}, Mg^{2+}) showed an increase in HA titer of the coelomic fluid. A drastic decrease in HA activity was observed in addition of 5 mM to 10 mM disodium EDTA.

Cross adsorption

Cross adsorption profile revealed the presence of a single agglutinin in the coelomic fluid of the sea urchin *Tripneustes gratilla* because there was decrease in HA activity after the first adsorption and disappearance of activity after second or third adsorptions with any of the erythrocytes specific for the agglutinin.

Hemagglutination inhibition (HAI) assay

α-lactose and D-fucose effectively inhibited the hemagglutinating activity. The inhibitory potency of tested sugar on HA of the coelomic fluid lectin was as follows: α-lactose > D-fucose > sucrose > trechalose = L-fucose > melibiose. Among glycoproteins PSM (porcine stomach mucin) was identified as the potent inhibitor with the HA titer of 1024, HAI was weakly inhibited by thyroglobulin > BSM = transferrin. Sugars such as N-acetyl D glucosamine and N-acetyl D-manosamine did not inhibit HA even at concentration as high as 100 mM (Table 2).

DISCUSSION

Lectins have been isolated from various marine invertebrates and great attention is being given regarding their application as tools in biotechnology. Invertebrate's lectin make an important contribution to innate immune protection and work along with epithelial barriers, cellular defense such as phagocytosis and pattern-recognition receptor that trigger pro-inflammatory signaling cascades [15].

The coelomic fluid agglutinin of *Tripneustes gratilla* agglutinated a variety of erythrocytes, with high affinity for dog > rat > rabbit = buffalo > pig erythrocytes and poor affinity towards human A, B, AB, O > cow and goat erythrocytes. The receptor components on the glycocalyx of dog erythrocytes is NeuGc / NeuAc [16] rat erythrocytes is NeuGc / NeuAc / 4 (7) O-acetylated sialic acids [17] buffalo erythrocytes is NeuGc [18].

The peak of the hemagglutinin activity was observed at pH 7.5 which got reduced below and above their optimum pH. In fact, this indicates that the extreme alkaline and acidic condition inactivate the lectin and this might be due to irreversible conformational changes which masks the available receptors thus reducing the biological activity, associated with partial defolding of protein. The lectin was active between the temperature ranges of 0-30°C. This may be due to the conformational changes that occur at the binding sites of the lectin at higher temperature which may accelerate or suppress the agglutinability. The HA titer also increased with the addition of low concentration of $CaCl_2$, Mg^{2+} up to 1- 10 mM which implies that the agglutinin depends on cations for its activity. Divalent cations are known to be important in stabilizing the structure of hemagglutinins [19-20]. The increased HA activity confirming the calcium dependency as reported by [21] in the Mangrove crab *Episesarma tetragonum*. The agglutinin of the sea urchin *T. gratilla* was inhibited by PSM, thyroglobulin, BSM and transferrin. PSM contains the sialic acid N-glycolyl neuraminic acid and NANA [22]. The hemagglutinin in coelomic fluid of sea urchin could be absorbed by any one of the erythrocytes recognized by the agglutinin. This property is similar to that of isohemagglutinin present in the mammalian serum. The data of adsorption test showed that a single hemagglutinin is present the coelomic fluid of *T. gratilla*. Hemagglutinins have been demonstrated in several orders of sea urchin (Mainly order Camarodonata) and therefore, it could be of interest to compare the hemagglutinins of each species of a sea urchin chemically and biologically. The present study revealed the coelomic fluid agglutinin of *Tripneusteus gratilla* could be further purified by affinity purification using PSM sepharose 4B column and further studies were made on coelomic fluid of sea urchin of *Tripneustes gratilla* on antimicrobial and antitumour activity.

Table 1: Hemagglutinating (HA) activity of coelomic fluid from the sea urchin *Tripneustes gratilla* against various mammalian erythrocytes

Erythrocytes	HA titre
Dog	1024
Rat	512
Rabbit	256
Buffalo	256
Pig	64
Human O	8
Human A	8
Human AB	8
Human B	8
Cow	4
Goat	4
Chick	0
Guinea pig	0

Table 2: Hemagglutination inhibition (HAI) of the coelomic fluid agglutinin of the Sea urchin *Tripneustes gratilla*

Inhibition by sugars			
Inhibitor	**HAI titer**	**Minimum Con. Required (mM/ `mg/ml)**	**Relative inhibitory potency (%)**
α-Lactose	256	0.39063	100
D-fucose	128	0.78125	50
Sucrose	16	6.25	6.25
Trechalose	4	25	1.56
L-fucose	4	25	1.56
Melibiose	2	50	0.78
N-acetyl D glucosamine	0	0	0
N-acetyl D-manosamine	0	0	0

Inhibition by glycoprotein			
Inhibitor	**HAI titer**	**Minimum Con.Required (mM/ `mg/ml)**	**Relative inhibitory potency (%)**
PSM	1024	4.882	100
Thyroglobulin	64	78.125	0.390
BSM	16	312.5	0.097
Transferrin	16	312.5	0.097
Fetuin	4	1250	0.024

Figure 1a: Impact of pH on HA titer of the coelomic fluid agglutinin of the sea urchin *Tripneustes gratilla*.

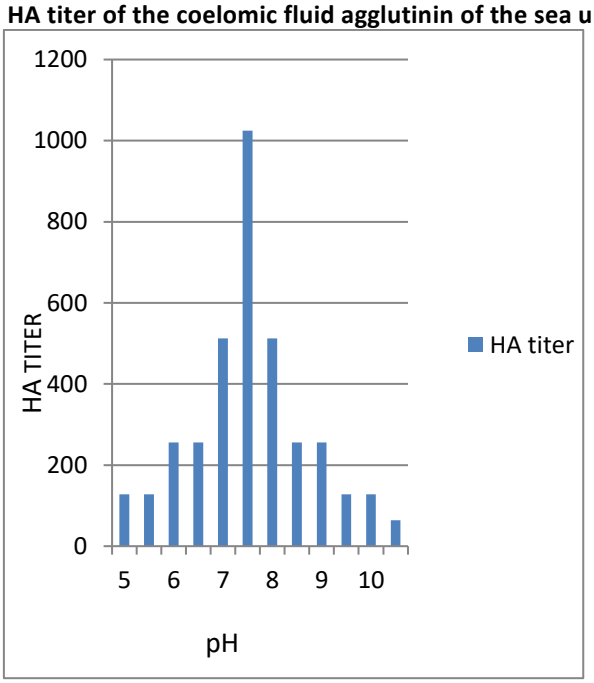
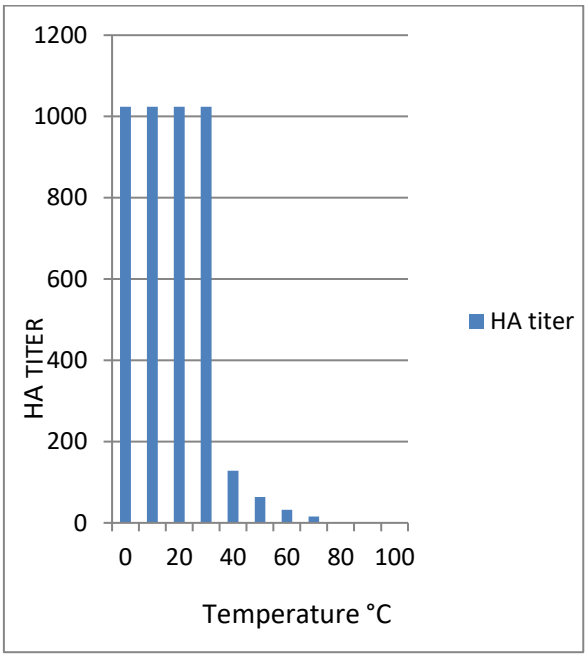


Figure 1b: Hemagglutination titer of *Tripneustes gratilla* agglutinin in relation to temperature



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