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RESEARCH ARTICLE

IMPACT OF HEAVY METALS ON HEMAGGLUTININ MOLECULE AND HEMOCYTES OF *VILLORITTA CYPRINOIDES*

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ABSTRACT

A naturally occurring hemagglutinin molecule detected in the hemolymph of black clam *Villoritta cyprinoides* was able to agglutinated various mammalian erythrocytes.

Reduction of activity was obtained when the animals were treated with Cd and Pb. As the hemocyte play a vital role in the innate immunity, this findings strongly confirmed that the heavy metals such as Cd and Pb affects the immune function of *V. cyprinoides*.

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INTRODUCTION

Estuaries are the most productive environment and also the most contaminated areas of water bodies. Bivalves which are having a backwater environment can be used as sentinel organism for pollution check because of their sessile nature and filter feeding habitation. Apart from this it is able to gather contaminants in their tissues. Natural and man-made toxicants/ pollutant come into aquatic ecosystems by various routes, such as direct discharge, land run-off, atmospheric deposition, in situ production, abiotic and biotic movements and food-chain transfer. All these activities are affecting the immune system of bivalves. *Villorita cyprinoides* (Gray) is a typical euryhaline clam consumed by humans all over West coast of India. It is suitable for human consumption and is rich with proteins and its shell used for the preparation of lime and cement. Invertebrates, lack adaptive immune system, but having a well developed host defense system which is called as innate immunity. A variety of humoral factors naturally occurring have been detected in the serum of non chordates which is include agglutinin, lysin, antibacterial and antifungal proteins, phenoloxidase system, β 1,3 glucan binding proteins (Jayaraj *et al.,* 2008).

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Lectins are di or multivalent carbohydrate binding proteins which helps non self recognition and clearance of invaded microorganisms in invertebrates. Apart from this, phagocytosis is considered as the primary defense mechanism against foreign invaders. The immune function of molluscs is based on innate mechanisms of cellular and humoral defense. According to Allam *et al.* (2001) the concentration of cells in the haemolymph is expressed as total haemocyte count (THC), is influenced by different pathogens or external factors. Disclosure of adult animals to lethal environmental contaminants such as heavy metals can lead to immune suppression (Fournier *et al.,* 2000). Agglutinins which are known to be distributed from unicellular to multi cellular organisms, has shown to possess diverse carbohydrates binding specificity. Molluscs are one of the basic group in animal kingdom which possess agglutinins. These agglutinins are distributed at varied levels depending on the developmental stage. Based on the literature survey, vertebrate erythrocytes were used as indicator cells for detection of hemagglutinating activity in molluscs belonging to various groups and these agglutinins are known to possess receptors for varied carbohydrates / carbohydrate moieties. One of the important function of this agglutinin molecule is the recognition of self and non-self within the cellular and humoral defense. It is demonstrated that the naturally occurring substance that are specific to agglutinate the RBCs are widely dispersed in animals and plants. In bivalves the natuarally occurring

hemagglutinins seem to display a considerable degree of specificity. Cheng and Sanders, 1962 demonstrated that blood of a fresh water snail (*Viviparus malleatus*) agglutinated only rabbit red blood cells, Johnson, 1964 reported that an extract of the butter clam (*Saxidomus giganteus*), a marine bivalve, agglutinated human cells of the phenotypes A1 and AIB only and Cushing *et al*. (1963) found that octopus serum reacted only with types Al and AZ. The possible value of these naturally occurring substances to the molluscs possessing them is unknown. Phagocytosis plays an important role in the defense mechanism in invertebrate against invading microorganisms. Cheng *et al.* (1981) reported that phagocytic molluscan hemocytes are able to contribute some other physiological functions such as wound healing, shell repair, transportation of nutrients etc. In this present study we are analyzing how the heavy metals are affecting the activity of immune molecule.

MATERIALS AND METHODS

Collection and maintenance of clams

Three different sites of backwaters were selected for the current study, (A) Padne, Kasaragod (latitude-12.18175, longitude-75.15054), (B) Kuppam, Kannur (latitude-12.05041, longitude-75.30880) and (C) Atholi, Kozhikode (latitude-11.3802, longitude-75.7527). The presence of heavy metals was analysed in water as well as tissue of clams using AAS. Based on those results clams were collected for subsequent studies from Atholi, Kozhikode since it was less contaminated compared to other two sites. The clams were transported to the laboratory with aerator and maintained in plastic tanks under laboratory conditions (26±2 C) with continuous aeration and the water was changed every day.

Collection of hemolymph and preparation of serum

Hemolymph was collected from the adductor muscle of clam *V. cyprinoides* using syringe. The hemolymph samples collected was centrifuged (400 x *g,* 15 min, 4° C) and the clear supernatant (=serum) was used for hemagglutinaton assay.

Reagents

Tris buffer saline (TBS*)*

Carbohydrates were purchased from sigma (St. Louis, MO, USA). Five types of tris-buffered saline (TBS) were used.1). TBS-I : 50 mM tris-HC1, 135 mM NaCl (300 mOsm); 2). TBS-I1 : 50 mM tris-HC1, 115 mM NaCl, 10 mM CaCl, (300 mOsm); 3). TBS-I11 : 50 mM tris-HC1, 5 mM NaCl, 30 mM CaCl, (135 mOsm); 4). TBS-IV : 50 mM tris-HC1,72 mM NaCl, 40 mM CaCl, (300 mOsm); 5). TBS-V : 50 mM tris-HC1,100 mM NaCl, 50 mM EDTA (300 mOsm). All the buffer solutions were adjusted to pH 7.5 and contained 0.02% NaN_3.

Acetate buffer (pH 3.0, 4.0, 5.0 and 6.0)

Sodium acetate (200 mM) was dissolved in 500 ml of autoclaved distilled water and 200 mM of acetic acid was made up to 500 ml using autoclaved distilled water. Aceteate buffer was prepared by mixing these two solutions in definite proportions to get a pH of 3.0, 4.0, 5.0 or 6.0

Tris-HCl buffer (pH 7.0, 8.0 and 9.0)

100 mM tris was dissolved in 500 ml of autoclaved distilled water and the pH was adjusted to pH 7.0, 8.0 or 9.0 using 2 N HCl.

Glycine-NaOH buffer (pH 10.0, 11.0 and 12.0)

100 mM glycine was dissolved in 500 ml of autoclaved distilled water. Glycine-NaOH buffer was prepared by this solution with 1N NaOH in definite proportions to get pH 10.0, 11.0 and 12.0 **Preparation of erythrocytes (RBC) suspension**

Human A, human B, human O, goat, sheep and rat blood samples were obtained by venous or cardiac puncture and collected in sterile Alsever's solution (Garvey *et al.,* 1979) containing 10 µg/ml streptomycin. This was stored at 4° C. Prior to use, the RBC were washed thrice using 0.9 % saline and once with TBS-II by centrifugation (400 x *g*, 5 min, RT). The RBC pellet was resuspended in TBS-II as 1.5 % suspension (v/v).

Determination of hemagglutination (HA) activity

HA assays were performed in V-bottomed microtiter plates (Tarsons, India) by two-fold serial dilution of 25 µl of serum sample (pooled) with equal volume of TBS-II. After dilution, 25 µl of 1.5 % RBC suspension was added to each well and incubated for 45 min at RT. The HA titer was recorded as the reciprocal of the highest dilution of the sample causing complete agglutination of RBC (Garvey *et al.,* 1979). Control for all assays consisted of the substitution of the serum sample by TBS-II.

Cross-adsorption assay

The initial HA titer for serum (unadsorbed) was determined using six RBC types. Cross-adsorption assay was performed by addition of 250 µl serum sample with an equal volume of freshly washed human A RBC pellet. This mixture was incubated for 1 h at RT with periodical gentle shaking to ensure thorough mixing. After 1 h, the mixture was centrifuged (400 x *g*, 5 min, RT) and the supernatant was collected and adsorbed for a second and third time under the same conditions using freshly washed human A RBC. The serum thus, adsorbed thrice was subsequently tested for HA activity against the same as well as other RBC types (Human B, O, rat, goat & sheep).

Effect of divalent cations and EDTA sensitivity

The serum samples (pooled) dialyzed against divalent cation-free TBS (TBS-I) and the HA activity tested in the absence of cations, resulted in complete loss of the activity. But on addition of Ca^{2+} the samples regained its activity, whereas Mg^{2+} and Mn^{2+} were not effective on HA activity regaining. Dialyzing the sample against TBS containing Ca2+, the

activity was unaffected. Serum samples dialyzed against TBS containing 50 mM EDTA and tested in absence of cations, the activity was completely lost and it did not resume in the presence of any of the cations tested.

Thermal stability and pH

Eleven aliquots of serum samples (each 100 µl) pooled from five clams (2.5 ±3 cm) were used to test the effect of temperature on HA activity. First serum sample was held at RT for 30 minutes which served as control. Two samples were held inside the refrigerator at 10 and 20º C. The remaining eight samples were held in a water bath maintained at 30, 40, 50, 60, 70, 80, 90 and 100º C. All the samples were held for 30 minutes and then centrifuged (400 x *g*, 5 min, RT). The clear supernatants were tested for HA activity using human A RBC. In another experiment, the effect of pH on HA activity was assessed by dialyzing serum samples (each 100 µl) against buffers with varying pH ranging from 3 to 12 for 24 h (2 x 100 ml) at 12º C in order to equilibrate the serum to a buffer with a specific pH. Then, the samples were re-equilibrated by dialysis against TBS-II for 24 h (2 x 100 ml). The samples were centrifuged (400 x *g*, 5 min, RT) and the clear supernatant were tested for HA activity using human A RBC.

Carbohydrate mediated HA-inhibition assay

The initial HA titer of serum (pooled) was determined. The HA titer was reduced to 4 by diluting the serum using TBS-II. The carbohydrates to be tested were serially diluted with equal volume (25 µl) of diluted serum in micro titer plates and incubated for 1 h at RT. After 1 h, 25 µl of 1.5 % human A RBC suspension was added to all the wells and incubated for 1 h at RT. The minimal concentration of test carbohydrate was completely inhibited agglutination of human A RBC was recorded.

Exposure of V. cyprinoides to heavy metals

The stock solution of cadmium and lead was prepared for mg/L using back water collected from the same biota. From this stock solution various concentration (10, 20, 40, 60, 80, 100 and 120µg) were prepared by serial dilution method using back water. The clams were divided in 8 groups and each group containing 10 animals. The last group serves as control. Remaining was exposed to various concentrations of heavy metals for 24 hours.

Determination of Hemagglutination (HA) activity

HA assays were performed in serum sample of V. cyprinoides exposed to Cd and Pb in V-bottomed micro titer plates (Tarsons, India) by two-fold serial dilution as described earlier.

Total hemocyte count (THC)

Hemocytes suspension of control and exposed to Cd and Pb , was used to determine THC. The hemocyte suspension was filled in hemocytometer by capillary action and was held in moist chamber for 5 minutes in order to allow the hemocytes for settling. The hemocytes in the four large corner squares were counted. The THC in 1 ml hemolymph was calculated using the formula given below:

$$\text{THC (cells/ ml)} = \frac{\text{Total number of cells in 4 corner Squares}}{4} \text{ X diluting factor X 1000}$$

Trypan blue dye exclusion test

Using the hemocyte suspension (from control and treated) six monolayers were made using 50 µl of suspension for each monolayer. The monolayer's were left in the moist chamber up to 2 h and the viability of cells was tested at 30, 60, 90, 120 minutes with various concentration of cadmium and lead (10, 20, 40 and 60µl). For viability assay, before 5 minutes of the specific time interval 50 µl of trypan-blue dye solution was overlaid on the monolayer and left in moist chamber for 5 minutes. After 5 minutes, the slide was viewed under bright-field at 20 X for the presence of any dead cells. The percentage of the viable cells for control and treated was calculated using the formula:

$$\text{Viable cells (\%)} = \frac{\text{Total number of cells} - \text{Total number of dead cells}}{\text{Total number of cells}} \text{ X } 100$$

RESULTS AND DISCUSSION

Hemagglutination (HA) profile

The occurrence of natural hemagglutinin molecule in the hemolymph of *V. cyprinoides* was detected using different types of mammalian RBC. Serum agglutinated all the RBC types tested. Among these RBC types, the highest HA titer of 32 was obtained against human A RBC (Table 1). Other RBC types tested were agglutinated by serum with relatively low titer values. Serum weakly agglutinated goat and sheep RBC with a HA titer of 2

Table 1. Hemagglutinating (HA) activity of serum of *V. cyprinoide* against various mammalian erythrocytes (RBC)

RBC types tested	HA titer*
Human A	32
Human B	8
Human O	16
Rat	8
Sheep	2
Goat	2

*Values are based on six determinations using serum from different preparation

Cross-adsorption

The HA activity in the serum may be allied with a single or multiple fraction which was examined by cross-adsorption tests. Cross-adsorption test was performed with human A RBC, since it showed high titer against serum. On adsorption of serum with human A RBC thrice, it completely removed the HA activity of the same as well as all the other RBC types tested (Table 2), Human A RBC were used as an indicator for all the following studies. A complete loss of the activity was observed when it tested in the absence of cations. But on

Table 2. Cross-adsorption of serum hemagglutinin (HA) of *V. cyprinoides* with human A RBC

Serum adsorbed with	HA titer against RBC types tested*					
	Human A	Human B	Human O	Rat	Sheep	Goat
None	32	8	16	8	2	2
Human A	0	0	0	0	0	0

* Determined using human A RBC as indicater cells and values are based on six determinations

Table 3. Effect of divalent cations and EDTA on the hemagglutinating (HA) activity of serum of *V. cyprinoides*

Treatment of serum sample	Cation in sample diluting medium and RBC suspension	HA titer*
Untreated serum (before dialysis)	$CaCl_2$	32
Serum dialyzed against cation-free TBS	None	0
(Control)	$CaCl_2$	32
	$MgCl_2$	0
	$MnCl_2$	4
Serum dialyzed against TBS+10 mM $CaCl_2$	$CaCl_2$	32
Serum dialyzed against TBS +50 mM EDTA	None	0
followed by dialysis against cation-free TBS	$CaCl_2$	16
	$MgCl_2$	0
	$MnCl_2$	0

* Determined using human A RBC as indicator cells and values are based on six determinations

Table 4. Carbohydrate mediated hemagglutination (HA) -inhibition assay of serum

Carbohydrate used	Minimum inhibitory concentration (µL)
Monosacharides	
L- Arabinose	-
D- glucose	100
D- galactose	100±50
D- mannose	100±50
Acetylated sugar	
N-acetyl- D- glucosamine (GluNAc)	-
Disaccharides	
Sucrose (glc α 1→2 glc))	-
Maltose (glc α 1→4 glc)	100
Lactose (gal β 1→4 glc)	50
Polysachrides	
Laminarin (a β 1 - 3, homopolymer of glucose 0.1%)	-

*Determined using human A RBC and the values are based on six determinations using various preparation of serum

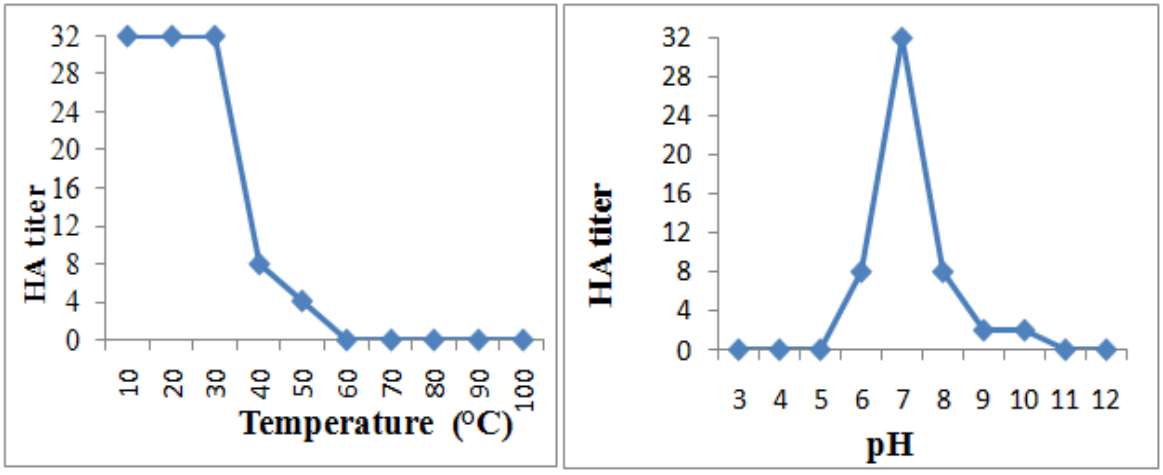


Figure 1. Effect of temperature and pH on the activity in the serum of V. cyprinoides

addition of Ca^{2+} the samples regained its activity, whereas Mg^{2+} and Mn^{2+} were not efficient on HA activity regaining. Dialyzing the sample against TBS containing Ca^{2+}, the activity was unaffected. After dialyzing the Serum samples against TBS with 50 mM EDTA and tested in absence of cations, the activity was completely lost and it did not take up again in the occurrence of any of the cations tested. In this present study, 9 carbohydrate tested for inhibition in which lactose is most effectively inhibited the HA activity at a comparatively lower concentration of 50mM indicating that the serum HA is

specific for lactose (gal β1-4 glc). The acetylated sugar and mono/polysaccharides are non inhibitory.

Thermal stability and pH

The HA activity in the serum of *V. cyprinoides* was stable between 10 and 30° C, it decreased at higher temperatures and was completely lost at 60° C and above (Fig. 1). The HA activity were remained stable between 10 and 30 C. the activity was completely lost at and above 60 C. When the serum of *V. cyprinoides* was dialyzed against buffers of varying pH, the HA activity was stable only at pH 7, it reduced below and above this and completely lost at pH 5 and 11 (Fig. 1). The HA activity in serum samples was destroyed at or above 40° C and the extreme pH above or less than 7 did not facilitate this activity. The occurrence of natural agglutinins or lectins has been recognized both in gastropods and bivalves (Ractliffe *et al*, 1985; Olafsen, 1986). In the hemolymph of black clam *V. cyprinoides* possess an agglutinin molecule with a highest reactivity with human A RBC among the other human RBC type tested. Only human A RBC were adsorbed all HA activity results the hemolymph of black clam share a common receptor in its HA binding sites. Imai et al (1993) also reported that the hemolymph of red rock lobster *Jasus novaeholland* also agglutinated with human ABO blood groups. Different lectins have been recognized in bivalves and they commonly belong to the C-type lectin family. Proteins from this group of calcium-dependent lectins have been reported in oysters (Minamikawa *et al.,* 2004, Yamaura *et al.,* 2008), scallops (Wang H *et al.,* 2007), and clams (Bulgakov *et al.,* 2004, Gourdine and Smith, 2007). Many lectins of marine invertebrates are reversibly or non-reversibly sensitive to EDTA and require Ca2+ for their activity (Ogawa *et al., 2011*). C-type lectins are characterized by a carbohydrate recognition domain (CRD) with a conserved fold and the involvement of a calcium ion in carbohydrate binding.

The crystal structure of mannose-binding protein was the first one to be described (Weis *et al.,* 1991). The CRD belongs to a larger family sharing a common fold and is referred to as the C-type lectin-like domain (Drickamer K, 1999). The agglutinin molecule in this current study is calcium dependent and sensitive to EDTA. Kim *et al* (2006) reported that the calcium dependent agglutinin molecule in the hemolymph of manila clam *Ruditapes philippinarum* has an opsonic role in the immune system. Acton *et al* reported that the hemagglutinins in the oyster are inactivated by the removal of calcium ions. When the hemolymph of clam treated with EDTA alters the structure of the agglutinin molecule and it prevents binding to RBC/ bacteria in a comparable manner. Fujoyoshi *et al*, (2015) reported that in endemic crab *Shinkia crosnieri*, HA activity were exceedingly thermo stable since it remained 100% active up to 70°C for 30 min and was visible even once heating at 100°C for 30 min against horse erythrocytes. The agglutinin was inactivated completely at 60°Cin this study, based on that we can confirm that it is a protein. Jayasree (2001) reported that in the case of *Penaeus indicus* HA activity was decreased when the temperature increased and completely lost at 85 C and supports our present findings. The serum samples was precipitated with ammonium sulphate and the agglutinin activity were inhibited by lectin having 1- 4 glycosidic linkage. The serum was non inhibitory to monosaccharides, acetylated sugar and laminarin.

Hemagglutination (HA) activity after exposure to heavy metals

In the agglutination activity of treated sample, there is a reduction of titer values which means there is a less agglutination was occurring in the serum with all RBC types; that is the heavy metal affects the activity of agglutinin molecule.

Table 6. Hemagglutinating activity in serum on exposure of *V. cyprinoides* to heavy metals

RBC type tested	HA titer*		
	control	Cadmium	Lead
Human A	32	8 ±16	16
Human B	8	4	4
Human O	16	2	2
Rat	8	2	0
Goat	2	2	4
Sheep	2	0	2

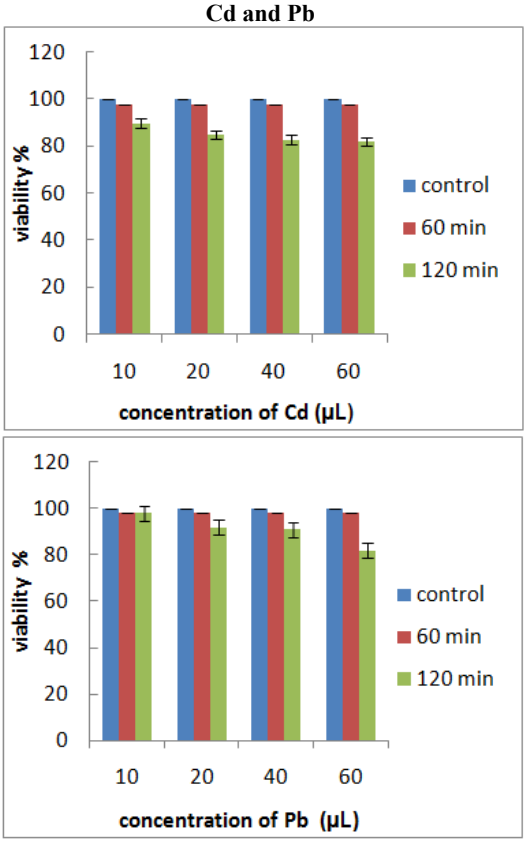
*Values are based on six determinations

Table 7. Total hemocyte count in clams after the exposure to Cd and Pb

Concentration(µL)	THC (× $10^{4\ cells}$/ ml) *		
	Control	Cd	Pb
10	336±8	322±4	320±7
20		288±5	319±3
40		279±2	317±6
60		209±8	245±2

*Values are based on six determinations using serum from different preparation

Graph 2. Percentage of viability after the exposure of animals to Cd and Pb



* Values are based on six determinations using serum from different preparation

Total Hemocyte Count (THC) and viability

The THC in the hemolymph of *V. cyprinoides* ranged between 320 and 345 x 10^4 cells per mL hemolymph. Results show that while increasing the concentration of heavy metals, the total hemocyte count was decreased (Graph 1) On testing the viability of hemocytes by trypan blue dye exclusion technique revealed that viability of cells become decreased while increasing the concentration of heavy metal and time (Graph 2 & 3). The serum was agglutinated with all RBCs in which agglutinating activity was high with human A RBC. But after treating with cadmium and lead there is a reduction in agglutination activity. The total hemocyte count was decreasing while increasing the concentration of heavy metals and time. Previous study shows that while exposing *V. cyprinoides* to cadmium the hemocyte count was decreasing. According to Cheng (1988), heavy metals are lethal to the hemocytes. This may be the reason for the decreased hemocyte count observed in the present experiment. George Frazier and (1983) have reported in cadmium exposed *Ostrea edulis* also results a decrease in hemocyte count.

A reduction in the hemocyte count will affect the immune system because phagocytosis was taking place in hemocyte only and the oyster *Ostrea edulis* from an uncontaminated environment is able to accumulate cadmium faster than the specimens from metal contaminated environment. It's already reported that the black clam *V. cyprinoides* is able to accumulate heavy metals in their body even though the absence in the surrounding water (Likhija and Nalini, 2016). The present study reveals that the activity of agglutinin molecule in the hemolymph of the clams was diminished due to the presence of heavy metals in their body.

Conclusion

From the above findings we can concluded that the agglutinin molecule present in the hemolymph of *V. cyprinoides* are proteins or glycoproteins which is able to recognize and respond to any foreign bodies and have the ability of carbohydrate binding specificity. Accumulation of Cd and Pb in the body of this black clam affects the activity of this agglutinin molecule, THC and viability indicates the turn down of immune response of this organism after the exposure of heavy metals.

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