

## Characterization of a natural hemolysin in the serum of a hermit crab *Clibanarius longitarsus* (Crustacea: Decapoda)

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### Abstract

A naturally occurring hemolysin (HL) was detected in the serum of the hermit crab *Clibanarius longitarsus* using mammalian RBC as indicator cells. The serum gave the highest HL titer with rabbit RBC. Cross adsorption of *C. longitarsus* serum with fixed rabbit RBC resulted in the complete removal of the lytic activity from the serum. The hemolytic activity in the serum of *C. longitarsus* was independent of divalent cations, reversibly sensitive to EDTA, thermolabile, stable between the pH 6-8. Further studies demonstrated that the HL activity is proteinaceous as it was precipitable by conventional deproteinizing agents and susceptible to the action of proteases and 2-mercapthoethanol. The HL-inhibition assay performed with several carbohydrates (mono, di, oligo, polysaccharides and Lipo polysaccharides) revealed that the serum HL was capable of specifically recognizing laminarin (a homopolymer of  $\beta$  1, 3-glucan). Thus this hemolysin appears to be unique among all the known crustacean hemolysins.

**Keywords:** Hemolysin, serum, *Clibanarius longitarsus*, laminarin.

### Introduction

The recent developments in intensive aquaculture practices have led to the outbreak of diseases in cultivable organisms, for the prevention of which the thorough knowledge of the invertebrate immune mechanism is essential. This has provided additional impetus and vigour to research on the mechanisms of immunity in invertebrates and has brought to light the presence of definitive immune reactions exhibiting a considerable degree of specificity and anamnestic quality involving both humoral and cellular component (Sindermann, 1971; Karp & Rhein, 1980; Cooper *et al.*, 1992; Osada *et al.*, 1993).

The cellular defense reactions in invertebrates primarily include coagulating, phagocytic encapsulating, nodule forming entrapment and cytotoxic responses depending on the size, number and nature of the invading foreign organism (Ratcliffe *et al.*, 1985). Invertebrate serum although lacking immunoglobulins has a range of factors which mediate lytic, agglutinating, cytotoxic and antimicrobial activities against various biological reagents. These factors may be naturally occurring or formed after antigenic stimulation. These factors involve in invertebrate defence reactions include lysins, agglutinins, bactericidins, lymphokine-like substances Prophenol oxidase cascade system and lipo polysaccharide binding proteins. Among these components, lysins which are referred to as hemolysins or cytotoxic molecules or killing factors have been reported infrequently and they have tended to receive rather less scrutiny (Smith & Chisholm, 1992).

In invertebrates lysis of invading microbial and macrobial parasites is primarily brought about by the

components of complement system. The presence of lytic activity has been demonstrated in a wide range of invertebrates and investigation into the lytic system of invertebrates has concentrated chiefly on their ability to lyse vertebrate erythrocytes *in vitro*. Lysins are thought to play an important role as serum factors in the immune mechanism of invertebrates (Osada *et al.*, 1993). There are a few reports based on *in vitro* and *in vivo* experiments suggested the possible role of lysins in immune mechanisms. In invertebrates it has been suggested that carbohydrates and lipids on the membrane of targets may be specifically recognised by the hemolysin. In the present study hemolytic activity of the serum of hermit crab *Clibanarius longitarsus* was tested for its ability to cause lysis of RBCs, the physicochemical properties and binding specificity.

### Materials and methods

#### *Experimental animals and laboratory maintenance*

The hermit crab *Clibanarius longitarsus* were collected from Royapuram, sea shore, Chennai. Both male and female hermit crabs were brought to the laboratory. These were maintained in plastic tanks containing marine water and the medium was changed every day. Only healthy uninjured hermit crabs weighing 8.4 to 12.6 g were used.

#### *Preparation of serum*

The hemolymph was withdrawn by inserting the needle of a syringe in to the arthroal membrane connecting the leg and the abdomen region. The samples were collected in polystyrene tubes held on ice and allowed to clot at room temperature (RT) ( $28 \pm 2^\circ\text{C}$ ) for 20 min. It was centrifuged (400 x g, 10 min at RT) and the

resulting clear supernatant (= serum) was used immediately.

#### *Preparation of erythrocyte (RBC) suspension*

Human and other mammalian blood samples were obtained by venous or cardiac puncture and collected in sterile Alsever's solution (Gravey *et al.*, 1979) containing 10 µg/ml of streptomycin. Prior to use, the RBCs were washed thrice with 0.9% saline and once with TBS-II (50 mM tris-HCl, 115 mM NaCl, 10mM CaCl<sub>2</sub> (300 mOsm)) by centrifugation (400 x g, 5 min at RT). Unless specified, the RBC pellet was finally resuspended in TBS-II as 1.5% suspension (v/v).

#### *Hemolytic (HL) assay*

HL assay were performed in V-bottom microtiter plates (Greiner, Nürtingen, Germany) by serial two-fold dilution of a 25 µl of serum sample with an equal volume of TBS-II. After dilution, 25 µl RBC suspensions was added to each well and incubated for 30 min at RT. The HL titers were recorded as the reciprocal of the highest dilution of the sample causing complete lysis of RBC (Garvey *et al.*, 1979). Controls for all assays consisted of the substitution of the sample by TBS-II. All the HL assays were performed in duplicate serum samples.

#### *Determination of percentage hemolysis*

100 µl of sample was mixed with an equal volume of 1.5% RBC suspension. Incubated at RT for 30 min, centrifuged at 400 x g, 5min at RT. A known amount of supernatant solution was diluted 10 fold using TBS-II and the amount of hemoglobin release was determined at 541 nm on a Shimadzu UV-160A spectrophotometer.

$$\% \text{ hemolysis} = \frac{\text{Test release} - \text{Spontaneous release}}{\text{Maximum release}} \times 100$$

Maximum release was obtained with double distilled water, spontaneous release was obtained with TBS-II.

#### *Cross absorption tests*

Serum samples (300µl) were mixed with an equal volume of washed and packed rabbit RBC and incubated for 1 hr at RT with occasional shaking. The suspension was centrifuged, supernatant collected and absorbed for a second and third time under the same conditions. The serum adsorbed thrice was subsequently tested for HL activity against fresh RBC types (Human A, B, O, Sheep and Rabbit RBC).

#### *Cation dependency and EDTA sensitivity*

Serum samples (500 µl) were dialysed (M W exclusion limit <10,000) extensively with TBS-II (to test cation dependency) or TBS-V (50 mM tris-HCl, 100 mM NaCl, 50 mM EDTA (300 Osm) to examine EDTA sensitivity at 17°C. The samples dialysed against TBS-V were subsequently re-equilibrated by dialysis against TBS-I (50 mM tris-HCl, 135 mM NaCl (300 mOsm)). The resulting dialysates were centrifuged and the remaining HL activity in the supernatant was determined using rabbit RBC (RRBC) in the presence of TBS that did or did not contain 10 mM CaCl<sub>2</sub>, MgCl<sub>2</sub>, or MnCl<sub>2</sub>. A serum sample, concurrently dialysed against TBS-II, was also tested for its HL activity against RRBC in TBS-II.

#### *pH and thermal stability*

The stability of HL activity in different pH was examined by dialyzing (24 hrs, 17°C) 300 µl serum samples against the following buffers at pH ranging from 3 to 12 (Lillie, 1954; Pearse, 1968), 0.2 M acetate buffer (pH 3 to 6), 0.2 M tris-HCl buffer (pH 7 to 9) and 0.1M glycine NaOH buffer (pH 10 to 12). After dialysis, all the samples were finally re-equilibrated by dialysis against TBS-II, and the HL titer determined with RRBC. In another experiment designed to study the thermal stability of HL, 300 µl serum samples were held for 30 min at temperatures ranging from 10 to 100°C, centrifuged and tested for the remaining HL activity with RRBC.

#### *Precipitation, susceptibility to proteases and 2-mercaptoethanol (2-ME)*

Precipitation of HL activity from serum was attempted using 20%, 40%, 60% and 80% ammonium sulphate [(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>] solutions as well as 10% trichloro acetic acid (TCA). The HL activity was finally measured using RRBC. Susceptibility of serum HL to exogenous proteases was studied using trypsin at a final enzyme concentration of 20 mg/ml. The serum-enzyme mixtures were incubated up to 3 hrs at 26°C. Controls consisted of serum and heat-inactivated (at 100°C, 10 min) enzyme. The samples collected immediately or after 3 hrs incubation were centrifuged and the supernatants were tested for HL activity using RRBC.

To test the effect of 2-ME, a reducing agent on HL activity, diluted serum samples were treated with 2-ME at a final concentration of 0.4 M up to 4 hrs at 37°C and then dialysed extensively against TBS-II to remove 2-ME. The samples before and after dialysis were tested for HL activity using RRBC.

#### *HL- inhibition assays*

Several carbohydrates (mono, di, oligo, polysaccharides and Lipo polysaccharides) were tested for ability to inhibit serum HL activity. They were dissolved in TBS-II and if necessary, the pH was adjusted to 7.5 using concentrated NaOH. Serum samples were diluted with TBS-II to a HL titer of 4 against RRBC. The inhibitor to be tested (25 µl) was serially diluted two-fold with an equal volume of diluted sample in microtiter plates and incubated for 1 h at RT. RRBC suspension (25 µl) was added to each well and kept for 30 min at RT. The minimal concentration of carbohydrate that completely inhibited HL activity was recorded.

## **Results**

#### *Serum HL profile*

The serum of hermit crab *C. longitarsus* possess a natural hemolysin capable of lytic activity against various mammalian red blood corpuscles (RBC). Among the various RBC types tested, rabbit RBC was found to be the most susceptible target cell, (lytic titer: 64) compared to human A, B, O and sheep RBCs. On the other hand, serum hemolytic titer against ox, buffalo, goat and rat was not detectable. However, the percentage lysis calculated

spectrophotometrically for these RBCs showed range of 25.6 to 48.8% (Table1).

*Table 1. Hemolytic activity of Clibanarius longitarsus serum against various mammalian erythrocyte types*

RBC Types	% Hemolysis	Hemolytic titer
Rabbit	76.2	64
Human O	71.1	16
Human B	69.9	16
Human A	65.5	16
Sheep	55.9	8
Buffalo	45.5	0
Rat	25.6	0
ox	48.8	0
goat	42.5	0

*Based on 5 determinations for each RBC type and the data for % hemolysis represent the mean and the hemolytic titer represents median values*

#### Cross adsorption tests

Cross adsorption tests using rabbit RBC was carried out to check whether the hemolytic activity observed against various RBC types was caused by a single fraction or multiple fractions. Rabbit RBC was used for cross adsorption test after being fixed in formalin to prevent lysis. Adsorption of diluted serum with formalin fixed rabbit RBC resulted in the complete removal of hemolytic activity against human A, B, O and sheep RBCs (Table 2).

*Table 2. Cross adsorption of Clibanarius longitarsus serum hemolytic activity with rabbit erythrocyte*

RBC types tested	Serum absorbed with			
	None		Rabbit	
	Hemolytic titer	% Hemolysis	Hemolytic titer	% Hemolysis
Rabbit	16	77.7	0	0
Human A	04	71.5	0	0
Human B	04	70.1	0	0
Human O	04	70.5	0	0
Sheep	02	52.2	0	0

*Based on 5 determinations and the data of hemolytic titer and % hemolysis represent median and mean values respectively.*

#### Cation dependency and EDTA sensitivity

It was observed that the assay of serum hemolytic activity in the presence of  $Ca^{2+}$ , dialysed against TBS-II remained unaffected as compared to that of undialysed serum. In the samples dialysed against TBS alone or dialysed against TBS-V followed by redialysis against TBS alone, the hemolytic activity was unaffected only in the presence of  $Ca^{2+}$ , whereas the absence of any cations and in the presence of  $Mg^{2+}$  or  $Mn^{2+}$  the hemolytic activity was reduced by 1 well as compared to that of undialysed serum (Table 3).

#### The pH and thermal stability

The hemolytic activity was stable between pH of 6 to 8. The pH tested below or above this range the hemolytic activity was distinctly lower and totally lost at pH 11 to 12. The activity remained stable between 36°C to 40°C and decreased at temperatures tested above and below this range. Noticeably, all hemolytic was lost at 60°C and above.

#### Precipitation, susceptibility to proteases and 2-ME

Both ammonium sulphate at 40% saturation as well as TCA at 10% precipitated all HL activity from serum. Incubation of serum with trypsin immediately reduced the hemolytic titer. No hemolytic activity could be detected in samples incubated for 2 hrs with trypsin. The treatment of serum with 2-ME (0.2 M) reduced the hemolytic titer and this reduced activity did not change after removal of 2-ME by dialysis.

#### Binding specificity of serum hemolysin

Out of 52 carbohydrates, 6 glycoproteins and 2 LPS tested only laminarin a homopolymer of  $\beta$  1, 3-glucan inhibited the serum hemolytic activity at a minimum concentration of 0.5%.

#### Discussion

The serum of the hermit crab *C. longitarsus* possesses naturally occurring hemolytic activity which showed the highest reactivity with RRBC among other RBC types tested. In addition, the hemolysis of mammalian RBCs at varying degrees, indicate a differential affinity of the hemolysin molecules for different mammalian RBCs. Cross adsorption of *C. longitarsus* serum with fixed rabbit RBC resulted in the complete removal of the lytic activity (both percentage lysis and hemolytic titer) from the serum, suggesting the presence of a single hemolysin fraction, with varying degree of specificity, responsible for the lytic activity observed against rabbit, human A, B, O and sheep erythrocytes.

The hemolytic titer of the serum dialysed against TBS alone was reduced by one well in the absence of cations and in the presence of  $Mg^{2+}$  or  $Mn^{2+}$  but in the presence of  $Ca^{2+}$  the hemolytic titer was restored to the level observed for undialysed serum and serum dialysed in TBS-II. Thus, the hemolytic activity in the serum of *C. longitarsus* was independent of divalent cations, in contrast to the hemolysins which have largely been reported to be cation dependant as in the case of echinoderms and tunicates (Canicatti and Parrinello, 1985; Canicatti, 1987; Canicatti and Ciulla, 1988; Canicatti, 1989; Perrinello *et al.*, 1993). In addition, Weinheimer *et al.*, (1969) have reported that the serum hemolysin of spiny lobster *P. argus* was stable up to 7 days in the presence of  $Ca^{2+}$  and  $Mg^{2+}$  and the hemolytic activity was inactivated by EDTA.

However, in the present investigation it was observed that the hemolytic activity in the serum of *C. longitarsus* was only partially susceptible to EDTA, since the serum dialysed against EDTA retained most of the hemolytic activity even in the absence of any cations and in the

Table 3. Effect of divalent cations and EDTA on hemolytic activity of *Clibanarius longitarsus* serum

Serum sample tested	Cation (10 mM) in diluting medium as well as in RBC suspension	Hemolytic titer	Hemolysis (%)
Before dialysis	None	4	70.1
	CaCl <sub>2</sub>	8	72.3
After dialysis against TBS+ 10 mM CaCl <sub>2</sub> (pH 7.5)	CaCl <sub>2</sub>	8	82.2
After dialysis against TBS alone (pH 7.5)	None	4	74.9
	CaCl <sub>2</sub>	8	77.8
	MgCl <sub>2</sub>	4	70.4
	MnCl <sub>2</sub>	4	69.3
After dialysis against TBS+ 50 mM EDTA followed by redialysis against TBS alone (pH 7.5)	None	4	73.9
	CaCl <sub>2</sub>	8	76.0
	MgCl <sub>2</sub>	4	69.1
	MnCl <sub>2</sub>	4	63.8

Determinate using rabbit RBC based on 5 determinations and the data for % hemolysis and hemolytic titer represent the mean and median values respectively.

presence of Mg<sup>2+</sup> or Mn<sup>2+</sup>, the loss in hemolytic activity could be recovered in the presence of Ca<sup>2+</sup>. These observations clearly indicate that the hemolytic activity in the serum of *C. longitarsus* was reversibly sensitive to EDTA and independent of cations, though maximum activity could be observed in the presence of Ca<sup>2+</sup>.

The hemolytic activity in invertebrates has been reported to be thermolabile (Dales, 1982; Canicatti, 1987 & 1989; Leippe and Renwranz, 1988), and the results obtained in the present investigation are in concordance with the above observation. Suggesting that, hemolytic activity observed in the serum of *C. longitarsus* was temperature dependent and may be enzymatic in nature. When dialysed against buffers of varying pH the hemolytic activity in the serum of *C. longitarsus* was found to be stable between the pH ranges 6-8, and drastically affected at pH relatively lower or higher than this range. This observation is in conformity with the report of Kisugi *et al* (1992) on the cytolytic factor in the whole body homogenate of *C. longitarsus*. The crustacean lytic principles are active only in a narrow range of pH.

A major part of the hemolytic activity in the serum of the hermit crab *C. longitarsus* was precipitable by 40% saturated ammonium sulphate and 10% TCA completely precipitated the hemolytic activity, indicating the proteinaceous nature of the hemolytic molecule. This suggestion gains further confirmation, from the fact that treatment of serum with trypsin (a protease) which is known to cleave the proteins specifically between the lysine-arginine residues (Lehninger, 1970), results in the complete loss of hemolytic activity. In this connection it may be noted that the hemolysin from the coelomic fluid of annelids and echinoderms (Parrinello *et al.*, 1979; Roch, 1979; Roch *et al.*, 1981; Canicatti, 1987; Osada *et*

*al.*, 1993), the cytolytic factor from hemocyte of the bivalve *Corbicula fluminea* and *Mytilus edulis* (Yoshino & Tailan Tuan, 1985; Leippe & Renwranz, 1988) are reported to be proteinaceous in nature. The present study indicates that the serum hemolytic factor in the hermit crab *C. longitarsus* is proteinaceous in nature.

The treatment of serum with β-mercaptoethanol, which is known to cleave the disulphide bonds, resulted in the loss of hemolytic activity within one hour; this effect could not be reversed upon removal of β-mercaptoethanol by dialysis. Thus indicating the presence of disulphide linkages on the molecule, and intact disulphide linkages are required for the activity. Studies on binding properties of hemolysin in annelids, molluscs and echinoderms (Decker *et al.*, 1981; Roch *et al.*, 1981; Dales, 1982; Canicatti & Parrinello, 1985; Canicatti, 1987, 1989, 1991) have shown the hemolysins could specifically recognize sugars or phospholipids. In the present investigation,

out of the wide range of sugars, glycoproteins and lipopolysaccharides tested, only laminarin a homopolymer of β 1, 3-glucan inhibited the hemolytic activity against rabbit RBC. Thus the present study constitutes the first report on the specific binding of a crustacean hemolysin to laminarin (an algal surface component), providing an insight into the molecular nature of hemolysin in host-defense in crustaceans.

From the results of the present investigation it may be concluded that the hemolysin of the serum of *C. longitarsus* is a unique cation independent homogenous protein with ability to cause lytic activity and capable of specifically recognizing laminarin (a homopolymer of β 1, 3-glucan).

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