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Original Research Article

Laboratory Studies on the effect of pronase induced melanization reaction in the haemocyanin of *Scylla serrata*

P.B.Harathi* and R.Radha

Department of Zoology, PSGR Krishnammal College for Women
Coimbatore, Tamilnadu, India
**Corresponding author*

A B S T R A C T

Crustaceans are a large and diverse invertebrate animal group that contains a complex and well-organized innate immune response against a variety of microorganisms. In crustaceans, the defense system against microbes rests largely on cellular activities performed by haemocytes such as adhension,phagocytosis, encapsulation, nodule formation and melanisation. Hence an investigation has been conducted to generate melanization reaction in the mud crab, *Scylla serrata* by treatment with pronase. Experimental studies performed *in vitro* had shown that inactive phenoloxidase could be activated directly by treatment with exogenous protease or detergents. The optical density of pronase treated samples increased from 0.127 to 0.424 at 540 nm. The serum of *S. serrata* was further fractionated into haemocyanin and clarified serum (depleted from haemocyanin) and tested their responsiveness to pronase-treatment. Pronase treatment of clarified serum did not significantly induce colour change as well as its optical density at 540 nm. Treatment of haemocyanin with pronase resulted in change in colour of the serum from light blue to black. The optical density of these samples also enhanced from 0.049 to 0.173. The relationship among melanization reaction and HA activity generated after treatment of serum of *S. serrata* with pronase was analyzed by adding phenyl thio urea to pronase treated serum. This inhibited melanization reaction but did not affect the induction of HA activity.

K e y w o r d s

Scylla serrata, Fractionated serum, Clarified serum, Haemocyanin, Melanization reaction

Introduction

Invertebrate immunity is based on cellular immune system comprising amoebocytes, coelomocytes or haemocytes (Gupta 1991) and humoral immune system, which include prophenol oxidase (pro PO) system, agglutinins and antimicrobial proteins (Asokan *et al* 1998, Mercy et al 2004). These humoral components appear to interact with haemocytes and potentiate haemocyte-mediated cellular immune responses against foreign invaders.

Crustaeans are the most numerous, diverse and prevalent animals on earth along with insects. Crustaceans lack the complex and highly specific adaptive immune system of

vertebrates, which is based on lymphocytes, immunoglobulins and immunological memory(Smith *et al*;1992). Their internal defenses rely only on innate immune responses that are relatively less specific, but are fast and efficient defenses against microbes. The innate immune system of crustaceans is primarily related to their blood or haemolymph and is comprised of cellular and humoral responses(Johansson *et al*;1985). Humoral defenses include pattern-recognition receptors/proteins that recognize pathogen-associated molecular patterns (PAMPs), the production of toxic oxygen and nitrogen metabolites, complex enzymatic cascades leading to melanization, clotting proteins and antimicrobial peptides.

Among invertebrates, insects and crustaceans have been extensively studied for the role of pro PO system in host immune responses (Cerenius & Soderhall, 1995). This system consists of inactive phenol oxidase and at least one serine protease. Pro PO could be activated through serine protease and microbial cell wall components are known to be potential activators of proPO through this pathway. The resulting PO appears to participate in haemocyte mediated cellular immune reactions such as melanotic and encapsulation, nodulation and generation of cytotoxic molecules. Experimental studies have shown that proPO could be activated directly by treatment with exogenous proteases or detergents (Asokan *et a*l 1997).

The proPO is an efficient non-self recognition system in invertebrates and recognizes foreign materials from microbes(Wang *et al*; 2001). Due to the activation of proPO by invading microorganism/parasite(s) the invaded parasites becomes blackened in the host haemolymph. This blackening is because of the melanin and the process is called melanisation which is a very important immune response in many invertebrates. On activation of this system the associated proteins achieve biological activity and thus involve themselves in the cellular defense reaction of the host animal (Sara Jones and Khan ,2010). Thus the crustacean immune system is primarily related to cellular responses and the production and release of important immune effectors into the haemolymph(Gargioni *et al*;1998). Hence, the objective of the present study is to generate pronase induced melanization reaction in the fractionated serum -clarified serum and haemocyanin of the mud crab, *Scylla serrata.*

Materials and Methods

Collection of test animals

The live mud crab, *Scylla serrata* were purchased from a local market, Saidapet, Chennai. The crabs were acclimated under laboratory conditions in plastic troughs with enough water at 19°C for one week prior to experimentation. They were fed ad libitum.

Collection of haemolymph

Haemolymph was collected from the cut end of dactylus region of the crab into a polystyrene tube, which was held on ice, left undisturbed for 15 min at 26°C. The clot was disturbed using a clean glass rod and centrifuged (300 µg; 5 min). The clear supernatant(serum) was used for the analyses. Serum was fractionated into clarified serum and haemocyanin following the procedure described previously (Murray & Jeffrey 1974).

Assay of melanization reaction: The supernatant obtained from each sample after reaction with appropriate reagents were diluted to 2 ml using TBS II. Its optical

density was measured at 540 nm against a blank consisted of 2 ml of TBS II in Shimadzu UV-160A Spectrophotometer.

HA activity: To study the effect of PTU on pronase induced HA activity, only unfractionated serum and hemocyanin solution were 32 fold diluted with TBS II whereas clarified serum was used as such. To these samples PTU and pronase were added and incubated at 37°C for 80 min in a water bath and supernatant from each sample was used for the assay of HA activity against rat RBC.

Result and Discussion

Pro-phenol oxidase is an important enzyme in cuticular sclerotisation and melanisation (Ashida *et al*;1990). It also plays an important role in the defense against pathogens and parasites, in insects and crustaceans.Naturally occurring proPO system exists in an inactive state and exogenous proteases triggers its activation. In preliminary studies, we found that the colour of the serum samples from *S. serrata* incubated with pronase, a non-specific protease turned black. This observation clearly indicated not only conversion of proPO into PO upon proteolytic action of pronase, but also the viability of naturally occurring phenolic substrates in the serum of *S. serrata* , which is readily oxidized by PO. The respiratory pigment haemocyanin represents 80-95% of total protein in the haemolymph of decapod crustaceans (Jeuniaux 1971). Moreover a study has demonstrated protease-mediated induction of PO activity in the haemocyanin of the tarantula *Eurypelma calcifornicum* (Decker & Rimke, 1998). Therefore, serum of *S. serrata* was further fractionated into haemocyanin and clarified serum (depleted from hemocyanin) and tested their responsiveness to pronase-treatment. Upon treatment with pronase, generation of melanization reaction, was found to be primarily associated with haemocyanin, as reported earlier in the tarantula (Decker & Rimke 1998). The relationship if any among melanization reaction and HA activity generated after treatment of serum of *S. serrata* with pronase was analysed by adding PTU to pronase treated serum. This inhibited melanization reaction but did not affect the induction of HA activity. These findings appear to indicate that PO, generated as a terminal product of ProPO activation cascades upon pronase treatment of serum, is not directly responsible for pronase-induced HA activity in the serum of *S. serrata*.

Treatment of unfractionated serum with pronase changed the colour of serum to black within 40 min. The optical density of pronase treated samples increased from 0.127 to 0.424 and this change is significantly higher than those observed with trypsin and alpha-chymotrypsin. ($p<0.001$) (Table1).

On the other hand, pronase treatment of clarified serum did not significantly induce colour change as well as its optical density at 540 nm. Treatment of hemocyanin with pronase resulted in change in colour of the serum from light blue to black. The optical density of these samples also enhanced from 0.049 to 0.173 and this change is statistically significant ($p<0.01$) compared to that observed with protease-treated clarified serum (Table 2).

In the presence of PTU, pronase did not induce visual colour change in unfractionated serum, clarified serum and hemocyanin. The optical density of unfractionated serum and hemocyanin in particular was significantly reduced in the presence of PTU (Table 3). The presence of

PTU strongly inhibited pronase-induced melanization reaction but did not affect pronase-induced generation of HA activity in clarified serum and hemocyanin (Table 4).

The outcome of this study, nevertheless, elucidates the presence of protease-sensitive inducible humoral immune systems in the serum of *S. serrata* . It is pertinent to note that several infectious organisms such as bacteria (Lee *et al* 1997) as well as parasitic helminthes are known to produce extracellular proteases. It is therefore, likely that the protease released by infectious organisms *in situ* might induce melanization reaction in the hemolymph of the infected host. Indeed, these reactions can very well serve adaptive immune functions against potential pathogens in the marine crab *S. serrata*

Table.1 Effect of various exogenous proteases on induction of melanization reaction in the serum of *S. serrata*

Serum treated with	Melanization reaction		
	Visual colour change	Untreated	Enzyme-treated
Trypsin(5mg/ml)	No change	0.084 ±0.005	0.107 ±0.005
α-chymotrypsin(5mg/ml)	Light blue to brown	0.077 ±0.017	0.127** ±0.023
Pronase(5mg/ml)	Light blue to black	0.127 ±0.026	0.424*** ±0.030

*- Asterisk indicates statistical significance : * $p<0.05$; ** $p<0.01$;
*** $p<0.001$; Mean difference Student *t*-test.

Table.2 Effect of pronase on induction of melanization reaction in the unfractionated serum, clarified serum and haemocyanin of *S. serrta*

Sample tested	Melanization reaction		
	Visual colour change	Untreated	Pronase-treated
Unfractionated serum (control)	Light blue to black	0.127 ±0.026	0.424 ±0.030
Clarified serum	No change	0.010 ±0.000	0.017 ±0.001
Haemocyanin	Light blue to black	0.049 ±0.003	0.173** ±0.006

*- Asterisk indicates statistical significance; ** $p<0.01$; Mean difference Student *t*-test.

Table.3 Effect of Phenylthiourea on pronase-induced melanization reaction in unfractionated serum, clarified serum and hemocyanin of *S. serrata*

S.No	Samples tested	Treatment of samples		
		Sample+Buffer	**Sample+Buffer**	**Sample+PTU+ Pronase**
1	**Unfractionated serum** Visual colour change	No Change	Light blue to black	No Change
	Δ 540 nm	0.055 ±0.018	0.169*** ±0.000	0.048*** ±0.021
2	**Clarified serum** Visual colour change	No Change	No Change	No Change
	Δ 540 nm	0.013 ±0.001	0.016 NS ±0.002	0014 NS ±0.001
3	**Haemocyanin** Visual colour change	No Change	Colourless to black	No Change
	Δ 540 nm	0.024 ±0.002	0.060** ±0.002	0.016*** ±0.000

Values represent mean SD from 4 determinations.
*- Asterisk indicates statistical significance : p<0.01;
*** p<0.002 or 0.001; NS: Not statistically significant;
Mean difference Student *t*-test.

Table.4 Effect of Phenylthiourea on pronase-induced hemagglutinating (HA) activity in unfractionated serum, clarified serum and haemocyanain of *S. serrata.*

S.No	Samples tested	Treatment of samples (HA titer)*		
		Sample+Buffer	**Sample+Buffer**	**Sample+PTU+ Pronase**
1	Unfractionated serum	0	256	256
2	Clarified serum	0	64	64
3	Haemocyanin	0	128	128

*- Assayed using rat RBC
Serum was diluted to a HA titer of 0 against rat RBC. Values given for enzyme-treated samples represent median titer values from 4 determinations.

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