Prophenoloxidase Activation Pathway and its Role in Freshwater Crab, Barytelphusa Cunicularis (West-wood) Immunity

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Abstract: The prophenoloxidase activating system (proPO-AS) in fresh water crab, Barytelphusa cunicularis is localized in the hemocytes. Using anticoagulant (EDTA-Citrate buffer) it was possible to isolate intact hemocytes without causing extensive degranulation & subsequent clotting. Activation and conversion of prophenoloxidase to phenoloxidase(PO) was carried out by serine proteinase. PO was found to have molecular range between 60-70kDa. PO showed increased activity in the presence of elicitors such as β-1, 3 glucan, SDS & Calcium. The activation was dependent upon Ca ++ ions and occurs with limited proteolysis. β-1, 3 glucan, SDS and Ca ++ activated the peptidase in HLS. Active PO strongly bound to foreign surfaces and this phenomenon may assist in providing opsonic properties for the proPO cascade.

Key words: proPO-AS, encapsulation, melanisation, opsonic, crab

INTRODUCTION

Invertebrate animals are widely distributed in nature and are found in all kind of habitat. The dispersal and survival of these invertebrates depends upon their adaptation to defend against the invading microorganisms/ parasites in environment. These adaptations are achieved by innate immunity, as invertebrate lack an adaptive immunity. However, Mendoza and Faye [26] reported proteins with immunoglobulin domain in invertebrate animals, yet these animals depend upon innate immunity.

One of the mechanisms in invertebrate immunity is the production of antimicrobial peptides in response to parasites entry. The regulation of genes for these antimicrobial peptides [12] and there response in insects [26] have been studied in great detail. The production of antimicrobial peptide is normally delayed for few hours after entry of bacteria and fungus. Thus, it becomes evident that some sort of recognition of foreign materials is taking place in order to transfer the message to the cell to synthesize appropriate immune factors. This recognition is made possible by recognition molecules called pattern recognition molecules present in the hemolymph. The pattern recognition molecules serve as biosensors for detection of invading pathogens in the innate immune system of vertebrate and invertebrate animals [14,23]. The pattern recognition molecules bind to certain pathogen associated molecular patterns that are not found in the host, such as, lipopolysaccharide or peptidoglycan from bacterial walls and β-1, 3 glucan from fungal cell walls. Upon binding foreign molecules, pattern recognition protein trigger defense pathways such as complement system in vertebrate [24] and the prophenoloxidase (proPO) activation pathway in insects and other arthropods[1]. These recognition molecules activate prophenoloxidase activating system (proPO-AS) and thus induce an activation of other defense system. The proPO system involves a protease cascade. Wang [33] reported that prophenoloxides activating enzyme is a serine proteinase catalyzing enzyme with a molecular mass of 36kDa, which converts prophenoloxidase to active phenoloxidase.

The proPO-AS, an efficient non-self recognition system in invertebrates and recognizes lipopolysaccharides (LPS)/ peptidoglycan from bacteria and fungi [29,30,11]. Due to the activation of proPO-AS by invading microorganism/parasite(s) the invaded parasites becomes blackened in the host hemolymph. This blackening is because of the melanin and the process is called melanisation. ProPO-AS consists of several proteins among them are proteinase, proteinase inhibitors and recognition molecules that recognize the structural features of bacterial and fungal components. On activation of this system the associated proteins gain biological activity and thus participate in the cellular defense reaction of the host animal.

Dularay and Lackie[9] reported that lysate derived proteins bind to negatively charged beads and this
could provide a useful model system to ascertain whether any of the adsorbed protein are opsonins. They further reported that the encapsulation in hemolymph was not formed in-vivo but in-vitro. The present investigation was carried out in fresh water crab, Barytelphusa cunicularis (West –Wood) to ascertain the properties of phenol oxidase from HLS and role of peptidase in prophenoloxidase activating system, opsonization and encapsulation.

**MATERIALS AND METHODS**

The fresh water crab, Barytelphusa cunicularis (West –Wood) were procured from Nathasagar Dam, Paithan near Aurangabad, Maharashtra, India. 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. Only male crabs of intermoult stage of uniform carapace length were used for the experiment. The proPO system in this crabs was studied in hemocytes by preparing hemocyte lysate supernatant (HLS).

1. **Preparation of Hemocyte Lysate Supernatant (HLS):** The hemolymph from fresh water crab was drawn by injecting 400ml of anticoagulant solution (0.08 M Tri-Sodium Citrate, 8mM Citric acid, 0.145 M Sodium Chloride, 0.01 Di-Sodium Salt of Ethylene Diamino Tetra Acetic Acid, pH 6) into the abdominal hemocoel through the arthropodial membrane of 4th walking leg. After three minutes, the hemolymph was collected by hypodermic syringe from chelicerae. The hemocytes were washed twice in buffer I and the final supernatant was centrifuged at 8000 x g for 5 minutes at 4°C. The collected supernatant was centrifuged at 10000 x g for 30 minutes at 4°C. The final supernatant thus obtained was used for the experiment. The proPO system in this crabs was studied in hemocytes by preparing hemocyte lysate supernatant (HLS).

2. **Assay of phenoloxidase activity (PO):** The Phenoloxidase activity was assayed according to the method described by Soderhall[31] with L-DOPA (4mg/ml in 0.01 M Sodium acetate buffer, pH6) as substrate. The unit of PO was defined as the amount of an enzyme, which produces an absorbance increase of 1 at 490nm. The protein concentration of HLS was assayed by U.V Spectrophotometer at 280nm. The Bovine Serum Albumin (Sigma) was used as standard.

2.1 **Effect of Calcium ions on PhenolOxidase (PO) Activity:** The concentration of Ca++ in buffer-II was varied from 5-25mM to determine the concentration required for the optimum PO activity. The optimum concentration thus determined was used for subsequent studies. The HLS without Ca++ was prepared after pooling the collected hemocytes in a modified buffer I to which 0.1 M EDTA was added at pH 7. The hemocytes was washed twice in the EDTA buffer (50mM EDTA, 10mM Sodium Cacodylate) and homogenized in EDTA buffer pH 7.0. The supernatant was dialysed against 10mM Cacodylate buffer, pH 7 to remove the EDTA, before assaying for PO activity.

2.2 **Effect of zymosan supernatant on proPO activity:** Zymosan (0.2%) was dissolved in distilled water by agitating the mixture and centrifuging them. The collected supernatant was centrifuged at 10000 x g for 5 minutes. The final supernatant thus obtained was of zymosan (Zs); a source of β –1, 3 glucan. The Zymosan was incubated with HLS in the volume ratio of 1:4 for 30 minutes at 19°C. In the control, same volume of buffer II was added in place of Zs and was assayed for PO activity.

2.3 **Effect of Bacterial Lipopolysaccharide (LPS) on Pro-PO:** The effect of bacterial lipopolysaccharides (LPS) from E.coli was studied on the proPO activity. Different concentrations of LPS were injected in different crabs of same carapace length. After one hour of injection, the HLS preparation was made and was analyzed for PO activity.

3. **Assay for Peptidase Activity:** An aliquot of 50ml of HLS was pre-incubated with an equal volume of laminarin or in case of control with cacodylate buffer for 1hour at 19°C. To this an aliquot of 300ml of 0.1M TrisHCl buffer pH 8.0 and 100µl of 2mM synthetic peptide (insulin) was added. After incubating at 30°C for 30 minutes, the reaction was terminated by adding 100ul of glacial acetic acid. The absorbance was read at 405nm. The enzyme activity was expressed as the change in absorbance at 405nm/min/ µg of protein. The activity of peptidase was tested with several elicitors like β-1, 3 glucan, Sodium dodecyl sulphate (SDS) and calcium ions.

4. **Preparation and Assay of PO and Protein Coated Sepharose Beads:** Negatively charged Sepharose beads [CM-Sepharose 6B-C1 Pharmacia] was coated by incubation of a known volume of beads [10-20ml] in 100ml of Ca++ activated HLS for 3hours at room temperature. To ascertain if PO had adhered to the beads the coated, washed beads was dispersed in 10% gelatin solution in a small petridish and the mixture was overlaid with L-DOPA (Sigma-Aldrich) as substrate solution.
5. Estimation of Carbohydrate: The estimation of the carbohydrate was carried out by converted Phenol Sulphuric acid methods [8].

6. Estimation of Protein: The protein concentration was estimated by UV spectrophotometer (Cole-parmer) at 280 nm.

RESULTS AND DISCUSSION

Properties of Phenoloxidase: The phenoloxidase activity in the presence of anticoagulant showed a 33% decrease /inhibition in plasma. The hemolymph harvested in anticoagulant was determined to ascertain whether the proPO system was cell associated or wholly or partially present in the plasma. It was found that most of the PO activity was present in the HLS preparation (Fig.1). This observation suggests that hemocytes are the main repository of the proPO system.

Effect of Calcium Ion Concentration: The role of calcium ion on the activation of proPO system was studied by adding different concentration of calcium ions to HLS aliquot, and the PO activity was assayed. Maximum PO activity was observed at a concentration of 10mM calcium ions. On increasing the concentration of Ca++ a decrease in PO activity was found. When HLS was treated with anticoagulant EDTA, a highly significant decrease was found (Fig. 2).

Effect of Zymosan Supernatant on Pro PO Activity: The zymosan treated HLS showed an increased specific activity of ProPO. The increase was statistically (P<0.01) significant (Fig. 3).

Effect of Lipopolysaccharide on Pro PO: The LPS treated fresh water crab showed a statistically significant response in the PO activity. However, the activity was dose dependent. At low dose, it showed a statistically (P<0.01) significant increase (Fig.4).

Peptidase Activity: The peptidase activity in the HLS was studied in the presence of elicitors like B-1, 3 glucan, sodium dodecyl sulphate and calcium It was found that SDS was the most potent elicitors of the peptidase followed by b-1,3,glucan and calcium ions (Fig.5) . When SDS and B-1, 3 glucan were added in the reaction mixture a significant increase in the peptidase activity was found. The increase in the peptidase activity with calcium, SDS and B-1, 3, glucan was 1.4, 3 and 6 times respectively when compared to control peptidase. 10mM calcium which showed highest PO activity was found to inhibit peptidase activity.

To Study the Adherence of PO to Foreign Particle: When negatively charged sepharose beads were used to study the adherence it was found that a dark brown color was formed around the beads suggesting that adherence of PO to beads to foreign particles. An attempt to find such an effect in in-vivo study failed, thus it appears that melanisation is brought about in-vitro and not in-vivo (Fig. 6).

Discussion: Phenol oxidase (Monophenyl L-dopa: oxygen oxido-reductase ;EC: 1.14.18.1) is an important enzyme in cuticular sclerotisation and melanisation. It also plays an important role in the defense against pathogens and parasites, in insects and crustaceans [1,30,32]. In arthropods this enzyme is synthesized as an inactive zymogen, prophenoloxidase (proPO) which is activated by proteolytic cleavage at a specific site near the proteins amino terminus. ProPO has been isolated and characterized from several invertebrate animals. In all the animals, where, proenzymes has been purified, the monomer has a mass of about 70-80 kDa after proteolytic activation. The active enzyme, has a mass of 60-70kDa [5,6,7,10,16,18].

The enzyme PO from freshwater crab, Barytelphusa cunicularis (West Wood) was purified and isolated from hemocyte lysate supernatant (HLS). It was difficult to obtain phenoloxidase in its proenzyme form as the enzyme was found to be frequently activated after bleeding. The anti coagulant EDTA –citrate, when used in the HLS showed a 33% decrease in PO activity. This confirms that hemocytes are the main repository of the proPO system.

Studies carried out to ascertain the optimum calcium ion required for the conversion of proPO to PO was found to be 10mM. Ashida and Soderhall [2] reported 15mM calcium as optimum concentration. The role of calcium in the conversion of proPO to PO was reported by various workers [2, 3]. It was reported that nonself molecules require calcium ions. However, the activating enzymes in B. mori proPO can convert proPO to PO in an active form in the absence of calcium ions [3]. Ashida and Soderhall [2] reported that calcium concentration was important at high concentration the proPO system was very stable and could not be activated. An inhibition in proPO activation at higher concentration of calcium ion was found in the present study. Similar, results were found in insects and crustaceans [3,27].

In arthropods proPO system, a threshold concentration of calcium is required for proPO activation [3,22,4] suggesting that calcium plays an important role in this process. Recently, Kwon [17] reported a gene coding for 45kDa protein. When this protein was incubated in the absence of calcium a limited proteolysis of 45kDa protein was observed with
Fig. 1: Phenoloxidase activity in hemocyte lysate supernatant (HLS)

Fig. 2: Effect of EDTA-citrate buffer (anti coagulant) and different concentration of calcium ions on the phenoloxidase activity in HLS

Fig. 3: proPO activity in HLS after treatment with Zymosan supernatant. (statistical significance: = P<0.01)

Fig. 4: Effect of different concentration of LPS on proPO activity
Fig. 5: Effect of elicitors on the peptidase activity

Fig. 6: Negatively charged Sepharose 6B beads incubated in HLS for 3 hours at room temperature, washed and dispersed in 10% gelatin solution and overlaid with substrate solution (L-DOPA) showed dark brown color around the beads.

Simultaneous PO activity. In the presence of calcium ions, the 45 kDa was not cleaved; however, PO activity was detected. At present the role(s) of calcium ions in these reactions are unclear. According to Kwon [17], the calcium ion may protect the cleavage of the 45kDa protein by conformational changes or calcium ions may mediate the binding of proPO. We require confirming the role(s) as suggested by Kwon [17]. It was found that at 10mM calcium the PO activity was highest, whereas, at low and higher concentration showed a less PO activity. The peptidase activity at 10mM calcium showed an decrease when compared to control. This suggests that at 10mM concentration of calcium, which showed highest PO activity, inhibit the peptidase activity by some mechanism, not yet known. When SDS and β-1, 3 glucan was added to the reaction mixture a significant increase in the peptidase activity was found. The increase in the peptidase activity in the presence of calcium, SDS and β-1, 3 glucan was 1.4, 3 and 6 times respectively more when compared to control peptidase. This raises a pertinent question as regard to the activation of peptidase by SDS and β-1, 3 glucan. The question is whether the mechanism of activation of peptidase by SDS and β-1, 3 glucan are different than the activation associated with calcium? It has been proposed that calcium may protect the cleavage of the protein by conformational changes or calcium may mediate the binding of proPO [21]. Recently, it was reported that calcium activates the proPO by cleaving the zymogen form of the serine proteinase [15]. We propose that activation of the peptidase must be restricted by some intracellular signals, since its activation may have different checkpoints. The higher concentration of 10mM may either bind proPO as suggested by Know [17] or the other elicitors, like SDS and β-1, 3 glucan may activate a path other than, the calcium pathway. Several kinds of serine proteinase homologues have been purified in vertebrates and invertebrates, and have been suggested to have different biological functions, such as antimicrobial activities [13], Pacifastacus masquerade-like protein [26]. It was reported that proPO activating system, which function as nonself recognition and defence responses in invertebrates is composed of an enzyme cascade consisting of several serine proteases and proPO [14, 1].

The native plasma of the B. cunicularis strongly enhanced the activation of the hemocytes derived proPO and Peptidase by zymosan, calcium and SDS. The plasma by itself did not trigger the peptidase or PO activity.
Yu and Kanost [34] reported that a plasma protein hemolin derived from Manduca Sexta has two binding sites from LPS, one that interact with phosphate group of lipid A and one that interact with O- specific antigens and the outer core carbohydrate of LPS. They also reported that the binding of the plasma protein hemolin is partially inhibited by calcium. Based on the observation we propose that a plasma protein from the crab may be synthesized to microbial challenges. The increased activity with low plasma protein must be binding LPS from the gram-negative bacteria. The increased activity with low concentration may not inhibit activation however, with increase in concentration it did. How exactly, this is brought about is not well understood.

Dularay and Lackie [9] reported that a variety of lysate derived proteins could bind to the negatively charged beads; this provided a useful model system to ascertain whether or not any of these adsorbed proteins were opsonic. The opsonin induced encapsulation. Often normally unencapsulated beads are easy to assay. The phenol oxidase and other protein in lysate may act as opsonin by adhering to foreign surfaces and thus encouraging adhesion of hemocyte. Similar, observation was reported by Soderhall and Smith [28]. This occurs because of hemocyte degranulation upon contact with a foreign substratum. The activated phenol oxidase is responsible for synthesis of melanin around invaded foreign material. It is well known that the quinine derivative generated by the activated PO might use endogenous protein components for melanin synthesis in arthropods. In our present study, the melanisation was found on sepharose granules; how activated PO synthesized melanin in in-vitro and its mechanism at molecular level is not understood. There is no report of protein components engaged in melanin synthesis induced by activated PO. The attempt to characterize this melanisation engaging protein was reported to be a 160kDa vitellogenin like protein, which is involved in arthropod melanin synthesis [31].

In conclusion our experiments demonstrated that hemocytes are the main repository of proPO system and that activated PO synthesized melanin in vitro. Elicitors like SDS, β-1, 3 glucans significantly increased peptidase activity but 10mM Calcium concentration which showed highest PO activity inhibited peptidase activity. The mechanism at molecular level is yet to be understood.

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REFERENCES


