

Study of prophenoloxidase activating system of freshwater crayfish (*Pontastacus leptodactylus*)

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Abstract: Phenoloxidase (Po) activity was measured spectrophotometrically in serum, plasma and haemocytes (HLS) of the freshwater crayfish (*Pontastacus leptodactylus*). The highest activity was found in HLS suggesting that the haemocytes are the major source of the Po or its proform prophenoloxidase (proPO) in crayfish. Furthermore, the enzyme activity in serum samples was reduced after freezing the samples for 20 days at -20°C. Po activity was cation-dependent and the peak of enzyme activity obtained using 5mM of CaCl₂ or MgCl₂. Also, the Po activity in the HLS samples treated with different elicitors of 10% sodium dodecyl sulphate (10%SDS), β 1-3-glucan, *Aeromonas hydrophila* lipopolysaccharids (LPS) and trypsin resulted in variable activities with the highest and the lowest activities measured in samples treated with trypsin and 10% SDS, respectively. The marked enhancement induced by trypsin suggest the role of an endogenous proteinase which is probably able to promote the enzyme activity. To confirm the role of the proteinase on the proPO activating system, trypsin activity was also measured in the crayfish HLS.

Keywords: Crayfish, *Pontastacus leptodactylus*, Prophenoloxidase system, Haemocytes proteinase, Trypsin, Lipopolysaccharids, Glucan

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Introduction

The crayfish (*Pontastacus leptodactylus*) is a dominant species in the Iranian aquatic environment and is economically an important resource of aquatic organisms. It is mainly distributed in the north-west rivers of Iran. Results of preliminary works by Iran Fishery Research Institute show that this animal is a valuable species as a new candidate for commercial aquaculture industry. Immunophysiological studies can be very useful not only to improve their growth conditions under intensive culture, but also to prevent and control the occurrence of the crayfish diseases on the farms.

The so-called proPO activating system in crustaceans consists of several components such as recognition molecules, serine proteinases and proteinase inhibitors (Soderhall & Soderhall, 2002 ; Aspan *et al.*, 1995; Soderhall & Cerenius, 1998 ; Cheng *et al.*, 2002). This system plays a key role in immune defence of crustaceans. The proPO activity can be triggered by several components of micro-organisms' cell wall including lipopolysaccharids, peptidoglycans and β -1,3-glucans which in turn can be recognized by recognition molecules (Stritunyalucksana & Soderhall, 2000 ; Holmblad & Soderhall, 1999 ; Lee, 2001). It is assumed that there are some receptors on the crustaceans haemocytes specific for the recognition molecules and their interaction leads to serine proteinase activation which in turn converts the proPO to Po, an oxidoreductase. Upon Po activation phenols are oxidised to quinones and quinones polymerized to melanin (Soderhall & Soderhall, 2002 ; Soderhall & Cerenius, 1998). Po is able to convert tyrosine to Dopa as well as Dopa to Dopa-quinone, followed by several intermediate steps which finally lead to the synthesis of a brown pigment, melanin that possesses an antimicrobial activity. Also during melanization some mediators are released which are toxic to micro organisms (Aspan *et al.*, 1995 ; Soderhall & Soderhall, 2002 ; Stritunyalucksana *et al.*, 2000 ; Soderhall & Cerenius, 1998 ; Holmblad & Soderhall, 1999).

The role of cations, Ca^{++} and Mg^{++} in defence mechanisms of crustaceans has been reported by some authors (Perazzolo & Barracco, 1997 ; Gollas-Galvan *et al.*, 1997). However there is no enough information on cation dependence activity of proPO system in *P. leptodactylus*. Therefore, the aim of this study was to investigate the proPO activating system of *P. leptodactylus* as a potential candidate for aquaculture industry in order to improve the crayfish immunity during cultivation period.

Materials and Methods

Animals

Forty adult males of crayfish (*P. leptodactylus*) with mean carapace length of $47 \pm 0.5 \text{ mm}$ and mean body weight of $60.4 \pm 1.9 \text{ g}$ were provided by Natural Resources Research Center, Urmieh, west Azerbaijan, Iran. Only intermoult and apparently healthy animals were used in this experiment. The animals were kept in a 1000 liter tank under normal day-night illumination and water temperature between $18\text{-}20^\circ\text{C}$ with a continuous water flow. They were fed with chicken gizzard twice a week. Other water quality parameters including NH_3 , NO_2 , dissolved oxygen and CO_2 were at acceptable levels.

Haemolymph Sampling and Serum preparation:

Samples (1ml) of haemolymph were withdrawn from the perioarthrodal membrane at the base of the walking legs using a 22.¼ gauge needle. Samples were then transferred to glass tubes allowed to coagulate and stored at 4°C for 24 hours and centrifuged at 2000g, for 20 minutes at 4°C to remove the sera. Samples were then frozen for 20 days at -20°C prior to use for determination of po activity level (Perazzolo & Barracco, 1997).

Haemocyte lysate supernatant (HLS) and plasma preparation:

Haemolymph of each crayfish (n=40) was withdrawn into a 2ml sterile syringe containing 0.5ml anticoagulant solution (0.015M potassium dihydrogen phosphate, 0.01M sodium citrate, 0.14 M NaCl, 0.15M glucose, 0.02M citric acid, pH 4.6). The diluted haemolymph of 6 crayfish (10ml) were centrifuged at 100g for 10 minutes at 4°C and the supernatant (plasma) was removed. The cell pellets were first washed in 0.01 M sodium cacodylate (100mM CaCl₂ pH 7.0) and then homogenized in 0.01M sodium cacodylate (5mM CaCl₂, pH 7.0). Homogenate centrifuged at 20000 x g for 20 minutes at 4°C and the resulting supernatant was used immediately in the experiments (Soderhall & Soderhall, 2002 ; Perazzolo & Barracco, 1997).

Phenoloxidase activity assay in serum, plasma and HLS:

The phenoloxidase activity of sera, plasma and HLS samples were measured using procedure described by Soderhall and Soderhall (2002) and Perazzolo and Barracco (1997). A 50μL of each sample of serum, plasma and HLS (n=40) were preincubated with 50μL of the elicitors (1mg/ml) consist of 10% SDS, β-1, 3-glucan (Sigma), *A. hydrophila* LPS and porcine trypsin (Sigma) at room temperature for 30 minutes. The samples then received 50 μL of chromogenic substrate L-DOPA (3mg/ml), and then 850μL of distilled water to slow the enzymatic reaction. The optical density was measured at 490nm after 5, 10 and 20 minutes spectrophotometrically. The control samples were used by replacing the cations with 0.14M NaCl. Each assay was repeated five times.

Effect of calcium and magnesium on Po activity in crayfish HLS:

To study the effect of calcium and magnesium on Po activity, the crayfish HLS was prepared using a cation-free homogenizing buffer as described by Perazzolo and Barracco (1997) and Gollas-Galvan *et al.* (1997). A 50μL of each sample (HLS) was preincubated with different concentrations of CaCl₂ and MgCl₂ (5, 10,

50 and 100 mM) for 30 minutes at room temperature. The samples were then diluted by adding 800 μ L of distilled water and then received 50 μ L of L-DOPA (3mg/ml). The Po activity was determined as described above. The control samples were used by replacing the cations with 0.14 M NaCl. Each assay was repeated 5 times.

Concentration of calcium and magnesium in sera samples:

Calcium and magnesium concentration of sera samples were determined using a modified sequential Auto Analyzer (EPOS-506 Eppendorf) (Burtis & Ashwood, 1999).

Trypsin activity assay in HLS:

Trypsin activity of HLS was undertaken by method of Perazzolo and Barracco (1997). A 100 μ L of each sample (HLS) was preincubated with 100 μ L of LPS (1mg/ml) for 15 minutes at room temperature. Each sample then received 500 μ L of Tris-buffered solution (TBS) pH 8.0 and 100 μ L of chromogenic peptide BAPNA (Na-Benzoyl-DL-arginine 4- Nitroanilide-hydrochloride) (Sigma) 0.05mol.L⁻¹ DMSO, and incubated at 30°C for one hour. The samples then received 200 μ L of 50% (v/v) acetic acid to stop the enzymatic reaction. Optical density was measured at 405nm. Control samples were used by replacing the HLS with TBS. Each experiment was repeated five times.

Data were analyzed using paired sample t-test, independent sample t-test and ANOVA subjected to SPSS software.

Results

The Po activity in fresh samples of HLS and plasma resulted in the highest (754 \pm 11) and the lowest (5.1 \pm 0.4) activities in HLS and plasma samples, respectively, while that of serum activity was 280 \pm 6 (p <0.05) (Table 1). Also, Po

activity in fresh samples of serum (280 ± 6) was significantly higher than the frozen ones (203 ± 6) ($p < 0.05$).

The results of Po activity in the HLS samples treated with different elicitors are shown in Table 2. The highest (1526 ± 29) and the lowest (820 ± 12) activities were measured in samples treated with trypsin and 10% SDS, respectively ($p < 0.05$). Also the Po activity in the samples induced by LPS (1388 ± 30) was significantly higher than the samples induced by β -glucan (1215 ± 30) ($p < 0.05$). In addition, all treated samples showed significantly higher Po activity compared to the controls (150 ± 4) ($p < 0.05$) (Table 2).

Proteinase activity in the HLS samples was measured by using a chromogenic peptide that is a specific substrate for trypsin. Optical density at 405nm for trypsin activity in crayfish HLS samples was 413.4 ± 5.9 as compared to the controls (15.7 ± 0.9) ($p < 0.05$). Results of Po activity in HLS samples treated with different concentrations of CaCl_2 are shown in Figure 1. The Po activity in HLS was cation dependent with the highest (919 ± 41) and the lowest (775 ± 36) activities at concentrations of 5mM and 100mM of CaCl_2 , respectively indicating that Po activity in the crayfish HLS is CaCl_2 dose dependent. Also, maximum level of Po activity (926 ± 40) occurred when the HLS was treated with 5mM of MgCl_2 while lower activity (799 ± 35) detected in the present of higher concentrations of 100mM MgCl_2 (Figure 2) The level of calcium and magnesium in the serum samples were 45.3 ± 2.3 and 4.4 ± 0.1 mg/dl, respectively.

Table 1: Po activity in HLS, serum and plasma of *P. leptodactylus* measured spectrophotometrically at 490 nm (OD $\times 1000$). (Means \pm SE, n=40)

Parameter	Po activity (OD $\times 1000$)
HLS	754 ± 11
Serum	280 ± 6
Plasma	5.1 ± 0.4
Control	0.0

Table 2: Po activity in the *P. leptodactylus* HLS treated with different elicitors and, measured spectrophotometrically at 490 nm (OD $\times 1000$) (Means \pm SE, n=40)

Elicitor	Po activity (OD $\times 1000$)
10% SDS	820 \pm 12
β -glucan	1215 \pm 30
LPS	1388 \pm 30
Trypsin	1526 \pm 29
Control	150 \pm 4

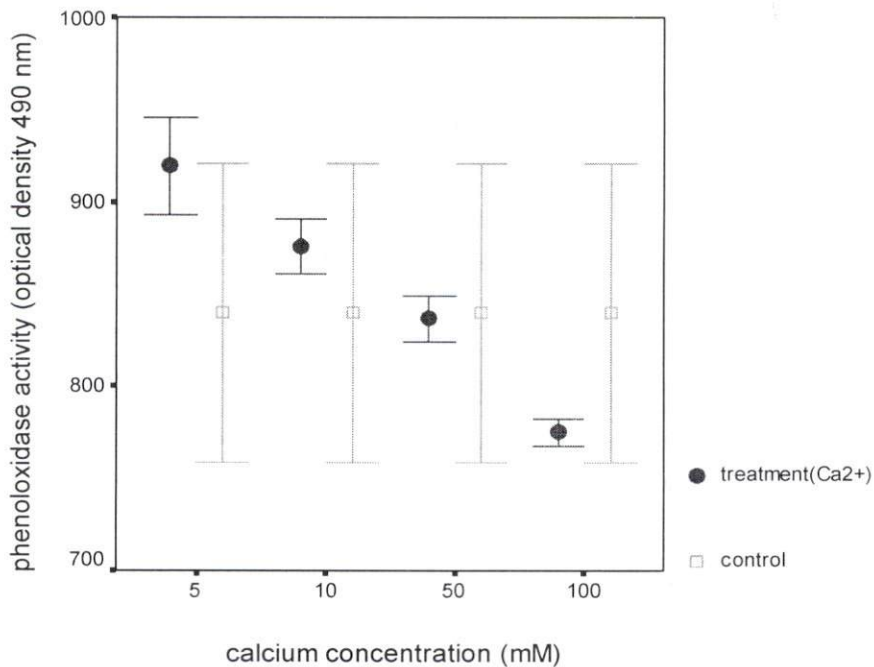


Figure 1: Po activity in *P. leptodactylus* HLS treated with different concentrations of CaCl_2 (5, 10, 50 and 100mM) and, measured spectrophotometrically at 490 nm (OD $\times 1000$) (Means \pm SE, n=40)

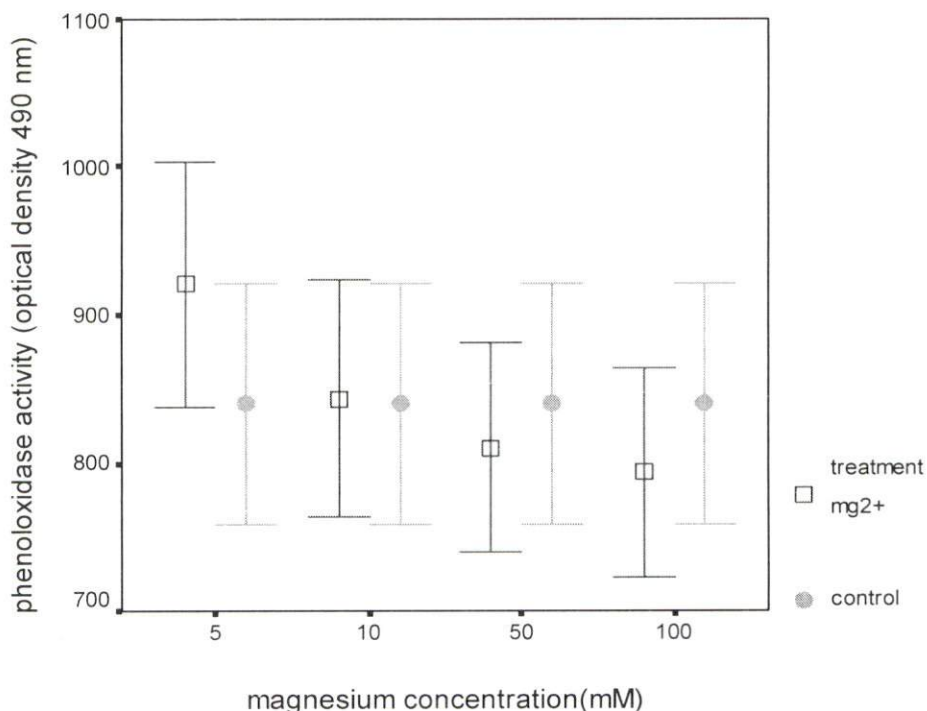


Figure 2: Po activity in *P. leptodactylus* HLS treated with different concentrations of MgCl_2 (5, 10, 50 and 100mM) and, measured spectrophotometrically at 490 nm ($\text{OD} \times 1000$) (Means \pm SE, $n=40$)

Discussion

The proPO activity has been mainly reported in crustacean's haemocytes with a low quantity in their plasma (Soderhall & Soderhall, 2002 ; Perazzolo & Barracco, 1997). However from the literature review few data are available concerning such activity in potentially commercial crayfish species such as *P. leptodactylus*. In this study the haemocytes of *P. leptodactylus* were recognised to be a crude source of Po or its proform proPO. Like other crustaceans (Soderhall & Soderhall, 2002 ;

Perazzolo & Barracco, 1997) very low quantities of Po activity were found in the plasma of *P. leptodactylus* compared to serum samples. Therefore, the adapted anti-coagulant used in this study was able to prevent the haemocytes lysis during HLS and plasma preparation procedure. Since the haemocytes samples of crustaceans are very fragile and the cell lysis occurs during serum preparation, the utilization of serum samples to detect proPO in the haemolymph of *P. leptodactylus* is possible and appears to be an alternative source for determination of proPO activating system for quick and preliminary analyses. This could be of interest for the monitoring of *P. leptodactylus* immunophysiological system status during cultivation in the enclosed areas as mentioned by Perazzolo and Barracco (1997).

In this study the components of the micro-organisms cell walls i.e. β -1, 3-glucan and bacterial LPS were able to promote the *P. leptodactylus* Po activity. However, LPS was more effective than β 1-3-glucan to induce the enzyme activity. Also, use of 10% SDS resulted in lower Po activity than both LPS and β 1-3-glucan (Table 2). Such activity by both bacterial LPS and β 1-3-glucan has been also demonstrated by other researchers in other crustaceans (Durliat 1985; Lee, 2001; Lee *et al.*, 2004 ; Wang *et al.*, 2001; Perazzolo & Barracco, 1997).

The role of proteinases in the proPO activating system of crustaceans has been reported by some workers (Soderhall & Soderhall, 2002 ; Stritunyalucksana *et al.*, 2001; Soderhall & Cerenius, 1998 ; Perazzolo & Barracco, 1997 ; Thornqvist & Soderhall, 1997; Durliat 1985 ; Lee, 2001; Lee *et al.*, 2004 ; Wang *et al.*, 2001). In this study marked enhancement of the Po activity induced by porcine trypsin suggests a role of an endogenous proteinase which is able to promote the enzyme activity. To confirm such endogenous role of proteinase, trypsin activity was also detected in *P. leptodactylus* haemocytes. Similar to other species of crustaceans (e.g. Perazzolo & Barracco, 1997 ; Gollas-Galvan *et al.*, 1997 ; Durliat 1985) ProPO activity in *P. leptodactylus* was considerably enhanced by the divalent ions Ca^{2+} and Mg^{2+} and strongly inhibited by higher concentrations of 50 and 100mM

(Figures 1 and 2). Therefore, low concentrations of these ions can play the role of the immunostimulators to enhance the Po activity in the animal, particularly during outbreaks by pathogenic micro-organisms.

In conclusion, the proPO activating system of *P. leptodactylus* is much similar to other examined crustaceans with the highest activity found in HLS. Such activity in serum samples was reduced when the samples were frozen at -20°C for 20 days. Also the Po activity was cation dependent and demonstration of marked enhancement induced by trypsin suggesting a role of an endogenous proteinase which is probably able to enhance the proPO activity.

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