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Phenoloxidase specific activity in the red swamp crayfish *Procambarus clarkii*

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The prophenoloxidase (proPO) system is considered an important mechanism of innate defence in arthropods. This enzymatic cascade has been studied in crustaceans such as the crayfishes *Astacus astacus* and *Pacifastacus leniusculus* and is located inside the haemocytes. An initial characterisation of this system in the commercially important red swamp crayfish *Procambarus clarkii* is described. The *P. clarkii* proPO system was activated by trypsin and also by zymosan A. This activation was calcium ion dependent. The calcium ion concentration also affected the background activation of the system and at 5 mM was highest as measured by the ability of phenoloxidase to oxidise L-3,4-dihydroxyphenylalanine. The effect of calcium ions appears to be related to the activation of an endogenous serine protease, but other calcium ion-dependent factors can also affect proPO activation. Lipopolysaccharides (LPS), glycolipids found in the outer leaflet of the outer membrane of Gram-negative bacteria, were also able to activate the proPO system in *P. clarkii* after a significant lag time of 25 to 30 min. However, LPS derivatives (deacylated LPS, lipid A and *β*-D-GlcNAc-[1→6]-D-GlcNAc) were not able to activate the enzymatic cascade in *P. clarkii*. Activation of the proPO system in other crayfishes by LPS has been shown to be mediated by serine protease-like enzymes. The observed effect of LPS and LPS derivatives on the activation of the *P. clarkii* proPO system suggests that a protease activity triggered by these molecules may be mediated through the recognition of a ''complete'' LPS molecule (polysaccharide and lipid A). The intermolecular recognition of LPS by a putative endogenous serine protease zymogen might explain the lag time observed in proPO activation.

Key words: prophenoloxidase, haemocyte lysate, lipopolysaccharide, *Procambarus clarkii*, serine protease, zymosan.

I. Introduction

The prophenoloxidase activating system (proPO) is an enzymatic cascade associated with the pathogen defence mechanisms of arthropods (Söderhäll & Smith, 1986; Rowley *et al*., 1986). In crustaceans proPO resides in the granular and semi-granular circulating haemocytes (Söderhäll & Smith, 1983; Smith & Söderhäll, 1991). The proPO can be transformed to the active enzyme phenoloxidase (PO) by compounds such as zymosan (carbohydrates from yeast

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cell walls), bacterial lipopolysaccharide (LPS), urea, calcium ions, trypsin, and heat (Söderhäll *et al*., 1979; Söderhäll & Unestam, 1979; Söderhäll, 1981; Söderhäll & Häll, 1984; Leonard *et al*., 1985; Söderhäll *et al*., 1986). Most of these compounds activate proPO through the action of a serine protease (Söderhäll, 1981; Leonard *et al*., 1985). Serine protease inhibitors prevent the activation for all of these compounds except for heat and urea (Leonard *et al*., 1985; Söderhäll *et al*., 1986). Proteins associated with the proPO system have been shown to present opsonic properties by provoking degranulation, encapsulation, cell adhesion of crayfish haemocytes and enhancing phagocytosis of foreign particles (Smith & Söderhäll, 1983*a*; Smith & Söderhäll, 1983*b*; Söderhäll *et al*., 1984; Söderhäll *et al*., 1986; Söderhäll *et al*., 1994).

The proPO activating system has been studied in the crayfish *Astacus astacus* and *Pacifastacus leniusculus* by Söderhäll as described above. The purpose of this work was to characterise the activation of the proPO system in the red swamp crayfish, *Procambarus clarkii*, as this system has been reported to be important in the innate immunity of crustaceans. *Procambarus clarkii* is a commercially important species and can suffer mass mortalities due to bacterial or other pathogenic infections (Thune *et al*., 1991; Diéguez-Uribeondo *et al*., 1995). Methods to determine the onset of disease are at present unknown, and it is possible that understanding the activation of the proPO system in the animals could lead to the development of diagnostic tools to assess animal health, or treatments to augment natural defence systems. The proPO system could provide an indicator system towards the development of methods to control infection. The similarity between proPO of *P. clarkii* and other species of freshwater crayfish has enabled us to examine some of the factors that activate this system and to test the specificity of activation by different types of molecules including trypsin, zymosan, and LPS derivatives.

II. Materials and Methods

CHEMICALS AND REAGENTS

Trypsin (bovine pancreas type XI), trypsin inhibitor (soybean type I-S), zymosan A from *Saccharomyces cerevisiae*, lipopolysaccharide (LPS) from *Salmonella minnesota* R5 Rc mutant, LPS detoxified (LPSdex) from *S. minnesota*, lipid A from *S. minnesota* Re 595, *β*-D-GlcNAc-[1→6]-D-GlcNAc (Compound X) (Rosner *et al*., 1979), L-3,4-dihydroxyphenylalanine (L-DOPA), and azocasein were obtained from Sigma Chemical Co. (St. Louis, MO). Reagents for protein assays were obtained from BioRad (Richmond, CA). Morpholinopropane sulphonic acid was purchased from United States Biochemical Corp (Cleveland, OH). All other chemicals and reagents were obtained from Fisher Scientific (Houston, TX).

PREPARATION OF HAEMOLYMPH LYSATE SUPERNATANT (HLS)

Procambarus clarkii were generously provided by Dr. J. Huner from the Crayfish Research Center, University of Southwestern Louisiana, Lafayette, LA, U.S.A. The animals were maintained in a water recirculating system at

22 °C before they were used for experimentation. Only healthy intermolt animals were used. Haemolymph was extracted with a 2 ml syringe and 22 gauge needle. The syringe contained 0·4 ml of ice-cold anticoagulant buffer (AB) consisting of 0·14 M NaCl, 0·1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA, pH 4·6 (Smith & Söderhäll, 1983*a*). For a given experiment, the extracted haemolymph from two or more animals was pooled into a sterile test tube and diluted with buffer to a final haemolymph to AB volume ratio of 1:1. Diluted haemolymph was distributed into 1·5 ml sterile Eppendorf tubes and centrifuged at 1300 *g* at 4 °C for 10 min. The supernatant was discarded and haemocytes were resuspended in an equal volume of ice-cold rinsing buffer (RB) consisting of 10 mM sodium cacodylate, 0·25 M sucrose and 20 mM $CaCl_2$, pH 7·0 (Leonard *et al*., 1985), and centrifuged as above for 10 min. The supernatant was discarded and 0·2 ml of ice-cold homogeniser buffer (HB) consisting of 10 mM sodium cacodylate and 20 mM $CaCl_2$, pH 7·0 (Leonard *et al*., 1985) was added to each tube. The haemocytes were homogenised with a motor driven drill and sterile polypropylene pestle. Homogenisation was performed on ice and at full drill speed for 1 min. HB was then added to the original haemolymph volume and centrifuged at 16 000 *g* for 20 min at 4 °C. The supernate was kept on ice, designated as haemocyte lysate supernate (HLS), and used as the proPO source. Additional experiments were performed using 10 mM MOPS (morpholinopropane sulphonic acid) in place of sodium cacodylate for the above buffers. No differences in activation or activity were noticed between the two buffers (not shown). Protein concentration of the HLS was determined using the BioRad Protein Assay System (Bio-Rad, Richmond, CA), with bovine serum albumin as the standard.

ProPO ACTIVATION AND MEASUREMENT OF PHENOLOXIDASE ACTIVITY

Measurements of PO activity were performed in 96 well ELISA plates (Corning, NY) and colour development was measured in a spectrophotometer (Spectra Shell Plate Reader, SLT Labinstruments). Phenoloxidase (PO) activity was assayed by mixing 50 μl of *P. clarkii* HLS with one of the following putative activators: trypsin (25 μl of a 0·5 mg ml^{-1} solution); zymosan (50 μl of the supernate of a 0·2% suspension of zymosan); LPS (10 μl of a 1 mg ml^{-1} solution); detoxified LPS (10 μl of a 1 mg ml^{-1} solution); lipid A (10 μl of a 1 mg ml^{-1} solution); and compound X (10 μl of a 1 mg ml^{-1} solution). HB was used when necessary to bring the final volume to 100 μl. Samples were incubated for 45 min in the ELISA plates in a constant environment chamber at 22 °C, followed by the addition of 50 μl of L-3,4-dihydroxyphenyl alanine (L-DOPA, 3 g l^{-1}) to each sample well. The absorbance at 492 nm was then measured with the reference wavelength of the spectrophotometer set at 620 nm. Enzyme activity was expressed as change in absorbance at 492 nm per min per mg of protein. The effect of calcium concentration on the proPO activation by trypsin was determined by using various levels of calcium chloride (0, 5, 10, 20, 30, 50, 100, and 150 mM) in both the RB and HB buffers for the preparation of the HLS. The effect of soybean trypsin inhibitor (STI) on proPO activation was determined by the addition of trypsin inhibitor (25 μl of a 0·5 mg ml^{-1} solution) to 50 μl of HLS and

incubation for 10 min before trypsin or zymosan was added to activate the system, followed by measuring for PO specific activity as described above. A control was run in all experiments and consisted of 50 μl HLS, 50 μl of HB, and 50 μl of L-DOPA.

PROTEOLYTIC ACTIVITY

The proteolytic activity of trypsin was determined by a modification of the method of Charney & Tomarelli (1947). This assay employs azocasein as substrate. The assay was run in plastic microcentrifuge tubes. The assay mixture consisted of 100 μl of 10 mg ml^{-1} azocasein dissolved in a 5 mg ml^{-1} $NaHCO_3$ solution (pH 8·3), trypsin 0 (blank), 5, 10, 20, 30, and 50 μl of a 0·5 mg ml^{-1} solution, and bicarbonate buffer (5 mg ml^{-1}, pH 8·3) necessary to complete a total reaction mixture of 200 μl. The reaction mixture (200 μl) was incubated at 38 °C for 5 min. Digestion was stopped and undigested azoprotein precipitated from solution by adding 800 μl of 5% trichloroacetic acid to each tube. Tubes were centrifuged at room temperature in a Micro-Centrifuge (Fisher-235B) for 5 min. Supernatant was transferred to an ELISA plate (100 μl) and mixed with 0·5 N NaOH (100 μl). Absorbance was measured in a spectrophotometer (Spectra Shell Plate Reader, SLT Labinstruments) at 450 nm. The velocity constant (K) of the enzymatic activity was calculated according to Charney & Tomarelli (1947). The effect of Ca^{++} ions on this constant was checked by the addition of $CaCl_2$ or EDTA, before the digestion step (5 min at 38 °C), in the assay to a final concentration of 10 mM.

DATA ANALYSIS

Phenoloxidase specific activity was obtained from the slope of regression line between the change in absorbance at 492 nm per mg protein and time in min. ANOVA analyses were applied for mean PO specific activity comparisons. Probability values $<0·05$ were considered significant. All statistical analyses were run in JMP software from SAS Institute Inc. (Cary, NC).

III. Results

Trypsin activation of proPO was affected by Ca^{++} concentration in the HLS processing buffers (Fig. 1). Mean PO specific activity showed variability at each level of Ca^{++} concentration, but the mean activity at 10 and 20 mM Ca^{++} was significantly higher than when 0 and 150 mM Ca^{++} was used ($P=0·006$). The background PO specific activity was also affected by Ca^{++} concentration (Fig. 1), and was found to be highest at 5 mM Ca^{++} ($P=0·025$). Protein concentration in the HLS at different Ca^{++} concentrations varied inversely according to PO specific activity [Fig. 2(a)]. This effect is not due to the protein assay, since comparison with BSA (1 mg ml^{-1}) detection at the same Ca^{++} concentrations used for HLS preparation did not show a similar pattern [Fig. 2(b)]. The mean enzymatic velocity constant of trypsin caseinolytic activity was not significantly affected under each trypsin concentration (2·5, 5, 10, 15, and 25 μg) due to Ca^{++} or EDTA (10 mM) addition ($P=0·2768$). The time course of activation of proPO by trypsin could not resolve a time point that

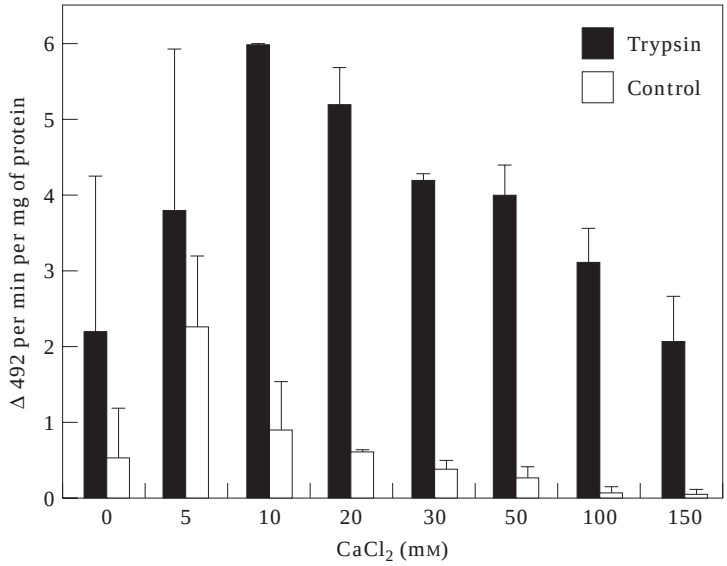

Fig. 1. Effect of $CaCl_2$ concentration on the trypsin-dependent activation of the *P. clarkii* proPO system. Control represents background PO specific activity. PO specific activity values at each $CaCl_2$ concentration are means of two experiments with three animals each. Error bars represent standard deviation.

showed less than full activity (Fig. 3). The mean PO specific activity was not different ($P = 0{\cdot}407$) over the period of incubation.

Trypsin and zymosan A were strong activators of the *P. clarkii* proPO system (Table 1). ProPO activated by trypsin and zymosan were significantly higher than the control ($P < 0{\cdot}001$). The activation of proPO by trypsin and zymosan was inhibited in the presence of trypsin inhibitor ($P < 0{\cdot}001$) (Table 1).

The activation of proPO by LPS and LPS derivatives was investigated with 10-fold dilutions of 1 mg ml^{-1} stock solutions. LPS was able to activate this system at concentrations of 10 and 100 μg ml^{-1} ($P < 0{\cdot}001$) [Fig. 4(a)]. LPSdex (deacylated LPS) was not able to activate the proPO system at all concentrations tested [Fig. 4(a)]. Lipid A, with a phosphoryl group at position 4′ in the β-(1→6) glucosamine disaccharide backbone, did not show any significant ($P = 0{\cdot}784$) activation of proPO at all concentrations tested [Fig. 4(b)]. Similarly, compound X, which is a synthetic backbone of lipid A formed by β-(1→6) linked glucosamine disaccharide with acetyl groups linked to amino moieties at positions 2 and 2′ and without acyl and phosphoryl groups, did not show any significant ($P = 0{\cdot}817$) activation of proPO [Fig. 4(b)]. The time course of proPO activation by ‘‘complete’’ LPS molecules (10 μg ml^{-1}) showed a delay in the activation up to 30 min, after which PO specific activity increased compared to the control levels (Fig. 5).

IV. Discussion

The observed effect of Ca^{++} ions on *P. clarkii* PO activity has been reported in other proPO systems (Söderhäll *et al.*, 1979; Söderhäll, 1981; Leonard *et al.*,

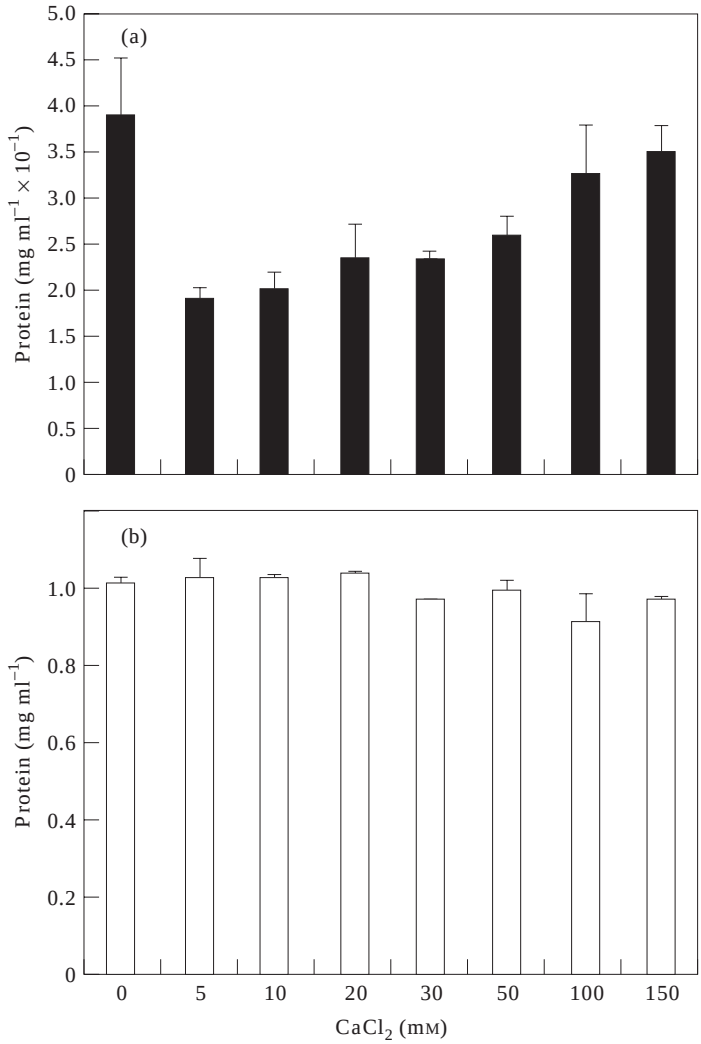

Fig. 2. Effect of $CaCl_2$ on (a) the yield of protein extracted in HLS preparations or (b) the colorimetric determination of the concentration of a 1 mg ml^{-1} solution of BSA. Error bars represent standard deviation.

1985). Trypsin enzymatic activity appears to be independent of Ca^{++} ions (Söderhäll *et al.*, 1979). The pattern of background activity of PO clearly shows the influence of Ca^{++} in the spontaneous activation of the proPO system (Fig. 1) that is not linked to trypsin addition. However, serine protease activity observed in crayfish HLS preparations seems to be regulated by Ca^{++} concentration (Söderhäll, 1981). A trypsin-like serine protease (ppA) that activates prophenoloxidase to its active form has been purified from crayfish haemocytes (Aspán *et al.*, 1990). This proteinase is believed to be present as an inactive precursor in crayfish haemocytes (Aspán *et al.*, 1990; Aspán & Söderhäll, 1991). Crystalline preparations of pancreatic trypsinogen can

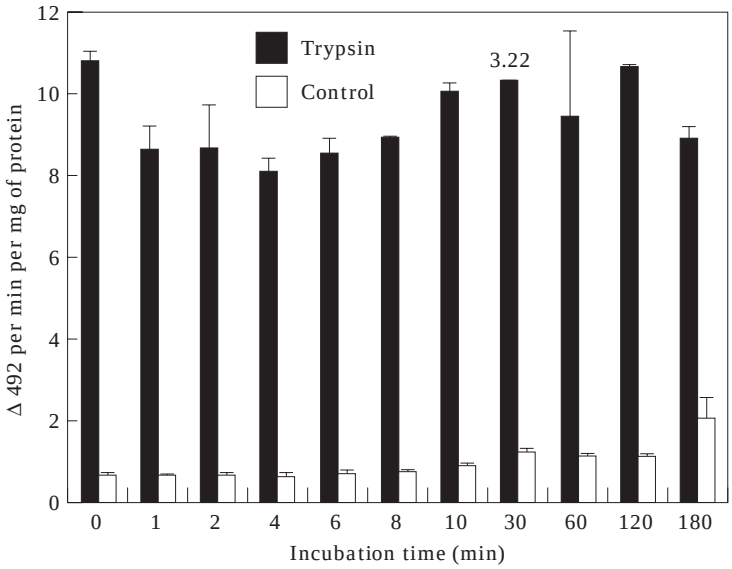

Fig. 3. Time course of proPO activation (20 mM Ca^{++} HLS preparation) by trypsin. Control refers to background PO specific activity. Value at 30 min (3·22) is standard deviation. Error bars represent standard deviation.

Table 1. Activation of *Procambarus clarkii* proPO by trypsin and zymosan A, and inhibition of activation by trypsin inhibitor. Values are means (± S.D.) of *n* experiments. Each experiment was run in duplicate

HLS treatment	PO specific activity Δ 492 per min per mg protein	*n*
Trypsin	7·75 (1·82)	8
Trypsin + Inhibitor	0·37 (0·18)	2
Zymosan A	4·88 (3·07)	6
Zymosan A + Inhibitor	0·94 (0·55)	2
Control	0·49 (0·16)	11
Control + Inhibitor	0·32 (0·09)	2

autocatalytically be transformed to active trypsin without the aid of activators when at a pH of 7 to 9 (Northrop *et al.*, 1948). This reaction can be greatly stimulated by Ca^{++} ions (Hadorn, 1974). This stimulation seems to be related to the ability of Ca^{++} to inhibit the formation of an inert protein product of trypsinogen in the presence of trypsin, thus enhancing the transformation of trypsinogen to trypsin (Northrop *et al.*, 1948). The effect of Ca^{++} ions observed in this and other studies on proPO activation might be related to the transformation kinetics of the putative zymogen into the active ppA. However, this argument does not explain the reduction of PO specific activity at higher Ca^{++} concentrations (>20 mM) unless Ca^{++} affects another factor (s) in the HLS preparation. Ca^{++} is not probably required for the active enzyme,

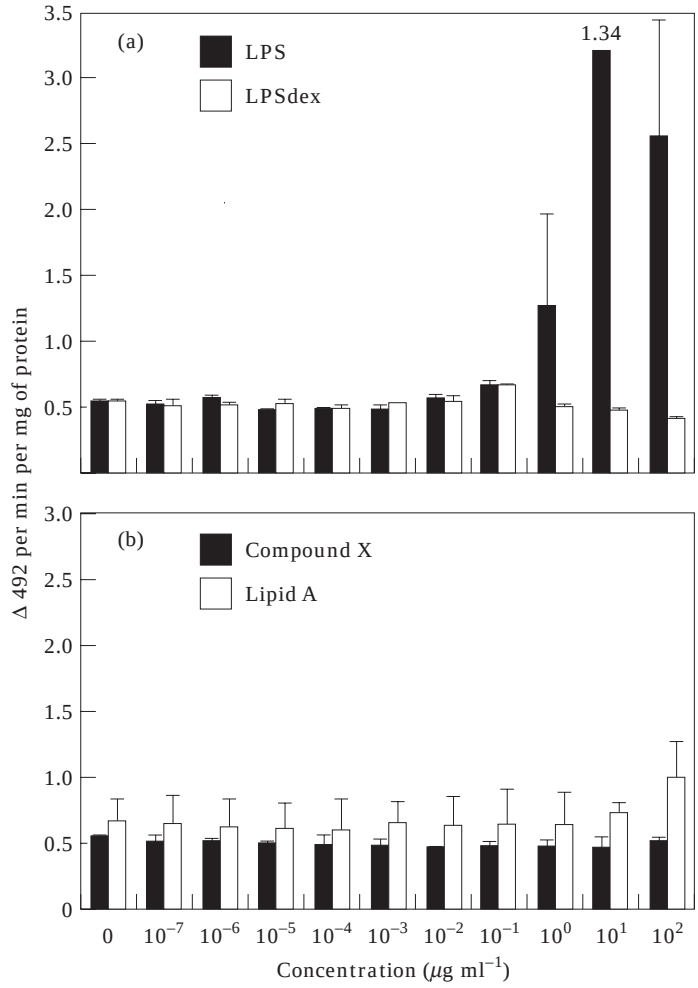

Fig. 4. Effect of different concentrations of LPS and LPS derivatives to activate the proPO system of *P. clarkii*. (a) Lipopolysaccharides (LPS) from *Salmonella minnesota* R5 Rc mutant and lipopolysaccharides detoxified (LPSdex) from *S. minnesota*, value at 10^1 μg ml^{-1} (1·34) is standard deviation, (b) Compound X and Lipid A from *S. minnesota* Re 595. PO specific activity values at each LPS and LPS derivative concentration are means of two experiments with three animals each. Error bars represent standard deviation.

since the proteolytic activity of a related serine protease (trypsin) does not require Ca^{++} for activity, as the rate constant for enzymatic activity was not dependent upon the presence or absence of free Ca^{++} ions. A $CaCl_2$ concentration of 5 mM can provoke a spontaneous coagulation process (Söderhäll, 1981). Polymerisation of proteins into high molecular weight aggregates at low Ca^{++} concentrations (5 mM) can reduce the crayfish HLS protein content to 50% after a 10 000 *g* centrifugation (Söderhäll, 1981). At higher Ca^{++} concentrations (100 mM) HLS protein concentration can be reduced to approximately 25% only after proPO and the clotting process are triggered by

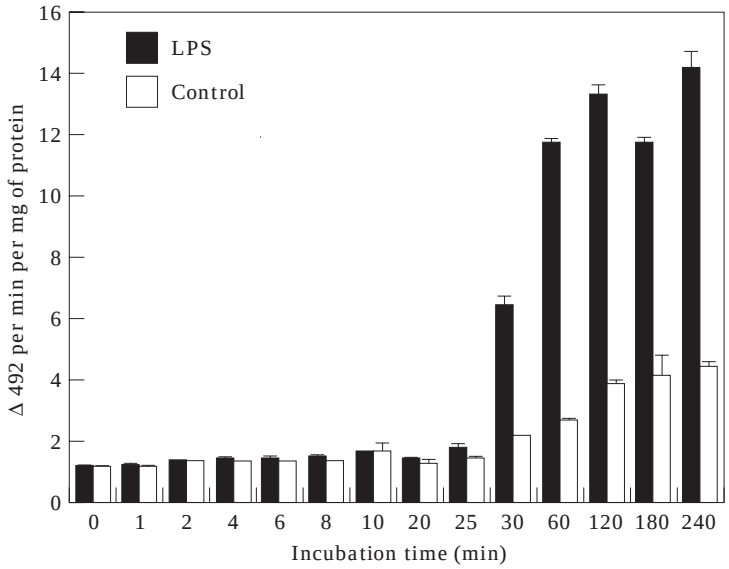

Fig. 5. Time course of proPO activation (20 mM Ca^{++} HLS preparation) by LPS (10 μg ml^{-1}). Control refers to background PO specific activity. Error bars represent standard deviation.

β-1,3-glucan addition (Söderhäll, 1981). It has been suggested that the clotting process and PO activity are not directly linked, but are the result of endogenous serine protease activation (Söderhäll, 1981). This suggests that the concentration of Ca^{++} during crayfish HLS preparation may have an indirect effect on its soluble protein concentration. Serine protease activation in low $CaCl_2$ (5 mM) could spontaneously trigger proPO activation and clotting processes. Polymerised HLS protein would be spun down during the 16 000 *g* centrifugation step (see Methods), resulting in the diminution of the soluble protein concentration. In the presence of increasing Ca^{++} concentrations, serine protease activation might be reduced, thus reducing spontaneous proPO activation, but the measured soluble protein detected would increase. This might explain the inverse pattern of HLS protein concentration and proPO activation by endogenous serine protease (control) observed in this study [see Figs 1 & 2(a)]. However, activation of proPO by an active exogenous serine protease (trypsin) followed the same pattern as background activity. Since this protease is already active, Ca^{++} ions might affect other unknown factor(s) of this enzymatic cascade that ultimately influence PO activity. Different biochemical aspects that involve Ca^{++} ions in the activation of the proPO system are still not fully understood. The lack of detectable delay in proPO activation by trypsin (at this time resolution) suggests that a serine protease exerts its proteolytic activity directly on the pro-enzyme. This activation is stable for at least 3 h (see Fig. 3).

The activation of the proPO system of *P. clarkii* by trypsin and zymosan, and its inhibition by a serine protease inhibitor (STI) supports the importance of a serine protease-like factor in the activation of this enzymatic cascade (see

Table 1). Activation by zymosan shows the capability of this system to recognise non-self molecules of microbial origin. The proPO activating compound in zymosan preparations has been shown to be *β*-1,3-glucan (Söderhäll & Unestam, 1979), and similar patterns of activation and inhibition of the *P. clarkii* proPO system observed in this study have been reported for other crayfish (Unestam & Söderhäll, 1977; Söderhäll *et al.*, 1979; Söderhäll & Unestam, 1979; Söderhäll, 1981; Söderhäll & Smith, 1983). The activation of proPO by zymosan and other microbial compounds can be used to test differences among species (*P. clarkii* and *P. zonangulus*) that can be important in the commercial production of these crustaceans.

Lipopolysaccharides, molecules of the outer membrane of Gram-negative bacteria, have been shown to activate the proPO systems of crayfishes (Söderhäll & Häll, 1984; Johansson & Söderhäll, 1985). This activation seems to be mediated by proteases in crayfish HLS preparations (Söderhäll & Häll, 1984; Aspán *et al.*, 1990). In *P. clarkii*, the activation of the proPO system by LPS was investigated. The specificity of the activation was also tested by using different moieties of LPS. The results of these experiments showed that LPS from a rough strain of *S. minnesota* (R5 Rc mutant) was able to activate *P. clarkii* proPO system. Rc mutant lipopolysaccharides are devoid of the O-somatic antigen and have an incomplete core structure formed mainly by glucose and heptose linked to lipid A via 2-keto-3-deoxy-D-*manno*-octonate (KDO) (Lüderitz *et al.*, 1966; Lüderitz *et al.*, 1982). LPSdex that bears a complete polysaccharide structure and deacylated glucosamine disaccharide backbone of lipid A was not able to activate the proPO system. Similarly, activation was not observed with lipid A nor with compound X. These observed effects of LPS and LPS derivatives on the activation of the *P. clarkii* proPO system suggest that serine protease-mediated activation by these molecules could require the specific recognition or interaction with both the polysaccharide and lipid A moieties since only the ''complete'' LPS molecule was able to provide for the activation of this system under these conditions. Ester-linked fatty acids in lipid A may be important in this protease recognition LPS, as a derivative unable to activate the proPO system (LPSdex) has been depleted of ester-linked fatty acids (Seid & Sadoff, 1981). Deacylated LPS has been shown to be up to 1000-fold less active than untreated LPS in causing *Limulus* amoebocyte lysate to gel (Seid & Sadoff, 1981; Ding *et al.*, 1990). These observations with deacylated LPS are similar to the lack of activation found in the *P. clarkii* proPO system by LPSdex reported here. The serine protease involved in *P. clarkii* proPO activation could be related to factor C from horseshoe crab haemocyte lysate (Nakamura *et al.*, 1986). Factor C, a glycoprotein serine-protease zymogen, can be activated by LPS and 4′-mono- or bi-phosphorylated acylated (*β*1-6)-D-glucosamine disaccharide (synthetic *Escherichia coli*-type lipid A) (Nakamura *et al.*, 1988). However, it was found that the *P. clarkii* proPO system could not be activated by the 4′-monophosphoryl lipid A. The lag time observed in proPO activation by LPS (see Fig. 5) implies the involvement of an intermediary molecule in proPO activation. A similar lag time in proPO activation by another molecule of microbial origin (*β*-1,3-glucans) has been reported in the crayfish *Astacus astacus* (Söderhäll, 1981). Serine protease activity is involved in crayfish

proPO activation by β-1,3-glucans and LPS (Aspán *et al.*, 1990). Serine proteases involved in cascade reactions are composed of two distinct parts: a recognition domain and a catalytic domain. It has been proposed that recognition domains are involved in the intermolecular recognition of these proteases (Sasaki & Ohkubo, 1994). These structures can be involved in the molecular recognition of microbial molecules which in turn activate the protease zymogen. The requirement for this recognition might explain the lag time observed in the crayfish proPO activation.

We are currently trying to isolate and identify LPS-binding proteins of *P. clarkii* through the use of LPS affinity chromatography of crayfish plasma and HLS. In this way it will be possible to determine if a serine protease of *P. clarkii* can recognise LPS itself, or if another as yet unidentified recognition factor works in conjunction with the protease to activate the proPO system. This would be required to define the sequence of events following a Gram-negative infection that triggers the activation of proPO system or other biochemical pathways involved in the innate defence of *P. clarkii* against potential bacterial pathogens.

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