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Cresolase, catecholase and laccase activities in haemocytes of the red swamp crayfish

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Phenoloxidase activity in crayfish haemocyte lysates and extracts of haemocyte membranes were studied using native PAGE and SDS-PAGE gels and staining for cresolase, catecholase and laccase activities. The activation of the proenzyme, prophenoloxidase to phenoloxidase, in native PAGE was demonstrated following exposure to SDS. By staining samples separated in SDS-PAGE followed by renaturation, a high molecular mass phenoloxidase activity was identified in both the soluble and membrane fractions of haemocyte preparations. The membrane-associated activity appeared at only relatively high molecular mass (>300 kDa), and could easily be eluted from membranes using detergents or NaCl. Further, this membrane-associated activity has a catecholase activity but not the cresolase activity seen in the soluble preparations. In addition, several other phenoloxidase enzymes were identified with different relative mobilities (250, 80, 72 and 10 kDa). Crayfish haemocytes also contained laccase activity, thought to be restricted to cuticle sclerotisation in the integument. Laccase activity in haemocytes might aid in the formation of capsule used to contain pathogens.

Key words: phenoloxidase, laccase, crayfish haemocytes, electrophoresis.

I. Introduction

Humoral and cellular components are key elements of the invertebrate innate immune system. Both components are enhanced by the action of the prophenoloxidase activating system (proPO) which has been identified in several invertebrates, and is also involved in the nonself recognition of these animals (Canicatti & Götz, 1991; Jackson *et al.*, 1993; Söderhäll *et al.*, 1994). This enzyme system appears to be a widespread defence mechanism also present in plants (Boss *et al.*, 1995; Thipyapong *et al.*, 1995).

Phenoloxidase (PO), the activated form of proPO, has received a great deal of attention in arthropods (Götz & Boman, 1985; Söderhäll *et al.*, 1994). This enzyme is present within circulating haemocytes (Smith & Söderhäll, 1991; Cárdenas & Dankert, 1997; Perazzolo & Barracco, 1997) and in haemolymph (Ashida, 1971; Andersson *et al.*, 1989). PO is also localised on haemocyte cell surfaces (Charalambidis *et al.*, 1996). Phenoloxidases associated with

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arthropod cuticles have been described for different species (Barrett, 1987a; Binnington & Barrett, 1988; Thangaraj & Aruchami, 1992). One of these enzymes appears to be involved in wound-healing reactions (Barrett, 1984) and is similar to haemocyte PO. Wound-healing and haemocyte POs are tyrosinase-type enzymes with cresolase (EC 1·14·18·1) and catecholase (EC 1·10·3·1) activities. Laccase (EC 1·10·3·2), another cuticle PO (Barrett & Andersen, 1981), can oxidise both *o*- and *p*-diphenols. Laccase seems to be restricted to integumental tissues and has not been reported in circulating haemocytes (Charalambidis *et al*., 1994).

Purified proPO and cuticle PO have been obtained as a single protein or as a dimer (Andersson *et al*., 1989; Aspán & Söderhäll, 1991; Hall *et al*., 1995; Kopácek *et al*., 1995).

Native forms of these enzymes are usually characterised by having molecular masses >100 kDa (Barrett, 1987b; Hall *et al*., 1995; Kopácek *et al*., 1995). Such studies have assayed substrate specificity of purified or crude extracts of PO spectrophotometrically. Detection of immobilised PO after gel electrophoresis has also been described (Nellaiappan & Vinayagam, 1986; Nellaiappan & Banu, 1991). This latter technique is useful to detect PO enzymes and the associated molecular mass in crude extracts without the necessity for enzyme purification. Furthermore, the effect of endogenous enzyme inhibitors can be avoided and detection of PO activity is highly sensitive.

To further understand the enzymatic properties of the PO, considered to be important in the innate immune system of arthropods, we have studied the activity of this enzyme system in the commercially important red swamp crayfish *Procambarus clarkii*. Enzymatic activity was detected in haemocyte lysate supernatants and extracts of haemocyte membranes after sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) to detect molecular mass differences. Substrate specificity (using various chromogenic substrates), enzyme inhibition and the effect of pH were also examined.

II. Materials and Methods

CHEMICALS AND REAGENTS

Substrates L-tyrosine methyl ester, 4-methyl catechol, L-3,4-dihydroxyphenylalanine (L-DOPA), dopamine, 3,3′-diaminobenzidine (DAB) and hydroquinone were obtained from Sigma (St. Louis, MO, U.S.A.). 3-Methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH), diethyldithiocarbamic acid (DETC), cholic acid, Triton X-100, trypsin, phenylmethyl sulfonyl fluoride (PMSF), and mushroom tyrosinase were also from Sigma. 3,3-dimethylglutaric acid (DMG) was from US Biochemical Corp. (Cleveland, OH, U.S.A.). Molecular weight standards BenchMark protein ladder and BenchMark prestained protein ladder were from Life Technologies (Grand Island, NY, U.S.A.).

Protein concentration was determined by the microtitre protein assay of Bio-Rad (Hercules, CA, U.S.A.), using BSA as standard.

HAEMOCYTE 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. Animals were maintained as described before (Cárdenas & Dankert, 1997). Haemolymph was extracted from one or more apparently healthy animals with a 5 ml syringe containing 2 ml of ice-cold anticoagulant buffer (AB) (Smith & Söderhäll, 1983). Diluted haemolymph was quickly centrifuged at 750 × *g* at 4° C for 15 min. Supernatant (plasma) was discarded and the cell pellet resuspended in 1 ml of AB and centrifuged at 725 × *g* at 4° C for 10 min. The supernatant was discarded and the cell pellet resuspended in a PBS-Vanadate buffer (10 mM potassium phosphate buffer, 150 mM NaCl, 1 mM Na_3VO_4, pH 6·8) and centrifuged under similar conditions. The supernatant was discarded and 200 μl of lysing buffer was added (LyB-PMSF, 20 mM Tris, 5 mM EDTA, 1 mM Na_3VO_4, 1 mM PMSF, pH 7·6). The haemocyte pellet was homogenised with a motor driven drill at full speed with a sterile polypropylene pestle, on ice for 1 min. LyB-PMSF was then added to complete 1 ml. The homogenate was centrifuged at 16 000 × *g* at 4° C for 30 min. The supernatant (HLS) was kept on ice until needed.

HAEMOCYTE MEMBRANES SUPERNATANT (HMS)

The pellet obtained after the 16 000 × *g* centrifugation was resuspended in 1 ml of LyB-PMSF, vortexed and centrifuged at 16 000 × *g* at 4° C for 10 min. This treatment was repeated. The cell membranes were resuspended a third time in LyB without PMSF and centrifuged. Then the membranes were resuspended in 1 ml of either: LyB, LyB with 1% cholic acid, LyB with 1% Triton X-100, LyB with 0·5 M NaCl, or LyB with 1 mg ml^{-1} trypsin. Tubes from each treatment were incubated at 4° C on an end-over-end shaker for 1 h. After incubation, all tubes were centrifuged at 16 000 × *g* as above. Supernatants (HMS) were kept on ice until needed.

ELECTROPHORESIS AND PHENOLOXIDASE ACTIVITY

Phenoloxidase activity was detected on polyacrylamide gels as described by Nellaiappan & Vinayagam (1986) with some modifications. HLS and HMS were run in a 4–20% gradient SDS-PAGE system using a Mini-PROTEAN II Cell (Bio-Rad) under non-reducing conditions. Samples were not boiled unless specified. HLS proteins were also separated in similar polyacrylamide gels, but under non-denaturing conditions (native gel, no SDS added). After electrophoresis, SDS-PAGE gel was washed 2 × 10 min in 2·5% Triton X-100 in 50 mM phosphate buffer, pH 6·5. A last 10 min wash was made in phosphate buffer without Triton. The gel was then incubated in a 4:1 ratio of substrate solution (10 mM) dissolved in phosphate buffer and MBTH (0·3%) dissolved in deionised water (DI). The gel was developed for 1 h and then rinsed with DI several times. The gel was submerged in a 7% acetic acid solution and dried. For inhibition studies, the gel was treated with DETC (10 mM) in phosphate buffer after Triton X-100 (see above). Before the gel was developed in the substrate mixture, it was washed 2 × 5 min with phosphate buffer. Native gels

were only treated with phosphate buffer before substrate addition. For proPO activation, native gels were exposed to SDS (0·1% in phosphate buffer) for about 15 min and then processed as SDS-PAGE gels.

The effect of pH on phenoloxidase activity was studied using 50 mM DMG. This wide range buffer allows testing to be performed at different pH values without changing the buffer used. The pH values ranged from 3·5–7·5 in 0·5 units of increment, giving a total of nine different values. When the electrophoresis was finished, the gel was treated with 2·5% Triton X-100 dissolved in DI. The gel was briefly (~10 min) incubated in each respective pH solution. The buffer solution was discarded and 10 mM of the required substrate dissolved in DMG at the corresponding pH was added. MBTH was added following the substrate and the whole mixture incubated for an hour for development.

The molecular mass of PO activity bands were estimated with prestained molecular weight markers that were run together with HLS and HMS proteins. Prestained markers were calibrated against Coomassie blue stained molecular weight markers.

III. Results

Phenoloxidase (PO) in this report makes reference to the enzyme that has both catecholase (oxidation of *o*-diphenols) and cresolase (*o*-hydroxylation of monophenols) activity. Laccase is a PO that can oxidise *o*- and *p*-diphenols, and does not have cresolase activity.

Native gels stained with 4-methyl catechol showed only a trace of PO activity (Fig. 1a), but this was greatly enhanced when polyacrylamide gels were exposed to SDS (Fig. 1b). Boiling of HLS proteins (100° C for 5 min) before native gel electrophoresis abolished any detectable PO activity as assessed by 4-methyl catechol oxidation with or without SDS pretreatment (Fig. 1c). The observed background PO activity (Fig. 1a) might be due to the nucleophile MBTH present in the substrate mixture (Nellaiappan *et al*., 1989).

PO activity detected through the use of catechol in HLS after SDS-PAGE showed a series of bands with molecular masses that ranged from 10 to >500 kDa (Fig. 2a). Most of the PO activity was developed at high molecular mass bands and concentrated on two bands of 400 and 370 kDa. In order to test for the presence of cresolase and laccase activity, tyrosine and DAB were used as substrates, respectively. One band of activity was developed for cresolase (Fig. 2d), and four or more bands were developed for laccase (Fig. 2g). DAB, a substrate for peroxidases, has been used to detect laccases in arthropod cuticle (Binnington & Barrett, 1988). Since peroxidases are able to oxidise catechol substrates (Okun *et al*., 1972), gel strips were preincubated with the copper-chelator and PO inhibitor diethyldithiocarbamic acid (DETC) before development in the respective substrates. This treatment was effective in abolishing any detectable cresolase, laccase and most of the catecholase activity (Fig. 2b, e, h). In order to test the specificity of the enzymatic assay, purified mushroom tyrosinase was included in the activity gels. Catechol staining of this enzyme depicted two doublet forms with fast and slow

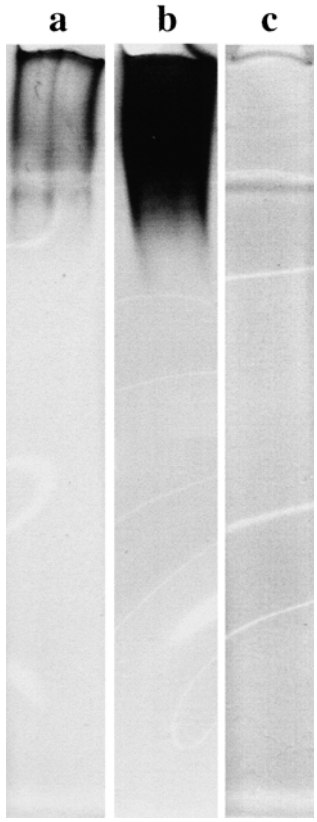

Fig. 1. P. clarkii HLS proteins separated on a 4·5% gel for native PAGE and stained with 4-methyl catechol: (a) HLS proteins electrophoresed and stained with indicated substrate; (b) gel was pretreated with SDS before substrate addition; (c) HLS sample was boiled (100° C for 5 min) before electrophoresis and pretreated with SDS before substrate addition. A total of 20 μg of protein was loaded in each well.

migration pattern (Fig. 2c). Cresolase activity was associated with the slow migrating bands (Fig. 2f), while laccase activity was associated with the fast migrating bands (Fig. 2i).

The thermostability of PO assayed with catechol and DAB was investigated at 20, 30, 50, 60, 70, 80, 90 and 100° C. HLS samples were pretreated at the indicated temperatures for 5 min before SDS-PAGE. Catecholase activity appeared to be inhibited above 70° C (Fig. 3a). A similar pattern of activity was observed for the laccase activity gel (Fig. 3b). The faint activity bands in both enzymatic assays seem to be more resistant to high temperatures.

The effect of pH on the detection of activity showed an increase of PO activity as pH was increased (Fig. 4a–c). Catecholase activity was observed at a wide range of pH values. Activity began to appear at pH 4 (Fig. 4a). Cresolase and laccase activities followed a similar pattern, but activity appeared at pH 5·5 (Fig. 4 b, c). Hydroquinone was used to test for laccase activity due to the oxidation of DAB at alkaline pH.

PO activity associated with haemocyte membranes (HMS) was restricted to high molecular mass bands in SDS-PAGE gels (Fig. 5). PO activity bands with

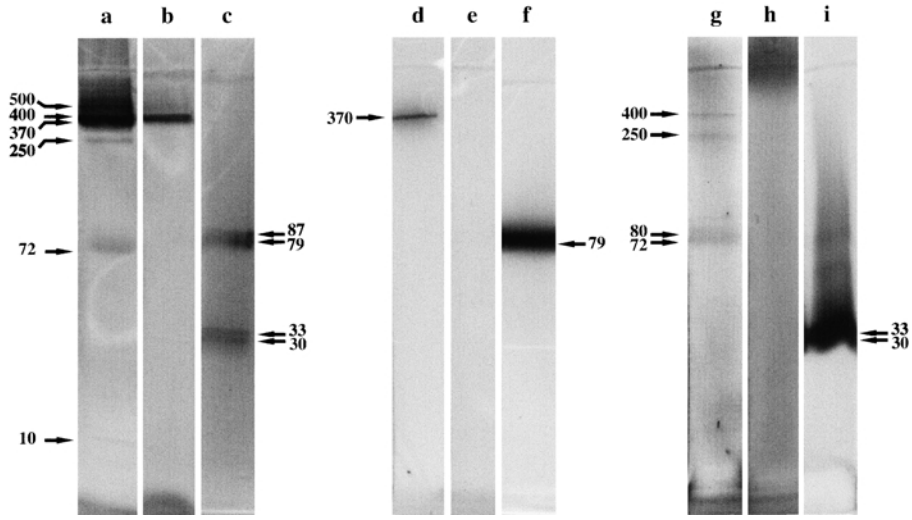

Fig. 2. P. clarkii HLS proteins separated on a 4–20% gradient gel for SDS-PAGE: (a) gel incubated with 4-methyl catechol for catecholase activity; (b) same as in (a), but pre-treated with 10 mM DETC; (d) gel incubated with tyrosine methyl ester for cresolase activity; (e) same as in (d), but pre-treated with 10 mM DETC; (g) gel incubated with DAB for laccase activity; (h) same as in (g), but pre-treated with 10 mM DETC; (c), (f) and (i), purified mushroom tyrosinase control incubated with 4-methyl catechol, tyrosine methyl ester and DAB, respectively. Protein loaded in catecholase, cresolase and laccase gels were 4·5, 2·5 and 2·5 μg, respectively. Mushroom tyrosinase was loaded at a concentration of 1 μg.

molecular masses less than 300 kDa were not observed in HMS, contrary to HLS (see Fig. 1). The different treatments used to elute PO activity from haemocyte membranes seems to result in different patterns of PO activity bands (Fig. 5 a–e). However, when all treatments were incubated with trypsin (1 mg ml^{-1}) before SDS-PAGE, two PO activity bands of 320 and 280 kDa were observed in all treatments (data not shown). HMS did not stain for cresolase and laccase activity.

IV. Discussion

The HLS preparation procedure and adapted PO activity gels were able to produce a 72 kDa PO band that is similar to the 76 kDa molecular mass of purified crayfish haemocytes proPO (Aspán & Söderhäll, 1991). The deduced amino acid sequence of a cDNA cloned proPo from *Pacifastacus leniusculus* codes for a protein of 80 kDa (Aspán *et al.*, 1995). This molecular mass corresponds with another PO activity band of approximately 80 kDa which stained with DAB, hydroquinone and with catechol in DMG (see Figs 2 and 4). Purified proPO from *Galleria mellonella* is composed of two subunits of 80 and 83 kDa which are detectable in the presence of SDS under non-reducing conditions (Kopácek *et al.*, 1995). The proPO gene, isolated from a cDNA library of *Hyphantria cunea*, encodes two polypeptides with molecular masses of 78·2 and 80·2 kDa (Park *et al.*, 1997). Purified *Manduca sexta* proPO contains

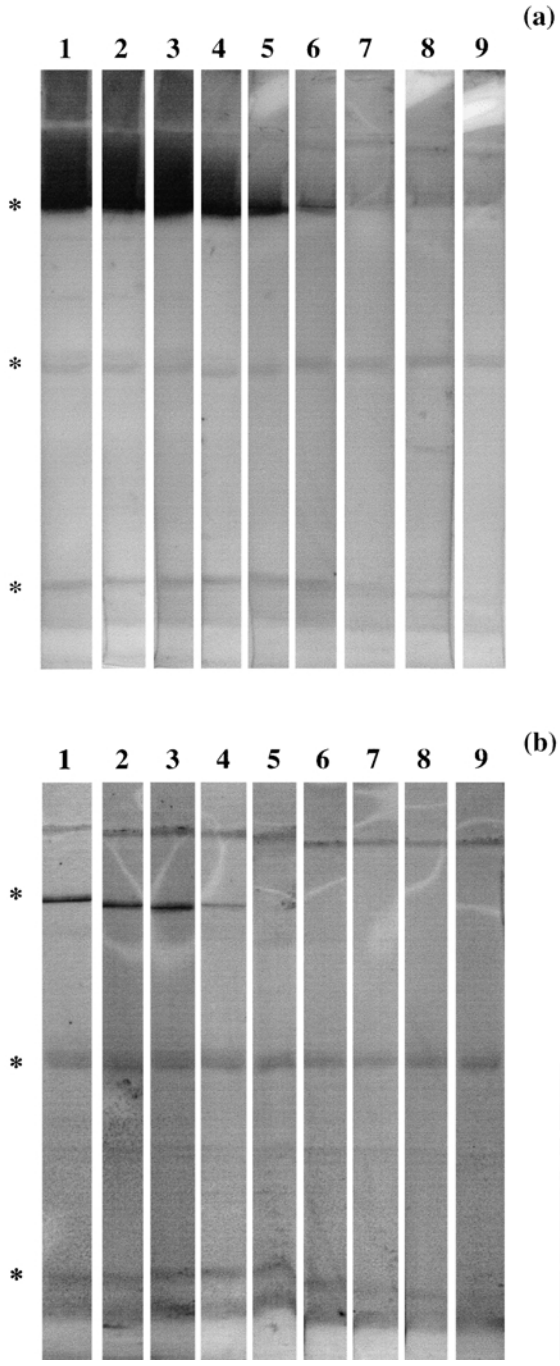

Fig. 3. P. clarkii HLS proteins separated on 4–20% gradient SDS-PAGE gels and developed in (a) 4-methyl catechol and (b) DAB. Before electrophoresis HLS samples were incubated at different temperatures for 5 min, starting at (1) 20° C up to (9) 100° C in 10 degree increments. Asterisk denote high, intermediate and low molecular mass PO activity bands depicted in previous figures. A total of 17 μg of protein was loaded in each well.

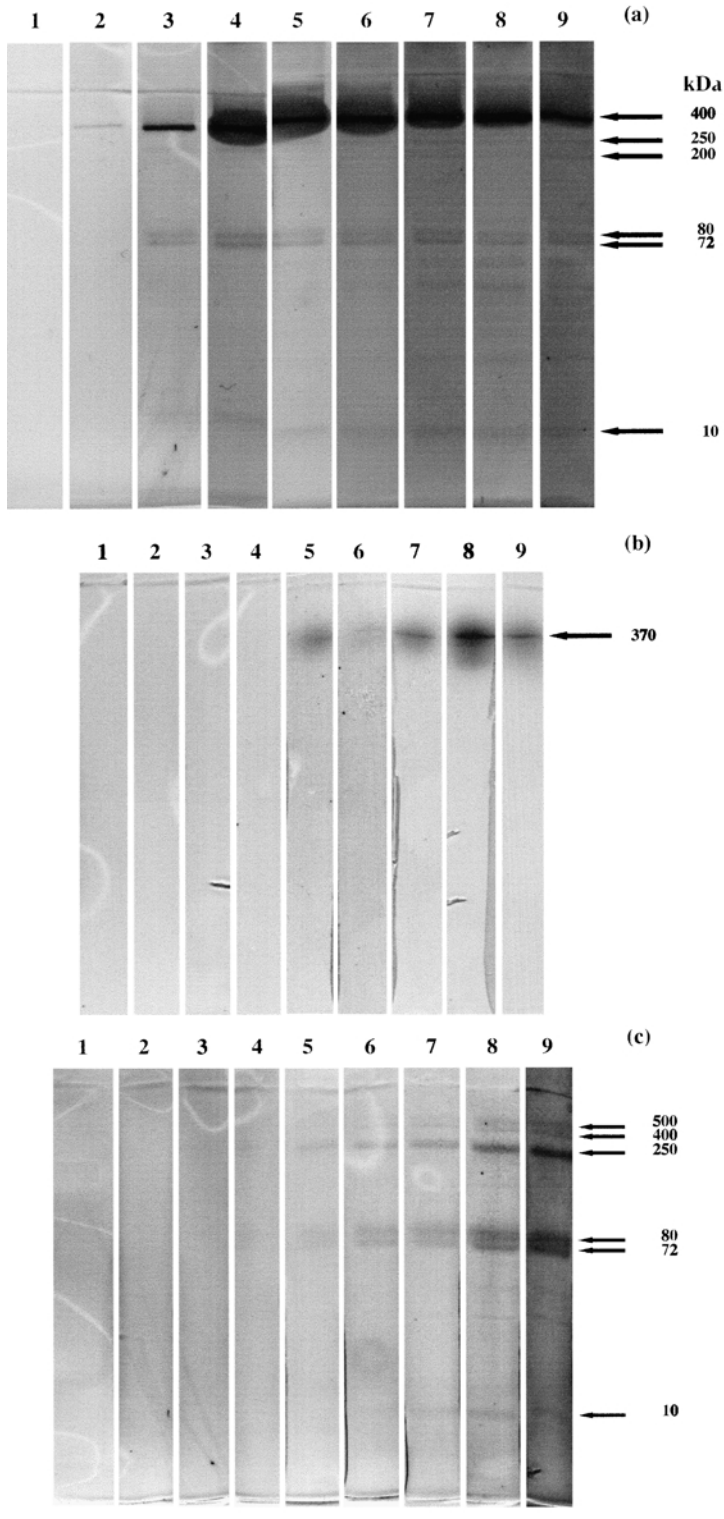

Fig. 4. For legend see facing page.

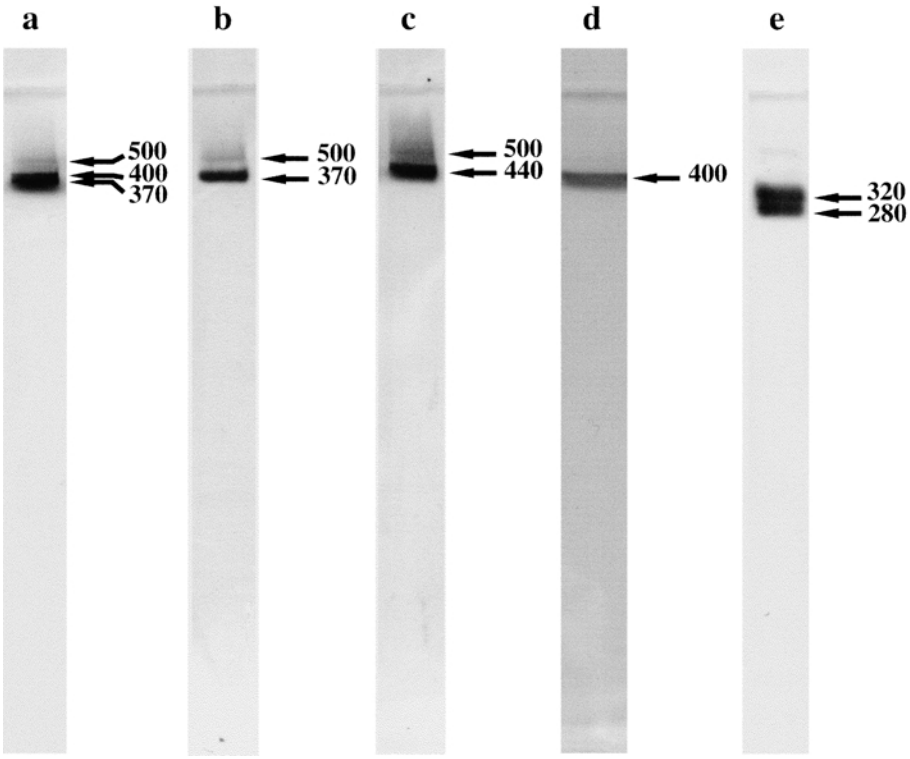

Fig. 5. P. clarkii HMS in 4–20% gradient SDS-PAGE. PO activity associated with membranes was assayed with 4-methyl catechol after the following treatments (see Materials and Methods): (a) LyB; (b) LyB plus 1% cholic acid; (c) LyB plus 1% Triton X-100; (d) LyB plus 0·5 M NaCl; (e) LyB plus 1 mg ml^{-1} trypsin. Protein determination was affected by extraction procedure, but same amount of extraction was loaded in each well.

two polypeptides of 78 and 80 kDa estimated from SDS-PAGE (Jiang *et al.*, 1997a). A cDNA library from *Anopheles gambiae* haemolymph produced two different proPO clones with deduced molecular masses of 78 and 78·6 kDa (Jiang *et al.*, 1997b). According to these reports, it is not surprising that bands of PO activity with similar molecular masses were detected in the present SDS-PAGE gels.

Partially or fully active PO seems to form large aggregates of higher molecular mass relative to inactive PO (Ashida & Yoshida, 1988; Andersson *et al.*, 1989; Aspán & Söderhäll, 1991). Aggregates of proPO, PO, and an interleukin 1-like protein has been shown to have a molecular mass of 400 kDa in *Manduca sexta* (Beck *et al.*, 1996). Partial activation of proPO in the present

Fig. 4. P. clarkii HLS in 4–20% gradient SDS-PAGE. PO activity was assayed in nine different pH values, starting with 3·5 (1) up to 7·5 (9) in 0·5 increment units. Gels were developed in three different substrates: (a) 4-methyl catechol; (b) tyrosine methyl ester and (c) hydroquinone. Protein loaded in each well for gels a, b and c, were 5·5, 15 and 14 μg, respectively.

HLS preparation or during SDS-PAGE would explain the high molecular mass observed in the PO activity gels. However, electrophoresis of the HLS preparation under non-denaturing conditions in native PAGE showed little PO activity unless the gel was subsequently treated with SDS when PO activity was enhanced. Thus, the presence of SDS is able to trigger the activation of proPO and hence its possible polymerisation to itself or other proteins under the denaturing conditions of SDS-PAGE. It is interesting to note that a monospecific antiserum preparation against crayfish proPO is able to bind to the 76 kDa protein (molecular mass of purified proPO), but is unable to recognise any other protein in crude extracts from crayfish haemocytes after SDS-PAGE (Aspán *et al.*, 1995). It is not clear whether in this last report crude extracts were boiled prior to SDS-PAGE, and thus protein aggregates might have been broken up. The epitope recognised by the monospecific antiserum might also have been masked by protein aggregates.

The presence of several PO activity bands in the HLS preparations can also be related to enzyme isoforms. In both the white shrimp *Penaeus setiferus* and the pink shrimp *P. duorarum*, two enzyme isoforms have been identified (Chen *et al.*, 1997). The white shrimp isoforms are 20 and 25 kDa, and the pink shrimp isoforms are 30 and 35 kDa. Tissue extracts from *Ceratitis capitata* larvae show several molecular forms with tyrosinase activity after SDS-PAGE (Charalambidis *et al.*, 1994). Mushroom tyrosinases have also been reported to be present in multiple forms (Bouchilloux *et al.*, 1963; Jolley & Mason, 1965). The PO band with lowest molecular mass in the present work on crayfish had a molecular mass of 10 kDa. This was present in the gels stained with catechol and hydroquinone. This molecular mass band region did not present cresolase activity. PO activity in this region is not likely to be an assay artifact since it stained differentially with catechol, DAB, hydroquinone and tyrosine. It was inhibited when the gel was pretreated with DETC as compared to non-pretreated controls, and its PO activity was impaired at temperatures higher than 70° C. It is possible that the 10 kDa band is a degradation product that retains some PO activity. This degradation product might only bear one of the two putative Cu-binding centers of crayfish proPO (Aspán *et al.*, 1995), important for enzyme activity. Based on a cDNA clone of crayfish proPO (Aspán *et al.*, 1995), a polypeptide that would contain the two putative Cu-binding centers would have a molecular mass>28 kDa.

The use of DETC, a copper chelator and PO inhibitor, readily abolished all detectable cresolase and laccase and most of the catecholase activities. This supports the action of phenoloxidases in the activity bands observed in the present study. Further, horseradish peroxidase was able to oxidise methyl catechol, but was not inhibited by DETC (data not shown). PO activity gels are particularly suitable for inhibition studies, because thiol compounds like DETC can be removed from the gels before substrate addition and thus avoiding the formation of colorless adducts (Sanada *et al.*, 1972). Therefore, only Cu-containing enzymes like PO can present inhibition of activity as opposed to iron-containing enzymes like peroxidase.

High temperature sensitivity of PO activity of high and low molecular mass bands supports the enzymatic nature of the substrate oxidation. High temperature resistance of PO intermediate molecular mass bands (i.e. 72 and 80 kDa)

resembles the cuticular laccase-type enzyme B of *Calliphora vicina* (Barrett & Andersen, 1981).

Substrate specificity of enzyme activity showed a strong catecholase reaction in all preparations. Methyl catechol was readily oxidised, followed by dopamine and L-DOPA estimated by the rate of visual development of activity bands on the gels (data not shown). Similar substrate preferences have been reported using different PO assay techniques (Barrett & Andersen, 1981; Hall *et al*., 1995). Cresolase activity confirmed the presence of a tyrosinase-type PO in *P. clarkii* haemocytes. However, detection of laccase activity is surprising since this enzyme is believed to be restricted to integumental tissues where its key function is thought to be involved in cuticle sclerotisation (Barrett, 1987b; Charalambidis *et al*., 1994). Interestingly, laccase activity assayed either with DAB or hydroquinone was not detected in the region corresponding to the cresolase band at 370 kDa. This agrees with the contention that phenoloxidases of the tyrosinase type have cresolase and catecholase activity, and laccases are able to oxidise *o*- or *p*-diphenols, but not monophenols. This enzymatic specificity was confirmed by the differential staining of purified mushroom tyrosinase included in the assays as a control. Haemolymph of immune reactive arthropods might benefit from the presence of laccase activity that could exert its action in the hardening of humoral or cellular capsules around invading parasites, which might explain its presence in crayfish haemocytes. Such a mechanism has been proposed for cellular capsule formation in *Drosophila* (Nappi *et al*., 1992).

Catecholase showed the widest range of pH activity. A similar pattern has been reported on isolated pre-phenoloxidase from the silkworm *Bombyx mori* (Ashida, 1971). In this last report, L-DOPA oxidation was reduced to 25% at pH 4·5, increased to 75% at pH5 and 100% at pH 6. Crayfish cresolase activity had a relatively higher pH value for optimum activity than the corresponding catecholase activity. This activity was inhibited below pH 5·5. In the table beet leaves cresolase activity increased at pH values above 4 and peaked at pH 5·5, maintaining high activity to about pH 7 (Escribano *et al*., 1997a). The optimum pH for insect cuticle-associated laccase activity ranges from 4·3–4·9 (Barrett, 1987a, b). In the present report, crayfish laccase activity was faint at pH 5 and peaked between pH 8 and 9. This higher pH preference might be related to its unusual localisation of this enzyme in crayfish haemocytes. Laccase is believed to be present only in arthropod cuticles where it mediates sclerotisation (Barret, 1987b).

Immunofluorescence analysis has demonstrated the presence of proPO on the surface of insect haemocytes (Charalambidis *et al*., 1996). In plants, the association of PO with membranes has been established (Escribano *et al*., 1997a, b). Our ability to elute PO easily from crayfish haemocyte membranes suggests this enzyme might be only weakly associated to the membranes. Similar weak associations of plant PO to thylakoid membranes has been documented (Robinson & Dry, 1991). If the putative haemocytic membrane PO activity is the result of unspecific binding of soluble PO during HLS preparation, then a similar banding pattern of PO activity should have been developed, as was seen in the HLS preparations. PO readily attaches to surfaces when active (Söderhäll *et al*., 1979). Since the HLS preparations were

shown to contain PO largely in an inactive form, PO binding to haemocyte membranes should have been avoided. Besides, the presence of EDTA in the lysing buffer should prevent PO attachment, because calcium ions are necessary for this property (Söderhäll *et al*., 1979). More importantly, the banding patterns of PO activity from membranes were dissimilar to HLS and cresolase activity was never observed in any of the membrane preparations, strongly suggesting that the membrane and HLS enzymes are different. The different PO enzymatic activity of membrane-bound and soluble PO has also been reported for the helminth, *Fasciola gigantia*, where PO is also believed to be involved in the pathogen defence mechanisms (Nellaiappan *et al*., 1989). A haemocyte surface PO might be important in the crayfish cellular defence against bacteria since it has been shown that cell-surface PO is a prerequisite for LPS internalisation in *Ceratitis capitata* haemocytes (Charalambidis *et al*., 1996).

In summary we have presented a preliminary characterisation of phenoloxidase activities in gel-immobilised proteins from *P. clarkii* HLS and HMS preparations. We demonstrate the presence of cresolase, catecholase and laccase activities in the crayfish HLS. The multiple bands of PO activity may be due to both protein aggregation and PO isoforms based on difference in substrate preferences, pH optima and temperature sensitivity. The detection of cresolase and catecholase activities in crayfish haemocytes agrees with the reports of tyrosinase-type activity that purified arthropod proPO has shown. However, laccase activity has not been reported before in arthropod haemocytes. Since this enzymatic activity is important in cuticle sclerotisation, it is possible that this enzyme might be important in the formation of capsules around parasites too big to be phagocytosed. A membrane bound PO activity is suggested, based on the PO activity of HMS preparations. The enzymatic properties of the different PO activities and associated molecular masses could be used to better determine the roles of these activities in the pathogen defence system of crayfish.

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