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Purification of the pro-phenol oxidase enzyme from haemocytes of the cockroach *Blaberus discoidalis*

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Pro-phenol oxidase was purified from the haemocytes of the cockroach *Blaberus discoidalis* by Blue Sepharose chromatography, hydrophobic-interaction chromatography on a Phenyl-Superose column and, finally, gel filtration on a Superose 6 column. Results suggest that the molecule exists as a polymer of identical 76 kDa monomeric units. The enzyme is a glycoprotein with pI of 5.2 and can be converted by trypsin into phenol oxidase.

INTRODUCTION

Phenol oxidase [PO; tyrosinase, phenolase, *o*-phenol monooxygenase, oxygen oxidoreductase (EC 1.14.18.1)] is a copper-containing enzyme involved in synthesis of the pigment melanin from phenolic substrates. It is a mixed-function oxidase, catalysing cresolase (*o*-hydroxylation of monophenols to *o*-diphenols) and catecholase (oxidation of *o*-diphenols to *o*-quinones) activities. In insects it has been detected in the haemolymph [1–3], cuticle [2,4,5], accessory glands [6,7], egg cases [8], salivary glands [9] and mid-gut [10]. Its immunological role is best understood in the haemolymph, where it is generally accepted to be present in a precursor form as pro-phenol oxidase (proPO) [11–16], either in the haemocytes [17–20] or in the plasma [15,16,21,22]. The activation of proPO occurs as a result of a Ca^{2+}-requiring enzyme cascade involving serine proteinases [21,23,24].

The proPO system has been implicated in immune defence [1,21,25,26], since the sheaths formed during the cellular responses of encapsulation and nodulation around microbial and macrobial parasites are often melanized [25–29]. In addition, activation of the proPO system is elicited by microbial components [2,25], during which other factors are released or activated, and may be responsible for processes such as opsonization and haemokinesis [21,25,30–35]. Furthermore, a number of agglutinins have recently been described and purified from the cockroach *Blaberus discoidalis* which are linked with the activation of the proPO system (H. J. Durrant, C. Chen and N. A. Ratcliffe, unpublished work).

In order to obtain a clearer understanding of the regulatory events involved in the proPO cascade system, it is necessary to isolate, purify and characterize the components involved. In the present paper we describe the purification and preliminary characterization of haemocytic proPO from *B. discoidalis*.

MATERIALS AND METHODS

Animals

Cockroaches (*B. discoidalis*) were reared at 34 °C and fed on dried cat food and water *ad libitum*. Adults of both sexes were used in experiments.

Preparation of a haemocyte-lysate supernatant (HLS)

Cockroaches were chilled at −20 °C for 10–15 min before being pre-injected with, and bled into, 0.4 ml of ice-cold anticoagulant (AC) (10 mM EDTA/100 mM glucose/145 mM NaCl/30 mM trisodium citrate/26 mM citric acid, pH 4.6; 370 mOsM) as described in Leonard et al. [18]. After centrifuging at 800 ***g***, for 5 min at 4 °C, the plasma was removed and the cell pellet washed twice in 10 mM sodium cacodylate buffer, pH 6.5 (cac buffer), containing 0.25 M sucrose and 35 mM $CaCl_2$. The pellet was then homogenized in cac buffer/35 mM $CaCl_2$, without sucrose, and centrifuged at 40000 ***g***, for 30 min at 4 °C, and the supernatant used as HLS.

Assay for PO

All purification samples and plasma were dialysed against 50 mM Tris/HCl/20 mM $CaCl_2$, pH 7.0, before assaying quantitatively for PO activity. Aliquots of 50 μl of HLS or purification fractions or plasma and 50 μl of trypsin (1 mg/ml in distilled water) were incubated together for 30 min at 30 °C. L-Dopa (L-β-3,4-dihydroxyphenylalanine) (50 μl; 3 mg/ml in distilled water) were then added for a further 5 min at 30 °C and the mixture diluted with 550 μl of distilled water before measuring absorbance at 490 nm using a Beckman DU-7 spectrophotometer. The specific activity of the enzyme was expressed as units of A_{490}/min per mg of protein.

For rapidity during purification, qualitative (i.e. yes/no) assays of PO activity were performed using equal quantities (15 μl) of purification sample (undialysed), trypsin and L-dopa mixed in 96-well microtitre plates (Nunc Life Technologies, Uxbridge, Middx., U.K.) at 30 °C until colour developed (15–30 min).

Purification of haemocytic proPO

All columns were run at room temperature, although samples were kept on ice or at 4 °C between stages. A 2 ml portion of HLS from 50 cockroaches was applied to a Blue Sepharose CL-6B (Sigma) column (20 cm × 1 cm) equilibrated in cac buffer containing 35 mM $CaCl_2$, and unbound eluate was collected immediately by washing with 20 ml of the same buffer at a flow rate of 0.3 ml/min. After collection, 0.05 M Tris/HCl, pH 7.3

Abbreviations used: AC, anticoagulant; HLS, haemocyte-lysate supernatant; proPO, pro-phenol oxidase; PO, phenol oxidase; L-dopa, L-β-3,4-dihydroxyphenylalanine; cac buffer, cacodylate buffer; i.e.f., isoelectric focusing.

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(Tris buffer), containing 5 mM $CaCl_2$ and 3.0 M $(NH_4)_2SO_4$, was gently stirred into the ice-cold eluate (~ 7 ml) to achieve a final concentration of 1.0 M $(NH_4)_2SO_4$. The suspension was then filtered through an Acrodisc (0.2 μm pore size) (Gelman Sciences, Northampton, U. K.) and applied, with a 10 ml superloop, to a phenyl-Superose HR 5/5 f.p.l.c. column (Pharmacia AB, Uppsala, Sweden), equilibrated in Tris buffer, containing 5 mM $CaCl_2$ and 1.0 M $(NH_4)_2SO_4$. Bound proteins were eluted with a 35 ml gradient of 1.0 M to 0 M $(NH_4)_2SO_4$ in Tris buffer, containing 5 mM $CaCl_2$, at a flow rate of 0.5 ml/min, and 0.5 ml fractions were collected. Active fractions were pooled and concentrated against 30% (w/v) poly(ethylene glycol) in Tris buffer, containing 35 mM $CaCl_2$, at 4 °C, and then 100 μl applied to a Superose 6 f.p.l.c. column (Pharmacia AB). Elution was carried out in Tris buffer made up with 35 mM $CaCl_2$ and 0.15 M NaCl, at a flow rate of 0.5 ml/min, and 1 ml fractions were collected. The native molecular mass of proPO was estimated by gel filtration using a Superose 6 column calibrated with proteins from molecular-mass kits (Pharmacia AB), containing chymotrypsinogen A (25 kDa), aldolase (158 kDa), ferritin (440 kDa), thyroglobulin (669 kDa) and Blue Dextran 2000.

SDS/PAGE

Vertical-slab electrophoresis was performed as described by Laemmli [36] in 10% (w/v)-polyacrylamide gels (9 cm × 6.5 cm) in the presence of SDS. A 20 μl portion of each of the samples, namely HLS (50 μg), unbound eluate from the Blue Sepharose CL-6B step (50 μg), phenyl-Superose eluate (10 μg) and Superose 6 eluate (5 μg) were loaded in wells on to a 3% concentrating gel. For determination of molecular mass, an electrophoresis calibration kit (Sigma Chemical Co., Poole, Dorset, U.K.) containing carbonic anhydrase (29 kDa), ovalbumin (45 kDa), BSA (66 kDa), phosphorylase *b* (97.4 kDa), β-galactosidase (116 kDa) and myosin (205 kDa) was used. Gels were stained with Coomassie Brilliant Blue or with the periodic acid/Schiff reagent as described in the Pharmacia PAGE handbook.

Staining for PO activity immobilized on nitrocellulose paper

SDS/PAGE was performed of an HLS sample (5 μg) together with a Pharmacia electrophoresis calibration kit containing α-lactalbumin (14 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa) and phosphorylase *b* (94 kDa). Electrotransfer of proteins was carried out by the method of Towbin et al. [37]. The gel was soaked in transfer buffer (20 mM Tris/150 mM glycine, pH 8.8) for 10 min and then the proteins electrotransferred using the same buffer on to nitrocellulose paper for 1.5 h at 0.8 A in a Bio-Rad Transblot cell (Bio-Rad Laboratories, Watford, Herts., U.K.). A lane run with molecular-mass markers was cut out and stained with Amido Black [1% Amido Black 10B in acetic acid/methanol/water (7:30:63, by vol.)] for 10–15 min and destained until bands were discernible. The rest of the paper was blocked by incubation in 50 mM Tris containing 150 mM NaCl, 5 mM EDTA, 0.05% Triton X-100 and 0.25% gelatin, pH 8.0, for 2 h at room temperature. It was then stained for PO activity by using the method of Nellaiappan & Vinayagam [38], which involves incubation of the nitrocellulose paper in 100 ml of cac buffer made up with 5 mM $CaCl_2$ containing 10 mM L-dopa mixed with 25 ml of 3-methyl-2-benzothiazolinone hydrazone (0.3% in ethanol) at room temperature, until staining developed (10–15 min).

Native PAGE and elution of protein from gels

Native PAGE was performed in 5% polyacrylamide gels (12–15 cm) as described by Davis [39] in a vertical-slab-electrophoresis system. The samples (~ 100 μl) consisted of HLS (3 μg) of Blue Sepharose CL-6B through-flow (3 μg). The gels were stained for enzyme activity by immediately immersing them in cac buffer with 5 mM $CaCl_2$, containing L-dopa (3 mg/ml), at 30 °C for 15–30 min until bands developed.

For elution, the HLS sample was loaded directly on to the surface of the gel without using wells, and after electrophoresis a vertical strip was cut out and stained with L-dopa. Horizontal strips were cut from the remainder of the gel, each corresponding to the three areas of enzyme activity detected (see the Results and discussion section), homogenized in Tris buffer and left stirring overnight before centrifuging to remove the gel pieces from the resulting protein solution. Each fraction was subjected to SDS/PAGE under the conditions detailed above.

Isoelectric focusing (i.e.f.)

I.e.f. was performed with a Pharmacia flat-bed apparatus at 10 °C using preformed LKB Ampholine PAG plates, pH 3.5–9.5 (Pharmacia AB), according to the manufacturer's instructions. The sample consisted of 5 μg of purified protein in 100 μl of Tris buffer. For pI determination, an Electran calibration kit (BDH Laboratory Supplies, Poole, Dorset, U. K.), containing c-phycocyanin (pI 4.75, 4.85), azurin (5.65), porcine trifluoroacetylated myoglobin met (5.9), porcine myoglobin met (6.45), equine myoglobin met (7.3), sperm-whale myoglobin met (8.3) and cytochrome *c* (10.6), was used.

Estimation of protein concentration

Protein concentration was determined by the method of Bradford [40], using dilutions of a solution of BSA as standards.

RESULTS AND DISCUSSION

Significant levels of proPO were detected in the haemocytes of *B. discoidalis*, but none was present in plasma. Similar results

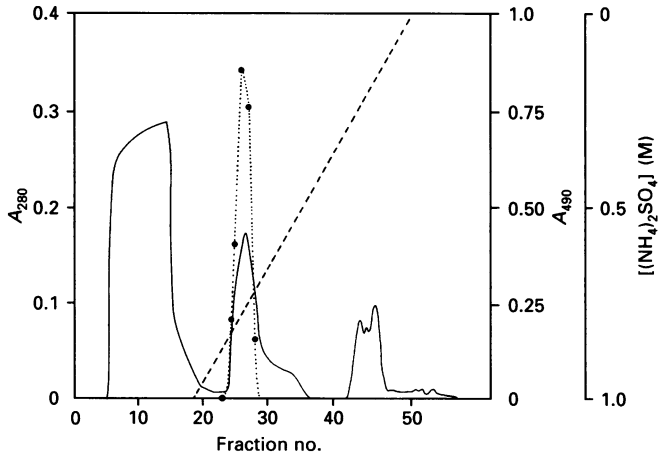

Figure 1 Hydrophobic interaction chromatography of *B. discoidalis* HLS proteins on phenyl-Superose

After Blue Sepharose chromatography, the sample was adjusted to a concentration of 1.0 M ammonium sulphate, and 10 ml was applied to a phenyl-Superose f.p.l.c. column. Adsorbed protein was eluted with a decreasing linear gradient from 1.0 M–0 M $(NH_4)_2SO_4$ in 0.05 M-Tris containing 5 mM $CaCl_2$ at pH 7.3. ——, Protein profile (A_{280}); ●, PO activity after trypsin activation (A_{490}); – – – –, concentration of $(NH_4)_2SO_4$.

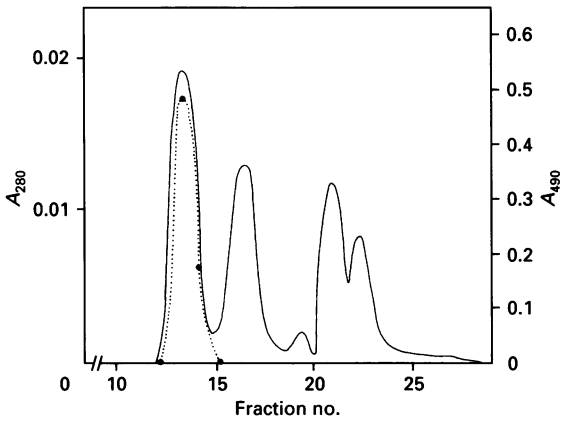

Figure 2 F.p.l.c. gel-filtration on Superose 6

After phenyl-Superose chromatography, active PO fractions were pooled and concentrated, and 100 μl was loaded on to a Superose 6 f.p.l.c. column and proteins eluted in the presence of 0.15 M NaCl in Tris buffer containing 35 mM $CaCl_2$. ——, Protein profile (A_{280}); ●, PO activity at A_{490} after trypsin activation.

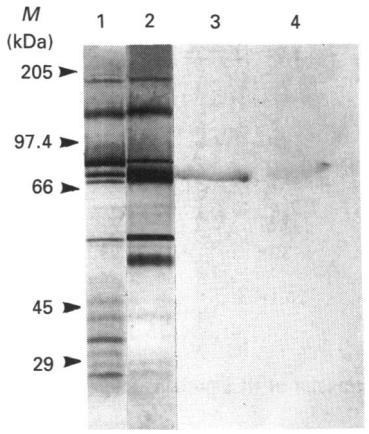

Figure 3 SDS/PAGE under non-reducing conditions of the stages of purification of proPO

HLS (50 μg; lane 1), Blue Sepharose eluate (50 μg, lane 2), phenyl-Superose eluate (10 μg, lane 3) and Superose 6 eluate (5 μg, lane 4) were subjected to SDS/PAGE (10% gel) with molecular-mass (*M*) markers. The gel was stained with Coomassie Brilliant Blue.

have been obtained by Leonard et al. [18] with the closely related species *B. craniifer*.

In order to purify proPO from the haemocytes of *B. discoidalis*, initially, the proPO-activating enzyme was removed by passing the crude HLS through a Blue Sepharose CL-6B column [41,42]. Subsequently, proPO free of the endogenous activating system was purified by f.p.l.c. employing phenyl-Superose hydrophobic-interaction chromatography, from which a major peak containing all the PO activity (fractions 23–28) and four minor peaks were eluted (Figure 1). This was followed by gel filtration using Superose 6, which separated the proPO sample into a further four peaks, of which the proPO (fractions 13 and 14) was found in the peak with the lowest retention time (Figure 2). Details of the purification are shown in Table 1. An HLS extract from 50 insects had a total protein content of 9.68 mg, and this yielded 27.0 μg of proPO with an apparent purification factor of 54-fold.

The proPO enzyme was more stable, in a form which could be activated to PO, after the Blue Sepharose column, and a high $CaCl_2$ concentration was used in the buffer to reduce the possibility of spontaneous activation of proPO. Preliminary results showed that the proPO appeared to be activated after $(NH_4)_2SO_4$ precipitation. For that reason, the percentage saturation of $(NH_4)_2SO_4$ which the sample was raised to, before phenyl-Superose chromatography, was below that at which it would precipitate. It was still, however, subject to some activation under these conditions. Preservation of activity at 4 °C for any length of time was variable at any stage of the purification, and the enzyme activity was not freeze–thaw-stable. That the final active extract after Superose 6 chromatography was mainly proPO was confirmed, since significant conversion of L-dopa was only apparent after fractions were treated with trypsin.

The purified enzyme was found to have a molecular mass of approx. 76 kDa as determined by SDS/PAGE under reducing and non-reducing conditions (Figure 3). This corresponded to a band staining for PO activity on nitrocellulose paper after SDS/PAGE and electrotransfer of proteins (Figure 4). The enzyme is unusual in that it has activity after exposure to SDS/PAGE conditions, as has been reported for proPO in other animals, e.g. the crayfish *Astacus astacus* [43]. During SDS/PAGE the proPO probably underwent a conformational change to produce PO activity. This assumption can be made, since similar observations have been made with the purified proPO from the crayfish *Pacifastacus leniusculus*, in which no proteolytic cleavage could have occurred in the pure sample. Furthermore, by addition of trypsin to the crayfish proPO, a change of molecular mass was then observed after electrophoresis [42]. The molecular mass of 76 kDa for the *B. discoidalis* enzyme is comparable with the molecular masses, estimated by SDS/PAGE, for other arthropod proPOs: *Bombyx mori* (silkworm), 80 kDa [11]; *Calliphora erythrocephala* (blow-fly), 87 kDa [44]; *Manduca sexta* (tobacco hornworm), 77 and 71 kDa [45]; *Hyalophora cecropia* (Cecropia moth), 76 kDa [46] and *Pacifastacus leniusculus*, 76 kDa [42].

Table 1 Summary of the purification of proPO from the haemocytes of *Blaberus discoidalis*

The data are calculated on the basis of a typical purification run as described in the Materials and methods section from an HLS made from 50 adult cockroaches.

	Volume (ml)	Total activity* (ΔA_{490}/min)	Percentage of PO† in total activity (%)	Total protein (mg)	Specific activity (ΔA_{490}/min per mg of protein)	Activity yield (%)	Purification (fold)
HLS	2	193	6.9	9.68	20	100	1
Blue Sepharose	7	165	14.4	2.31	72	86	3.6
Phenyl-Superose	4	31	6.1	0.25	126	16	6.3
Superose 6	2	29	3	0.03	1070	15	53.9

* Total activity is the activity of the sample with trypsin followed by the substrate, L-dopa.
† Percentage of PO is the activity of sample without trypsin activation.

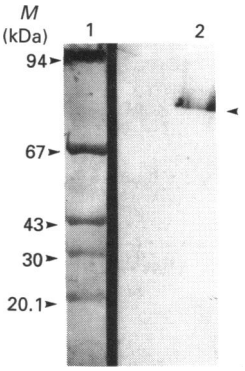

Figure 4 Electrotransfer of HLS proteins after SDS/PAGE

HLS (5 μg, lane 2) was subjected to SDS/PAGE with molecular-mass (*M*) markers (lane 1) and then electrotransferred to nitrocellulose paper. The HLS proteins were stained with L-dopa and 3-methyl-2-benzothiazolinone hydrazone to detect PO activity (arrow).

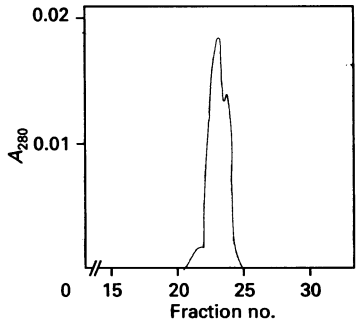

Figure 5 Re-chromatography on Superose 6, in the absence of NaCl, of PO active fractions from a peak resolved on Superose 6 in the presence of NaCl

Purified proPO was subjected to gel filtration on f.p.l.c. Superose 6 in Tris buffer containing 35 mM $CaCl_2$ in the absence of NaCl. ——, Protein profile (A_{280}).

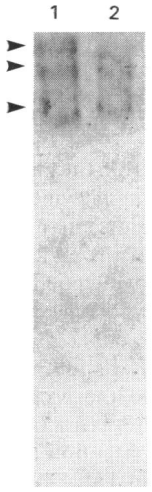

Figure 6 Native PAGE of *B. discoidalis* HLS and Blue Sepharose eluate

HLS (3 μg, lane 1) and Blue Sepharose eluate (3 μg, lane 2) were subjected to native PAGE (5% gel) and the gel stained with L-dopa to detect PO activity. The arrows indicate the positions of the three bands with PO activity.

After gel filtration, the molecular mass of *B. discoidalis* proPO was found to be approx. 300 kDa. This is also in agreement with the results of other studies utilizing native PAGE: *Musca domestica* (housefly) larvae, 310 kDa [47]; *M. domestica* prepupae, 178 kDa inactive, 340 kDa active form [48], and *M. domestica* pupae, 330 kDa [49]. Interestingly, Aspan & Söderhäll [42] reported a very similar molecular mass after gel filtration for the proPO from crayfish. Our interpretation of these results is that, *in vivo*, the enzyme may occur as a polymeric protein.

When the purified fractions in the peak with proPO activity on the Superose 6 column were pooled and rechromatographed on Superose 6 in the absence of NaCl, a major protein peak with two shoulders was obtained (Figure 5). At this stage, however, all proPO activity had been lost. In addition, when the HLS or the Blue Sepharose through-flow fractions were subjected to non-denaturing PAGE and stained for enzyme activity, three bands of low mobility were obtained (Figure 6). An identical result was obtained whether or not trypsin was included with L-dopa in the incubation mixture, indicating that proPO had been activated during electrophoresis as proposed above for SDS/PAGE. When the three bands obtained by native PAGE were cut out from the gel and the protein was eluted by diffusion and subjected to SDS/PAGE, however, they co-migrated with the chromatographically purified proPO preparation at 76 kDa. These observations suggest that proPO can be present in different polymeric forms *in vivo*. Native PAGE of purified POs of *H. cecropia* pupae [46] and *B. mori* larvae [12], two lepidopterans, and now the results obtained with the proPO purified from a dictyopteran, described here, indicate that multiple forms of the protein may be present in various insect groups. In contrast, native PAGE of purified PO from larvae [47], pupae [49] and prepupae [48] from *M. domestica*, a dipteran, revealed only one band. In the case of the prepupae preparation, however, this required electrophoresis of the proPO sample in the presence of EDTA.

Purified *B. discoidalis* proPO could also be revealed on SDS/PAGE gels by staining with periodic acid/Schiff reagent, which indicates that it is a carbohydrate-containing protein. ProPO has been reported as a glycoprotein from all preparations that have been tested for carbohydrate [e.g. 42,45,50,51]. Mannose and sialic acid are reported to be two of the constituent carbohydrates in both an insect cuticular-granule PO and a human tyrosinase [45,52]. Differences in the extent of glycosylation could also explain the minor differences between the native molecular-mass forms observed in *B. discoidalis*.

Only one protein band was revealed after isoelectric focusing and this corresponded to pI 5.2. This is similar to that obtained for other arthropod haemolymph proPO, e.g. 4.98 for *B. mori* [11], 4.7 for *M. domestica* [47] and 5.4 for *P. leniusculus* [42]. This result suggests that no proPO isozymes are present in *B. discoidalis* haemocytes.

Now that purification of the proPO in *B. discoidalis* and the study of some of its physical properties have been achieved, it should be possible to proceed with functional studies of its activation and role in insect immunity.

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