The Isolation of an *o*-Diphenol Oxidase from Third-Instar Larvae of the Blowfly *Calliphora erythrocephala*

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1. The isolation of an *o*-diphenol oxidase from an acetone-dried powder of late-third-instar larvae of *Calliphora erythrocephala* was investigated. An insoluble and microcrystalline fraction containing the enzyme activity was obtained after fractionating extracts of the acetone-dried powder with $(\text{NH}_4)_2\text{SO}_4$ and acetone. 2. This fraction can be solubilized in 0.1% sodium dodecyl sulphate without loss of activity. 3. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate shows that the *o*-diphenol oxidase is a minor component of the extracts from the acetone-dried powder. 4. The *o*-diphenol oxidase was purified by zonal centrifugation on a sucrose density gradient in the presence of sodium dodecyl sulphate. 5. The amino acid composition of the purified enzyme resembles that of some other *o*-diphenol oxidases. 6. The subunit composition of the *o*-diphenol oxidase is discussed.

o-Diphenol oxidases (EC 1.14.18.1, *o*-diphenol- O_2 oxidoreductase) are important components of the blood and cuticle of insects, and their characterization is necessary if we are fully to understand the roles they play. Karlson and his co-workers carried out a series of studies on the phenolic metabolism of the larvae of blowflies. One of these studies (Karlson & Liebau, 1961), which described the purification of an *o*-diphenol oxidase from an acetone-dried powder of third-instar larvae of the blowfly *Calliphora erythrocephala*, has been prominently noted in reviews (Cottrell, 1964; Brunet, 1965). This enzyme has been considered to play a key role in the oxidation of *o*-diphenols involved in cross-linking the puparium. It does not readily oxidize monophenols. We decided to reinvestigate the method of purifying this insect *o*-diphenol oxidase, because Karlson & Liebau (1961) claimed that it gave a pure crystalline enzyme. During the course of our work, Munn & Bufton (1973) demonstrated that extracts of acetone-dried powders of *Calliphora* larvae contained substantial amounts of the blood protein calliphorin (Munn *et al.*, 1971) and another protein (Protein II) (Munn & Greville, 1969) as well as the *o*-diphenol oxidase activity.

In the blood of *Calliphora* larvae the *o*-diphenol oxidase is in the form of an inactive pro-enzyme which can be activated by a specific proteolytic enzyme (Schweiger & Karlson, 1962). Methods for separating the pro-enzyme in the blood from the two major contaminating proteins by means of agar-gel electrophoresis and density-gradient centrifugation

were described by Munn & Bufton (1973), though on the evidence of gel electrophoresis in the presence of sodium dodecyl sulphate, the purified pro-enzyme was not homogeneous. The present paper demonstrates, by another method, that the active *o*-diphenol oxidase in extracts of acetone-dried powders of late-third-instar larvae of *Calliphora erythrocephala* is, on a protein basis, a minor component of the extract. A method for purifying the active *o*-diphenol oxidase is described, and its amino acid composition resembles that of other *o*-diphenol oxidases. The results of polyacrylamide-gel electrophoresis of the active protein give information about its subunit composition. An abstract of some of the results presented in this paper has been published (Pau & Eagles, 1973).

Experimental

Materials

Insects. Larvae of *Calliphora erythrocephala* (Meig.) were grown on ox spleen at 28°C. They were collected in sawdust at the late wandering stage, which precedes pupation, and stored at 4°C for as long as 3 weeks, until the required quantity of larvae had been accumulated.

Chemicals. All chemicals were obtained from BDH, Poole, Dorset, U.K., and were of AnalaR grade.

Buffers. Unless otherwise stated, buffer solutions were 10mM-Tris-HCl, pH 7.0, made by diluting a stock solution with water. The stock solution was

made by adjusting the pH of 0.5M-Tris with 11.6M-HCl at 20°C.

Methods

Enzyme assays. *o*-Diphenol oxidase activity was assayed spectrophotometrically by measuring the rate of formation of dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) from DL-dopa (DL-3,4-dihydroxyphenylalanine) at 25°C. For this 50 μ l of enzyme in Tris-HCl buffer was mixed with 2.5 ml of 25 mM-DL-dopa in Tris-HCl buffer in a cuvette (1 cm light-path), and the initial increase in absorbance at 475 nm was measured by using a Gilford 240 spectrophotometer. The unit of enzyme activity was taken as the amount of enzyme which will catalyse the oxidation of 1 μ mol of DL-dopa/min at 25°C, and it was assumed that 1 μ mol of dopachrome is produced for every 2 μ mol of dopa oxidized (Evans & Raper, 1937). The molar extinction of dopachrome was taken as 3600 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ (Mason, 1948).

Protein concentrations. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (containing less than 1.5% water, w/w) as a standard.

Acetone-dried powders of larvae. Larvae (250 g) were homogenized in 2 litres of acetone at -20°C by using a 2.5-litre Waring blender for 30 s at top speed. The homogenate was filtered under vacuum in a 5-litre Buchner funnel at 4°C until a firm filter cake was obtained. The filter cake was rehomogenized twice under the same conditions, after which it was homogenized in 2 litres of a mixture of cold (-20°C) acetone and diethyl ether (1:1, v/v) and filtered. The filter cake was then spread out on a sheet of filter paper and dried in air at room temperature (20°C). The dried powder was ground with a mortar and pestle and stored at room temperature in an airtight container.

Extraction of the acetone-dried powder. Acetone-dried powder (2 g) was mixed with 50 ml of Tris-HCl buffer containing 0.1 M-ascorbic acid neutralized with a saturated aqueous solution of NaOH. The suspension was thoroughly mixed and then sonicated for 30 s, at a peak-to-peak amplitude of 6 μ m, by using an MSE 100W ultrasonic disintegrator. The sonicated suspension was centrifuged at 20000g (r_{av} , 7.38 cm) for 20 min to give a supernatant which contained the bulk of the enzyme activity.

Purification by zonal centrifugation. The supernatant was adjusted to 35% saturation by the addition of solid (NH₄)₂SO₄ (20.9 g to 100 ml) and centrifuged at 20000g (r_{av} , 7.38 cm) for 30 min. The precipitate was dissolved in 30 ml of Tris-HCl buffer containing 0.1% (w/v) sodium dodecyl sulphate and 2.5% (w/v) sucrose. Zonal centrifugation was performed with a MSE B-14 titanium zonal rotor at 20°C. Gradients were formed with an MSE variable gradient former, the flow rate into the rotor being

15 ml/min. The rotor was loaded at 2500 rev./min with a 500 ml linear gradient of 5–30% (w/v) sucrose in Tris-HCl buffer containing 0.1% sodium dodecyl sulphate. The remaining volume was filled with 60% (w/v) sucrose in Tris-HCl buffer. The solution of the (NH₄)₂SO₄ precipitate was then introduced into the core of the rotor and moved away from the centre by an overlay of 100 ml of Tris-HCl buffer containing 0.1% sodium dodecyl sulphate. After centrifugation at 125000g (r_{av} , 4.23 cm) for 6.5 h at 18°C, the rotor was decelerated to 2500 rev./min and unloaded by pumping 60% sucrose in Tris-HCl buffer at a flow rate of 15 ml/min to the periphery of the rotor. The effluent from the core of the rotor was collected in 7.5 ml fractions. The density of sucrose in the fractions was estimated by measuring their refractive index at 20°C with a Bellingham and Stanley refractometer.

Ultrafiltration. Protein solutions were concentrated by ultrafiltration by using Amicon XM-100 Diaflo membranes and an Amicon model 52 ultrafiltration cell (volume 65 ml).

Gel electrophoresis. Polyacrylamide-disc-gel electrophoresis in the presence of sodium dodecyl sulphate was performed as described by Weber & Osborn (1969), except that the final concentrations of acrylamide and methylenebisacrylamide were varied as indicated. The proteins used as standards of known molecular weight were: myosin, 200000; phosphorylase, 94000; bovine serum albumin, 68000; catalase, 60000; ovalbumin, 43000; lactate dehydrogenase, 36000; haemoglobin, 15600; and lysozyme, 143000. Unless otherwise indicated, the samples were not heated. *o*-Diphenol oxidase activity in the gels was detected by incubating the gels in 2.5 mM-DL-dopa in Tris-HCl buffer at room temperature. When the gels were stained for activity, the enzyme solutions were not treated with 2-mercaptoethanol before electrophoresis at room temperature. Enzyme activity was clearly visible as a red band developing within 5 min. Gels were stained for protein with Coomassie Brilliant Blue R250.

Electron microscopy. The protein suspension was negatively stained with 1% (w/v) uranyl acetate. A drop of the protein suspension was placed on a carbon-coated grid and excess drawn off with filter paper. One drop of uranyl acetate solution was immediately applied and the excess quickly removed. The grid was examined in a Hitachi HU 11-ES electron microscope working at 50 kV.

Amino acid analysis. Samples (about 1 mg) were dialysed overnight against water, freeze-dried, and then dissolved in 1 ml of 5.8 M-HCl (BDH AristaR grade). The solutions were heated at 108°C for 24 and 72 h in sealed tubes which had been flushed with N₂. The hydrolysates were evaporated to dryness under vacuum over NaOH and H₂SO₄. The residues were then dissolved in 0.2 M-sodium citrate buffer, pH 2.2, and analysed on a Locarte amino acid analyser.

Table 1. Preliminary purification of *o*-diphenol oxidase from 2 g of acetone-dried powder

Stage of purification	Total volume (ml)	Activity (units/ml)	Protein (mg/ml)	Total activity (units)	Specific activity (units/mg of protein)
1. Extract of acetone in Tris-HCl buffer	49.2	114	9.35	5609	12
2. Solution, in Tris-HCl buffer, of precipitate after centrifugation of extract which was 35% satd. with $(\text{NH}_4)_2\text{SO}_4$	25.0	96	2.13	2400	45
3. Solution from stage 2, mixed with cold acetone (30% v/v), centrifuged, and the precipitate resuspended in Tris-HCl buffer	8.0	86	0.6	688	143

Fig. 1. Zonal centrifugation of *o*-diphenol oxidase

Fractionation of the precipitate from an extract of 2 g of acetone-dried powder of larvae, 35% satd. with $(\text{NH}_4)_2\text{SO}_4$, by zonal centrifugation in a sucrose gradient. The details are described in the Experimental section. ■, E_{280} ; □, *o*-diphenol oxidase activity; ○, density profile in terms of concentration (w/v) of sucrose.

Results

Initial purification of the extract of the acetone-dried powder can be achieved by precipitating the *o*-diphenol oxidase activity with $(\text{NH}_4)_2\text{SO}_4$ at 35% saturation, or with cold (-20°C) acetone (30%, v/v) or by adjusting the pH of the extract to 5.5 by the addition of 0.1M-acetic acid (at 0°C). Whichever of these methods of purification was used first, the second stage of purification, using the same or one of the other procedures, always resulted in a precipitate which could not be solubilized in 10mM-Tris-HCl buffers at either pH 7.0 or 8.5. Although the precipi-

tate contained less activity than the supernatant, it had a higher specific activity. The results of a typical purification are shown in Table 1. This suspension appeared to be microcrystalline when swirled, and, when negatively stained with uranyl acetate and viewed with the electron microscope, it contained particles which resembled the protein calphorin (Munn *et al.*, 1971). The precipitate is readily solubilized in 0.1% sodium dodecyl sulphate with only slight loss in enzyme activity. Electrophoresis of the precipitate in the presence of sodium dodecyl sulphate showed a major band after staining for

protein (Plate 1a). The mobility of this band corresponded to a mol.wt. of 75000.

Sodium dodecyl sulphate, up to a concentration of 0.15% (w/v), slightly inhibited the activity of the *o*-diphenol oxidase from the acetone-dried-powder extract. About 90% of the activity remained after incubation for 66h at 4°C. About 80% of the activity remained after incubation in 0.5% (w/v) sodium dodecyl sulphate for 66h at 25°C. The K_m for the oxidation of DL-dopa from Lineweaver-Burk plots (2.4mM) was found to be unchanged in the presence of 0.1% sodium dodecyl sulphate.

The retention of activity in sodium dodecyl sulphate makes it possible to detect *o*-diphenol oxidase activity after electrophoresis on polyacrylamide gels run in the presence of sodium dodecyl sulphate. If sodium dodecyl sulphate gels of the enzyme prepared by $(\text{NH}_4)_2\text{SO}_4$ and acetone fractionation are stained for enzyme activity, the activity is not at the position of the major protein band, but instead it migrates more slowly with a mobility equivalent to that of a protein of mol.wt. 210000 (Plates 1a and 1b). Staining for protein reveals no protein band at the position where the active enzyme migrated. The enzyme is therefore only a minor component of this fraction. Because the molecular weight of the active enzyme differed significantly from the molecular weight of the major protein band, it was decided to separate the contaminant protein by density-gradient centrifugation in a zonal rotor. The

results of a typical run on a sucrose zonal gradient in the presence of 0.1% (w/v) sodium dodecyl sulphate are shown in Fig. 1. About 85% of the activity applied to the gradient was generally recovered, and there was 106-fold purification of the applied sample.

A sample of the pooled fractions (about 75ml), which contained *o*-diphenol oxidase activity after zonal centrifugation, gave a single band when incubated in 2.5mM-DL-dopa after electrophoresis in sodium dodecyl sulphate (Plate 1c). In order to observe the protein band after staining, the pooled fractions were concentrated about 13-fold by ultrafiltration before performing electrophoresis (Plate 1d). The band of *o*-diphenol oxidase activity corresponded to the single protein band. The amino acid composition of the pooled fractions which showed *o*-diphenol oxidase activity resembled that of the *o*-diphenol oxidases from mushrooms (Jolley *et al.*, 1969), silkworm (Ashida, 1971) and mouse melanoma (Burnett, 1971) (Table 2). When the purified enzyme was concentrated, and heated to 100°C in the presence of 1% (w/v) sodium dodecyl sulphate, with or without 0.1% (v/v) mercaptoethanol, and then electrophoresed in the presence of sodium dodecyl sulphate, it migrated with a mobility equivalent to that of a protein of mol.wt. 75000 (Plate 1e). In order to establish whether the purified enzyme, which had been heated to 100°C in the presence of sodium dodecyl sulphate, could be dissociated further, it was dialysed overnight against a solution of 8M-urea in 0.1M-sodium

Table 2. Amino acid compositions of *o*-diphenol oxidases

The compositions are expressed as percentage of total residues. For the *o*-diphenol oxidase from *Calliphora*, serine and threonine values were extrapolated to zero time from values obtained from 24 and 72h hydrolyses. Values for tryptophan were not obtained, and no separate analysis, by using a special procedure to avoid the destruction of cysteine by acid hydrolysis, was performed.

Amino acid	<i>Calliphora</i> larvae <i>o</i> -diphenol oxidase	Mushroom β isoenzyme (Jolley <i>et al.</i> , 1969)	Mouse melanoma T2 isoenzyme (Burnett, 1971)	Silkworm pro-(<i>o</i> -diphenol oxidase) (Ashida, 1971)
Lys	4.8	3.9	5.2	2.9
His	2.9	2.5	1.9	2.6
Arg	5.1	4.3	3.9	7.4
Asx	13.8	12.9	10.8	14.9
Thr	6.3	6.8	7.6	5.3
Ser	7.4	5.4	6.4	7.1
Glx	10.5	11.1	11.6	10.2
Pro	4.8	6.8	7.1	6.2
Gly	10.6	7.9	7.7	5.6
Ala	7.9	6.1	7.2	6.0
1/2 Cys	0.1	0.7	1.3	0.5
Val	6.5	5.7	5.8	5.8
Met	1.9	1.8	1.8	3.0
Ile	4.4	5.0	3.4	5.0
Leu	6.7	7.1	8.0	7.6
Tyr	3.5	3.9	3.0	3.4
Phe	7.1	5.0	4.2	6.3
Try	—	2.9	3.2	—

EXPLANATION OF PLATE I

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate

(a) Fraction obtained from an extract of an acetone-dried powder of *Calliphora* larvae after fractionation with $(\text{NH}_4)_2\text{SO}_4$ and acetone (Table 1, stage 3), stained for protein. (b) As in (a), but stained for *o*-diphenol oxidase activity. (c) *o*-Diphenol oxidase (0.23 units), purified by zonal centrifugation, and stained for *o*-diphenol oxidase activity. (d) Sample (100 μl) of the active peak from the sucrose zonal gradient after 13-fold concentration by ultrafiltration (87 enzyme units); stained for protein. The above samples were not heated before electrophoresis. (e) As in (d), mixed with 100 μl of 2% sodium dodecyl sulphate (w/v) in 0.1 M-sodium phosphate buffer, and heated to 100°C for 10 min; stained for protein. (f) As in (d), mixed with 100 μl of 2% (w/v) sodium dodecyl sulphate–0.2% (v/v) 2-mercaptoethanol in 0.1 M-sodium phosphate buffer, heated to 100°C for 10 min and dialysed overnight against 8 M-urea. The fraction was electrophoresed in a gel containing 8 M-urea and stained for protein. Gels (a)–(e) contained 5% (w/v) acrylamide and 0.0675% (w/v) *NN'*-methylenebisacrylamide. Gel (f) contained 10% (w/v) acrylamide and 0.27% (w/v) *NN'*-methylenebisacrylamide.

phosphate buffer, pH7, containing 0.1% (w/v) sodium dodecyl sulphate, and 0.1% (v/v) mercaptoethanol, and then electrophoresed in the presence of sodium dodecyl sulphate in a polyacrylamide gel containing 8M-urea. No extensive dissociation to lower-molecular-weight species was observed, though a faint band with a mobility corresponding to a mol.wt. of 32000 appeared (Plate 1f).

Discussion

The purification of an *o*-diphenol oxidase from extracts of acetone-dried powders of *Calliphora* larvae described by Karlson & Liebau (1961) relied to a great extent on high-speed centrifugation and gave a protein of mol.wt. 530000. Munn & Bufton (1973) demonstrated that these extracts contain large amounts of the blood protein calliphorin, and they suggested that Karlson & Liebau (1961) assigned the properties of calliphorin to the enzyme. The results presented in the present paper are in agreement with this suggestion, and show that during the preliminary purification of the *o*-diphenol oxidase, a protein which appears to be microcrystalline is significantly purified together with the *o*-diphenol oxidase activity. This protein resembles calliphorin in appearance under the electron microscope and in molecular weight, as estimated by its mobility on polyacrylamide gels in the presence of sodium dodecyl sulphate. Estimates of the subunit molecular weight of calliphorin vary between 72000 and 87000 (Munn *et al.*, 1971). After the preliminary purification of the inactive contaminating protein by the method described in the present paper, the accompanying *o*-diphenol oxidase was found to represent a very small fraction of the total protein in the extract (less than 1%). We have shown that the *o*-diphenol oxidase can be purified simply by density gradient centrifugation, and we consider that the protein isolated by this method is essentially pure, because the active enzyme migrates as a single protein band on electrophoresis, and all the activity is associated with this band. The amino acid composition of the purified protein resembles that of other *o*-diphenol oxidases. Purification of the enzyme has been made possible because calliphorin is dissociated in the presence of 0.1% sodium dodecyl sulphate (w/v), whereas the *o*-diphenol oxidase remains as an active high-molecular-weight protein.

The retention of activity of an *o*-diphenol oxidase in sodium dodecyl sulphate was first observed with the enzyme from mushrooms. In the presence of 0.27% (w/v) sodium dodecyl sulphate the mushroom enzyme was found to be 33% dissociated into subunits with no loss of activity (Bouchilloux *et al.*, 1965). In 1% (w/v) sodium dodecyl sulphate the enzyme was 80% dissociated with some loss of activity in 8M-urea (Zito & Kertesz, 1966). When the inactive

pro(*o*-diphenol oxidase) of insects is enzymically activated, it aggregates and appears to form a series of components with different molecular weights (Munn & Bufton, 1973; Ashida & Ohnishi, 1967). The enzyme which we have isolated also aggregates readily when active, and this accounted for the initial difficulties with purification.

The results of the present paper show that, in the presence of 0.1% (w/v) sodium dodecyl sulphate, the purified *o*-diphenol oxidase forms only one active component, with an apparent mol.wt. of 210000. When heated in 1% (w/v) sodium dodecyl sulphate, the enzyme dissociates to a mol.wt. of 75000, which is similar to the subunit molecular weight of the major contaminating protein; dissociation of the enzyme under these conditions results in complete loss of activity. We were unable to dissociate the species of mol.wt. 75000 to any significant extent by using 8M-urea in the presence of sodium dodecyl sulphate and mercaptoethanol, though some indications of a species with mol.wt. 32000 were observed. This is interesting because the subunit molecular weights of a number of *o*-diphenol oxidases, including one from an insect, range between 29000 and 36000 (Jolley *et al.*, 1969; Ashida, 1971; Burnett, 1971). In *Calliphora*, the enzyme species with a mol.wt. of 75000 appears to be very stable. The subunits of the pro(*o*-diphenol oxidase) of the silkworm *Bombyx mori* (mol.wt. 36000) readily dimerize to give a mol.wt. of 72000 (by ultracentrifugation) or 80000 (by electrophoresis in sodium dodecyl sulphate; Ashida, 1971). By analogy, the species with a mol.wt. of 75000 from *Calliphora o*-diphenol oxidase is likely to be a dimer.

It is unfortunate that we cannot accurately determine the molecular weight of the active species in 0.1% (w/v) sodium dodecyl sulphate. Since this protein is active, it is unlikely to bind the maximum number of sodium dodecyl sulphate molecules. The apparent molecular weight from electrophoresis in sodium dodecyl sulphate-polyacrylamide gels is therefore likely to be an overestimate. Glucose oxidase and papain, which retain their activities in the presence of sodium dodecyl sulphate, do not bind measurable amounts of this detergent (Nelson, 1971). However, it was found that they both migrate at the proper rates, according to their molecular weight, as if they were binding to sodium dodecyl sulphate. If this is also the case with the *o*-diphenol oxidase from *Calliphora*, the simplest interpretation is that the active form in 0.1% (w/v) sodium dodecyl sulphate is likely to be a hexamer. In order to obtain further information on the structure of *o*-diphenol oxidase from *Calliphora*, it would be necessary to obtain the pure enzyme in the absence of sodium dodecyl sulphate. This can be achieved by first isolating the inactive pro-enzyme.

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