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PURIFICATION OF PROPHENOL OXIDASE FROM *DAUCUS CAROTA* CELL CULTURES

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Key Word Index—*Daucus carota*; Umbelliferae; carrot; latent enzyme; phenol oxidase; prophenol oxidase; purification.

Abstract—The enzyme phenol oxidase was purified from carrot cells in an apparent inactive form, prophenol oxidase, to electrophoretic homogeneity. The proenzyme had a M_r of *ca* 36000 under non-reducing as well as reducing conditions as judged by SDS-PAGE. Phenol oxidase activity of the purified prophenol oxidase could be induced by the addition of calcium chloride at millimolar concentration, or by trypsin treatment.

INTRODUCTION

The redox enzyme phenol oxidase (EC 1.14.18.1) has been isolated in a latent state from several higher plant species [1, 2] and enhancement of phenol oxidase activity *in vitro* can be achieved by a variety of treatments such as low pH [3, 4], and exposure to fatty acids [5], detergents [6–8] and proteases [9–11]. Solubilization [1, 12], induction of a limited conformational change [6], as well as dissociation of an enzyme-inhibitor complex [7, 13], have been proposed to account for this activation of latent phenol oxidase. However, neither the presence of latent phenol oxidase *in vivo* has been conclusively shown, nor has the mechanism by which the enzyme is activated *in vitro* been clearly elucidated. Recently, we have reported the isolation of phenol oxidase from carrot cells in a completely inactive form as a soluble prophenol oxidase (proPO) [10]. This proenzyme could be converted into active phenol oxidase (PO) by millimolar concentrations of Ca^{2+}- or Mn^{2+}-ions or by trypsin at lower Ca^{2+}-concentrations [10]. In this paper, we now report the purification of prophenol oxidase to electrophoretic homogeneity from a *Daucus carota* L. cell culture.

RESULTS

The enzyme phenol oxidase was purified in an apparent inactive form (proPO) from *D. carota* cells. Since the proenzyme is easily activated, several precautions had to be taken during the purification procedure. The carrot cells used as starting material had to be actively growing since the presence of dying cells resulted in activation of the proenzyme. Therefore the suspension cultures were subcultured at latest every fourth day. After homogenization in a buffer containing EDTA the cell extract was immediately centrifuged and rapidly precipitated with 65% ammonium sulphate. This procedure resulted in a stable inactive proPO-preparation which resisted freezing and thawing several times. This preparation was further purified by DEAE cellulose chromatography. To avoid activation of the proenzyme during this purification step, it was necessary to run several small columns, as rapidly as possible, for if a large amount of protein was applied to one big column, proPO was activated and as a result phenoloxidase was irreversible bound to the matrix, and only a small amount of the active enzyme could be eluted even if the ionic strength of the elution buffer was increased to 1.5 M NaCl. This could be demonstrated by adding the phenoloxidase substrate L-dihydroxyphenylalanine (L-dopa) to the column which then turned red (not shown). The proPO obtained after DEAE cellulose chromatography could be bound to a Cu^{2+}-Sepharose column whereas most other proteins were washed off the column (Fig. 1). The proenzyme could then be eluted in an inactive form with 5 mM imidazole.

After the final gel filtration *ca* 35% of the initial enzyme activity was recovered and the purification was about 1300-fold (Table 1). As judged by SDS-PAGE, this preparation was pure and migrated in the gel as a single polypetide chain with a M_r of 36000 under reducing as well as non-reducing conditions (Fig. 2a). Electroblotting and staining for phenol oxidase activity of a crude carrot homogenate run on SDS-PAGE gave one single band at this M_r (36000) (Fig. 2b).

PO-activity of the purified proPO-preparation could be induced by addition of calcium chloride and maximal activity was reached at *ca* 1 mM (Fig. 3). At Ca^{2+}-concentrations below 0.5 mM the proenzyme was stable but after preincubation with trypsin PO-activity was induced without addition of calcum chloride. Optimum pH for PO-activity of the purified enzyme was a broad peak in the range 6.7–7.2 regardless of whether the proenzyme was activated by Ca^{2+}-ions or by trypsin. The optimum pH for activation by calcium chloride coincided well with this activity optimum whereas activation by trypsin was most efficient at pH 7.1.

DISCUSSION

A prophenol oxidase (proPO) has been purified to electrophoretic homogeneity from carrot cells. The proenzyme consists of one single polypeptide with a M_r of 36000 as judged by SDS-PAGE and this 36000

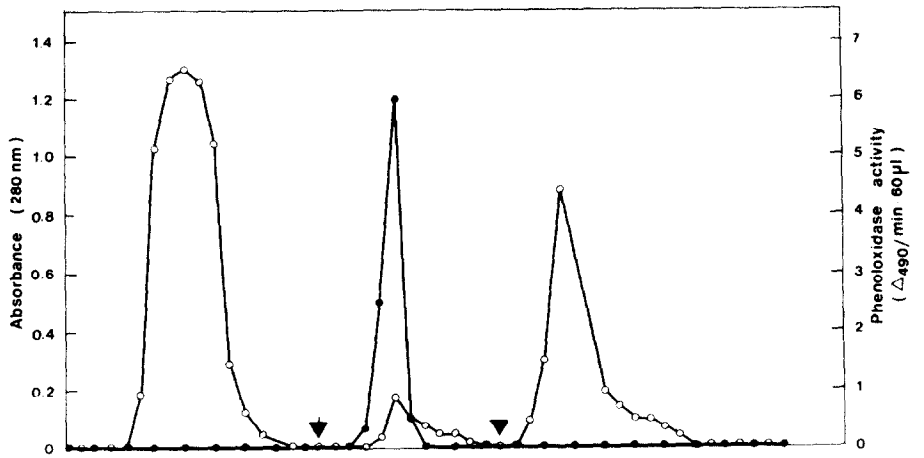

Fig. 1. Cu^{2+}-Sepharose chromatography of prophenol oxidase from *D. carota* cells. Absorbance at 280 nm (○); phenoloxidase activity (●). First arrow indicates the application of 5 mM imidazole in buffer C and second arrow the application of 50 mM imidazole in buffer C.

Table 1. Purification of prophenol oxidase from *D. carota* cells

Purification step	Volume (ml)	Total activity (ΔA_{490}/min)	Total protein (mg)	Specific activity (ΔA_{490}/min × mg protein)	Yield (%)	Enrichment (-fold)
Crude homogenate*	272	304	380	0.8	100	1
$(NH_4)_2SO_4$-ppt. dialysis	15	338	140.8	2.4	110	3
DEAE-cellulose	26.4	240	1.38	174.0	79	218
Cu^{2+}-Sepharose	2.0	142.8	0.294	468.0	47	605
Sephacryl S-200	1.5	106.4	0.1	1064	35	1330

*The activity of the crude homogenate was assayed after dialysis against 0.01 M sodium cacodylate buffer, pH 7.0.

protein had phenoloxidase activity. This is in close agreement with that reported for the mung bean, *Vigna radiata*, phenoloxidase [14], but somewhat lower compared to several recently reported phenol oxidases [5, 15–18].

However, phenol oxidases have been partially purified from several plant species [17, 19–22] and M_rs ranging from 26000 to 180000 have been reported [1] although the high $-M_r$ forms have been proposed as aggregates or multiple forms of the same protein [1]. Aggregation of plant phenol oxidase and its tendency to attach to different substances has frequently been observed [10, 23–25] and similar properties are associated with phenol oxidase in arthropods [26]. This property has made it difficult to purify the active enzyme since phenol oxidase may be irreversible bound to several gel materials and consequently low recovery of the enzyme activity is often reported (18–20].

However, in a recent study we have shown that carrot phenol oxidase appears to be a soluble enzyme but upon activation by Ca^{2+}-ions or trypsin the active phenol oxidase easily aggregates and can be pelleted [10]. Jolley *et al.* [27] also reported reversible association of mushroom tryosinase which was induced by Ca^{2+}-ions, but this association did not affect the enzyme activity [27]. Purified carrot proPO was converted into active enzyme by high concentrations of calcium chloride or by trypsin in the absence of calcium chloride present. The mechanism by which Ca^{2+} exerts its effect is not known, but the high concentration of Ca^{2+}-ions may induce aggregation of the proenzyme [10] and an active conformation is likely to be generated. The exact mechanism for the ways in which proPO is converted to PO by Ca^{2+} still remains to be investigated in detail.

Trypsin could also induce activity of the purified proPO and as reported for a partially purified proPO [10] this activation took *ca* 10 min. This is in contrast to the trypsin induced phenoloxidase activity in spinach chloroplasts [9] where maximal activity was reached in less than 30 sec. As this rapid activation could also be achieved by heat-inactivated trypsin, it may not be due to proteolytic cleavage of phenol oxidase [9]. Heat-inactivated trypsin could not activate carrot proPO. Recently, King and Flurkey [11] showed that trypsin treatment of a crude preparation of broad bean phenol oxidase generated an active form of the enzyme concomittant with a change in M_r from 45000 to 43000. However, their crude preparation did exhibit phenol oxidase activity even without trypsin treatment. Several

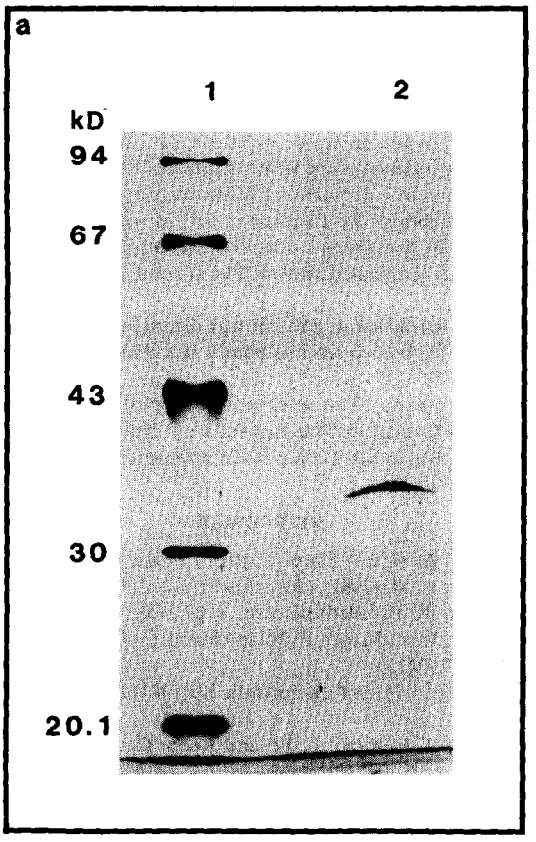

Fig. 2.a. SDS-PAGE under reducing conditions of a purified prophenoloxidase from *D. carota* cells. Lane 1: Marker proteins; phosphorylase b (94 000), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000) and trypsin inhibitor (20 100). Lane 2: Purified prophenoloxidase (5 μg of protein). SDS-PAGE was run as described in Experimental and the proteins were detected by silver stain according to Marshall [30].

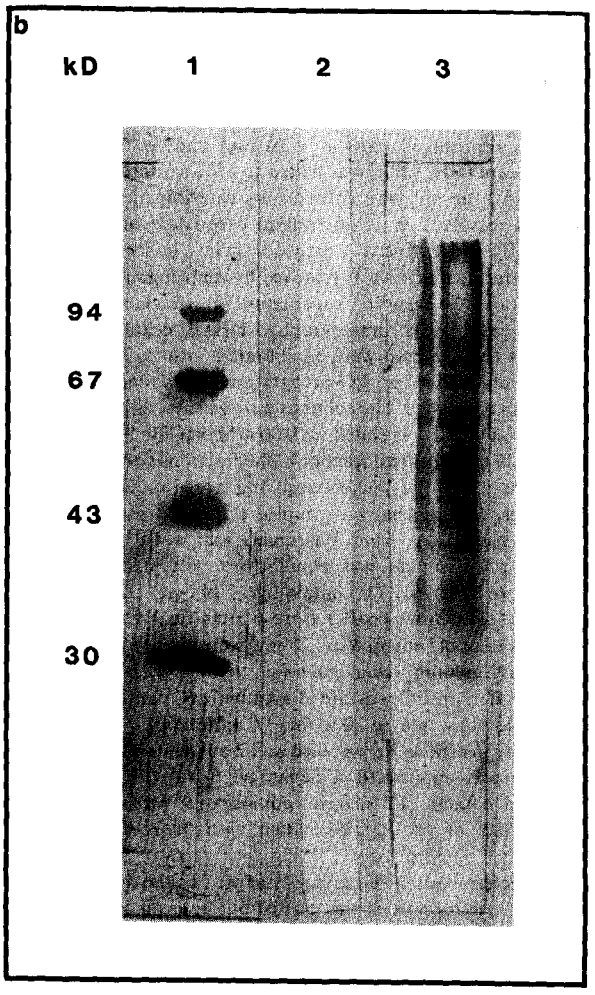

Fig. 2b. Crude carrot homogenate subjected to SDS-PAGE, electroblotting and enzyme- or protein staining as described in Experimental. Lane 1: Marker proteins, phosphorylase b (94 000), albumin (67 000), ovalbumin (43 000) and carbonic anhydrase (30 000). Lane 2: crude carrot homogenate (50 μg protein) stained for phenoloxidase activity. Lane 3: Crude carrot homogenate (50 μg protein) stained for protein.

other proteases could also increase this activity but only trypsin enhancement resulted in a lower M_r of the enzyme [11].

Our purified proPO from carrot was not activated by other proteases such as chymotrypsin or subtilisn, but at present we have not clarified whether trypsin induces PO-activity by a specific cleavage of the proenzyme resulting in a lower M_r, or if the activation is due to a conformational change of the proenzyme. However, the purification described in this paper of an inactive prophenol oxidase may enable more detailed studies on the mechanism by which the enzyme activity is triggered *in vitro* as well as *in vivo*.

EXPERIMENTAL

Cell material. An embryogenic cell strain of *Daucus carota* initiated from root tissue was maintained in liquie B_5-medium [28] containing 0.1 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) in 100 ml Erlenmeyer flasks as described earlier [10]. The suspension cultures were kept in darkness at 25° and were subcultured every 4th day.

Purification of prophenoloxidase. Throughout the purification procedure, all operations were carried out at 0–4°. Three days after subculturing, the cells (*ca* 600 ml cell suspension) were

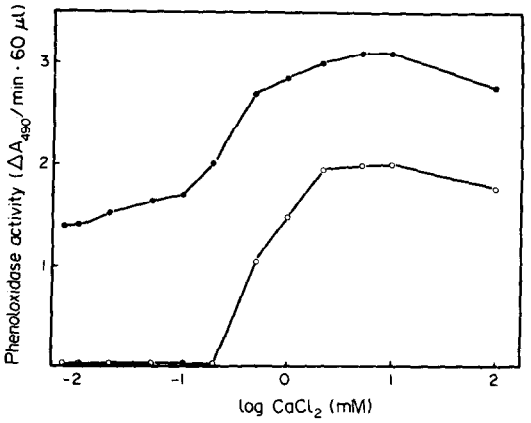

Fig. 3. Activation of a purified proPO at different concentrations of calcium chloride. The proPO was preincubated with calcium chloride for 10 min without trypsin (○) or with trypsin (●).

collected by centrifugation (1000 *g* 5 min). The cell pellet was washed once in 300 ml 0.01 M sodium cacodylate buffer, pH 7.0, containing 5 mM EDTA (Buffer A) and then homogenized in *ca* 275 ml of this buffer with a glass piston homogenizer and passed once through a French Pressure cell (110 MPa). The homogenate was centrifuged (10000 *g*, 20 min) and to the resulting supernatant $(NH_4)_2SO_4$ was added to 65% saturation. After *ca* 2 hr at 4°, the ppt. was collected by centrifugation (10000 *g*, 20 min), dissolved in 15 ml sodium cacodylate buffer, pH 7.0 (Buffer B), and extensively dialysed against buffer B. The ppt. formed during dialysis was removed by centrifugation (50000 *g*, 20 min) and the resulting supernatant was further purified by DEAE cellulose chromatography. DEAE cellulose (Sigma) columns (30 × 16 mm) were equilibrated with buffer B and to each column 2.5 ml proPO-preparation (containing *ca* 23.5 mg protein) was applied. The columns were washed with the equilibrating buffer and was eluted with 80 mM NaCl in buffer B (flow rate *ca* 20 ml/hr, 1.0 ml/fraction). The fractions containing proPO were pooled (*ca* 4 ml/column) and stored on ice. Chelating Sepharose (Pharmacia) was coupled with Cu^{2+}-ions according to the instructions from Pharmacia and a Cu^{2+}-Sepharose column (30 × 16 mm) was equilibrated with 0.01 M sodium cacodylate buffer, pH 7.0, containing 0.2 M NaCl (Buffer C). The fractions containing proPO from 6 runs on DEAE cellulose (total amount of protein was 1.38 mg) were applied to the Cu^{2+}-Sepharose column. After extensive washing with the equilibrating buffer, proPO was eluted with buffer C containing 5 mM imidazole (flow rate *ca* 20 ml/hr, 1.0 ml/fraction). The proPO-containing fractions were pooled and 2 ml (containing *ca* 0.3 mg protein) was applied to a Sepharcyl S-200 (Pharmacia) gel filtration column (1.5 × 80 cm) equilibrated with buffer C. A single peak of proPO was eluted with buffer C (flow rate 9.0 ml/hr, 1.5 ml/fraction).

For determination of the purity as well as the M_r of proPO, SDS gel electrophoresis was carried out according to Laemmli [29] at pH 8.3 using a 10% polyacrylamide gel containing 0.1% SDS. SDS-PAGE was run under non-reducing as well as reducing (sample containing 2.5% mercaptoethanol) conditions and a purified proPO-preparation containing *ca* 5 μg protein was applied to the gel For M_r determination phosphorylase b (94000), albumin (67000), ovlbumin (43000), carbonic anhydrase (30000) and trypsin inhibitor (20100) (Pharmacia low $-M_r$ calibration kit) were used as reference proteins. After electrophoresis (50 mA, 4 hr) the gel was fixed in 10% HOAc dissolved in 50% MeOH overnight and then stained for proteins according to Marshall [30].

Assay of phenoloxidase activity. The PO-activity was assayed by incubating 60 μl proPO-preparation, 20 μl (1 mg/ml) trypsin (Sigma) and 20 μl 6 mM $CaCl_2$ for 10 min at 22° prior to the addition of 20 μl (3 g/l) L-dopa (Sigma) as substrate. The enzyme reaction was slowed down after 2 min by the addition of 580 μl of buffer A and the absorbance at 490 nm was immediately recorded. In addition PO-activity was assayed after high Ca^{2+}-treatment [10] by incubating 60 μl proenzyme, 40 μl 0–600 mM $CaCl_2$ and 20 μl L-dopa as substrate for 1–2 min at 22° and then the absorbance at 490 nm was determined as above. Controls containing no trypsin or $CaCl_2$ as well as controls without substrate were always run in parallel.

Electroblotting and enzyme staining. SDS-PAGE of a crude carrot homogenate (50 μg protein) was performed at 4° in the presence of 0.1% SDS as described above for the purified proPO. After electrophoresis the gel was equilibrated for 30 min in transfer buffer (25 mM Tris, 192 mM glycine pH 8.3) at 4°. A "sandwich" was then prepared consisting of a synthetic sponge pad, 3 Whatman No 1 filter papers, the polyacrylamide gel, a nitrocellulose (NC) membrane, 3 Whatman No 1 filter papers and finally a synthetic sponge pad. The proteins were subjected to electroblotting from cathode to anode in a BioRad Transblot Cell (0.1 A, *ca* 15 V) for 16 hr. After electroblotting the NC membrane was soaked in 1% KOH for 5 min and then rinsed extensively in phosphate buffered saline (0.02 M K-Pi, 0.9% NaCl, pH 7.4) containing 0.3% Tween 20 (PBS-tween). Some lanes on the NC membrane were then stained for protein using a 0.1% solution of Indian ink in PBS-tween. For enzyme staining the NC membranes were soaked in 0.01 M sodium cacodylate buffer, pH 7.0, containing L-dopa (1.5 g/l) and 5 mM $CaCl_2$. The reaction was terminated after *ca* 2 hr at room temp. by rinsing with H_2O.

Protein determination. Protein was determined according to ref. [31] using bovine serum albumin as a standard.

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