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PROPERTIES OF CARROT POLYPHENOLOXIDASE

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Key Word Index—*Daucus carota*; Umbelliferae; carrot; latent enzyme; phenoloxidase; prophenoloxidase; purification.

Abstract—A latent phenoloxidase (PPO) was purified in the presence of protease inhibitors from carrot cells to electrophoretic homogeneity. The inactive enzyme had a M_r of *ca* 59 000 under denaturating conditions as judged by SDS–PAGE. When stored in the absence of protease inhibitors, PPO in a crude extract was converted to forms with a lower M_r. Phenol oxidase activity of the purified PPO was induced by the presence in the assay medium of 12.5 mM Tris, and 6 mM $CaCl_2$ increased the activity further.

INTRODUCTION

Phenoloxidase (EC 1.14.18.1) which catalyses the oxidation of orthodiphenols to orthodiquinones is a ubiquitous, apparently membrane associated, chloroplast enzyme with an as yet unknown function [1–3]. However, this enzyme is not only present in the chloroplast since it is found in large amounts in trichomes of some Solanaceae species [4, 5], where it is soluble and responsible for the polymerization of trichome exudate upon insect attack. PPO is frequently reported in a latent stage and it can be activated *in vitro* by a number of different treatments such as detergents [6–8], proteases [9, 10], low as well as high pH [11, 12] and exposure to fatty acids [13]. However, the most commonly used treatment to obtain activity in routine assays is by the use of SDS. Moore and Flurkey [14] proposed for *Vicia faba* PPO that SDS at an optimal concentration of 0.8 mM activates the enzyme by opening or unblocking the active site of the enzyme making it accessible for the substrate.

PPO has been purified to homogeneity from several species [13, 15–18] and a M_r of 45×10^3 has been frequently reported. Similarly the *Vicia faba* enzyme was previously thought to be synthesized as a M_r 45×10^3 polypeptide [19], but recently it has been conclusively shown that the enzyme is synthesized as a $60–65 \times 10^3$ protein, and that the 45×10^3 form is a result of proteolytic breakdown during preparation and/or due to insufficient reduction prior to separation on SDS–PAGE [20, 21]. Later the isolation and cloning of the PPO genes from tomato, potato as well as broad bean has revealed that cDNA from these species encodes for polypeptides which are processed to mature proteins of $57–59 \times 10^3$ [22–24].

We have previously purified a 36×10^3 PPO in an inactive state (prophenoloxidase) from embryogenic carrot cells [25]. This latent enzyme exhibits PPO activity on addition of Ca^{2+}-ions at mM concentrations or by trypsin at lower Ca^{2+}-concentrations. However, when separating a crude carrot extract by SDS–PAGE followed by immunoblotting and probing with polyclonal antibodies to purified *Vicia faba* PPO we detected one band at 36×10^3 as well as one band at a higher M_r, about $58–60 \times 10^3$. During purification this higher band disappeared and when separating a crude extract by SDS–PAGE followed by staining for PPO activity with L-dopa only the 36×10^3 band showed PPO activity. The purpose of this study was to purify the large M_r polypeptide and reveal whether it is a PPO or another protein cross-reacting with the antibodies against *Vicia faba* PPO.

RESULTS AND DISCUSSION

A completely latent PPO was found in a carrot cell extract when isolated in a buffer containing the serine protease inhibitors PMSF and benzamidine (buffer I). Since several plant PPOs have been shown to be activated by anionic detergents such as SDS [6–8, 14] this was also tested for the carrot PPO. However, SDS in the range 0.1 mM–10 mM could not induce any PPO activity of either the purified or the crude latent enzyme when SDS solutions of pH 3.5–8.5 were tested. Furthermore, PPO activity did not appear after treatment of PPO with different proteases such as trypsin, chymotrypsin, subtilisin or *Streptomyces* protease. Instead optimum activation of this latent enzyme occurred after treatment with 12.5 mM Tris pH 8 containing 6 mM $CaCl_2$. To test whether this activation was due to the high pH of the Tris buffer, several buffers were tested for their ability to induce activation at different pH. As shown in Fig. 1 the

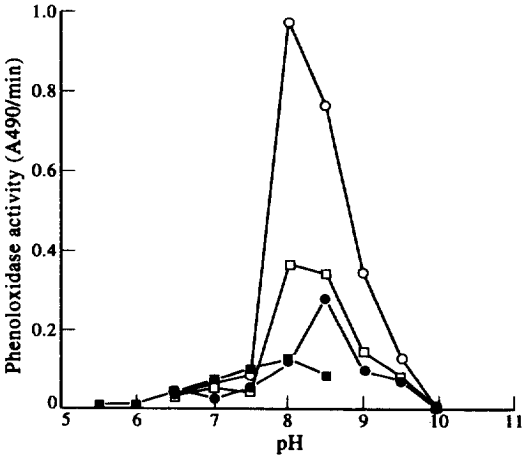

Fig. 1. Activation of purified PPO from carrot cells by treatment with buffers at different pH as described in the Experimental. Phenoloxidase activity after treatment with Tris (○); sodium borate (●); Hepes (□); sodium cacodylate (■).

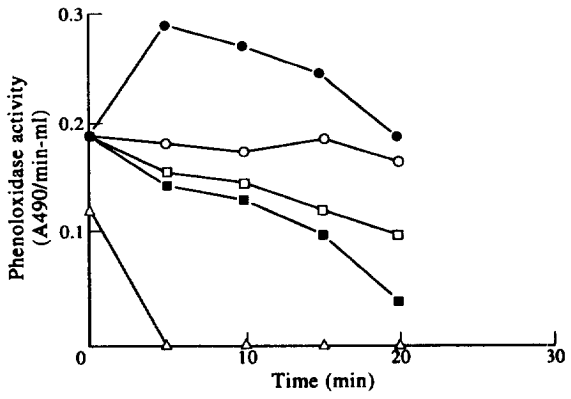

Fig. 2. Phenoloxidase activity of partially purified PPO incubated at 50° (○); or at 65° (●); phenoloxidase activity of a partially purified PPO activated by Tris/Ca^{2+} prior to incubation at 50° (□); or at 65° (■); phenoloxidase activity of a partially purified PPO dialysed against buffer II after incubation at 50° (△).

latent PPO was activated when buffer at pH 8.0–8.5 was added to the assay solution, but treatment of the latent enzyme with Tris did result in at least three times higher activity compared with other buffers tested (Fig. 1). When pH above 9 was tested, the activity measurements became unreliable due to spontaneous oxidation of the substrate at high pH.

The presence of $CaCl_2$ increased the activity of the purified as well as of the crude enzyme. Ca^{2+} alone could not induce any activity, as was the case when isolated according to ref. [25]. It is apparent from work by Moore and Flurkey [14] that SDS activation involves a limited conformational change of the enzyme and this is probably the case also for *D. carota* PPO (not shown). The Tris treatment of the 59×10^3 PPO as well as Ca^{2+} or trypsin treatment of the 36×10^3 PPO may induce an alteration in the conformation making the active site accessible to the substrate.

Using a buffer containing protease inhibitors for the isolation of PPO resulted in a very stable preparation. A dialysed homogenate (against buffer II) as well as a non-dialysed extract was subjected to high temperatures. At 50° the non-dialysed enzyme was stable for 20 min while the dialysed sample rapidly lost its activity. When incubated at 65° a minor activation occurred for the non-dialysed sample. When this preparation was activated by Tris/Ca^{2+} prior to incubation, the stability of the enzyme rapidly decreased (Fig. 2). These results indicate that the PPO in its inactive state is an extremely stable molecule, and when activated, the molecule is sensitive to denaturating temperatures. Also a slight activation was recorded by short treatment at 65°. Similar heat activation was earlier shown for proPO from the crayfish *Pacifastacus leniusculus* [26].

The stability after storage of the carrot PPO preparation was investigated and compared with PPO isolated in other buffers. Isolation of PPO in a cacodylate/EDTA buffer according to ref. [25] resulted in a three times less active preparation compared to buffer I, but both these preparations were inactive and stable for 24 hr when stored at +20° as well as at −20°. However, when a buffer containing neither protease inhibitors nor EDTA (buffer II) was used, the preparation was apparently inactive immediately after isolation, but after storage at −20° for 4–5 hr activity of the enzyme had been induced, and after storage for further 20 hr at 4° or at −20° the enzyme activity was completely destroyed.

Since the presence in the homogenization buffer of protease inhibitors resulted in a very stable preparation, the presence of proteolytically modified enzymes in crude carrot extracts was investigated using immunoblotting technique. After probing with anti *Vicia faba* PPO, one major band is found at $58–59 \times 10^3$ when buffer I was used (Fig. 3A). After preparation in buffer II followed by storage for 4 hr at room temperature a carrot extract seemed to contain several forms of PPO, one faint band at 59×10^3 as well as one at 45×10^3 and one band at 36×10^3 (together with several faint bands) indicating proteolytic breakdown of the proenzyme (Fig. 3A). The use of buffer III resulted in one major band at 36×10^3 (Fig. 3A). All these extracts were fully denaturated, boiled for 5 min in a sample buffer containing 100 mM DTT and 2% SDS prior to separation. Staining for PO activity was performed with these extracts under partially denaturating conditions in a sample buffer containing 0.1% SDS (no DTT, no boiling). One major band at 36×10^3 was found together with minor bands at 45×10^3 when buffer II was used as isolation medium, while the use of buffer III resulted in one band at 36×10^3 (Fig. 3B). However using buffer I no visible bands appeared when staining the nitrocellulose membranes with L-dopa, but if 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH, which binds to the quinone produced by the enzymatic reaction), was included in the assay solution

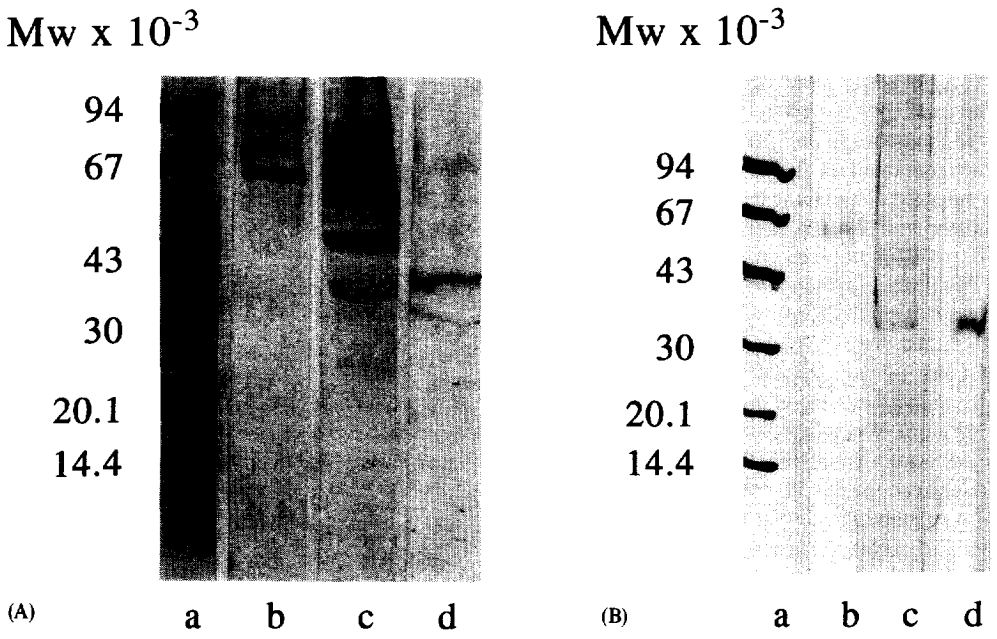

Fig. 3. A. Western blots of crude carrot cell extracts probed with anti *Vicia faba* PPO. The proteins were boiled for 5 min in the presence of 100 mM DTT and 2% SDS prior to separation by SDS–PAGE. Lane **a**: Amido Black staining of M_r standards; lane **b**: extract isolated in buffer I; lane **c**: extract isolated in buffer II; lane **d**: extract isolated in buffer III. B. Phenoloxidase activity of crude carrot cell extracts separated by SDS–PAGE under partially denaturating conditions (0.1% SDS, no DTT, no boiling) and electroblotted to NC membranes. Lane **a**: Amido Black staining of M_r standards; lane **b**: L-dopa staining of extract isolated with buffer I; lane **c**: L-dopa staining of extract isolated with buffer II; lane **d**: L-dopa staining of extract isolated with buffer III. In this experiment the staining procedure described in ref. [26] was used.

[27] a faint band at *ca* 59×10^3 appeared (Fig. 3B). If an extract homogenized in buffer I was dialysed against buffer II to eliminate the protease inhibitors followed by separation by SDS–PAGE and immunoblotting as above, the staining pattern was as for buffer II (not shown). These results show that latency and activation of carrot PPO is highly dependent upon the isolation procedure. When isolated in a buffer containing no protease inhibitors and no EDTA the enzyme preparation is fully active after a short period of storage, while in the presence of EDTA a stable latent enzyme preparation is obtained [25]. This preparation contained mainly a 36×10^3 protein and is activated by the addition of Ca^{2+} ions or by trypsin [25]. So even though this form is proteolytically broken down it is isolated in a latent stage.

Since these results indicated a proteolytic breakdown of the 59×10^3 PPO, attempts were made to purify this latent enzyme. Although PPO did not bind to Q-Sepharose at pH 7, the fractionation of a carrot extract using this material resulted in a nine-fold purification (Table 1). The increase in total activity after this purification step may indicate the removal of some inhibitory substances. One peak of activity was eluted in the void

Table 1. Purification of PPO from carrot cells

Purification step	Volume (ml)	Total activity*	Total prot. (mg)	Specific activity (ΔA 490 $min^{-1} \times$ mg protein)	Yield (%)	Enrichment (-fold)
Crude homogenate	150	960	99	9.7	100	1
Q-Sepharose	300	1360	15	90.6	142	9
Phenyl-Seph.1.	310	812	12	67.7	85	7
Phenyl-Seph.2.	63	233	0.2	117.0	24	120

*ΔA 490 min^{-1}.

volume and these fractions were pooled and run through a phenyl Sepharose column. This purification step resulted in a great loss of activity and no apparent purification (Table 1). However by this step the small portion of PPO which was broken down to 36×10^3 was eliminated since this PPO form remained on the column in the absence of ammonium sulphate. The latent PPO was then eluted in the void volume and the fractions were combined and made 1 mM with ammonium sulphate and then fractionated by hydrophobic interaction chromatography on a second phenyl Sepharose column. One peak of PPO activity was eluted with 35% ethylene glycol (Fig. 4). This eluted peak of purified PPO when analysed by SDS–PAGE showed a single band with an M_r of $58–59 \times 10^3$ (Fig. 5). When 70% ethylene glycol was applied to the column, a small peak of activity was eluted which also contained the $58–59 \times 10^3$ PPO. However this peak was also contaminated with other proteins.

No amino acid sequence at the *N*-terminal could be deduced due to blockage. However after treatment of the purified PPO with TFA vapour, which may be used to cleave *N*-terminal acetyl groups, the following sequence of the *ca* 59×10^3 PPO was obtained; LVEXKGTSG, but as reported by Hulmes and Pan [28] TFA treatment may lead to cleavage of peptide bonds as well as cleavage of *N*-terminal acetyl groups. Searching for similarities with other sequenced proteins in the NBRF Database as well as GenBank Database did not give any significant sequence similarities.

Phenoloxidase has been purified to apparent homogeneity from several plant species and shown as a single polypeptide of $40–45 \times 10^3$ for spinach [13, 15], broad bean [19], grape [17] and cell cultures of *Mucuna pruriens* [18]. However recent work on broad bean has revealed that purification in the presence of protease inhibitors results in a single 60×10^3 protein [20] or a $65–68 \times 10^3$ dimer [21]. Similarly we have previously purified an apparently inactive PPO with M_r of 36×10^3 [25] and now in the presence of protease inhibitors a 59×10^3 (as judged by SDS–PAGE) polypeptide was purified to homogeneity.

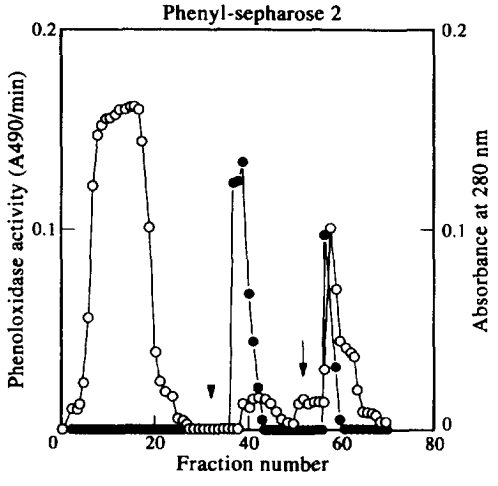

Fig. 4. Phenyl-Sepharose chromatography of PPO from carrot cells in the presence of 1M $(NH_4)_2SO_4$. *A* at 280 nm (○); phenoloxidase activity (●). First arrow indicates the application of 35% ethylene glycol in buffer I and second arrow the application of 70% ethyleneglycol in buffer I.

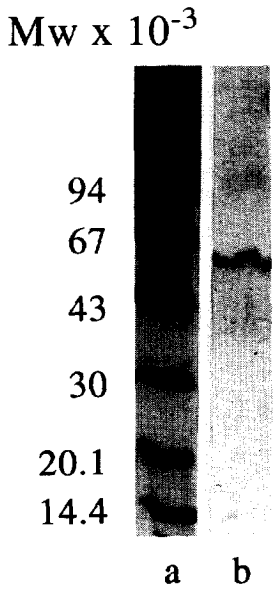

Fig. 5. Proof of electrophoretic homogeneity and estimation of M_r of PPO from carrot cells. PPO (*ca* 5 μg) was run on SDS–PAGE under reducing conditions and was stained for protein using Coomassie Brillant Blue.

Taken together, these results indicate that phenoloxidase in carrot cells is present as a 59×10^3 protein *in vivo*. However whether this form is active within the plant tissue or whether it is transformed into an active lower M_r protein *in vivo* cannot be deduced from this study. In grapevine, activation by proteolytic cleavage seems to be the case since a variegated mutant, Bruce's Sport contains a large amount of a 60×10^3 PPO and little or no apparent PPO activity *in vitro*, while in a green variety, Sultane, PPO was present mainly as an active 40×10^3 enzyme [29]. It is not conclusively shown whether this is due to conversion of the 60×10^3 enzyme to the 40×10^3 PPO during isolation and different susceptibility to proteolytic breakdown in different varieties. Several PPOs which have been isolated and purified to homogeneity including PPO from *Solanum tuberosum* [24], *Vicia faba* [23] and *Lycopersicum esculentum* [22] have been cloned and sequenced, the physiological function of PPO in plants still remains unclear. From recent work on *Vicia faba* [20, 21] and Solanaceae species [4, 22] as well as on *D. carota* it seems justified to propose that the PPO enzyme is present as a $59–60 \times 10^3$ protein *in vivo*, and depending on the preparation technique, different active as well as inactive forms can be produced *in vitro*. These active forms are most likely produced by proteolysis after disrupting the plant tissue during isolation, but whether

proteolytic cleavage of the high M_r PPO is related to an *in vivo* function of the enzyme as is the case in arthropods [30] still remains to be elucidated.

EXPERIMENTAL

Plant material. *Daucus carota* cell cultures, initiated from root tissue were maintained in liquid B5-medium [31] containing 0.1 mg l^{-1} 2,4-dichlorophenoxy acetic acid in 100 ml conical flasks as previously described [32]. The suspensions used for purification of phenoloxidase were subcultured every 4th day.

Purification of latent phenoloxidase. Throughout the purification procedure all operations were carried out at 0–4°. One week after subculturing, the cells from *ca* 900 ml cell suspension were collected by filtration and rapidly washed on the filter with 0.01 M Na cacodylate buffer pH 7 containing 1 mM PMSF and 1 mM benzamidine (buffer I). The cells were rapidly homogenized in 180 ml of buffer I with a glass piston homogenizer. The homogenate was centrifuged (40 000 g, 20 min) and the resulting supernatant was stored at −20°. Aliquots of 15 ml of this prepn were loaded onto a 5 × 5-cm column of Q-Sepharose Fast Flow equilibrated with buffer I (flow rate 2.5 ml min^{-1}) and then the column was washed extensively with buffer I, and the PPO-containing frs were pooled (*ca* 30 ml). This active fr. was loaded onto a phenyl-Sepharose column (1 × 11 cm, flow rate 0.5 ml min^{-1}) equilibrated with buffer I and frs containing PPO in the throughflow were pooled and adjusted to 1 M $(NH_4)_2SO_4$ before application onto a new phenyl-Sepharose column (1 × 11 cm, flow rate 0.5 ml min^{-1}) equilibrated with buffer I containing 1 M $(NH_4)_2SO_4$. After extensively washing the column with the equilibration buffer the bound latent enzyme was eluted with buffer I containing 35% ethylene glycol.

Assay of phenoloxidase activity. PPO-activity was routinely assayed by incubating 100 μl proPO-prepn, 25 μl 0.1 M Tris–HCl pH 8 (or other substances for the activation studies), 25 μl 6 mM $CaCl_2$ (or 0.01 M Na cacodylate buffer pH 7, buffer II, for the controls) and 50 μl L-dopa for 3–5 min and then the reaction was stopped by the addition of 800 μl 10 mM of buffer II and the *A* at 490 nm was immediately recorded. Activation studies were performed using Na cacodylate buffer (pH 5.5–8.5) and Hepes (4-(2-hydroxyethyl)-1-piperazin-ethan-sulphonicacid), Na borate or Tris (pH 6.5–10). Thermal inactivation studies were carried out by incubating the different enzyme prepns (with or without the addition of 0.1 M Tris–HCl) at 50° or 65° and then aliquots were removed at various times and assayed for PPO-activity as above.

Electrophoresis and immunoblotting. For determination of the purity as well as the M_r of the purified latent enzyme, SDS slab gel electrophoresis was performed according to ref. [33] in 5–15% gradient gels using the Mini-protean slab gel system (Bio-Rad). Gels were stained for protein with Coomassie Brilliant Blue R250. Prior to electrophoresis, the samples were boiled for 5 min in Laemmli sample buffer containing 100 mM DTT as a reductant and 2% SDS according to ref. [20]. For the immunoblotting experiments, different prepns of crude carrot extracts were treated as above and subjected to SDS–PAGE. The gels were equilibrated for 30 min in a transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) MeOH pH 8.3) and then transferred to nitrocellulose using a Mini Trans blot Unit (BioRad) operated at 0°, 100 V, 250 mA for 35 min. After transfer, the NC-membranes were treated with blocking buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.05% Triton-X 100 and 0.25% gelatin, pH 8.0) for 4 hr at 37°. Proteins were detected on the NC-membranes with 0.5% Amido Black in MeOH–H_2O–HOAc (5:5:1). For immunodetection of phenoloxidase the membranes were probed with polyclonal *Vicia faba* anti PPO (a kind gift from W. H. Flurkey) and identified by peroxidase-conjugated sheep antirabbit IgG. For detection of phenoloxidase activity the membranes were incubated in L-dopa (10 mM Na cacodylate buffer pH 7 containing 1 mM $CaCl_2$ and 1 mM Tris at room temp. until bands appeared on the membrane. In some experiments the membranes were stained for PPO activity according to ref. [26].

Amino acid sequencing. Purified PPO was run by SDS–PAGE and electroblotted onto polyvinylidene difluoride membranes (PVDF, Millipore corporation, Bedford, U.S.A.). The membranes were stained with 0.1% Coomassie Blue in 50% MeOH, destained in 30% MeOH 10% HOAc, washed extensively with H_2O, dried and stored at −20°. The stained bands were excised, and to unblock the aminoterminal amino acid the membranes were placed inside small test tubes which then were enclosed in a glass tube containing anhydrous CF_3COOH (TFA). The tube was heated at 60° for 1 hr to expose the membranes to TFA vapour. The amino terminal amino acid sequence of this band (4 replicates) was determined by automated degradation on an Applied Biosystems model 470A gas phase sequencer with an on-line phenylthiohydantoin derivative analyser.

Protein determination. Protein was determined according to ref. [34] using bovine serum albumin as a standard.

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