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ISOLATION AND CHARACTERIZATION OF THE POLYPHENOLOXIDASE FROM SENESCENT LEAVES OF BLACK POPLAR

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Key Word Index—*Populus Nigra*; black poplar; Salicaceae; polyphenoloxidase; peroxidase; senescent leaves; transition metals; oligoelements; phenols; inhibition; activation.

Abstract—The polyphenoloxidase (PPO) from black poplar senescent leaves has been purified to almost complete homogeneity by a combination of ammonium sulphate precipitation, Sephadex G75 filtration and DEAE-cellulose chromatography. The purified enzyme has a MW of 60 000 and is probably a Cu^{2+} enzyme. Peroxidase (PO) activity co-purifies with PPO and has the same MW as it. The two enzymes differ in pH optimum and in response to the effect of ionic strength. Natural phenols are either substrates, inhibitors or activators of black poplar PPO. This enzyme is an *o*-diphenoloxidase which binds substrates with K_m in the millimolar range. With caffeic and chlorogenic acids inhibition by excess substrate is observed. Benzoic acid phenols and cinnamic acid phenols are either competitive or non-competitive inhibitors of PPO. Hydroquinone is a highly potent non-competitive inhibitor of the enzyme (K_i = 90 μM). Ferulic acid is a potent activator of the PPO-catalysed oxidation of catechol (K_a = 0.34 mM, v_{sat}/v_o = 7.7).

INTRODUCTION

The ability of fruits or vegetables to brown is related to their content of polyphenols and/or PPOs (EC 1.10.3.1) [1, 2]. Meyer and Biehl [3] found an increase in PPO activity during spinach leaf senescence. This increase is not due to a *de novo* synthesis but to a release of active enzyme from thylakoïd membranes. We have recently shown that PPO activity is present in senescent leaves from various tree species [4]. This activity is still present after the leaves fall and is responsible for the fast deoxygenation of aqueous leaf macerates. This deoxygenation triggers the formation of polyphenols by a process identical to that which is responsible for the browning of fruits and vegetables [5].

Among the tree species we have studied [4] the black poplar is particularly important because of the deleterious action of its senescent leaves on fish living in pools surrounded by it. This natural pollution is probably mediated by the PPO contained in the leaves [4]. We have therefore isolated this enzyme and investigated some of its properties. Another phenoloxidase, PO, co-purified with PPO during all purification steps. The distinguishing properties of the two enzymes will also be reported.

RESULTS

Purification of PPO and PO

Without Triton X-100 very little enzyme activity could be solubilized, suggesting that PPO and PO are membrane-bound enzymes. The ethanol precipitate was poorly soluble in phosphate buffer (only 25% of the enzyme activity contained in this precipitate could be solubilized). On ammonium sulphate precipitation, the total PPO activity increased by 17% whereas the total PO activity decreased by 50%. During this step, the specific activity of PPO increased by a factor of 7 whereas that of PO increased only by a factor of 2. On Sephadex G75 the two enzyme activities eluted almost simultaneously. A similar pattern was observed after DEAE-cellulose chromatography (Fig. 1).

The closeness of the two elution peaks and the low enzyme concentration in the starting material precluded further attempts to separate the two enzyme activities. After this step the specific activities of PPO and PO were 290 and 150 Units/mg respectively (1 Unit = Δ absorbance/min 10^3). After the Sephadex step the preparation showed four bands on polyacrylamide gel electrophoresis whereas it showed only one major band and a few minor bands after DEAE cellulose chromatography. The R_f value of this band was the same as that of the PPO and PO activity bands indicating that the major part of the proteins of our preparation are PPO and PO. Whether SDS is present or absent in the electrophoretic system, both PPO and PO activities migrate with an R_f corresponding to a MW of 60 000 ± 5000.

Comparative enzymatic properties of PPO and PO

PPO and PO react with their classical substrates catechol and guaiacol. The K_m values for the PPO/catechol and the PO/guaiacol systems are 5 mM and 8 mM respectively. Whereas the former system obeys Michaelis–Menten kinetics, the latter exhibits the phenomenon of inhibition by excess substrate. Figure 2 shows that the two enzyme activities have quite different optimum pH values: 7.5 for PPO and 5.5 for PO.

PPO activity is insensitive to sodium chloride or potassium chloride up to at least 1 M if tested immediately after addition of salts. However, after a 1 hr preincubation of PPO with 0.3 M sodium chloride, the enzyme activity is decreased by 50%. On the other hand, phosphate ions increase PPO activity: in 1 M phosphate

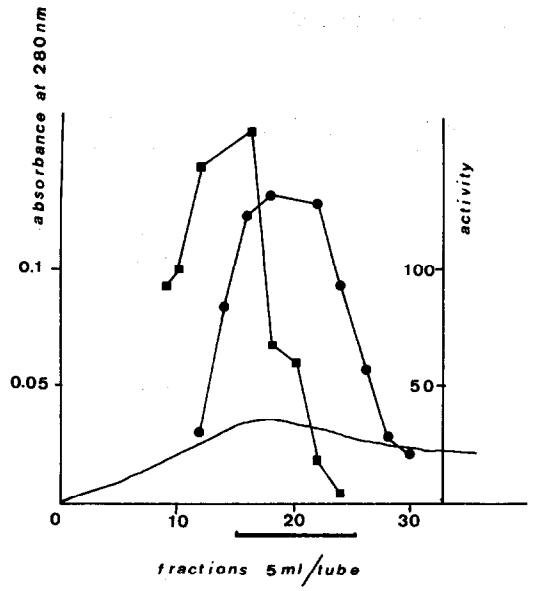

Fig. 1. DEAE-cellulose chromatography of the active fractions collected after Sephadex G75 gel filtration. After sample application, the column (3.5 × 50 cm) was washed with 0.1 M phosphate buffer, pH 6 and subsequently developed with a linear gradient of phosphate concentration (150 ml of 0.1 M phosphate + 150 ml of 1 M phosphate pH 6). The fractions (volume = 5 ml) indicated by a black bar were pooled. The activity is expressed in ΔA per min × 10^3. (PPO, —●—●—; PO, —■—■—). (———) = absorbance at 280 nm.

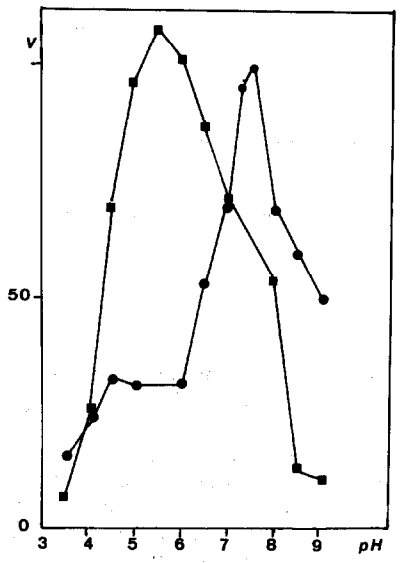

Fig. 2. pH dependency of PPO (—●—●—) and PO (—■—■—) activities at 25°. The buffers were: citrate phosphate 3.5–6; phosphate Na–Na_2 6–7.5; glycine–NaOH 8–9.5.

the activity is twice as high as in 0.1 M buffer. Phosphate ions as well as sodium chloride do not influence PO activity. By contrast, potassium chloride increases this activity.

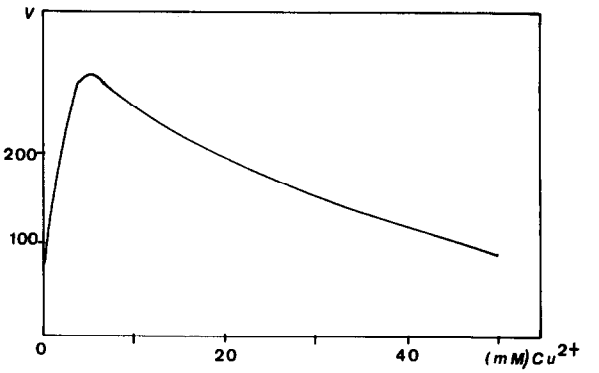

Fig. 3. Effect of Cu^{2+} ions on PPO activity. $V = \Delta A/\text{min} \times 10^3$.

Metallo-enzyme properties of PPO

The activity of PPO was decreased by chelating agents like potassium cyanide and *o*-phenanthroline. When the enzyme activity is tested immediately after the addition of potassium cyanide, the I_{50} value of this reagent is 0.6 mM. The two chelating agents also inhibit PPO in a time-dependent fashion. The half-time of inhibition of *o*-phenanthroline (1 mM) is *ca* 30 min whereas it is *ca* 15 min for potassium cyanide (0.1 mM). Cu^{2+} activates PPO but becomes an inhibitor for concentrations higher than 5 mM (Fig. 3). Manganese has no effect on PPO. By contrast cobalt and nickel ions accelerate the catalysis. The acceleration factors are *ca* 20% and 360% respectively for 50 mM concentrations of Co^{2+} and Ni^{2+}.

Substrate and inhibitor specificity of PPO

Among the naturally occurring phenols we have tested, some are substrates and others are inhibitors or activators (Table 1). With some substrates classical Michaelian kinetics were observed, while some others exhibited the phenomenon of inhibition by excess substrate (e.g. Fig. 4). The inhibitors may also be classified into two groups: those which behave as competitive inhibitors (e.g. Fig. 5) and those which exhibit non-competitive inhibition. In addition, two compounds are activators of PPO catalysis (Fig. 6).

DISCUSSION

The MW of black poplar PPO is about the same as that of the PPO from oak (*Quercus robur* = *Q. pedunculata*) [6]. Our data also suggest that it is a copper enzyme like other PPO [7] or like tyrosinase [8, 9]. The biphasic shape of the Cu^{2+} saturation curve (Fig. 3) may be explained in the following manner. The initial increase of PPO activity may result from the fact that some of the enzyme molecules had lost their metal during isolation. The inhibitory effect of higher Cu^{2+} concentrations may be due to non-specific binding of Cu^{2+} to the protein with a resultant unfavourable conformational change.

The co-purification of PPO and PO from black poplar reported in this paper was also noticed a few years ago by Signoret and Crouzet [10] during the isolation of PPO from tomato. These authors found that the two enzymes eluted at identical positions from a DEAE-cellulose

Table 1. Substrate and inhibitor properties of phenols on PPO at pH 6.0 and 25°

No.	Compounds: Chemical name	Footnote	K_m (mM)	V_m (μM/sec)	K_i (mM)
	Monophenols				
1	Tyrosine		no interaction		
2	4-Hydroxybenzoic acid	*			2.0
3	2-Hydroxybenzoic acid	†			1.75
4	3,5-Dimethoxy-4-hydroxy benzoic acid (syringic acid)		no interaction		
5	3-Methoxy-4-hydroxy benzoic acid (vanillic acid)		no interaction		
6	4-Hydroxycinnamic acid	‡			0.24
7	3-Hydroxycinnamic acid	†			2.5
8	4-Hydroxy-3-methoxy cinnamic acid (ferulic acid)	§	activation + inhibition		
	Diphenols				
9	Catechol	‖	5.0	2.9	
10	4-Methylcatechol	‖	5.0	7.6	
11	Hydroquinone	*			0.09
12	3,4-Dihydroxybenzoic acid (protocatechuic acid)	†			4.3
13	2,5-Dihydroxybenzoic acid (gentisic acid)	†			0.35
14	3,4-Dihydroxycinnamic acid (caffeic acid)	¶,**	0.7		
15	Chlorogenic acid	¶,**	5.0		
16	Dihydroxyphenylalanine (DOPA)	††	> 100	> 0.5	
17	Dihydroxycoumarin		substrate		
	Triphenols				
18	Pyrogallol	**	7		
19	3,4,5-Trihydroxybenzoic acid (gallic acid)		activation + inhibition		

*Non-competitive inhibition.
†Competitive inhibition.
‡Inhibition type difficult to diagnose.
§See Fig. 6 for the activation process, $K_a = 0.34$ mM; for the inhibition process, $I_{50} > 10^{-2}$ M.
‖Michaelian kinetics.
¶Non-Michaelian kinetics: inhibition by excess substrate.
** V_m could not be quantitated because the product was so unstable that its ε could not be measured.
††No saturation kinetics with this substrate.

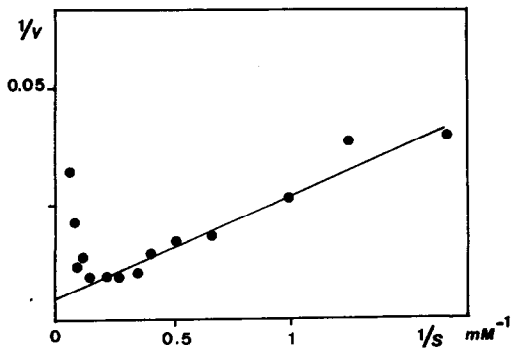

Fig. 4. The PPO-catalysed oxidation of chlorogenic acid at pH 6 and 25°: Lineweaver–Burk plot [20] demonstrating inhibition by excess substrate.

column, and had identical optimum pH activity, isolectric point and electrophoretic mobilities. They suggested that PPO is probably a peroxidase able to oxidize phenolic substrates with oxygen as the second substrate [10]. Although PPO and PO from black poplar have identical MW they do not appear to be identical enzymes because (i) they do not co-elute on DEAE cellulose chromatography (Fig. 1) (ii) their pH optimum is different by two units (Fig. 2) (iii) they respond differently on varying ionic strength. In addition, guaiacol, a substrate of PO, does not inhibit PPO. PPO may be considered as an *o*-diphenoloxidase since it catalyses the oxidation of *o*-di- or -triphenols only. The K_m values for these substrates (Table 1) are in the same order of magnitude as those reported for the oxidation of identical substrates by catecholoxidases from higher plants [11].

PPO appears to possess an extended substrate-binding site since, on the one hand, the enzyme–substrate affinity is

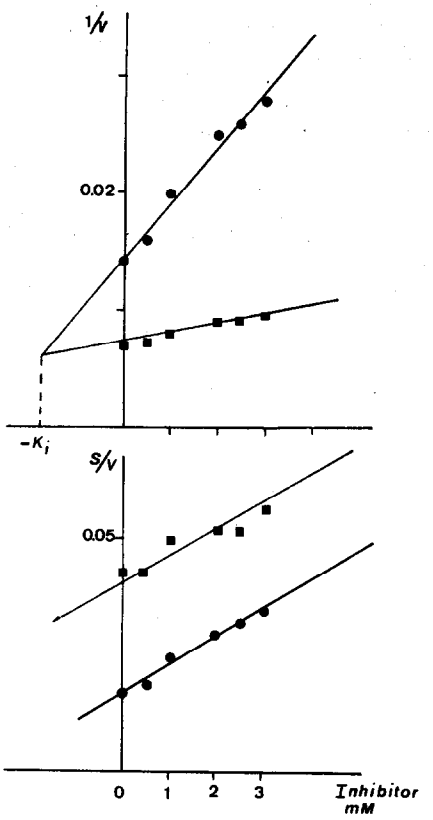

Fig. 5. Inhibition of PPO by protocatechuic acid (3,4-dihydroxybenzoic acid) at pH 6 and 25°. Upper part: Dixon plot [21]. Lower part: Cornish-Bowden plot [22] demonstrating that the inhibition is purely competitive.

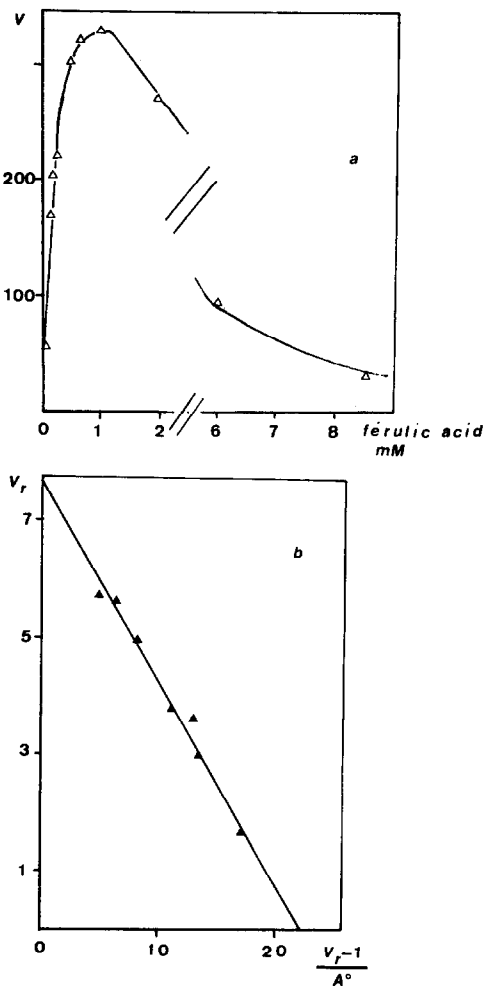

Fig. 6. Activation and inhibition of PPO activity by 4-hydroxy-3-methoxycinnamic acid (ferulic acid) at pH 6 and 25°. (a) Direct plot of rate (Δ A per min $\times 10^3$) vs ferulic acid concentration; (b) Inagami plot [23] for the activation effect (v_r = rate in the presence of activator/rate in the absence of activator; ($A°$) = total activator concn). The substrate used in this experiment was 1.2 mM catechol.

relatively insensitive to the substrate's bulkiness (compare compounds **9** and **15**) and on the other hand, inhibition by excess substrate is observed with compounds **14** and **15** (see Fig. 4). The latter phenomenon is usually rationalized by assuming that at high substrate concentrations a second substrate molecule binds non-productively to the active center of the enzyme and inhibits the transformation of the productively bound one [12]. It may therefore be hypothesized that black poplar PPO will also oxidize diphenolic oligomers as does the tea PPO [13].

Catechol and 4-methylcatechol (compounds **9** and **10**) react with PPO with identical affinities but are oxidized at significantly different maximum rates: the presence of a methyl group in the *para* position increases the catalytic efficiency of the enzyme. This group is an electron donor ($\sigma = -0.17$). Therefore, our result is in agreement with data showing that electron-attracting substituents decrease the rate of enzymatic reaction [14]. Our view is supported by the observation that protocatechuic acid (compound **12**) which bears an electron-attracting carboxylic group is not oxidized at all by PPO although it binds to the enzyme.

Only minor structural changes are necessary to transform substrates into inhibitors (compare compounds **9** or **10** to **12** and **14** to **8**). Steric hindrance of the binding of some inhibitors also exists (compare compounds **4** or **5** to **2** or **12**). The presence of the unsaturated –CH=CH–COOH residue leads to a large increase in the affinity of some ligands for the active center of black poplar PPO (compare compounds **2** and **6**, **5** and **8**, **9** or **12** and **14**). This effect has already been noticed by Walker and Wilson [15] for PPO from another source.

The ferulic acid induced activation of the PPO-catalysed oxidation of catechol (Fig. 6) deserves special comments. First, this activation is very pronounced ($K_a = 0.34$ mM, $v_{sat}/v_o = 7.7$). Second, it does not take place when the PPO substrate is caffeic acid which also bears the –CH=CH–COOH residue. Since activation of catalysis implies the occurrence of a ternary enzyme–substrate–activator complex, our data show that ferulic acid binds strongly to PPO at a site different from the substrate binding site of the enzyme. The activator binding site must however be very close to the substrate binding site since the activator does not interact when the substrate is bulky. The inhibition of the activation process

observed for ferulic acid concentrations higher than 1 mM (Fig. 6) may be due to a direct competition between this compound and the substrate (competitive inhibition). Gallic acid (compound **19**) yields the same phenomenon of activation and inhibition but the amplitudes of the effects are smaller than with ferulic acid.

The activation phenomenon is mechanistically similar to non-competitive inhibition (compounds **2** and **11**) or to inhibition by excess substrate (compounds **14** and **15**) since a ternary enzyme–substrate–effector complex is postulated in the three cases to account for the observed effects [16]. Whether all effector molecules (activators, inhibitors or substrates) bind to one single regulatory site of PPO is unknown at present. It is likely that several regulatory sites exist because (i) the effector molecules have different structures (ii) activation and inhibition are probably mediated by different sites. Oligomeric diphenols might use these regulatory sites as secondary substrate binding sites.

EXPERIMENTAL

Senescent leaves from black poplar were harvested as soon as possible after their fall, lyophilized, powdered, sieved (mesh diameter: 500 μm) and stored at $-20°$ in stoppered bottles (H_2O content *ca* 7%).

Purification. This was started by suspending 50 g of leaf powder in 0.1 M NaPi buffer pH 6 containing 0.1% mercaptoethanol (to avoid autoxidation of phenols), 1% Triton X-100 [17] and 1% PEG 6000 [6]. After homogenizing (Ultra-Turrax, 30 sec) and centrifuging (20 min, 40000 *g*) the proteins were precipitated with 60% (v/v) EtOH resolubilized by homogenization in 0.1 M NaPi buffer pH 6 and submitted to fractional $(NH_4)_2SO_4$ precipitation. The protein fraction which precipitates between 70% and 90% $(NH_4)_2SO_4$ satn was dissolved in and dialysed against 0.1 M NaPi buffer pH 6. The soln was then concd *ca* 5 × (Amicon PM 10) and loaded on a Sephadex G 75 column (3 × 35 cm) equilibrated and developed with the above buffer. Fractions showing substantial PPO and PO activities were pooled and submitted to DEAE-cellulose chromatography as described in the legend to Fig. 1. Active fractions were pooled, dialysed against 0.01 M NaPi buffer pH 6, concd *ca* 10 × (Amicon PM 10) and stored frozen. Protein concns were measured by the method of ref. [18].

Polyacrylamide gel electrophoresis. This was performed with 9% gels at pH 7.4 in the presence of 0.1% SDS [19]. Protein bands were detected by Coomassie blue staining. Enzymatic activities were revealed by immersing the gels for *ca* 1 hr in the appropriate substrate solns (see above). BSA (MW = 67000), ovalbumin (MW = 43000), chymotrypsinogen (MW = 25000) and myoglobin (MW = 17200) served as markers.

Enzymatic activities. These were measured spectrophotometrically at pH 6 (0.1 M NaPi) and 25° in 500 μl reaction vols containing 5 μl (*ca* 0.6 μg protein) of enzyme preparation. For PPO the substrate was 3 mM catechol and $\lambda = 420$ nm. PO activity was measured with 3 mM guaiacol, 9 mM H_2O_2 and $\lambda = 470$ nm. The substrates and the inhibitors were dissolved just before the assay. The absorbancies were recorded for *ca* 5 min and initial rates were calculated by drawing tangents to the kinetic curves. The action of transition metals on PPO activity was measured in a 5 mM Na barbital/HOAc buffer, pH 5 with 2 mM catechol (the pH 6 buffer was not used in this case to avoid formation of metal hydroxides). The reference cuvette contained the same reagents as the sample cuvette except the enzyme.

The inhibition of PPO activity was tested using two substrate (catechol) concs (1.2 and 6 mM) and variable inhibitor concn. The graphical method of ref. [20] was used to determine K_m and V_m values of substrates. The latter were calculated using ε values for products calculated according to ref. [14]. Dixon [21] and Cornish-Bowden [22] plots were used for measuring K_i values of inhibitors and ascertaining the inhibition type (see Fig. 5). The activation of the PPO-catalysed oxidation of catechol by ferulic acid was quantitated using an Inagami plot [23] which is based on the following equation:

$$v_r = \frac{v_{sat}}{v_o} - \frac{v_r - 1}{(A^\circ)} \cdot K_a$$

where v_o = rate in the absence of activator,
v_r = rate in the presence of activator/v_o,
$v_{sat}/v_o = v_r$ for infinite concn of activator,
(A°) = activator concn,
K_a = equilibrium dissociation constant for the enzyme–activator complex.

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