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CHARACTERIZATION OF CATECHOLASE AND CRESOLASE ACTIVITIES OF MONASTRELL GRAPE POLYPHENOL OXIDASE

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Key Word Index—*Vitis vinifera*; Vitaceae; Monastrell grapes; polyphenol oxidase; *p*-cresol; cresolase; 4-methyl catechol; catecholase; lag period.

Abstract—The polyphenol oxidase of Monastrell grape was purified 126-fold by ammonium sulphate fractionation. The partially purified enzyme has both cresolase and catecholase activity. The latter activity has temperature and pH optima within the ranges 20–40° and 3.5–5.0, respectively. The apparent K_m for 4-methyl catechol is 9 mM. Cresolase activity exhibits a lag period, which is modulated by different factors. An increase in enzyme concentration, temperature or pH (in a range 3.5–7.5) shortens the lag period. By contrast, an increase in the substrate concentration increases the lag period. The presence of *o*-diphenols in the reaction medium abolishes the lag period, by acting as co-substrates. The apparent K_m towards *p*-cresol and the activation constant for *o*-diphenol (K_{act}) for cresolase activity are 0.5 mM and 1.6 μM, respectively.

INTRODUCTION

Polyphenol oxidase (monophenol, dihydroxy-L-phenylalanine: oxygen oxidoreductase, EC 1.14.18.1.) (PPO) is a widely distributed copper-containing protein, which is responsible for the darkening of damaged plant tissue. It uses molecular oxygen and catalyses the *o*-hydroxylation of monophenols (like *p*-cresol) to *o*-diphenols (like 4-methyl catechol), cresolase activity, and the further oxidation of *o*-diphenols to *o*-quinones, catecholase activity.

PPO has been characterized from various fruits [1–3]. Its high activity in grapes is responsible for a rapid browning during juice and wine production. This phenomenon causes a marked change in the colour and flavour, which greatly reduces the quality of the processed products.

In spite of the fact that a number of investigations of grape polyphenol oxidase have dealt with the subcellular location of the enzyme [4], the reaction mechanism [5], the variation of PPO activity during grape maturation and wine production [6–8] and the specificity of *o*-diphenol substrates from different grape varieties, there is no detailed study of cresolase activity.

The present paper describes a kinetic study of catecholase and cresolase activities of partially purified PPO of Monastrell grape (*Vitis vinifera* L. cv Monastrell). The cresolase acitivity has a long lag period, which depends on different factors, such as substrate and enzyme concentrations, the presence of *o*-diphenols, pH and temperature.

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RESULTS AND DISCUSSION

Purification of polyphenol oxidase

PPO of Monastrell grape was purified using a modification of the method of ref. [9]. A typical purification is summarized in Table 1. The purification achieved was 126-fold with 70% recovery of PPO activity (assayed at pH 5.0 using 4-methyl catechol as substrate). The principal modification was in step 2, where the Triton X-100 treatment was increased from 30 min to six hr and phenols were precipitated with insoluble polyvinyl pyrrolidone

Table 1. Purification of polyphenol oxidase from Monastrell grape

Purification step	Volume (ml)	Protein (mg/ml)	Specific activity (units/mg protein)*	Purification fold	Recovery (%)	Laccase activity
Crude extract	410	16.4	78	1	100	ND
Triton X-100 extract	6.2	19.83	4458	57	104†	ND
45–95% $(NH_4)_2SO_4$	3	12.36	9854	126	70	ND

*Assayed at pH 5.0 with 4-methyl catechol as substrate.
†Values obtained without dialysis. The value after dialysis was 95%.
ND = Not detected.

(PVP). The latter treatment produced a noticeable increase of PPO activity, which was attributed to the removal of phenolic compounds which would otherwise inhibit and inactivate many enzymes [10], including PPO [11] and the activation of PPO by Triton X-100, so that, when the Triton extract was dialysed, a decrease in the recovery was observed (from 104 to 90%) (Table 1). The removal of the insoluble PVP by centrifugation avoided the inhibitory effect of PVP reported in ref. [12].

No laccase activity was detectable by polarographic or spectrophotometric methods [13, 14] at any stage of the purification. This reflects the facts that the grapes employed were mould-free and that the whole purification was carried out at a pH (7.3) at which laccase loses 90% of its activity in 40 min [13].

Partially purified PPO has both catecholase (Fig. 1a) and cresolase activity (Fig. 1b–f). The latter is characterized by a lag period, defined as the intercept on the abscissa obtained by extrapolation of the linear part of the product accumulation curve. This lag period has been reported for other polyphenol oxidases from varying sources when cresolase acitivity is measured [15, 16]. The lag period and the steady-state rate (defined as the slope of the linear part of the accumulation product curve) are affected by substrate and enzyme concentrations, pH and temperature (Fig. 1).

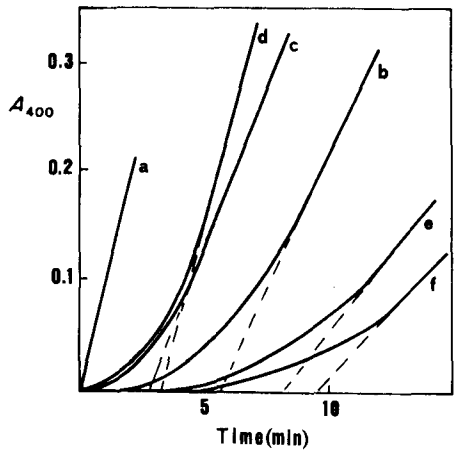

Fig. 1. Enzyme activities of Monastrell grape PPO. (a) Catecholase activity at 30°. The reaction mixture included, in a total volume of 2.5 ml: 0.75 μg/ml of PPO and 30 mM 4-methyl catechol in 10 mM acetate buffer, pH 5.0 (b–f) Cresolase activity at 30°. The reaction medium contained in a final volume of 1 ml: (b) 0.5 mM *p*-cresol, 480 μg/ml of partially purified PPO, pH 7.0; (c–f) The same as (b) except: (c) 50°, (d) 1200 μg/ml of partially purified PPO, (e) 0.25 mM *p*-cresol and (f) pH 6.0.

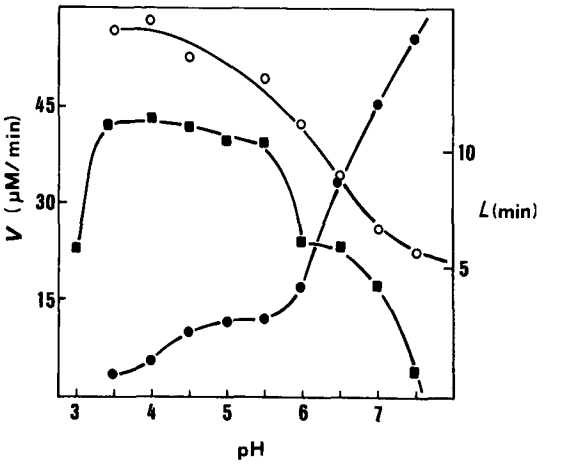

Fig. 2. Effect of pH on the activities of Monastrell grape PPO. (a) Catecholase activity (■ —— ■). The reaction mixture at 30° included, in a total volume of 2.5 ml: 3 μg/ml of partially purified PPO and 30 mM 4-methyl catechol in 10 mM Na-Pi or acetate buffers, at different pHs. (b) Cresolase activity (● —— ●). The reaction medium at 30° included, in a total volume of 1 ml: 480 μg/ml of partially purified PPO and 0.5 mM *p*-cresol in a 10 mM Na-Pi or acetate buffers, at different pHs. (c) Influence of pH on the lag period of cresolase activity (○ —— ○). The conditions are the same as (b).

Characteristics of catecholase activity

Catecholase activity of Monastrell grape polyphenol oxidase exhibited a broad pH optimun, 3.5–5.0, with a marked decrease in activity above pH 6.0 and below pH 3.5 (Fig. 2). This broad optimum is a remarkable characteristic of Monastrell grape PPO since most grape polyphenol oxidases have optimum pHs between pH 5.0–7.0, and a bell-shaped curve, usually with a small shoulder [17–20].

The optimal temperature for maximal PPO activity was 25–40°, with a slight decrease in activity at higher temperatures. This result is in contrast with the optimum temperature (25–30°) reported for other grape PPOs [19, 20].

The apparent K_m for 4-methyl catechol as calculated by the Hanes plot (data not shown) was 9 mM, which is an intermediate value among the values found for this substrate for grape PPO (3–24 mM) [5, 12]. However, this K_m is high when compared to other plant polyphenol oxidases, and very high when compared to the values reported for fungi and bacteria [1].

Characteristics of cresolase activity

Cresolase activity showed a lag period which was increased when the substrate concentration was increased (Fig. 1b, e). An inverse dependence was found when enzyme concentration was increased (Fig. 1b, d). Both results are qualitatively similar to those results obtained for other PPOs [21–23].

Cresolase activity rises from pH 3.5 to pH 7.5 without reaching a defined maximum (Fig. 2). At pH values above 7.5 it is difficult to follow the formation of 4-methyl-*o*-benzoquinone spectrophotometrically, because of its great instability, so we chose an assay pH of 7.0. The optimum temperature for this activity was found to be between 40–60°. A decrease in the lag period was observed when either pH or temperature was increased (Fig. 1b, f and Fig. 1b, c).

It is a well-known fact that the lag period of cresolase activity can be shortended or eliminated by addition of reducing agents or *o*-diphenols which act as co-substrates [24–26]. The 'activation constant' (K_{act}) with 4-methyl catechol was 1.6 μM. This is relatively high when compared with other polyphenol oxidases [25, 26], and very small when compared with the apparent K_m for 4-methyl catechol.

The apparent K_m for *p*-cresol was evaluated by Hanes plot, a value of 0.5 mM being obtained. This result cannot be compared with any published data, because K_m values for cresolase activity have not been reported for other varieties of grapes. This may be due to loss of cresolase activity during extraction and purification found by some investigators [17–20]. According to ref. [27], banana PPO loses its cresolase activity when it is purified from an acetone powder, while it preserves this activity when it is fractionated by ammonium sulphate.

The results obtained in the present paper show that rather than a loss of the activity, there is a masking of cresolase activity due to the loss of phenols during the purification steps. These phenols may act by shortening or eliminating the lag period in crude extracts.

In general, the optimal assay conditions for this activity (pH, temperature, etc.) have not been determined. The conditions used are the same as those used to assay catecholase activity and it is possible that the assays have not been carried out over a long enough time period for cresolase activity to leave its lag period, which can last more than one hr, if the same enzyme concentration as in the catecholase assay is present.

EXPERIMENTAL

Enzyme purification. Mould-free Monastrell grapes were harvested at the maturation stage at Jumilla, Murcia, Spain. Extraction of the enzyme from the frozen grapes was carried out using a modification of the method of ref. [9]. The grapes (350 g) were defrosted in 175 ml 100 mM Pi buffer, pH 7.3, containing 10 mM sodium ascorbate, homogenized in a blender for 15 sec, filtered through 8 layers of gauze and centrifuged at 4000 *g* for 15 min. The ppt. was extracted for 6 hr with 1.5% Triton X-100 and 2% insol.-PVP in 0.1 M Pi buffer, pH 7.3, and then centrifuged at 15 000 *g* for 1 hr. The supernatant was subjected to $(NH_4)_2SO_4$ fractionation between 45 and 95% saturation at 4°. The ppt. was resuspended and after dialysis was used as the enzyme source.

Protein concentrations of the samples at different stages of purification were determined using the method of ref. [28] after ppting phenols with PVP.

Enzyme assay. Both cresolase activity towards *p*-cresol and catecholase activity towards 4-methyl catechol were measured spectrophotometrically by the appearance of 4-methyl-*o*-benzoquinone (λ_{max}400 nm; $\varepsilon = 1350\ M^{-1}\ cm^{-1}$), as has been described in ref. [29].

One unit of enzyme activity was defined as the amount of enzyme that produce 1 μmol of 4-methyl-*o*-benzoquinone per min. Laccase activity was routinely assayed towards *p*-quinol using an O_2-electrode and spectrophotometrically using syringaldazine as substrate, as described in refs [13, 14], respectively.

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