Cresolase Activity of Potato Tuber Partially Purified in a Two-Phase Partition System

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The cresolase activity of partially purified potato polyphenol oxidase extracted by a two-phase partition method in Triton X-114 (Sánchez-Ferrer, A.; Laveda, F.; García-Carmona, F. *J. Agric. Food Chem.* **1993**, previous paper) has been characterized without using ascorbic acid. The purification method avoids the loss of cresolase activity described by other potato polyphenol oxidases. The activity was characterized by a lag period, whose duration depended on the substrate concentration, the pH, and the presence of catalytic amounts of *o*-diphenol. By increasing the concentration of *o*-diphenols, it was possible to evaluate the enzyme activation constant, K_{act}, which showed a value of 4.5 μM. A general kinetic mechanism for this enzyme is used to explain the loss of activity which normally occurs during potato tuber polyphenol oxidase purification.

INTRODUCTION

The production of slow-browning potatoes is highly desirable to avoid or minimize postharvest waste (Matheis, 1987a). Of the constituents responsible for browning, the concentration of polyphenol oxidase (PPO) appears to be dependent on the variety of potato, whereas the concentration of phenolic compounds seems to depend mainly on location and climatic factors (Adams, 1989). Of all the phenolic compounds, the concentration of monophenols is the most important in the browning process, since all PPO forms exhibit monophenolase activity in crude extracts. There is no lag period in this activity in the extracts because of the presence of *o*-diphenols, mainly chlorogenic acid and caffeic acid (Matheis, 1987a).

Despite its importance in potato, there has been only one incomplete study of this activity (Lavollay et al., 1975) measured in the presence of ascorbic acid. This absence of data stems from the lability of the cresolase activity in potato tubers during the purification process (Matheis, 1987a). This phenomenon is well-known in other plant PPOs (Mayer and Harel, 1979) and results from changes in the structure of the protein during purification (Walter and Purcell, 1980).

This loss in cresolase activity has been reported by Batistuti and Lorenço (1983) in the Tebere potato tuber during the purification process. In constrast, Macrae and Duggleby (1968) point out that the potato tuber, var. Orion, shows no capacity to catalyze the hydroxylation of *p*-coumaric acid.

The present paper shows that the use of the two-phase partitioning method described in the previous paper (Sánchez-Ferrer et al., 1993) preserves cresolase activity, permitting it to be characterized and enabling discussion of the experimental problems associated with measuring it without the need of reducing agents, such as ascorbic acid (Lavollay et al., 1975), which can alter the real activity expressed by the enzyme.

MATERIALS AND METHODS

The potato tubers (*Solanum tuberosum* cv. Desiree) used in this study were harvested at maturation stage in Murcia, Spain, and stored at 10 °C in the dark with a relative hummidity of 50–60%. Substrates were purchased from Sigma Chemical Co. (Madrid, Spain), and the other reagents were of analytical grade.

Potato PPO was partially purified in a two-phase system and catecholase activity measured as described in the previous paper (Sánchez-Ferrer et al., 1993).

Cresolase activity was also determined spectrophotometrically at 400 nm at 25 °C. The steady-state rate was calculated from the linear zone of the product accumulation curve after the lag period. The standard reaction mixture included in a total volume of 1 mL 40 μg/mL soluble potato PPO and 2.5 mM *p*-cresol in 50 mM sodium phosphate buffer (pH 6.5). One unit of enzyme was taken as the amount that produced 1 μmol of 4-methyl-*o*-benzoquinone/min (ϵ = 1433 M^{-1} cm^{-1}; Mayer et al., 1966).

Experiments were performed in triplicate and the mean and SD plotted.

RESULTS

Potato PPO was partially purified by using two-phase partitioning in Triton X-114 (Sánchez-Ferrer et al., 1993). The enzyme has both catecholase (Figure 1a) and cresolase activities (Figure 1b–e). 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 various sources when cresolase activity is measured (Lerch, 1981; Robb, 1984; Sánchez-Ferrer et al., 1988). The lag period and the steady-state rate (defined as the slope of the linear part of the accumulation product curve) were affected by the substrate concentration, the pH, and the presence of catalytic concentrations of *o*-diphenols (Figure 1b–e).

Effect of pH. Cresolase activity toward *p*-cresol increased as the pH increased from pH 3.5 and showed a maximum at pH 6.5 (Figure 2). At pH values above 7.0, it was difficult to follow the formation of 4-methyl-*o*-benzoquinone spectrophotometrically because of its great instability. This maximum obtained for cresolase activity differed substantially from that reported when tyrosine was used as substrate (pH 8.2) (Matheis, 1987b).

The pH affected not only enzyme activity but also the lag period, and a decrease in the latter was observed when pH was increased (Figure 2). These results are similar to those described for other plant PPO (Valero et al., 1988; Sánchez-Ferrer et al., 1988; Cabanes et al., 1987).

Effect of Substrate Concentration. An increase in *p*-cresol concentration from 0.1 to 5 mM (Figure 3)

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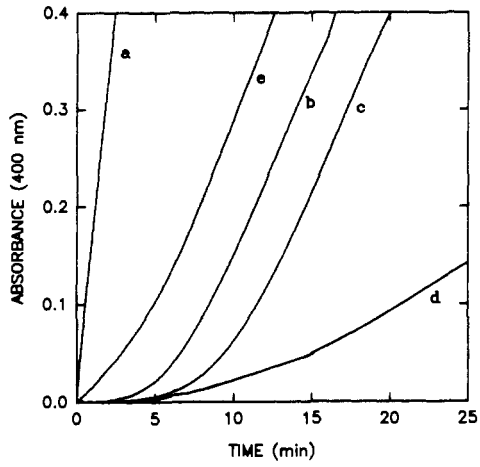


Figure 1. Enzymatic activities of soluble potato PPO. (a) Catecholase activity at 25 °C. The reaction medium included 2 μg/mL PPO and 5 mM 4-methylcatechol in 50 mM phosphate buffer (pH 6.5). (b–e) Cresolase activity at 25 °C. The reaction medium contained (b) 40 μg/mL PPO and 2.5 mM *p*-cresol in 50 mM sodium phosphate buffer (pH 6.5). (c,d) The same reaction medium as (b) except for (c) different substrate concentration (5 mM *p*-cresol), (d) different pH (pH 4.5), and (e) the addition of *o*-diphenols to the reaction medium (15 μM 4-methylcatechol).

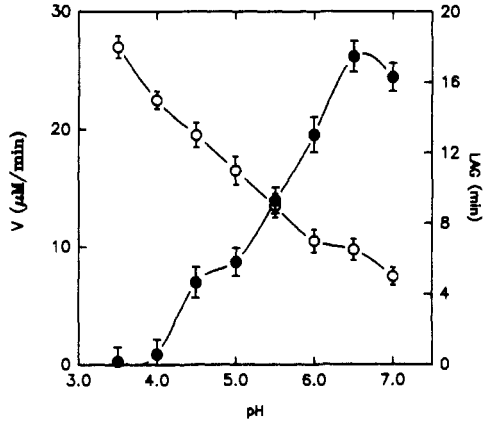


Figure 2. Effect of pH on cresolase activity (●) and on its lag period (○). The reaction medium at 25 °C included 40 μg/mL partially purified PPO and 2.5 mM *p*-cresol in 50 mM sodium acetate (pH 3.5–5.5), and sodium phosphate (pH 5.5–7.0) buffers.

produced an increase in the lag period of partially purified potato PPO, similar to that described for other plant PPO (Lavollay et al., 1975; García-Carmona et al., 1988; Sánchez-Ferrer et al., 1988). However, the explanation of the increase in the lag period profile is complex since the shape of the curve depends on the enzyme source used.

The kinetic constants, K_m and V_{max}, were evaluated from the data of Figure 3 (inset) by a Lineweaver–Burk plot and gave values of 0.56 mM and 33 μM/min, respectively. The K_m value was similar to that obtained by Lavollay et al. (1975) for partially purified potato PPO in the presence of ascorbic acid and by Macrae and Duggleby (1968) for the PPO found in the crude extract of the variety Orion.

The catalytic efficiency (V_{max}/K_m) between catecholase and cresolase activities was evaluated after the respective values of K_m and V_{max} were obtained for 4-methylcatechol (4MC) in 50 mM sodium phosphate buffer (pH 6.5). This diphenolic substrate showed inhibition at a high concentration, and it was necessary to evaluate the kinetic parameters, V_{max} and K_m, and substrate inhibition constant (K_{si}) as described in the previous paper (Sánchez-Ferrer et al., 1993). The values obtained for 4MC were 3.8 mM/min, 18 mM, and 31 mM, respectively.

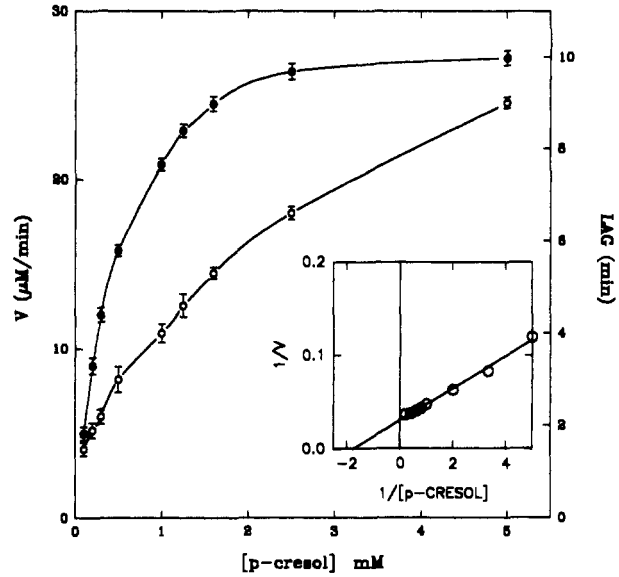


Figure 3. Effect of substrate concentration on cresolase activity of PPO (●) and on the lag period (○). The reaction medium at 25 °C included 40 μg/mL of partially purified PPO in 50 mM sodium phosphate buffer (pH 6.5) with different *p*-cresol concentrations. (Inset) Lineweaver–Burk plot.

The catecholase/cresolase ratio was 3.6. This appears to indicate that at pH 6.5, which is close to the physiological pH of potatoes, the oxidation of diphenols is favored over that of monophenols. This is in agreement with the assumption of Matheis (1987a), who suggests that chlorogenic acid oxidation is favored over tyrosine oxidation.

Effect of *o*-Diphenols. It is well-known that the cresolase activity lag period can be shortened or eliminated by the addition of reducing agents or *o*-diphenols, which act as cosubstrates (Pomerantz and Warner, 1967; Duckworth and Coleman, 1970; Lerch and Ettlinger, 1972; García-Carmona et al., 1979). The effect of the latter compounds on the cresolase activity of partially purified potato PPO was studied according to the protocol of Pomerantz and Warner (1967), assuming that the *o*-diphenol bound to the "activation center" by a simple isotherm and that the lag period was proportional to the nonactive enzyme concentration at zero time, using the empirical formula (Pomerantz and Warner, 1967)

$$\frac{1}{l} = \frac{1}{L} + \frac{1}{L}\frac{[o\text{-diphenol}]}{K_{act}}$$

where l, L, and K_{act} represent the lag period in the presence of *o*-diphenol, the lag period in the absence of *o*-diphenol, and the diphenol activation constant, respectively. Pomerantz and Warner (1967) interpreted the K_{act} as the *o*-diphenol affinity constant for the activation center.

Figure 4 shows the decrease in the lag period as *o*-diphenol concentration was increased up to 50 μM, at which stage the lag was abolished. To check that the *o*-diphenol added was used as cosubstrate and not as substrate, the steady-state rate was plotted against *o*-diphenol concentration (Figure 5), and no changes were observed up to 50 μM. Above this concentration the rate increased proportionally, indicating that the *o*-diphenol was being used as substrate for the catecholase activity. The activation constant K_{act} was calculated from the reciprocal of the lag period in the presence of diphenol (Figure 4, inset). The value obtained was 4.5 μM, which was significantly lower that the K_m of the 4-methylcatechol (18 mM), when acting as substrate for the catecholase activity, and higher than the K_{act} found for other plant

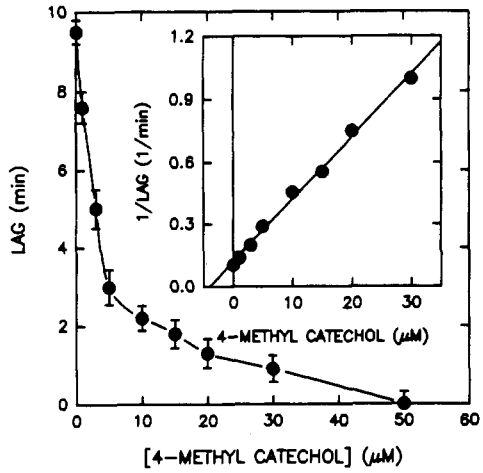


Figure 4. Influence of 4-methylcatechol on the shortening of lag period. In the standard reaction medium with 20 μg/mL PPO, the concentration of 4-methylcatechol ranged from 0 to 50 μM. (Inset) Evaluation of the activation constant, K_{act}, by the Pomerantz equation (Pomerantz and Warner, 1967).

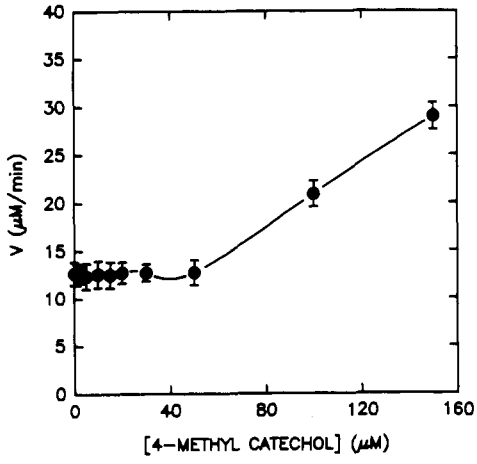


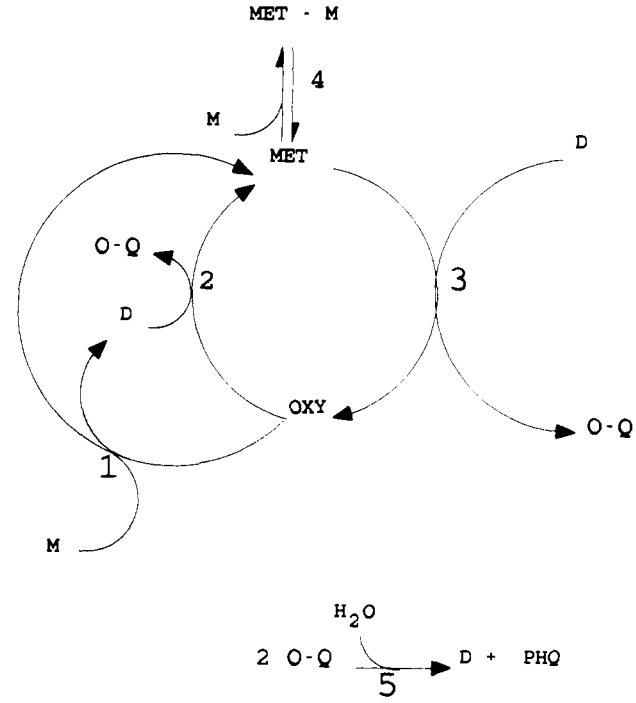
Figure 5. Effect of catalytic amounts of 4-methylcatechol on the steady-state rate of cresolase activity. The conditions are the same as in Figure 4.

PPO (Duckworth and Coleman, 1970; Lerch and Ettlinger, 1972; Sánchez-Ferrer et al., 1988).

DISCUSSION

The results presented for the cresolase activity of partially purified potato PPO are in agreement with the mechanism described for mushroom PPO (Cabanes et al., 1987). The lag period may be interpreted as the result of the enzymatic step, the competition between the mono- and *o*-diphenols for the *oxy* and *met* forms of the enzyme and the chemical addition of water to the quinones that regenerates the original *o*-diphenol (García-Carmona et al., 1987, 1988). This dynamic adjustment reaches the steady state when the sum of the *o*-diphenol production rates is equal to the rate of transformation of the *o*-diphenol into *o*-quinone. This can be easily understood from Scheme I, where reactions 1 and 5 represent the enzymatic and chemical generation of the *o*-diphenol and reactions 2 and 3 the destruction steps. In fact, the lag period means that a certain level of *o*-diphenol production is needed in the reaction medium before steady-state rate is reached. Thus, if the level of *o*-diphenol is present in the reaction medium before the enzymatic reaction starts, the lag period is abolished (Figure 4) without the steady-state rate being increased (Figure 5).

Scheme I. General Reaction of the Enzymatic–Chemical–Chemical Mechanism Proposed To Explain the Lag Period of the Cresolase Activity[a]



[a] M, *p*-cresol; D, 4-methylcatechol; O–Q, 4-methyl-*o*-benzoquinone; PHQ, 2-hydroxy-*p*-benzoquinone.

The above results explain why the lack of cresolase activity in other potato varieties may be due to increases in the catecholase/cresolase ratio caused by the loss of natural diphenols during purification and the drastic methods used to purify the enzyme. In fact, 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 sulfate (Thomas and Janave, 1986). The above factors avoid the transformation of the *met* form into the *oxy* form of the enzyme and produce a long lag period of more than 1 h when the same optimal conditions are used for following catecholase activity as for cresolase activity.

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