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MONOPHENOLASE ACTIVITY OF STRAWBERRY POLYPHENOL OXIDASE

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Key Word Index—*Fragaria* × *ananassa*; Rosaceae; strawberry; 4-hydroxyanisole; 3-methyl-2-benzothiazolinone hydrazone; monophenolase; polyphenol oxidase.

Abstract—Strawberry polyphenol oxidase (PPO) was extracted and partially purified by using three sequential phase partitionings with Triton X-114 (TX-114) reported here for the first time. Monophenolase activity of strawberry PPO was reported for the first time. The enzyme fulfilled all the tests of the kinetic reaction mechanism previously proposed for PPOs from other sources. Therefore, this mechanism was also applicable to monophenolase activity of strawberry PPO. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Enzymatic browning in fruits and vegetables is often an undesirable reaction which is responsible for unpleasant sensory qualities and losses in nutrient quality. The prevention of this reaction has always been a challenge to food scientists [1]. The main enzyme involved in this reaction is tyrosinase or polyphenol oxidase (EC 1.14.18.1; PPO) which has been the subject of several reviews [2–4]. PPO catalyses two different reactions: the hydroxylation of monophenols to *o*-diphenols (monophenolase activity) and the oxidation of *o*-diphenols to *o*-quinones (diphenolase activity) which, in turn, are polymerized to brown, red or black pigments [5, 6].

The red colour in strawberries is due to the presence of two main anthocyanin pigments, pelargonidin-3-glucoside (PGN) and cyanidin-3-glucoside (CYN). It has been suggested that the anthocyanins are not degraded directly by the enzyme, but via *o*-quinones formed by PPO [7] or by a degradation pattern similar to that occurring to pigments in wines [8, 9].

In most PPOs, including strawberry PPO, diphenolase activity has been widely characterized [10–12]. However, there are only a few reports on monophenolase activity [13–18], and this activity has not been shown for strawberry PPO. This scarce information about the monophenolase activity of PPO stems from the lability of the enzyme during the purification process [1]. This phenomenon is well known in other plant PPOs [2] and results from changes in the structure of the protein during purification [9].

The aim of this work was the detection and kinetic characterization for the first time of the monophenolase activity of strawberry PPO, after its extraction and partial purification by using the method with Triton X-114 previously applied for extracting other PPOs [15–18]. The monophenolase activity was assayed with 3-methyl-2-benzothiazolinone hydrazone (MBTH) as coupled chromogenic nucleophile [15–18, 20] on *p*-hydroxyphenyl propionic acid (PHPPA) and 4-hydroxyanisole (4HA), as new substrates for strawberry PPO. From the kinetic characterization of the monophenolase activity of this enzyme, the applicability to strawberry PPO of the same reaction mechanism previously proposed for PPO from other sources [13–18, 21] was tested.

RESULTS AND DISCUSSION

Strawberry PPO was extracted and partially purified by using several sequential phase partitionings with TX-114 [15–18]. The use of this surfactant enabled the removal of both pectic material and anthocyanins from the extract in a simple and fast procedure. The enzyme had both diphenolase [Fig. 1(A), curve a] and monophenolase [Fig. 1(A), curve b] activities. The latter was characterized by a lag period (τ) prior to the attainment to the steady state rate (V_{ss}) [16, 18].

Drastic extraction methods provoke the loss of the monophenolase activity [1, 2, 19]. The non removal of pectic material can cause co-aggregation with proteins

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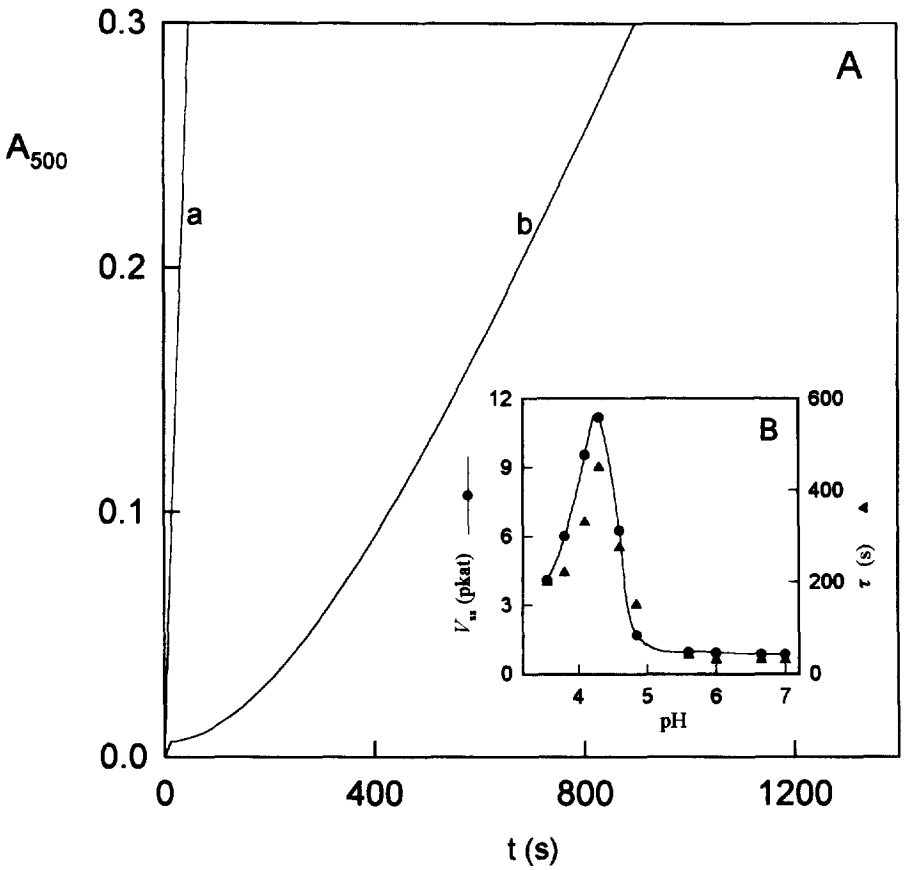

Fig. 1. (A) Enzymatic activities of soluble strawberry PPO. (a) Diphenolase activity. The reaction medium included 21 μg ml^{-1} protein, 15 mM DHPPA, 1 mM MBTH and 2% DMF in 0.15 M AB (pH 4.3); (b) Monophenolase activity. The reaction medium contained 210 μg ml^{-1} protein, 1 mM MBTH, 2% DMF and 5mM PHPPA in 0.15 M AB (pH 4.3). (B) Insert: effect of pH on monophenolase activity (●) and on its lag period (▲). The reaction medium included 60 μg ml^{-1} protein, 3 mM MBTH, 2% *N,N,*-dimethyl formamide and 0.5 mM 4HA in 50 mM AB (pH 3.6–5.6) and PB (pH 5.8–7.0).

and enzymes such as PPO [22, 23]. The catalytic power (V_{max}/K_m) for the diphenolase activity is much higher than that for the monophenolase activity of PPO [13, 14]. Thus, losses and/or dilutions in the PPO activity lead to a very low catalytic power for the monophenolase activity even to the limit of detection.

Another factor which limits the detection of the monophenolase activity is the assay method. The MBTH method previously used in other works [15–18, 20] is very sensitive and reliable. Moreover, the monophenol that we use in this work (4HA) [24] is a very good substrate for strawberry PPO because the effect of the methoxyl group on the hydroxyl group of the aromatic ring facilitated the nucleophilic attack of the oxygen to the copper of the active site of the enzyme.

Monophenolase activity toward 4HA increased as the pH was increased from pH 3.5 and showed a maximum at pH 4.3. The pH affected not only the enzyme activity, but also the lag period. Both plots, V_{ss} vs pH and τ vs pH, showed the same profiles [Fig. 1(B), insert]. These results differed from those described for some plant PPOs [21, 25, 26], but were in agreement with those obtained for certain other plant PPOs [14, 15, 18]. When the PHPPA/3,4-dihydroxyphenol propionic acid (DHPPA) pair was assayed a similar optimum pH was obtained. The K_m values for PHPPA and DHPPA were 0.3 and 1.5, respectively. The V_{max} values for PHPPA and DHPPA were 11.7 pkat and 1.6 nkat, respectively; with 210 μg protein in the assays. The K_m value for 4HA was 2 mM and the V_{max} value was 90 pkat with 90 μg protein in the assays. Changes in the pH affected the K_m but not the V_{max} values, indicating the existence of two significant pK_a values of the free enzyme forms but not of the enzyme–substrate complexes. The profile of τ vs pH was explained because the E_{met} form of the enzyme (inactive on monophenols) had more affinity, at the optimum pH, for monophenols and therefore τ increased. However, when the pH was either lower or higher than the optimum the affinity, of the E_{met} form of the enzyme on monophenols was lower, and τ decreased.

An increase of the enzyme concentration produced a linear increase in V_{ss} as well as a hyperbolic shortening in the lag period. This behaviour has been widely described from several PPO sources [14–18, 21]. The data obtained can be explained by the PPO reaction

mechanism [13]. Increasing PPO concentrations produce a proportional increase of the E_{oxy} form in the native state which means that there is more enzymatic activity and the level of *o*-diphenol in the steady state is reached more quickly, and so τ diminishes.

An increase of monophenol concentration produced an increase in both the steady state rate and lag period, similar to that described for other PPOs [14–18]. This behaviour can be explained by the PPO reaction mechanism [13]. The E_{met} form of the enzyme is saturated when the monophenol concentration is raised and there is more enzyme in the dead-end complex $E_{met}M$ requiring more time to reach the steady state. Moreover, the dimensionless parameter R, which is the ratio between the *o*-diphenol concentration in the steady state ($[D]_{ss}$) and the initial monophenol concentration ($[M]_o$), ($[D]_{ss}/[M]_0$), must be considered [13, 14]. Therefore, an increase in the monophenol concentration (at the same enzyme concentration) produces an increase in the *o*-diphenol concentration and more turnovers are needed for the E_{oxy} form of the enzyme to accumulate the necessary *o*-diphenol in the steady state.

The addition of different *o*-diphenol concentrations in the monophenolase activity diminished the lag period. Again the data obtained can be explained by the PPO reaction mechanism [13]. The R value, ($[D]_{ss}/[M]_0$) is independent of the enzyme concentration and is constant when the initial monophenol and enzyme concentrations are constant [13, 14]. Therefore, a rise in the initial *o*-diphenol concentration ($[D]_0$) shortened the time required for the steady state rate level *o*-diphenol ($[D]_{ss}$) to be reached. When $[D]_0$ is higher than $[D]_{ss}$, the system must first consume the excess of *o*-diphenol and then gradually consume the monophenol and *o*-diphenol before the steady state rate is finally reached. In these conditions, there was a burst in the activity. Depending on the assay conditions a ($-\tau$) or ($+\tau$) appeared which corresponded with an over-steady state or sub-steady state rate level of $[D]_0$, respectively.

Consideration of the above factors allows for the determination of the optimal monophenolase assay conditions on 4HA. Thus, a limit of detection (LOD) of 0.07 pkat ml^{-1} and a limit of quantitation (LOQ) of 0.2 pkat ml^{-1} were obtained. Furthermore, from ten assays at 1.53, 7.65 and 15.3 pkat ml^{-1}, coefficients of variation (CV) of 6.4, 1.4 and 0.5%, respectively, were obtained for the corresponding V_{ss} data values.

EXPERIMENTAL

Chemicals. Strawberries (*Fragaria* × *ananassa Duchesne*, cv. Chandler) picked in Huelva (Spain) at commercial maturity and stored at 4° were used as enzyme source. 4HA was purchased from Aldrich (Spain), DHPPA, PHPPA and MBTH from Sigma (Spain) and all other reagents were of analytical grade and supplied by Fluka (Spain). All other experimental conditions (prepn of PPO, enzymatic assays, data analysis) were as described in refs [15–18].

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