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Polyphenoloxidase in Wild Rice (*Zizania palustris*)

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Polyphenoloxidase (PPO) was isolated from wild rice (*Zizania palustris*). Partial purification by acetone precipitation and dialysis of the crude extract yielded a 2.8-fold purification of the activity of the extract. The optimum pH and temperature of the enzyme were 7.8 and 25 °C, respectively. The enzyme catalyzed the oxidation of *o*-diphenols but not monophenols. Thermal inactivation data indicated first-order kinetics with an apparent activation energy of 20 kJ/mol. Polyacrylamide gel electrophoresis showed the PPO to be isoenzymes with molecular weights of 116 000, 48 000, 42 000, and 35 000, respectively.

Polyphenoloxidase (PPO; *o*-diphenol:O_2 oxidoreductase, E.C. 1.14.18.1) is one of the enzymes involved in the oxidation of phenolic compounds to brown pigments. The enzyme has been extensively studied because of its relative ubiquity and importance in the food and agricultural industry. It has been found in green olives (Sciancalepore and Longone, 1984), bananas (Palmer, 1963; Galeazzi et al., 1981), avocados (Kahn, 1977), peaches (Jen and Kahler, 1974; Flurkey and Jen, 1978), mushrooms (Jolley et al., 1969; Yamaguchi et al., 1970), potatoes (Hyodo and Uritani, 1966; Weaver et al., 1968; Mondy and Koch, 1978; Batistuti and Lourenço, 1985), and artichokes (Zawistowski et al., 1986). In all these commodities, the enzyme is implicated in the undesirable browning of the products, which ultimately reduces their marketability. Except for tea fermentation, the browning reaction catalyzed by PPO has not been positively associated with food quality.

Wild rice is a cereal of the *Zizania* family growing in the shallow and sluggish waters of Northern United States and Canada. The exotic crop, which commands premium price, requires unique processing protocol compared to other cereals. After harvest, the rice is cured, a process that involves biochemical transformation of the rice grain to give its characteristic color and aroma. Qualitative presumptive tests (Withycombe, 1974) suggest that the curing process involves polyphenolase-mediated reactions in which inherent free phenolic acids in the rice are oxidized into brown and black pigments responsible for the color of the rice. The presence of polyphenolase and the characteristics of the enzyme in wild rice have not been clearly demonstrated. This study was undertaken to confirm the presence of polyphenolase activity in wild rice and to characterize the enzyme.

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MATERIALS AND METHODS

Materials. Freshly harvested wild rice was obtained from the La Ronge Wild Rice processing plant. The rice was separated into maturity groups at the La Ronge plant by a combination of the following tests: visual color examination, moisture content, and bulk density. The fully matured rice, with a moisture content range of 25–30%, a bulk density range of 0.40–0.50 g/cm^3, and olive green to brown color, was shipped on solid carbon dioxide in a cooler and was kept frozen until used.

Methods. *Enzyme Extraction.* This was performed essentially as described by Flurkey and Jen (1978). The wild rice samples were thawed, and 100-g samples were homogenized for 2 min in a Waring blender with 200 mL of cold acetone (−20 °C) and 5.0 g of poly(ethylene glycol) as a phenolic scavenger. The slurry was suction-filtered and the pellet reextracted with cold acetone (−20 °C). The pellet was quantitatively transferred to a 500-mL beaker and homogenized for 2 min on a Polythron homogenizer with 200 mL of cold acetone. The slurry was suction-filtered and washed with another 200 mL of cold acetone. The powder was air-dried overnight at room temperature in a fumehood to remove residual acetone. The acetone powder was kept frozen at −20 °C until use. A 5-g portion of the acetone powder thus obtained was suspended in 200 mL of 0.05 M potassium phosphate buffer, pH 6.2, containing 1 M KCl. The suspension was stirred for 30 min at 4 °C and then centrifuged at 12000*g* for 20 min at 4 °C. The supernate was carefully decanted and was considered the "crude enzyme extract".

Partial Purification Procedure. The partial purification was performed essentially as described by Galeazzi et al. (1981). Two volumes of cold acetone (−20 °C) were slowly added to 50-mL aliquots of the crude enzyme extracts, and the resulting precipitate was separated by centrifugation at 12000*g* and 4 °C for 15 min. The precipitate was resolubilized with 0.2 M sodium phosphate buffer, pH 7.0, containing 0.25% Triton X-100 and recentrifuged. The supernate was dialyzed against 0.05 M phosphate buffer, pH

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Table I. Effect of Partial Purification on the Activity of PPO in Wild Rice (*Z. palustris*)

enzyme extract	protein content, mg/mL	sp act., units/mg protein	act. purifn, fold
crude	1.32	186.4	1.0
partially purified	0.44	527.3	2.8

6.2, in dialysis tubes with molecular weight cutoff of 6000–8000. The dialysis was carried out at 4 °C with three changes of the dialyzing solution. The supernate was termed "partially purified enzyme extract". All the extracted fractions were analyzed for enzyme activity using catechol as substrate.

Measurement of Enzyme Activity. The enzyme activity was measured with a Shimadzu double-beam scanning spectrophotometer. The reaction mixture consisted of catechol (5 mM) dissolved in phosphate buffer (0.1 M, pH 7.2) plus 50 μL of the enzyme extract; the final volume was 3.0 mL. One unit of enzyme activity was defined as a change of 10^{-3} AU/min at 410 nm.

Protein Determination. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Characterization Studies. Substrate specificity of the enzyme was determined on 1 mM solutions of the following compounds dissolved in 0.1 M phosphate buffer, pH 7.2: chlorogenic acid, 4-methylcatechol, catechol, caffeic acid, sinapic acid, *p*-coumaric acid, and tyrosine.

The effect of pH on the enzyme activity was determined using a phosphate buffer (pH 2–9.0). Phosphoric acid was used to adjust the pH of the buffer to obtain the acidic pH ranges.

The effect of temperature on the activity of the wild rice PPO was tested by separately incubating aliquots of the extract and catechol solution (5 mM) at the appropriate temperature in a water bath. After equilibration, the enzyme extract was assayed at the desired temperature in temperature-controlled jacketed cuvettes.

The temperature stability of the enzyme was determined on crude extracts that were heated at 60, 70, 80, and 90 °C for varying time periods in a temperature-controlled bath. The extracts after incubation at the respective temperatures were cooled in an ice bath and assayed at 25 °C with catechol as substrate. The stability of the enzyme was expressed as the logarithm of the percent remaining activity. The activation energy was estimated from an Arrhenius plot of log reaction rate against the reciprocal of the absolute temperature.

The molecular weight of the partially purified polyphenolase was determined by gel electrophoresis according to the method of Laemmli (1970). The protein zones were detected by staining the gels in 0.1% Coomassie brilliant blue (R-250) in 25% 2-propanol, 10% acetic acid, and distilled deionized water. PPO activity was observed by staining electrophoretic gels in 10 mM catechol solution for 30 min. Protein standards used for the molecular weight estimation were myosin (200 000 Da), phosphorylase B (92 500 Da), bovine serum albumin (66 200 Da), ovalbumin (45 000 Da), carbonic anhydrase (31 000 Da), soybean trypsin inhibitor (21 000 Da), and lysozyme (14 400 Da).

RESULTS AND DISCUSSION

The wild rice PPO could easily be extracted in phosphate buffer and partially purified by acetone precipitation. The partial purification yielded 2.8-fold purification of the activity as compared to the crude extract (Table I) with catechol as substrate. A similar extraction procedure has been used on banana PPO system (Augustin et al., 1985), and a 4.9-fold purification was observed. Relatively lower fold of activity was observed in this study.

Data presented in Table II show that the PPO in wild rice had activity toward *o*-diphenols but not toward monophenols. This observation is indicated by the lack of activity toward *p*-coumaric acid and tyrosine in both the crude and partially purified systems. PPO systems that have mainly *o*-diphenol activity have been reported for various fruits (Halim and Montgomery, 1978; Rivas and Whitaker, 1973; Stelzig et al., 1972; Wong et al., 1971) and

Table II. Substrate Specificity of Crude and Partially Purified PPO from Wild Rice (*Z. palustris*)[a]

substrate	rel activity (%) for enzyme type: crude	rel activity (%) for enzyme type: partially purified
4-methylcatechol	100.0	99.5
catechol	72.6	60.8
chlorogenic acid	34.6	100.0
caffeic acid	32.9	28.6
sinapic acid	14.4	20.3
tyrosine	0.0	0.0
p-coumaric acid	0.0	0.0

[a] Protein contents of crude and partially purified extracts were 1.32 and 0.44 mg/mL, respectively.

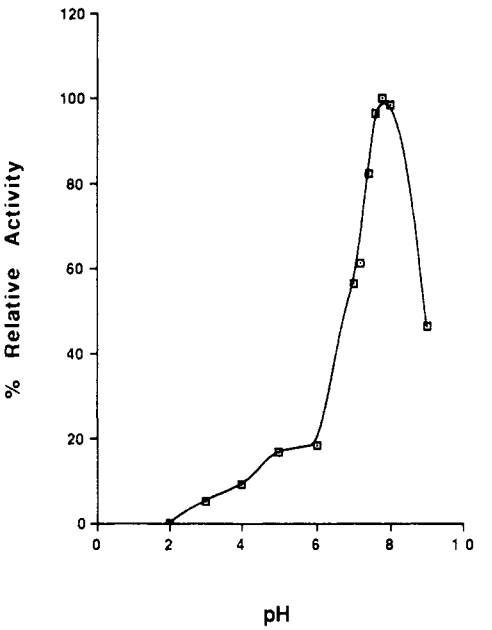

Figure 1. Optimum pH of crude wild rice PPO with catechol as substrate. Each point is an average value of duplicate results from two experiments. The pooled estimate of the standard deviation was ±3.2%.

tubers (Zawistowski et al., 1986; Batistuti and Lourenço, 1985). The PPO in wild rice seemed to behave similarly as the reported systems. Among the tested *o*-diphenols, the crude enzyme system showed the highest activity for 4-methylcatechol followed by catechol. On the other hand, the partially purified system showed the highest activity for chlorogenic acid, but the relative activity for 4-methylcatechol was almost equal to that of the chlorogenic acid. The repressed activity of the crude enzyme toward chlorogenic acid might be due to the presence of inhibitory compounds in the crude extract. Phenolic acids such as cinnamic acid and ferulic acid act as inhibitors of PPO activity when chlorogenic acid is the substrate (Batistuti and Lourenço, 1985). The presence of such compounds in the crude extract could account for the repressed activity of the enzyme toward chlorogenic acid. Partial purification and dialysis apparently could remove the inhibitory compounds in the crude extract, thus increasing the relative specificity of the enzyme for chlorogenic acid.

Figure 1 shows that, with catechol as substrate, the optimum pH of the crude PPO system was 7.8. The enzyme showed repressed activities from pH 2 to 6 and then increased rapidly after pH 6, peaking at pH 7.8 and then falling rapidly at pH 9. The wild rice PPO was relatively more stable in the alkaline region (pH 7–8) than in the acid pH (pH 2–6). Various PPO systems have been charac-

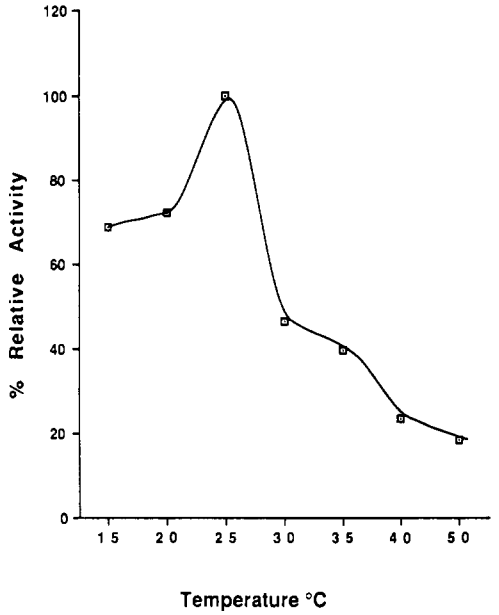

Figure 2. Optimum temperature of crude wild rice PPO with catechol as substrate. Each point is an average value of duplicate results from two experiments. The pooled estimate of the standard deviation was ±4.3%.

terized to show similar alkaline pH preferences (Thomas and Janave, 1973; Park and Luh, 1985). The optimum pH range of the wild rice PPO was very short (around pH 7.8–8.0), suggesting that pH changes could be a critical factor in the curing of wild rice. Some PPO from other sources such as artichokes (Zawistowski et al., 1986) showed a broad pH activity range (5.0–8.0). The difference between such PPO systems and the wild rice PPO may be due to the sources of enzymes and might support the observations of Aylward and Haisman (1969) that the optimum pH of PPO activity varies with the source of enzyme and substrate.

The optimum temperature and heat stability of the PPO system from wild rice are shown in Figures 2 and 3, respectively. The relative activity of the enzyme increased with temperature from 15 to 25 °C, with the optimum occurring at 25 °C. After 25 °C, the activity declined very rapidly with increases in temperature, presumably from partial heat inactivation of the enzyme.

The enzyme was reasonably stable at 60 °C, but the activity declined rapidly at 90 °C. The linear time course for the thermal inactivation of the wild rice PPO suggested first-order kinetics. Several researchers (Dimick et al., 1951; Chan and Yang, 1971; Benjamin and Montgomery, 1973; Halim and Montgomery, 1978) have observed similar first-order inactivation kinetics for PPO from various sources. An Arrhenius plot of the thermal stability data indicated an apparent activation energy of approximately 20 kJ/mol for the crude enzyme. The activation energy of the enzyme seemed similar to that of PPO from bananas (18.5 kJ/mol) reported by Padron et al. (1975) but considerably lower than that of artichokes (54 kJ/mol) reported by Zawistowski et al. (1986). The relatively lower stability of the wild rice enzyme may be related to its relatively lower optimum temperature and might be an important factor modulating the curing of wild rice with respect to curing temperature regimen.

A schematic diagram of the electropherograms of the partially purified wild rice PPO system is shown in Figure 4. Six protein bands were observed but only four of them showed PPO activity when the gels were soaked in catechol solution (Figure 4b). The apparent molecular weights of

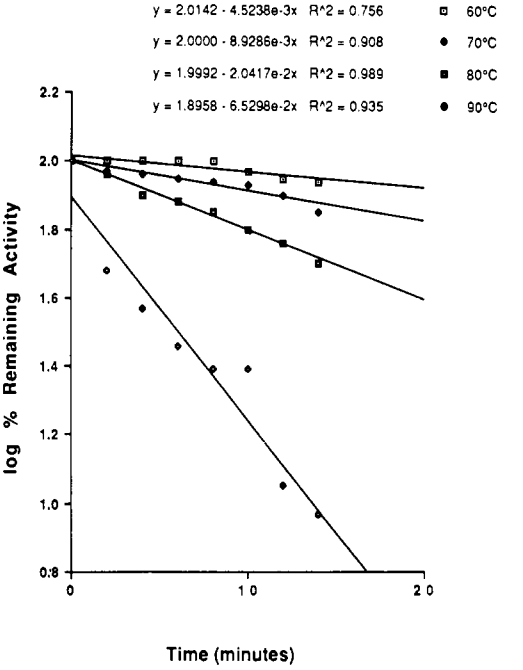

Figure 3. Heat inactivation of crude wild rice PPO with catechol as substrate. Each point is an average value of duplicate results from two experiments. The pooled estimate of the standard deviation was ±2.8%.

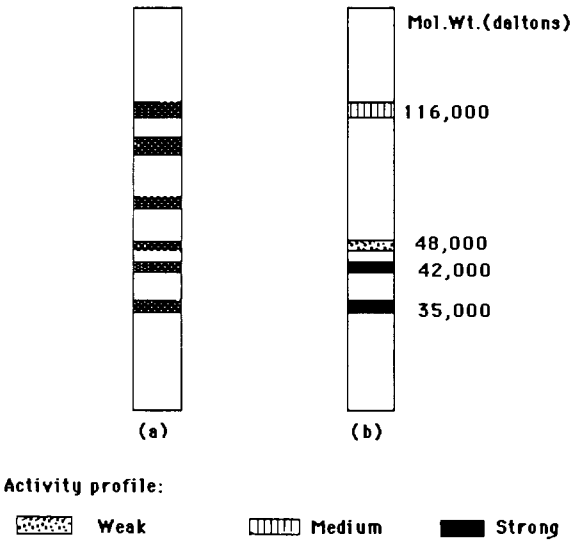

Figure 4. Gel electrophoresis diagram of the isoenzymes of wild rice PPO: (a) protein bands, (b) PPO activity bands.

the bands showing PPO activity were approximately 116 000, 48 000, 42 000, and 35 000, respectively. Heterogeneous PPO enzyme systems have been reported in various studies. Halim and Montgomery (1978) observed eight isoenzymes for the PPO system in pears. Flurkey and Jen (1978) observed five isoenzymes for peaches after their gels were fixed in acetic acid. Although it is not known why such heterogeneity exists, it has been speculated that multiple enzyme forms might provide greater resistance to substrate or product inhibition (Constantinides and Bedford, 1967). For fruits, it has been observed that isoenzyme patterns change during maturation, with the disappearance of some isoenzymes and subsequent appearance of new ones at various stages of ripening (Frenkel, 1971). It is not known whether a similar phenomenon exists in wild rice. The rice samples used in this study were matured grains ready for curing. If the isoenzyme pattern in wild rice is dependent on maturation,

then the observed pattern could be considered to be for matured wild rice.

The observations made in this study confirm the presumptive test of Withycombe (1974) that there is polyphenolase activity in wild rice. Whether the enzyme plays any dominant role, particularly in the development of the characteristic black color, during the curing of wild rice remains to be demonstrated.

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