Purification and Characterization of a Polyphenol Oxidase from the Kew Cultivar of Indian Pineapple Fruit

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Three isoforms of polyphenol oxidase (PPO) were purified to apparent homogeneity from the Kew cultivar of Indian pineapple fruit in a four-step procedure. The major isoenzyme, with a yield of 45%, was found to be a tetramer of identical subunits of molecular mass ≈25 kDa. An ionic strength dependent association−dissociation equilibrium was observed with pineapple PPO. Amino acid analysis of the major isoenzyme indicated the presence of a high content of glutamic acid, glycine, and serine and a low content of the sulfur-containing amino acids. The enzyme was optimally active between pH 6 and 7. The PPO did not show any cresolase activity, and the preferred substrates were diphenols. Ascorbic acid, L-cysteine, and potassium metabisulfite were found to be potent inhibitors of PPO.

Keywords: *Catecholase; pineapple fruit; polyphenol oxidase; PPO-isoenzyme; association−dissociation*

INTRODUCTION

Pineapple fruit is widely distributed in tropical regions and in recent years has become one of the most demanded exotic fruits. However, the browning after mechanical or physiological injury, suffered during either harvest or cold storage, affects consumer acceptability and palatability. Most browning in fruits is caused by enzymatic oxidation of natural phenolic compounds. Polyphenol oxidase (EC 1.10.3.1; PPO) is the major enzyme that catalyzes the oxidation of phenolic compounds to quinones, which further polymerize to brown pigments (Lee, 1991). Techniques have been developed to prevent browning and PPO activity, each requiring a different approach depending on the characteristics of the plant tissue and the PPO (Martínez and Whitaker, 1995; Walker and Ferrar, 1995).

PPO characteristics have been thoroughly investigated in apple (Janovitz-Klapp *et al*., 1989), grape (Sánchez-Ferrer *et al*., 1988), potato, and mushroom (Chen *et al*., 1992). Although a number of studies on the morphological characteristics and physical and biochemical changes during pineapple fruit development are available (Gortner and Singleton, 1965; Kermasha *et al*., 1987; Bartolome *et al*., 1995), there is little or no information on PPO. In order to understand the role of PPO in the browning of pineapple fruit during cold storage, purification of PPO from pineapple was attempted. Herein we report a four-step purification and properties of a PPO, isolated from the Kew cultivar of Indian pineapple fruit.

MATERIALS AND METHODS

Plant Materials. The pineapple fruits (*Ananas comosus* L. Merr. cv. Kew, 12−13° Brix) used in this study were obtained from the local market.

Chemicals. Catechol, catechin, L-3,4-dihydroxyphenylalanine (L-DOPA), chlorogenic acid, gallic acid, *p*-coumaric acid, L-tyrosine, cinnamic acid, resorcinol, *p*-phenylenediamine, polyvinylpolypyrrolidone (PVPP), and SDS-PAGE molecular weight markers were obtained from Sigma Chemical Co., St. Louis, MO. Phenyl Sepharose CL-4B, Sephadex G-150, and gel filtration markers were from Pharmacia Fine Chemicals, Uppsala, Sweden. All other chemicals used were of analytical grade.

Experimental Procedures. All procedures were done at 4 °C unless otherwise noted.

Enzyme Extraction. The frozen pulp (150 g) was ground in a stainless steel Waring blender and suspended in 100 mL of extraction buffer [0.01 M sodium phosphate (NaPi), pH 7, containing 1% PVPP] for 60 min with occasional stirring and then filtered through cheese cloth. The filtrate was centrifuged at 10500*g* for 30 min. The supernatant was extensively dialyzed against the same buffer used for extraction (5 × 1000 mL) at 4 °C, overnight.

Purification Procedures. Solid $(NH_4)_2SO_4$ (16.4 g/100 mL) was added to the dialyzed crude extract to obtain 30% saturation and then centrifuged at 10500*g* for 30 min. Solid $(NH_4)_2SO_4$ (32.3 g/100 mL) was added to the supernatant to obtain 80% saturation. The precipitate obtained was redissolved in extraction buffer containing 1 M $(NH_4)_2SO_4$ and loaded onto a Phenyl Sepharose CL-4B (15 × 2.5 cm) column, previously equilibrated with the extraction buffer containing 1 M $(NH_4)_2SO_4$, and washed with the same buffer to remove unbound proteins. Protein was eluted in gradient by decreasing the concentration of $(NH_4)_2SO_4$ from 1 to 0 M, followed by H_2O and then 50% ethylene glycol in a stepwise manner. Fractions of 3 mL were collected. The fractions containing PPO activity were appropriately combined, dialyzed against extraction buffer, and concentrated. The three enzyme fractions were labeled F_1, F_2, and F_3 in order of their elution (Figure 1).

Enzyme Assay. Polyphenol oxidase was assayed according to the spectrophotometric procedure of Coseteng and Lee (1987). The assay mixture consisted of 2.6 mL of 0.01 M NaPi buffer, pH 6.5, 0.3 mL of 0.5 M catechol, and 0.1 mL of enzyme extract. The increase in absorbance at 420 nm was measured. One unit of enzyme activity is defined as the amount of the enzyme that causes an increase in absorbance of 0.001/min at 25 °C.

Protein Determination. Protein concentration was determined according to the dye binding method of Bradford (1976) with bovine serum albumin as standard.

Electrophoresis. Polyacrylamide gel electrophoresis (PAGE, 7.5% T and 2.7% C) was performed by following the method of Hames (1981). Duplicate samples were run for simultaneous protein and enzyme staining. The gels were

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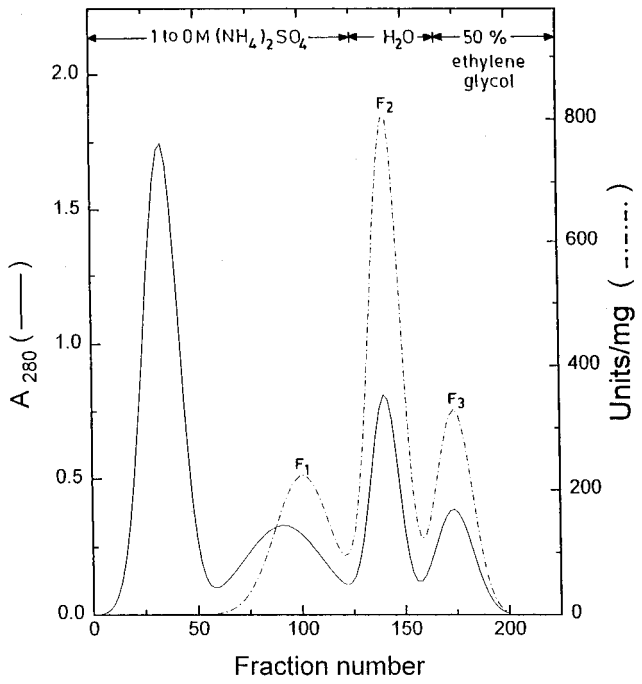

Figure 1. Phenyl Sepharose CL-4B chromatography elution profile of pineapple fruit PPO. F_1, F_2, and F_3 represent peaks with PPO activity.

stained for protein with Coomassie Blue and for PPO following the method of Lee (1991).

Molecular Weight Determination. The molecular weight of the native enzyme was determined using a calibrated Sephadex G-150 (80 × 1.5 cm) column according to the method of Andrews (1970). The subunit molecular weight was determined by SDS-PAGE (10% T, 2.7% C) in the discontinuous buffer system of Laemmli (1970). The molecular weight was also determined by HPLC (Shimadzu LC6A) using a Zorbax G-250 column (25 cm × 9.4 mm i.d.). The eluent used was 0.2 M NaPi, pH 7.4, at a flow rate of 2 mL/min. The proteins were detected at 280 nm. The column was calibrated using β-galactosidase (540 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease (13.7 kDa).

Amino Acid Composition. The enzyme was subjected to RP-HPLC to remove salts using a Shimpak octadecyl column (25 cm × 4.6 mm i.d.). The protein was eluted using a linear gradient of 0.1% trifluoroacetic acid/acetonitrile (7:3) and detected at 280 nm. A 24 h vapor phase hydrolysis (using the Pico-Tag workstation) of the purified protein (1−5 μg) was performed *in vacuo* at 110 °C using 5.8 M HCl. Amino acid analysis was performed by precolumn derivatization using phenylisothiocyanate. The phenylthiocarbamoyl amino acids were analyzed using a Waters Pico-Tag amino acid analysis system (Bidlingmeyer *et al.*, 1984).

UV−Vis Absorption Spectrum. The absorption spectrum of the purified enzyme was determined from 200 to 600 nm, taking 0.6 mg of the enzyme in 1 mL of 0.01 M NaPi buffer. The protein was precipitated using 5% TCA at 4 °C. The precipitate was collected by centrifugation at 10000*g* for 30 min. The protein was redissolved in 1 mL of 0.01 M NaPi buffer and scanned between 200 and 600 nm at a speed of 600 nm/min.

Effect of pH. The effect of pH on PPO activity was determined under the assay conditions described using various buffers at a concentration of 0.01 M: sodium acetate (pH 3.5−6.0), phosphate (pH 5.5−8.0), and Tris-HCl (pH 7.5−9.0) at 25 °C.

Substrate Specificity. To study the substrate specificity, 0.3 mL of the various substrates (0.5 M) was added to 2.6 mL of assay buffer, followed by the addition of 0.1 mL of purified enzyme. Enzyme activity was measured at the optimum wavelength of each product at 25 °C.

Effect of Inhibitors. To determine the effect of inhibitors on PPO activity, the purified enzyme (17 893 units/mg protein) was preincubated with the various inhibitors (final concentration of 0.5−3 mM) for 5 min at 25 °C. Residual PPO activity was measured under standard assay conditions.

RESULTS AND DISCUSSION

Extraction and Purification of PPO. The enzyme activity of the crude extract was not detected owing to the presence of endogenous inhibitors, like ascorbic acid. However, when these extracts were dialyzed overnight, the PPO activity could be measured. The results of the purification of PPO are shown in Table 1. A 76% recovery of PPO activity was obtained by 30−80% $(NH_4)_2SO_4$ fractionation. Chromatography on Phenyl Sepharose CL-4B, which has been used for the purification of PPO from peaches (Flurkey and Jen, 1980), grapes (Wissemann and Lee, 1980), apples (Janovitz-Klapp *et al.*, 1989), and strawberries (Wesche-Ebeling and Montgomery, 1990), was also employed to purify the ammonium sulfate precipitated PPO from pineapple. The elution profile of PPO on Phenyl Sepharose CL-4B (Figure 1) shows three peaks with PPO activity. At very low $(NH_4)_2SO_4$ concentration the first isoform F_1 eluted. The major fraction F_2 eluted in water, whereas fraction F_3 was obtained with 50% ethylene glycol. The recovery of the major isoenzyme F_2 was 45%, after the 25-fold purification, showing highest PPO specific activity (17 893 units/mg). PAGE of the three isoenzyme fractions (F_1, F_2, and F_3) showed single protein bands using both Coomassie Blue (data not shown) and PPO activity stain (Figure 2). Harel *et al.* (1965) found four isoenzymes of PPO in apple flesh (three from chloroplast and one from the mitochondria), whereas in the peel only two have been isolated (Zhou *et al.*, 1993) using Phenyl Sepharose CL-4B.

Molecular Weights. The native molecular mass (M_r) of the major isoenzyme (F_2) by gel filtration on a Sephadex G-150 column was ≈104 kDa. However, SDS-PAGE indicated the presence of a single polypeptide of ≈25 kDa (Figure 3). The results suggested that the enzyme is a tetramer of identical subunits. The problem of M_r and quaternary structure of higher plants' PPOs is complex, and the values reported cover a wide range (Mayer and Harel, 1979, 1991). Harel and Mayer (1968) observed three isoforms of PPO from apple chloroplast having molecular masses in the range 30−40, 60−70, and 120−130 kDa, respectively. Multiplicity of M_r's has also been observed in PPOs from avocado, potato tubers, and banana (Mayer and Harel, 1979). An association−dissociation phenomenon was observed when the predominant form of pineapple fruit PPO (F_2) was exposed to solutions of varying ionic strength or denaturing solvents (Figure 4). The equilibrium could be shifted toward association by decreasing the ionic strength, whereas dissociation was facilitated by increasing ionic strength. The presence of SDS (1%) caused complete dissociation of the tetramer. Harel *et al.* (1973) observed that the predominant form of grape PPO (M_r 55−59 kDa) undergoes dissociation upon storage or when exposed to acid pH and urea. A concentration dependent association−dissociation equilibrium of mushroom PPO has been reported by Jolley *et al.* (1969).

Amino Acid Composition. The amino acid composition of the pineapple fruit PPO (F_2) showed the presence of Asp (8 mol %), Glu (16 mol %), Ser (12 mol %), Gly (14 mol %), His (2 mol %), Arg (6 mol %), Thr (7 mol %), Ala (5 mol %), Pro (2 mol %), Tyr (3 mol %), Val

Table 1. Purification of Pineapple Fruit Polyphenol Oxidase

purification step	vol (mL)	activity (units/mL)	total activity (units)	protein (mg/mL)	spec activity (units/mg of protein)	recovery (%)	purifn (fold)
dialyzed crude extract	100	224	22 400	0.309	725	100	
30−80% $(NH_4)_2$ SO_4	35	486	17 010	0.246	1976	76	3
Phenyl Sepharose CL-4B fraction							
F_1	25	102	2550	0.015	6800	11	9
F_2	20	501	10 020	0.028	17 893	45	25
F_3	15	213	3195	0.048	4438	14	6

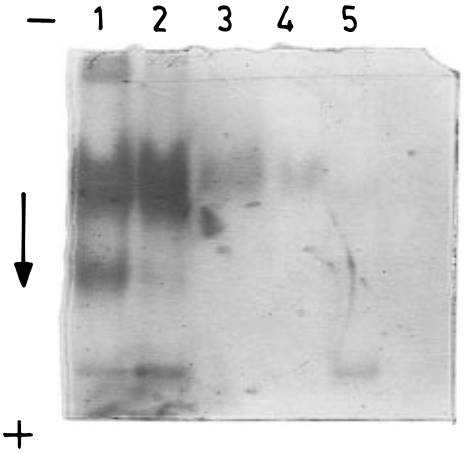

Figure 2. PAGE (7.5% T, 2.7% C) of pineapple fruit PPO. The gels were stained for PPO activity: (lane 1) dialyzed crude extract; (lane 2) $(NH_4)_2SO_4$ fraction; (lane 3) F_2; (lane 4) F_1; (lane 5) F_3 fraction from Phenyl Sepharose CL-4B chromatography.

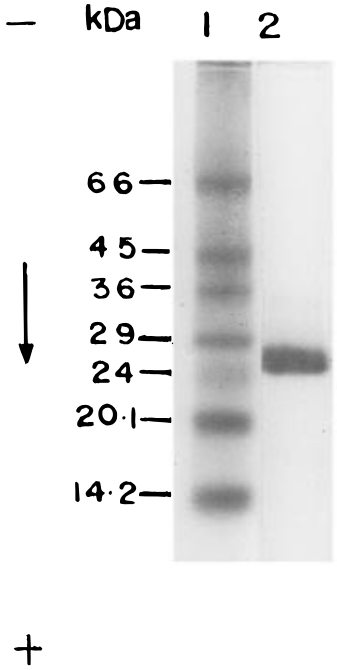

Figure 3. SDS-PAGE (10% T, 2.7% C) of pineapple fruit PPO: (lane 1) molecular weight standards and (lane 2) F_2 fraction from Phenyl Sepharose CL-4B chromatography.

(5 mol %), Met (1 mol %), Cys (1 mol %), Ile (4 mol %), Leu (6 mol %), Phe (4 mol %), and Lys (4 mol %). The amino acid composition of the pineapple fruit PPO (F_2) when compared with that of grape (Kidren *et al*., 1977) shows considerable similarities. The similarities include the high content of glutamic acid, serine, glycine, alanine, threonine, and isoleucine and the relatively low content of sulfur-containing amino acids cysteine and methionine. Cysteine was present in enzymes from

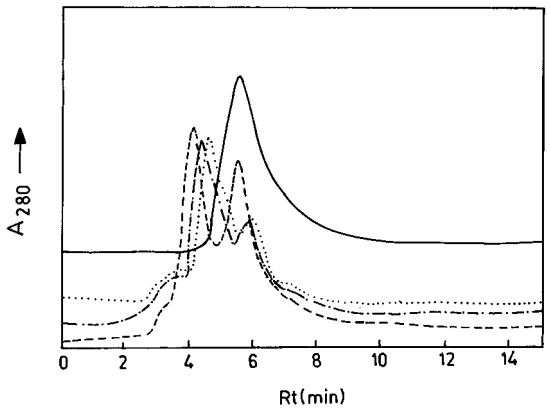

Figure 4. HPLC (gel filtration) profile of F_2 fraction from pineapple fruit PPO on a Zorbax G-250 column: (—) in sodium phosphate buffer, pH 7, 0.2 M containing 1% SDS and 1% β-mercaptoethanol; (· · ·) in 0.1 M; (— · —) in 0.05 M; and (- - -) in 0.01 M.

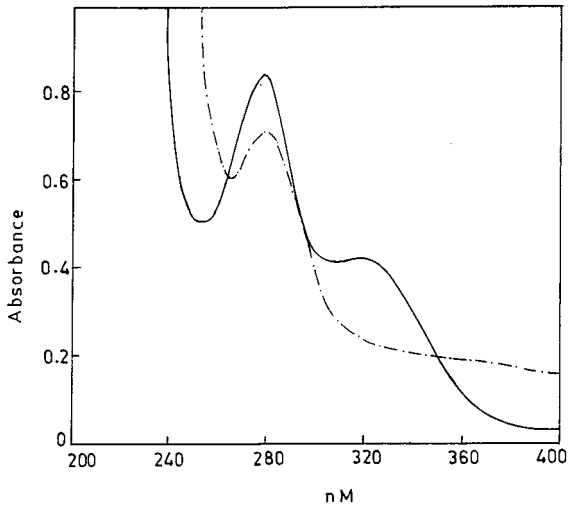

Figure 5. UV−vis absorption spectra of pineapple fruit PPO: (—) fraction F_2 from Phenyl Sepharose CL-4B chromatography; (— · —) fraction F_2, TCA-precipitated protein.

grape (Kidren *et al*., 1977) and potato tuber (Balasinghan and Ferdinand, 1970).

UV−Vis Absorption Spectra. The UV−vis absorption spectrum of the purified enzyme fraction (F_2) had a peak of maximum absorption at 278.5 nm and a shoulder at 320 nm, which is not characteristic of a protein spectrum (Figure 5). Absorption between 330 and 350 nm has been reported for banana, apple chloroplast, potato, and coffee PPO (Mayer and Harel, 1979; Galeazzi *et al*., 1981). However, when the purified pineapple enzyme was precipitated with trichloroacetic acid, the shoulder at 320 nm disappeared (Figure 5). This shoulder could be due to bound endogenous polyphenols/polyphenolic acids, which show an absorption maxima between 300 and 350 nm. Galeazzi *et al*. (1981) identified the bound phenolic compound of banana PPO to be chlorogenic acid. The $E^{1\%}_{1cm}$ of the precipitated

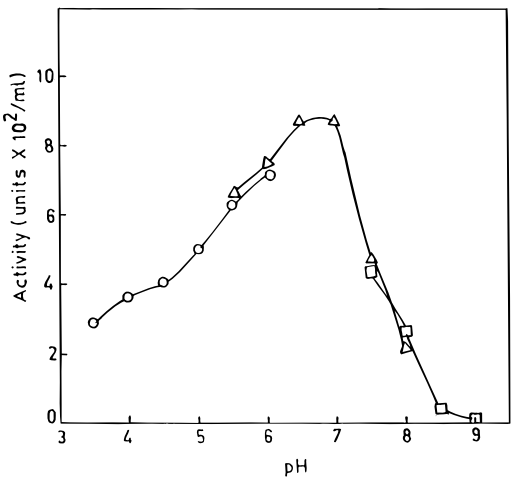

Figure 6. Effect of pH on pineapple fruit PPO activity: (○) sodium acetate buffer (0.01 M, pH 3.5−6); (△) sodium phosphate buffer (0.01 M, pH 5.5−8.0); and (□) Tris- HCl (0.01 M, pH 7.5−9).

Table 2. Effect of Various Substrates on Purified Pineapple Fruit Polyphenol Oxidase

substrate	wavelength (nm)	spec activity (units/mg of protein)	rel activity[a]
catechol	420	17 643	100
L-DOPA	480	6500	37
catechin	420	4464	25
chlorogenic acid	340	2000	11
gallic acid	420	0	0
p-coumaric acid	420	0	0
L-tyrosine	480	0	0

[a] Relative activity calculated taking catechol to be 100%.

protein at 280 nm was found to be 18.0, indicating the presence of aromatic amino acids in the purified protein.

Effect of pH. The pH activity profile for the oxidation of catechol by purified PPO (F_2) is shown in Figure 6. A broad optimum pH of 6−7 was found for the enzyme. The pH curve is characterized by a rapid loss of activity below pH 5 and above pH 7. Differences in optimum pH with several substrates have been reported for PPO from strawberries (Wesche-Ebeling and Montgomery, 1990) and other sources (Mayer and Harel, 1979). Aylward and Haisman (1969) reported that the optimum pH for maximum activity of PPO varies from about 4 to 7 depending upon the extraction methods, substrates, and the localization of the enzyme in the cell.

Substrate Specificity. PPO (F_2) activities using various substrates are shown in Table 2. Maximum activity was found when catechol was used as the substrate. Relative activities of PPO (F_2) measured at the absorption maximum of each product were calculated using catechol for comparison. The results indicate that pineapple PPO was more active toward diphenols. Relatively no activity toward monophenols was detected, suggesting the absence of cresolase activity in pineapple PPO. Low activities were measured for chlorogenic acid (11%) and catechin (25%). Wesche-Ebeling and Montgomery (1990) reported 11% of the maximum activity for chlorogenic acid in strawberries, but a very high (100%) activity for D-catechin. The substrate specificity of the fungal and plant PPOs are wide and varied when compared to the oxidase from animal tissue, where the specificity for optical isomers also is clear-cut (Mayer and Harel, 1979). The K_m for catechol calculated from a Lineweaver−Burk plot (data not shown) was 1.1×10^{-5} M. The K_m is similar to that reported for apple peel (Zhou *et al*., 1993) and twice that reported for apple flesh (Harel *et al*., 1965; Janovitz-Klapp *et al*., 1989) but lower than that reported for banana PPO (Galeazzi and Sgarbieri, 1981).

Table 3. Effect of Various Inhibitors on Purified Pineapple Fruit Polyphenol Oxidase

inhibitor	concn (mM)	lag phase (min)	inhibition (%)
	0.5	0.8	15
ascorbic acid	1.0	1.5	64
	1.5	2.5	97
L-cysteine	0.5	0.6	20
	1.0	1.5	60
	1.5	2.0	87
	1.0	0.6	18
potassium metabisulfite	2.0	1.4	55
	3.0	2.2	88
cinnamic acid	1.0	0	60
	3.0	0	85
resorcinol	1.0	0	52
	3.0	0	75
EDTA	1.0	0	45
	3.0	0	75

Effect of Inhibitors. The effect of various inhibitors on the purified PPO activity is shown in Table 3. Among these inhibitors, ascorbic acid was the most effective inhibitor of purified PPO (F_2) followed by L-cysteine and potassium metabisulfite. The lag period observed increased as the inhibitor concentration increased. This phenomenon has also been observed for PPO from banana (Galeazzi and Sgarbieri, 1981). The inhibitors' reaction mechanisms differ depending on the reducing agent used. The inhibition by thiol compounds is due to an addition reaction with the quinones, resulting in stable colorless products (Sanada *et al*., 1972; Janovitz-Klapp *et al.*, 1990) and/or binding to the active center of the enzyme as in the case of metabisulfite (Valero *et al*., 1992). Ascorbate reduces the initial quinone formed by the enzyme to the original diphenol, before it undergoes secondary reactions which lead to browning (Matheis and Whitaker, 1984). The apparent K_i for ascorbic acid and potassium metabisulfite were 1.8×10^{-5} and 4.3×10^{-5} M, respectively. Golan-Goldhirsh and Whitaker (1984) have reported ascorbic acid to cause irreversible inhibition of mushroom PPO.

CONCLUSION

PPO from pineapple fruit was purified 25-fold, to apparent homogeneity, adopting a four-step purification procedure. The enzyme is a tetramer of ≈104 kDa molecular mass. The optimum pH ranges between 6 and 7. The enzyme has no cresolase activity. Among the substrates used, the enzyme shows maximum activity toward catechol. The properties of the enzyme closely resemble PPOs from other fruits such as apple, grape, and banana.

ABBREVIATIONS USED

L-DOPA, L-3,4-dihydroxyphenylalanine; PPO, polyphenol oxidase; PVPP, polyvinylpolypyrrolidone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; NaPi, sodium phosphate.

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