



ISOLATION AND CHARACTERIZATION OF A MUNG BEAN LEAF POLYPHENOL OXIDASE

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Abstract—Crude extracts from mung bean leaves, roots, stems and dark grown seedlings contained multiple forms of polyphenol oxidase (PPO) with subunit M_r values of 65×10^3 , 59×10^3 , 52×10^3 , 47×10^3 , 31×10^3 and 21×10^3 . Only two of these forms were present in broken and intact leaf chloroplasts. A mature form of PPO, purified to apparent homogeneity from leaf chloroplasts, contained two proteins with subunit M_r values of 65×10^3 and 59×10^3 , respectively. The purified enzyme had a pH optimum of 6.0, a pI of 5.1, and oxidized a variety of *o*-diphenols. The K_m for L-dopa was 24 mM. *o*-Diphenol oxidation was inhibited by salicyl hydroxamic acid and 2,3-naphthalenediol, whereas methimazole, tropolone and 4-hexylresorcinol only resulted in partial inhibition. © 1997 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Polyphenol oxidase (EC 1.10.3.1: PPO) is found in many plants and fungi [1–6]. In higher plants, the enzyme has been localized to the thylakoid membranes of chloroplasts or other plastid organelles by cytochemical and immunochemical localization [3–6]. In its active form, PPO catalyzes the hydroxylation of monophenols to *o*-diphenols (cresolase/monophenolase oxidase activity) and the oxidation of *o*-diphenols to *o*-diquinones (catecholase/*o*-diphenolase activity). The enzyme can also exist in a partially or completely latent form, depending upon the plant or fungal species.

The mature form of PPO has been purified to apparent homogeneity from only a few sources. Mature PPO, with subunit M_r values ranging from 59×10^3 to 68×10^3 , has been isolated from apples [7, 8], broad beans [9, 10], carrots [11], potato trichomes [12], and potato tubers [13]. Similar subunit M_r values (57×10^3 to 64×10^3) have been proposed for the mature PPO, based on the sequence of PPO genes [14–22]. PPO has also been purified to homogeneity from edible Burdock [23], grapes [24], *Mucuna* [25], mung bean seedlings [26], spinach [27], and sunflower seeds [28], with subunit M_r values smaller than the 59×10^3 to 68×10^3 of mature forms. Little information was available on the subunit M_r of PPO purified to apparent homo-

geneity from lettuce [29], pears [30], and yams [31].

Mature forms of apple, carrot, and broad bean PPO have been shown to be susceptible to proteolysis, generating a proteolysed form having a M_r 40×10^3 to 45×10^3 [8–10, 32]. In addition, proteolysis of the major forms of PPO (60×10^3 to 63×10^3) found in peaches, apricots, almonds, plums, and cherries generated a proteinase-resistant form with an M_r of approximately 43×10^3 [33]. Fraignier *et al.* [33] also suggested that some of the observable multiple forms of PPO were a result of this proteolysis. This proteolysis has been shown to occur at the carboxy terminal end of PPO, to yield polypeptides of 40×10^3 to 45×10^3 and 15×10^3 to 20×10^3 in size [10, 19]. Thus, lower M_r forms of PPO may arise from proteolysis.

Mung bean PPO has not been isolated or characterized in a mature form with subunit M_r values (59×10^3 to 68×10^3) consistent with other purified plant PPOs. Polyphenol oxidase activity has been observed in hypocotyl cell walls of mung beans, but the enzyme was suggested to be more related to laccase than PPO [34, 35]. A PPO isolated from dark-grown mung bean seedlings by Takeuchi *et al.* [26] has also been characterized with regard to its M_r , pI, isoforms, and kinetic properties. Even though PPO appears to be very active and abundant in mung bean leaves [36, 37], it has not been characterized in detail. In the present study, we report the first isolation and characterization of a mature form of PPO from mung bean leaves, and show that it is dissimilar in many respects to the PPO isolated from dark-grown mung seedlings.

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RESULTS AND DISCUSSION

Crude homogenates were prepared from leaves, roots, and stems of mung beans grown under greenhouse conditions, as well as dark-grown mung bean seedlings. PPO activity was determined in these homogenates using enzyme assays without sodium dodecyl sulfate (SDS), since different extracts were not activated by SDS to the same extent (data not shown). Under these assay conditions, leaf homogenates contained the greatest activity per gram fresh weight (250 units g^{-1}) followed by roots, stems, and dark-grown seedlings with 15, 14, and 9 units g^{-1} fresh weight, respectively.

Samples from extracts of leaves, roots, stems, dark-grown seedlings, ruptured mung bean chloroplasts (S1 fractions), and intact mung bean chloroplasts (S3 fractions) were subjected to partially denaturing SDS polyacrylamide gel electrophoresis (PAGE) to detect active PPO isoforms [36]. S1, S3, leaf, stem, and dark-grown seedling samples showed a major PPO isoform in common after electrophoresis and enzyme staining with L-dopa (Fig. 1A). Only small amounts of PPO were detected in roots and stems (lanes 4 and 5). S3 fractions (lane 2) from intact chloroplasts contained a minor form with slightly slower mobility. Root and dark-grown mung bean seedling fractions (lanes 4 and 6) showed a minor PPO form with much slower mobility under these conditions. The number of active enzyme forms is similar to that reported by Vaughn and Duke [6], Angleton and Flurkey [36] and Lanker *et al.* [37].

Similar amounts of protein from each homogenate were subjected to denaturing SDS-PAGE and stained for protein (Fig. 1B). These same proteins were transferred to polyvinylidene difluoride (PVDF) membranes and subjected to Western blotting (Fig. 1C). All homogenates contained two PPO isoforms, with M_r values of 65×10^3 and 59×10^3 , in common. Crude extracts from leaves, roots, stems, and dark-grown mung bean seedlings also contained another major isoform with a M_r of 52×10^3 . This form was not present in S1 and S3 homogenates (lanes 1 and 2). Faint bands of immunological cross-reacting proteins were present in S1 extracts (M_r 37×10^3), leaf extracts (M_r 37×10^3 , 31×10^3 , and 21×10^3), root extracts (M_r 37×10^3), stem extracts (M_r 47×10^3 , 31×10^3 , and 21×10^3) and dark-grown seedling extracts (M_r 47×10^3 and 31×10^3). Another faint band at M_r 45×10^3 was observed in root and dark-grown seedling extracts. The presence of more immunological cross-reacting PPO isoforms in crude tissue extracts compared to S3 (intact chloroplast) extracts suggests the generation of 'artifactual' PPO isoforms, even when extracts are prepared in the presence of ascorbate and protease inhibitors. Based on the greater activity per gram fresh weight and the fewer number of PPO forms identified after Western blotting, we attempted to purify PPO from intact and ruptured mung bean leaf chloroplasts.

PPO was purified to apparent homogeneity from supernatants of ruptured chloroplasts (S1) and from intact chloroplasts (S3 supernatants). A large amount of PPO activity was present in the S1 supernatant, indicating breakage of the chloroplasts during initial homogenization (Table 1). Varying the type of buffer, pH, osmotic medium, and temperature had little effect on decreasing the amount of PPO released into the S1 supernatant or increasing the yield of intact chloroplasts (data not shown). Enzyme obtained from ruptured chloroplasts was isolated in very low yields (1%). Most of the enzyme loss occurred during the two ion-exchange steps. Further purification by size exclusion chromatography resulted in low yields, decreased the specific activity, and resulted in apparent degradation of the PPO, as evidenced by lower molecular weight forms after Western blotting.

Using intact chloroplasts as starting material, higher yields of PPO were recovered starting from the S3 supernatant, but the overall yield from the crude homogenate was still very low (Table 1). Much of the activity was lost during the ion-exchange steps. Even though other proteins were separated from PPO during hydroxyapatite chromatography, the specific activity of the enzyme decreased. Based on protein assays, approximately 80 μg of PPO was isolated. Electrophoresis followed by protein staining indicated yields to be much lower and of the order of 10–20 μg per 50 g of leaves. The latter estimates were similar to that reported for mung bean seedling PPO [26]. The PPO isolated from intact chloroplasts also lost activity rapidly during isolation and storage (Table 1, data not shown).

Native isoelectric focusing of the S3 PPO showed PPO had a pI of 5.1 (data not shown). This pI is slightly lower than that reported by Takeuchi *et al.* [26] for mung bean seedling PPO. Purified broad bean PPO has been shown to have multiple isoelectric points having pI values in the range 5–7 [9].

Denaturing SDS-PAGE, followed by Western blotting, was used to identify PPO in crude homogenates and at each step in the purification schemes for PPO isolated from S1 (ruptured chloroplasts) and S3 (intact chloroplast extracts). The apparent subunit M_r of the major PPO was estimated to be approximately 65×10^3 . A minor band having an M_r of 59×10^3 was also present in each sample. Estimations of the M_r values these forms were based on nonprestained markers. Prestained M_r markers were used to indicate approximate size and transfer efficiency, but not subunit M_r (Fig. 2). These PPO forms were similar in size to those reported for broad bean, tomato, potato, carrot, and apple PPO [7–13], but were much larger than the M_r of 40×10^3 reported for mung bean seedling PPO [26]. SDS-PAGE followed by protein staining of the enzyme isolated from S3 supernatants showed proteins with subunit sizes of M_r 65×10^3 and 59×10^3 . The distribution of protein staining was similar to that observed for Western blotting (Fig. 2, lane f, data not shown). We also observed lower M_r forms

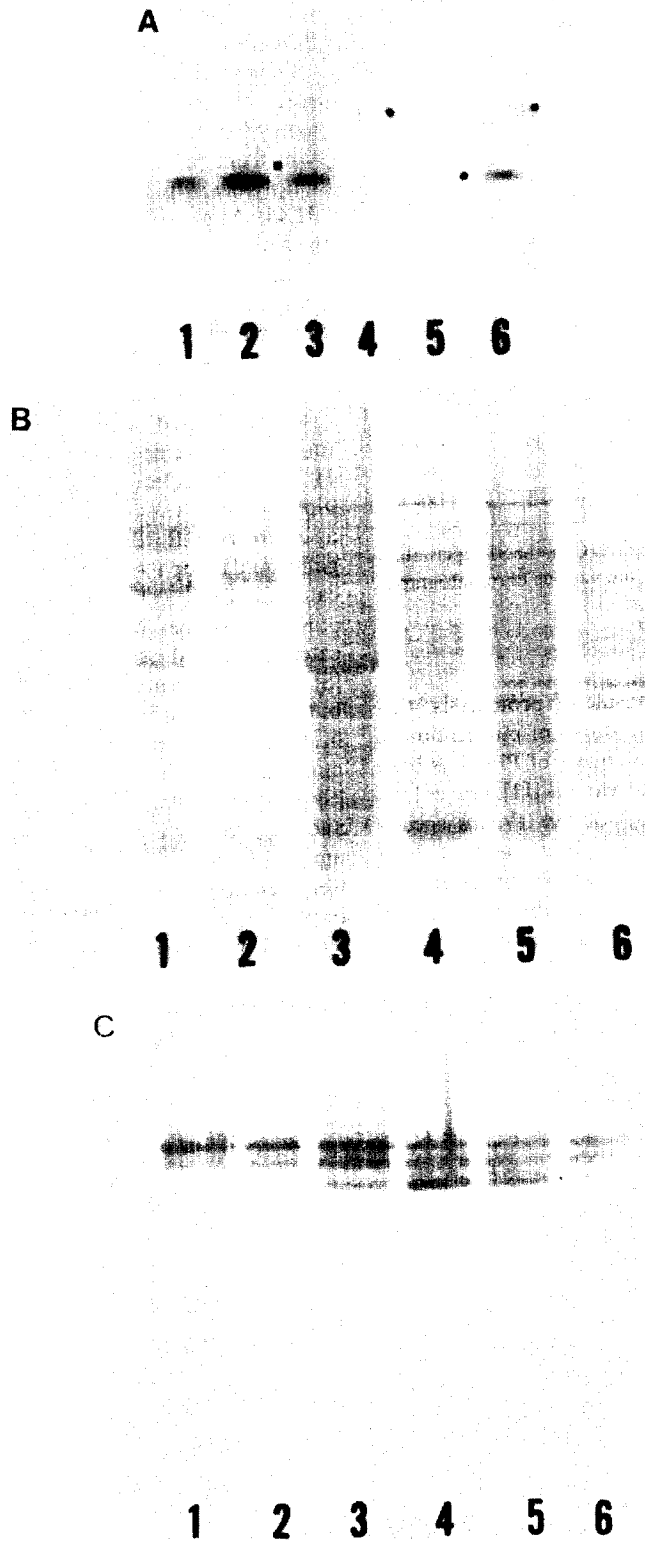


Fig. 1. (A) PPO isoforms detected after partially denaturing SDS-PAGE and staining with L-dopa. Lanes: (1) S1 supernatant, 0.1 units; (2) S3 supernatant, 0.1 units; (3) leaf homogenate, 0.1 units; (4) root homogenate, <0.01 units; (5) stem homogenate, 0.01 units; (6) dark-grown seedling homogenate, 0.1 units. Faint bands of staining are located by a dot. (B) Protein stained gel of mung bean samples. Approximately equal amounts of protein from each sample were subjected to denaturing SDS-PAGE and stained with Coomassie Blue R-250. Lanes are numbered and designated as is (A). Molecular weight markers in the far left lane are (top to bottom, $\times 10^3$: 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31, 21.5, 14.4, 6. (C) Western blot of mung bean samples. Proteins in gel (B) were transferred onto PVDF membranes, destained, and probed with anti-broad-bean PPO. Lane designations are the same as in (B).

Table 1. Purification of PPO from ruptured chloroplasts (S1) and intact chloroplasts (S3)

Step*	Protein (mg)	Enzyme activity (units)	Yield (%)	Specific activity (units mg ⁻¹)
S1 purification				
CE	2500	1300		
S1	1900	1000	100	0.53
DEAE	200	420	42	2.1
PS	19	370	37	19.5
HA	0.5	50	5	100
G-150	0.4	12	1	30
S3 purification				
Ce	2600	1300		
S3	320	59	100	0.18
DEAE	0.11	32	55	290
HA	0.08	15	25	188

* Samples from the crude extract (CE), the S1 supernatant, and combined fractions from the various chromatographic columns.

of PPO, presumably the result of proteolysis, that were generated during some stages of purification of enzyme (Fig. 2, lane j). Proteolysis of PPO has been observed in broad beans [10], carrots [11], grapes [38], apples [32] and in the fruits of five *Prunus* species (peach, apricot, almond, plum, and cherry) [33] in which the mature polypeptide can be converted into active fragments with M_r values of about 40×10^3 to 45×10^3 and smaller in size. Mung bean leaf PPO, therefore, appears to be similar to other plant PPO

with respect to its mature size and susceptibility to proteolysis.

The pH optimum for mung leaf PPO purified from intact chloroplasts was determined to be approximately 6.0, although the activity was practically constant from pH 5.5 to 6.5. The activity dropped off sharply below pH 5.0 and no activity was present at pH 4.0. At pH 8.5, the enzyme displayed only 40% of the activity.

Takeuchi *et al.* [26] reported that PPO purified from mung bean seedlings contained both monophenolase and diphenolase activities. Duke and Vaughn [39] also showed that mung bean PPO could hydroxylate *p*-coumaric acid. The mung bean leaf PPO isolated in this study lacked monophenolase activity, although this could have been lost during purification (Table 1). Mung bean leaf PPO did oxidize a variety of *o*-diphenols and was most active using 4-methylcatechol as a substrate. Rates of oxidation for catechin, pyrogallol, and L-dopa were much lower than for 4-methylcatechol (Table 2).

Kinetic studies were carried out using 4-methylcatechol and L-dopa as substrates. A plot of v versus $[S]$ for 4-methylcatechol appeared to be hyperbolic, but a similar plot for dopa appeared to be sigmoidal in shape. Apparent K_m and V_{max} constants were determined using nonlinear regression analysis of v versus $[S]$ plots using Enzyplot software. The apparent K_m and V_{max} for 4-methylcatechol were 4 mM and 0.11 units min⁻¹, respectively. The K_m and V_{max} for L-dopa were determined to be 24 mM and 0.13 units min⁻¹, respectively. The K_m for L-dopa is similar to that reported by Takeuchi *et al.* [26], but the V_{max} differs approximately 10-fold. This difference might be due

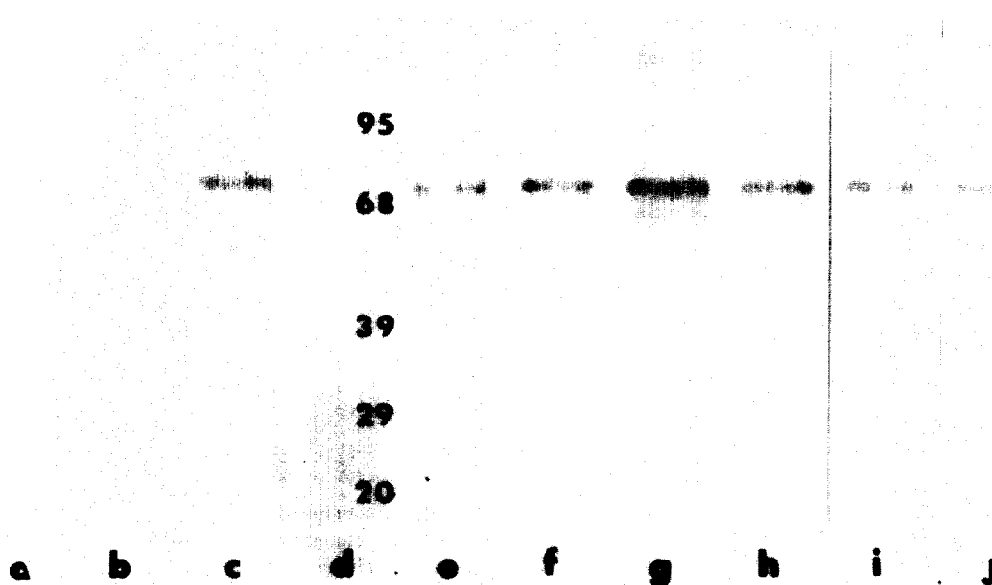


Fig. 2. SDS-PAGE and Western blotting of samples from the purification of PPO from S1 and S3 supernatants. Lanes: (a) crude extract; (b) supernatant; (c) S3 supernatant; (d) prestained M_r markers; (e) S3, PPO fractions from the DEAE column; (f) S3, PPO fractions from the HA column; (g) S1, PPO fractions from the DEAE column; (h) S1, PPO fractions from the PS column; (i) S1, PPO fractions from the HA column; (j) S1, PPO fractions from the G-150 column.

Table 2. Substrate specificity of PPO*

Substrate	[S] (mM)	Activity (units)	Activity relative to catechol (%)
4-Methylcatechol	5	0.063	208
Chlorogenic acid	5	0.042	140
Catechol	5	0.030	100
Catechin	5	0.012	40
L-Dopa	5	0.012	40
Pyrogallol	5	0.011	37

* All assays were carried out as described in the Experimental section.

Table 3. Effect of various inhibitors on mung leaf PPO*

Inhibitor	Activity (units)	Inhibition (%)
None	0.043	0
SHAM	0	100
Naphthalenediol	0.013	70
Kojic acid	0.017	60
Methimazole	0.020	53
Tropolone	0.025	42
4-Hexylresorcinol	0.032	25

* PPO was assayed as described in the Experimental section, using 10 mM 4-catechol and 200 μ M of each inhibitor.

to altered kinetic characteristics of intact PPO versus proteolyzed PPO, since Marques *et al.* [32] demonstrated altered biochemical properties of PPO associated with proteolysis. We also attempted to determine the K_m using 3-methyl-2-benzothiazolinone hydrazone (MBTH) to monitor the quinones that were formed when either dopa or 4-methylcatechol was used as substrate [40]. Under these conditions, an apparent K_m of 16 mM was obtained for L-dopa, and an apparent K_m of 3 mM was obtained for 4-methylcatechol. The estimations for the K_m of L-dopa appear higher than those reported for other plant PPO.

Oxidation of 4-methylcatechol by mung bean leaf PPO was completely inhibited by 200 μ M salicyl hydroxamic acid (SHAM) (Table 3). Oxidation of this substrate by PPO was also inhibited by 2,3-naphthalenediol, kojic acid, methimazole, tropolone, and 4-hexylresorcinol, but to a lesser extent. In fact, inhibition of the enzyme by methimazole, tropolone, and 4-hexylresorcinol was much lower than expected considering the potency of these inhibitors with respect to the enzyme from other sources.

The PPO isolated and purified from mung bean leaf chloroplasts is different from that reported by Takeuchi *et al.* [26] for mung bean seedlings. The enzymes differ in subunit size, *pI*, substrate specificity,

and catalytic activity. They are, however, similar in some of their kinetic properties. Because mung bean leaves and dark-grown seedlings contain similar PPO forms (Fig. 1A and C), the difference in properties reported by Takeuchi *et al.* [26] for mung bean seedling PPO and those in this report for mung leaf PPO may have resulted from changes in PPO during isolation of the enzyme. However, the subunit size of mung bean leaf PPO isolated in this study is similar to that of other plant PPOs, suggesting that the PPO isolated by Takeuchi *et al.* [26] may have been a proteolyzed form of PPO. If this is the case it would suggest that the intact mung bean PPO has different properties relative to the proteolyzed form of PPO.

EXPERIMENTAL

Crude extracts. Crude extracts from leaves, stems, roots, and dark-grown seedlings (whole plants) were prepared as rapidly as possible by blending tissues in 10 mM Pi (pH 6.0) containing 1 mM *p*-aminobenzamidine, 1 mM ϵ -aminocaproic acid, 1 mM PMSF, and 5 mM ascorbate. The homogenates were filtered through cheesecloth and centrifuged at 9000 *g* for 20 min. Portions from each of the supernatants were assayed for PPO activity. Portions from each supernatant were also precipitated with 10% trichloroacetic acid, washed with acetone, and subjected to denaturing SDS-PAGE and Western blotting.

Isolation of PPO. Leaves (50 g) from 10-day-old mung bean plants were homogenized in 20 mM Tris (7.5), 100 mM KCl, 10 mM MgCl₂, 200 mM mannitol or sucrose, and 5 mM ascorbate using 5 $\times$ 3 sec bursts in a Waring blender. The homogenate was filtered through two layers of cheesecloth and 2 layers of Miracloth and then centrifuged at 6000 *g* for 5 min at 4 $^{\circ}$ C to pellet chloroplasts. The supernatant was labelled S1. The pellet was washed once with the homogenization buffer and centrifuged as above. The pellet was blended in 10 mM NaPi (6.0) and centrifuged at 20 000 *g* for 20 min at 4 $^{\circ}$ C. The supernatant was labelled S3.

PPO was isolated from lysed and broken chloroplasts (S1 supernatants). Ascorbic acid and PMSF were added to S1 at final concentrations of 5 mM and 1 mM, respectively. One milligram of pepstatin A, antipain, and leupeptin were added to the S1 supernatant along with 0.2 mg of bestatin, 20 mg of ϵ -aminocaproic acid, and 20 mg of *p*-aminobenzamidine [9]. DEAE cellulose was added to S1 until most of the color in the supernatant was adsorbed onto the resin. The slurry was then filtered and the cake washed with 10 mM NaPi (6.0), 100 mM NaPi (6.0), and 500 mM NaPi (6.0). Most of the enzyme was found in the 500 mM wash and this fraction was concentrated by ultrafiltration to 10 ml. The concentrate was brought to 25% saturation of (NH₄)₂SO₄ and applied to a 1 $\times$ 3 cm Phenyl Sepharose column equilibrated in 50 mM NaPi (6.0) and 25% saturated (NH₄)₂SO₄. The column was washed with buffer and the enzyme eluted with

water. The eluted enzyme was pooled and concentrated by ultrafiltration to 2 ml and then dialysed against 5 mM NaPi (6.0). The dialysate was applied onto a 1 × 2 cm hydroxyapatite (HA) column equilibrated in 5 mM NaPi (6.0). The HA column was washed with buffer followed by 50 ml of a 10–300 mM NaPi gradient. Fractions containing the bulk of the PPO activity were pooled and concentrated by ultrafiltration. The concentrate was applied to a 1 × 60 cm Sephadex G-150 column equilibrated in 100 mM NaPi (6.0) and eluted with buffer. Active fractions were pooled and concentrated by ultrafiltration.

PPO was isolated from intact chloroplasts, the S3 supernatant, using ion exchange on DEAE and HA columns. The S3 supernatant was passed through a 3 × 20 cm column of DEAE cellulose equilibrated in 10 mM NaPi (6.0). The column was washed with buffer followed by 100 mM and 500 mM NaPi (6.0) washes. The 100 mM wash was concentrated and dialysed against 10 mM NaPi (6.0). The dialysate was applied to a 1 × 2 cm HA column equilibrated in 10 mM NaPi (6.0). The column was washed with buffer followed by 50 ml of a 10–300 mM NaPi gradient. Active PPO fractions were pooled and concentrated.

Enzyme and protein assays. PPO activity was monitored by following the oxidation of L-dopa (5 mM) or 4-methylcatechol (5 mM) in 100 mM NaPi (6.0) at 475 or 410 nm, respectively. The total assay volume was 3 ml. The linear portion of the absorbance vs time curve was used to determine the initial rates. One unit of enzyme activity was defined as a change in one absorbance unit min^{-1} at 475 or 410 nm. Protein content was determined using the Lowry method [41].

Electrophoresis, isoelectric focusing and Western blotting. Electrophoresis under native, partially denaturing SDS-PAGE, and denaturing SDS-PAGE was carried out as described previously [9, 36, 37]. Active enzyme was located by incubating the gels in 100 mM NaPi (6.0) for 10 min followed by incubation in buffer containing 5 mM L-dopa. Patterns of enzyme staining were recorded using Kodak EDF duplicating film. Western blotting, using a polyclonal anti-broad bean PPO as a probe, was performed as described in [9, 37], and isoelectric focusing was carried out using precast Agarose IEF plates (pH 3–10) at 10° [9]. After focusing was complete, gels were incubated in 100 mM NaPi (pH 6.0) containing 5 mM L-dopa. Orange colored bands were marked with ink. The pH gradient was determined by incubating 1-cm slices of the agarose gels in 2 ml of distilled water and measuring the pH 2 hr later. The pH gradient was also determined using protein pI markers (BioRad Laboratories, Richmond, CA; Pharmacia Biotech, Piscataway, NJ).

Substrate specificity. The following substrates were used to monitor PPO activity spectrophotometrically: 4-methylcatechol (410 nm), catechol (410 nm), chlorogenic acid (400 nm), catechin (380 nm), pyrogallol (400 nm), and L-dopa (475 nm). All substrates were used at a concentration of 5 mM in 100 mM NaPi (6.0). The slopes from the linear portion of each curve

were used to calculate initial rates. One unit of enzyme activity was defined as one absorbance change min^{-1} .

Kinetics. Mung PPO was assayed in varied concentrations of either 4-methylcatechol, catechol, or L-dopa. Oxidation reactions were monitored as described above and rates calculated from the linear portion of the absorbance vs time curves. Assays were also conducted using MBTH as described in [40]. Reaction rates were calculated from the linear portion of the absorbance (525 nm) vs time curves. All data were analyzed using the Enzpack 3 (Biosoft, U.S.A.) or Enzyplot software.

pH optimum. The pH optimum of mung bean PPO was determined using 100 mM buffers from pH 4 to 8.5 using 10 mM catechol as the substrate. Acetate buffers were used from pH 4 to 5.5 and, phosphate buffers from pH 6 to 8.5.

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