# Purification and Characterization of a Polyphenol Oxidase from the Seeds of Field Bean (*Dolichos lablab*)

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The polyphenol oxidase from field bean (*Dolichos lablab*) seeds has been purified to apparent homogeneity by a combination of ammonium sulfate precipitation, DEAE−Sephacel chromatography, phenyl agarose chromatography, and Sephadex G-200 gel filtration. The purified enzyme has a molecular weight of 120 ± 3 kDa and is a tetramer of 30 ± 1.5 kDa. Native polyacrylamide gel electrophoresis of the purified enzyme revealed the presence of a single isoform with an observed pH optimum of 4.0. 4-Methyl catechol is the best substrate, followed by catechol, and L-3,4-dihydroxyphenylalanine, all of which exhibited a phenomenon of inhibition by excess substrate. No activity was detected toward chlorogenic acid, catechin, caffeic acid, gallic acid, and monophenols. Tropolone, both a substrate analogue and metal chelator, proved to be the most effective competitive inhibitor with an apparent $K_i$ of $5.8 \times 10^{-7}$ M. Ascorbic acid, metabisulfite, and cysteine were also competitive inhibitors.

**Keywords:** *Polyphenol oxidase; field bean seed; Dolichos lablab; catecholase; tetramer; substrate inhibition; tropolone*

## INTRODUCTION

Polyphenol oxidase (EC. 1.10.3.1, PPO) is a widely distributed enzyme in the phylogenetic scale. PPO, a bifunctional copper-containing enzyme in the presence of molecular oxygen catalyses the hydroxylation of monophenols to *o*-diphenols (monophenolase or cresolase activity) and the further oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). The generated, unstable highly reactive *o*-quinones, subsequently react with themselves, amino acids, or proteins, evolving into brown, black, or red heterogeneous polymers responsible for quality loss in many foods (Matheis and Whitaker, 1984; Garcia-Carmona et al., 1988; Sanchez-Ferrer et al., 1995). The considerable economic and nutritional loss induced by enzymatic browning is of concern to food processors and researchers. Although numerous studies have been devoted to the biochemical and catalytic properties of PPO (Mayer and Harel, 1991; Zawistowski et al., 1991) and to the inhibition of PPO activity (Ferrar and Walker, 1996), the physiological function of PPO in plants remains obscure.

PPO has been studied in many fruits and vegetables including apple (Murata et al., 1992; Janovitz-Klapp et al., 1989), potato (Sanchez-Ferrer et al., 1993a, 1993b; Chen et al., 1992), pineapple (Das et al., 1997), grape (Sanchez-Ferrer et al., 1995; Wisseman and Lee, 1981), cabbage (Fujita et al., 1995), avocado (Kahn, 1976; Espin et al., 1997), oilbean (Chilaka et al., 1993), peaches (Luh and Philthakphol, 1972) and plantain (Ngalani, 1993). However, among the leguminous plants, reports are available only on the PPO of broad bean leaves (*Vicia faba* L) (Ganesa et al., 1992), mung bean leaf (*Vigna radiata*), and seedlings (*Vigna mungo*) (Shin et al., 1997; Takeuchi et al., 1992). In this laboratory during the affinity purification of a glucose−mannose-specific lectin from field bean (*Dolichos lablab*) seeds, severe browning of the extracts was observed. A similar browning reaction of the oilbean (*Pentaclethra macrophylla* Benth) seeds has been reported (Chilaka et al., 1993). Preliminary investigations in our laboratory revealed the presence of a single PPO, in crude extracts of field bean seeds. The purification of PPO from higher plants continues to be a problem compounded by the presence of multiple isoforms. The single form in field bean seeds is ideally suited for structural characterization and X-ray crystallography studies. As a primary step to understanding the structure, regulation, and function of seed PPO, we report here the isolation and characterization of a PPO from field bean seeds.

## MATERIALS AND METHODS

**Plant Materials and Reagents.** Field bean (*Dolichos lablab* var. lignosus) seeds were purchased from the local market. Catechol, 4-methyl catechol, L-3,4-dihydroxyphenylalanine (L-DOPA), chlorogenic acid, gallic acid, catechin, caffeic acid, *p*-phenylenediamine, polyvinylpolypyrrolidone (PVPP), tropolone, DEAE−Sephacel, phenyl agarose, and cysteine−HCl were obtained from Sigma Chemical Co., St. Louis, MO. Sephadex G-100 and gel filtration markers were from Pharmacia Fine Chemicals, Uppsala, Sweden. SDS−PAGE markers were from Bangalore Genei Pvt. Ltd., Bangalore, India. All other chemicals used were of analytical grade.

**Enzyme Extraction and Purification.** Defatted field bean powder (20 g) was extracted for 16 h at 4° C with 0.1 M Tris−HCl buffer, pH 7.0 (100 mL) containing 2% (w/v) PVPP and 1.2% (w/v) NaCl. The extract was centrifuged at 8500*g* for 30 min. To the supernatant, solid ammonium sulfate (22.6 g/100 mL) was added to obtain 40% saturation at 4 °C. The precipitated protein was removed by centrifugation at 8500*g* for 30 min and discarded. Solid ammonium sulfate (25.8 g/100 mL) was added slowly to the supernatant at 4 °C and allowed to stand overnight. The precipitate thus obtained was redissolved in 0.01 M Tris−HCl buffer, pH 8.2, containing 1.2%

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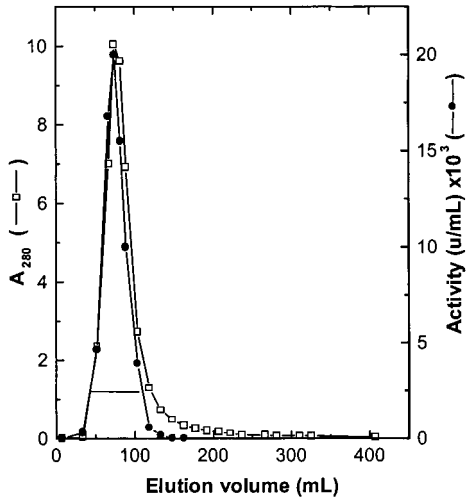


**Figure 1.** DEAE−Sephacel chromatography of field bean PPO. Elution buffer, 0.01 M Tris−HCl pH 8.2 containing 1.2% NaCl; flow rate, 35 mL/h. PPO activity peak was pooled as indicated (−).

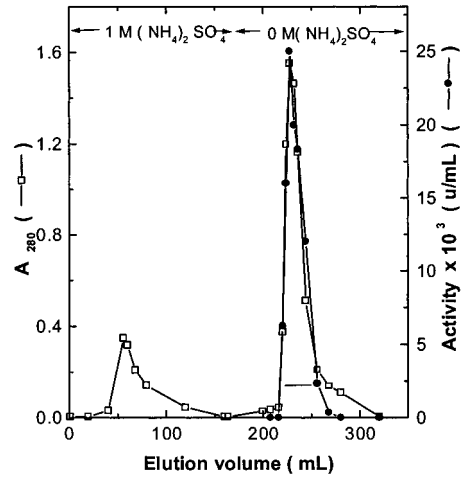


**Figure 2.** Phenyl agarose elution profile of field bean seed PPO. The PPO was eluted with 0.25 M Tris−HCl without $(NH_4)_2SO_4$. The active fractions were pooled as shown (−).

NaCl (w/v) and dialyzed against the same buffer (3 × 500 mL). The dialyzed solution was loaded onto a DEAE−Sephacel column (12 × 3.5 cm) previously equilibrated with the same buffer, and then the column was washed with the same buffer. The PPO activity was recovered as the unbound protein fraction in the buffer wash (Figure 1). The active fractions were pooled, the pH was adjusted to 7.0, and solid $(NH_4)_2SO_4$ was added to a final concentration of 1 M and loaded onto a phenyl agarose column (11.5 × 3.5 cm), equilibrated with 0.025 M Tris−HCl buffer, pH 7.0 containing 1 M $(NH_4)_2SO_4$ and 1.2% (w/v) NaCl. The column was washed with the same buffer to remove proteins unbound to the column. The bound PPO was eluted using the same buffer minus the $(NH_4)_2SO_4$. The PPO active fractions were pooled (Figure 2) and subjected to 0−80% $(NH_4)_2SO_4$ precipitation. The 80% precipitate was loaded onto a Sephadex G-100 column (100 × 2 cm), equilibrated in 0.025 M Tris−HCl buffer pH 7.0 containing 1.2% NaCl. The PPO active fractions pooled (Figure 3) and stored at 4 °C was used for further studies.

**Enzyme Assay.** PPO was assayed according to the spectrophotometric procedure of Cosetang and Lee (1987). The assay mixture consisted of 0.9 mL of 0.05 M Sodium acetate buffer, pH 4.0, 0.1 mL of 0.5 M catechol, and 10−100 $\mu$g of enzyme. The increase in absorbance at 420 nm was measured as a function of time for 3 min. 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.

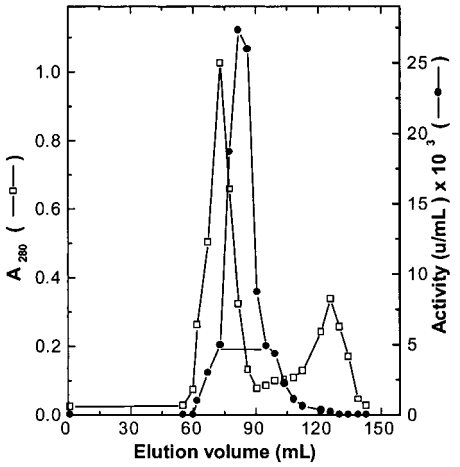


**Figure 3.** Sephadex G-100 chromatography elution profile of field bean seed PPO. The active fractions were pooled as shown (−).

**Protein Estimation.** Protein concentration was determined by the dye-binding method of Bradford (1976) and Zor and Selinger (1996). Bovine serum albumin was used as the standard.

**Polyacrylamide Gel Electrophoresis (PAGE).** Native PAGE (7.5% T, 2.7% C) was performed as described by Zhang and Flurkey (1997). Duplicate samples were run for simultaneous protein and enzyme staining. The gels were stained for protein with Coomassie Brilliant Blue R-250. The method of Lee (1991) using catechol and *p*-phenylenediamine was followed for PPO staining. SDS−PAGE of the purified PPO was carried out according to the method of Laemmli (1970).

**Capillary Electrophoresis.** Purified PPO was electrophoresed on a Prince Technologies capillary electrophoresis system with a coated capillary (Prince Technologies B. V., The Netherlands) (i.d. = 75 $\mu$m, length =100 cm), at 28 °C by applying a voltage of 30 kV. The running buffer was 0.025 M Tris−0.192 M glycine (pH 8.3) or 0.05 sodium acetate (pH 4.0). Prior to analyses, the capillary was flushed for 2 min with running buffer. The samples were injected at 20 mBar pressure for 10 s.

**Molecular Weight Determination.** The apparent molecular weight of the native enzyme was determined by HPLC equipped with a TSK G2000 SWXL, (7.8 mm i.d. × 30 cm) column. The eluent used was 0.1 M NaPi, pH 7.0, at the flow rate of 1 mL/min. The proteins were detected at 230 nm. The column was calibrated using thyroglobulin (660 kDa), ferritin (450 kDa), aldolase (158 kDa), bovine serum albumin (66.3 kDa), ovalbumin (43.5 kDa), $\beta$-lactoglobulin (36.8 kDa), carbonic anhydrase (25.0 kDa), myoglobin (16.9 kDa), and ribonuclease (13.7 kDa). The molecular weight was also determined using a calibrated Sephadex G-100 column according to the method of Andrews (1970).

**Amino Acid Composition and N-Terminal Sequence Analysis.** The purified protein was transferred from SDS−PAGE to polyvinylidene diflouride (PVDF) membrane in 10 mM CAPS−10% methanol buffer (pH 11) by electroblotting at 0.8 A/cm$^2$ of constant current for 1.5 h. The membranes were stained with Coomassie Blue R-250 and destained according to the method of Matsudaira (1989). The bands corresponding to field bean PPO were excised. The excised PPO band was washed with methanol and loaded directly to the automated gas-phase sequenator (Shimadzu PSQ1) for amino-terminal sequencing by automated Edman degradation.

The protein band was hydrolyzed in vacuo at 110 °C in constant boiling HCl for 24 h using the Pico Tag Workstation. Amino acid analysis was performed by precolumn derivatization using phenylisothiocyanate. The phenylthiocarbamoyl amino acids were analyzed by Rp HPLC (Bidlingmeyer et al., 1984).

**Effect of pH.** PPO activity as a function of pH was determined using 0.05 M of catechol, 0.05 M 4-methyl catechol,

**Table 1. Purification of Field Bean Seed Polyphenol Oxidase[a]**

| purification step | total activity (units) × $10^5$ | total protein (mg) | specific activity (U/mg) | yield (%) | fold purif. |
|---|---|---|---|---|---|
| crude extract | 24.3 | 1207 | 2 013 | | |
| $(NH_4)_2SO_4$ (40−80%) | 17.7 | 431 | 4 176 | 72.8 | 2.1 |
| DEAE−Sephacel chromatogr. | 15.6 | 135 | 11 556 | 64.2 | 5.7 |
| phenyl agarose chromatogr. | 10.9 | 36.5 | 29 863 | 45 | 14.8 |
| size exclusion chromatogr. (Sephadex G-100) | 7.4 | 10.87 | 68 077 | 30.5 | 33.8 |

[a] These are the results of a typical purification starting from 20 g of defatted field bean flour. These values were reproduced in five separate purifications.

and 0.01 M of DOPA as substrates. The buffers used were McIlvaines (citric acid−$Na_2HPO_4$, pH 2.5−7.5), glycine−HCl (pH 2.5−3.5), sodium acetate (pH 3.5−6.0) and sodium phosphate (pH 6.0−8.0) at 25 °C.

**Substrate Specificity.** Catechol, 4-methyl catechol, chlorogenic acid, DOPA, catechin, caffeic acid, gallic acid, *p*-cresol, and pyrogallol, at a final concentration of 0.01 M, were used to monitor PPO activity. The rate of the reaction was measured in terms of the increase in absorbance at the absorption maxima of the corresponding quinone products for each substrate at 25 °C (Zhou et al., 1993).

**Effect of inhibitors.** Tropolone (0.2−1 $\mu$M), potassium metabisulfite (2−10 $\mu$M), ascorbic acid (2−10 $\mu$M), and cysteine-HCl (5−50 mM) were evaluated for their effectiveness as inhibitors of PPO activity using catechol as the substrate.

## RESULTS AND DISCUSSION

**Purification and Extraction.** Enzyme purification in plant extracts is hampered by the presence of a large variety and quantity of secondary products that can bind tightly to the enzymes and change their native characteristics (Loomis, 1974). The use of acetone powders, ammonium sulfate fractionation, salts, insoluble polymers and detergents partially circumvent this problem. The purification method described here involves the use of an insoluble polymer PVPP, which can complex endogenous polyphenols and prevent the crude extract from browning at 4 °C.

The ammonium sulfate precipitate obtained between 40 and 80% saturation of the crude extract exhibited the maximum PPO activity. The field bean seed PPO was purified to apparent homogeneity after three steps of purification by column chromatography. The results of the purification are summarized in Table 1. Ion-exchange chromatography on DEAE−Sephacel was effective in removing inactive compounds. PPO eluted as a single peak unbound to the ion-exchange matrix, in the column wash (Figure 1). The PPO was further purified by hydrophobic interaction chromatography on phenyl agarose. Phenyl Sepharose CL 4B chromatography has been successfully used for purification of PPO from various plant sources (Wisseman and Lee, 1980; Janovitz-Klapp et al., 1989; Zhou et al., 1993; Das et al., 1997). In this step, an inactive protein fraction eluted in the void volume during the washing with equilibrating buffer. Protein was eluted with equilibrating buffer containing no ammonium sulfate. The elution profile of field bean PPO from phenyl agarose is presented in Figure 2. All of the PPO activity eluted as a single peak with a low-ionic-strength elution buffer, containing no ammonium sulfate, with a 3-fold increase in specific activity. The protein fractions containing PPO were appropriately combined and precipitated by 80% ammonium sulfate fractionation. Finally, size exclusion chromatography on Sephadex G-100 (Figure 3) resulted in an apparently homogeneous form of PPO. The recovery of PPO was ~30%, after a 34-fold purification, with a specific activity of 68 077 units/mg protein (Table 1).

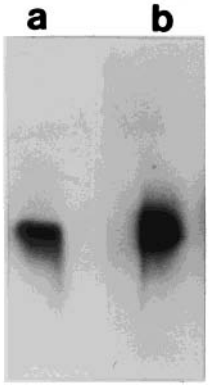


**Figure 4.** PAGE (7.5% T, 2.7% C) of field bean PPO. The gels were stained for protein (lane a) and for PPO activity (lane b).

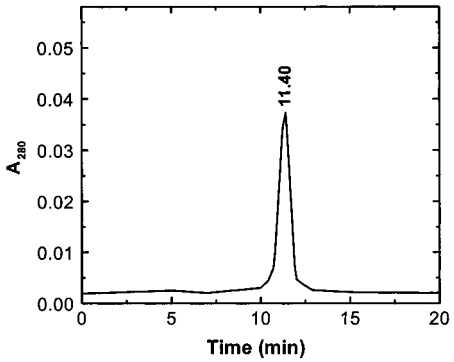


**Figure 5.** Capillary electropherogram of field bean seed PPO in Tris−glycine buffer, pH 8.3 detected at 230 nm.

**Criteria of Homogeneity.** The homogeneity of the purified protein was assessed by native PAGE and capillary electrophoresis. The purified enzyme was electrophoresed in a 7.5% polyacrylamide gel in Tris−glycine buffer pH 8.3 and located by enzyme staining. The purified enzyme revealed a single band (Figure 4, lane b) by specific enzyme staining with catechol and Coomassie Blue (Figure 4, lane a), indicating the presence of a single isoform. The mobility of the enzyme electrophoresed in $\beta$-alanine−acetic acid buffer pH 4.5 (results not shown) was similar to that in Tris−glycine buffer pH 8.3. The diffused bands of field bean seed PPO is due to the general glycoprotein nature of PPOs. The purified PPO electrophoresed as a single peak by capillary electrophoresis in pH 8.3 at 20 mbar pressure (Figure 5), confirming the presence of a single isoform. Several bands of PPO activity were found in the crude extracts of mung bean seedlings (Takeuchi et al., 1992) of which the major isozyme had a pI of 5.4. The release of a single amino-terminal amino acid, asparagine, of both the native enzyme and the denatured enzyme indicated the enzyme to be homogeneous. Further

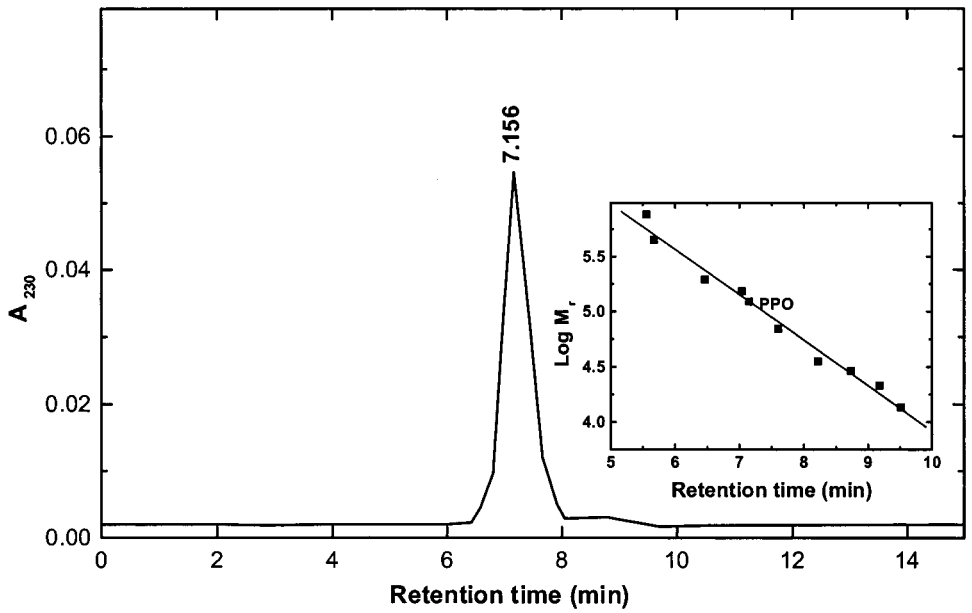


**Figure 6.** HPLC (gel filtration) profile of PPO on Progel TSK G2000 SWXL column.

sequence analysis of the native enzyme, showed the following sequence from the amino-terminus, $NH_2$−NNLISFT....

**Molecular Weight Determination.** The gel filtration of the purified enzyme was carried out by HPLC using a TSK G2000 SWXL (Figure 6) column and a calibrated Sephadex G-100 column as described (Andrews 1964). The $M_r$ of the purified enzyme was estimated to be 120 ± 3.0 kDa (Figure 6 inset) and is in close agreement to a general molecular weight of 110.0 kDa for most plant PPOs (Mayer and Harel, 1979). The size of field bean PPO is similar to that reported for pineapple fruit (Das et al., 1997), oilbean (Chilaka et al., 1993), yam (Anosike and Ayaebene, 1982), and cocoyam (Anosike and Ojimelukwe, 1982) but was larger than that reported for broad bean leaf PPO (Flurkey, 1989), tomato, potato, carrot, and mung bean seedling PPO (Takeuchi et al., 1992).

SDS−PAGE of the purified PPO, followed by protein staining showed a single subunit of 30 ± 1.5 kDa (Figure 7). The apparent subunit size was the same irrespective of the enzyme being reduced with $\beta$-mercaptoethanol or not (results not shown). These results suggest that the enzyme is a tetramer of identical subunits with no intermolecular disulfides. Both the oilbean seed (Chilaka et al., 1993) and pineapple fruit PPO (Das et al., 1997) are tetramers of identical subunit size. PPOs, although ubiquitous in angiosperms, also display a complex, heterogeneous quartenary structure, and the values reported cover a wide range. Flurkey (1989) resolved the broad bean leaf PPO into doublets of $M_r$ = 61.5 and 60.0 kDa and 44.5 and 43.0 kDa, all of which have the same amino-terminal sequence.

**Amino-Terminal Sequence and Amino Acid Composition.** SDS- and heat-denatured PPO was transferred from SDS−PAGE to PVDF membrane and subjected to amino-terminal sequencing. The sequence obtained after 17 cycles of automated Edman microsequencing is $NH_2$−NNLISFTMKEFSXTIIA.... Native PPO was sequenced by loading the purified enzyme directly to the Polybrene-washed filter of the sequenator. The amino-terminal sequence obtained up to 11 cycles, $NH_2$−NNLISFTMKE... is identical to that of the

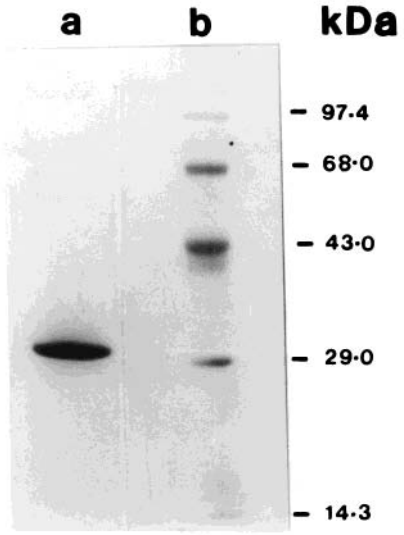


**Figure 7.** SDS−PAGE (10% T, 2.7% C) of field bean seed PPO: (lane a) field bean seed PPO and (lane b) molecular weight standards: phosphorylase b (97.4 kDa), bovine serum albumin (68.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (29.0 kDa), soyabean trypsin inhibitor (20.0 kDa), lysozyme (14.3 kDa).

denatured PPO. These results reflect the identical status of the subunits of field bean seed PPO.

The amino acid composition of the purified PPO is shown in Table 2. Glu and Gln are grouped as Glx, and Asp and Asn are grouped as Asx. The contribution of Trp residues was not determined as they are destroyed by acid hydrolysis. The high content of aspartate, serine, glycine, threonine, alanine, and leucine are similar to that of PPO in grape (Kidren et al., 1977) and in glandular trichomes of *Solanum* and *Lycopersicon* species (Yu et al., 1992).

**Effect of pH.** Aylward and Haisman (1969) reported that the optimum pH for maximum PPO activity in plants varies from about 4 to 7, depending on the extraction methods, the substrates used for assay, and the localization of the enzyme in the plant cell. The maximum activity of the purified PPO with catechol as

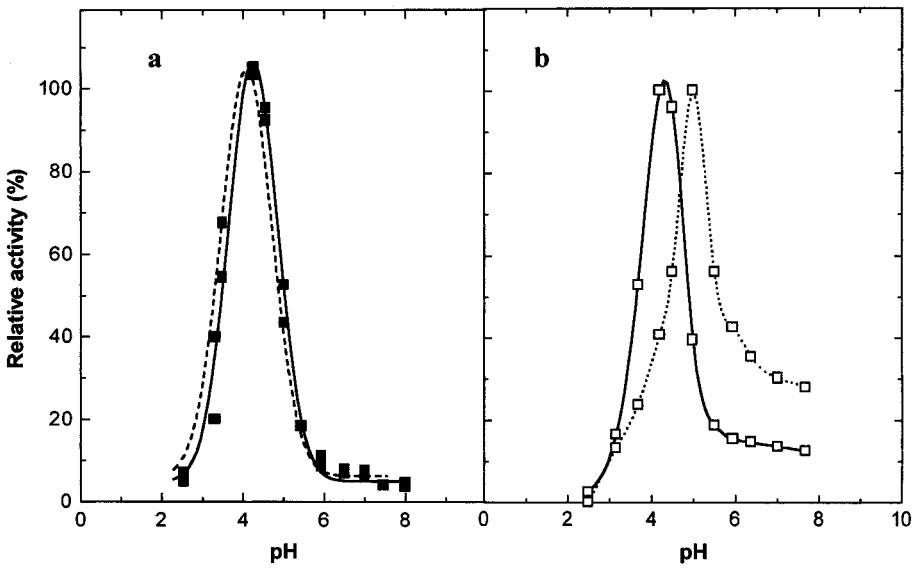


**Figure 8.** Effect of pH on field bean seed PPO activity: (a) PPO activity with catechol as the substrate. (−) McIlvaines (pH 2.5−7.5); (- - -), glycine−HCl buffer (pH 2.5−3.5); sodium acetate (pH 3.5−6.0) and sodium phosphate buffer (pH 6.0−8.0). (b) PPO activity with 4-methyl catechol (−) and DOPA (• • •) as the substrate.

**Table 2. Amino Acid Composition of Field Bean Seed Polyphenol Oxidase**

| amino acid | rel. amino acid comp. (mol %)[a] |
|---|---|
| Asx[b] | 12.6 |
| Glx[c] | 7.9 |
| Ser | 10.3 |
| Gly | 12.6 |
| His | 1.1 |
| Arg | 4.5 |
| Thr | 8.5 |
| Ala | 7.8 |
| Pro | 4.4 |
| Tyr | 2.2 |
| Val | 6.8 |
| Met | 1.0 |
| Cys | 0.2 |
| Ile | 4.0 |
| Leu | 7.8 |
| Phe | 5.8 |
| Lys | 3.0 |

[a] Average of duplicates. [b] Aspartate and asparagine. [c] Glutamate and glutamine.

the substrate was found at pH 4.0, irrespective of the buffers used for assay (Figure 8a). The enzyme is relatively active at low pH. Half the maximal activity was still present at pH 3.5 and 5.5, and 25% was present at pH 3.0. PPO purified from blueberry fruit is also very active at pH 3.0, which is also the pH of the blueberry fruit per se (Kader et al., 1994). We have not determined the pH of the seed. Although the maximum activity with catechol and 4-methyl catechol (Figure 8 a and b) occurred at pH 4.0, for DOPA maximum activity was measured at pH 5.0 (Figure 8b). Differences in pH optima have been reported for partially purified strawberry PPO (Wesche-Ebeling and Montgomery, 1990) and tea-leaf PPO (Gregory and Bendall, 1966). The single pH optimum exhibited by these three substrates further evidences the presence of a single isoform as demonstrated by native PAGE (Figure 4).

**Substrate Specificity.** PPO activity of the purified enzyme for various substrates is shown in Table 3. Relative activities of PPO measured at the absorption maximum of each oxidation product were calculated using catechol for comparison. Maximum activity was achieved with 4-methyl catechol as the substrate, followed by catechol and DOPA. No activity could be measured with chlorogenic acid, caffeic acid, gallic acid, and catechin as substrates. Field bean PPO also showed no activity toward the monophenols, *p*-cresol and tyrosine, suggesting the absence of monophenolase (cresolase) activity. The field bean seed PPO appears to have a small and compact substrate-binding site with a high affinity for small *o*-diphenols such as catechol and 4-methyl catechol and no affinity to bulky *o*-diphenols such as caffeic, chlorogenic acid, catechin, and diphenolic oligomers. Black poplar leaf PPO possesses an extended substrate site, as the enzyme−substrate affinity is relatively insensitive to the substrate's bulkiness, wherein the $K_m$'s for catechol, 4-methyl catechol, chlorogenic acid, and caffeic acid are similar (Tremolieres and Bieth, 1984). Substrate specificities of plant and fungal PPOs are wide and varied when compared to those of from animal tissue where stereospecificity for optical isomers is clear-cut (Mayer and Harel, 1979). The field bean PPO efficiently uses pyrogallol, a triphenol, as a substrate similar to oilbean seed PPO (Table 3).

**Table 3. Effect of Various Substrates on the Activity of Purified Field Bean Seed PPO**

| substrate | wavelength[a] (nm) | rel. activity (%) | $K_m$ (mM) |
|---|---|---|---|
| catechol | 420 | 100 | 10.5 |
| 4-methyl catechol | 420 | 140 | 4.0 |
| L-DOPA | 480 | 22.6 | 1.18 |
| chlorogenic acid[b] | 325 | 0 | ND[c] |
| catechin | 420 | 0 | ND |
| caffeic acid | 420 | 0 | ND |
| tyrosine | 480 | 0 | ND |
| *p*-cresol | 420 | 0 | ND |
| pyrogallol | 334 | 24 | 12.5 |
| gallic acid | 420 | 0 | ND |

[a] Absorption maxima of the corresponding oxidation product. [b] Decrease in absorbance was monitored as the index of PPO activity. [c] ND = not determined.

The purified enzyme displayed Michaelis−Menten kinetics with catechol, 4-methyl catechol, pyrogallol, and DOPA (Figure 9, only catechol shown). Linear regression analysis of *v* versus [S] (Figure 9) indicated apparent $K_m$'s of 10.5, 4.0, 12.5, and 1.18 mM for

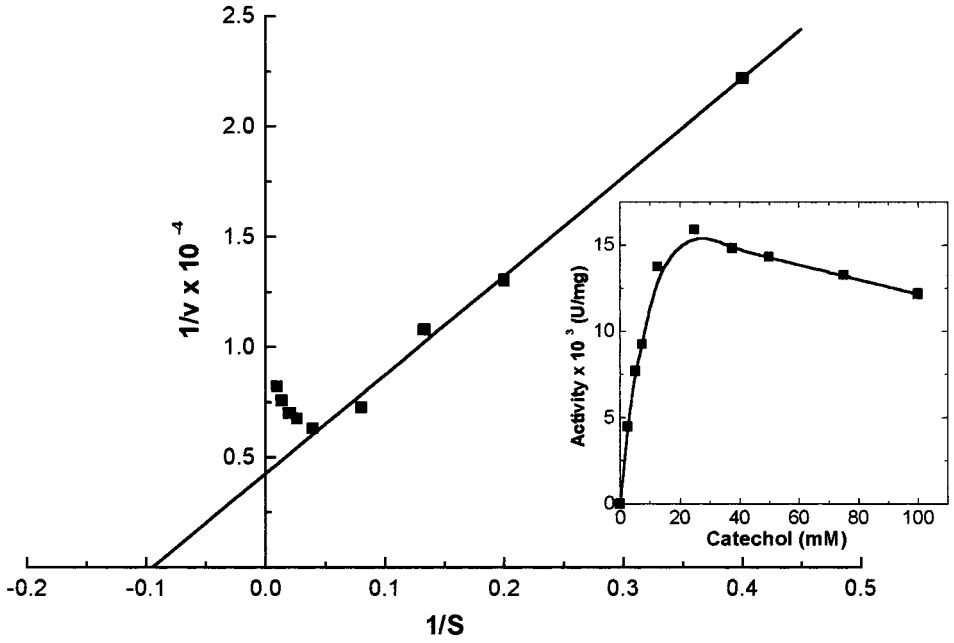


**Figure 9.** Double-reciprocal plot of the effect of catechol concentration on field bean seed PPO. Inset: Michaelis−Menten plot of the above.

**Table 4. Effect of Various Inhibitors on the Activity of Purified Field Bean Seed PPO**

| inhibitor | inhibition | $K_i$ (M) |
|---|---|---|
| tropolone | competitive | $5.8 \times 10^{-7}$ |
| potassium metabisulfite | competitive | $5.5 \times 10^{-6}$ |
| ascorbic acid | competitive | $7.5 \times 10^{-6}$ |
| cysteine−HCl | competitive | $1.95 \times 10^{-2}$ |

catechol, 4-methyl catechol, pyrogallol, and DOPA, respectively (Table 3). Catechol and 4-methyl catechol react with field bean PPO with similar affinities but are oxidized with different $V_{max}$, with 4-methyl catechol being oxidized at a much higher rate. The presence of a methyl group, an electron donor at the para position in 4-methyl catechol, greatly increases the catalytic efficiency. Gallic acid which bears the carboxylic group, an electron-withdrawing group, is not oxidized (Table 3). Gallic acid binds to the enzyme and is an activator at low concentrations and an inhibitor at high concentrations (unpublished results). Protocatechuic acid, with an electron-withdrawing COOH group is not a substrate for poplar leaf PPO (Tremolieres and Bieth, 1984) and blueberry PPO (Kader et al., 1997). On one hand the enzyme−substrate affinity for field bean PPO is high for catechol, 4-methyl catechol, pyrogallol, and DOPA, and on the other, substrate inhibition is observed at concentrations greater than 25, 15, 75, and 10 mM, respectively (Figure 9, only catechol shown). This phenomenon can be rationalized by assuming that a second substrate molecule binds nonproductively to the active site, thereby inhibiting the productively bound one (Trowbridge et al., 1963).

**Inhibitor Studies.** Various inhibitors were examined to determine their potential for inhibition of PPO activity. These inhibitors included a substrate analogue, which is also a metal chelator, and reducing agents. Lineweaver−Burk plots of $1/v$ versus $1/[S]$ at three inhibitor concentrations determined the type of inhibition. The inhibition constant $K_i$ for these inhibitors was deduced from the Dixon plots. Table 4 shows the inhibition results with catechol as the substrate. All of the inhibitors used in this study inhibited PPO competitively. Tropolone (2-hydroxy-2,4,6-cycloheptatrien-1-one), the progenitor of a group of compounds called tropolones, is the most potent inhibitor, with an apparent $K_i$ of 0.58 $\mu$M. It is both structurally analogous to the *o*-diphenolic substrates of PPO and an effective copper chelator (Bryant et al., 1953), which explains the high inhibition potency. Tropolone inhibits field bean PPO in a classical manner, although it is reported to be a slow binding nonclassical competitive inhibitor of grape PPO (Valero et al., 1991). A mixed type of inhibition with tropolone was reported for soluble potato PPO (Sanchez-Ferrer et al., 1993a) and mushroom tyrosinase (Kahn and Andrawis, 1985). Diethyldithiocarbamate, a potent inhibitor of plant PPOs (Anosike and Ayaebene 1981), complexes the copper prosthetic group at the active center.

The inhibition constants for the reducing agents ascorbic acid and metabisulfite are similar and severalfold lower than that for cysteine. Cysteine−HCl, potassium metabisulfite, and ascorbic acid exhibited a lag period, which increased as the inhibitor concentration increased. A similar increase of lag period with increasing inhibitor concentrations has been observed in banana (Galeazzi and Sgarbieri, 1981) and pineapple fruit (Das et al., 1997) PPO. The inhibitor reaction mechanism differs depending on the reducing agent employed. Inhibition by thiol compounds is attributed to either the stable colorless products formed by an addition reaction with *o*-quinones (Ikediobi and Obasuyi, 1982) or binding to the active center of PPO, like metabisulfite (Valero et al., 1992). Ascorbate, acting as an antioxidant, reduces the initial *o*-diquinone product prior to it undergoing secondary reactions which lead to browning (Whitaker, 1972).

## CONCLUSIONS

The mature form of PPO from leguminous plants has been purified to apparent homogeneity from only broad bean leaf (Ganesa et al., 1992), mung bean leaf (Shin et al., 1997), and mung bean seedlings (Takeuchi et al., 1992). We report the purification and characterization

of a PPO from a legume seed. PPO from field bean seed was purified 34-fold to apparent homogeneity using a four-step procedure. The enzyme exists as a single isoform of $M_r$ 120 ± 3.0 kDa and is a tetramer of identical subunits. Apparent pH optima were 4.0 for catechol and 4-methyl catechol and 5.0 with DOPA as a substrate. Field bean seed PPO is a catecholase, active toward small *o*-diphenols, indicating a compact substrate-binding site. The Michaelis constant $K_m$ for catechol, 4-methyl catechol, pyrogallol, and DOPA are 10.5, 4.0, 12.5, and 1.18 mM, respectively. Tropolone was the most effective competitive inhibitor of the enzyme, with a $K_i$ of 0.58 $\mu$M. Ascorbic acid, potassium metabisulfite, and cysteine inhibited the enzyme.

## ABBREVIATIONS USED

L-DOPA, L-3,4-dihydroxyphenylalanine; PPO, polyphenol oxidase; PVPP, polyvinylpolypyrrolidone; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene diflouride

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