Purification and Characterization of Pea Seedling Amine Oxidase for Crystallization Studies

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Pea (Pisum sativum L.) seedling amine oxidase (EC 1.4.3.6) is the first amine oxidase to be crystallized that diffracts to atomic resolution (2.5 Å). Extensive modifications of a published purification procedure were necessary to obtain protein that would give diffraction-quality crystals. Here we report the improved purification and also use this high-purity protein to reexamine some fundamental characteristics of pea seedling amine oxidase. The extinction coefficient at 280 nm (ε280) and the molecular mass of the protein are investigated by a variety of techniques, yielding ε280 = 20 cm/mM and a mass of 150 ± 6 kD. In addition, the stoichiometry of the metal and organic cofactors, Cu(II) and 6-hydroxy dopa (Topa) quinone, respectively, is examined. The ratio of Cu(II):Topa:protein monomer is found to be 1:1:1.

Copper-containing amine oxidases (EC 1.4.3.6) catalyze the oxidative deamination of primary amines:

\[
\text{RCH}_2\text{NH}_2 + \text{O}_2 + \text{H}_2\text{O} \rightarrow \text{RCHO} + \text{NH}_3 + \text{H}_2\text{O}_2.
\]

Amine oxidases are ubiquitous in nature, occurring in microorganisms (fungi and Gram-negative and Gram-positive bacteria), plants, and mammals (Cooper et al., 1992; McIntire and Hartmann, 1993). In peas (Pisum sativum L.), amine oxidases are found primarily in the extracellular fluid in the epicotyl, being absent from the seed (Smith, 1985). The enzyme is present in greatest concentration during the first week of growth. Studies suggest that one of its functions may be providing H2O2 to peroxidase-catalyzed reactions that occur within the cell wall (Federico and Angelini, 1986). Such reactions are responsible for the coupling of lignin subunits, which control wall stiffening. Amine oxidases may also be involved in the biosynthesis of certain plant growth factors (Smith, 1985).

Amine oxidases are dimeric proteins (ε280) whose subunit size is approximately 70 kD. They contain one type II Cu(II) center per monomer; the copper is essential for activity and is believed to play a redox role in substrate turnover (Dooley et al., 1991; Turowski et al., 1993). An organic cofactor identified as Topa quinone is also present and serves as the site of substrate binding (Janes et al., 1990; Brown et al., 1991). Plant and mammalian amine oxidases are glycoproteins reported to contain 3 to 15% carbohydrate (Rossi et al., 1992; McIntire and Hartmann, 1993). Although discrepancies concerning the cofactor:protein stoichiometry and reactivity have been reported in the literature (McIntire and Hartmann, 1993), the more recent studies on a variety of amine oxidases as well as this investigation support a Cu(II):Topa:monomer ratio of 1:1:1 (Collison et al., 1989; Janes and Klisman, 1991). However, the question of the reactivity of half of the sites in certain amine oxidases has not been resolved (see, for example, Morpurgo et al., 1992).

PSAO is the first amine oxidase to be crystallized that diffracts to atomic resolution (2.5 Å) (Vignevich et al., 1993). The purity of the protein sample was a critical factor in successfully obtaining diffraction-quality crystals. Here we report an improved procedure for the purification of PSAO and a detailed investigation of the Cu(II):Topa:protein stoichiometry. The molecular mass of the protein, reported to be 190,000 D by Kleutz et al. (1980), is reexamined, because crystallographic results indicate a much lower value of 132,000 D.

### MATERIALS AND METHODS

PMSF and benzylhydrazine were obtained from Sigma. DE52 (Whatman) was purchased from VWR Scientific (Seattle, WA), Biogel HT Hydroxylapatite from Bio-Rad, and Ultrogel AcA34 from IBF (Columbia, MD). Alaskan variety pea (Pisum sativum L.) seeds were obtained from Hart Seed Co. (Hartford, CT). All other reagents used were of reagent-grade purity. UV and visible spectra were recorded on a Cary-219 (Varian, Houston, TX) interfaced with a PC using software by On-Line Information Systems (Jefferson, GA) or on a Hewlett-Packard HP8452 spectrophotometer. Trp content was determined on a Jasco (Easton, MD) J710 spectrophotometer equipped with an Alpha Scientific (Hayward, CA) electromagnet using lysozyme as a standard (Barth et al., 1972). Copper content was determined by flame atomic absorption spectroscopy on a GBC 908 spectrophotometer (Chicago, IL). Cu(II) EPR spectra at X-band were measured on a Bruker (Billerica, MA) 220D-SRC run by EPRWare from Scientific Software Services (Bloomington, IL). Activity measurements during purification were carried out at 37°C on the Cary-219 using p-N,N-dimethylaminomethyl benzylamine as the substrate (Bardsley et al., 1972). For the cofactor quanti-

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**Abbreviations:** Bhyd, benzylhydrazine; EPR, electron paramagnetic resonance; ε280, extinction coefficient at wavelength 280 nm of a 10 mg/mL protein solution; LD, laser desorption; MCD, magnetic circular dichroism; pl, isoelectric point; PSAO, pea seedling amine oxidase; Topa quinone, 6-hydroxy dopa quinone.


Purification

PSAO was purified by modifying a published procedure (Kleutz et al., 1980). Three kilograms of seeds were soaked for 10 h in tap water and then spread in a monolayer on trays lined with moistened filter paper. The trays were covered tightly with aluminum foil and left for 32 h. The germinated seeds were then thickly planted on top of 1 inch of wet vermiculite in 10 dish buckets, lightly covered with dry vermiculite, and placed on a heating mat at 25°C in the dark. The seedlings were watered every other day with approximately 500 mL of tap water per bucket. After growing in vermiculite for 10 d, the etiolated seedlings were harvested above the roots, typically yielding 4 to 6 kg of seedlings. To purify protein suitable for crystallization, the seedlings were harvested, purified via MS technique (kindly provided by Dr. Lowell Ericsson at the University of Washington, Seattle, WA), and by SDS-PAGE in vermiculite for 10 d, the etiolated seedlings were harvested approximately 500 mL of tap water per bucket. After growing in vermiculite for 10 d, the etiolated seedlings were harvested above the roots, typically yielding 4 to 6 kg of seedlings. To purify protein suitable for crystallization, the seedlings were used immediately; however, they may be kept frozen at -20°C for up to 2 weeks. After harvesting the seedlings, all procedures were carried out at 4°C.

The seedlings were homogenized to a slick slurry in a 4-L Waring blender. Seedlings (1.5 kg) were blended with 596 mL of 0.2 M KPO₄, pH 7.2, plus 6 mL of the protease inhibitor PMSF (0.1 M in 95% ethanol). The slurry was squeezed through muslin cloth, and the liquid was treated with ammonium sulfate as described by Kluetz et al. (1980). Organic solvent precipitation and 80% ammonium sulfate recovery followed (Kleutz et al., 1980). In the latter process, gentle stirring for no longer than 1 h proved to be critical to solid curd formation.

On the second day, the centrifuged, dialyzed PSAO sample was applied at a flow rate of 15 mL/min to a 9 x 15 cm DE52 column equilibrated with 0.015 M KPO₄, pH 7.2, and was immediately eluted with 0.02 M KPO₄, pH 7.2. The eluting protein, pink in color, was collected in 100-mL fractions. Those of highest specific activity were pooled and concentrated to <100 mL. The sample was then applied at a flow rate of 1.25 mL/min and 40-cm operating pressure to a 5 x 5.5 cm hydroxylapatite column equilibrated with 0.015 M KPO₄, pH 7.2. The column was immediately eluted with a linear gradient of 650 mL each of 0.03 to 0.18 M KPO₄, pH 7.2, and 12-mL fractions were collected overnight. The pink color of PSAO was readily detected in the tubes; these fractions were analyzed by specific activity measurements. The tubes with the highest specific activity were pooled, concentrated to 50 mg/mL, and applied to a 1.6 x 100 cm Ultrogel AcA34 gel filtration column. The protein was eluted at 8 mL/h with 0.1 M KPO₄, pH 7.2, and collected in 3-mL fractions. Each pink fraction was checked for purity by specific activity, visible absorbance, and SDS-PAGE (Coomassie blue stain). The purest fractions were pooled and used for crystallization studies without freezing.

Cofactor Determination

PSAO was titrated both in air and anaerobically in 0.1 M KPO₄, pH 7.2, at 25°C with Bhyd. PSAO (290 μL, 15.8 μM) was reacted with 2-μL aliquots (0.3 eq per dimer) of a 0.69 mM Bhyd stock solution. The reaction was followed by A₃₅₀, after each addition of reactant, 2 μL of the mixture was removed and its activity was determined with benzylamine.

Copper Determination

Adventitious metals were removed from PSAO by dialyzing 1 mL of PSAO three times versus 2 L of chelexed 0.1 M KPO₄, pH 7.2, with 2 mM EDTA, followed by dialysis three times versus chelexed 0.1 M KPO₄, pH 7.2. The A₂₈₀ of an approximately 1 mg/mL sample was determined and the sample was then analyzed by flame atomic absorption spectroscopy. A buffer blank was also analyzed and subtracted from the protein reading. Another sample was prepared to approximately 40 mg/mL; its A₂₈₀ was measured, and then it was examined by X-band EPR at 77 K. The concentration of EPR-detectable Cu(II) in PSAO was calculated by double integration of the Cu(II) signal, using Cu(ClO₄)₂ in 2 mM NaClO₄ as a standard.

Extinction Coefficient Determinations

The electronic A₂₈₀ and A₄₅₀ of approximately 1 mg/mL native, oxidized PSAO were measured. This sample was then titrated anaerobically with a solution of 0.80 mM benzylamine, prepared from freshly distilled benzylamine. The amount of substrate required to completely bleach the 500-nm band was used to determine the amount of cofactor present in the protein solution.

The actual ε₁₅₀ was determined by the dry weight method described below. The UV electronic A₂₈₀ and MCD signal at 293 nm of the sample were carefully measured at each stage of the protocol. The UV electronic spectrum of Trp in proteins usually blue shifts and its molar absorbptivity decreases when the proteins are unfolded in urea or guanidine (Barth et al., 1972). In addition, pH also influences the absorption spectrum of Trp (Wetlaifer, 1962). Thus, protein UV A₂₈₀, due mostly to Trp, may be affected by tertiary structure, since the microenvironment of the Trp residues (i.e. solvent accessibility and pH) may be altered. The MCD of proteins at 293 nm, due almost entirely to Trp electronic transitions, is an intrinsic property of the electronic structure of the indole group in the Trp chromophore itself, and is therefore practically independent of its environment (Barth et al., 1972; McFarland and Coleman, 1972). Thus, MCD spectroscopy provides an alternative approach to measuring protein concentration when procedures that may alter tertiary structure are used (i.e. lyophilization or changes in pH and ionic strength).
Measurements of the UV $A_{280}$ and the MCD at 293 nm were made on 800 µL of approximately 10 mg/mL PSAO in 0.1 M KPO$_4$, pH 7.2. The sample was then extensively dialyzed versus HPLC-grade water and the measurements were repeated. Seven hundred microliters of the sample were transferred (using a Pipetman P1000) into a preweighed 15-mL sterile plastic conical tube. The tube was frozen at $-80^\circ$C, covered with a Kimwipe, and placed in a 500-mL lyophilizing bottle. The sample was lyophilized overnight and the tube was reweighed. The freeze-dried protein was reconstituted in water and centrifuged and its activity, MCD, and UV $A_{280}$ were checked.

**RESULTS AND DISCUSSION**

The results from a typical purification are shown in Table I and a profile of the hydroxylapatite fractions is displayed in Figure 1. A small but reproducible drop in specific activity occurred early in the PSAO elution. Although no further attempt has been made to understand this phenomenon, the fractions corresponding to this decreased specific activity were omitted from crystallization samples.

Figure 2 shows an analysis of the gel filtration column. Again, only the purest fractions were used for crystallization, although those of somewhat lower purity were still suitable.

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**Table I. Purification of PSAO**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol (mL)</th>
<th>Total Units</th>
<th>Total Protein* (mg)</th>
<th>Specific Activity* (IU)</th>
<th>Percent Yield</th>
<th>Purification Factor</th>
</tr>
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<tbody>
<tr>
<td>Homogenate</td>
<td>4,650</td>
<td>2,446</td>
<td>75,678</td>
<td>0.0326</td>
<td>100</td>
<td>1X</td>
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<tr>
<td>60% ammonium sulfate</td>
<td>670</td>
<td>2,034</td>
<td>26,178</td>
<td>0.0777</td>
<td>83</td>
<td>2.4X</td>
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<td>pellet</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol/CHCl$_3$</td>
<td>665</td>
<td>1,507</td>
<td>8,159</td>
<td>0.185</td>
<td>61.6</td>
<td>6.7X</td>
</tr>
<tr>
<td>Triturations</td>
<td>119</td>
<td>1,073</td>
<td>516.8</td>
<td>2.08</td>
<td>43.9</td>
<td>63.6X</td>
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<td>Dialysis (DE52 load)</td>
<td>156</td>
<td>994</td>
<td>284.2</td>
<td>3.5</td>
<td>40.6</td>
<td>107X</td>
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<td>DE52 pool</td>
<td>316</td>
<td>885</td>
<td>69.4</td>
<td>12.75</td>
<td>36.2</td>
<td>391X</td>
</tr>
<tr>
<td>HT$^a$-pool A (for Ultrogel)</td>
<td>194</td>
<td>569</td>
<td>25.9</td>
<td>21.9</td>
<td>23.3</td>
<td>672X</td>
</tr>
<tr>
<td>HT-pool B</td>
<td>91</td>
<td>218</td>
<td>10.7</td>
<td>20.45</td>
<td>8.9</td>
<td>627X</td>
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<tr>
<td>Ultrogel-pool A (for crystallization)</td>
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<td>253.9</td>
<td>12.5</td>
<td>20.3</td>
<td>10.4</td>
<td>623X</td>
</tr>
<tr>
<td>Ultrogel-pool B</td>
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<td>102</td>
<td>5.2</td>
<td>19.6</td>
<td>4.2</td>
<td>600X</td>
</tr>
</tbody>
</table>

* Calculated using $d_{280} = 20$ cm$^{-1}$. $^a$ HT, Hydroxylapatite.

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*Figure 1.* Profile of a typical hydroxylapatite column showing protein concentration (■) and specific activity (x) of the eluted fractions. Inset, Visible absorption spectra of PSAO before hydroxylapatite chromatography (—) and from pool A (--) and pool B (--.--). Pool A was further purified for crystallization studies.
Figure 2. Visible absorption spectra of the PSAO fractions collected from Ultrogel AcA34 chromatography. S.A., Specific activity in IU. Pool A was used for crystallization studies.

for most spectroscopic studies. Purity was judged by specific activity, peak-to-trough ratio (500:400 nm) in the visible spectrum, and, most importantly, the lack of minor bands detected by SDS-PAGE (Fig. 3, lane 1). By severely overloading the SDS gel and using Coomassie blue stain, a small amount of a 30-kD impurity could be detected in the sample before the gel filtration column and in wing fractions of the eluting protein. Silver stain, even at extremely high loadings, showed no detectable bands other than PSAO after hydroxylapatite chromatography (data not shown). Prior to the addition of PMSF to the purification procedure, an increase in low molecular mass bands could be detected after the purified protein had been stored for several days at 5°C. The best preparations of PSAO had specific activities of 19 to 21 IU (based on $\epsilon_{280}^\text{nm} = 20 \text{ cm}^{-1}$; 13–14 IU based on $\epsilon_{280}^\text{nm} = 13.5 \text{ cm}^{-1}$) and are at least 98% pure as judged by SDS-PAGE.

The molecular mass of PSAO was determined by SDS-PAGE to be 72 kD per subunit. This is much lower than the literature value of 85 kD but is within the error margins of 66 ± 7 kD estimated from the crystal structure and derived by measuring changes in the density of the crystals as a function of changes in the density of the mother liquor. To provide an independent check of molecular mass, the protein was examined by LD/MS and found to have a subunit mass of 75.7 ± 1 kD. This number agrees with the size determined by the translated gene sequence and carbohydrate content (M. McPherson, personal communication). Indeed, the recently published translated amino acid sequences of several other amine oxidases indicate subunit sizes in the 67- to 77-kD range (Brunenber et al., 1989; Rossi et al., 1992; Zhang et al., 1993), which are significantly smaller than those deduced by the more traditional techniques of gel filtration.
Figure 4. Visible absorption spectral changes accompanying the titration of PSAO with Bhyd. [PSAO], 15.8 μM; [Bhyd] (μM): A, 0; B, 4.74; C, 9.5; D, 14.4; E, 19.3; F, 24.3; G, 29.3; H, 34.4. Inset, Percent activity (□) and change in A₃₈₃ (×) versus [Bhyd].

Figure 5. Visible absorption spectral changes accompanying the anaerobic titration of PSAO with substrate. Seven hundred microliters of PSAO (A₃₈₃ = 0.5847 mm⁻¹) was titrated with 0.80 mM benzylamine in 0.1 M KPO₄, pH 7.2, at room temperature. Substrate added (nmol): A, 0; B, 4; C, 8; D, 12.8; E, 16.8; F, 20.8; G, 25.2; H, 29.2; I, 32.4. Inset, A₃₈₃ versus nmol benzylamine added.

The pl of PSAO was found to be 7.3. This value is higher than those of other amine oxidases, which tend to be acidic and have pl values in the 4 to 6 range (McIntire and Hartmann, 1993). Unlike pig plasma amine oxidase (Falk et al., 1983), PSAO shows no isoenzymic behavior and migrates as one band during IEF.

Bhyd has been reported to be a slow substrate for bovine plasma amine oxidase (Morpurgo et al., 1989), yet it reacts irreversibly and stoichiometrically with the amine oxidase isolated from lentil seedlings (Padiglia et al., 1992). With PSAO, the Bhyd reaction was also irreversible and stoichiometric. The reaction between the Topa quinone cofactor and Bhyd, shown in Figure 4, was complete within 1 min of the hydrazine addition. As with lentil seedling amine oxidase, the magnitude of the $A_{383}$ at the endpoint of the reaction indicated the presence of two quinones per protein dimer. Moreover, the decrease in the activity of the protein correlated with formation of the Topa-Bhyd product and showed that both quinones were active toward substrate. Anaerobic titrations yielded the same results as those performed aerobically.

Given the evidence for a lower molecular mass of PSAO than was previously assumed, a reinvestigation of the extinction coefficients at 500 and 280 nm was in order. Figure 5 shows the anaerobic titration of 700 lL of PSAO ($A_{280} = 0.5847$ mm$^{-1}$) with benzylamine and demonstrates that 27 nmol of a monoamine substrate are required to completely bleach the Topa band at 500 nm (formation of the Topa semiquinone species may also be seen). Using these data, the extinction coefficient at 500 nm was determined to be 4.9 mm$^{-1}$ cm$^{-1}$ per dimer. The ratio of $A_{280}/A_{500}$ is 61.4, implying that $e_{280} = 300$ mm$^{-1}$ cm$^{-1}$.

The dry-weight determination provides an $e_{280}$ that is based on protein concentration as expressed in mg/mL. The results of the dry-weight determination on PSAO indicate that $e_{280} = 20$ cm$^{-1}$. Using this value and the molar extinction coefficient, $e_{280} = 300$ mm$^{-1}$ cm$^{-1}$, from the substrate-titration experiment, the molecular mass of PSAO is estimated to be 150 kD, in excellent agreement with the direct measurements.

Whereas extinction coefficients at 280 nm may be somewhat influenced by protein conformation, the MCD extinction of Trp is independent of tertiary structure. The constant ratio of $A_{280}$ to Trp content (as measured by MCD) of the sample before and after lyophilization, seen in Figure 6, as well as the recovery of specific activity, indicate that the protein was not denatured or altered by lyophilization. The total Trp content of PSAO was estimated to be 26 ± 4 per dimer.

Figure 6 also shows that the Topa visible-absorbance band is sensitive to pH and red shifts when the protein is taken from phosphate-buffered pH 7.2 solution (maximum wavelength = 500 nm) into water (pH 5.7). In acetate-buffered pH 5.2 solution maximum wavelength shifts to 515 nm (data not shown).

The copper content of PSAO was determined by flame atomic absorption on a sample that had an $A_{280}$ of 1.6076 cm$^{-1}$. The sample analyzed had 0.77 ppm (12.0 $\mu$M) copper. Using the previously published values for molecular mass and $A_{280}$, the dimeric protein concentration was 6.3 $\mu$M and the Cu(II):protein stoichiometry was 1:9.1. The values determined in this paper indicate a protein concentration of 5.4 $\mu$M and a Cu(II):protein stoichiometry of 2.2:1. Double integration of X-band EPR measurements at 77 K of PSAO give a Cu(II):protein dimer ratio of 2.0 - 2.2:1, the former derived from previous literature values and the latter from those published here.

CONCLUSIONS

Pea seedling amine oxidase is readily prepared to homogeneity with just three chromatographic steps. Highly purified PSAO has a specific activity of 19 to 21 IU (based on the extinction coefficients determined in this paper) and a pl of 7.3. The methods used in this investigation suggest a subunit molecular mass of 75 ± 3 kD. Reexamination of the extinction coefficients of PSAO yields values of $e_{280} = 20$ cm$^{-1}$ (300 mm$^{-1}$ cm$^{-1}$) and $e_{500} = 4.9$ mm$^{-1}$ cm$^{-1}$. There are two type-2 Cu(II) ions and two reactive Topa quinones per protein dimer.

Received April 25, 1994; accepted July 27, 1994.
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LITERATURE CITED

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