

**PARTIAL PURIFICATION AND CHARACTERIZATION OF THE  
SOLUBLE POLYPHENOL OXIDASES FROM SUSPENSION CULTURES  
OF PAPAVER SOMNIFERUM**

AN-FEI HSU, EDWIN B. KALAN and DONALD D. BILLS

*Eastern Regional Research Center\**, 600 E. Mermaid Lane, Philadelphia, PA 19118  
(U.S.A.)

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**SUMMARY**

Suspension cultures of *Papaver somniferum* contained at least four heterogeneous forms of polyphenol oxidase (PPO) by polyacrylamide gel electrophoresis (PAGE). Two were purified approx. 100-fold by ammonium sulfate precipitation, ultracentrifugation, carboxymethylcellulose chromatography and Sephacryl S-200 chromatography; they had the same  $K_m$ -value ( $5 \times 10^{-3}$  M) for catechol, similar heat stability and both were active toward *o*-diphenols but not toward monophenols, and were inhibited similarly in vitro by sodium cyanide,  $\beta$ -mercaptoethanol, sodium sulfite, sodium ascorbate and L-cysteine. However, diethyldithiocarbamate greatly inhibited one purified form compared to the other.

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*Key words:* *Papaver somniferum* — Callus — Polyphenol oxidase

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**INTRODUCTION**

PPO (EC 1.14.18.1) has been implicated in the biosynthesis of opium alkaloids in the opium poppy, *P. somniferum* [1], apparently in the pathway from tyrosine to the opium alkaloids [2-4]. PPO from acetone powder of the whole plant has been isolated and partially purified [5], although its

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\*Agricultural Research Service, U.S. Department of Agriculture.

Abbreviations: DIECA, diethyldithiocarbamate; DOPA, dihydroxyphenylalanine; PAGE, polyacrylamide gel electrophoresis; PPO, polyphenol oxidase; PVP, polyvinylpyrrolidone.

direct involvement in the biosynthesis of morphine alkaloids has not been demonstrated.

Although crude extracts from *P. somniferum* were shown to convert ( $\pm$ )-[3-<sup>3</sup>H] reticuline to [<sup>3</sup>H] salutaridine in the presence of hydrogen peroxide [6], peroxidase isolated from seedlings of *P. somniferum* was ineffective in catalyzing oxidation of reticuline [7]. However, horseradish peroxidase and a crude poppy enzyme fraction were able to transform morphine alkaloids into N-oxides and morphine to pseudomorphine [8]. Since the involvement of PPO or peroxidase in the oxidation step of morphine biosynthesis is not clear, the results of this study should contribute to a better understanding of the nature of PPO occurring in opium poppy and its relationship to phenanthrene alkaloid biosynthesis is needed.

#### MATERIALS AND METHODS

Suspension cultures of *P. somniferum* were induced from seedling callus as previously reported [9]. Sephacryl S-200\* was purchased from Pharmacia Company and carboxymethylcellulose was obtained from Whatman Company. Acrylamide (*N, N'*-methylene bisacrylamide) was purchased from Eastman Kodak Company.

##### *Isolation and purification of PPO*

Suspension cells obtained by filtration were homogenized in a motor-driven glass-Teflon Potter-Elvehjem homogenizer with 3 vols. of sodium phosphate buffer (pH 6.8, 5 mM) containing 20 mM sodium ascorbate and 0.2 M sucrose. The homogenate was passed through four layers of cheese cloth and centrifuged at  $3000 \times g$  for 10 min. Solid ammonium sulfate was added to the supernatant to 85% saturation, and the mixture was centrifuged at  $48\,000 \times g$  for 20 min. The pellet was redissolved in homogenization buffer (minus sodium ascorbate) to a protein concentration of 10 mg/ml.

Dialysis was performed overnight with 0.01 M sodium phosphate buffer (pH 6.8) containing 0.2 M sucrose. The dialysate was centrifuged at  $100\,000 \times g$  for 1 h and the supernatant was used as the crude soluble PPO.

##### *PPO assay procedure*

Enzyme assays were performed in 1.0 ml vol. containing 10 mM catechol in 0.1 M sodium phosphate buffer (pH 6.8 and saturated with O<sub>2</sub>) at room temperature. An appropriate amount of enzyme was added and activity was monitored at 430 nm with a Beckman Model 22 recording spectrophotometer. Under these conditions, linearity was maintained for 5 min with one unit of PPO-activity defined as a change in absorbance of 0.001/min at 430 nm at 25°C.

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\*Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

### *Ion exchange chromatography*

Activated preswollen carboxymethylcellulose was packed into a  $2.5 \times 20$ -cm column and equilibrated at  $4^{\circ}\text{C}$  with 0.01 M sodium phosphate buffer (pH 6.8) containing 0.2 M sucrose. The sample was applied and followed with three void volumes of the buffer. A sodium chloride gradient was applied in a stepwise fashion with concentrations of 10, 60, 100 and 200 mM sodium chloride in 200 ml of buffer. Three millimeter fractions were collected and 200- $\mu\text{l}$  aliquots were used to determine PPO-activity. Protein concentration was determined by the Lowry procedure [10].

### *Sephacryl S-200 chromatography*

Each fraction containing PPO-activity from CM-cellulose column was separately pooled, concentrated to 1 ml in an Amicon apparatus and then loaded on a Sephacryl S-200 column ( $1 \times 60$  cm), equilibrated with 0.05 M sodium phosphate buffer (pH 6.8) containing 0.2 M sucrose. The eluent was continuously monitored at 215 nm and PPO-activity was assayed as described before.

### *PAGE*

Discontinuous PAGE was carried out on 7.5% acrylamide gels as described previously [11]. During electrophoresis, the temperature was maintained at  $0$ – $2^{\circ}\text{C}$  by circulating ice-water through the electrophoretic cells. PPO-activity in the gels was detected by immersing the gel for 30 min in 20 ml of 10 mM catechol (0.1 M of sodium phosphate buffer, pH 6.8) containing 0.5% *p*-phenylenediamine as described by Benjamin and Montgomery [12]. PPO mobilities was expressed as a ratio of the mobility of bromophenol blue.

## RESULTS

Four distinct heterogenous forms of PPO were separated by CM-cellulose chromatography followed by increasing ionic strength from 10 mM to 200 mM of sodium chloride (Fig. 1). No further PPOs were eluted from the column with salt concentrations above 200 mM.

Fractions obtained by CM-cellulose were separately pooled and concentrated to a small volume with an Amicon ultrafiltration apparatus and subjected to PAGE. Staining with *p*-phenylenediamine, peak I of Fig. 1 contained two PPO bands ( $R_f$  0.30 and 0.24), peak II contained only one PPO ( $R_f$  0.24, designed as PPO-A), peak III contained one PPO ( $R_f$  0.16, designed as PPO-B) and peak IV contained a mixture of PPO ( $R_f$  0.16 and 0.10). Attempts to separate two PPOs in peak I and IV by either cation or anion exchange resins with continuous or step gradients were not successful.

Further purification of PPO-A and B was achieved by chromatography on a Sephacryl S-200 column which separated PPO-activity from the major protein peak. This step resulted in the greatest purification.

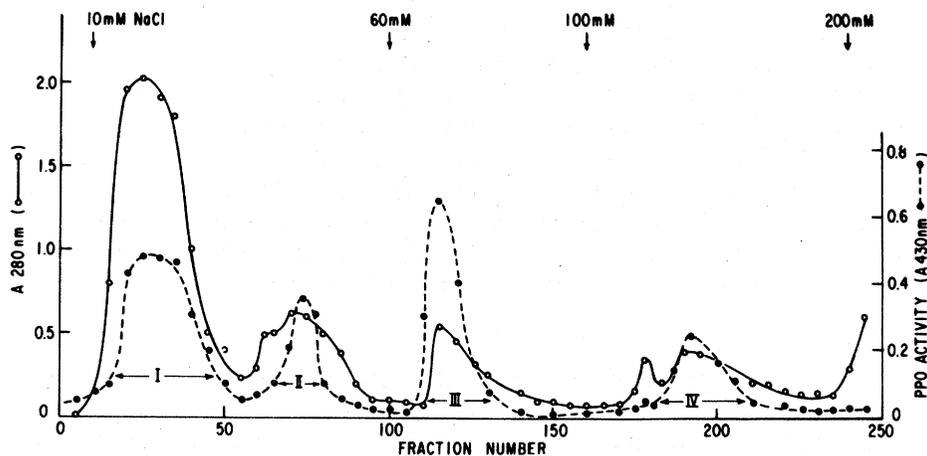


Fig. 1. CM-cellulose chromatography of soluble polyphenol oxidase. The crude preparation of soluble PPO was loaded on the CM-cellulose column. The separation of proteins was described under Materials and Methods. Protein peak (○—○) was determined by  $A_{280 \text{ nm}}$ , and PPO-activity ( $A_{430 \text{ nm}}$ , ●—●) was detected as described in the text.

Table I provides a summary of the purification steps for PPO. Ammonium sulfate precipitation and ultracentrifugation produced little purification of the isoenzymes. Only 60% of the total activity was recovered in the soluble fraction (100 000  $\times$  g supernatant), while the remainder (40%) was detected

TABLE I

PURIFICATION OF PPO FROM SUSPENSION CULTURES OF *P. SOMNIFERUM*

Purification step	Total act. (units) <sup>a</sup>	Total protein (mg) <sup>b</sup>	Spec. act. (units/mg)	Yield <sup>c</sup> (%)	Purification fold
1. Crude PPO extract	286	283	1.01	100	1
2. Ammonium sulfate precipitate	440	230	1.91	150	2
3. Soluble fraction	330	100	3.30	115	3
4. CM-cellulose					
Peak I	135	40	3.38	47	3
Peak II	120	15	8	41	8
Peak III	70	7	10	24	10
Peak IV	12	2	6	4	6
5. Sephacryl S-200					
PPO-A	58	0.62	92	20(13) <sup>d</sup>	92
PPO-B	34	0.35	97	12(08) <sup>d</sup>	96

<sup>a</sup>Units: absorbance change of 0.001 at 430 nm per min.

<sup>b</sup>Protein determined by Lowry assay.

<sup>c</sup>Based on the comparison of total activity to crude PPO extract.

<sup>d</sup>Based on ammonium sulfate precipitate fraction.

in precipitates obtained by low speed centrifugation. A greater degree of purification was obtained by chromatography on Sephacryl S-200 (Table I) which yielded roughly 92- and 96-fold of purification based on the specific activity of the crude PPO.

#### *Effect of catechol concentration on PPO activity*

The rate of formation of quinone products by PPO-A increased with increasing concentration of catechol up to 20 mM with linearity according to classical kinetics (Fig. 2). A double reciprocal plot yielded a straight line with a  $K_m$  of  $5 \times 10^{-3}$  M for catechol. PPO-B had the same  $K_m$  as PPO-A when catechol was used as a substrate.

#### *Inhibitor studies*

All compounds used in this study inhibited PPO-activity (Table II), and the extent of inhibition depended on the concentration of the inhibitor. Sodium cyanide and  $\beta$ -mercaptoethanol were most effective for both PPO-A and PPO-B, while diethyldithiocarbamate (DIECA) had different inhibition effects on PPO-A and PPO-B. At a concentration of 1 mM, DIECA almost completely inhibited PPO-B activity, while it inhibited less than 40% of PPO-A activity. With the same concentration of inhibitors, the order of

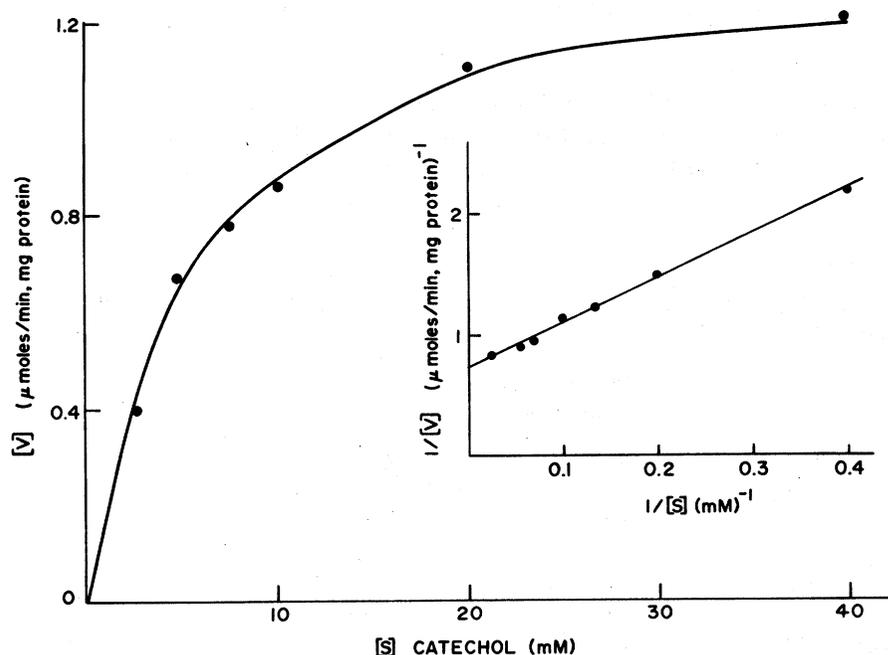


Fig. 2. Effect of catechol concentration on PPO-A activity. Constant amount of PPO-A (100  $\mu$ g) was incubated with different concentrations of catechol. The values of  $V_{max}$  and  $K_m$  were determined by a least-squares fit of the data on a double reciprocal plot. The product concentration was calculated based on  $\log \epsilon (M^{-1} cm^{-1}) = 3.324$ .

TABLE II  
EFFECT OF PPO INHIBITORS OF THE PPO-A AND B

Inhibitor	Inhibitor conc. (mM)	Percent inhibition	
		PPO-A	PPO-B
Control <sup>a</sup>	0	0	0
Diethyldithiocarbamate	10	100	100
	1	34	95
Na-Ascorbate	10	100	100
	1	20	8
L-Cysteine	10	100	100
	1	18	49
Na-Sulfite	10	100	100
	1	24	40
$\beta$ -Mercaptoethanol	10	100	100
	1	61	68
Na-Cyanide	10	100	100
	1	59	83

<sup>a</sup>Enzyme activity was determined as described under Materials and Methods, and the control experiment was the incubation of enzyme and substrate without the addition of inhibitors.

effectiveness of the inhibitors for PPO-A was:  $\beta$ -mercaptoethanol  $\cong$  sodium cyanide > DIECA > sodium sulfite  $\cong$  sodium ascorbate and L-cysteine. The degree of effectiveness on PPO-B was DIECA > sodium cyanide >  $\beta$ -mercaptoethanol >  $\alpha$ -cysteine  $\cong$  sodium sulfite > sodium ascorbate.

TABLE III  
SUBSTRATE SPECIFICITY OF PPO-A AND B FROM SUSPENSION CULTURES OF *P. SOMNIFERUM*

Substrate	PPO activity <sup>a</sup> (percentage)	
	PPO-A	PPO-B
Catechol	100 <sup>b</sup>	100 <sup>b</sup>
P-Cresol	3	0
Tyrosine	0	3
Pyrogallol	61	40
Caffeic acid	11	14
Chlorogenic acid	18	7

<sup>a</sup>Enzyme activity was determined as described under Materials and Methods.

<sup>b</sup>The highest enzyme activity was assigned a value of 100%.

### *Substrate specificity*

PPO-A and B preparation from culture cells of *P. somniferum* had strong activity toward *o*-diphenol (catechol), but little or no activity toward monophenols (*p*-cresol and tyrosine) (Table III).

### DISCUSSION

The multiplicity of polyphenol oxidase [13,14] has been reported previously, but this is the first report containing evidence that PPO from *P. somniferum* exists in multiple forms. At least four forms are evident on PAGE. Two forms of PPO were obtained in a high degree of purity, whereas the other two forms could not be purified. The finding in this study of the multiple forms of PPO raises questions regarding differences in their properties and the relationship of these properties to their physiological role. Although such differences might be physiologically significant, the establishment of this requires further evidence.

Although many attempts have been devoted to obtaining homogenous PPO from various plant sources, there is no evidence in the literature [1] that this has been accomplished. Difficulty in securing homogenous enzymes results from the considerable heterogeneity of PPO and rapid loss of activity during purification. In our case, our attention was directed to the separation of some of these heterogenous forms of PPO and exploration of some of its properties. Further purification after Sephacryl S-200 was very difficult due to rapid loss of activity.

During preparation and purification, the addition of sodium ascorbate was found to be superior to polyvinylpyrrolidone (PVP), protamine sulfate or acetone powder for recovery of PPO-activity. This enable recovery of two forms of PPO in amounts that allowed characterization and comparison of the two PPO. After ammonium sulfate precipitation, the total PPO-activity was greater than the crude homogenate. The reason may have resulted from the removal of some inhibitors such as phenolic compounds in the crude preparation. Both PPO-A and B have a broad pH maximum from 6.5 to 7.2 as well as similar Michaelis Menten constants, much lower than the  $K_m$ -value for PPO isolated from latex or the whole plant of *P. somniferum* [5].

Peroxidase from seedlings of *P. somniferum* did not catalyze oxidative ring closure of (–) reticuline to salutaridine [7]. The PPO described here may be involved in the oxidative steps in morphine alkaloid biosynthesis. The direct demonstration of PPO in the biosynthesis of opium alkaloids is not presented here, however, our preliminary data indicate that both purified PPO can each metabolize [<sup>3</sup>H]morphine to morphine N-oxide.

The best substrate for both PPO-A and B was catechol. PPO from latex or the whole plant of *P. somniferum* was more active against dihydroxyphenylalanine (DOPA) or dopamine than catechol [5].

Sodium cyanide and  $\beta$ -mercaptoethanol inhibited PPO-activity very effectively. The mode of action of inhibitors on PPO, however, is far from clear.

PPO contains  $\text{Cu}^{2+}$  [26], and cyanide and DIECA are  $\text{Cu}^{2+}$ -chelating agents [1]. With mushroom catechol oxidase, however, cyanide was competitive to molecular oxygen, a participant in the reaction [16]. The inhibition of PPO by  $-\text{SH}$  compounds and other reducing agents is well documented [17]. Some  $-\text{SH}$  compounds appear to react with the enzyme's  $\text{Cu}^{2+}$ , but reducing agents can affect the enzyme reactions in several ways [1]. For example, reducing agents may act on the quinoid products of PPO-activity and convert them non-enzymatically back to the phenolic precursors, thus decreasing the amount of product and increasing the amount of substrate without involvement with the enzyme. Ascorbic acid is a reducing agent, but our preliminary results suggest that it functions at least in part as a competitive inhibitor of PPO from *P. somniferum*.

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