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PEROXIDASES OF *PAPAVER SOMNIFERUM*

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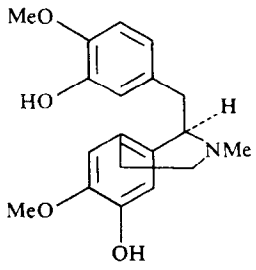
Key Word Index—*Papaver somniferum*; Papaveraceae; poppy, peroxidase; alkaloids; reticuline; IAA.

Abstract—Extracts of *Papaver somniferum* that had peroxidase activity were ineffective in catalysing oxidation of reticuline. Two peroxidases were purified from young seedlings and their properties examined. Only one of them was active toward indole-3-acetic acid (IAA).

INTRODUCTION

The enzymology of the oxidative ring-closure step that converts tetrahydrobenzylisoquinoline precursors to either aporphine or morphine alkaloids has remained obscure despite many attempts to elucidate this reaction. A chemical mechanism for oxidative phenol coupling first proposed by Barton [1] has been generally accepted as a model for the process; and both enzymatic and chemical oxidation have been successful in transforming certain tetrahydrobenzylisoquinolines to aporphines [2–5]. The enzymes used, however, were not obtained from alkaloid-containing plants. Neither chemical nor enzymatic processes have been used to synthesize the morphinan nucleus. Both young seedlings of *Papaver somniferum* and the latex of mature plants undoubtedly synthesize morphinan compounds from the precursor (−)-reticuline (**1**), and the first identified product is (+)-salutaridine (**2**) [6–8].



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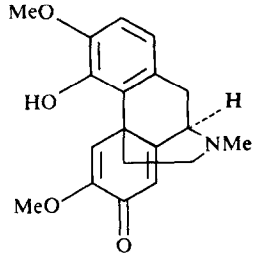

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Although enzymes of either the peroxidase type (EC 1.11.1.7) or laccase type (EC 1.10.3.2) appear to be likely candidates for catalysing such a reaction, no peroxidase activity has been found in latex of *P. somniferum*, and phenol oxidizing preparations from this source are inactive toward (−)-reticuline [9]. The present work was intended to show whether suitable oxidizing enzymes might be present in young seedlings. Peroxidases were found to be present in seedlings at a time when morphinan alkaloids are being synthesized but characterization of them did not indicate any role in alkaloid formation.

RESULTS AND DISCUSSION

The initial extracts of 10-day-old seedlings were brown-colored and browning could not be prevented by homogenizing in the presence of Polyclar-AT or removed by extensive dialysis. Extracts from both etiolated and light-grown seedlings showed peroxidase activity by the *o*-dianisidine assay within a few days of planting.

Electrophoretic analysis of the extracts on polyacrylamide gels gave the best resolution in acidic buffer (pH 4.5) rather than the customarily used basic buffers. An ontogenetic study of the peroxidase pattern in developing seedlings showed no peroxidase bands at first, then the appearance of one band (B) at *ca* 5 days in both etiolated and light-grown plants. In the latter there was no further change through 19 days; but in etiolated seedlings by 14 days a new slower-moving band (A) appeared; and the first band diminished in intensity. Since there appeared to be no qualitative or quantitative difference in peroxidase activity between etiolated and light-grown plants, etiolated plants were used in the purification experiments.

Three column chromatography steps were used: the first on Sephadex G-150 gave a large central peak of activity (B) which was then chromatographed on CM-Sephadex. Elution of the latter column resulted in most activity being recovered before starting the KCl gradient with some additional activity appearing at about 0.13 M KCl. Only the major peak was used for the final chromatography on DEAE Sephadex, and again two peaks of activity were found. The first of these (P_s-2) represented the most highly purified peroxidase obtained in this work (Table 1).

PAGE analysis was done at each stage of enzyme purification. In addition to the two peroxidase bands seen in the original extract a third, faster-moving band (C) appeared after the protamine fractionation step. The fraction retained by CM-Sephadex (P_s-CM) contained only band C. The fraction that was not

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Table 1. Purification of peroxidases from *Papaver somniferum*

Step	Volume (ml)	Total activity	Protein (mg/ml)	Yield (%)	Specific activity
Crude	452	8340	41	100	0.45
Protamine sulfate	452	7200	16	87	1
30% $(NH_4)_2SO_4$ supernatant	452	6100	11.7	74	1.16
80% $(NH_4)_2SO_4$ precipitate	25	2250	35	27	2.56
G-150 Sephadex peak (B)	16	1696	30	21	3.5
CM-Sephadex eluant	9	531.0	53.5	6.4	1.1
CM-Sephadex peak (P_s-CM)	1	10.8	2.4	0.13	4.5
DEAE eluant (P_s-2)	2	438	1.2	5.3	183

retarded on CM-Sephadex contained A and B. The non-retarded material from DEAE-Sephadex (P_s-2) showed only band B. The fraction retarded on DEAE-Sephadex presumably represented band A, but not enough could be recovered for further characterization. In no case did a band of peroxidase activity coincide with a discrete band of protein detected by protein stains.

The appearance of new peroxidase entities in the course of fractionation makes correlation of final fractions with the peroxidase bands found in the first extracts difficult. Unaccounted for are the early and late peaks of activity eluted from the Sephadex G-150 column. All three of the electrophoretic bands present in material applied to the G-150 column were present in the one central fraction from this column that was selected for further work-up. The three fractions perhaps represent degrees of aggregation that coalesced to a single species under the conditions of acidic gel electrophoresis.

MW estimation was done for fraction P_s-2 by SDS gel electrophoresis and yielded an approximate value of 3.2×10^4 daltons. Although this is lower than the MW of horseradish peroxidase, it is similar to peroxidases from some other plants [10].

The activity of crude and dialysed extracts of *P. somniferum* seedlings toward the oxidation of (±)-reticuline was assayed in several different ways. The hypothetical first morphinan structure produced by oxidative ring closure is salutaridine (2), and this conversion would be signalled by a shift in the absorption spectrum from a strongly absorbing peak at 280 nm to two peaks at 240 (log ϵ 4.25) and 277 nm (log ϵ 3.75). Under no conditions was any such spectral change detected over time periods up to several hours. A second method of detecting any salutaridine that might be formed depended on its reaction with dinitrophenylhydrazine in basic solution to give a hydrazone absorbing strongly (log ϵ 3.57) at 480 nm [11]. Application of this procedure also showed no formation of salutaridine up to 3 hr of incubation time. In a third approach 25 ml of seedling extract was incubated for 13 hr with (±)-reticuline-*N*-[^{14}C]methyl (11 900 cpm), with and without H_2O_2, at pH 6.0. Aliquots were chromatographed, and no alkaloids other than starting reticuline were revealed. However, radioactivity measurement showed a shifting of ^{14}C towards lower R_f values. This shift was not affected by the presence or absence of H_2O_2 was therefore not due to a peroxidase reaction, nor did the region of increased radioactivity coincide with salutaridine or thebaine. There is therefore no evidence that this cell-free extract from plants that make the latter two alkaloids from reticuline is able to effect the conversion. Oxygen measurements with the highly purified peroxidases designated P_s-CM and P_s-2 (Table 1) also showed no metabolism of reticuline whether or not H_2O_2 or 2,4-dichlorophenol were present.

Kinetic studies on the two purified peroxidases used *o*-dianisidine, pyrogallol, and IAA as substrates. With the first two substrates there was activity with no addition other than H_2O_2; but for IAA oxidation 2,4-dichlorophenol at 1.8×10^{-5} M was also required: K_m values for the two enzymes and several substrates are given in Table 2. All plots of velocity vs substrate concentration were hyperbolic except for slight inhibition of P_s-2 at *o*-dianisidine concentrations above 10^{-3} M. The most striking difference between the two enzyme fractions was the complete absence of activity toward IAA exhibited by P_s-CM. Even if the concentration of P_s-CM was increased to seven-fold the amount used in dianisidine assays no activity toward IAA was seen. With P_s-2 and *o*-dianisidine as substrate in 0.1 M potassium phosphate–citrate buffer the pH optimum was pH 5.5, half maximal at 4.4 and *ca* 7.5. With IAA as substrate the pH optimum was 5.0, half maximal at 3.7 and 6.2.

In conclusion, although young etiolated seedlings of *Papaver somniferum* synthesize morphinan alkaloids [7] and were shown in the present work to

Table 2. K_m values for peroxidases from *Papaver somniferum*

Substrate	Peroxidase*	
	P_s-2	P_s-CM
o-Dianisidine	1.1×10^{-4} M	4×10^{-5} M
H_2O_2 (with pyrogallol)	2.9×10^{-4} M	1.1×10^{-3} M
Pyrogallol	2.3×10^{-4} M	1.6×10^{-3} M
IAA	3.2×10^{-5} M	No activity
H_2O_2 (with IAA)	5.5×10^{-5} M	No activity

contain peroxidases, no indication could be found that the peroxidases function as catalysts in the oxidative ring closure of (−)-reticuline (**1**) to salutaridine (**2**). Characterization of two of the peroxidases showed that both were active toward *o*-dianisidine and pyrogallol, but only one was active with IAA as substrate.

EXPERIMENTAL

Seeds of *Papaver somniferum* were obtained from the USDA, Fort Collins, Colorado (Lot B40-Li-5b). Reticuline was synthesized by published methods [12, 13]. For radioactive reticuline the same synthetic procedure was followed with the substitution of [^{14}C]methyl iodide at the *N*-methylation step. *o*-Dianisidine obtained from Nutritional Biochemicals was recrystallized several times from hot EtOH. Oxygen consumption reactions were measured with the Gilson Oxygraph. Radioactivity measurements were made in the Packard Model 3310 scintillation counter. PAGE used the Buchler apparatus.

Growth of plants and extraction. Seeds were planted in sterile sea sand to a depth of 1–2 mm and kept watered with sterile H_2O supplied through fiberglass wicks. Growth was either at a constant temp. of 29° or alternating 12 hr periods at 29 and 21°. Plants were harvested 10 days after planting, when average height was 2–3 cm. They were washed first in tap H_2O and then with cold deionized H_2O. They were blotted dry, weighed and homogenized with an equal weight of cold 0.1 M KPi buffer, pH 8.0 for 60 sec. The homogenate was filtered through 4 layers of cheesecloth, adjusted with 2 N NaOH to pH 7.5 and centrifuged at 27 000 *g* for 30 min. Centrifugation and all subsequent fractionation steps were performed at 4°.

Enzyme purification. 1% solid protamine sulfate was added and stirring continued for 30 min. The solution was centrifuged at 20 000 *g* for 30 min and the dark brown pellet discarded. Solid $(NH_4)_2SO_4$ was added slowly to the supernatant soln to 30% saturation. Stirring was continued for another 30 min and the soln then centrifuged. The ppt. was discarded and the supernatant soln brought to 80% saturation. After stirring overnight the ppt. was collected by centrifuging, redissolved in a minimal amount of 0.01 M KPi buffer, pH 6.0 and chromatographed on Sephadex G-150 using the same buffer. The major peak of activity from this column with a protein content of *ca* 2 mg/ml was chromatographed on CM-Sephadex in 0.01 M KPi buffer pH 6.0, containing 0.05 M KCl. After all colored material had been eluted, a linear gradient of KCl up to 0.75 M was started. The most active fraction was concentrated in a dialysis bag surrounded with dry Sephadex G-200 and chromatographed on a column of A-25 DEAE Sephadex with 0.01 M KPi buffer, pH 8.0.

Gel electrophoresis. At the various stages of enzyme purification samples were analysed by PAGE on acidic, 7.5% gels at pH 4.5 [14]. Protein bands were detected with Coomassie Brilliant Blue or Amidoschwarz. Peroxidase activity bands were detected with 0.003% H_2O_2 and 1% *o*-dianisidine. For MW determination electrophoresis was performed by the method of ref. [15].

Enzyme assay and kinetics. Peroxidase activity was assayed routinely with *o*-dianisidine and H_2O_2 [16]. In some expts activity of the enzyme preparation was also measured with pyrogallol as substrate by monitoring the increase in A_{410}. The spectrophotometric assay for IAA oxidation was found to be insufficiently sensitive, and so an oxygen electrode procedure was used. For kinetics measurements spectrophotometric assays (*o*-dianisidine and pyrogallol) were done at 29° or 30° ± 0.1°, and oxygen electrode assays (IAA) at 25° ± 0.1°. H_2O_2 concn was determined by titration just before each expt [17] and was normally in the range 5×10^{-5}–10^{-4} M in the assay mixture.

Protein measurement. Protein was routinely determined by $A_{280/260}$ measurement [18] although this method gives spurious values at the initial stages of enzyme purification because of the presence of phenols.

Paper chromatography. Alkaloids were analysed by ascending PC on sheets buffered with Pi–citrate at pH 6.5 and water-saturated *n*-BuOH as the developing solvent [19]. Spots were detected with Dragendorff reagent [20] and gave R_f values: reticuline 0.35, thebaine 0.45, salutaridine 0.72. In experiments with ^{14}C-labelled compounds radioactivity was detected by cutting the chromatogram at intervals of 0.5 R_f unit and measuring radioactivity in each section by scintillation counting.

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