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OXIDATION OF TYROSINE BY *PAPAVER SOMNIFERUM* LATEX*

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Abstract—The polyphenolase complex isolated from the organelles which sedimented at 1000 *g* from the latex of *Papaver somniferum* was found to be composed of soluble and membrane-bound fractions Partial purification resolved two polyphenolases, only one of which utilized tyrosine, a probable precursor of the alkaloid morphine Activity of these two polyphenolase fractions was shown to change during the development of the capsule

INTRODUCTION

PREVIOUS WORK[1] has shown that the whole of the polyphenolase activity of the opium poppy, *Papaver somniferum* var Halle, is located in organelles which sediment at 1000 *g* in which Fairbairn[2,3] has demonstrated alkaloid biogenesis Recently Matile[4] has isolated organelles from *Chelidonium majus* latex which appear to contain alkaloids, and also enzymes which are characteristic of lysosomes However, *P somniferum* latex 1000 *g* organelles do not contain lysosomal enzymes which are found in the "supernatant" (the rest of the latex other than the 1000 *g* organelles[5]) This is in contrast to *Chelidonium* latex where the catecholase activity was found in the supernatant and not in the alkaloid-containing lysosomes Since polyphenolases have been implicated in the biogenesis of the opium alkaloids,[6] detailed studies[1 7] of polyphenolase in opium poppy have shown that this enzyme oxidized a number of substrates including catechol, *p*-cresol, tyrosine and DOPA The fact that this enzyme complex oxidizes tyrosine, a probable precursor of morphine, was of particular interest The experiments[1] also demonstrated that polyphenolase activity, whilst negligible in intact 1000 *g* organelles was readily released by mechanical disintegration, such as freeze-thawing or sonication, and also by exposure to the surface-active agent, Triton X100 This detergent has been used by other workers for the activation and solubilization of latent enzymes, particularly those of lysosomes [8–10] During the course of these experiments[1] inexplicable variations in the polyphenolase of the 1000 *g* organelles were observed and the present work therefore considers the

* Part II in the series 'Enzymic Studies with *Papaver somniferum*" For Part I see ROBERTS, M F (1971) *Phytochemistry* **10,** 3021

[1] ROBERTS, M F (1971) *Phytochemistry* **10,** 3021
[2] FAIRBAIRN, J W, PALMER, J M and PATERSON A (1968) *Phytochemistry* **7,** 2117
[3] FAIRBAIRN J W and DJOTE M (1970) *Phytochemistry* **9,** 739
[4] MATILE, PH, JANS, B and RICHENBACHER R (1970) *Biochem Physiol Pflanzen* **161,** 447
[5] ROBERTS, M F and ANTOUN M to be published
[6] BARTON, D H R and COHEN, T (1957) *Festschrift A Stoll, Basel* 117
[7] ASGHAR, A S and SIDDIQI, M (1970) *Enzymologia* **39,** 289
[8] BENDALL, D S and DE DUVE, C (1960) *Biochem J* **74,** 444
[9] BEAUFAY, H and DE DUVE, C (1959) *Biochem J* **73,** 604
[10] PUNJARNISCLE, A (1968) *Physiol Veg* **6,** 27

variations in enzyme activity during the development and ripening of the poppy capsule, together with the effect of nonionic detergents on the release and activation of the membrane bound enzyme A partial purification of the soluble and membrane-bound polyphenolase of the latex 1000 *g* organelles has been made in an attempt to study further the polyphenolase activity with the substrate tyrosine

RESULTS AND DISCUSSION

The changes in enzyme activity with pH show a broad peak of major activity at pH 8 0 with a small shoulder around pH 5 0 Enzyme activity measured using conventional manometric techniques and catechol as the principal substrate was several fold higher that the catecholase activity of apple chloroplasts,[11] but lower than the activity recorded for grape catechol oxidase [12] However unlike grape catecholase, poppy latex polyphenolase showed greater activity with *p*-cresol than with catechol

Most of the polyphenolase activity of the poppy latex sedimented at 1000 *g* was found to be readily released with the mechanical disintegration Part of the polyphenolase activity of these organelles was associated with the sediment obtained after mechanical disintegration and centrifugation at 10000 *g* and it was assumed that this activity was associated with the organelle membranes since treatment of this residue with 0 1% Triton X100 released most of the activity into solution

Partial purification of the soluble latex 1000 g organelle polyphenolase

Most of the alkaloid content of poppy latex is associated with the 1000 *g* organelles and can vary from 61 mg g latex at day 1 to 127 mg/g at day 15 from petal opening [13] The extraction and purification of the polyphenolase of the 1000 *g* organelles necessitates the early removal of these alkaloids and phenolic material The possibility of removing these substances from the organelles and at the same time affecting some purification of the enzyme was investigated using column chromatography After mechanical disintegration of the organelles from 3 ml of latex with sonication the insoluble material was removed by centrifugation at 10000 *g* The phenolics and alkaloids were removed using a Sephadex G25 column and the eluate treated as described in the Experimental Of the total activity 97% was recovered in the supernatant and 90% of this supernatant activity was recovered between 50 and 80% saturation with ammonium sulphate The eluate after treatment with Sephadex G25 was applied to a DEAE-Sephadex A25 column and two active fractions were obtained The first peak (fraction I) was eluted at 5 mM Tris (pH 8 0) soon after the void volume and prior to the application of a gradient Using a linear sodium chloride gradient a second peak (fraction II) was eluted at 90 mM This extraction procedure gave a 20 fold increase in polyphenolase activity (Table 1) The two fractions obtained were compared for substrate specificity and for variations in the activity of the fractions with the development and ripening of the capsule Fraction I was most active in the latex immediately after petal opening but 3 weeks later more activity was found in fraction II Eight days from petal opening an intermediate stage occurred in which increases in the activity of fraction II were observed particularly with DOPA and *p*-cresol as substrates Although

[11] Harel, E., Mayer A M and Shain Y (1965) *Phytochemistry* **4**, 783
[12] Harel E and Mayer A M (1971) *Phytochemistry* **10**, 17
[13] Hakim F private communication

most of the substrates are oxidized by both fractions, tyrosine was only oxidized by fraction I The detailed results are given in Table 2

TABLE 1 PURIFICATION OF SOLUBLE CATECHOL OXIDASE FROM *Papaver somniferum* LATEX 1000 *g* ORGANELLES

Step	Total protein (mg)	Sp act	Purification	Yield (%)
Disintegrated 1000 *g* organelles after dialysis	8 0	90		
Disintegrated 1000 *g* organelles after centrifuging at 10 000 *g* and treatment with Sephadex G25	2 4	290	3 2	97
50–80% saturated ammonium sulphate ppt	1 3	703	7 7	90
DEAE Sephadex A25 eluate (5 mM fraction)	0 32	1840	20	69

Solubilization of the membrane-bound polyphenolase with surface active agents

In previous work[1] with the poppy latex 1000 *g* organelles, 0 1% Triton X100, which is a non-ionic detergent effective in the solubilization of enzymes[8,10,11,14] was used to disintegrate the organelles Large increases in polyphenolase were observed, particularly in experiments where 1000 *g* organelles were pretreated overnight with this detergent Most of the increased activity may be attributed to the release of soluble polyphenolase from the organelles However, greater activity was observed than that obtained with mechanical disintegration, and this could have been due either to the release of further activity which was membrane bound or to activation of soluble polyphenolase Two anionic surface active agents sodium dodecylsulphate (SDS) and sodium dioctyl sulphosuccinate (Manoxol OT) had been previously found to activate latent polyphenolase in broad bean [15]

TABLE 2 VARIATIONS WITH CAPSULE DEVELOPMENT OF LATEX POLYPHENOLASE FRACTIONS FROM A DEAE SEPHADEX A25 COLUMN

Days	Sample from petal opening	Substrate	Activity Crude Prep	†Ammonium sulphate ppt	Fractions from a DEAE Sephadex A25 column eluted with 5 mM Tris buffer pH 8 0 containing NaCl Fraction I	Fraction II
1	(Soluble enzyme)	Catechol	342	535	1416	132
		Tyrosine	-	50	400	0
		DOPA	—	812	1840	544
		p Cresol	- -	820	2360	500
8A	(Soluble enzyme)	Catechol	290	703	1840	232
		Tyrosine	—	40	424	0
		DOPA	—	942	2400	1228
		p Cresol	—	900	3496	1000
8B	(Membrane bound enzyme Triton X100 extracted)	Catechol	4 6*	5 2	0 58	75 8
		Tyrosine	—	--	0 70	0
		DOPA	—	3 0	3 3	111 2
		p Cresol	—	8 6	4 1	182
21	'(Soluble enzyme)	Catechol	300	1093	1800	2600
		Tyrosine	—	16	130	0
		DOPA	—	1200	1240	5000
		p Cresol	-	1550	1720	3000

Activity = μl O_2 absorbed/min/mg protein

* This value is for the ' membrane bound" enzyme in buffer suspension prior to extraction with 0 1% Triton X100

† Values for Samples 1, 8A, 21 were for protein precipitated with ammonium sulphate 50 to 80% saturation Sample 8B was protein precipitated with ammonium sulphate at 0–80% saturation

[14] WALKER, J R L (1964) *Australian J Biol Sci* **17,** 360

[15] KENTEN, R H (1958) *Biochem J* **68,** 744

In a study of the effect on latex polyphenolase activity of SDS, Manoxol OT and of Triton X100, the soluble and membrane-bound enzyme fractions of the 1000 *g* organelles prepared as described in the Experimental were treated with these detergents at a concentration of 0·1% for 1 hr prior to assay. Results given in Table 3 show that Triton X100 at this concentration does not significantly activate the polyphenolase of either the soluble or insoluble fractions of the 1000 *g* organelles in the presence of the substrates catechol and *p*-cresol. However, the detergents SDS and Manoxol OT showed very significant activation of the polyphenolase of the insoluble fraction. Since no activation was observed with Triton X100 this detergent was used in an attempt to solubilize the apparently "membrane-bound" activity of the 1000 *g* latex organelles. The residue obtained after disintegration of these organelles by sonication was further treated with 50 mM phosphate buffer (pH 7·0) containing 5 mM sodium metabisulphite and 0·1% Triton X100 for 1 hr. Two extractions of the residue were made and it was found after centrifugation that most of the polyphenolase activity resided in the Triton X100 extract. However, much of the protein extracted in this manner and some of the polyphenolase activity could not be separated from the Triton X100. The protein in these solutions after dialysis was concentrated by the addition of ammonium sulphate to 80% saturation. The protein so precipitated was redissolved in 5 mM phosphate buffer (pH 8·0) and applied to a

TABLE 3. THE EFFECT OF DETERGENTS ON THE ACTIVITY OF THE SOLUBLE AND INSOLUBLE POLYPHENOLASES OF THE 1000 *g* ORGANELLES OF *Papaver somniferum* latex

Substrate	Activity μl O_2 min ml latex	
	Catechol 10^{-4} M	*p*-Cresol 10^{-4} M
Soluble enzyme	113	226
Soluble enzyme + 0·1% Triton X100	150	236
Soluble enzyme + 0·1% SDS	155	237
Soluble enzyme + 0·1% Manoxol OT	194	284
Insoluble enzyme	14	15
Insoluble enzyme + 0·1% Triton X100	11	14
Insoluble enzyme + 0·1% SDS	24	120
Insoluble enzyme + 0·1% Manoxol OT	21	119

The protein content of the soluble and insoluble polyphenolase fractions was 1300 and 6000 μg ml latex respectively. Detergent was added to the reaction media such that the concentration with respect to the total volume was 0·1%.

Sephadex G25 column. The polyphenolase-containing eluate was applied to a DEAE Sephadex A25 column and, as with DEAE-cellulose columns, a large proportion of the extracted protein and some polyphenolase activity was eluted from both columns with the Triton X100. The majority of the polyphenolase activity was eluted from the DEAE Sephadex A25 column in a second peak with 90 mM sodium chloride. The detailed results are given in Table 2 (Sample 8B). These results suggest that most of the enzyme with tyrosine as substrate is soluble and that activity is greatest in the early stages (0–7 days) of capsule development. The existence of this activity with tyrosine as substrate at this stage may well play a significant role in the biosynthesis of the opium alkaloids from tyrosine. It is of interest to note that Fairbairn and Hakim[13] have shown that the incorporation of DL-3(3,4-dihydroxyphenylalanine-[2-^{14}C])(DOPA) into the total alkaloid fraction of poppy latex is most successful at this stage of development but no experiments with radioactive

tyrosine have yet been made The results also indicate that some changes in protein structure must occur with capsule development and ripening since a fall in overall activity of fraction I (Table 2) occurs during the 3-week period opening with a corresponding increase in activity developing in fraction II

EXPERIMENTAL

Latex of the opium poppy *Papaver sommiferum* var Halle was collected as described previously [1]

Extraction of polyphenolase from the latex 1000 g *organelles* The 1000 *g* organelles from 3 ml latex were isolated by centrifugation and the polyphenolase enzyme released by placing the organelles in 50 mM phosphate buffer (pH 7 6) containing 5 mM sodium metabisulphite followed by sonication for 1 min The samples were finally dialysed overnight Details of this dialysis procedure have been described elsewhere [1] Soluble and "membrane-bound" polyphenolase were separated by centrifuging the dialysed samples at 10000 *g* for 20 min The insoluble residue was washed with 2 × 2 ml phosphate buffer (pH 7 6) for each ml of latex used and the washings added to the supernatant solution which was made to a total of 6 ml for 1 ml of latex used The residue was finally resuspended in 4 ml phosphate buffer (pH 7 6) These two solutions after dialysis were used as the soluble and insoluble, polyphenolase preparations used in determining the effect of surface active agents

Purification of the soluble polyphenolase fraction The soluble polyphenolase prepared as above was applied to a Sephadex G25 column (2 × 60 cm) equilibrated with 5 mM Tris (pH 8 0) in place of the dialysis procedure The polyphenolase in the eluate (40 ml) was precipitated with $(NH_4)_2SO_4$ at 50–80% saturation The protein so obtained was redissolved in 5 mM Tris buffer (pH 8 0 1 ml) and desalted on a Sephadex G25 column (1 × 27 cm) equilibrated with the same buffer The resulting polyphenolase eluate (4 ml) was finally applied to a DEAE Sephadex A25 column (1 × 30 cm) Elution with 5 mM Tris buffer (pH 8 0) was maintained during the collection of the first 10 × 4 ml fractions A linear gradient of 5-600 mM NaCl (150 ml) was then applied to the column Fractions were collected using an automatic LKB fraction collector with UV monitoring device The linearity of the gradient was determined by quantitative estimation of the NaCl in the various fractions The polyphenolase activity of the eluate was estimated semi-quantitatively as follows To 1 ml eluate, 3 ml 5 mM Tris buffer (pH 8 0) and 0 5 ml 1% catechol soln were added The mixture was incubated for 10 min at room temp and Absorbance was measured at 410 nm Protein was determined according to Lowry *et al* [16]

Solubilization and ion-exchange chromatography of the membrane-bound fraction The residue after sonication and centrifugation of the latex 1000 *g* organelles was extracted with 2 × 6 ml of 5 mM phosphate buffer (pH 7 0) containing 5 mM sodium metabisulphite and 0 1% Triton X100 over a period of 90 min The extracts were centrifuged at 10000 *g* for 20 min and after dialysis $(NH_4)_2SO_4$ was added to 80% saturation The resulting ppt was redissolved in 5 mM Tris buffer (pH 8 0, 1 ml) and treated according to the procedure used for the ion-exchange chromatography of the soluble fraction

Measurement of polyphenolase activity The activity of the various polyphenolase preparations was measured by manometry at 30° as μl O_2 absorbed/min/mg of protein except where samples with the same protein content are compared Unless otherwise stated the enzme (0 2 ml) was placed in the side arm of the reaction vessel and the other components (to a total of 3 ml) in the main compartment These included, where applicable, the substrate 10^{-4} M (0 2 ml) detergent 0 1% (0 1 ml) and 50 mM phosphate buffer (pH 7 6) Controls were used which were without the enzyme solution

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[16] LOWRY, O H, ROSEBROUGH, N J, FARR, A L and RANDALL, R J (1951) *J Biol Chem* **193**, 265