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THE MULTIPLICITY OF PYRIDINIUM OXIDASES IN *RICINUS COMMUNIS*

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Abstract—The crude extract of pyridinium oxidizing enzymes from *Ricinus communis* seedlings was resolved into three enzyme entities by DEAE column chromatography Their optimal pH, temperature stability, activation energies and substrate specificities are similar Analysis of their enzymatic products have shown that each of these enzymes catalyses the oxidation of 1-methyl-nicotinonitrile perchlorate to form 4- and 6-pyridones

INTRODUCTION

In the previous papers[1,2] we have reported that at least seven species of the family Euphorbiaceae have shown pyridinium oxidizing activity on 1-methyl nicotinonitrile perchlorate Furthermore, the finding of a pyridone-forming enzymatic system in *Trewia nudiflora* correlates with its pyridone alkaloid, nudiflorine. This observation suggests that this enzyme system is probably phylogenetically specific It also implies that this pyridinium oxidase might play a significant role in the biogenesis of pyridine alkaloid and the pyridine nucleotide cycle of NAD metabolism.[3] Purification and characterization of the crude enzyme extracts from *Ricinus communis* have been undertaken, and some of the results are discussed here.

RESULTS

Separation of Three Pyridinium Oxidases

Table 1 shows the purification scheme for one experiment. The purification achieved by isoelectric point precipitation was usually about 50-fold, this being one of the major purifying steps. The resultant supernatant was very clear. After dialysis, the clear dialysed enzyme solution was applied to a DEAE-cellulose column, and by means of gradient elution (0·05–0·4 M phosphate buffer) pyridinium oxidases A, B and C were clearly resolved. (Fig. 1) Only inactive protein was eluted with 0·01 M phosphate buffer. A consistent

[1] P Fu and T Robinson, *Phytochem* 9, 2443 (1970)
[2] P Fu and T Robinson, *Abstracts*, Northeastern Section of the American Society of Plant Physiologists at Harvard University, Cambridge, Massachusetts (1967).
[3] T Robinson, *Phytochem* 4, 67 (1966)

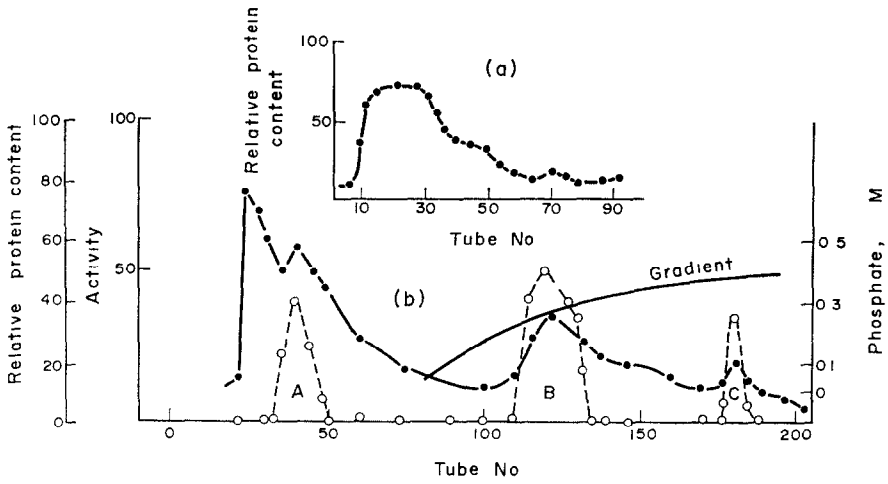

Fig 1 Separation of pyridinium oxidases A, B and C on DEAE cellulose chromatography

A 300-ml (4 6 mg/ml) dialysed enzyme solution was applied to a 2 5 × 50 cm column The relative protein content was measured by UV absorbance The enzyme assay was defined according to Robinson [3]

(a) Elution with 0 01 M potassium phosphate buffer No activity detected Fractions collected were 15 ml/5 min/tube (b) Separation by gradient elution Fractions collected were 6 ml/2 min/tube (●) Protein profile, (○) Pyridinium oxidaae activity

pattern was invariably observed among many experiments on DEAE-cellulose The inclusion of a proteolytic inhibitor such as phenylmethylsulfonylfluoride did not alter either the elution pattern or the relative amount of these three enzymes Gel filtration showed that

Table 1 Purification of pyridinium oxidases from seedlings of *Ricinus communis*

Procedure	Volume (ml)	Activity per ml	Total activity	Specific activity	Yield (%)	Purification
Whole extract	517	30	15,510	0 65		1
Supernatant 12,000 rev/min, pH 8 5	517	30	15,510	3		4
Dialysed supernatant pH 8 5	517	150	77,550	14	100	22
Supernatant I E P pH 6 3	480	150	72,000	33	93	50
DEAE column						
Enzyme A	42	17	693	7	0 9	11
Enzyme B	425	35	14,875	80	19 2	112
Enzyme C	220	35	7700	103	9 9	158
			23,268		30 0	
Sepharose 4B column						
Enzyme A	9	20	180	63	0 4	99
Enzyme B	45	150	6750	652	8 7	1000
Enzyme C	33	100	3280	278	4 1	430
			10,210		13 2	

The protein content was determined by absorbance at 280 and 260 nm The three enzyme activities were measured by difference spectrum (see Experimental) Activity was defined according to Robinson [3]

active enzyme fractions of enzymes A, B and C came out through the void volume of Sephadex G25, G75, G150 and G200.[4] A similar elution pattern of pyridinium oxidases A, B and C was again obtained by using Biogel 5A and Sepharose 4B The gel filtration step usually gave a 500–1000-fold purification It is important to note that the degree of purification is dependent upon the number of fractions pooled and the stability of the purified enzyme The total activity was observed to have increased after dialysis

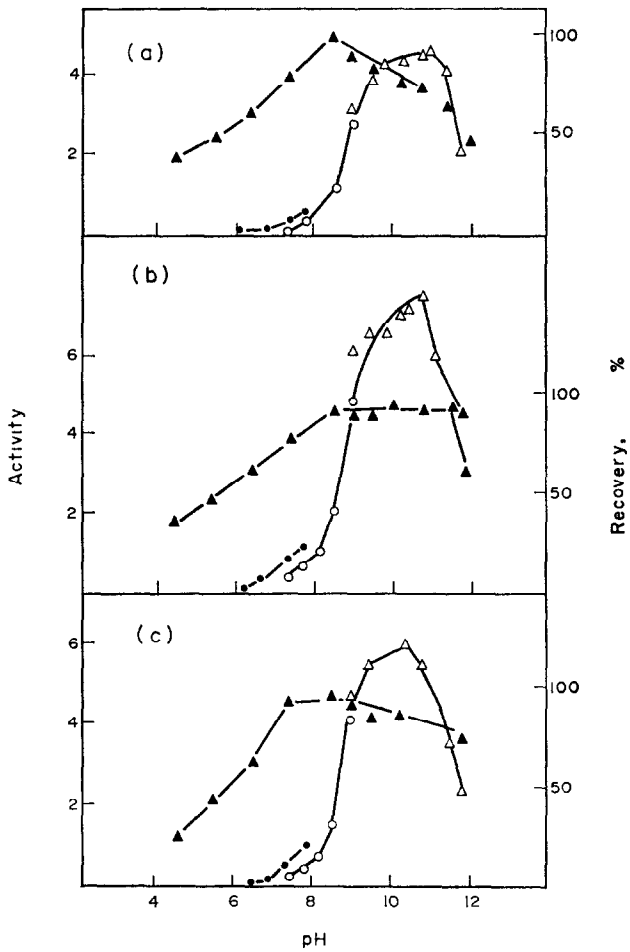

FIG 2 THE EFFECT OF pH ON THE ACTIVITY AND STABILITY OF PYRIDINIUM OXIDASES A, B and C The enzyme reaction mixtures incubated at the indicated pH values were measured spectrophotometrically at 255 nm as described in the Experimental To test the effect of pH on stability the enzymes were exposed to the pH values given and the activity was then tested at pH 9 65 (●) 0 05 M Potassium phosphate buffer 10^{-3} M EDTA, (○) 0 05 M Tris-HCl buffer, 10^{-3} M EDTA, (△) 0 05 M sodium carbonate buffer, 10^{-3}M EDTA, (▲) per cent activity recovery

[4] P Fu, Ph D Thesis. University of Massachusetts (1969)

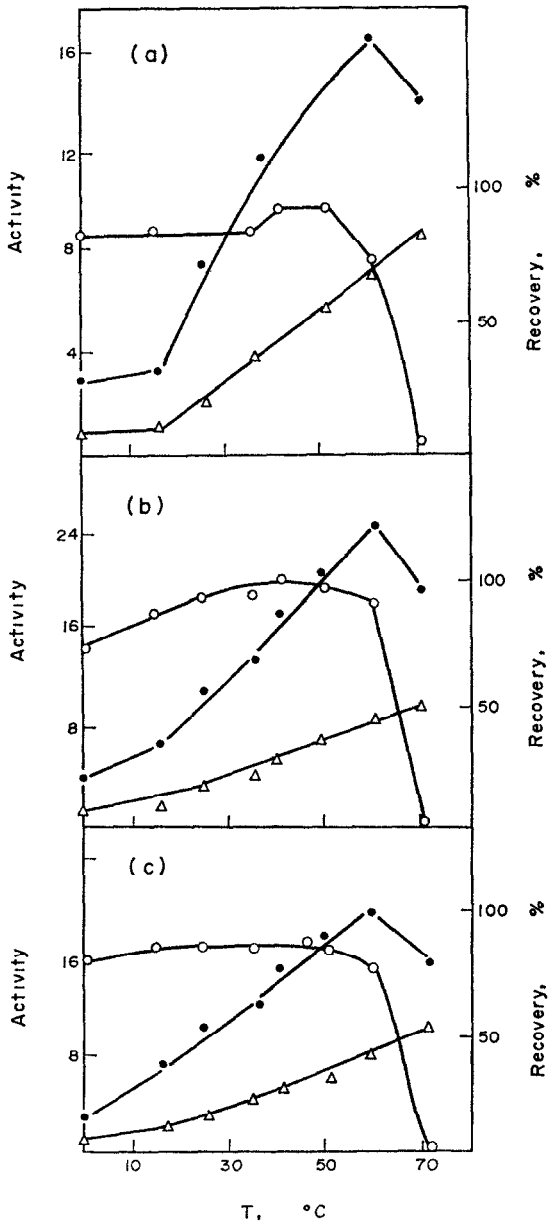

FIG. 3 THE EFFECT OF TEMPERATURE ON PYRIDINIUM OXIDASE A, B AND C

The enzymes and the reaction mixture were preincubated at the indicated temperature for 5 min. The activity was assayed spectrophotometrically immediately after adding the enzyme solution. To test the effect on enzyme stability the enzymes were exposed to a given temperature for 2 hr before measuring activity at 40°

Δ) Activity at 1 min, (●) activity at 3 min, (○) per cent stability

Properties of Pyridinium Oxidases A, B and C

Effect of pH. The optimal activity for pyridinium oxidases A, B and C was between pH 9·5 and 10·5. The activity above pH 11 and below 9·5 fell sharply. Pyridinium oxidases A, B and C appeared fairly stable in the pH range from 8·0 to 10·5. This is shown in Fig. 2.

Effect of temperature The initial velocity increased steadily as the temperature was elevated. The optimal temperature obtained from the initial velocity was 70°, however, such optimal temperature shifted to 60° if the activity was assayed 3 min after the enzyme solution was introduced Pyridinium oxidases A, B and C were fairly stable from 10° to 60°. This is shown in Fig 3. Based on the initial velocity with respect to various temperatures, activation energies of 5940, 5470 and 6150 cal/mole were obtained for pyridinium oxidase A, B and C respectively

Substrate specificity The ability of aldehyde oxidase and xanthine oxidase to oxidize a large variety of substrates such as aromatic quaternary nitrogen compounds, purines and several aldehydes is well documented.[5] The pyridinium oxidases A, B and C, with an average of 500- to 1000-fold purification were used to test for activity with various substrates (Table 2) A trace of xanthine oxidase activity was observed only for pyridinium oxidase A

TABLE 2 SUBSTRATES OXIDIZED BY PYRIDINIUM OXIDASES A, B AND C

pH 7 3	pH 9 5
1-Methyl-3-cyano-pyridinium iodide*	1-Methyl-3-cyano-pyridiniumperchlorate
Acetaldehyde	1-Methyl-3-aldehyde-pyridinium iodide
Benzaldehyde	1-Methyl-3-acetyl-pyridinium iodide
Xanthine†	1-Ethyl-3-cyano-pyridinium bromide
	1-Benzyl-3-cyano-pyridinium chloride
	1-Methyl-nicotinamide iodide‡

* Nonenzymatic reaction observed at pH 9 5.
† Trace activity seen in pyridinium oxidase A
‡ Trace activity seen

at pH 7·3 but not at pH 9·5, and pyridinium oxidase B and C showed no oxidation of xanthine. In this case, this would be the only difference with respect to substrate specificity found among pyridinium oxidases A, B and C A trace of activity in the oxidation of 1-methyl nicotinamide iodide by the pyridinium oxidases was observed In this study of substrate kinetics for pyridinium oxidases 1-methyl nicotinonitrile perchlorate was used as substrate, incubating at 40° for 5 min before addition of the enzyme. Similar K_m values were found for pyridinium oxidases A, B and C, respectively $6{\cdot}65 \times 10^{-4}$ M, $5{\cdot}0 \times 10^{-4}$ M and $5{\cdot}0 \times 10^{-4}$ M Substrate analogues such as trigonelline sulfate, pyridine methiodide, quinoline methiodide, nicotinic acid, nicotinonitrile, 1-methyl-3-cyano-2-pyridone, 1-methyl-3-cyano-4-pyridone and 1-methyl-3-cyano-6-pyridone were not oxidized by pyridinium oxidases. The failure to oxidize trigonelline and pyridine methiodide indicated the necessity of a strong electron withdrawing group at the 3 position There appeared to be no further oxidation on 1-methyl-3-cyano-2, 4 and 6 pyridones. Nicotinonitrile itself did not serve as a substrate for pyridinium oxidase. This further emphasized the importance of a quaternary nitrogen in the ring for enzymatic oxidation

[5] L GREENLEE and P HANDLER, *J Biol Chem* **239**, 1090 (1964)

Identification of Products of Oxidation of Quaternary Compounds

Robinson[3] had previously reported that castor bean pyridinium oxidase catalysed the hydroxylation of the ring of quaternary heterocyclic compounds, yielding pyridones, yet the question remained whether a single enzyme catalysed the formation of 4- and 6-pyridone The purified pyridinium oxidases A, B and C were separately incubated with substrate The oxidation products which were further separated by TLC on silica gel showed compatible R_f values [1] This strongly suggests that pyridinium oxidases A, B and C all catalysed a similar oxidation process on 1-methyl-nicotinonitrile perchlorate

TABLE 3 EFFECT OF VARIOUS INHIBITORS ON PYRIDINIUM OXIDASES A, B AND C

Inhibitors	Concentration (M)	Inhibition (%) A	B	C
p-Chloromercuriphenylsulfonic acid	2.5×10^{-4}	56	51	75
Phenylmercuric nitrate	10^{-5}	96	97	95
Mercuric chloride	10^{-4}	96	100	95
Iodoacetate	2×10^{-3}	72	56	58
Benzaldehyde	10^{-5}	4	17	4
Quinacrine	3×10^{-5}	77	46	80
Methanol	10^{-3}	70	70	65
Sodium cyanide	10^{-6}	88	100	96

Effect of Various Inhibitors on Pyridinum Oxidase A, B and C

Various recognized inhibitors were tested with the pyridinium oxidases Table 3 summarizes our findings Comparable results were observed among all three preparations. Sulfhydryl inhibitors in general exerted a rather strong inhibition. Of particular interest were the findings with quinacrine, methanol and sodium cyanide, since they have been reported to inhibit aldehyde and xanthine oxidases [6,7] Inhibition due to benzaldehyde might have resulted from a competitive inhibition effect since benzaldehyde acted also as a substrate

DISCUSSION

The crude extract from *Ricinus communis* seedlings was resolved into three active enzyme entities, namely the pyridinium oxidases A, B and C Table 1 summarizes the results of a typical purification procedure Prolonged blending in the extraction step was not desirable since it gave a large quantity of impurities which not only made the protein determination difficult, but also strongly inhibited the enzyme itself This was shown by an increment of total enzyme activity after dialysis Sephadex G200, Biogel 5A and Sepharose 4B gave similar resolution in purification An approximately 500- to 1000-fold purification was achieved after gel filtration, and yet the percentage yield of the active protein resulting from the various steps was still rather low

[6] K V RAJAGOPALAN, I FRIDOVICH and P HANDLER, *J Biol Chem* **237**, 922 (1962)
[7] J HURWITZ, *J Biol Chem* **212**, 757 (1955)

The occurrence of multiple molecular forms of an enzyme is now well documented,[8,9] more than 100 enzymes having been reported to exist isozymic forms This multiplicity not only extends to many enzymes but is found in nearly all organisms [10] A recent review by Shannon[11] summarizes a number of isozyme systems in plants These three pyridinium oxidases A, B and C exhibited different behavior on DEAE cellulose columns suggesting that they are different protein molecules Alteration of elution method from gradient to linear or stepwise did not change their elution pattern, and their relative amounts remained constant These are illustrated in Fig 1 Multiplicity has been observed in xanthine dehydrogenase[12,13] and aldehyde dehydrogenase [14,15] Conversion from one form to the other has been reported in the aldehyde dehydrogenase system Glassman and Jakoby[13,15] related such phenomena to the purification process Although Jakoby showed that addition of esterase inhibitors in the enzyme preparations would abolish one peak seen in the DEAE column, in our preparations in the presence of esterase inhibitor all three peaks of enzyme activity were seen consistently These three pyridinium oxidases have many properties in common Results from gel filtration chromatography indicated that they have similar molecular weights Their optimal pH, pH stability, temperature stability and activation energies were not significantly different Their substrate specificities as well as kinetics were alike Preliminary experiments using xanthine as substrate at near neutral pH showed that only pyridinium oxidase A exhibited a trace of xanthine oxidase activity However, contamination of other enzymes in the pyridinium oxidase A fraction could not be excluded. Among these three enzymes pyridinium oxidase A appeared to be the least stable Analysis of products showed that all three enzymes catalysed the formation of 4- and 6-pyridones. The degree of inhibition of pyridinium oxidase activity by various inhibitors were nearly the same for these three enzymes This implies that pyridinium oxidases A, B and C perhaps have similar active sites The observed inhibition by quinacrine suggests that the pyridinium oxidases are possibly flavoproteins Thus far pyridinium oxidases A, B and C in many ways seem almost the same The question posed by this multiplicity is simply whether they constitute an isozyme system Moreover, do they come from a single tissue or from different ones? Further studies will be necessary in order to answer any one of these questions.

EXPERIMENTAL

Enzyme assay The substrate 1-methyl-3-cyano-pyridinium perchlorate and its analogues were synthesized by published methods with slight modifications[3] and preparation of the known pyridones of 1-methyl nicotinonitrile was reported by Robinson and Cepurneek [16] Other compounds used were analytical grade, obtained commercially Enzyme activity was routinely assayed as described previously [1] The appearance of products was measured spectrophotometrically either at 255 nm or at 282 nm at 40° 1-methyl-3-nitropyridinium iodide oxidation was followed at 300 nm at pH 7 5 in 0 05 M potassium phosphate buffer with 10^{-3} M EDTA Xanthine oxidation was assayed at 290 nm at pH 7 8 Acetaldehyde and benzaldehyde oxidation were measured spectrophotometrically at 600 nm by the reduction of dichlorophenolindophenol (2×10^{-4} M) 1-methyl nicotinamide oxidation was also measured spectrophotometrically at 300 nm at pH 7 5 in 0 05 M potassium phosphate buffer containing 10^{-3} M EDTA Protein was assayed

[8] E S Vessell, *Ed Ann N Y Acad Sci* **151** (1968)
[9] F. Wroblewski, *Ed Ann N Y Acad Sci* 94 (1961)
[10] C L Markert, *Ann N Y Acad Sci* **151**, 14 (1968)
[11] L M Shannon, *Ann Rev Plant Physiol* **19**, 187 (1968)
[12] E C Keller, P Saverance and E Glassman, *Nature, Lond* **198**, 286 (1963)
[13] T T Yen and E Glassman, *Genetics* **52**, 977 (1965)
[14] J K Raison, G Henson and K G Rienits, *Biochim Biophys Acta* **118**, 285 (1966)
[15] C R Steinman, W B Jakoby, *J Biol Chem* **242**, 5019 (1967)
[16] T Robinson and C Cepurneck, *Phytochem* **4**, 75 (1965)

either by a colorimetric procedure[17] or by the method of Warburg and Christian [18] Activity was defined as absorbance change at a designated wavelength per ml enzyme per min

Purification of enzyme pyridinium oxidase [5] Heat and isoelectric point precipitation The dialysed crude enzyme solution was rapidly warmed to 55° and after 10 min, the enzyme solution was cooled to 10–15° with continuous stirring The solution was centrifuged at 12,000 rev/min for 20 min near 0° The precipitate was discarded and the brown supernatant was adjusted to pH 6 3 by dropwise titration with 0 5 N HCl The dark suspension was centrifuged at 12,000 rev/min for 30 min The supernatant solution appeared clear pale yellow and was carefully adjusted to pH 6 7 by the addition of 0 5 N KOH The resultant clear enzyme solution was then dialysed overnight against 0 01 M potassium phosphate buffer (pH 6 7) containing 10^{-3} M EDTA

Ion exchange chromatography DEAE-cellulose (DE 11, Whatman) was treated with alkali and acid before equilibrating with an appropriate buffer solution Usually a 2 5 cm dia column was used for DEAE-cellulose Both stepwise and gradient elution were employed The ionic strength usually ranged from 0 01 to 0 4 M potassium phosphate In some cases, a gradient of both pH and ionic strength were carried out in the elution process Protein was monitored at 280 nm

Ammonium sulfate precipitation Enzyme grade, crystalline ammonium sulfate was added to the enzyme solution from the DEAE column The cloudy solution was allowed to stand in a refrigerator for 2 hr The precipitate from 20 to 70% saturation was collected by centrifugation at 12,000 rev/min for 20 min, and dissolved in a minimal volume of an appropriate buffer containing 0 001 M EDTA This solution was then dialysed overnight against 1000-fold vol of buffer

Molecular-sieve chromatography The pre-swollen Sephadex Gels (25, 75, 150, 200), Sepharose 4B and Bio-Gel A5M (Bio-Rad Laboratories) were used in columns of 2 5 × 95 cm or 1 8 × 65 cm eluting in an upflow manner The operating pressure for Sephadex 200 was kept below 10 cm Enzyme activity and A_{260} A_{280} readings were obtained from aliquots of the fractions

Analysis of enzymatic products The enzymatic products were isolated and characterized as previously described [1]

Acknowledgements—This work was partially supported by NSF Grant GB-3993

[17] G L Ellman, *Anal Biochem* **3**, 40 (1962)
[18] D Warburg and W Christian, *Biochem J* **2**, 310, 384 (1941)

Key Word Index—Ricinus communis, Euphorbiaceae, pyridinium oxidases, multiple enzymes