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PURIFICATION AND PROPERTIES OF PEA COTYLEDON AND EMBRYO DIAMINE OXIDASE

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Key Word Index—*Pisum sativum*, Leguminosae; pea; diamine oxidase; purification; kinetics.

Abstract—Diamine oxidase was purified separately from cotyledon and embryo of pea seedlings germinated for 6 days. The K_m of the cotyledon enzyme for putrescine was 1.6×10^{-4}M while that for the embryo enzyme was 9×10^{-5}M. On heating for 15 min at 70° the embryo enzyme retained about 90% activity whereas the cotyledon enzyme retained only 20% activity. The electrophoretic mobility of the cotyledon enzyme was *ca* twice that of the enzyme from embryo.

INTRODUCTION

We have shown [1] that diamine oxidase of pea cotyledon differs from that of the embryo in certain properties such as inhibition by 2,4-D, activation by GA, induction by putrescine, spermidine and ornithine and the effect of cycloheximide. A comparison of some of the properties of the diamine oxidases purified separately from cotyledon and embryo has now been made.

RESULTS AND DISCUSSION

Purification

The enzyme has been purified 471 and 158 fold from cotyledon and embryo respectively (Tables 1 and 2). Kenteen and Mann [2], Hill [3] and Smith [4] used 6–18 days germinated pea seedlings as the starting material. McGowan and Muir [5] however, used 4–5 days germinated epicotyls for purification. In the present study the cotyledon and embryo from seeds germinated for 6 days were used. Treatment with $CHCl_3$–EtOH mixture [3] had no apparent harmful effect on the cotyledon enzyme but the embryo enzyme was completely inactivated, even on reducing the temperature or raising the protein content.

Table 1. Purification of diamine oxidase from pea cotyledons

Fraction	Total volume (ml)	Total activity (units) nkat	Total protein (mg)	Specific activity (units/mg protein)	Purification (fold)	Recovery (%)
1. Crude extract	600	6730	39600	0.17	—	100
2. $CHCl_3$–EtOH supernatant	600	6180	3440	1.8	11	92
3. Dialysed fraction after $(NH_4)_2SO_4$ fractionation	50	1690	150	11.2	67	25
4. After precipitation at pH 5	32	1300	18	72.4	428	20
5. Calcium gel eluate	32	685	8.6	79.6	471	10

Table 2. Purification of diamine oxidase from pea embryos

Fraction	Total volume (ml)	Total activity (units) nkat	Total protein (mg)	Specific activity (units/mg protein)	Purification (fold)	Recovery (%)
1. Crude extract	100	1300	3400	0.38	—	100
2. Dialysed fraction after $(NH_4)_2SO_4$ fractionation	125	1240	250	4.9	13	95
3. Alumina C_γ gel eluate	125	1150	94	12.3	32	88
4. Cellulose phosphate column supernatant	125	1120	75	14.9	39	85
5. Calcium gel eluate	125	902	15	60.1	158	69

pH optimum

The pH optimum for both cotyledon and embryo enzyme was 7.5 with either phosphate or barbitol buffer. Though the cotyledon enzyme shows higher activity with barbitol buffer at the optimum pH, the embryo enzyme at the same pH shows more activity with phosphate buffer. Phosphate buffer has been used for both enzymes for the present studies.

Kinetics

The K_m for cotyledon and embryo enzyme was 1.6×10^{-4}M and 9×10^{-5}M respectively at pH 7.5. Smith has reported [4] a K_m of 4×10^{-5}M at pH 7.5 while Yamasaki *et al* [6] have reported a K_m of 7.4×10^{-5}M at pH 8. McGowan and Muir [5] found a K_m of 8.2×10^{-5}M at pH 7. The K_m of the embryo enzyme in the present studies resembles that reported by other workers but that of the cotyledons is somewhat higher. The possibility that the higher K_m value is due to the accumulation of H_2O_2 during the assay is unlikely since no increase in activity was obtained by adding catalase to the system. The energy of activation was 7440 and 8730 cal respectively for cotyledon and embryo enzymes. The two enzymes differ in their heat stabilities. The cotyledon enzyme retains only *ca* 20% activity when exposed to 70° for 15 min whereas the embryo enzyme under the same conditions retains *ca* 90% activity. The two enzymes do not however, differ in their response to inhibitors.

Electrophoresis

Polyacrylamide gel electrophoresis was performed and 3 mm bands were cut from the gel for enzyme activity. The relative mobilities of cotyledon and embryo enzyme were 0.45 and 0.26 respectively. This difference in mobility is not likely to result from dimerization or dissociation of a dimer due to difference in the purification procedure since the differences in electrophoretic pattern were obtained even with the crude extract.

The differences in kinetics and other properties observed in the present study suggest that there are two distinct proteins and this may account for their differential response to various effectors as reported in ref. [1].

EXPERIMENTAL

Plant material. Pea seeds (*Pisum sativum*) were soaked and germinated for 6 days as described in ref. [1].

The purification of cotyledon enzyme up to pH fractionation was carried out essentially as described in ref. [3]. The enzyme fraction obtained by pH fractionation was mixed with calcium phosphate gel (20 mg dry wt/ml) in a gel–enzyme vol ratio of 2:5, stirred for 30 min and then centrifuged. The adsorbed enzyme from the gel residue was eluted by 0.2 M Pi buffer, pH 7.

Purification of embryo enzyme. 70 g embryos were homogenized in a chilled blender using 25 mM Pi buffer, pH 7. The extract was passed through cheese-cloth and made up to vol $(NH_4)_2SO_4$ (21 g/100 ml) was added to the crude extract with stirring. After 30 min it was centrifuged and the supernatant was again fractionated with $(NH_4)_2SO_4$ (16 g/100 ml) with stirring. After standing for 30 min it was again centrifuged. Residue obtained was dissolved in Pi buffer, pH 7 and dialysed against 5 mM Pi buffer, pH 7 for 18 hr at 10°. Dialysed enzyme was mixed with alumina gel in a gel–enzyme vol ratio of 1:2. The mixture was stirred for 30 min and centrifuged. The enzyme from the gel residue was eluted with 0.1M Pi buffer, pH 7. The eluate was put on a 10 cm column of cellulose phosphate equilibrated with 10 mM Pi buffer, pH 7 for negative adsorption. It was then mixed with calcium phosphate gel in a gel–enzyme vol ratio of 1:2, stirred for 30 min and then centrifuged. The adsorbed enzyme from the gel residue was eluted with 0.2M Pi buffer, pH 7. The enzymes thus purified from cotyledon and embryo were stored at − 5° and diluted with cold H_2O × 30 and × 10 respectively before use.

Polyacrylamide gel electrophoresis was carried out as described in ref. [7]. Purified enzyme (100 μg protein) was applied using 5% sucrose soln on top of the gel. Electrophoresis was carried out at 10° using 2.5 mA per tube in 0.1M Tris–glycine buffer (pH 7.5) for 4 hr. For the elution of enzyme fractions the gel columns were removed from the tubes, frozen and sliced into 3 mm sections. Each section was extracted with 1 ml of 0.1M buffer, pH 7.5.

Enzyme activity was determined as described in ref. [1] except that 0.1 ml of diluted enzyme was used for all studies.

Protein content of the enzyme fractions was determined by the method of ref. [8].

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REFERENCES

1. Srivastava, S. K., Prakash, V. and Naik, B. I. (1977) *Phytochemistry* **16**, 185.
2. Kenten, R. H. and Mann, P. J. G. (1952) *Biochem. J.* **50**, 360.
3. Hill, J. M. (1971) in *Methods in Enzymology*, (Tabor, H. and Tabor, C. W. eds.) Vol. 17B, pp. 730.
4. Smith, T. A. (1974) *Phytochemistry* **13**, 1075.
5. McGowan, R. E. and Muir, R. M. (1971) *Plant Physiol.* **47**, 644.
6. Yamasaki, E. F., Swindell, R. and Reed, D. J. (1970) *Biochemistry* **9** 1205.
7. Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 204.
8. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.