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Purification and Characteristics of Diamine Oxidase from the Apoplast of Pea Epicotyls

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Abstract

Diamine oxidase was solubilized by stirring with a buffer from the apoplast of pea epicotyls and purified to homogeneity by one-step technique. The specific activity of the purified diamine oxidase was 22.5U/mg protein. SDS gel electrophoresis showed a single band at a molecular weight of 70KDa (1 %SDS and 5 %mercaptoethanol, at 100℃ for10min) and main band at a molecular weight of 140KDa (1 % SDS only, at 100℃ for10min). The enzyme activity was inhibited strongly by carbonyl reagents but was little inhibited by chelaters. The effect of the copper addition was not admitted at all.

1. Introduction

Damine oxidase is contained voluminously in cell walls of pea epicotyls. The enzyme catalyses the following reactions:

$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$

McGuirl et al. reported simple purification and properties of the active site of the enzyme[1]. The enzyme was reported to occurrence in the apoplast of pea epicotyls by Federico and Angelini[2]. It is a first paper of purifying diamine oxidase from the apoplast. Our purification procedure is an isoelectric point chromatography[3]; one-step purification is possible by the procedure with high yield. As for the specific activity of diamine oxidase, it varies widely in pea[4,5], and half-sites reactivity is uncertain.

In this paper, we report the purification procedure of diamine oxidase in the apoplast of pea epicotyls. And we examined some properties of the purified enzyme.

2. Materials and methods

2. 1. Plant material

Seeds of pea (*Pisum cv. Alaska*) were soaked for 24hour at 25℃. And then pea was cultivated in reticulation plastic trays under tap water in the dark for a total of 7days at 25℃ (water culture). Epicotyls were cut out to the length of 2 and 3 cm, disinfected with 0.1% benzalkonium chloride for 10min and thoroughly washed with deionized water.

2. 2. Measurement of diamine oxidase activity

Diamine oxidase activity was assayed by measuring the absorption at 435nm[6]. The incubation mixture consisted of 1.5 mL of 0.2M K Pi buffer pH7.0, 0.2mL of 0.1% o-aminobenzaldehyde (in ethanol), 0.3mL of 10mM putrescine, and appropriate enzyme in a total volume of 3.0mL. The incubation temperature was 37℃. The reaction was terminated by adding 0.1mL of 50% TCA. Effect of some compounds was examined by using this assay method after preincubation (37℃, 5min). Protein was determined according to the method of Bensadoun and Weinstein[7] with BSA as a standard.

2. 3. Purification of diamine oxidase

The operation was done at 4℃. Epicotyls segments of pea (125g) were stirred with 500mL of 0.2M Tris succinate buffer, pH8.5 in a 1L beaker overnight (the nylon mesh was pulled so that the bar and the segments should not touch.). (Step 1) After the buffer was separated, the pH was brought to 6.5 with saturated succinic acid, and then the solution was diluted to 0.1M Tris succinate buffer pH6.5 with distilled water. SP-Sephadex C-50 (fresh weight 1g) equilibrated with 0.1M Tris succinate buffer pH6.5 was added with the enzyme solution. After gentle stirring for 1hour, The SP-Sephadex C-50 was recovered with a Buchner funnel and washed with 300mL of 0.1M Tris succinate buffer pH6.5. The enzyme was eluted with 100mL of 0.1M

Tris succinate pH8.0. The enzyme was concentrated with centrifuge dialysis method or in cellophane tube in contact with polyethylene glycol 20,000, and was kept at 4℃ until use.(Step 2)

2. 4. SDS electrophoresis and enzyme staining

SDS electrophoresis was performed as described by Laemmli[8]. As standard protein markers, Mark 11 was used.(Tefco) Proteins were visualized with a silver staining kit.(Nacalai tesque)

3. Results and discussion

3. 1. Enzyme purification

The enzyme was purified by SP-Sepadex C-50. The results of purification are summarized in Table1. The yield of the enzyme was 88.4%, the purification resulted in 84-fold, and the specific activity of the enzyme was 22.5U/mg protein, the specific activity of DAO that has been reported is 69.6U/mg protein[9] and 45.9U/mg protein[10], and the specific activity of this report is the lowest though the purified enzyme is a single protein(Fig.1).

Table 1. Purification of diamine oxidase from the apoplast of pea epicotyls

Step	Total activity	Total protein	Specific activity	Purification	Yield
	(Unit)	(mg)	(U/mg)	(−fold)	(%)
1. Apoplasts Fraction					
	45.8	171	0.268	1	100
2. eluate from SP-Sephadex C-50					
	40.5	1.8	22.5	84	88.4

3. 2. SDS electrophoresis

To examine the purity and subunit structure of the enzyme, SDS electrophoresis was performed. SDS electrophoresis showed that the purified enzyme was single and the molecular weight of the subunits with 1% SDS and 5% 2-mercaptoethanol treatment at 100℃ for10min is 70KDa. And a molecular weight of main band with 1% SDS treatment at 100℃ for 10min is 140KDa(Fig.1). The purified enzyme was a dimmer. These results agreed with other diamine oxidases[11, 12, 13]. The molecular weight of the purified enzyme was different from the previous report[9] though the origin was the same. Because DAO is known as a glycoprotein, glycans obtained by different purification methods might be different.

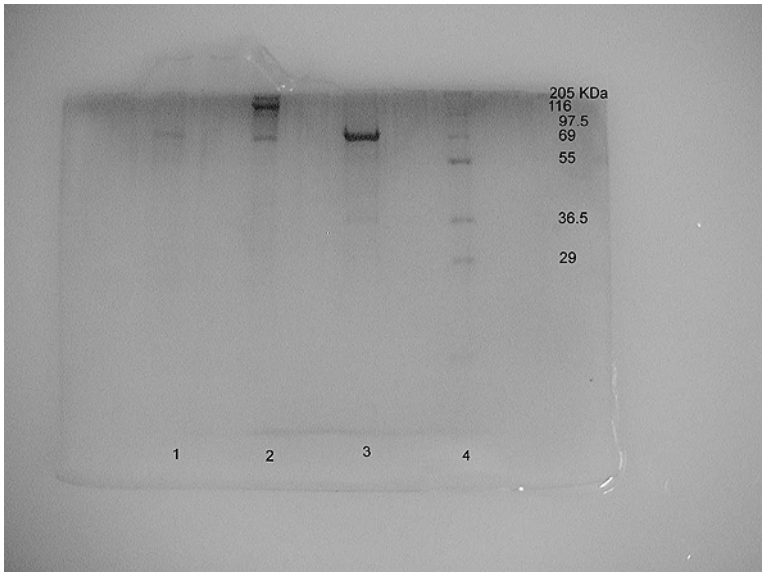

FIG. 1. SDS electrophoresis of diamine oxidase
Line1, apoplast fraction12. 6μg; Line2, eluate from SP-Sephadex 24. 9μg(1%SDS at 100℃ for10min); Line3, eluate from SP-Sephade x 24. 9μg(1% SDS and 5% mercaptoethanol at 100℃ for 10min); Line4, standard protein markers.

3. 3. Inhibitions of carbonyl reagents and copper chelaters

In general, diamine oxidase contains topaquinone(TPQ)and copper[14], the purified enzyme was strongly inhibited by phenyl hydrazine, semicarbazide, and hydroxylamine. It was suggested that the purified enzyme contained TPQ. And the purified enzyme was weakly inhibited by copper chelaters.(Table 2) The purified enzyme might contain not copper but other metals.

Table 2. Effect of inhibitors

	mM	Relative Activity (%)
Control		100
Carbonyl Reagents		
Phenyl Hydrazine	0.5	0
Semicarbazide	0.5	0
Hydroxylamine	0.5	0
Chelaters		
Diethyldithiocarbamate	0.05	76.3
	0.1	54.7
Cuprizone	0.05	87.0
	0.1	46.7

3. 4. Effect of copper addition

The copper addition did not activate the enzyme activity.(Table 3) It was suggested that the purified enzyme did not contain apo-enzyme(enzyme to lack copper).

Table 3. Effect of copper addition

	mM	Relative Activity (%)
Control		100
$CuSO_4$	0.005	79.3
	0.05	46.8
	0.5	20.2

4. Conclusions

Neither homogenization nor high speed centrifugation was used this time for enzyme purification. Diamine oxidase was purified to homogeneity easily using SP Sephadex C-50. The specific activity of the purified enzyme was relatively low regardless of a single protein. And because inihibition of copper chelaters is weak, the purified enzyme doesn't seem to contain copper. Two kinds of diamine oxidases are likely to exist. One is an enzyme containing copper and another one containing metals other than copper.

It might be interesting to compare the feature of the enzyme purified from the plant cells and the enzyme purified this time.

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