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The Isolation and Purification of Diamine Oxidase of Pea Seedlings and Pig Liver

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ABSTRACT

Enzymes have been extensively used in many industries for the last 20 years. They are becoming more common because of new areas of application. The limitations of applications of enzymes are activity, specificity, stability, and price. Higher enzyme activities and less susceptibility of process conditions are desirable. Therefore, in some cases, purified enzyme extracts are needed. The purpose of this study is the isolation and purification of diamine oxidase (DAO) of pea seedlings and pig liver. The relationship between pea seedlings growth rate and enzyme activity is established. DAO of pea seedlings and pig liver is prepared by way of tissue disruption with homogenization, centrifugation, fractionation with ammonium sulfate, precipitation of inert components, column electrophoresis, and DEAE-cellulose column chromatography. The specific activity of disrupted pea seedlings cells was measured as 0.017 (U/mg protein), and the pig liver DAO activity was measured as 0.00037 (U/mg protein). The specific enzyme activity from pea seedlings was increased to 6.750 (U/mg protein). On the other hand, the specific enzyme activity from pig liver was increased to 0.30 (U/mg protein). The final enzyme extract from pea is 400-fold purer than raw material, and the final enzyme extract from pig liver is 820-fold purer than raw material.

Index Entries: Enzyme; diamine oxidase; enzyme purification; pea seedlings; pig liver.

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INTRODUCTION

It is now common to use enzymes in industries and medical technology because of the advances in enzyme biochemistry. Although the cheese production process has been known for many centuries, use of pure enzyme is relatively known.

Research extending over the past 20 years has established the presence in animal tissues of two oxidizes or groups of oxidizes that catalyze the oxidation of amines. Diamine oxidase (DAO) catalyzes the oxidation of diamines of the general formula $NH_2(CH_2)_nNH_2$ where n = 2–8 and of certain substituted diamines, such as histamine and agmatine. DAO, in contrast to monoamine oxidase, is inhibited by cyanide and by a carbonyl reagent. A plant enzyme differing in distribution from the plant DAO and catalyzing the oxidation of aliphatic monoamines and phenylalkylamines was described by Werle and Roewer (*1*).

The sensitivity of animal DAO to carbonyl reagents led to the suggestion that the enzyme contains a functional aldehyde or ketone group (*2*).

Much evidence has since been reported that the prosthetic groups of both animal DAO and the related plant enzyme contain pyridoxal phosphate. Both the plant enzyme and animal DAO are inhibited by sodium diethydithiocarbamate. Pea seedlings provide a very rich source of plant DAO. Though the DAO and spermine oxidase of animal tissues have been extensively purified, no such pig liver evidence has been reported (*3*). This enzyme is used for meat protection and medical applications.

The present work describes a method for obtaining a crystalline preparation of the DAO from pig liver and pea seedlings, and some properties of the crystalline enzyme.

MATERIALS AND METHODS

Material

Pea seedlings were supplied from Unwins Seeds Ltd., Cambridge, England, and the pig liver was obtained from the market. Pure DAO for analysis was obtained from Sigma Chemical Co., England. The other chemicals (ammonium sulfate, phosphate [$K_2H_2PO_4$], riboflavin, temed, acrylamide, bisacrylamide) were obtained from Merck Chemical Co., Germany. The Sepharose series (DEAE-Sepharose, Sepharose CL-GB, Pharmacia P.L. Sweden), the Sephadex series (G25, G200, Pharmacia LKB. Bucks/England), and the cellulose series (AE-Cellulose, DEAE-Cellulose (Sigma Chemical Co., England) were used for column chromatography.

Methods

Optimum growth conditions were obtained by comparing seeds that were germinated both under the sunlight and in darkness. The efficiency

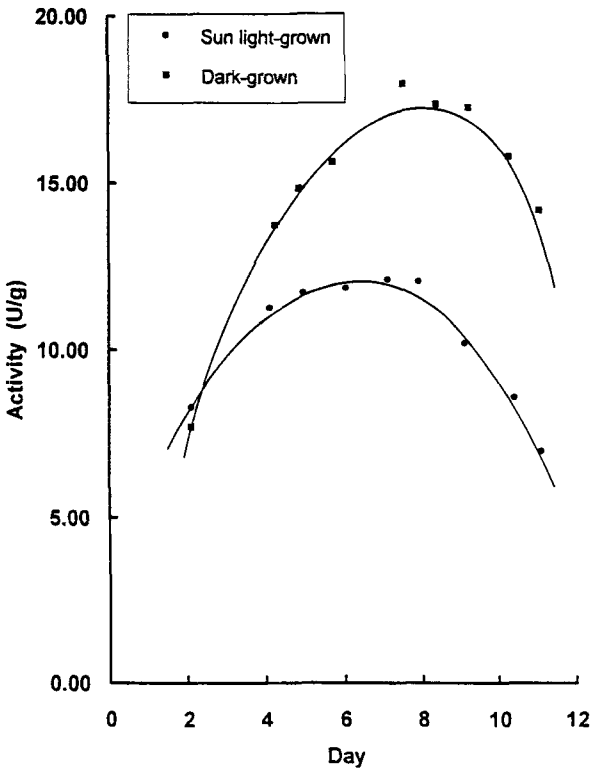

Fig. 1. The growth rate/activity profile of pea seedlings.

of DAO production was determined spectrophotometrically by measuring the absorbancy at 280 mμ over a 2-wk period of growth (*4*).

The preparation in the dark so obtained was about two times as active (activity/g). This value is seen to be constant under these conditions. The germinated seeds are a yellow color when grown in the dark, but green when grown in the light. Seeds germinated in the dark were about two times the weight. The highest efficiency of DAO production was between the 7th and 10th d of growth in the dark (Fig. 1).

Purification

The steps for purification were precipitation, centrifugation, extraction, electrophoresis, and chromatography, which are described below (*5–7*). However, the present work describes a method for obtaining a crystalline preparation of the DAO from pig liver and pea seedlings. It may be calculated that the plant preparations were about 22 times as active as the animal preparations. These preparations of the plant enzyme have activities that are much higher than those so far reported for the purified preparations of the related animal DAO, though exact comparison is possible because same methods are used to express the specific activities (*8–10*).

Pea seedlings (7–10 d old) were washed free from soil, and any obviously diseased material was removed. The weighed seedlings (200 g) were in a chilled domestic meat mincer, and the residual pulp was homogenized with 500 mL 0.20*M* phosphate buffer, pH 7.2, and 200 mL iced water for 5 min. The combined extracts were cooled in an iced-salt freezing mixture to 0–5°C. The crude homogenate was centrifuged for 15 min at 12,000 rpm, and supernatant solution was obtained. The insoluble particles in the suspension were separated. Then 30% was brought to saturation with ammonium sulfate (16.4 g/100 mL). The stirring was continued for 30 min, and the mixture was then centrifuged for 10 min at 12,000 rpm. It was then treated with ammonium sulfate (60% = 18.1 g/100 mL). The almost clear yellow supernatant was poured off from the bulky white precipitate and 0.20*M* phosphate buffer and again cooled. During vigorous mechanical stirring, 0.1*M* KOH was slowly added. The stirring was continued for 15 min, and the suspension was brought to pH 9 by dropwise addition 0.1*M* KOH. It was then centrifuged for 30 min at 18,000 rpm, and the supernatant solution recovered. The suspension was dialyzed for 24 h against 0.02*M* phosphate buffer, pH 7.2.

The dialyzed suspension was centrifuged for 30 min at 18,000 rpm, and a mixture of ethanol (200 mL) and chloroform (100 mL), previously cooled to −10°C, was slowly added with vigorous mechanical stirring. The stirring was continued for 30 min, and the mixture was then centrifuged for 30 min at 18,000 rpm. The almost clear yellow supernatant was removed from the white precipitate and from the bottom layer of chloroform, and again cooled. It was then treated with ammonium sulfate (85% = 55.9 g/100 mL) and was stirred for 30 min. The mixture was then centrifuged for 30 min at 18,000 rpm, and precipitate was in the centrifuge with a further small amount of 0.2*M* phosphate buffer, pH 7.2. The suspension was dialyzed for several hours against running tap water and then overnight at 0–2°C against (solution volume × 100) L of 0.02*M* phosphate buffer, pH 7.2. The dialyzed suspension was centrifuged for 30 min at 18,000 rpm, and these enzyme preparations were stored at −10°C until there was enough for the next stage in the purification.

A small sample of the supernatant solution was reserved for initial estimations, and the rest was applied to a DEAE column (2.5 × 100 cm) equilibrated with 0.02*M* phosphate buffer, pH 7.2. The rate of flow varied from 50 to 80 mL/h in different experiments. The eluate was collected in 5-mL fractions. The fractions of main DAO were showing the highest DAO activity. $E_{280\ mM}$ was combined, and solution was passed through a column (2.5 × 100 cm) of ultragel AcA 34, which was than washed with 0.02*M* phosphate buffer, pH 7.2. The column was equilibrated with 0.02*M* phosphate buffer, pH 7.2, before use.

On the other hand, DAO was purified from pig liver. Fresh pig liver (298.1 g) was homogenized in 357.72 mL of distilled water for 10 min at 55°C in a Warring blender and centrifuged for 1 h at 8000 rpm. The supernatant solution was fractionated with $(NH_4)_2SO_4$ (33% saturation) and

Table 1
The Steps of DAO of Pea Seedlings Isolation and Purification

Step	Total protein, mg	Volume, mL	Specific activity, U/mg protein	Total unit, U	Yield, %	Purification degree
Tissue disruption, filtration	12,600	340	0.017	215	100	1
Centrifugation	3600	290	0.052	190	88	3
Treatment with 30/60% $(NH_4)_2SO_4$	1300	150	0.130	170	79	8
Treatment at high pH, 24-h dialysis	360	70	0.430	155	72	25
Treatment with EtOH/chloroform	70	72	1.860	130	60	110
Saturation with 85% $(NH_4)_2SO_4$	—	—	—	—	—	—
Dialysis at the weak ionic polyelectrolyte	35	15	3.420	120	56	200
Adsorption in DEAE-Cellulose column	25	15	4.600	115	53	270
Gel filtration in the Ultragel AcA 34 column	16	15	6.750	108	50	400

centrifuged for 15 min at 11,000 rpm, and then again fractionated with $(NH_4)_2SO_4$ (60% saturation) and stirred for 30 min. The extract was centrifuged for 15 min at 12,000 rpm and suspended in 0.25*M* phosphate buffer, pH 7. The supernatant solution was fractionated with ammonium sulfate (33–60%), followed by dialysis against 0.20*M* phosphate buffer, pH 5.3.

The dialyzate was centrifuged for 15 min at 20,000 rpm, and the supernatant was applied to a DEAE-Sepharose column (4.5 × 12 cm) equilibrated with 0.025*M* phosphate buffer, pH 7.0. The active fractions were combined and stored at 4°C. A summary of typical purification procedure from pea seedlings and pig liver is shown in Tables 1 and 2.

RESULTS AND DISCUSSION

The optimum germinating conditions for peas both under the sunlight and in the darkness were determined by comparing their activities for producing DAO enzyme efficiently. For this purpose, the growth rate

Table 2
The Steps of DAO of Pig Liver Isolation and Purification

Step	Total protein, mg	Volume, mL	Specific activity. 10^4, U/mg protein	Total unit, U	Yield, %	Purification degree
Tissue disruption, filtration	289,000	1700	3.73	108	100	1
Centrifugation	30,000	1440	25	75	69	7
Treatment with 30/60% $(NH_4)_2SO_4$	4300	296	168	72.4	67	45
Treatment at low pH, 24-h dialysis	950	240	632	60	55	170
Column electrophoresis	175	100	2257	39.5	37	605
Column chromatography in DEAE-Sepharose column	81.55	30	3065	25	23	820

activity profile of peas was described (Fig. 1). The most efficient production of DAO was observed in between 7 and 10 d after germination in dark. DAO was isolated and purified from pea seedlings by disruption in a homogenizer, centrifugation, treatment with ammonium sulfate, precipitation of inert material, column electrophoresis (PAGE), DEAE-Cellulose, AE-Cellulose, and Ultragel AcA 34 column chromatography.

On the other hand, DAO was isolated and purified from pig liver by disruption in a homogenizer, centrifugation, treatment with ammonium sulfate, precipitation of inert material, column electrophoresis, DEAE-Sepharose, and Sephadex G25 column chromatography.

Specific activity of raw material from pea seedlings was determined as 0.017 (U/mg protein). The specific activities of DAO from pea seedlings at each of the isolation and purification steps are given below:

0.052 After the centrifugation;
0.130 After the treatment with 30/60% ammonium sulfate;
0.430 At the end of the 24 h of dialysis;
1.860 After the treatment with ethanol/chloroform;
3.420 After the saturation 85% with ammonium sulfate and dialysis at low ionic strength;
4.600 After the column electrophoresis and the column chromatography in DEAE-Cellulose column; and
6.750 After the gel filtration by passing through ultragel column.

It was found that the final enzyme was 400-fold purer than the raw material, and the final enzyme activity was determined as 6.750 (U/mg

protein). In comparison of this value with that reported in literature, which is 1.079 (U/mg protein), the specific activity of DAO from pea seedlings obtained from this study is more suitable for applications.

On the other hand, specific activity of raw material from pig liver was determined as 0.00037 (U/mg protein). The specific activities of DAO from pig liver at isolation and purification steps are given below:

0.0025 After the centrifugation;
0.0168 After the treatment with 33/60% ammonium sulfate and dialysis at low ionic strength;
0.0632 After the treatment with 0.20*M* phosphate buffer at pH 5.3;
0.2257 After the column electrophoresis; and
0.3065 After the column chromatography in DEAE-Sepharose column.

The purest preparations of pea seedling DAO reported were made by the method of Mann, but the initial preparations were unstable and lost all their activity within 24 h. The method now described, involving chromatographic fractionation on DEAE-Cellulose, yields purer and more stable preparations. These preparations of the plant enzyme have activities that are much higher than those so far reported for the purified preparations of the related animal DAO. Although exact comparison is possible, the same methods are used to express the specific activities.

The highly purified preparations of the animal (pig liver) enzyme obtained by the present method had a specific activity (enzyme U/mg protein), calculated from the rate of oxidation of putrecine at 37°C, of 3605×10^{-4}. The preparations of the plant (pea seedlings) enzyme obtained by the present method had a specific activity (enzyme U/mg of protein) calculated from the same reaction at 37°C of 6.750. It may be calculated that the plant preparations were about 22 times as active as the animal preparations.

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