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0273-2289/95/5301—0083$05.60

# Purification and Properties of the Diamine Oxidase of Pea Seedlings

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Received August 2, 1994; Accepted August 11, 1994

## ABSTRACT

Enzymes have been used extensively in many industries for the last 20 yrs. The purpose of this study was the isolation, purification, and specification of diamine oxidase (DAO) of pea seedlings. The relationship between enzyme activity and growth conditions has been investigated.

DAO that was extracted from pea seedlings was purified by centrifugation, thermal denaturation, fractionation with ammonium sulfate, precipitation of inert components, column electrophoresis, and DEAE-cellulose column chromatography. It was found that the final enzyme preparation is 400-fold purer than the original extract at the end of the purification steps. The molecular weight, isoelectric point, and copper content of the purified enzyme also were determined.

**Index Entries:** Pea seedlings; diamin oxidase; purification of DAO; specification of DAO.

## INTRODUCTION

Research work extending over the past 26 yrs has proven the presence of enzymes that catalyze the biochemical reactions of living systems in animal tissues, plant tissues, and microorganisms. Diamine oxidase catalyzes the oxidation reaction of diamines of the general formula

$$NH_2(CH_2)_nNH_2 \quad (1)$$

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where $n = 2 - 8$, and of substituted diamines, such as histamine and agmatine (*1*). The studies on the isolated enzyme yield information on its specificity for substrates, kinetic parameters for the reaction, and possible means of regulation. The limitations of various enzyme applications can be low activity, lack of specificity, low stability, and high price. Higher enzyme activity and less susceptibility to process conditions are desired. All enzyme are produced by the plants, animals, or microorganisms. A complete purification may not be needed for some enzymes. On the contrary, the enzyme complex, which is a mixture of several enzymes, may be preferred to purified enzymes in serial reactors. However, most applications require purified enzymes (*2*).

The aim of a purification procedure should be to isolate a given enzyme with the maximum possible yield, based on the percentage of recovered activity compared with the total activity of the original extract. In addition, the preparation should possess the maximum catalytic activity, i.e., there should be no degraded or other inactivated enzymes present. It should also be of the maximum possible purity with little or no other enzymes or other large molecules (*3,4*).

The purpose of this paper was the purification and specification of DAO that was isolated from pea seedlings.

## MATERIALS AND METHODS

### The Isolation of DAO of Pea Seedlings

The pea seedlings used in this study were obtained from Unwins Seeds Ltd., Cambridge/Leeds, England.

A number of the studies reported here required the use of large quantities of DAO from the pea seedlings. In order to satisfy this requirement, a purification procedure had to be developed to obtain a homogeneous enzyme preparation with high yield and in large quantity. Optimum growth conditions were obtained by comparing seeds that are germinated both under the sunlight and in the dark. The efficiency of DAO production was determined by measuring enzyme activity for a 2-wk period (*5–7*). It was found that the highest efficiency of DAO production was between d7 and d10 of growth in the dark. The pea seedlings (7–10-d-old) were washed to remove soil and any other debris. The weighed seedlings were homogenized with 0.02*M* phosphate buffer solution, pH 7.2, and cold water (0°C) in a homogenizer, and squeezed through cloth to prepare a crude homogenate. The enzyme activity of the crude homogenate was measured by the manometric method (*5,6,8*) (Table 1).

### The Purification of DAO of Pea Seedlings

The aim of a purification procedure should be to isolate a given enzyme with the maximum possible yield. In addition, the enzyme preparation

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

| Step | Total protein, mg | Volume, mL | Specific activity, U/mg protein | Total unit, U | Yield, % | Purification, degree |
|---|---|---|---|---|---|---|
| Tissue disruption Filtration | 12600 | 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 |

should possess the maximum catalytic activity, i.e., there should be no degraded or inactivated enzyme present, and it should be of the maximum possible purity, without any other enzymes or large molecules. The steps for purification were centrifugation, precipitation, extraction, electrophoresis, and chromatography, which are described later (*9–11*).

### *Centrifugation*

The insoluble particles in suspension were separated by centrifuging at 8000, 11,000, 12,000, and 18,000 rpm.

### *Precipitation*

Ammonium sulfate was added to change the solubilities of proteins and to form insoluble particles.

### *Extraction*

Phosphate ($K_2H_2PO_4$) solution was used for the extraction of the DAO from the insoluble particles.

*Electrophoresis*

Polyacrylamide gel electrophoresis was performed.

*Chromatography*

Ion exchange and gel filtration were performed. DEAE-Cellulose, DEAE-Sepharose, and Utragel AcA 34 were used for this purpose.

The activity of DAO at the end of each preparative step was measured by the manometric method. All purification and isolation steps were performed at 0–5°C to sustain enzyme activity. The measured values during the isolation and purification of DAO are given in Table 1.

## Stability of DAO of Pea Seedlings and Activity Profiles

Ammonium sulfate concentration, pH, temperature, and ionic strength (of phosphate buffer solution) and stability profiles were determined for the purified DAO of pea seedlings.

*Effect of Ammonium Sulfate Concentration*

Ammonium sulfate concentration affects the enzyme activity. Figure 1 shows the effect of ammonium sulfate concentration on DAO activity. According to this, ammonium sulfate concentration should be 60% for the maximum activity.

*Effect of pH*

DAO of pea seedlings activity as a function of pH at 37°C is shown in Fig. 2. It was found that the optimum pH range is 6.5–7.5 for maximum enzyme activity.

*Effect of Temperature*

The relationship between temperature and activity is given in Fig. 3. It was determined that the optimum temperature is 37°C.

*Effect of Ionic Strength of Phosphate Buffer Solutions*

Figure 4 shows the effect of ionic strength of buffer solution on DAO of pea seedlings activity. The optimum concentration of buffer ($K_2H_2PO_4$) solution is 0.02*M*.

## Properties of the DAO of Pea Seedlings

Molecular weight, copper content, and isoelectric point of the purified DAO of pea seedlings were experimentally studied. The molecular weight of purified enzyme was determined by SDS (sodium dodezil sulfate)-acrylamide gel electrophoresis with and without pretreatment by using a crosslinking agent and gel filtration in both low ionic strength (nondenaturing conditions) and high ionic strength (denaturing conditions) (*12,13*). The results indicate that the molecular weight of the purified DAO of pea seedlings is approx $18 \times 10^4$ Daltons and that native enzyme is composed of two identical units of $9 \times 10^4$ Daltons. The copper content of

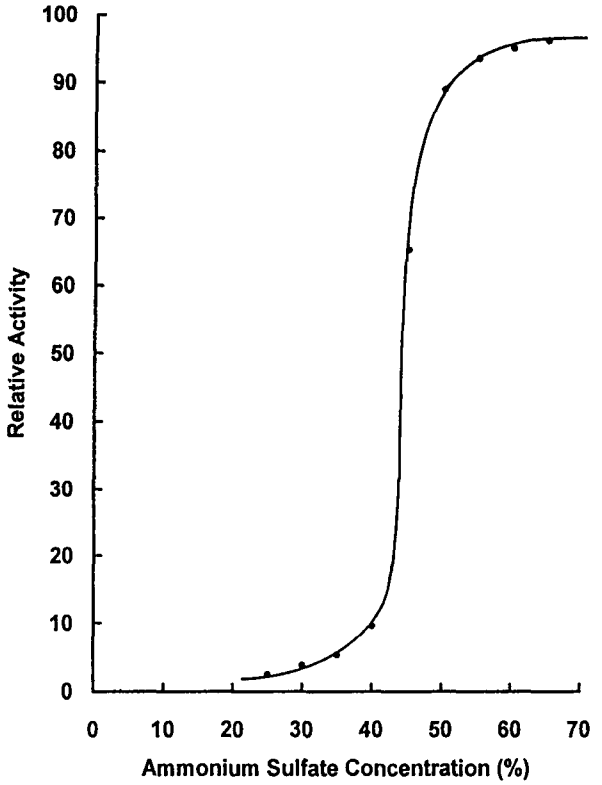


Fig. 1. Effect of ammonium sulfate concentration on DAO of pea seedling activity.

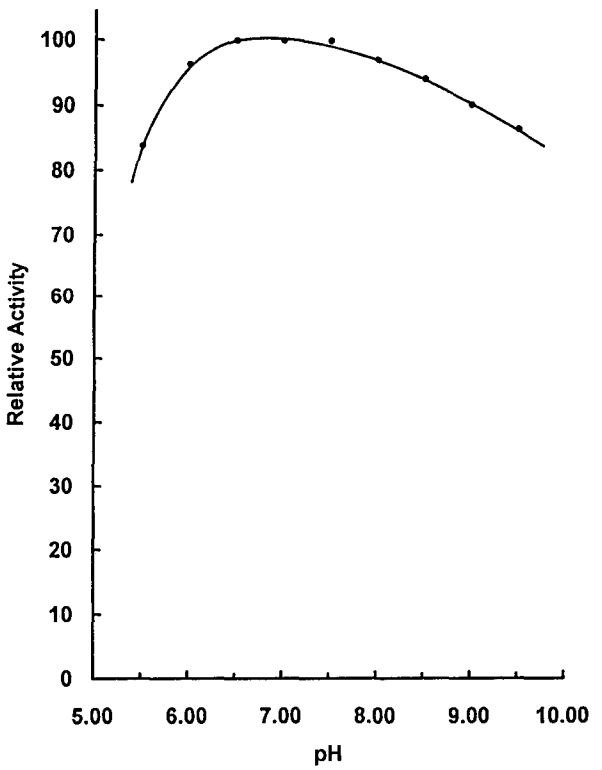


Fig. 2. Effect of pH on DAO of pea seedling activity at 37°C.

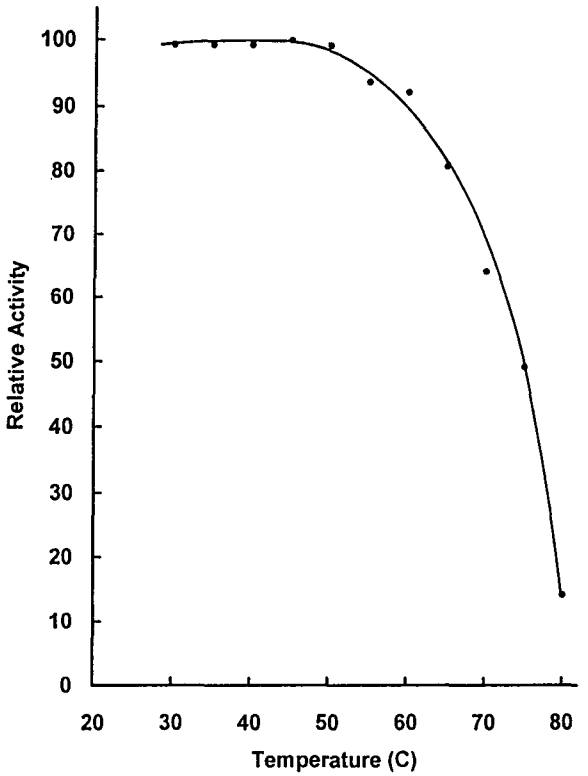


Fig. 3. Effect of temperature on DAO of pea seedling activity of pH 7.2.

the purified enzyme was determined by anodic strip voltmeter and atomic absorption spectrophotometry (*14,15*). The isoelectric point of the purified enzyme was also investigated (*8,13*) (Fig. 5).

## RESULTS AND DISCUSSION

DAO of pea seedlings was purified from crude homogenate by the following steps—centrifugation, treatment with ammonium sulfate, precipitation of inert material, column electrophoresis, and DEAE-cellulose column chromatography.

The specific activity of the crude homogenate was found to be 0.017 U/mg protein. The specific activity of the final enzyme was determined to be 6.75 U/mg protein at the end of the purification steps. According to these values, the final enzyme preparation was 400-fold purer than the crude homogenate.

The stability and activity profiles were also determined for the purified DAO. The following are the conditions for maximum enzyme activity:

1. Ammonium sulfate concentration should be 60%;
2. pH Range should be 6.5–7.5;
3. Temperature should be 37°C; and
4. Concentration of buffer ($K_2H_2PO_4$) solution should be 0.02*M*.

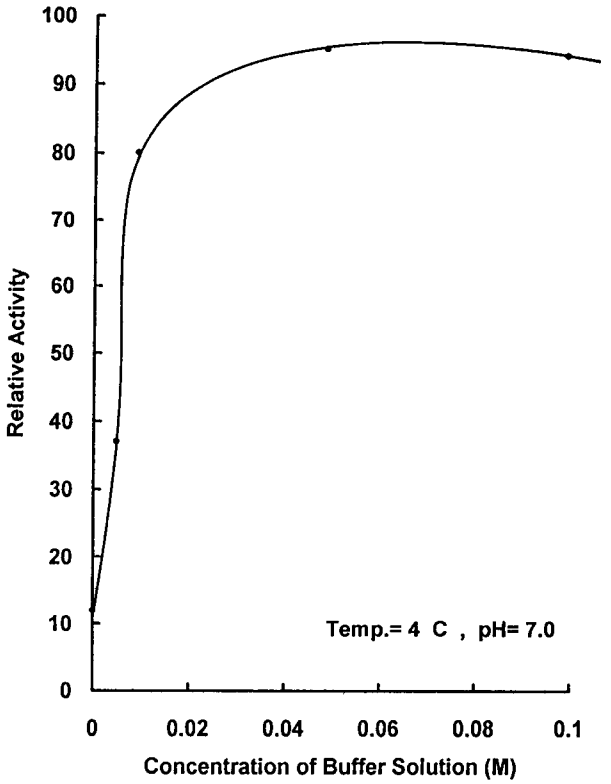


Fig. 4. Effect of buffer ($K_2H_2PO_4$) solution concentration on DAO of pea seedling activity.

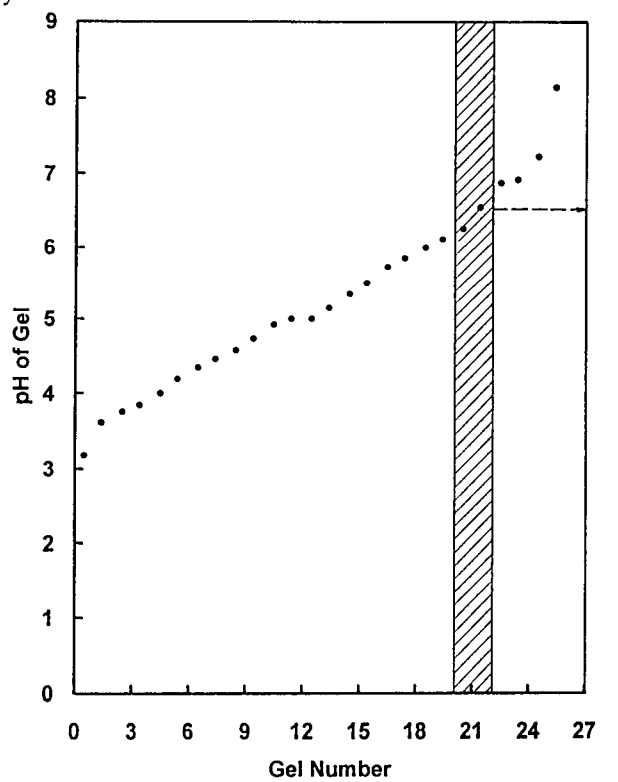


Fig. 5. Isoelectric point of DAO of pea seedlings.

The crystallized final enzyme was homogenized electrophoretically and purified by ultracentrifugation. The molecular weight of prepared enzyme was determined. It was found that the weight of the subunit was 95–97 $\times$ $10^3$ daltons without pretreatment; 1 $\times$ $10^5$ daltons with a cross-linking agent by using SDS-polyacrylamide gel electrophoresis; 18–20 $\times$ $10^4$ daltons by gel filtration in low ionic strength (nondenaturing conditions); 9 $\times$ $10^4$ daltons by using gel filtration in high ionic strength (denaturing conditions). The results indicate that the molecular weight of the enzyme is approx 18 $\times$ $10^4$ daltons and that the native enzyme is composed of two identical units of 9 $\times$ $10^4$ daltons.

Copper analysis showed that the native enzyme contains two strongly bound to $Cu^{2+}$ ions, presumably one per subunit. Apoenzyme first treated with an excess of $Cu^{2+}$ ions and then treated with 1.0 m*M* EDTA to remove the loosely and unspecifically bound $Cu^{2+}$ ions gives approx one $Cu^{2+}$ ions per subunit.

The value of the isoelectric point is 6.5.

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