ISOLATION AND PURIFICATION OF OXALATE OXIDASE FROM BARLEY SEEDLING

by :

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Abstract

A procedure of isolation of oxalate oxidase from barley seedling and a new method for purification is described.

A purification step was accomplished by the second partition using poly ethylene glycol (PEG-6000) and Dextran-500 (4.5%:14), and affinity chromatography using oxalate immobilized on activated CNBR-Sepharose 6MB as a ligand.

After partition some protein were separated, and the specific activity were increased by 5.30 fold. The affinity chromatography using Oxalate-Sepharose effectively separated the oxalate oxidase. The specific activity of pure enzyme was 154.3 U/mg protein and the purity was 41 fold.

Introduction

The crude extracts of oxalate oxidase from barley root and seedling (Chiriboga, 1966), beet stem (Obanski, 1981) and moss (Laker, 1980), have been used for the assay of oxalic acid in biological liquids. The enzyme catalyses the reaction producing two moles of carbon dioxide and one mole of hydrogen peroxide from one mole of oxalic acid.

$$(COOH)_2 + O_2 \rightarrow 2\,CO_2 + H_2O_2$$

Chiriboga (1966) found that barley (*Hordeum vulgaris*) seedling bear a soluble oxalic acid oxidase. It was also found that a crude extract of barley seed metabolize oxalic acid in the presence of yeast kochsaft (Fodor, 1930). Many factor influence the activity of the enzyme system and therefore the accuracy of the assay in crude extracts is limited.

The purification steps covering repeated fractionation using ammonium sulphate followed by ion-exchange chromatography and gel filtration on Bio gel A 0.5 and affinity chromatography using Oxalate-Affi gel have been done. The purified enzyme occur after affinity Chromatography using oxalate, immobilized on Affi-Gel 10 as a ligand (Pietta, 1982).

The affinity chromatography has been described as an adsorbent containing ligand attached by bonds susceptible to specific chemical cleavage. The application of affinity chromatography in many cases will critically depend on placement of the ligand at a considerable distance from the matrix back bone.

In this paper the partition for preliminary purification and the application of the enzyme to the next step of purification by Oxalate-Sepharose is reported.

Experimental Methods

Materials

Barley used was of the commercial variety obtained from local market at Taipei. Oxalic acid (Hayashi) used as a substrate

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was dissolved in 50 mM of succinic acid Buffer at pH 3.6. Activated CNBR-Sepharose-6MB (Pharmacia) was applied for preparing the Oxalate-Sepharose gel. Polyethyleneglycol (PEG-6000) (Merck) and Dextran 500 were used in the partition procedure.

Methods

Isolation of enzyme:

Barley seed was soaked overnight and then planted in the small plate containg a moistened paper towel, incubated at room temperature (20 — 30°C). The seedling resulted were removed.

The sprouts and the roots were weighed and homogenized separately using a Waring Blendor with an addition of two volumes of 10 mM succinate buffer at pH 3.6. After passing through a cheese cloth, the homogenate was spun using a Beckman Centrifuge Model J2-21 at 25.000 G for 30 minutes at 4°C. The enzyme activity was measured during 6 days of seedling.

Enzyme and protein assay

Oxalate oxidase analysis kit consisting of:

R-1 : 0.3 M of aminoantipyrine in 10 mM of succinate buffer pH 3.6.

R-2 : peroxidase solution 169 U/ml in 10 mM succinate buffer at pH 3.6.

R-3 : 20 mM 3,5-dichloro-hydroxybenzene sulfonate (DHBS) in 10 mM of succinate buffer pH 3.6.

Preparation: 20 mg of potassium oxalate in 100 ml flask, added with 0.1 ml of R-1 and 0.1 ml of R-2 and 2.0 ml of R-3 solutions and 7.7 ml of 10 mM succinate buffer. The solution was adjusted at pH of 3.6 using 1 N HCl.

The measurement involving the addition of 1 ml of the oxalate oxidase analysis kit with 0.1 ml of the enzyme extract produces a red color (chromogen) measured at 520 nm.

Reaction of the enzyme assay:

$$(COOH)_2 + O_2 \xrightarrow{\text{oxalate oxidase}} CO_2 + H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyrine} + \text{DHBS} \xrightarrow{\text{peroxidase}} \text{chromogen} + H_2O \text{ (red color)}$$

Protein content was determined by the method of Lowry *et al.* (1951).

Preparation of Oxalate-Sepharose gel

A modified procedure by O'Carra and Barry (1974) was applied. The activated CNBR-Sepharose-6MB (1.5 g) was washed with water (3 x 30 ml), filtered and reacted with 230 mg of diaminohexane per milliliter of the gel (pH 10.5 at room temperature). The resulted amino-hexyl-Sepharose gel rinsed with water. The degree of substitution checked by subjecting a small amount of the gel to trinitroben-benzene sulfonate test for free amine group as described by Inman and Dintzis (1969). One milliliter of saturated sodium borate was added to a slurry (0.2 — 0.5 ml in distilled water) of the gel. Three drops of 3% aqueous solution of sodium 2,4,6-trinitrobenzene sulfonate (TNBS) were then added.

The color reaction of the gel bead was completed within 2 hours. The degree of substitution was estimated from the relative color intensity of the washed gel. The formation of orange color indicated the presence of derivative containing a primary amine.

Oxalic acid was dissolved in water and added to the gel, adjusted to pH 7.5 using potassium oxalate solution, followed by addition with the same amount of 1-ethyl-3-carbodiimide immideiately before it was used and by dropwise over a period of about

5 minutes. Over this periode, and for the following hour, the mixture was stirred gently for further 20 hours at 4°C without further adjustment of the pH. The gel was finally washed on a sintered glass funnel with about 100 ml of 0,1 M NaCl per ml of gel. The substitution of oxalate was checked by subjecting a small amount of the gel as described above. No color formation indicated that oxalate link to the gel.

Purification step

The supernatant was separated using the second partition of PEG 6000-Dextran 500 (4.5:14.0). The upper layer containing enzyme having higher activity was subjected to second partition with the same concentration. This upper layer was then applied to the oxalate-Sepharose column equilibrated with 10 mM of succinate buffer at pH 3.6. The elution was started with the same buffer and chromatographic fractionation was conducted in an automatic step gradient device. The following potassium chloride 0.5 M was passed. The effluent protein was monitored by a double beam Beckman DU-40 Spectrophotometer at 280 nm. The subsequent affinity chromatography was stopped after the second elution had passed through.

The disc-PAGE contained acrylamide and bis-acrylamide (Merk chemical Co.) in Tris Buffer solution. The protein pattern was stained with coomassie Blue R-250. The homogenate protein appeared only as a single band.

Results

Isolation of enzyme during barley seedling

In order to colum a particular part of the six days ole barley seedling bearing a higher enzyme activity, the leaves were separated from the roots. It was found that specific activity of the enzyme from the leaves was 0.026 U/ml and that from the roots was 5.48 U/ml respectively. Therefore the roots were used for the following purification. The oxalate oxidase activity during six days seedling were shown in the Table 1.

Purification of oxalate oxidase

In order to obtain a suitable amount of partially purified enzyme, the supernatant was fractionated with ammonium sulfate, and partitioned with PEG: Dextran. The resulting partial purification was as shown in Table 2.

Table 1. **Oxalate oxidase activity during seedling**

Sample	Activity (U/ml)	Protein (mg/ml)	Specific Activity (U/mg protein)
Barley grain	1.63	1.92	0.83
Soaked for 12 h	3.30	1.86	1.77
Seedling:			
1st day	8.60	2.13	4.04
2nd day	13.40	2.05	6.54
3rd day	16.30	2.10	7.76
4th day	10.6	1.94	5.46
5th day	9.4	1.87	5.03
6th day	8.9	1.86	4.78

Note: 1 unit activity expressed as U mol of H_2O_2.

Gel-chromatograph of the supernatant after second partition on oxalate-sepharose 6MB was as shown in Fig 1, and the purification scheme appreared in Table 3.

The different fractions obtained were assayed for oxalate oxidase activity. The activities were indicated by the peaks resulted from elution in the tubes number 16 to 20. Elution using succinate buffer did not release oxalate oxidase from the affinity column, therefore the enzyme was then eluted with a linear gradient of KCl (0.1 — 0.5 M).

Analysis of crude and purified enzyme preparation by SDS-poly acrylamide gel electrophoresis

As shown in Fig 3, fractions resulted from each purification step were analyzed by SDS-polyacrylamide gel electrophoresis. The molecular weight was determination by comparison with a group standard subjected to SDS-electrophoresis. It was 150.000 being in good agreement with the previously reported data (Sugiura, *et al.*, 1979).

Table 2. **Partial purification of supernatant**

Sample	Protein (mg/ml)	Total Vol. (ml)	Total Prot. (mg)	Total Act. (U)	Spec. Act. (U/mg)	Recov. (%)	Fold
Crude extract							
Unheated	2.3	20	46	172	3.7	100	1
Heated	1.9	20	38	198	5.2	115	1.4
$(NH_4)_2$ SO_4 treatment	0.9	5	4.5	72	16.0	41.9	4.3
Precipitate partition							
I. Upper	0.85	10	8.5	85.7	10.1	49.8	2.7
Bottom	1.35	20	27.0	32.4	1.2	18.8	0.3
II. Upper	0.36	4.5	1.62	21.6	19.6	12.6	5.3
Bottom	0.84	11.5	9.66	0	0	0	0

Table 3. **Purification of oxalate oxidase from barley seedling**

Sample	Protein (mg/ml)	Total Vol (ml)	Total Prot (mg)	Total Act. (U)	Spec Act (U/mg)	Recov (%)	Fold
Crude ext.	2.3	15	34.5	129	3.7	100	1
Part. I	0.85	10	8.5	85.7	10.1	66.4	2.7
Part. II	0.36	3	1.1	21.6	19.6	16.7	5.3
Affinity	0.023	3	0.07	10.8	154.3	8.4	41.7

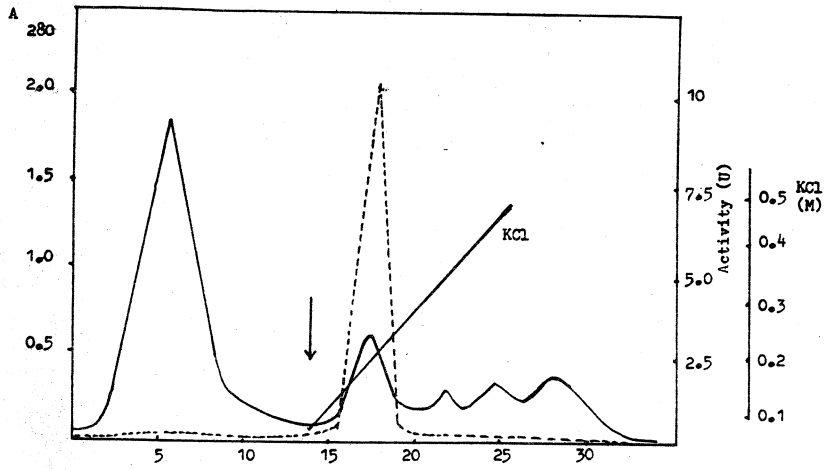

Figure 1. **Affinity chromatograph of fraction from Oxalate-Saphorose coloumn. The active fraction from second partition was applied on a coloumn of 3,5 ml bed volume. A fraction of 1 ml was collected. The coloumn was subsequently eluted with 25 ml of 5 mM succinate buffer at pH 3.6 and then with a KCl gradient in the same buffer as expressed in the text. The starting point of the gradient was shound by the arrow.**
(——) Absorbance at 280 nm
(. . .) U/ml axalate oxidase activity expressed as U mol H_2O_2

Discussion

Spectrophotometric detection of oxalate oxydase using the kits was rapid, simple and accurate. Hydrogen peroxide produced through catalysis by oxalate oxydase, oxydatively reacted with 4-amino antipyrine and 3,5 dichloro-2 hydroxybenzene sulfonate (DHBS). In the presence of peroxidase red color of complex chromogen was resulted. It was found that the optimum incubation time was three minutes.

Seedling of the third day contained oxalate oxidase having higher activity. Apparently seedling time effected the production of enzyme.

The homogenous preparation of oxalate oxidase had been obtained by Sugiura (1979) using preparative isoelectrofocusing. This technique was useful for assessing the purity in protein preparation, though it was not applicable for obtaining a large amount of the enzyme. Affinity chromatography seemed to be a suitable method to purify enzymic proteins on a preparative scale.

In this study, partition for partial purification followed by the affinity chromatography procedure was proved to be very effective for removal of the bulk of inactive proteins as shown in Table 3. Using this method it was possible to obtain apparently homogenous oxalate oxidase as shown in Figure 1 and 2.

By the affinity chromatography on oxalate-Sepharose 6MB a large amount of inactive protein could be separated. After second partition the specific activity of oxalate oxidase increased 5.3-fold, and after affinity chromatography 41.7-fold, with a recovery of 8.4% from the total enzymic activity. This was also remarkable, in comparison with the conventional and laborious procedure of frac-

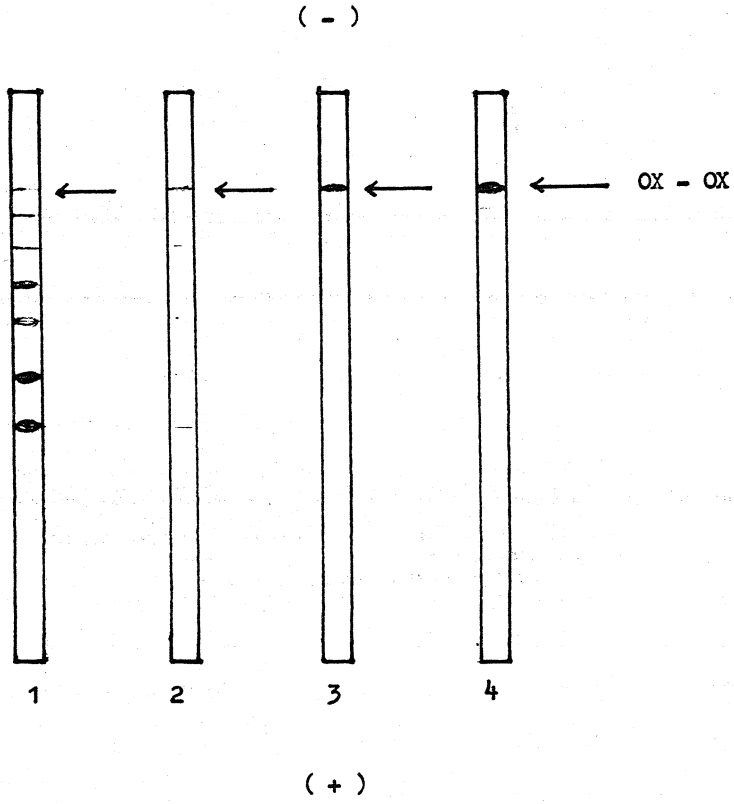

Figure 2. **Sodium dodecyl sulfate poly acrylamide gel electrophoresis of fractions from: (1) crude extract; (2) first partition; (3) second partition; and (4) affinity chromatography.**
OX-OX represented the band of oxalate oxidase after being stained with coomasie blue R-250.

tional precipitation using ammonium sulfate, which caused a considerable loss of enzyme.

It was also worth to be noted that the effectiveness of hydrophilic spacer-arm of oxalate-Sepharose in the affinity chromatography step, was considered an improvement over that of similar procedure using hydrophobic arm in the gel.

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