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PURIFICATION AND CHARACTERIZATION OF OXALATE OXIDASE FROM SPINY PIGWEED

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Abstract

Oxalate oxidase activities were determined in various tissues of the spiny pigweed (Amarantus spinosus L.). *The highest acivity was found in the leaves. Partial purification of this enzyme from the leaves of spiny pigweed was accomplished by heat treatment, ammonium sulfate fractionation, high speed centrifugation and DEAE-Sephacel chromatography. The enzyme was shown to be heat stable and to exhibit substrate specificity towards oxalic acid with a Km of 2.1 × 10^{-5}M. The optimal pH was 4.5 and the optimal temperature was about 35-50°C. The molecular weight as determined on a Sepharose 6B column was 265,000. The enzyme was moderately inhibited by azide and strongly inhibited by fluoride and 2 β-mercaptoethanol. The high specific activity and recovery of oxalate oxidase obtained from spiny pigweed suggests that spiny pigweed is a good source for preparation of this enzyme.*

Introduction

In recent years the measurement of oxalic acid in urine is of clinical interest in patients with urinary tract stones and hyperoxaluric syndrome. Numerous methods have been proposed for the measurement of oxalic acid in serum and urine. These techniques include colorimetry[1], fluorometry[2], enzymatic measurement[3-12] and advanced chromatographic techniques[13-15]. Many of these techniques are technically demanding or time consuming. However, measurement of oxalic acid by oxalate oxidase (EC.1.2.3.4) coupled with peroxidase is rapid, sensitive, precise[6,7,10] and needs only a simple spectrophotometer. However, although oxalate oxidase is now commercially available, its high price precludes its use for routine analysis, especially in a developing country such as Thailand. The discovery of oxalate oxidase from various sources has been reported : moss[16], barley seedling[17-19], mold[20] and spinach beet[21]. A preliminary survey of oxalate oxidase from various plants in Khon Kaen showed that spiny pigweed *(Amarantus spinosus L.)* was the best source of the enzyme. In this paper, the partial purification and some kinetic properties of oxalate oxidase from spiny pigweed are reported.

Materials and Methods

Chemicals

Horseradish peroxidase, 3-methyl-2-benzothiazolinone hydrazone (MBTH), N, N-dimethyl aniline (DMA) were purchased from Sigma. DEAE-Sephacel, Sepharose 6B and standard molecular weight markers were from Pharmacia Fine Chemicals. Other reagents used were reagent grade.

Assay of enzyme activity

The activity of oxalate oxidase was measured spectroscopically by conversion of oxalic acid to H_2O_2 and CO_2. H_2O_2 was determined enzymatically with horseradish peroxidase by oxidative coupling of MBTH with DMA. The resulting indamine dye was determined at 595 nm.[5]

Stock solutions of MBTH 1 g/L and DMA 2.5 g/L were prepared in 0.1 M hydrochloric acid. Working reagent was freshly prepared by diluting 2.5 ml of stock MBTH solution and 5 ml of stock DMA to 100 ml with 50 mM citrate buffer, pH 4.5. Oxalate oxidase activity was assayed in 1 ml of working reagent, 0.2 mM oxalic acid, and 18 U of horseradish peroxidase. The reaction mixture was incubated at room temperature for 30 min. The absorbance at 595 nm was recorded in a Varian Techtron series 634 spectrophotometer. H_2O_2 (5-20 nmole) was used as standard. One unit of the enzyme activity was defined as the amount of the enzyme which formed 1 μmole of hydrogen peroxide per min in the standard assay system.

Extraction and purification

Fresh tissues of the spiny pigweed were weighed and homogenized in a blender with two volumes of cold water. The homogenate was filtered through cheese cloth and then centrifuged in a Sorvall RC-5 Superspeed refrigerated centrifuge at 10,000 rpm for 15 min, at 4°C. The supernatant was heated for 3 min at 80°C under continuous agitation and then quickly chilled in ice. The homogenate was centrifuged again at 12,000 rpm for 15 min. Solid ammonium sulfate was added to the supernatant to 70% saturation with constant stirring at 4°C. The precipitate from the 70% saturation with ammonium sulfate was dissolved in water and dialyzed through three changes of water in a cold room for 24 h. Any precipitate formed during dialysis was removed by centrifugation at 12,000 rpm for 20 min. For further purification, the supernatant was centrifuged in a Beckman L8-80 Ultracentrifuge at 40,000 rpm for 1 h at 4°C. The supernatant was then dialyzed against 5 mM phosphate buffer, pH 7.0 for 24 h and applied to the DEAE-Sephacel column equilibrated with the same buffer. Fractions of 2 ml were collected at a flow rate of 20 ml/h. The loaded column was washed until the absorbance at 280 nm was close to zero and then eluted by a gradient of 10-500 mM phosphate buffer, pH 7. The protein and oxalate oxidase activity were determined as described above.

Tissue distribution of oxalate oxidase

To find out the tissue distribution of the enzyme, fresh spiny pigweeds were separated into flowers, leaves, roots and stems. 45, 50, 60 and 150 g of fresh flowers, leaves, roots and stems respectively, were partially purified as described above. The 70% ammonium sulfate fractions were assayed for protein content and oxalate oxidase activities. The specific activities and total activities were compared.

Effect of pH and temperature on oxalate oxidase activity

To study the effect of pH on the activity of oxalate oxidase, the activities of the enzyme were measured after incubating in various buffers for 30 min as described in the text. The buffers included: 0.1 M citrate buffer, pH 3.0-6.0; 0.1 M phosphate buffer, pH 6.5-8.0 and 0.1 M borate buffer, pH 9.5-10.0 The reagent mixtures without the enzyme oxalate oxidase, at various pHs, were used as blanks.

Effect of temperature on the activity of oxalate oxidase was studied by incubating the enzyme mixture at various temperatures (25-90°C) for 30 min. The reaction mixture without oxalate oxidase incubated at the same temperature was used as blank. The amount of peroxidase used in these studies was previously tested to be excess for coupling the hydrogen peroxide produced by oxalate oxidase reaction.

Determination of protein

Protein was determined by the method of Lowry *et al.*, using bovine serum albumin as standard.

Molecular weight determination by Sepharose 6B

A Sepharose 6B (1.8 × 56 cm) column was equilibrated with 10 mM phosphate buffer, pH 7.4 and pre-calibrated with standard molecular weight markers: blue dextran (2,000,000), thyroglobulin (669,000), ferritin (440,000), catalase (232,000) and DNP-lysine (366). Fractions of 2 ml were collected at a flow rate of 8.5 ml/h.

Results

Tissue distribution of oxalate oxidase

The oxalate oxidase activities of the 70% ammonium sulfate fractions obtained from roots, stems, leaves and flowers of spiny pigweed were compared. The leaf-fraction exhibits the highest activity when the enzyme was expressed as units/g wet weight and units/g dry weight. On the other hand, the enzyme from the stem has the highest specific activity when compared to the other tissues (Table 1).

TABLE 1 TISSUE DISTRIBUTION OF OXALATE OXIDASE FROM SPINY PIGWEED.

Enzyme activity	Roots	Stems	Leaves	Flowers
Unit/g wet wt.	8	13	306	2
Unit/g dry wt.	46	131	1248	18
Unit/mg protein	30	63	29	2

TABLE 2 PARTIAL PURIFICATION OF OXALATE OXIDASE FROM 50 G WET WEIGHT OF SPINY PIGWEED LEAVES.

	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	% yield
Crude homogenate	1148	643	1.6	1	-
70% ammonium sulfate fractionation	15295	509	29	17	100
High speed centrifugation	10615	114	92	55	69
DEAE-Sephacel column	6017	3	1629	977	39

TABLE 3 EFFECT OF VARIOUS REAGENTS ON OXALATE OXIDASE ACTIVITY

Reagent tested* (1mM)	Remaining activity (%)
1. Control	100
2. Glucose	127.37
3. EDTA	96.65
4. NaF	9.49
5. Na_2CO_3	84.92
6. NaN_3	41.89
7. Na_2SO_4	67.04
8. NaCl	75.42
9. 2 ß-mercaptoethanol	0

* All the reagents used were shown to have no effect on peroxidase activity.

Partial purification

The 70% ammonium sulfate fraction of spiny pigweed leaves was further purified by high speed centrifugation and DEAE-Sephacel column chromatography (Fig. 1). The results of partial purification are summarized in Table 2. The recovery of enzyme activity during purification was 39% and the final specific activity was 1630 unit/mg, corresponding to a 977-fold purification when compared to the crude homogenate.

The enzyme obtained from the DEAE-Sephacel column was pooled, lyophilized and kept for further studies.

Properties of oxalate oxidase

Substrate specificity: The enzyme showed a high substrate specificity towards oxalic acid and did not react with other acids (citric acid, glycine, aspartic acid, gluconic acid and malonic acid). The Km for oxalic acid, determined from the Lineweaver-Burk plot, was 2.1×10^{-5}M (Fig. 2).

Optimal pH: Oxalate oxidase activities at various pHs were determined. The optimal pH of the enzyme is shown to be 4.5.

Optimal temperature and heat stability: The enzyme exhibits maximum activity at 35-50°C. The heat stability study shows that the enzyme is markedly stabel to heat, with more than 80% of the activity remaining after 3 min at 80°C.

Molecular weight: The molecular weight of oxalate oxidase was estimated by the Sepharose 6B column, using thyroglobulin, ferritin and catalase as standard molecular weight markers, and found to be 265,000.

Effect of various reagents on the enzyme activity: As shown in Table 3, EDTA at 1 mM has no effect on oxalate oxidase activity. The enzyme is strongly inhibited by fluoride and 2 ß-mercaptoethanol, and moderately inhibited by azide.

Discussion

Oxalate oxidase activity in the spiny pigweed was found to be higher in the leaves compared to the roots, stems and flowers. Partial purification of the enzyme by heat treatment, ammonium sulfate fractionation, high speed centrifugation and ion-exchange column chromatography yielded oxalate oxidase in 39% yield with 977-fold purification. Chiriboga[18] used ammonium sulfate and DEAE-cellulose chromatography to purify oxalate oxidase from barley seedling and obtained only 6.8% recovery. Sugiura, *et al.*[19] also purified oxalate oxidase from barley seedlings by using ammonium sulfate fractionation and isoelectrofocusing with 31.2% recovery. In this report, high specific activity oxalate oxidase could be obtained from spiny pigweed leaves in good yields, suggesting that spiny pigweed is another good source for the preparation of this enzyme.

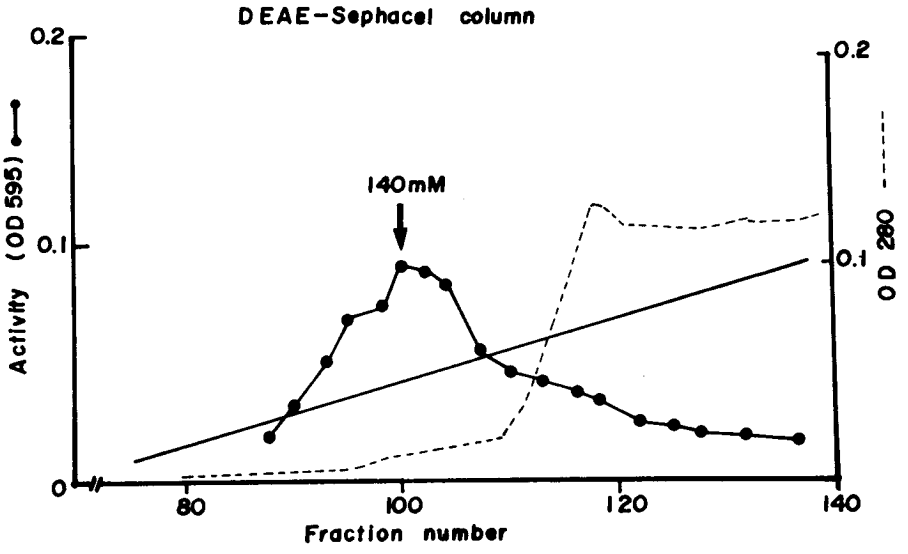

Fig. 1 Activity profile of oxalate oxidase from spiny pigweed. The enzyme obtained after high speed centrifugation was applied to a DEAE-Sephacel column (1.8 × 11 cm). The enzyme was eluted with 10-500 mM phosphate buffer gradient, pH 7. The phosphate gradient is represented by the straight line without symbols.

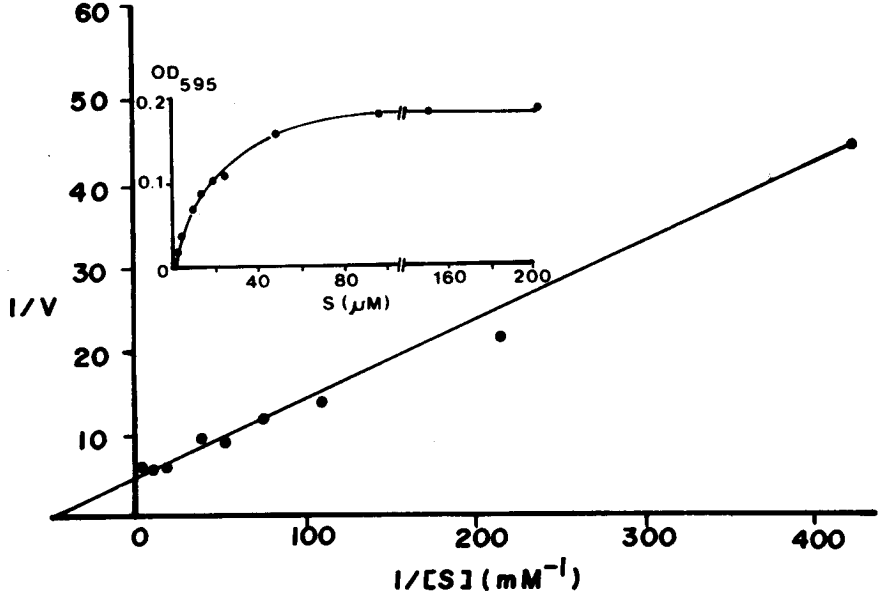

Fig. 2 The reciprocal plot of oxalate oxidase activities versus oxalic acid concentration. The enzyme activities were assayed as described in the text, with varying concentrations of oxalic acid. The saturation curve is illustrated in the small figure.

The optimal pH of the enzyme from spiny pigweed was 4.5, which differs from the results obtained with enzyme from barley seedlings (pH 3.5[18], 3.2[19]), mold (pH 5.0)[20] and spinach beet (pH 4.0)[21]. The molecular weight of oxalate oxidase determined here (2.6×10^5) was higher than that of barley seedlings (1.5×10^5)[19].

Like the enzyme obtained from other sources, oxalate oxidase from spiny pigweed is heat stable and shows strict substrate specificity towards oxalic acid. The Km of oxalic acid of the enzyme obtained here (2.1×10^{-5} M) is lower than that of barley seedling (4.2×10^{-4}M)[18].

Oxalate oxidase from barley seedlings was demonstrated to be activated by EDTA[18], but EDTA has no effect on the activity of the enzyme reported here. Strong inhibition of azide to the enzymes obtained from barley seedlings[15] and moss[16] has been reported, but azide only showed moderate inhibition of the enzyme from spiny pigweed. On the other hand, fluoride which moderately inhibits enzymes from the other sources[19,20], strongly inhibited the enzyme from spiny pigweed. Like the enzyme from the other sources, oxalate oxidase from spiny pigweed is strongly inhibited by 2 ß-mercaptoethanol. Comparison of the properties of oxalate oxidase from spiny pigweed to the enzymes from the other sources[15-20], suggests that oxalate oxidases obtained from different sources exhibit different properties, and the only common characteristics of these enzymes are the substrate specificity to oxalic acid, the heat stability and the strong inhibition by 2 ß-mercaptoethanol.

A number of analytical methods have been developed for determination of oxalate in urine and plasma[1-15]. Among these techniques, enzymatic measurement is potentially the most suitable for routine assay of oxalate. So far, oxalate oxidase has been prepared from various sources[16-21]. Some of these are commercially available but at a high expense. Recently, a cheap source of this enzyme prepared from banana peel has been reported[11]. Spiny pigweed, the source of enzyme reported here, is a weed commonly found in the field. Our simple purification procedure yielding high specific activity suggests that spiny pigweed is another inexpensive source for the preparation of this enzyme. Oxalate oxidase prepared from barley seedlings has been tested for clinical diagnosis of oxalate in urine[5,6,10,11] and plasma[10,12]. Attempts to use the enzyme from spiny pigweed to determine oxalate in urine and serum for clinical diagnostic tests are now under investigation.

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บทคัดย่อ

จากการศึกษาแอคติวิตี้ของเอ็นไซม์ออกซาเลทออกซิเดส จากส่วนต่าง ๆ ของผักโขมหนาม *(Amarantus spinosus L.)* พบว่า ใบเป็นส่วนที่มีเอ็นไซม์ดังกล่าวอยู่มากที่สุด การศึกษานี้ได้สกัดและแยกเอ็นไซม์ออกซาเลทออกซิเดสจากใบผักโขมหนามโดยการผ่านความร้อน การตกตะกอนด้วยแอมโมเนียมซัลเฟต การปั่นเหวี่ยงด้วยความเร็วสูง และการผ่าน DEAE-Sephacel column การศึกษาคุณสมบัติของเอ็นไซม์ดังกล่าวพบว่าเอ็นไซม์นี้มีความเสถียรต่อความร้อน มีความจำเพาะสูงต่อการใช้ออกซาเลทเป็นสับสเตรทและมีค่า Km = 2.1×10^{-5}M pH และ อุณหภูมิที่เหมาะสมสำหรับเอ็นไซม์นี้คือ 4.5 และ 35-50°ซ น้ำหนักโมเลกุลเมื่อศึกษาโดยผ่าน Sepharose 6B column มีค่า 265,000 dalton นอกจากนี้ยังพบว่าเอไซด์ ฟรูออไรด์ และ เมอร์แคปโตเอทานอล สามารถยับยั้งปฏิกิริยาของเอ็นไซม์นี้ได้ การศึกษานี้มีข้อบ่งชี้ว่าผักโขมหนามจะเป็นแหล่งที่สำคัญแหล่งใหม่ในการผลิตเอ็นไซม์ออกซาเลทออกซิเดสในอนาคต