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PARTIAL PURIFICATION AND PROPERTIES OF PEROXISOMAL OXALATE OXIDASE IN *HELIANTHUS ANNUUS* L. CALLUS CULTURES

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Peroxisomal preparations from hypocotyls of Helianthus annuus L. catalysing the oxidation of oxalate to CO_2 was described. Purification of oxalate oxidase was accomplished by ultrasonic and heat treatments, ammonium sulphate fractionation and DEAE cellulose chromatography. Specific activity in crude extract was 0.59 µmol H_2O_2/mg protein which was 5 fold higher than the maximum reported for other preparations. The enzyme exhibits a definite substrate inhibitory effect with Km $5x10^{-4}$ M. The rate of H_2O_2 formation was linear up to first 7 min. and showed maximum activity at pH 5 and 40°C. The enzyme was inhibited by EDTA and diethyldithiocarbamate (DEDTC) but α-α'-dipyridyl had no effect. The inhibition by DEDTC was specifically reversed by Cu^{2+}, while flavins (riboflavin, flavin adenine dinucleotide "FAD" and flavin mononucleotide "FMN") stimulate the enzyme in presence of Cu^{2+} only. The enzyme was unaffected by Na^{+}, nitrate and some metal ions.

Keywords :*Oxalate oxidase; Helianthus annuus L. callus culture; peroxisome purification properties.*

Introduction

Oxalate oxidase (EC 1.2.3.4) catalyzes the oxidation of oxalate into CO_2 with production of H_2O_2. Recently the interest in oxalate oxidase arised from the need of the purified enzyme preparation for oxalate determination in biological fluids and clinical diagnosis (Pundir and Usha 1993; Mazzuchin et al., 1990; Morgan 1987; Sidhu et al., 1987). The purified enzyme will also be useful in determination of oxalate content of common foods, which is necessary for prescription of low oxalate diet to patients with urinary and kidney stones (Kasidas et al., 1980; Hodgkinson, 1981).

Investigations on intracellular localization of oxalate oxidase do not permit for a definite conclusion about solubility or particulate nature of the enzyme. Soluble enzyme preparations appear to be concentrated in the roots of barley seedlings (Chiriboga, 1963, 1966) and in sorghum leaves and roots (Pundir and Nath, 1984; Pundir and Kuchhal, 1989; Kuchhal et al., 1993). Leek et al. (1972) suggested that some oxalate oxidase activity is released from peroxisomes isolated from spinach and beet leaves during the extraction of the enzyme. Oxalate oxidase is also associated with constituents of the cells in beet extract probably due to mitochondrial fraction (Meeuse and Campbell, 1959). A particulate oxalic acid oxidase in homogenates of Bougainvillea and Begonia leaves was associated with chloroplast fraction (Srivastava and Krishnan, 1962; Sasaki, 1963).

It is obvious therefore that the nature and the mechanism of action of this enzyme is not fully understood. The present report involves the purification and some properties of this enzyme from *Helianthus annuus* L. callus tissues.

Materials and Methods

Growth and maintenance of Helianthus annuus L. callus tissues

Four days old (Helianthus annuus L. cv. Mayak) seedlings (0.5-2.0 cm high) were used for callus

initiation. The hypocotyls were cut into sections approximately 0.5 cm long, surface sterilized and transferred to Mason Jars containing 30 ml modified Murashige and Skoog (1962) medium. Callus normally began to form in about 1 week and was ready for subculturing after two weeks. All cultures were maintained in a growth chamber at 25 1°C with 16 hour light period (cool white fluorescent lamps) under 500 Lux illumination.

Isolation of enzyme

Oxalate oxidase from sunflower callus tissue was prepared as following: Frozen callus (50 g) was homogenized with 120 ml 0.05 M phosphate buffer (pH 7.5) containing 20% glycerol in a prechilled mortar and pestle. The cell debris was removed by centrifugation at 500 X g and the supernatant was designated as crude extract.

Ultrasonic treatment

The crude extract was sonicated for 2 min. in Fisher's Sonic Dismembrator Model 150, followed by centrifugation at 15000 X g.

Heat treatment

The sonicated fraction was heated for 5 min at 55°C under continuous agitation and then quickly chilled in ice. The preparation was centrifuged again for 15 min at 15000 X g.

Ammonium sulphate fractionation

Solid $(NH_4)_2SO_4$ was added to the supernatant to 40% saturation with stirring for 30 min. and then this mixture was centrifuged at 15000 X g for 10 min. The pellet was discarded and the supernatant was brought to 60% saturation of $(NH_4)_2SO_4$. Sedimented proteins were collected by cantrifugation (15000 X g, 10 min), dissolved in the same buffer and dialyzed overnight against buffer glycerol mixture. The bulk of activity remained in 40-60% of ammonium sulphate precipitate.

DEAE cellulose column chromatography

The dialyzed sample was applied to DEAE cellulose column (70x2.5 cm) equilibrated with 0.05 M phosphate buffer (pH 7.5) containing 20% glycerol. Proteins were eluted by increasing concentrations of KCL from 0 ta 0.3 M in the same buffer. Most of the activity was eluted with 0.05 M KCl, the active fractions were pooled used for studying the properties of the enzyme.

Assay of enzyme

The method used was that reported by Pundir and Nath (1984). The enzyme was assayed in glass tubes wraped with black paper. To each tube was added 80 µmol Na succinate buffer (pH 5), 1 µmol Na oxalate and 0.5 mg enzyme protein in a total vol. of 2 ml. One µmol $CuSO_4$ was also added to the reaction mixture expect when the effects of metals, metal chelators and co-enzyme was studied. After incubation at 40°C for 5 min, 1 ml colour reagent was added, the tubes were shaken and then allowed to stand at room temperature for 30 min to develop the colour. The absorbance was read at 520 nm.by a Perkin Elmer spectrophotometer (Model 200 Hitachi) and the content of H_2O_2 generated during the reaction was extrapolated from the standard curve of H_2O_2 prepared in 0.05 M Na succinate buffer (pH 5).

One enzyme unit is defined as the amount of enzyme protein required to produce 1 µmol H_2O_2 per min under standard conditions of assays.

The colour reagent was prepared as described by Bais et al. (1980) and consisted of 0.05 g 4-aminophenazone, 0.1 g phenol and 1 mg horseradish peroxidase per 100 ml 0.4 M Na Pi buffer, pH 7.

Protein was estimated according to Lowry et al. (1951).

Preparation of the particulate fractions by differential centrifugation

Fresh sunflower callus were used for preparation of cell fractions by the method described by Leek et al. (1972). All operations were carried out at 0-4°C. Ten grams of callus tissue were homogenized in Waring Blendor for 30 sec. with 20 ml of 0.5 M sucrose in 20 ml of 0.02 M glycylglycine buffer adjusted to pH 7.5. The homogenate was squeezed by hand through a double layer of muslin. The extract was subjected to differential centrifugation (20 min. for each step). The first pellet (500 X g) contained mostly large cell fragments, starch grains. The second pellet (6000 X g) contained some mitochondria and large part of the peroxisomes. The third pellet (35000 X g) designated as mitochondria contained the remaining microbodies. The remaining solution was designated as "supernatant". Each pellet was resuspended in the grinding medium.

The precepitate sedimented at 6000 X g, was resuspended in 2 ml of the medium used for homogenization and layered above 5 ml of 1.8 M sucroce in 0.02 M glycylglycine buffer (pH 7.5). The extract was centrifuged for 3 hours at 4°C at 25000 rpm (44700 X g to 106900 X g) in swinging bucket rotor SW 25.2 of the Spinco Centrifuge Model L.

Results and Discussion

The present report describes partial purification and some properties of a highly active oxalate oxidase in the peroxisomal preparation of sunflower callus tissue. The intial activity in crude extract prepared by centrifugation at 500 X g was 3.8 units/g fresh

weight. Most of this activity was lost in supernatant after recentrifugation at 15000 X g while 85% of this activity was recovered in the resuspended pellet. This indicates the particulate nature of the enzyme, so the last step was omitted from the purification scheme. A simple reproducible method for partial purification of oxalate oxidase is shown in Table 1. The specific activity in the crude extract was 0.59 µmol H_2O_2/mg protein which was almost 10 fold higher than the reported specific activities for barley seedling (69 n mol/mg protein) (Chiriboga, 1966). The specific activity of oxalate oxidase in the roots, stem and leaves of grain sorghum were 28.6, 14.1, 10.4 n mol/mg protein respectively (Pundir and Kuchhal, 1989). The ultrasonic treatment (Table 1) raised the specific activity up to 0.7 and 86% of the activity was recovered. Heat treatment (Table 1) led to slight increase in specific activity and the yield was 80%.

Concerning $(NH_4)_2SO_4$ fractionation (Table 1) three fold increase in specific activity over crude extract, was obtained while 60% of the activity was lost. Purification on DEAE cellulose (Table 1 and Fig. 1) reveals that active fraction was eluted with 0.05 M KCl. Five fold increase in specific activity over crude extract was recorded. Properties of the enzyme were studied using partially purified enzyme (DEAE cellulose pooled fractions).

Table 1. Purification of oxalic acid oxidase.

Fraction	Volume (ml)	Total Protein (mg)	Total units	*Specific activity	Recovery %
Crude homogenate	100	325	192	0.59	-
Ultrasonic treatment	97	230	165	0.71	85.9
Heat treatment	95	190	155	0.81	80.7
Ammonium sulphate (40-60%)	12	40	72.8	1.82	37.9
DEAE cellulose	50	7.1	21.2	2.98	11

See text for description of treatment.

*Specific activity Units (µmol H_2O_2 (5 min)/mg protein.

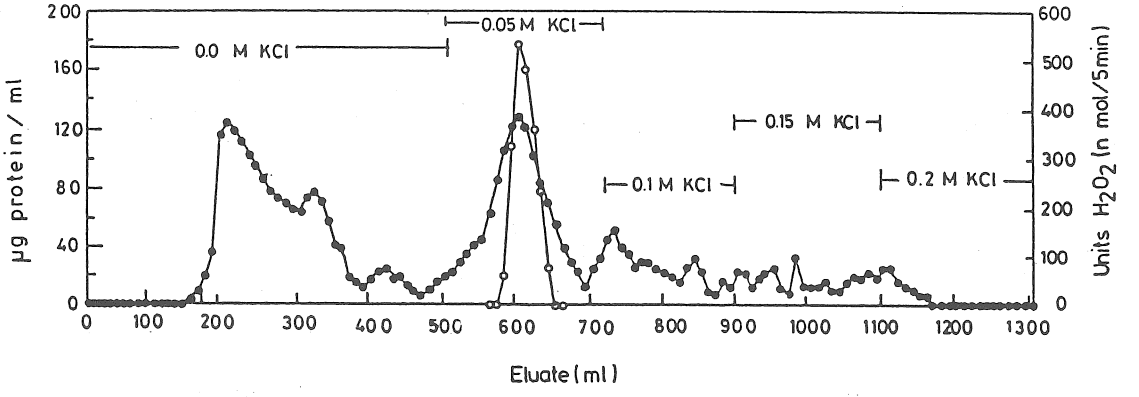

Fig 1. Typical elution profile for the behaviour of oxalate oxidase in DEAE cellulose column. Enzymatic activity and procedures were mentioned in text.
●—● Protein, o—o Units

Effect of incubation time and enzyme concentration on the partially purified enzyme

The enzyme activity was linear up to first 7 min., after 20 min. the reaction rate slightly decreased (Fig. 2). This is comparable to that reported for barley roots and sorghum

leaves (10 min. incubation) by Suguira et al. (1979), Pundir and Nath (1984) and for sorghum roots (5 min. incubation) by Pundir and Kuchhal (1989). Pundir and Nath (1984) attributed this decrease to the possibility of either enzyme inactivation with accumulation of product or disappearance of H_2O_2 with increased incubation time. There was a linear relationship between the enzyme activity and protein content of the purified enzyme in the concentration range 0.01 - 0.1 mg/assay (Fig. 3).

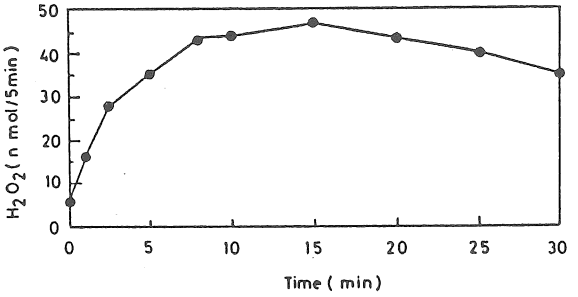

Fig. 2. Effect of incubation period on purified sunflower oxalate oxidase activity. Standard assay conditions were used except for the incubation period which was varied as indicated.

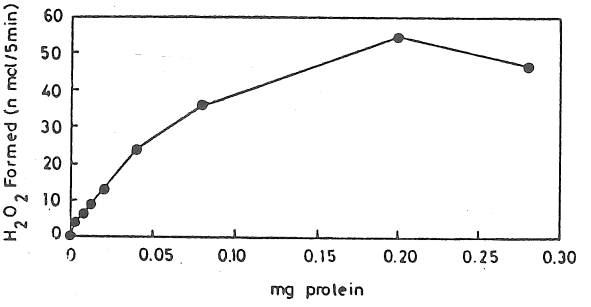

Fig.3. Dependence of reaction rate on enzyme concentration.

Effect of oxalate concentration

The activity of the partially purified enzyme was measured with increasing oxalate concentrations (from 1x10-4 M to 5x10-3 M). A hyperbolic relationship between oxalate concentration and enzyme activity was obtained up to a concentration of 1x10-3 M, above which the system exhibits a definite substrate inhibitory effect (Fig. 4). A similar stituation has been reported for barley roots and sorghum leaves enzymes (Chiriboga, 1966; Pundir and Nath, 1984). Km for oxalic acid determined from lineweaver and Burk plot shown in Fig. 4 (inset) is 5x10-4 M. The reported Km values for oxalate of barley root and sorghum leaves were 4.2x10-4 M and 2.4x 10-5 M respectively (Chiriboga, 1966; Pundir and Nath, 1984).

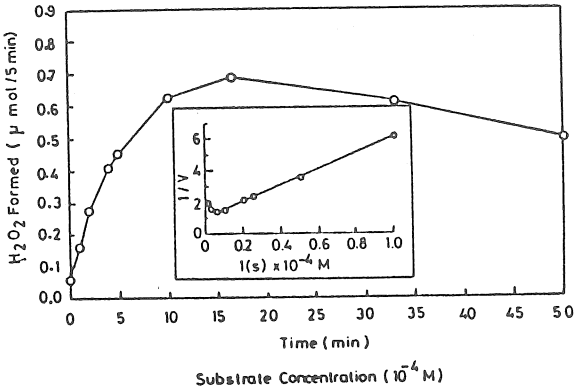

Fig. 4. Dependence of initial velocity of purified oxalate oxidase reaction on substrate concentration. Standard assay conditions were used for the incubation period, which was varied as indicated. The inset shows the double reciprocal plot for oxalate. All points were derived from Fig.4.

Effect of pH and temperature

The optimum pH for the purified oxalate oxidase enzyme was 5 (Fig. 5). Most

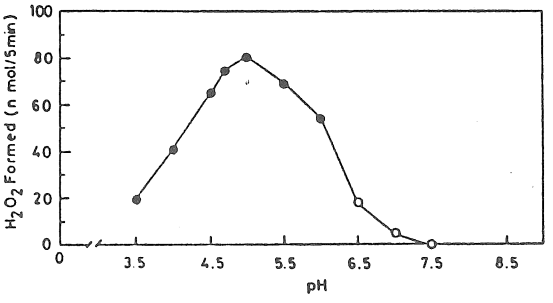

Fig. 5. Effect of pH on sunflower oxalate oxidase activity. Standard assay conditions were used except for the pH which was varied as indicated (●—● pH 3.5-6 sodium succinate, o—o pH 6-7.5 sodium phosphate, each at a concentration of 0.05M).

reported pH optima are in acidic range as for T. controversa (pH 2.6), barley root (pH 3.5), mosses (pH 4.0) shorghum leaves (pH 5), Bougainvillea leaf (pH 6.8), (Vaisey, et al., 1961; Chiriboga, 1966; Datta and Meeuse, 1955; Pundir and Nath, 1984; Srivastava and Krishnan, 1962 respectively).

The enzyme exhibited a maximum activity at a temperature of 40°C, after which it declined rapidly, most probably because of thermal denaturation of the enzyme.

Effect of chelating agents, metals and co-enzymes

The strong inhibition of enzyme by EDTA indicated the metal ion requirement for enzyme activity (Table 2). Among the various metal ions tested, only Cu^{2+} stimulated the enzymatic reaction of the dialyzed enzyme (Table 2). Other metals (Fe^{2+}, Co^{2+}, Sr^{2+}, Pb^{2+}, Mg^{2+}, Zn^{2+}, Na^{+} and K^{+} at $5x10^{-4}$ M) had no effect.

Table 2. Effect of metal chelators and metals on oxalate oxidase activity.

Reagent added	Relative activity
None	100
EDTA	65
α, α'-Dipyridyl	100
Diethyldithiocarbamate	60
Fe^{2+}	100
Cu^{2+}	195
EDTA+Cu^{2+}	90
α, α'-Dipyridyl + Cu^{2+}	220
Diethyldithiocarbamate+Cu^{2+}	230

Standard assay conditions were used except for the addition of metal chelator and metals as indicated. The final concentration of each compound(s) added was $5x10^{-4}$ M.

Diethyldithiocarbamate caused 40% inhibition of the enzyme activity, this inhibition was specifically reversed by Cu^{2+}. On the other hand α, α'-dipyridyl had no effect which excluded Fe^{2+} requirement for enzyme activity (Table 2). The metal requirement for stimulation of oxalate oxidas was variable as: Fe^{2+} was reported for sorghum leaves, while Cu^{2+} for sorghum roots. (Pundir and Nath, 1984; Pundir and Kuchhal, 1989; Kuchhal et al., 1993). Suguira et al. (1979) reported 36% stimulation by Cu^{2+} of oxalate oxidase purified from barley seedling. Stimulation (75%) for enzyme preparation from pseudomonas sp. OX-53 by Mn^{2+} but 36% by Cu^{2+} at 10^{-4} M was reported by Koyama (1988).

Effect of sodium and nitrate ions

Both of sodium and nitrate ions did not show any inhibitory effect on callus oxalate oxidase up to 0.25 M. The same situation was reported for this enzyme prepared from sorghum leaves (Pundir and Nath, 1984; Kuchhal et al., 1993). On the other hand previous reports have revealed that oxalate oxidase from barley and *Bougainvillea* are sodium sensitive and an inhibition of more than 50% has been observed by 0.1 M sodium chloride (Chiriboga, 1963, 1966; Leek, 1972). Nitrate concentration as low as $5x10^{-5}$ M gave severe inhibition of oxalate oxidase in beet extract (Meeuse and Campbell, 1959). For studying the requirement of co-enzyme, the purified enzyme was dailysed against metal-free water in cold for 24 hr with constant stirring. The dialysed enzyme lost almost all its activity, which was restored by addition of Cu^{2+} while Fe^{2+} had no effect (Table 3). All the flavins such as riboflavin, FMN and FAD had slight stimulatory effect on the enzyme activity which was increased by addition of Cu^{2+} but not Fe^{2+}. These observations suggest that the enzyme is most probably present as copperflavoprotein in nature. Moss, fungi and sorghum leaves oxalate oxidase were classified as flavoproteins. On the other hand all the flavins (riboflavins, FMN and FAD) had almost no effect on the activity of sorghum root oxalate oxidase (Pundir and Kuchhal, 1989).

Intracellular Localization

The activities of enzyme in the fractions obtained from the centrifugation procedure

Table 3. Effect of flavins on partially purified dialyzed oxalate oxidase activity.

Reagent added	Relative activity
None	100
Cu^{2+}	330
Fe^{2+}	105
FMN	120
FAD	110
Riboflavin	135
FMN+Cu^{2+}	340
FAD+Cu^{2+}	330
Riboflavin+Cu^{2+}	310
FMN+Fe^{2+}	125
FAD+Fe^{2+}	110
Riboflavin+Fe^{2+}	140

Partially purified dialysed enzyme was used. Standard assay conditions were used except for the addition of $FeSO_4$, $CuSO_4$ and/or flavin as indicated. The final concentrations of Cu^{2+} and Fe^{2+} were $5x10^{-4}$ M and of flavins 10^{-4}.

are shown in Table 4. Almost 85% of the activity detected in initial homogenate was associated with peroxisomal preparation (second pellet) and most of the activity (82%) was accompanied by the peroxisomal precipitation through 1.8 M sucrose. However 15% of activity was detected in the supernatant after centrifugation at 6000 X g Tolbert et al. (1969) concluded from fractionation studies with a number of species that these enzymes were readily released from peroxisomes during extraction procedure. Leek et al. (1972) suggested that peroxisomal preparation from leaves of spinach beet catalyzed the oxidation of oxalate to CO_2.The presence of plastid bound oxalate oxidase in the leaves of *Bougainvillea spectabilis* was reported by Srivastava and Krishnan (1962). The association of the oxalate oxidase with mitochondrial preparation in beet extract was reported by Meeuse and Campbell (1959). On the other hand the soluble preparations of oxalate oxidase were carried out using barley roots and leaves (Chiriboga, 1963, 1966; Pundir and Nath, 1984; Pundir and Kuchhal, 1989; Kuchhal et al., 1993). Pundir and Nath (1984) do not permit for a definite conclusion about the solubility or particulate nature of the enzyme because of the possibility of breaking the membrane of cell organells during the extraction.

Table 4. Intracellular distribution of oxalate oxidase isolated from sunflower callus tissue.

Cellular fraction	Total activity (Units/g fr. wt.)*	%
A- Differential centrifugation	3840	100
a- Initial homogenate (500 X g)		
Cell debris	ND	-
b- Second pellet (6000 X g)	3264	85
Supernatant (6000 X g)	192	5
c- Third pellet (3500 X g)	ND	-
Supernatant	ND	-
B- Centrifugation through 1.8 M sucrose (second pellet)	3148	82

*One unit of activity is defined as the amount of enzyme required to produce 1 n mol H_2O_2/5 min under standard assay condition, ND=Not detected.

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