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DETECTION OF AN OXALATE OXIDASE IN GRAIN SORGHUM ROOTS

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Key Word Index—*Sorghum vulgare*; Gramineae; sorghum roots; oxalate oxidase; oxalate oxidation; oxalate; Cu^{2+}; Fe^{2+}.

Abstract—An oxalate oxidase (EC 1.2.3.4) was detected in 15000 *g* supernatant of 10-day-old roots of grain sorghum variety CSH-5. The enzyme showed optimal activity at pH 5 and 40° and required five min incubation. The enzyme was strongly inhibited by EDTA and diethyldithiocarbamate. The activity of the dialysed enzyme could be restored by adding Cu^{2+} only. Cu^{2+} specific chelator partly reversed the stimulation caused by Cu^{2+}. Flavins had almost no effect on dialysed enzyme. The enzyme was inhibited both by sulphydryl reagents and —SH group blocking agents which was reversed by addition of Cu^{2+}. The possibility of sulphydryl-copper ion complex at the active centre of the enzyme is discussed.

INTRODUCTION

Oxalate catabolism in higher plants involves activation and decarboxylation by oxalate decarboxylase [1] and oxidation by oxalate oxidase [2]. The latter enzyme is of greater interest, as it has been recently used in the direct estimation of oxalate in biological fluids [3–5]. Although an oxalate oxidase from barley seedlings [6–7] has been purified and characterized, the nature of this enzyme and mechanism of oxalate oxidation in higher plants are not fully understood. Recently, Pundir and Nath [8] reported the occurrence of an oxalate oxidase in 10-day-old leaves of grain sorghum variety CSH-1 and suggested the essentiality of Fe^{2+} for its activity. We have detected this enzyme in 10-day old seedlings of grain sorghum variety CSH-5 which required smaller time of incubation and Cu^{2+} for its optimal activity as compared to that of CSH-1 variety [8]. Because roots showed a higher specific activity than leaves and stems of the seedlings, the root enzyme was chosen for further characterization. The present report, deals with some of the properties of this enzyme, its cofactor requirement and possible mechanism of oxalate oxidation.

RESULTS

The specific activity of oxalate oxidase in 15000 *g* supernatant of roots, stems and leaves of 10-day-old seedlings of grain sorghum variety CSH-5 is shown in Table 1. The specific activity of the enzyme was found in the order roots > stems > leaves.

The effects of pH on the activity of enzyme was studied in the pH range 2–7 using the following buffer solutions: pH 2, KCl–HCl; pH 2.5, glycine–HCl; pH 3–6, Na succinate and pH 6.5–7, Na phosphate, each at a concentration of 0.05 M. The enzyme had a broad pH optima at pH 5. The enzyme activity at pH 2 and 6 was 25 and 60% of the activity at pH 5, respectively.

The enzyme showed optimum activity within five min of incubation which was constant up to seven min, after which there was a slight decline in the activity of enzyme.

Table 1. Specific activity* of oxalate oxidase in 15000 *g* supernatant of various tissues of 10-day-old seedlings of grain sorghum variety CSH-5

Tissue	Specific activity
Seedlings	12.8
Leaves	10.4
Stems	14.1
Roots	28.6

*Standard assay conditions were used except for the addition of 1 μmol ferrous sulphate. Specific activity denotes the number of enzyme unit per mg protein. One unit of enzyme is defined as the amount of enzyme required to generate 1 n mol of hydrogen peroxide per min.

Therefore, in all enzyme assays, the incubation time was five min. There was a linear relationship between the enzyme activity and protein contents of the 15000 *g* supernatant in the concentration range 0.1–0.5 mg/assay.

When enzyme activity was assayed at different temperatures ranging from 25 to 70°, optimum activity was at 40° and this was stable up to 55° after which a decline in the activity was observed, probably due to thermal denaturation of the enzyme. When the enzyme was stored at 0–4°, it lost *ca* 50% activity within 30 days.

The strong inhibition of enzyme by EDTA indicated the metal(s) ion requirement for enzyme activity (Table 2). Among the various metal ions tested on the activity of partially purified dialysed enzyme at a concentration of 5×10^{-4} M, Cu^{2+} caused three-fold, stimulation which was more pronounced ($\times 6$) when undialysed crude enzyme (15000 *g* supernatant) was used as a source of

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

Reagent added	Relative activity (%)
None	100
EDTA	62
α,α′-Dipyridyl	100
Diethyldithiocarbamate	70
8-Hydroxyquinoline	150
Cu^{2+}	329
EDTA + Cu^{2+}	95
α,α′-Dipyridyl + Cu^{2+}	200
Diethyldithiocarbamate + Cu^{2+}	233
8-Hydroxyquinoline + Cu^{2+}	467

*The partial purification was done as described in experimental. Standard assay conditions were used except for the addition of chelators and/or Cu^{2+} as indicated. The final concentration of each compound(s) added was 5×10^{-4} M.

Table 4. Effect of flavins on partially purified dialysed* oxalate oxidase activity

Reagent added	Relative activity (%)
None	100
Cu^{2+}	310
Riboflavin	130
FMN	125
FAD	100
Riboflavin + Cu^{2+}	337
FMN + Cu^{2+}	330
FAD + Cu^{2+}	312

*The enzyme was partially purified and dialysed as described in the Experimental. Standard assay conditions of assays were used except for the addition of Cu^{2+} ($CuSO_4$) and/or flavins as indicated. The final concentration of Cu^{2+} and flavins was 5×10^{-4} and 10^{-4} M, respectively.

enzyme (Table 3). The stimulation by Cu^{2+} was partially reversed by addition of various metal chelators such as α,α′-dipyridyl or diethyldithiocarbamate, a Cu^{2+} specific chelator. However, 8-hydroxyquinoline both alone and in the presence of Cu^{2+} caused *ca* 50 and 42% stimulation, respectively (Table 2).

All the flavins such as riboflavin, FMN and FAD both alone and in the presence of Cu^{2+} had almost no effect on the activity of partially purified dialysed enzyme (Table 4).

Iodoacetate and *N*-ethylmaleimide caused *ca* 30 and 24% inhibition respectively. However, the addition of Cu^{2+} could reverse the inhibition caused by these –SH group inhibitors. The enzyme lost almost all the activity in the presence of L-cysteine, which was restored slightly by addition of Cu^{2+}. 2-Mercaptoethanol with and without Cu^{2+} inhibited the enzyme by 53 and 28% respectively. GSH caused *ca* 55% inhibition of enzyme reaction which was reversed partially by addition of Cu^{2+} (Table 5).

Table 3. Effect of metals on partially purified dialysed* oxalate oxidase activity

Reagent added	Relative activity
None	100
$HgCl_2$	100
NaCl	102
KCl	95
$CaCl_2$	101
$ZnSO_4$	100
$NiSO_4$	103
$MgCl_2$	105
$FeCl_3$	105
$BaCl_2$	98
$SrCl_2$	100
$CdCl_2$	103
$PbCl_2$	100
$CoCl_2$	100
$CuSO_4$	310
$CuCl_2$	300
$FeSO_4$	115
$CuSO_4$ (Undialysed crude enzyme)	666

*The enzyme was partially purified and dialysed as described in the Experimental. Standard assay conditions were used except for the addition of metals. The final concentration of each metal ion was 5×10^{-4} M.

Sodium dithionite in the presence of Cu^{2+} strongly inhibited the enzyme activity. However, sodium dithionite alone had no effect. Sodium arsenite stimulated the enzyme activity both alone and in the presence of Cu^{2+}. Sodium azide in the presence of Cu^{2+} showed strong inhibition which was less at higher concentration (Table 5).

DISCUSSION

The present report describes the occurrence of a highly active oxalate oxidase activity in the 15000 *g* supernatant of roots of 10-day-old seedlings of grain sorghum variety CSH-5. The enzyme has optimum pH 5 and is stable in the acidic pH range (pH 3–6). The pH optima in the acidic pH range have been reported for oxalate oxidase from barley seedlings (pH 3.5) [6], mosses (pH 4) [9], spinach beet (pH 4) [10], *Pseudomonas* sp. OX-53 (pH 4.8) [11] and sorghum leaves variety CSH-1 (pH 5) [8]. The enzyme had a temperature optima at 40° which is comparable to that reported for *Bougainvillea* leaves (37°) [12], *Pseudomonas* sp. OX-53 (37°) [11] and barley roots (35°) [7] oxalate oxidases. However, the enzyme requires a shorter incubation time (5 min) for optimum activity as compared to barley roots (10 min) [7] and sorghum leaves, variety CSH-1 (10 min) [8] and is thus more suitable for quantitative analysis of oxalate in biological fluids. After 10 min of incubation there was a slight decline in the activity of enzyme which might be either due to enzyme inactivation with accumulation of product or disappearance of hydrogen peroxide with increased incubation period. The enzyme required Cu^{2+} for its activity, as is evident from its inhibition by diethyldithiocarbamate, a Cu^{2+} specific chelator and strong stimulation of dialysed enzyme by Cu^{2+} only. The Cu^{2+} caused more pronounced stimulation in the activity of crude enzyme than ammonium sulphate precipitate dialysed enzyme which might be due to the partial removal of some unknown factor required for enzyme stimulation by Cu^{2+}. Earlier the metal ion requirement for oxalate

Table 5. Effect of inhibitors on partially purified* oxalate oxidase activity

Reagent added	Relative activity
None	100
N-Ethylmaleimide (5×10^{-3} M)	76
Iodoacetate	70
Iodoacetate (5×10^{-3} M)	64
GSH	45
GSH (5×10^{-3} M)	40
L-Cysteine	8
2-Mercaptoethanol	72
Sodium arsenite	147
Sodium dithionite	100
Sodium azide (NaN_3)	150
Cu^{2+}	310
N-Ethylmaleimide + Cu^{2+}	300
Iodoacetate + Cu^{2+}	305
GSH + Cu^{2+}	229
L-Cysteine + Cu^{2+}	ND†
2-Mercaptoethanol + Cu^{2+}	145
Sodium arsenite + Cu^{2+}	474
Sodium dithionite + Cu^{2+}	117
Sodium dithionite (5×10^{-3} M) + Cu^{2+}	86
Sodium azide (5×10^{-3} M) + Cu^{2+}	263
Sodium azide + Cu^{2+}	161

*Enzyme was partially purified and dialysed as described in Experimental. Standard assay conditions were used except for the addition of inhibitors and/or Cu^{2+} ($CuSO_4$) as indicated. Unless otherwise noted, the final concentration of each compound(s) added was 5×10^{-4} M.

†Activity not detected.

oxidase was suggested in *Tilletia controversa* [13]. Suguira *et al.* [7] reported 36% stimulation by Cu^{2+} (1 mM) of oxalate oxidase purified from barley seedling. Recently, Koyama [11] found 75% stimulation by Mn^{2+} but 36% inhibition by Cu^{2+} at 10^{-4} M concentration for *Pseudomonas sp.* OX-53 oxalate oxidase.

Of the sulphydryl groups blocking agents, iodoacetate and *N*-ethylmaleimide decreased the rate of reaction which was completely reversed by adding Cu^{2+}. This suggests that the sulphydryl group(s) blocking agent does not block sulphydryl group(s) of the enzyme in the presence of Cu^{2+}. GSH strongly inhibited the reaction which was partly reversed in the presence of Cu^{2+}. As Cu^{2+} is known for its strong binding with sulphydryl groups and therefore, could bind with the sulphydryl group(s) involved at the active centre of the enzyme. Addition of GSH in the absence of exogenous Cu^{2+} removes Cu^{2+} from its binding to the sulphydryl group(s) of the enzyme and thus releases the free sulphydryl group(s) on the enzyme. When exogenous Cu^{2+} is added to the reaction mixture, it binds free sulphydryl group and forms a sulphydryl-copper ion complex at the active centre of the enzyme which favours the binding of substrate to the enzyme.

Interestingly L-cysteine was found to be a more potent inhibitor than GSH and inhibited the reaction completely. This may be due to the formation of a disulphide linkage between the sulphydryl group(s) of the enzyme and the L-cysteine molecules. 2-Mercaptoethanol inhibited the enzyme activity which was more pronounced in the presence of Cu^{2+}. Inhibition by 2-mercaptoethanol was also reported by Suguira *et al.* [7] for oxalate oxidase from barley seedlings. It is speculated that sorghum root oxalate oxidase might have more than one sub-unit which are joined by disulphide linkages to maintain the active conformation of the enzyme. Addition of 2-mercaptoethanol might break these disulphide bridges between these sub units of the enzyme and thus render it inactive. Earlier, Chiriboga [6] reported two sub-units for barley oxalate oxidase while Koyama [11] suggested eight sub-units for *Pseudomonas* sp. OX-53 oxalate oxidase.

Flavins did not show any effect on the enzyme. The stimulation of oxalate oxidase activity by flavins had been reported in mosses [9], fungi [13], and barley seedlings [6]. Datta and Meeuse [9] classified moss oxalate oxidase as flavoprotein whereas Koyama [11] classified *Pseudomonas* sp. OX-53 oxalate oxidase as a metalloprotein (Mn^{2+}). Because the enzyme is strongly stimulated by Cu^{2+}, it could therefore be classified as a Cu^{2+} requiring enzyme.

EXPERIMENTAL

Chemicals. 4-Amino-phenazone was from Koch Light. GSH and iodoacetate was supplied by SRL, India. 2-Mercaptoethanol, L-cysteine and *N*-ethylmaleimide were from Sigma. Horse radish peroxidase was supplied by CSIR centre for Biochemicals, India. α,α'-Dipyridyl, 8-hydroxyquinoline, Na arsenite and NaN_3 were from BDH. All other chemicals were of AR grade.

Plant material. The seeds of grain sorghum (*Sorghum vulgare* L-hybrid CSH-5) were supplied by Andhra Pradesh Seed Development Corporation Hyderabad, India.

Collection of leaves, roots and stems. Seeds were surface-sterilized with 0.1% $HgCl_2$ and washed with H_2O and germinated in petri dishes (diam. 20 cm) lined with a double layer of moist filter paper at room temp. (max. 33° ± 2°; min 23° ± 2°). After 4 days germination the seedlings were irrigated daily with Hoagland's nutrient soln and maintained in a photoperiod of 10–12 hr. After 10 days germination, the seedlings were removed from the filter paper and their leaves, roots and stems collected separately with sharp scissors. These tissues were washed with cold H_2O, dried between filter paper and stored immediately at −20°, until use.

Isolation of enzyme. Oxalate oxidase from various tissues of sorghum seedlings was prepared according to the method of ref. [8]. The frozen plant tissues were homogenized with cold H_2O in a ratio of 1:3 in a chilled pestle and mortar. The homogenate was squeezed through a double layer of cheesecloth and filtrate centrifuged at 15000 *g* for 30 min in a refrigerated centrifuge at 0–4°. The 'yellow brown supernatant was collected and distributed in 2 ml aliquots which were stored at −20°. The 15000 *g* supernatant was used as a source of crude enzyme.

Partial purification of the crude enzyme was as described in ref. [7]. Solid $(NH_4)_2SO_4$ was added to the crude enzyme to obtain 70% satn. The contents were stirred constantly for 1 hr at 2° and the pellets collected by centrifugation at 6000 *g* for 20 min and dissolved in desired vol. of 0.05 M Na succinate buffer, pH 5. The $(NH_4)_2SO_4$ ppt. showed 4-fold purification. The enzyme was dialysed at 2° against 0.05 M Na succinate buffer (PH 5) for 20 hr with constant stirring and 1–2 changes of buffer.

Assay of enzyme. The enzyme was assayed in 15 ml test tubes wrapped with black paper. Each tube contained 1.8/1.7 ml of 0.05 M Na succinate buffer, pH 5; 0.1 ml of enzyme (0.1–0.5 mg enzyme protein). Reaction mixture was preincubated at 40° for

2 min and the reaction started by adding 0.1 ml oxalic acid (10 mM). To study the effect of various compounds with and without metal ions, 0.1 ml of each compound of desired concn was also added to the reaction mixture before adding oxalic acid and the vol. of the buffer reduced accordingly, so that total vol. was 2 ml. After incubation at 40° for 5 min, 1 ml of colour reagent was added and kept in the dark at room temp. for 30 min to develop. A_{520} was read and the H_2O_2 generated during the reaction extrapolated from the standard graph of H_2O_2 prepared in 0.05 M Na succinate buffer, pH 5.

Unit of enzyme. One enzyme unit is defined as the amount of enzyme protein required to produce 1 n mol of H_2O_2 per min under the standard conditions of assay.

The colour reagent, which was prepared according to the method of ref. [4], consisted of 50 mg 4-aminophenazine, 100 mg phenol and 1 mg of horseradish peroxidase per 100 ml of 0.4 M Na-Pi buffer pH 7. It was stored in a bottle wrapped with black paper at 0–4°. Fresh reagent was prepared every week. The soluble protein in various enzyme preparations was estimated as described in ref [14].

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