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# OCCURRENCE OF AN OXALATE OXIDASE IN *SORGHUM* LEAVES

CHANDRA S. PUNDIR and RAVINDRA NATH*

Department of Biochemistry, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

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**Key Word Index**—*Sorghum vulgare*; Gramineae; sorghum leaf; oxalate oxidation; oxalate oxidase; flavoprotein, $Fe^{2+}$; -SH groups.

**Abstract**—An oxalate oxidase found in the 15 000 *g* supernatant of 10-day-old sorghum leaves exhibited a pH optimum of 5 and a temperature optimum of 45° and was unaffected by $Na^+$. The enzyme activity remained linear up to 10 min and the apparent $K_m$ for oxalate was $2.4 \times 10^{-5}$ M. The enzyme activity was strongly inhibited by sodium dithionite and α,α'-dipyridyl. Inhibition by the latter was specifically reversed by $Fe^{2+}$. The activity of the dialysed enzyme was restored by the addition of $Fe^{2+}$ and FAD. Inhibition of the enzyme by iodoacetate, *p*-chloromercuribenzoate and *N*-methylmaleimide revealed that -SH groups at the active site are essential.

## INTRODUCTION

Mechanisms for oxalate metabolism in micro-organisms have been reviewed by Jacoby and Bhat [1]. Mosses and fungi most probably metabolize oxalate into $CO_2$ and $H_2O_2$ [2, 3]. Oxalate metabolism in higher plants involves activation and decarboxylation by oxalate decarboxylase [4] and oxidation by oxalate oxidase [5–10]. The latter enzyme is of greater interest, as an oxalate oxidase from barley has been recently used in the direct estimation of oxalate in biological fluids [11–13] but with limited success due to the inhibition of the enzyme by sodium ions, normally present in the biological fluids [9, 14].

Extensive characterization of oxalate oxidase from various species of mosses [2] and *Tilletia controversa*, a pathogenic fungus from wheat [3], revealed that the enzyme was of flavoprotein nature. Although Chiriboga [14] purified and characterized a soluble oxalate oxidase from barley roots, the nature of this enzyme from higher plants is not fully understood. We report herein the occurrence of oxalate oxidase activity in cell-free extracts of 10-day-old leaves of *Sorghum vulgare*. Some properties of the enzyme and its cofactor requirements are also described.

## RESULTS AND DISCUSSION

The present investigations revealed a highly active oxalate oxidase in the 15 000 *g* supernatant of 10-day-old sorghum leaves. The assay of the enzyme was based on the formation of $H_2O_2$, which was estimated by a colour reaction with the 4-aminophenazone and peroxidase system [12]. The assay method developed in the present study was sensitive to as low as 10 nM $H_2O_2$ and was highly reproducible. The stoichiometry of the reaction could not be established satisfactorily using Warburg manometry because of the small quantity of $O_2$ uptake and $CO_2$ released [10]. The enzyme was stable at 0–4° for 2 weeks.

*To whom correspondence should be addressed

### *Effect of pH and temperature*

The enzyme activity was assayed at different pHs from 2 to 8 using the following buffer solutions: pH 2, KCl–HCl; pH 2.5–3, sodium citrate; pH 3.5–6, sodium succinate and pH 6.5–8, sodium phosphate, each at a concentration of 0.05 M. The enzyme was stable in the acidic pH range 3–6, with a single but broad pH optimum at pH 5.0. Enzyme activity at pH 5 was 60 and 24% higher than at pH 3 and 6, respectively (Fig. 1). The pH optima in the acidic pH range have been reported for barley root (pH 3.5) [14], mosses (pH 4.0) [2] and *T. controversa* (pH 2.6) [3]. Srivastava and Krishnan found a value of pH 6.8 for *Bougainvillea* leaf [7].

The enzyme exhibited a maximum activity at a temperature of 45°, after which it declined rapidly, most probably because of thermal denaturation of the enzyme.

### *Effect of incubation time and enzyme concentration*

The enzyme activity was linear up to the first 10 min, after which it remained constant. However, after 15 min, a

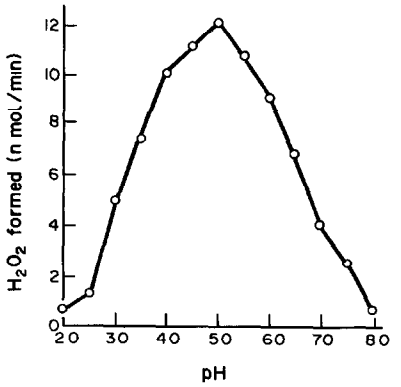


Fig. 1 Effect of pH on *Sorghum* oxalate oxidase activity (15 000 *g* supernatant). Standard assay conditions were used except for the pH, which was varied as indicated.

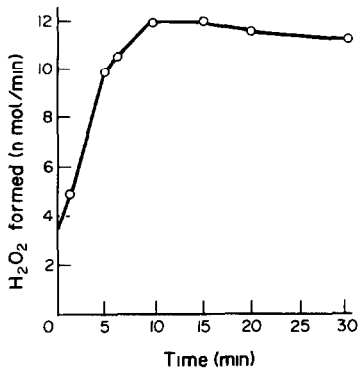


Fig. 2. Effect of incubation period on *Sorghum* oxalate oxidase activity (15 000 *g* supernatant). Standard assay conditions were used except for the incubation period, which was varied as indicated.

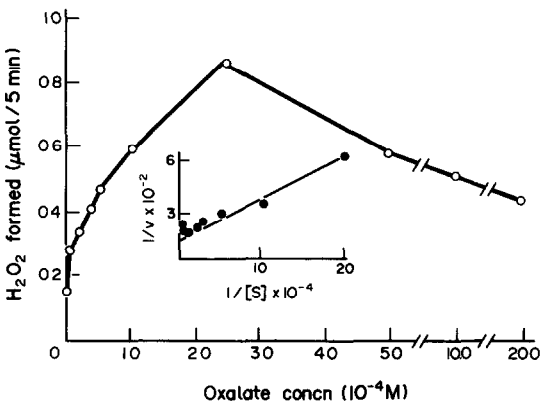


Fig. 3. Effect of oxalate concentration on *Sorghum* oxalate oxidase activity (30–65% ammonium sulphate precipitate). Standard assay conditions were used except for the oxalate concentration, which was varied as indicated. The inset shows the double reciprocal plot for oxalate. All points are derived from Fig. 3.

slight decrease was observed in the rate of reaction (Fig. 2). This indicated the possibility of either enzyme inactivation with accumulation of products or disappearance of $H_2O_2$ with increased incubation time. Therefore, in all the enzyme assays, the incubation time was 10 min. The increase in oxalate oxidase activity was directly proportional to the increase in protein content of 15 000 *g* supernatant in the range of protein concentration 0.1–1.5 mg/assay.

*Effect of oxalate concentration*

The activity of the partially purified enzyme was measured with increasing concentrations of oxalate (from $10^{-5}$ to $2 \times 10^{-3}$ M). A hyperbolic relationship between oxalate concentration and enzyme activity was obtained only up to a concentration of $2.5 \times 10^{-4}$ M, above which the enzyme showed substrate inhibition (Fig. 3). A similar situation has been reported for barley root enzyme [14]. A double reciprocal plot of $1/s$ vs $1/v$ revealed an apparent $K_m$ of $2.4 \times 10^{-5}$ M for oxalate (Fig. 3, inset), which is 20 times lower than the $K_m$ for oxalate of barley root enzyme ($K_m = 2.1 \times 10^{-4}$ M) [14]. This comparison leads to the speculation that the use of oxalate oxidase from sorghum in enzymatic determination of oxalate in biological fluids could enhance the sensitivity of the method over the barley root enzyme [11, 13].

*Effect of sodium ions*

To observe the effect of $Na^+$ on the enzyme activity, sodium chloride solution was added to the assay mixture. The $Na^+$ did not show any effect on the activity up to 0.25 M. Previous reports have revealed that oxalate oxidase from barley and *Bougainvillea* are $Na^+$-sensitive, and an inhibition of more than 50% has been observed by 0.1 M sodium chloride [9, 10, 14]. This observation lends weight to the suggestion that the oxalate oxidase from sorghum is a more suitable enzyme than barley [13] and moss [15] for the direct estimation of oxalate in biological fluids.

*Effect of chelating agents, metals and co-enzymes*

An absolute metal ion requirement for the enzyme was evident from the inhibition of enzyme activity by EDTA and fluoride. Cyanide resulted in only 12% inhibition of the enzyme activity. The chloride salts of divalent and trivalent metals were added to the reaction at a final concentration of $5 \times 10^{-4}$ M. Of all the metals tested, only $Fe^{2+}$ stimulated the enzymatic reaction (Table 1). Other metals ($Mg^{2+}$, $Mn^{2+}$, $Cu^{2+}$, $Cd^{2+}$, $Co^{2+}$, $Sr^{2+}$, $Zn^{2+}$ and $Fe^{3+}$) had no effect. $Hg^{2+}$ caused 13% inhibition of the enzyme activity, probably by precipitation of the enzyme protein. Both α,α'-dipyridyl and β-hydroxyquinoline (iron-specific chelators), each at $10^{-4}$ M concentration, caused 52% inhibition of the enzyme activity. The inhibition of enzyme caused by α,α'-dipyridyl was partly reversed by $Fe^{2+}$, but not by other metals ($Mg^{2+}$, $Mn^{2+}$, $Zn^{2+}$, $Ni^{2+}$, $Co^{2+}$ and $Fe^{3+}$) (Table 1). These findings suggest the absolute requirement of $Fe^{2+}$ for the enzyme activity. This is supported by the observation that the addition of $Fe^{2+}$ alone caused *ca* 78 and 87% stimulation in the dialysed enzyme activity using 15 000 *g* supernatant and 30–65% ammonium sulphate precipitate as the source of enzyme, respectively. Vaisey *et al.* [3], from inhibition studies, reported that metal is necessary for the *T. controversa* enzyme. On the other

Table 1. Effect of metal chelators and metals on oxalate oxidase activity (15 000 *g* supernatant)*

| Compound added | Final concentration (M) | Effect (%) |
|---|---|---|
| EDTA | $1 \times 10^{-4}$ | −30 |
| NaF | $5 \times 10^{-3}$ | −55 |
| NaCN | $1 \times 10^{-4}$ | −12 |
| α,α'-Dipyridyl | $1 \times 10^{-4}$ | −52 |
| 8-Hydroxyquinoline | $1 \times 10^{-4}$ | −52 |
| $FeSO_4$ | $5 \times 10^{-4}$ | +42 |
| $HgCl_2$ | $5 \times 10^{-4}$ | −13 |
| α,α'-Dipyridyl + $FeSO_4$ | $1 \times 10^{-4}$ + $5 \times 10^{-4}$ | −16 |

*Standard assay conditions were used except for the addition of metal chelators and metals as indicated.
− = Inhibition; + = stimulation.

Table 2. Effect of $Fe^{2+}$ and FAD on dialysed* oxalate oxidase activity

| Enzyme preparation | Addition | Final concentration (M) | Effect (%) |
|---|---|---|---|
| 15000 *g* supernatant | $FeSO_4$ | $5 \times 10^{-4}$ | +78 |
| | FAD | $10^{-4}$ | 0 |
| | $FeSO_4$ + FAD | $5 \times 10^{-4} + 10^{-4}$ | +100 |
| 30–65% $(NH_4)_2SO_4$ precipitate | $FeSO_4$ | $5 \times 10^{-4}$ | +86 |
| | FAD | $10^{-4}$ | 0 |
| | $FeSO_4$ + FAD | $5 \times 10^{-4} + 10^{-4}$ | +99 |

*The enzyme was dialysed against metal-free water in the cold for 20 hr with constant stirring. Standard conditions of assay were used except for the addition of $FeSO_4$ and/or FAD as indicated.

+ = Stimulation; 0 = no effect.

hand, Chiriboga observed that metal is not required for the activity of barley enzyme [14].

For studying the requirement of co-enzyme, both 15000 *g* supernatant and 30–65% ammonium sulphate precipitate were used as the source of enzyme. The enzyme was dialysed against metal-free water (2 l.) in cold for 20 hr, with constant stirring. In both cases, the dialysed enzyme lost almost all the activity, which was restored by the addition of $Fe^{2+}$ and FAD only. $Fe^{2+}$ was more effective than FAD in restoring the activity in both cases. However, FAD was effective only in the presence of $Fe^{2+}$ and caused 22 and 13% stimulation in the activities of 15000 *g* supernatant and 30–65% ammonium sulphate precipitate, respectively (Table 3). This difference in FAD stimulation was possible because of the removal of $Fe^{2+}$ during ammonium sulphate fractionation; therefore a larger stimulation by $Fe^{2+}$ was found in the activity of dialysed enzyme associated with 30–65% ammonium sulphate precipitate as compared to the 15000 *g* supernatant. These observations suggest that the enzyme is most probably iron-flavoprotein in nature. Previously moss and fungal oxalate oxidases were classified as flavoproteins [2, 3].

*Effect of inhibitors*

To study the involvement of –SH groups at the active site of the enzyme, various –SH group inhibitors were added separately to the reaction mixture at a concentration of $5 \times 10^{-3}$ M. *p*-Chloromercuribenzoate and iodoacetate inhibited the enzyme activity. However, *N*-methylmaleimide was a more potent inhibitor than *p*-chloromercuribenzoate (Table 3). These results suggest that –SH groups are essential at the active site of the enzyme, confirming the observations of Chiriboga for barley root enzyme [14].

The enzyme was strongly inhibited by sodium azide but was unaffected by β-mercaptoethanol (Table 3), which indicates that catalase does not take part in the reaction [7, 10]. The inhibition of enzyme activity by sodium dithionite indicates that the reaction is $O_2$-dependent (Table 3).

Table 3. Effect of inhibitors on oxalate oxidase activity (15000 *g* supernatant)*

| Compound added ($5 \times 10^{-3}$ M) | Inhibition (%) |
|---|---|
| Iodoacetate | 47 |
| *p*-Chloromercuribenzoate | 73 |
| *N*-Methylmaleimide | 89 |
| β-Mercaptoethanol | 0 |
| Sodium arsenite | 12 |
| Sodium dithionite | 77 |
| Sodium azide | 80 |

*Standard assay conditions were used except for the addition of inhibitor as indicated.

0 = No effect.

Table 4. Intracellular distribution of oxalate oxidase isolated from *Sorghum* leaves*

| Cellular fractions | Total activity (units/g fr. wt) | (%) |
|---|---|---|
| Crude homogenate | 357 | 100 |
| Cell debris, nuclear and chloroplasts (1600 *g* precipitate) | N.D. | — |
| Cell-free extracts (1600 *g* supernatant) | 355 | 99 |
| Mitochondria (15000 *g* precipitate) | N.D. | — |
| Cell-free extract (15000 *g* supernatant) | 351 | 98 |

*The assay of the enzyme was carried out as described in the Experimental. One unit of activity is defined as the amount of enzyme required to produce 1 nmol $H_2O_2$ per min under the standard conditions of assay.

N.D. = Not detected.

*Intracellular localization*

As shown in Table 4, we were able to detect almost all activity of the leaf homogenate in the 1600 *g* supernatant, which was further removed entirely into the 15000 *g* supernatant. However, there was practically no activity of oxalate oxidase associated with the particulate fractions sedimenting at 1600 and 15000 *g*. These results do not permit a definite conclusion about the soluble or particulate nature of the enzyme because of the possibility of breaking the membrane of cell organelles during the extraction. Tolbert *et al.* [16] reported the release of marker enzymes from peroxisomes during extraction. Leek *et al.* [10] suggested that some oxalate oxidase activity is released from peroxisomes isolated from spinach beet leaves during the extraction of enzyme.

## EXPERIMENTAL

*Chemicals.* 4-Aminophenazone and iodoacetate were supplied by Koch-Light, and *p*-chloromercuribenzoate and *N*-methylmaleimide were from BDH. Na dithionite and β-mercapto-

ethanol were supplied by Merck; FAD was from Cal-Biochem; and horseradish peroxidase was from Sigma. All other chemicals were of AR grade.

*Seeds.* Grain sorghum (*Sorghum vulgare* L. hybrid CSH-1) was obtained from the Indian Agricultural Research Institute, Regional Station, Hyderabad and stored at 0–4°, until use.

*Collection of leaves.* Seeds were surface-sterilized with 0.1% $HgCl_2$, washed with $H_2O$ and germinated in Petri dishes (diameter 20 cm) lined with a double layer of moist filter paper, at room temp. (33° ± 2°). After 5 days of germination, the seedlings were irrigated daily with Hoagland's nutrient soln and maintained in a photoperiod of 12–13 hr. After 10 days of germination, the seedlings were removed from the filter paper and their leaves separated with sharp scissors, weighed and immediately stored at −20° until use.

*Isolation of enzyme* Oxalate oxidase from sorghum leaves was prepared according to the method of ref. [14], with slight modification. Frozen leaves (30 g) were homogenized with 90 ml cold $H_2O$ in a chilled mortar and pestle. The homogenate was squeezed through a double layer of cheesecloth and the extract centrifuged at 15000 *g* for 30 min. The yellowish brown supernatant was collected and used as the source of crude enzyme and was stored at 0–4° until use.

*Partial purification.* Solid $(NH_4)_2SO_4$ was added to the crude enzyme extract to obtain 0–30 and 30–65% saturation. The pellet from the latter fraction was collected by centrifugation in the cold at 10000 *g* for 30 min and dissolved in 0.05 M Na succinate buffer, pH 5. An increase of *ca* 4-fold in the specific activity of crude enzyme was achieved.

*Assay of enzyme.* The enzyme was assayed in 10 ml stoppered glass tubes wrapped with black paper. To each tube was added 80 μmol Na succinate buffer (pH 5), 1 μmol Na oxalate and 0.7 mg enzyme protein in a total vol of 2 ml 1 μmol $FeSO_4$ was also added to the reaction mixture, except when the effect of metals, metal chelators and co-enzymes was studied. After incubation at 40° for 10 min, 1 ml colour reagent was added, the tubes were shaken and then allowed to stand at room temp. for 30 min to develop the colour. The *A* was read at 520 nm 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.

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

*The colour reagent* was prepared as described in ref. [12] and consisted of 0.05 g 4-aminophenazone, 0.1 g phenol and 1 mg horseradish peroxidase per 100 ml 0.4 M NaPi buffer, pH 7. It was stored in an amber bottle at 0–4°. Fresh reagent was prepared every week.

*Protein* was estimated according to the method of ref. [17].

*Intracellular localization of enzyme.* All operations were carried out at 0–4°. Fresh leaf homogenate prepared in $H_2O$ or 0.5 M sucrose containing $H_2O$ by grinding in a Waring blendor for 3 min was centrifuged at 1600 *g* for 30 min. The sedimented material was washed once with a small vol. of grinding fluid and the wash liquid was combined with the main supernatant. This supernatant was further centrifuged at 15000 *g* for 30 min. The residues sedimenting at 1600 and 15000 *g* were suspended in a small vol. of grinding fluid.

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