Comparative study of oxalate oxidase in three genotypes of *Sorghum vulgare*

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Summary. The presence of an oxalate oxidase (EC 1.2.3.4) has been demonstrated in 15,000 × g supernatants prepared from 10-day-old seedlings of three genotypes of *Sorghum vulgare*: grain sorghum hybrid (CSH-5), grain-cum-forage sorghum (PC-6) and forage sorghum (PC-1). The specific activity of the enzyme in the different tissues of seedlings was found to be present in the order leaves > stems > roots in PC-6 and PC-1, but this order was reversed in CSH-5. A comparison of the different properties of the leaf enzyme of these three genotypes of sorghum revealed that the enzyme has maximum activity in the acidic pH range from 4.0 to 5.0 and in the temperature range from 37 °C to 40 °C. The enzyme was stimulated by Cu^{2+} and Fe^{2+}. The rate of H_2O_2 formation in the enzyme reaction was linear up to 5 min and was stoichiometrically related to oxalate consumption. The enzyme is unaffected by Na^+ at physiological concentration (0.15 M). The superiority of this enzyme over moss and other plant enzymes for enzymic determination of urinary oxalate is discussed.

Key words. Sorghum vulgare; Gramineae; oxalate oxidation.

Oxalate oxidase (oxalate: oxygen oxidoreductase, EC 1.2.3.4), known to catalyze the generation of two moles of CO_2 and one mole of H_2O_2 from one mole of oxalate and one mole of aerobic O_2, was discovered in a mold by Houget, Mayer and Plantefol[1] about six decades ago. Since then, the enzyme has been found in *Pseudomonas* sp. OX-53[2], in *Tilletia controversa*, a pathogenic fungus from wheat[3], in mosses[4] and in higher plants[5–10]. The extensive characterization of oxalate oxidase from a dozen species of mosses[4], and from teliospores of *T. controversa*[3], revealed the flavoprotein nature of the enzyme. Very recently, Koyama purified an oxalate oxidase from *Pseudomonas* sp. OX-53 and classified it as a metalloprotein (manganese)[2]. In higher plants, oxalate oxidase has been purified to homogeneity from barley seedlings and some of its properties have been studied[11,12]. During the past decade, more attention has been paid to this enzyme, especially in higher plants, possibly because of its clinical applications. Oxalate oxidase prepared from barley seedlings[13], beet stems[9] and banana peel[10] has been employed for specific measurement of oxalate in urine, which is of critical importance in the diagnosis and treatment of hyperoxaluria and idiopathic renal stones[14]. These enzymatic methods for oxalate determination include pretreatment of urine prior to oxalate assay in order to remove various endogenous enzyme inhibitors, mainly Na^+. Recently, Pundir and Nath found an oxalate oxidase in the 15,000 × g supernatant of leaves of 10-day seedlings of the grain sorghum hybrid CSH-1, which is unaffected by Na^+ in the physiological concentration range[15]. Pundir et al.[16] demonstrated the use of this sorghum enzyme in the direct measurement of oxalate in urine that had not undergone pretreatment. The potential usefulness of this enzyme prompted us to make a detailed study of the oxalate oxidases of three different genotypes of sorghum.

Materials and methods

Chemicals: 4-Aminophenazone was supplied by Koch-Light, Laboratories, USA. Horseradish peroxidase was from CSIR Centre for Biochemicals, Delhi, India. 1-^{14}C-Oxalate (1–5 μmol/mCi) was obtained from N.E.N., Mass., USA. All other chemicals were of AR grade.

Seeds: The seeds of grain sorghum hybrid genotype CSH-5 were a gift from Andhra Pradesh State Seed Development Corporation, Hyderabad, India, and Nath Seeds Ltd. Aurangabad, India. The seeds of grain-cum-forage sorghum, genotype PC-6, and forage sorghum genotype PC-1 were obtained from IARI, New Delhi, India. The seeds were stored at 0–4 °C until used.

Collection of plant material: The seeds of each genotype of sorghum were surface-sterilized with 0.1% (w/v) $HgCl_2$ for 1 min, washed with distilled H_2O several times, and germinated in petri dishes lined with a double layer of moist filter paper, at 33 ± 4 °C. After 5 days of germination, the seedlings were irrigated daily with Hoagland's nutrient solution and maintained in a light period of 8–10 h/day. After 10 days of germination, the seedlings were removed from the filter paper, and their leaves, stems and roots were separated, washed in chilled distilled water, blotted between filter paper sheets, weighed and stored immediately at −20 °C until use.

Extraction of oxalate oxidase: Oxalate oxidase from whole seedlings, leaves, roots and stems of each genotype sorghum was prepared in the cold as described by Pundir and Nath[14]. Frozen tissues were homogenized with cold distilled H_2O in the ratio 1:3 (w/v) in a chilled mortar and pestle. The homogenate was squeezed through a double layer of cheese cloth and the filtrate was centrifuged at 15,000 × g for 30 min in the cold (4 °C). The supernatant was collected and used as the source of enzyme and stored at −20 °C until use.

Assay of oxalate oxidase: The assay of oxalate oxidase was carried out as described by Pundir and Nath [14]. The enzyme was assayed in 15-ml stoppered glass tubes wrapped in black paper. To each tube was added 80 µmol of sodium succinate buffer pH 5.0, 1 µmol of sodium oxalate and 0.5–0.7 mg crude enzyme protein in a total volume of 2.0 ml. 1 µmol of $FeSO_4$ was also added to the reaction mixture except when the effect of $CuSO_4$ was studied. After incubation at 40 °C for 10 min, 1 ml of the color reagent was added to each tube. The tubes were shaken and then kept at room temperature for 30 min in the dark to develop the color. The absorbance at 520 nm was read and the amount of H_2O_2 generated during the reaction was extrapolated from a standard curve for H_2O_2 prepared in 0.05 M sodium succinate buffer pH 5.0.

Unit of enzyme: One enzyme unit is defined as the amount of enzyme required to produce one nmole of H_2O_2 per min under the standard conditions of the assay.

The color reagent was prepared according to the method of Bais et al.[17] and consisted of 0.05 g 4-aminophenazone, 0.1 g solid phenol and 1 mg horseradish peroxidase per 100 ml of 0.4 M sodium phosphate buffer pH 7.0. It was stored in an amber-colored bottle at 0–4 °C. Fresh reagent was prepared every week. The protein was estimated according to the method of Lowry et al.[18].

Leaf cell-free extract (15,000 × g supernatant) of all three genotypes of sorghum were used for further study of oxalate oxidase, as this tissue from two of the genotypes showed maximum activity at pH 5.0. To study the optimum pH, the activity was assayed at varying pH (3.0–7.0) in different buffers each at 0.05 M conc.: sodium citrate, pH 3.0–3.5; sodium succinate, pH 4.0–6.0 and sodium phosphate, pH 6.5–7.0. To measure the oxalate consumption in the enzyme reaction, 1-^{14}C-oxalate (1 µmol/mCi) was used in the assay and its level at different times was measured according to the method of Chiriboga [19].

Results and discussion

The presence of a highly active oxalate oxidase was detected in the 15,000 × g supernatant of 10-day-old whole seedlings of the three different genotypes of sorghum. The enzyme assay was based on the measurement of H_2O_2 generated in the enzyme reaction by a color reaction [16]. Table 1 shows the specific activity of the enzyme in different tissues of seedlings of the three genotypes, which was found in this order: leaves > stems > roots in PC-6 and PC-1 and roots > stems > leaves in CSH-5. Comparatively, the enzyme activity was lower in the tissues of PC-1 and higher in tissues of CSH-5. Some properties of the leaf enzyme in the three genotypes of sorghum were studied further.

Stability and optimum pH: The crude enzyme from all genotypes could be stored in aqueous solution at 0–4°C for 15 days without any loss of its activity. However, the enzyme lost 50 % of its activity when stored at 0–4 °C for 30 days.

Table 1. Specific activity * of oxalate oxidase in 15,000 × g supernatant of 10-day-old tissues of sorghum genotypes

Tissue	Genotype CSH-5	PC-6	PC-1
Seedlings	12.85	14.86	10.81
Roots	28.57	4.16	5.19
Stems	14.13	7.98	7.59
Leaves	10.40	17.97	11.41

* Figures are the means of three separate determinations. Standard assay conditions were used for measurement of enzyme activity. Specific activity is reported in units per mg protein. One unit of enzyme is defined as the amount of enzyme required to produce 1 nmol H_2O_2 per min. Genotypes CSH-5, PC-6 and PC-1 are the grain sorghum hybrid, grain-cum-forage sorghum and forage sorghum, respectively.

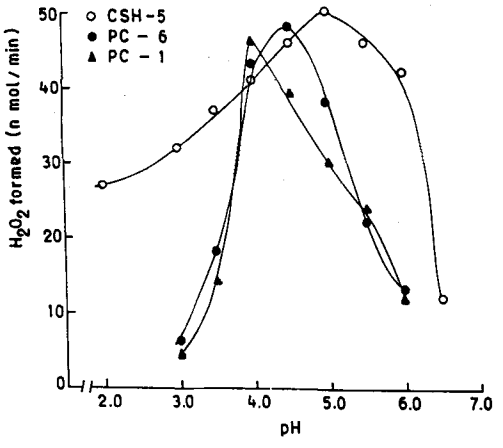

Figure 1. Oxalate oxidase activity of leaves of 10-day-old seedlings of sorghum genotypes at different pH values. Standard assay conditions were used except that pH was varied as indicated. Each point is the mean of three determinations.

The enzyme showed a single but broad pH optimum at pH 4.0, 4.5 and 5.0 for PC-1 PC-6 and CSH-5 genotypes, respectively (fig. 1). The enzyme was stable in the acidic pH range from 3.0–6.0 in all three genotypes. A pH optimum in the acidic pH range has previously been reported for leaf oxalate oxidase in from spinach beet (pH 4.0)[8] and grain sorghum genotype CSH-1 (pH 5.0)[15]. Srivastava and Kirshnan [5] reported a value of pH 6.8 for Bougainvillea leaf.

The enzyme from the PC-1 and PC-6 genotypes exhibited its highest activity at 37 °C, and that from CSH-5 at 40 °C. In this respect the sorghum enzyme resembles those that have been described in other higher plants [5, 12]. The enzyme activity showed a rapid decline at temperatures above 45° in all genotypes.

Time course study: The rate of the enzyme reaction as measured by formation of H_2O_2 was linear up to 5 min, after which it levelled off in all the three genotypes (fig. 2). Therefore in all subsequent assays, the reaction mixture was incubated for 5 min only.

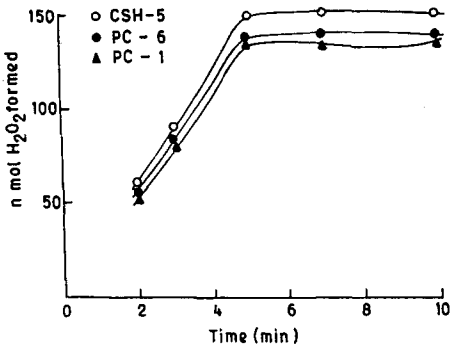

Figure 2. Oxalate oxidase activity of leaves of 10-day-old seedlings of sorghum genotypes at various times. Standard assay conditions were used except that the incubation time was varied as indicated. Each point is the mean of three determinations.

Stoichiometry: Both oxalate consumption and formation of H_2O_2 were measured at several time intervals. The results (table 2) showed that one mole of oxalate was consumed in the formation of one mole of H_2O_2 in all the three genotypes.

Effects of substrate concentration: The data given in table 3 show that the enzyme activity increased as the concentration of oxalate was increased in all the three genotypes. The optimum concentration of oxalate was found to be 2.5×10^{-4} M in CSH-5 and PC-6 but 1×10^{-4} M in PC-1. However, the concentration used in the reaction mixture for the assay of enzyme in all three genotypes was 5×10^{-4} M. When the substrate was not added to the reaction mixture, a low level of enzyme activity was observed, which was probably due to the presence of endogenous substrate in the 15,000 × g supernatant.

Effect of Na^+: To observe the effect of Na^+ on the enzyme, NaCl was added to the reaction mixture at levels ranging from 10 mM to 200 mM. Na^+ up to 200 mM had no effect on the enzyme activity in all the three genotypes of sorghum, as had been found previously in the leaf enzyme of grain sorghum, genotype CSH-1[15]. Earlier, about 85% inhibition by 0.1 M Na^+ was reported for barley seedling[6] enzyme, and 50% for that from Bougainvillea leaves[5].

Effect of divalent metal ions: To study the effect of divalent metal ions on the enzyme activity, the chloride or sulphate salts of the divalent metals were added to the reaction mixture at a final concentration of 5×10^{-4} M. Of the several metals tested, only Cu^{2+} caused strong stimulation, ranging from 325 to 543% in the following order: PC-6 < PC-1 < CSH-5. Fe^{2+} also produced slight stimulation, in an increase of 66% in both CSH-5 and PC-6 and 90% in PC-1 (tables 4 and 5). Other cations (Mg^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Sr^{2+}) had no effect. Vaisey et al.[3] suggested that a fungal oxalate oxidase had a metal ion requirement, because it was inhibited by CN^- and F^-. Suguira et al.[11] observed 36% stimulation by Cu^{2+} (1 mM) of the activity of barley oxalate oxidase. Pundir and Nath[15] suggested the essentiality of Fe^{2+} for this enzyme from grain sorghum leaves, genotype CSH-1. Very recently, Koyama[2] showed the presence of Fe^{2+} (0.09 atoms/subunit) in the oxalate oxidase purified from *Pseudomonas* sp. OX-53.

The properties of oxalate oxidase from the leaves of 3 genotypes of sorghum have been summarised in table 5 and compared with those of enzymes from other sources, which have been used up to now for the enzymic determination of urinary oxalate. A comparison of these enzymes reveals that the sorghum enzyme requires a shorter time (5 min) for assay than other enzymes. In addition,

Table 2. Rates of oxalate consumption and H_2O_2 formation in the reaction of oxalate oxidase in 15,000 × g supernatant of leaves of 10-day-old seedlings of sorghum genotypes

Genotype	Reaction time in min	Oxalate disappearance in nmol	H_2O_2 formation in nmol
CSH-5	2	52.6	60.0
	3	82.4	90.0
	5	137.7	150.0
	7	138.5	151.0
PC-6	2	48.6	55.0
	3	78.4	84.0
	5	130.2	138.0
	7	132.7	139.0
PC-1	2	46.5	53.0
	3	76.8	84.0
	5	128.4	135.0
	7	130.6	137.0

Standard assay conditions were used except that the incubation period was as indicated above, and ^{14}C-oxalate was added for measurement of oxalate disappearance as described by Chiriboga[19]. The data are the means of three observations.

Table 3. Effect of substrate concentration on oxalate oxidase in 15,000 × g supernatant of leaves of 10-day-old seedlings of sorghum genotypes

Final concentration of oxalate added to reaction mixture (M)	H_2O_2 formation (nmol/min) Genotype CSH-5	PC-6	PC-1
None	23	21	22
10^{-5}	85	80	76
0.5×10^{-4}	93	87	82
1.0×10^{-4}	100	96	92
2.5×10^{-4}	105	99	85
5.0×10^{-4}	101	96	83

Standard assay conditions were used except that the concentration of oxalate was varied as given above. The data are the means of three observations.

Table 4. Effect of iron and copper on oxalate oxidase in 10-day-old leaves of sorghum genotpyes

Substance added	H_2O_2 formation (nmoles/min) Genotype CSH-5	PC-6	PC-1
None	30.0	30.0	25.0
Fe^{2+}	50.0	50.0	47.5
Cu^{2+}	193.0	127.5	135.0

Standard assay conditions were used except for the addition of sulphates of iron or copper to the final conc. of 5×10^{-4} M. The data are the means of three observations.

Table 5. Comparison of oxalate oxidase from leaves of sorghum genotypes and from other sources

Property	Sorghum gentopyes CSH-5	PC-6	PC-1	CSH-1 [15]	Other plant tissues Barley seedlings [12]	Beet stem [9]	Banana peel [10]	Mosses [20]
pH optimum	5.0	4.5	4.0	5.0	3.2	4.3	5.2	4.1
Temperature for highest activity [°C]	40	37	37	37	35	37	37	25
Incubation time [min]	5	5	5	10	10	30	30	50
Inhibition by Na^+ (1×10^{-1} M) [%]	–	–	–	–	85	ND	ND	ND
Stimulation by Cu^{2+} (5×10^{-4} M [%]	543	325	440	ND	36 at 10^{-3} M	ND	ND	ND
Stimulation by Fe^{2+} (5×10^{-4} M) [%]	66	66	90	42	ND	ND	ND	ND

ND = Not detected. Genotypes CSH-5 and CSH-1, PC-6 and PC-1 represent grain sorghum hybrids, grain-cum-forage sorghum and forage sorghum, respectively.

it is not inhibited by Na^+ ions, which are normally present in urine. The available enzymic methods of urinary oxalate analysis employing oxalate oxidase from mosses [20], barley seedlings [13], beet stem [9], and banana peel [10] require pretreatment of urine sample to remove endogenous substances which would otherwise interfere in the assay, especially Na^+. This problem could be overcome by using the sorghum enzyme. Pundir et al. [16] have already demonstrated the usefulness of this enzyme from the leaves of the grain sorghum hybrid, genotype CSH-1, for the direct measurement of urinary oxalate. The purification of this enzyme from leaves of the grain sorghum hybrid genotype CSH-5 is in progress in this laboratory for use in direct enzymic determination of urinary oxalate.

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