Characterization of cell wall oxalate oxidase from maize roots

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Abstract

Oxalate oxidase activity was detected in the cell wall fraction isolated from maize roots (Zea mays L.). The enzyme was active at acidic pH with optimal activity at pH 3.2. It was thermally extremely stable and resistant to high salt concentration, SDS and pepsin. The enzyme activity was inhibited by sulphhydryl reagents 2-mercaptoethanol (2-ME), N-ethyl maleimide (NEM) and dithiotreitol (DTT), but was insensitive to EDTA, KCN and metal ions. Measurements of enzyme activity were performed using colorimetric assay of H2O2, as well as polarographic detection of O2 consumption. Maximal activity was obtained with 5 mM oxalic acid for the colorimetric method, and 10 mM oxalic acid for the polarographic method. Both methods were applicable in oxalate oxidase characterization, the polarographic method being more suitable under conditions of H2O2 interaction with some of the analyzed substances. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Cell wall; Oxalate oxidase; Root; Zea mays

1. Introduction

Oxalate oxidase (oxalate:oxygen oxidoreductase, EC 1.2.3.4) catalyzes the oxidation of oxalate by molecular oxygen, yielding CO2 and H2O2. Such an enzyme activity was demonstrated in a number of higher plant species and in various plant tissues: in grain sorghum seedlings, roots, leaves and stems [1], barley seedlings [2] and leaves [3], wheat embryo [4] and leaves [5], Ama- ranthus leaves [6], beet stem [7], etc. The characteristics of the enzyme varied significantly depending on the plant species used for the investigation.

The discovery that germin, a developmentally regulated protein in wheat, is in fact an oxalate oxidase [4], focused the scientific attention to this enzyme. Although sorghum oxalate oxidase activity was found in the 15 000 g supernatant of leaves and roots [1,8], wheat and barley germins are at least partly associated with the cell wall [3–5,9]. This enzyme would have a role in developmental processes by producing H2O2 which is involved in the oxidative cross-linking of a cell wall polymers [10]. Additionally, increased transcription of a gene for germin-like oxalate oxidase in wheat and barley leaves following pathogen attack [3,5], and modulation of germin gene expression in barley roots by salt stress [11] or in wheat seedlings by heavy metal ions [12], suggested the involvement of this enzyme in plant response to stress.

In this report, we describe some characteristics of the cell wall-bound oxalate oxidase in maize roots, and compare its activity in different parts of the plant and early stages of development of maize. Enzyme activity was determined by the standard enzyme assay, measuring H2O2 generation, as well as by measuring the oxygen consumption rate. The results obtained using both methods were compared.
2. Materials and methods

2.1. Plant material

Seeds of maize (Zea mays L., inbred line Oh43) were germinated on water and transferred after 3 days to 50% Knop [13] nutrient solution. Plants were grown up to 9 days in controlled environment under a 12-h light/dark regime at 22/18°C, with a light intensity of 40 W m⁻² and relative humidity at 70%. Before experiments, roots were washed with H₂O, dried between filter papers and immediately used for cell wall isolation. Additionally, the cell wall was isolated from leaves and embryonic axis (embryo without scutellum) of imbibed seedlings.

2.2. Isolation of cell wall

Tissue was homogenized with medium (0.25 M sucrose in 0.05 M Tris–HCl, pH 7.2) in 1:11 ratio (w/v) in a chilled mortar and pestle. The homogenate was squeezed through 0.5 mm nylon mesh and the filtrate centrifuged at 1000 × g for 10 min in the cold. The pellet was washed twice by resuspension in 10 vol. of the homogenization medium containing 1% (w/v) Triton X-100 and four times with 30 vol. of the same medium without Triton X-100. After each wash the pellet was collected by centrifugation at 1000 × g for 10 min. The final pellet was considered to be the purified cell wall fraction, and was used for most of the experiments. In order to obtain the ionically bound fraction, cell wall was incubated in 1 M NaCl or 1 M CaCl₂ for 30 min with continuous stirring at 4°C. After centrifugation at 1000 × g for 10 min, the resulting supernatant contained the ionically bound proteins and the pellet the tightly bound cell wall proteins. The enzyme activity was assayed in the dialyzed supernatant, as well as in the pellet which was washed three times and resuspended in homogenization medium. SDS treatment was performed by incubation of the purified cell wall fraction in 0.4% SDS at 37°C for 30 min. After washing three times in the homogenization medium, the resulting pellet was resuspended in the same medium, the enzyme activity assayed, and compared to enzyme activity in the control experiment performed without SDS. Pepsin susceptibility was analyzed by treatment of the cell wall fraction with 50 µg/ml⁻¹ of pepsin at pH 2 adjusted with HCl at 37°C for 30 min, and then neutralized with NaOH before measurement of enzyme activity. In the control experiment pepsin was omitted.

2.3. Enzyme assay

Colorimetric assay of oxalate oxidase was carried out as described by Pundir [8]. The assay mixture (1 ml), containing 50 mM Na succinate, pH 3.2 and 50–100 µg protein was incubated at 37°C in test tubes wrapped with black paper. In case of experiments where the effect of pH was studied, Na succinate buffers with pHs in the range of 2–5 were used. The reaction was started by adding oxalic acid to a final concentration of 10 mM, unless otherwise specified. After 5 min 0.5 ml of color reagent for H₂O₂ measurement was added to stop the reaction, and the color was allowed to develop for 30 min at room temperature. The color reagent consisted of 50 mg 4-aminophenazine, 100 mg solid phenol and 500 U of horseradish peroxidase per 100 ml of 0.4 M Na–Pi buffer, pH 7.0. The absorbance of the solution was measured at 520 nm and corrected for absorbance obtained when oxalic acid was omitted from assay mixture. Hydrogen peroxide generated during the reaction was determined by interpolation from a standard curve in the range from 0.01 to 0.25 µmol H₂O₂ prepared in 50 mM succinate buffer. Enzyme activity is expressed as the amount of H₂O₂ produced per min and mg of protein. The effect of various compounds on the enzyme activity was studied by their addition to the reaction mixture before adding oxalic acid. Additional control experiments were performed by substituting the cell wall isolate and oxalic acid in a standard reaction mixture with H₂O₂ to a final concentration of 0.1 mM, in order to test the possible effect of analyzed compounds with H₂O₂ measurements. The reaction was performed under standard assay conditions, with or without compound added, and its effect calculated as the difference in the absorbances.

2.4. Oxygen consumption

Measurement of oxygen consumption was performed using a Clark-type polarographic electrode (Hansatech Ltd., England), at 25°C. Cell wall (50–100 µg protein) was added to 1 ml of the
assay mixture containing 50 mM Na succinate, pH 3.2, unless otherwise specified, and oxygen consumption initiated by the addition of oxalic acid. The rate of O₂ consumption was calculated and expressed as the amount of O₂ consumed per min and mg of protein.

### 2.5. Protein determination

Protein content was determined according to Lowry et al. [14] using bovine serum albumin as the standard.

### 3. Results

Oxalate oxidase activity was detected in the cell wall fraction but not in the soluble fraction (20 000 g supernatant) of maize seedlings. The enzyme activity could be detected in the embryonic axis after 10 h of imbibition, and it increased significantly after 48 h. Such high activity persisted with a maximum at 72 h (Fig. 1). At this time, seedlings were transferred to the nutrient solution, and the activity of the oxalate oxidase in the root and leaf was analyzed. The activity in the leaf cell wall was an order of magnitude lower, compared to the root cell wall activity in the case of 6-day-old plants. Further analysis was performed only on the root cell wall isolates, and the data presented show that there was a gradual decrease of activity with development, 9-day-old plants having only 20% of the maximal activity for the root. In further work, the characterization of the enzyme was done on root cell wall isolates obtained from plants grown for 3–5 days on the nutrient solution.

Colorimetric assay showed an optimum of enzyme activity within 5 min of incubation. A 40% decrease in activity was observed after 10 min of incubation (data not presented). Oxygen consumption by cell wall was not observed in the absence of added substrate. The addition of oxalic acid induced oxygen consumption at a constant rate (Fig. 2, insert), no changes being observed over 15 min.

The effect of pH on the activity of the enzyme was studied in the pH range of 2.1–5.1 using the succinate buffer. Both assays used yielded similar results showing that maize oxalate oxidase has a pH optimum around 3.2 (Fig. 3). Optimal concentration of substrate was determined using both assays as shown in Fig. 4. A total of 5 mM oxalic acid gave maximal reaction rates, in the case of colorimetric assays, and 10 mM in the case of polarographic assays. The relationship between oxalate concentration and reaction rates revealed $K_m$ of 2.54 and 1.31 mM for oxalate obtained by

Fig. 1. Activity of the cell wall oxalate oxidase in early stages of development of maize plant determined in the embryonic axis (embryo without scutellum), roots and leaves. The activity was assayed polarographically and expressed as nmol O₂ consumed mg protein⁻¹ min⁻¹. The time was measured from the beginning of the imbibition. The vertical arrow indicates the moment of transfer of seedlings from water to the nutrient solution.

Fig. 2. Oxalate oxidase activities expressed as nmol H₂O₂ produced mg protein⁻¹ min⁻¹, determined by colorimetric assay, plotted against the oxalate oxidase activities expressed as nmol O₂ consumed mg protein⁻¹ min⁻¹, determined polarographically, of the same samples. The correlation coefficient ($r^2$) is presented. Insert: an example of time course of oxygen consumption induced by the addition of oxalate (figure next to trace is expressed in nmol O₂ min⁻¹ mg protein⁻¹).

Fig. 3. Effect of pH on oxalate oxidase activity assayed by colorimetric method, measuring \( \text{H}_2\text{O}_2 \) production (○), and by polarographic oxygen consumption measurements (●). Enzyme activities are expressed as percent of maximal activity ± S.E. (indicated by vertical bars).

Fig. 4. Effect of oxalate concentration on oxalate oxidase activity assayed by colorimetric method, measuring \( \text{H}_2\text{O}_2 \) production (○), and by polarographic oxygen consumption measurements (●). Enzyme activities are expressed as percent of maximal activity ± S.E. (indicated by vertical bars).

O\(_2\) consumption and colorimetric measurements, respectively. Substrate inhibition was evident with 20 mM oxalic acid, in the case of \( \text{H}_2\text{O}_2 \) measurement, while \( \text{O}_2 \) consumption was not inhibited at concentrations higher than 10 mM. Because the values obtained by the colorimetric assay were not significantly different with 5 and 10 mM oxalic acid, 10 mM oxalic acid was used in all further experiments. By comparing the specific activities of oxalate oxidase determined in the same cell wall isolates by using two methods in a number of experiments (Fig. 2) a good correlation was obtained \( (r^2 = 0.9592) \). Thus, oxygen polarography was used as an alternative method to express the activity of oxalate oxidase.

In order to examine the degree of association of oxalate oxidase with cell wall, we subjected purified cell wall fraction to salt washing with 1 M NaCl or 1 M CaCl\(_2\). After both of the treatments, ~90% of the enzyme activity was detected in tightly bound-cell wall fraction (Table 1), demonstrating that binding of oxalate oxidase to the cell wall was more than an ionic association. The heat stability of oxalate oxidase was investigated by heating the cell wall fraction at various temperatures in the range of 37–80°C for 30 min. Full activity remained at 80°C (data not presented), demonstrating the very good heat-stability of the enzyme. The enzyme was resistant to SDS and pepsin treatment, 90 and 100% of the activity remaining after SDS and pepsin treatment, respectively (data not presented).

In order to examine the possible inhibitory effect of some chemical compounds (KCN, 2-mercaptoethanol, dithiothreitol, EDTA, glutathione, \( N \)-ethyl maleimide and L-cysteine) on the activity of oxalate oxidase we added them to the assay mixture in final concentrations of 0.5 and 5.0 mM and the reaction was performed under standard assay conditions, both detection methods being employed. The effect of these chemical compounds on the control (\( \text{H}_2\text{O}_2 \) measurements performed on a system where external \( \text{H}_2\text{O}_2 \) was added instead of oxalic acid and the cell wall preparation pro-

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Effect of salt washing (1 M NaCl and 1 M CaCl(_2)) on cell wall-bound oxalate oxidase( ^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total oxalate oxidase activity (nmol ( \text{H}_2\text{O}_2 ) min(^{-1} ))</td>
</tr>
<tr>
<td>Cell wall fraction before treatment</td>
<td>129.5 ± 8.1</td>
</tr>
<tr>
<td>Cell wall fraction after treatment (pellet)</td>
<td></td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>115.9 ± 6.7</td>
</tr>
<tr>
<td>1 M CaCl(_2)</td>
<td>117.8 ± 2.3</td>
</tr>
<tr>
<td>Ionomically-bound fraction after treatment (supernatant)</td>
<td></td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>7.9 ± 0.7</td>
</tr>
<tr>
<td>1 M CaCl(_2)</td>
<td>7.2 ± 2.1</td>
</tr>
</tbody>
</table>

\( ^a \) Cell wall was isolated from 5-day-old maize seedlings. Colorimetric assay was used in enzyme activity determination. One milliliter of the cell wall isolate was used for each of the treatments and the means with ± S.E. of assays in triplicate are shown.
Table 2
Effects of some chemical compounds on the oxalate oxidase activity determined colorimetrically by assaying H$_2$O$_2$ and polarographically by assaying O$_2$ consumption$^a$

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Colorimetric assay of H$_2$O$_2$</th>
<th>Polarographic assay of O$_2$ consumption by cell wall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control with H$_2$O$_2$ substituting cell wall + oxalate</td>
<td>H$_2$O$_2$ produced enzymically</td>
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<tr>
<td></td>
<td></td>
<td></td>
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<tr>
<td>KCN</td>
<td>5 mM 41.3 ± 0.5 (2)</td>
<td>75.7 ± 1.9 (2)</td>
</tr>
<tr>
<td></td>
<td>0.5 mM 107.7 ± 2.6 (2)</td>
<td>105.7 ± 2.0 (2)</td>
</tr>
<tr>
<td>2-mercapto-</td>
<td>5 mM 6.0 ± 1.9 (2)</td>
<td></td>
</tr>
<tr>
<td>ethanol</td>
<td>0.5 mM 7.7 ± 0.3 (2)</td>
<td></td>
</tr>
<tr>
<td>Dithiothreitol</td>
<td>5 mM 1.9 ± 0.0 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 mM 0.7 ± 0.0 (2)</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>5 mM 93.6 ± 1.4 (2)</td>
<td>81.0 ± 2.8 (2)</td>
</tr>
<tr>
<td></td>
<td>0.5 mM 93.6 ± 0.8 (4)</td>
<td>88.9 ± 7.6 (2)</td>
</tr>
<tr>
<td>Glutathione</td>
<td>5 mM 3.0 ± 0.4 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 mM 5.2 ± 0.0 (2)</td>
<td></td>
</tr>
<tr>
<td>N-ethyl maleimide</td>
<td>5 mM 99.1 ± 0.7 (2)</td>
<td>43.3 ± 7.3 (2)</td>
</tr>
<tr>
<td></td>
<td>0.5 mM 99.8 ± 1.9 (2)</td>
<td>82.9 ± 1.5 (2)</td>
</tr>
<tr>
<td>L-cysteine</td>
<td>5 mM 1.9 ± 0.6 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 mM 3.8 ± 0.4 (2)</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ The results are presented as remaining activity in % ± S.E. with number of experiments in parentheses.

$^b$ n.d., not determined.

Producing H$_2$O$_2$ along with their effect on oxalate oxidase activity, measured as H$_2$O$_2$ production and O$_2$ consumption, are presented in Table 2. The presence of reagents containing SH groups in both concentrations strongly affected H$_2$O$_2$ determination in colorimetric assay. Also, higher concentration of KCN inhibited strongly this reaction (60%), while inhibition by both concentrations of EDTA was not so pronounced (6%). In such cases, where reagents interfered with H$_2$O$_2$ measurements, it was not possible to examine their effect on oxalate oxidase activity measured colorimetrically. By using oxygen consumption rate to express oxalate oxidase activity, inhibitory effect of KCN, EDTA, glutathione (GSH), L-cysteine and 0.5 mM DTT was not detected, while higher concentration of DTT inhibited approximately 35% and 2-ME inhibited almost completely enzyme activity. Only the results of inhibitory effects of NEM were comparable for both methods, inhibition being ~20 and 50% with 0.5 and 5.0 mM NEM, respectively. Thus, in some cases the colorimetric method was not applicable because of the interaction of various compounds with the H$_2$O$_2$ produced by oxalate oxidase or with reagents used in the colorimetric assay. This problem was overcome by oxygen consumption measurements.

We also tested the effect of various metal ions and chloride salts on the enzyme activity. Addition of 1 mM NaCl to the assay mixture did not show any effect on the enzyme activity. Also, none of metal ions tested (Cu$^{2+}$, Fe$^{3+}$, Zn$^{2+}$, Mn$^{2+}$, Mg$^{2+}$, Al$^{3+}$) stimulated the enzyme activity, nor reversed the rate of reaction after inhibition by sulphydryl reagent, 2-ME (data not shown).

4. Discussion

In this report we demonstrated the occurrence of oxalate oxidase in cell walls isolated from maize seedlings. A significant increase in activity in the embryonic axis was obtained 2–3 days post-imbibition, subsequently decreasing in the plant organs that develop. Oxalate oxidase activity associated with cell wall is not unexpected since it was established that germin, a protein marker of early plant development, is an oxalate oxidase [4], and wall-bound form accounts for ~40% of the total germin in wheat embryos during germination [9].
Also, oxalate oxidase from barley leaves was found to be located in the cell wall [3]. However, contrary to the results obtained for sorghum [1], barley [2] and wheat [15], we did not detect any enzyme activity in the soluble fraction.

Maize oxalate oxidase was shown to be tightly bound to the cell wall since it was not extracted by either NaCl or CaCl2. It was also shown that the enzyme is heat stable since it remained fully active at 80°C. Our data show that it is even more stable than the oxalate oxidase purified from barley seedlings, which retained 80% of its activity at 75°C [2]. The resistance of maize enzyme to pepsin proteolysis is similar in characteristics to germin of wheat embryos [16]. Also, the resistance to SDS treatment, observed in our experiments, was shown previously for wheat embryo germin [16] and oxalate oxidase from barley seedlings [17].

Oxalate oxidase was active only at acidic pHs, with a pH optimum around 3.2. Previously, different values for pH optimum in the acidic range have been reported for different plant species. Our result for maize root oxalate oxidase is close to the results obtained for barley seedlings (pH 3.5) [2] and wheat embryo (pH 3.5) [3], and different from sorghum leaves (pH 5.0) [18] and roots (pH 5.0) [1]. The $K_m$ values obtained in our experiments were similar to $K_m$ obtained for *Amaranthus* leaves [6], while they were an order of magnitude higher than $K_m$ obtained for barley root [19] and two orders higher than for sorghum leaves [18] and beet stem [7].

The insensitivity of the enzyme to the tested metal and chloride ions that we observed is similar to the result obtained with membrane-bound oxalate oxidase from *Amaranthus* leaves [6]. Contrary to these results, the oxalate oxidase from sorghum leaves [18] and roots [1] required Fe$^{2+}$ and Cu$^{2+}$ for maximal activity, respectively, and chloride exhibited an inhibitory effect on oxalate oxidase from barley seedlings [2]. EDTA did not inhibit maize root oxalate oxidase. A similar result was obtained for barley seedlings [2] and roots [20], contrary to the strong inhibition of the enzyme from sorghum roots [1] and leaves [18]. Among the sulphhydryl reagents investigated, only 2-ME completely inhibited oxalate oxidase, while DTT and NEM exhibited a less pronounced inhibition. Inhibition by 2-ME was also reported for oxalate oxidase in barley seedlings [2] and roots [20] (more than 80%, with 1 mM 2-ME), and in sorghum roots (28% with 0.5 mM 2-ME) [1], but not in sorghum leaves [18].

Oxalate oxidase activity has been demonstrated in a number of plant species. Of these, only the wheat and barley enzymes have been classified as germin-like oxalate oxidases [4,21] until now. Our experiments indicate that maize root cell wall oxalate oxidase exhibits most of the characteristics of germins: heat-stability, protease-resistance, SDS-tolerance and similar pH optimum to that of barley and wheat (as opposed to that of sorghum whose oxalate oxidase is not classified as germin). An increase of enzyme activity 48-h post-imbition, similar to the observed increase in activity in the early stages of wheat embryos [9], suggests its significant role in the early stages of maize development and also argues in favor of such a contention.

Because oxalate oxidase catalyzes the reaction: 

$$\text{HOOC-COOH} + \text{O}_2 \rightarrow 2\text{CO}_2 + \text{H}_2\text{O}_2,$$

consuming oxygen, we have employed the polarographic method to measure its activity, besides the conventional colorimetric method for determination of the generated H2O2. A comparison of the two methods has been made using the same cell wall isolates, the results obtained demonstrating a good correlation for optimal assay conditions. However, differences have been observed in the values of the optimum concentrations, $K_m$, substrate inhibition and rate dependence on the incubation time. The optimal incubation time in colorimetric reaction of 5 min and decline of activity after prolonging the incubation period to 10 min was also observed in sorghum root oxalate oxidase [1]. Substrate inhibition of oxalate oxidase activity was observed in our experiments above 10 mM oxalic acid in the case of colorimetric assay. A similar inhibition was observed with partially purified enzyme from sorghum leaves [18], although at lower concentrations of oxalate (above 0.25 mM). The fact that oxalate oxidase activity assayed polarographically did not show substrate inhibition or decline of activity for periods up to 15 min indicates that the observed phenomena are indeed caused by the colorimetric assay, rather than by inhibition of the enzyme activity. On the other hand, our results show that the colorimetric method is not useful in studies of the effect of several chemical compounds on the activity of oxalate oxidase. In this method, oxalate oxidase activity was assayed by detection of the resulting H2O2 using horseradish peroxidase-de-
pendent staining method. KCN is a known inhibitor of peroxidase [22], and it interfered also in our colorimetric assays. Also, our results demonstrated that this method is not applicable in the studies of the effect of reagents containing SH groups (L-cysteine, GSH, 2-ME and DTT) on the oxalate oxidase activity. Their effect on H₂O₂ measurements can be explained by nonenzymic reaction of SH groups with H₂O₂ [23]. In such instances oxygen polarography proved to be a more convenient method for measuring the oxalate oxidase activity.

References