Oxalate oxidase (EC 1.2.3.4) catalyses the conversion of oxalate and dioxygen to carbon dioxide and hydrogen peroxide (for reviews see [1-3]). Oxalate oxidase activity has been detected in a number of organisms but the most biochemically characterised enzymes are from barley and wheat. The plant enzyme is isolated as a glycosylated homopentamer which is remarkably resistant to heat, SDS and proteinase treatment. It has recently been shown that the plant protein germin [4,5] and a few members of the germin-like protein family are oxalate oxidases and that these have been detected during plant germination, fungal stress and salt stress. It has been proposed that the activity may be involved in providing hydrogen peroxide for cell wall biosynthesis. The hydrogen peroxide producing enzymes that have been described to date require either a flavin cofactor, a copper (II) ion or heme for catalysis [6]. Purified oxalate oxidases from plant and microbial sources exhibit no detectable absorbance in the visible spectrum [7-9], clearly showing the complete absence of flavin and heme. The only metal analysis of an oxalate oxidase that has been reported is that of a Pseudomonas oxalate oxidase, which has somewhat different physical properties to the plant enzymes [8]. A total of 1.12, 0.36 and 0.99 atoms of manganese, zinc and iron were detected per polypeptide monomer and no copper, molybdenum, cobalt or nickel were detected. The authors did not point out that an observed 1.75-fold increase in activity on addition of manganese (II) ions suggested that the enzyme requires two (1.96) manganese ions per polypeptide for full activity and that the Pseudomonas oxalate oxidase appears to be in its own new class of hydrogen peroxide producing enzymes. There is some controversy about the identity of any metal ions that might associate with plant oxalate oxidase activity. Some authors have suggested copper [2,10], some iron [11,12] and others have suggested the involvement of Ca²⁺ and even Pb²⁺ [9]. With a view to a better understanding of the chemistry of oxalate oxidation and the physiological requirements of the plant enzyme, we are investigating the identity of the cofactors and structure of plant oxalate oxidases.

Our initial studies have been with the commercial, partially purified barley seedling oxalate oxidase from Boehringer which appears to be about 30 % pure. In order to reduce the level of any contaminating metal ions in this preparation, a 1 mg ml⁻¹ protein solution was desalted by 3 successive 10-fold dilutions and concentrations, using 10 mM acetate buffer, pH 5.0, and Amicon Microcon devices with 30 kDa cut-off ultrafiltration membranes at room temperature. Given that the molecular mass of the homopentamer is 125 kDa, the remarkable stability of the plant enzyme and the lack of evidence for monomers being active, the enzyme was expected to remain in the retentate. To our surprise, about 60 % of the activity was reproducibly detected in the filtrate. Microcon devices with a cut-off of 10 kDa were required to successfully desalt the enzyme. It would therefore appear that the pentamers are able to reversibly dissociate completely to 25 kDa monomers under these conditions. This unexpected phenomenon is the subject of further investigation.

Incubation of the desalted commercial enzyme preparation with the metal ions Zn²⁺, Mn²⁺, Fe³⁺ and Fe⁴⁺, in the concentration range 10 μM to 1 mM, did not lead to any increase in enzyme activity. Metal analysis of the preparation using Inductively Coupled Plasma techniques gave a molar ratio of 1.0:0.5:0.3:0.1:<0.01 for the metals zinc, manganese, iron, copper and lead. These values take into account control determinations without added protein. This result is consistent with those for the microbial enzyme [8] in that zinc, manganese and iron were considerably more abundant than copper. Although Pb²⁺ ions have been reported to stimulate the activity of the plant enzyme [9], no lead was detected.

Contaminating proteins, which may bind metal ions themselves, were removed from this partially purified enzyme preparation by ion exchange FPLC. Since the enzyme is most stable at pH 5.0 and the pH is predicted to be about 5.5 from the primary sequence [4], the enzyme was applied to a MonoS column pre-equilibrated with 10 mM acetate buffer, pH 5.0, and eluted with a salt gradient at about 0.14 M NaCl. This afforded a 2.7-fold purification with a 47 % recovery to give a specific activity of 6.0 U mg⁻¹ using a peroxidase-coupled spectrophotometric assay [22, 23]. The assay used 0.2 M sodium acetate pH 6.0, 0.1% 3, 3-diaminobenzidine and 0.001% H₂O₂. The specific activity compares reasonably well with that determined by others using alternative assay procedures at 37 °C (9 and 3.4 U mg⁻¹ [4,9]). The purified enzyme appeared to be at least 90 % pure according to SDS PAGE with Coomassie blue staining. The enzyme gave replicate gels using an activity stain containing oxalate, peroxidase and 4-chloro-1-naphthol confirmed that the major band was oxalate oxidase with samples that were not heated before electrophoresis.

The absorbance spectrum of the purified enzyme was determined at liquid helium and nitrogen temperatures. The typical six line spectrum of manganese (II) was observed, (2.03 at 4 K). Integration of this signal, based on a copper EDTA standard, gave about 0.4 manganese ions per monomer. This is not only consistent with the metal analysis, but also indicative that all of this metal is in the manganese (II) oxidation state. None of the typical signals associated with iron (III) ions, organic free radicals or copper (II) ions were detected. The nature of the iron ions in the sample is the subject of further investigation.

In this paper, we have shown for the first time that active plant oxalate oxidase contains essentially no copper ions. We can conclude therefore, that the plant enzyme catalyses the conversion of dioxygen to hydrogen peroxide using chemistry quite different from that used by galactose and amine oxidase. At this stage, it is not clear what the catalytically essential cofactors are for the plant enzyme, but it would appear that the most likely candidate metal ions are iron and manganese. It is possible that only a single metal ion is required for catalysis along with a one electron oxidised and modified tyrosine residue in a manner analogous with the copper containing oxidases. Many of the germin-like protein family share a conserved tyrosine residue. Sequence homology between plant oxalate oxidases and auxin binding proteins, which to our knowledge has not been reported previously, also lead one to consider the requirement for a small, diffusible organic cofactor with important regulatory consequences. However, we can not rule out the possibility that two metal ions are required for this two electron process. It therefore remains to be seen whether the plant and microbial enzymes utilise the same cofactors. We are currently purifying and characterising the enzyme from barley seedling roots that are grown in our laboratory.

We thank the BBSRC for a research studentship for L.R. and Drs. S.A. Fairhurst and D.J. Lowe for guidance with EPR spectroscopy.

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