# Barley oxalate oxidase is a hexameric protein related to seed storage proteins: evidence from X-ray crystallography

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**Abstract The oxalate oxidase enzyme expressed in barley roots is a thermostable, protease-resistant enzyme that generates $H_2O_2$. It has great medical importance because of its use to assay plasma and urinary oxalate, and it has also been used to generate transgenic, pathogen-resistant crops. This protein has now been purified and three types of crystals grown. X-ray analysis shows that the symmetry present in these crystals is consistent with a hexameric arrangement of subunits, probably a trimer of dimers. This structure may be similar to that found in the related seed storage proteins.**



*Key words:* Germin; Cupin; Seed storage protein; Abiotic stress; Pathogenesis-related protein; Hydrogen peroxide

## 1. Introduction

Oxalate oxidase (EC 1.2.3.4) (ox-ox), the enzyme that degrades oxalate to hydrogen peroxide and carbon dioxide, was first described by Zaleski and Reinhard [1] in a study of wheat flour, and it has since been shown to be widespread in microbes and plants [2]. The particular form of this enzyme expressed in barley roots is of great medical importance because of its use in kits to assay plasma and urinary oxalate [3]. In addition, it has been used to produce transgenic crops with improved tolerance to those fungal pathogens such as *Sclerotinia sclerotiorum* that use oxalic acid as a toxin ([4], Dunwell et al., unpublished). The barley isoform was recently [5,6] found to be homologous to the well-known wheat protein germin, a glycosylated, thermostable, protease-resistant protein, present at high levels in the extracellular matrix, produced during germination, and presumed to be the enzyme first identified more than 80 years ago [1]. In addition to the isoform highly expressed during embryo germination in cereals, there are a large number of related germin-like proteins (GLPs) [7,8], many of them induced during exposure of plants to important pathogens such as powdery mildew [9,10]. Some of these proteins are also induced by abiotic stresses, for example by saline conditions in barley [11] and *Mesembryanthemum crystallinum* (GenBank sequences gi|2725646, gi|2995935), by manganese deficiency in tomato (gi|2979494), by aluminium treatment in wheat [12], and by submergence in rice (gi|2952338, gi|3201969). The biochemical connection between these stress responses is unclear but it may be associated with the link between oxalate, its precursor ascorbate, and its product $H_2O_2$ (ox-ox itself is highly resistant to $H_2O_2$). Specifically, oxalate and peroxide are likely to be part of a signalling cascade [9], and in addition, ascorbate and $H_2O_2$ are known to be involved in the generation of hydroxyl ($^{\bullet}OH$) radicals capable of cleaving cellulose and other cell wall polysaccharides [13].

It was proposed recently, on the basis of sequence similarities, that ox-ox, the GLPs, and the seed storage proteins (these latter proteins in particular have extreme desiccation tolerance) have all evolved from identifiable microbial progenitors [14,15], and are members of a new superfamily of functionally diverse proteins designated as cupins (from the Latin term *cupa*, a small barrel or cask) [2] on the basis of their conserved β-barrel structural element found by X-ray crystallography of the bean storage protein vicilin [16]. Cupins exist in two forms, one (c. 100–200 AAs) with a single domain (e.g. ox-ox, phosphomannose isomerase, polyketide synthase [14]) and the other (c. 400–500 AAs) having two domains (e.g. oxalate decarboxylase [14], sucrose binding protein [17], storage proteins [16]). It is intended to establish the validity of this hypothesis by determining the tertiary structure of other members of this proposed superfamily.

Sequence analysis of wheat germin and its closely related ox-ox from barley show a protein size of about 200 AA (plus a targeting sequence to direct secretion to the cell wall), and a molecular mass of 21.2 kDa for the non-glycosylated form. (The native protein is glycosylated with a mass of about 26 kDa.) Both proteins are multimeric and enzyme activity is only associated with the assembled protein, and not with the monomer [5]. Additionally, although the oligomeric form of the enzyme survives treatment with trypsin, chymotrypsin, or *Staphylococcus aureus* protease in the presence 27 mM SDS, the monomeric form is digested in these conditions [18]. Despite these detailed biochemical studies, biophysical measurements of the exact oligomeric status have proved inconclusive. Early results [18] on the barley enzyme suggested a dimeric structure with 75 kDa subunits, whereas later studies either favoured a tetrameric structure (MW of 100 kDa, [6]) or did not try to estimate the oligomeric status because of the uncertainties of gel-based assays [19]. In contrast, wheat germin is usually reported to have a homopentameric structure with a molecular mass of about 125 kDa [5], and a barley GLP has been described as tetrameric or pentameric [8]. The distant homology of these proteins to the seed storage proteins [14,20] (trimeric proteins with two-domain subunits) favours a hexameric composition, a conclusion supported by a recent homology modelling study which used the known crystal structure of the storage protein vicilin to produce a model of the wheat germin [21]. This model also provided a valuable insight into the possible active site residues by identifying three histidines which form a cluster of adjacent side chain

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imidazole groups lying on neighbouring anti-parallel β-strands.

In an attempt to solve the dilemma about the oligomeric status of this important enzyme, the present study was designed to use the latest method for purification of barley ox-ox [22], to generate crystals, and to use these for X-ray analysis. Information on subunit composition would also be of immediate value in relating its structure to the known crystal structure of the storage proteins [16].

## 2. Materials and methods

### 2.1. Isolation, purification and assay of oxalate oxidase from barley roots

The partially purified ox-ox was a gift of Sigma/Aldrich plc. All procedures were performed at 4°C. Lyophilised powder (11 g) was dissolved in 110 ml of S16 buffer (100 mM potassium acetate, 3 mM magnesium acetate, 20 mM HEPES, pH 7.5) with 1% polyvinylpyrrolidine, and centrifuged at 20 000×*g* for 1 h. Solid $(NH_4)_2SO_4$ was then added slowly to the supernatant and the less dense brown layer in the fraction between 30% and 80% was removed carefully before redissolving in 90 ml succinate buffer (20 mM, pH 3.8). This viscous brown layer contains the ox-ox, together with polysaccharides and phenolic compounds. Ice-cold acetone was slowly added to this solution and the resulting pellet in the fraction between 25 and 50% was dissolved in 100 ml S16 buffer. The solution was centrifuged at 20 000×*g* for 1 h to remove insoluble material before filtration through a centrifugation filter kit (VIVA SPIN 15) with 0.2 μm clarification cup, and dialysis with 20 mM sodium acetate buffer pH 5.0. The acetone precipitation and filtration steps aided removal of the brown colour, but some colour remained. The resultant enzyme solution was applied (flow rate 0.36 ml/min) to a dye-affinity column comprising Procion turquoise MX-G (commonly known as Blue 140, a dichlorotriazine dye with a copper phthalocyanine group) immobilised on Sepharose CL-4B (Pharmacia, code no. 17-0150-01) previously equilibrated in 20 mM sodium acetate at pH 5.0 (method adapted from [22]). The column was washed with 50 ml buffer to remove unbound material, before bound ox-ox enzyme was eluted with 15 ml equilibration buffer containing 1 M $CaCl_2$. Those fractions with ox-ox activity were pooled, concentrated and dialysed against 10 mM Tris-HCl pH 8.5 before being subject to FPLC. The resulting enzyme solution (1 ml) was injected into an FPLC Mono Q column and the bound enzyme eluted with equilibration buffer containing 1 M NaCl. Those fractions with ox-ox activity were pooled and dialysed against 10 mM sodium acetate buffer (pH 5.0). The final yield of enzyme was 0.4 mg/g of crude extract and the protein was homogeneous as estimated by SDS-PAGE analysis.

### 2.2. Assay of oxalate oxidase activity

The procedure adopted was that of Zhang et al. [23]. The assay solution was prepared as follows: 40 mM succinic acid/KOH (pH 3.8), 60% (v/v) ethanol, 2 mM oxalic acid, 5 U/ml horseradish peroxidase (Sigma, P-8000), 20 μl/100 ml *N*,*N*-dimethylaniline, 8 mg/100 ml 4-aminoantipyridine. The above solution (1.5 ml) was preincubated at 30°C for 5 min, and then the enzyme solution (20 μl) was added. After 10 min, the reaction was stopped by addition of 20 μl of 1 M NaOH, and the absorbance of the resulting solution was measured at 555 nm. One unit of enzyme activity was defined as the amount of enzyme which produced 1 μmol $H_2O_2$ per min in the standard assay system.

### 2.3. Crystallisation

Ox-ox was concentrated with a Centricon (Amicon), using a 10K cut-off membrane, to 8.2 mg/ml (assuming an $OD_{280}$ of 1.0 corresponds to a final concentration of 1 mg/ml). Crystallisation experiments were conducted using the hanging drop method at 18°C, and the Hampton Screening Kits I and II [24]; crystals grew with PEG 4000 or 8000 in the pH range 4.6–8.5. Depending on the particular combination of conditions, three types of crystals were produced (Table 1). Type I crystals were rectangular with a size of 0.20×0.17×0.10 mm, type II were long needle-shaped (0.80×0.10×0.12 mm), and type III were cubic (0.20×0.20×0.20 mm). It should be noted that no attempt was made to deglycosylate the enzyme before crystallisation.

### 2.4. Data collection and processing

X-ray diffraction data from a type I crystal were collected at room temperature using the beam line DW42 at LURE, Orsay, France, and data from type II and type III crystals were obtained using an in-house MacScience (Siemens) rotating anode generator with double mirrors and a DIP 1030 image plate system. Because rapid radiation damage occurred when crystals were irradiated at room temperature, they were transferred to a cryosolution consisting of mother liquor supplemented with 20% 2-propanol and 10% 2-methyl-2,4-pentanediol (MPD) (for type II crystals) or 8% 2-propanol and 20% glycerol (type III) before being cryocooled in the nitrogen stream of an Oxford Cryosystems Cryostream at 100 K. The data were processed using DENZO and SCALEPACK [25]. All crystallographic calculations were made using the CCP4 program suite [26] unless otherwise stated.

## 3. Results

Details of the three crystal types are summarised in Table 1. Only a few images were collected using type I crystals but quite complete data were collected from crystal types II and III. Data from a type II crystal showed that it belonged to the space group $P42_12$. The merged data were 97.7% complete (98.6% for the highest resolution shell), had a merging $R_{sym}(I)$ of 0.079 (0.191), and 45% (42%) were measured 9–12 times. The unit cell parameters give a $V_m$ of 3.03 or 2.02 $Å^3$/Da assuming two or three monomers in the asymmetric unit, respectively; these values are within the range considered to be acceptable [27]. The self rotation function was calculated using the program POLARRFN. Using a resolution of 5–10 Å, there are significant peaks on the κ = 120° and κ = 180°

Table 1
Summary of the three types of ox-ox crystal

| | Type I | Type II | Type III |
|---|---|---|---|
| Growth conditions | PEG 4000 18%<br>$(NH_4)_2SO_4$ 0.2 M<br>pH 8.5 (0.1 M Tris) | PEG 4000 16%<br>$(NH_4)_2SO_4$ 0.06 M<br>pH 8.5 (0.1 M Tris) | $(NH_4)_2SO_4$ 2 M<br>2-propanol 5%<br>(initial pH 5.3) |
| Cryoprotectants | – | MPD 10%<br>2-propanol 20% | glycerol 20%<br>2-propanol 8% |
| Space group | P1 (4.0 Å) | $P42_12$ (5.0 Å) | R32 (2.5 Å) |
| Crystal parameter | $a$ = 61.4 Å, $b$ = 67.3 Å<br>$c$ = 72.1 Å<br>α = 72.1°, β = 79.3°<br>γ = 87.3° | $a$ = 71.5 Å<br>$c$ = 237.7 Å<br>α = 90°, β = 90°<br>γ = 90° | $a$ = 127.7 Å<br>$c$ = 73.1 Å<br>α = 90°, β = 90°<br>γ = 120° |
| Cell volume | $2.78\times10^5$ $Å^3$ | $1.22\times10^6$ $Å^3$ | $1.03\times10^6$ $Å^3$ |
| Number of monomers in asymmetric unit | (6)[a] | 3 | 1 |

[a]Compatible with six molecules per unit.

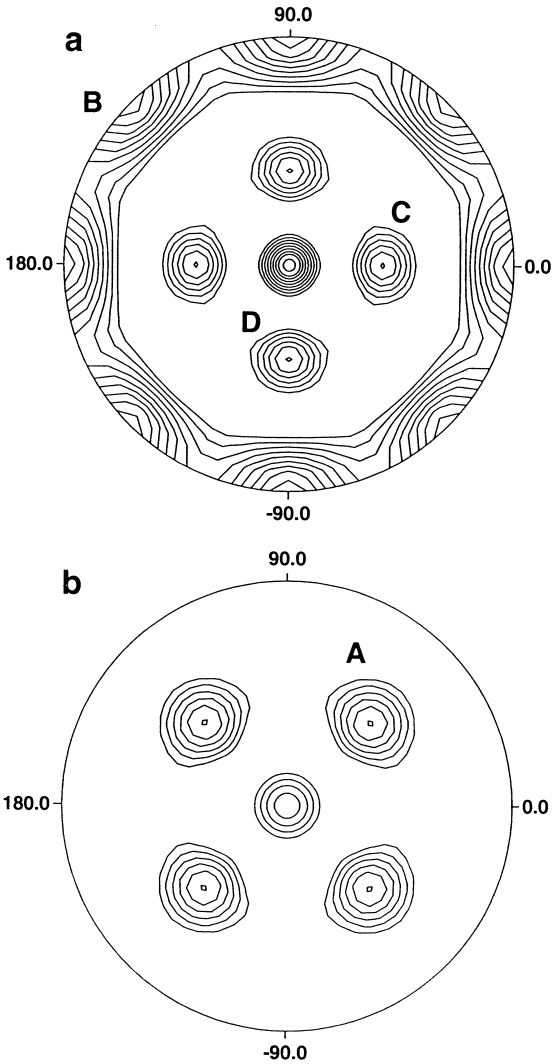


Fig. 1. Results of the self-rotation function calculation for type II crystals. a: $\kappa = 180°$ section. b: $\kappa = 120°$ section. If the three-fold axis of the ox-ox hexamer is consistent with peak A (125°, 45°, 120°) and one of the three two-fold molecular axes is placed according to the crystallographic peak B, then the other two non-crystallographic two-fold axes correspond to peaks C and D.

sections (Fig. 1). The heights of the peaks corresponding to the non-crystallographic two-fold and three-fold axes have identical values of 0.61, compared with values of 1.00 for the peaks corresponding to the crystallographic symmetry. Two non-crystallographic two-fold axes occur at $\omega$, $\phi$, $\kappa$ angles of (45°, 0°, 180°, respectively) and (135°, 270°, 180°) (Fig. 1a) and a single non-crystallographic three-fold axis occurs at (125°, 45°, 120°) (Fig. 1b). The native data from the type III crystal were 91.3% complete (58.0% for the highest resolution shell) to 2.5 Å with an $R_{sym}$(I) value of 0.092 (0.123); a total of 7253 reflections were used in scaling, and the number of data measured five times was 31% (3.7%). This crystal type belongs to space group R32 with a single molecule in the asymmetric unit and a $V_m$ value of 2.3 Å$^3$/Da.

A hexameric structure is clearly supported by the data from the type III crystals. Such a structure would have its molecular three-fold axis coincident with the crystallographic three-fold axis and its three two-fold axes coincident with the crystallographic two-fold axes. Consideration of the crystallographic and non-crystallographic symmetry of type II crystals also leads to the conclusion that a hexameric structure is likely. In this case, the hexameric molecular three-fold axis is non-crystallographic; this three-fold axis relates the three molecules in the asymmetric unit. One of the three molecular two-fold axes is crystallographic and generates the hexamer, whilst the other molecular two-fold axes are non-crystallographic (Fig. 1a). The angles between these axes are consistent with this proposal. The available data from type I crystals tentatively supports a hexameric structure.

The cross-rotation function calculated using the ALMN program and type II and III data in the range 15 Å to 6 Å with Patterson radii of 20 Å to 5 Å shows a single significant peak at 3$\sigma$. This suggests that the packing in type II and type III crystals is similar.

## 4. Discussion

The results from this X-ray analysis are of importance for a number of reasons. First, by showing that in the crystalline form, ox-ox packs as a hexamer of subunits, they substantiate the previous prediction from homology modelling [21] that ox-ox may be a trimer of dimers, and they thus provide evidence to support the discovery [14,20] of the evolutionary link between germin (and the GLPs) and the seed storage proteins; these latter proteins are trimeric with two-domain subunits [16]. The present results are therefore incompatible with the long-held belief that wheat germin, and the equivalent ox-ox from barley are pentameric [5,22] or tetrameric [6] in composition; they endorse the conclusion that gel-based assays are not a reliable means of estimating the total molecular mass of this particular multimeric protein [8,9]. (B.G. Lane and E.F. Pai, personal communication, have previously reported that they have evidence incompatible with a pentameric structure for germin.)

The second important conclusion concerns subunit dissociation. As the hexameric state of ox-ox does not vary in the pH range 4.8–8.5, dissociation of the oligomeric structure cannot account for the known loss of enzymatic activity at high pH [22]. Such loss of activity is therefore more likely to be associated with a change in ionisation state of a catalytic amino acid or in amino acid binding to a metal co-factor.

Final resolution of the detailed structure of this enzyme at the atomic level must await additional investigations. When available, such data will be of particular significance in confirming the identity of the active site ligands and the role of the histidine cluster defined recently [21]. Such information would be of considerable commercial significance as it may facilitate site directed mutagenesis as a means of improving the efficiency of the enzyme used in medical diagnosis. It will also aid the analysis of the structure/function relationships of the ox-oxs and GLPs, many of which are induced by a range of plant pathogens [8–10] and abiotic stress responses [11,12]. Since the trimeric storage proteins are known to bury a large amount of non-polar surface area during oligomerisation, it will be of particular interest to study the relationship between the monomer-monomer interfaces and the unique stability of this family of proteins in physical and chemical conditions that promote the degradation of most other proteins.

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