Purification, crystallisation and preliminary X-ray analysis of the vanadium-dependent haloperoxidase from *Corallina officinalis*

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Abstract The vanadium-dependent haloperoxidase from the seaweed *Corallina officinalis* has been purified to homogeneity and crystallised. The protein is reported to be a hexamer of 12 × 64,000 Da, contains no haem, and is dependent on vanadium for activity. The crystals are grown from polyethylene glycol (PEG) 6,000 and 0.4 M potassium chloride. They are stable and diffract to better than 2 Å resolution. They are of a cubic space group *I*23 (or *I*2$_1$3) with cell dimensions *a* = *b* = *c* = 310 Å.

Key words: Haloperoxidase; Vanadium enzyme; Crystal; X-ray diffraction; Biotransformation

1. Introduction

Vanadium-dependent haloperoxidases are able to utilise hydrogen peroxide to oxidise halides (Cl^-, Br^-, I^-) and to halogenate suitable organic substrates [1]. They are thought to be responsible for the production of a variety of halometabolites identified in marine algae, including environmentally significant volatile halocarbons such as bromoform [2]. Vanadium-dependent haloperoxidase was first identified in the brown macroalga *Ascophylum nodosum* [3]. Subsequently, vanadium-dependent haloperoxidases were isolated and partially purified from the red macroalgae *Ceramium rubrum* [4] and *Corallina officinalis* [5]. The native enzyme is oligomeric with a M_r of 740,000 and a denatured subunit size of 64 kDa supporting the dodecameric structure of two superimposed hexagonal rings presented for an apparently similar enzyme from *Corallina pilulifera* [6], although vanadium dependence for this enzyme was not examined. Extracellular haloperoxidase from *Curvularia inaequalis* also required activation by vanadium [7] and had previously demonstrated high stability to oxidised halide HOCl, a putative halogenation reaction product [8]. A vanadium-dependent haloperoxidase has also been isolated from the lichen *Xanthoria parientina* [9]. The use of the *C. officinalis* enzyme for biotransformations has been demonstrated by its ability to carry out regiospecific halogenations of cinnamyl substrates [10]. Regiospecific halogenation and typical high stability towards oxidants, organic solvents [11] and temperature [12] make vanadium-dependent haloperoxidases interesting catalysts for use in future biotransformations.

Crystallisation of vanadium-dependent haloperoxidase has been carried out on the monomeric enzyme from the phaeophyte *Ascophyllum nodosum* [13]. The crystals were in the monoclinic space group $P2_1$. Here we report the crystallisation of the *C. officinalis* enzyme. Structural studies will further our understanding of the multimeric arrangements of the protein subunits and the role of vanadium in the catalytic action. One property of the vanadium cofactor in haloperoxidases is that it can be removed at low pH in the presence of EDTA, rendering the enzyme inactive. Incubation of the enzyme at neutral pH with vanadate restores activity. This has led to the suggestion, which is confirmed by spectroscopic studies, that the vanadium is in the valance state 5+ ($3d^0$) [14].

2. Materials and methods

2.1. Isolation of haloperoxidase

Haloperoxidase was extracted by initial maceration of 450 g of *C. officinalis* collected from the Devon shoreline. This was carried out by grinding fresh fronds with acid-washed sand in buffer A (50 mM Tris-H_2SO_4), pH 8.0. All purification steps were carried out at 4°C. The crude protein extract was made 40% saturated with respect to enzyme-grade ammonium sulphate. After stirring for 30 min the extract was centrifuged at 15,000 rpm for 30 min and the resulting precipitate was discarded. Ammonium sulphate was added to the supernatant to 60% saturation, centrifuged as above and the supernatant discarded. The precipitate was dissolved in buffer A, pH 8.0, and dialysed overnight against the same buffer before being loaded onto a DEAE-cellulose column (Whatmann; 2.5 × 25 cm) equilibrated with the same buffer. Protein was eluted with a linear gradient of 0–1.0 M potassium bromide. Active fractions were pooled and made to 20% (w/v) ammonium sulphate. The enzyme solution was loaded onto a phenyl-Sepharose CL-4B column (Pharmacia; 5 × 5 cm) equilibrated with 20% (w/v) ammonium sulphate in buffer A, pH 8.0. Protein was eluted with a negative linear gradient from 20% (w/v) to 0% ammonium sulphate. Fractions rich in haloperoxidase were pooled and concentrated by precipitation with 80% ammonium sulphate. The enzyme was redissolved in a minimal volume of buffer A, pH 8.0, and loaded onto a Sephacryl-S400 (Pharmacia) gel-filtration column (1 × 40 cm). Fractions rich in haloperoxidase activity were collected and applied to a Sephacryl S-1000HR column (1 × 25 cm). Active fractions were concentrated using 80% ammonium sulphate and dialysed overnight against 1 mM sodium orthovanadate and buffer A, pH 6.8.

2.2. Haloperoxidase activity assays

Haloperoxidase activity was assayed by the monochlorodimedone assay. One unit of enzyme activity is defined as the amount of enzyme which converts 1 μmol of monochlorodimedone to products in 1 min at ambient temperature. MES buffer, pH 6.0, containing 0.1 mM monochlorodimedone, 1 mM hydrogen peroxide and 200 mM potassium bromide, was mixed in a 1 ml quartz curvette. After addition of enzyme the absorbance decrease at 290 nm was recorded spectrophotometrically using the extinction coefficient of monochlorodimedone of 19.9 $mM^{-1} \cdot cm^{-1}$ [16].

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2.3. Stability studies

The enzyme was heated to temperatures between 70 and 90°C for 15 min without 10 mM sodium orthovanadate and activity checked by the monochlorodimedone assay. The enzyme was deactivated by dialysis against 50 mm citrate buffer, pH 4.0, 10 mM EDTA. The long-term effect of chaotropic agents upon haloperoxidase activity was determined by incubation of the enzyme with 0–80% v/v of ethanol, propanol-2-ol and acetone for 21 days at 4°C. The remaining activity was checked using the monochlorodimedone assay described above. The effect of pH on the enzyme was tested by incubation at pH values ranging from 4 to 10 for 1 month at 4°C. The resultant activity was tested as described above.

2.4. Haloperoxidase crystallisation

Protein was concentrated on Amicon Centrion 10 microconcentrators to a concentration of 4.2 mg·ml^{-1}. Crystals were grown at 18°C by vapour-phase diffusion using the hanging-drop technique. The protein concentration was 4.2 mg·ml^{-1} in a solution containing buffer A, pH 6.8, 0.4 M potassium chloride and 5% (w/v) PEG 6000. The hanging drops were suspended in sealed compartments above a 25% (w/v) solution of PEG 6000 containing 0.4 M potassium chloride. The crystals which grew in 1–2 weeks were harvested into a mother liquor containing 30% PEG 6,000 and 1 M potassium chloride.

2.5. X-ray data collection

Data were collected on the Synchrotron beamline X 11 at the EMBL Hamburg outstation at a wavelength of 0.92 Å. Native crystals diffract to beyond 2.0 Å resolution. 50° of data, to a resolution of 3.15 Å were collected in 0.5° frames and processed with the MOSFLM software [17].

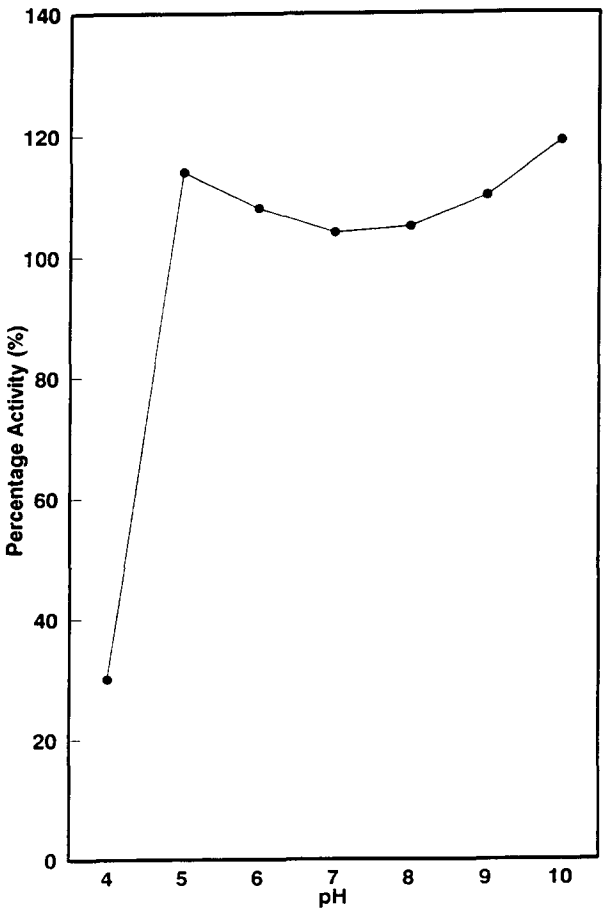

Fig. 1. Graph showing the percentage of activity remaining after storage of the haloperoxidase after incubation at pH values 4–10 for 1 month at 4°C.

Fig. 2. Crystals of vanadium-dependent haloperoxidase from *Corallina officinalis*, grown from PEG 6,000. Crystal dimensions were typically 0.3 × 0.3 × 0.3 mm.

3. Results and discussion

Protein prepared as described in section 2 ran as a single band on SDS/PAGE with a molecular weight of 64,000 Da [15]. The stability studies showed that the enzyme was stable to high temperatures, with reversible activity loss at a tempeature of 90°C. Loss of haloperoxidase activity at 90°C followed by reactivation at 20°C was consistent with the results of Sheffield et al. [12]. In the presence of excess sodium orthovanadate (10 mM), activity loss was not observed at 80°C. When the enzyme was deactivated by dialysis against 50 mM citrate buffer at pH 4 in the presence of 10 mM EDTA the activity was fully restored by vanadium orthovanadate. The long-term effect of storage of the enzyme in chaotropic agents is shown in Table 1. Haloperoxidase activity was unaffected by storage at 4°C in 40% (v/v) ethanol and was increased by 28% when incubated for 1 month 40% (v/v) propan-2-ol.

Vanadium-dependent haloperoxidase could chlorinate monochlorodimendone with a pH optimum of 4.5. The enzyme was stable for 1 month at pH values ranging from 5 to 10 (Fig. 1). The stability of this enzyme to temperature, solvents and pH makes it ideally suited for use industrially in large scale biotransformation reactions.

Cubic crystals grew from PEG 6,000 in about 1–2 weeks and

Table 1
Long-term effect of chaotropic agents upon haloperoxidase activity

Solvent % (v/v)	Percentage of activity remaining		
	Ethanol	Propan-2-ol	Acetone
0	100	100	100
20	107	132	59
40	104	128	57
60	34	11	0
80	34	0	0

Enzyme was incubated with an appropriate amount of solvent for 21 days at 4°C. The values represent the amount of haloperoxidase activity remaining.

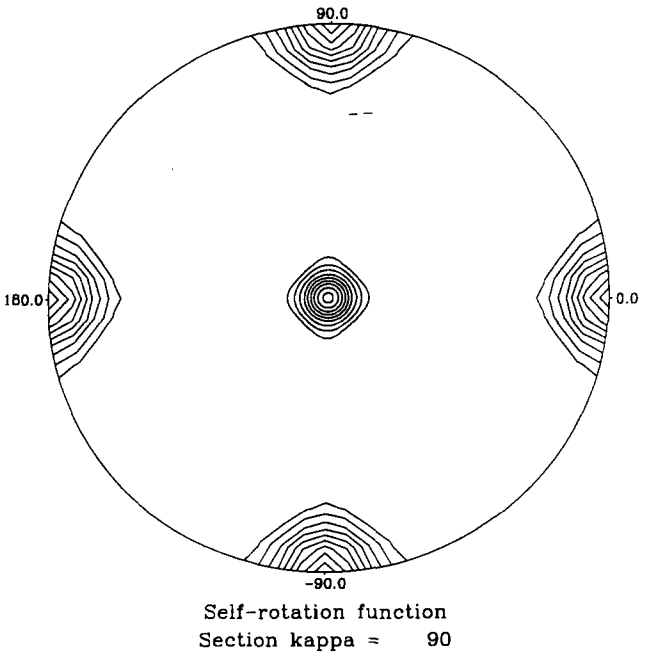

Fig. 3. $\kappa = 90$ section of the self-rotation function calculated with the CCP4 program POLARRFN. Data from 10.5 Å were used together with an outer radius for Patterson integration of 25 Å. The calculation has been performed such that crystallographic symmetry results in a peak height of 100 units. The plot is contoured from 10 to 100 in steps of 10. The non-crystallographic 4-fold axis indicates that the space group is pseudo $I432$ (or $I4_132$), as is also indicated by the reasonable merging statistics in this space group (see text).

were typically $0.3 \times 0.3 \times 0.3$ mm in dimension (Fig. 2). The crystals diffract to beyond 2.5Å resolution and are in the cubic space group $I23$ (or $I2_13$) with cell dimensions $a = b = c = 310$ Å. Taking into account the molecular weight of the protein (64,000 Da) and the asymmetric unit volume of 1,205,254 Å^3, it is possible that the asymmetric unit contains 6, 7, 8 or 9 molecules with packing densities of 3.1, 2.7, 2.3 or 2.1 Å^3/Da. The final data contained 519,194 observations of 84,368 unique reflections. Only 146 experimental observations were rejected during the data reduction procedure. The final data are 98% complete to 3.15 Å resolution, with an R_{merge} of 0.065, and a mean multiplicity of observation of 6.1 observations/reflection. The crystals diffract extremely well, considering the asymmetric unit volume, which suggests that the lower solvent contents are more likely. Inspection of the native diffraction amplitudes or the self-rotation function (Fig. 3) indicates the presence of a strong non-crystallographic 4-fold axis. The crystals therefore have a pseudo space group of $I432$ or $I4_132$. Indeed the diffraction data can be reduced in this space group to give an R_{merge} of only 0.12, but this requires that 21% of the experimental observations are rejected compared to 0.028%. With the present information, it is not clear how the proposed molecular structure of two stacks of 6 molecules can be related to the observed crystallographic and non-crystallographic symmetry. It is hoped that further X-ray studies will help to solve this problem. Information on the structure of the enzyme will be of interest in understanding the role of vanadium in the catalytic mechanism. The potential to modify the substrate specificity of the enzyme by site-directed mutagenesis will extend the use of vanadium haloperoxidase in large scale biotransformation reactions.

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References

[1] Itoh, N., Izumi, J. and Yamada, H. (1987) J. Biol. Chem. 262, 11982–11987.
[2] Collén, J., Ekdahl, A., Abrahamsson, K. and Pedersén, M. (1994) Phytochemistry 36, 1197–1202.
[3] Vilter, J. (1984) Phytochemistry 23, 1387–1390.
[4] Krenn, B.E., Plat, H. and Wever, R. (1987) Biochim. Biophys. Acta 912, 287–291.
[5] Yu, H. and Whittaker, J.W. (1989) Biochem. Biophys. Res. Comm. 160, 87–92.
[6] Itoh, N., Yashikazu, I. and Yamada, H. (1986) J. Biol. Chem. 261, 5194–5200.
[7] van Schijndel, J.W.P.M., Vollenbrock, E.G.M. and Wever, E. (1993) Biochim. Biophys. Acta 1161, 249–256.
[8] Liu, T.N.E., M'Timkula, T., Geigert, J., Wolf, B., Neidleman, S.L., Silva, D. and Hunter-Cevera, J.C. (1987) Biochem. Biophys. Res. Commun. 142, 329–333.
[9] Plat, H., Krenn, B.E. and Wever, R. (1987) Biochem. J. 248, 277–279.
[10] Coughlin, P., Roberts, S., Rush, C. and Willetts, A. (1993) Biotec. Lett. 15, 907–912.
[11] De Boer, E., Plat, H., Tromp, M.G.M., Wever, R., Franssen, M.C.R., van der Plas, H.C., Meijer, E.M. and Shoemaker, H.E. (1987) Biotech. Bioeng. 30, 607–610.
[12] Sheffield, D.J., Harry, T., Smith, A.J. and Rogers, L.J. (1993) Phytochemistry 32, 21–26.
[13] Müller-Fahrnow, A., Hinrichs, W., Saenger, W. and Vilter, H., (1988) FEBS Lett. 239, 292–294.
[14] Arber, J.M., De Boer, E., Gainer, C.D., Hasnain, S.S. and Wever, R. (1989) Biochemistry 28, 7968–7973.
[15] Laemmli, U.K. and Favre, M. (1973) J. Mol. Biol. 80, 575–599.
[16] De Boer, E. and Wever, R. (1988) J. Biol. Chem. 263, 12326–12332.
[17] Leslie, A.G.W., Brick, P. and Wonacott, A.J. (1986) CCP4 Newsl. 18, 33–39.