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Purification, crystallization and preliminary crystallographic analysis of DehIVa, a dehalogenase from Burkholderia cepacia MBA4

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Purification, crystallization and preliminary crystallographic analysis of DehIVa, a dehalogenase from *Burkholderia cepacia* MBA4

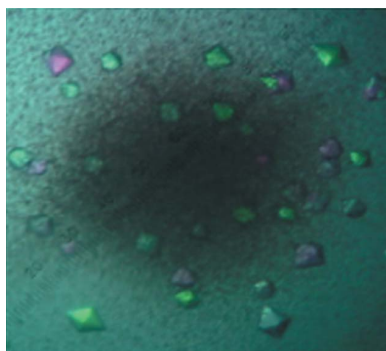
DehIVa is one of two dehalogenases produced by the soil- and water-borne bacterium *Burkholderia cepacia* MBA4. It acts to break down short-chain halogenated aliphatic acids through a nucleophilic attack and subsequent hydrolysis of an enzyme–substrate intermediate to remove the halide ions from L-enantiomers substituted at the C2 position (e.g. L-2-monochloropropionic acid). Dehalogenases are an important group of enzymes that are responsible for breaking down a diverse range of halogenated environmental pollutants. The *dhlIVa* gene coding for DehIVa was expressed in *Escherichia coli* and the protein was purified and crystallized using the hanging-drop method. Crystals grown in PEG 4000 and ammonium sulfate diffracted to 3.1 Å. The crystals had a primitive hexagonal unit cell, with unit-cell parameters $a = b = 104.2$, $c = 135.8$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. Determining this structure will provide valuable insights into the characterization of the catalytic mechanisms of this group of enzymes.

1. Introduction

Halogenated organics are an industrially important group of chemicals that in recent decades have been the focus of considerable international attention regarding their detrimental effects on the environment. Their harmful qualities stem from the recalcitrant, carcinogenic and toxic properties of many of these compounds and there is an obvious need to determine strategies for their removal from contaminated systems (Schwarze *et al.*, 1997). Numerous microorganisms have been isolated that degrade halogenated organics through the action of enzymes called dehalogenases. These enzymes form a diverse group with a broad range of substrate specificities and catalytic mechanisms (Slater *et al.*, 1995). Within this group are the L-2-haloacid dehalogenases (L-2HADs), which catalyze the dehalogenation of the L-enantiomers of C2-substituted short-chain organic acids. The reaction proceeds with the inversion of configuration at the chiral centre to produce a D-2-hydroxyacid. This stereospecificity, common throughout dehalogenases, also makes them useful in stereoselective industrial applications (Kurihara *et al.*, 1995) in addition to their usefulness in bioremediation.

Burkholderia cepacia MBA4 was isolated by Tsang *et al.* (1988) by growing cultures containing monobromoacetate (MBA) as the sole carbon source and was shown to produce two dehalogenases, though only the dehalogenase DehIVa was expressed under batch conditions. DehIVa is a member of the L-2HAD family of enzymes and exists as a homodimer with a molecular weight of ~45 kDa. Each monomer is 231 amino acids in length (Tsang & Pang, 2000).

Structural information is available for other dehalogenases, including a number of haloalkane dehalogenases (Streltsov *et al.*, 2003; Oakley *et al.*, 2004; Verschuere *et al.*, 1993) and two haloacid dehalogenases, DhIB and L-DEX YL (Ridder *et al.*, 1997; Hisano *et al.*, 1996). DehIVa has a high amino-acid sequence identity with the latter two enzymes (40 and 39%, respectively; Slater *et al.*, 1997). The structures and reaction mechanisms of these enzymes have been determined to be very different from those of the haloalkane dehalogenases (Ridder *et al.*, 1997, 1999; Janssen *et al.*, 2001; Liu *et al.*, 1995). It should be noted that there is still some uncertainty surrounding the roles of specific catalytically important residues; in particular, histidines 20 and 56 and arginines 42 and 156 (Liu *et al.*, 1994; Pang & Tsang, 2001; Ridder *et al.*, 1999; Li *et al.*, 1998). L-HADs



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are an important group of enzymes; further structural characterization of related proteins would benefit our understanding of the groups' reaction mechanisms.

Although there have been a number of rigorous biochemical studies reported regarding this enzyme (Murdiyatmo *et al.*, 1992; Tsang *et al.*, 1988; Tsang & Pang, 2000; Tsang & Sze, 2002), this is the first report of the crystallization and preliminary crystallographic analysis of the enzyme DehIVa.

2. Materials and methods

2.1. Overexpression and purification of DehIVa

The construct pHKU201 (Tsang & Pang, 2000) bearing the structural gene for DehIVa (*hdIIVa*) was cloned into *Escherichia coli* BL21 (DE3). Tsang & Pang (2000) have developed protocols for the overexpression and purification of DehIVa. After confirming the sequence of the *hdIIVa* gene, our own protocols were developed from those of Tsang and Pang. In summary, three 2 l cultures were grown in Luria Broth media at 310 K until the OD₆₀₀ reached 2.7 and were then induced by the addition of IPTG (to 1 mM). The culture-growth temperature was not modified. After a 4 h induction, cells were spun down at 7500 rev min⁻¹ for 8 min. Cell pellets were harvested and resuspended in 275 ml 50 mM Tris buffer pH 7.4. PMSF was added to a final concentration of 1 mM and the cell suspension then lysed in a French Pressure cell. Cell lysate (270 ml) was then spun down at 15 000 rev min⁻¹ for 15 min to remove the insoluble fraction.

The overexpression and purification of DehIVa was monitored as the dominant 25 kDa band on SDS-PAGE as described by Tsang & Pang (2000). The 25 kDa band was clearly evident after induction with IPTG (Fig. 1a).

The estimated percentage purity of DehIVa throughout its purification is summarized in Table 1. As the pI of DehIVa is ~5.1 (determined experimentally), the lysate supernatant was applied to a Q-Sepharose (Pharmacia/Pfizer, New York, USA) anion-exchange chromatography column pre-equilibrated with 50 mM Tris pH 7.4. DehIVa was eluted with a linear salt gradient from 0 to 1 M NaCl at a flow rate of ~1.0 ml min⁻¹ for 5 h in the same Tris buffer. Samples containing DehIVa as indicated by SDS-PAGE were combined. This was then treated with two rounds of ammonium sulfate precipitation, first at 50% (the precipitate was discarded) and then at 90% ammonium sulfate. Precipitate was resuspended in 50 mM Tris pH 7.4 and run on a 400 ml Sephacryl (Pharmacia) column. The DehIVa peak was diluted down in a 1:5 ratio with double-distilled H₂O to a buffer concentration of 10 mM Tris pH 7.4 and then run through a weak anion-exchange column (DEAE Sephacel, Pharmacia) and eluted with a linear salt gradient to 10 mM Tris pH 7.4, 0.5 M NaCl

Table 1
Purification of DehIVa from overexpression in *E. coli* strain BL21.

Step	Purity† (%)
Lysate	5
Q-Sepharose	15
(NH ₄) ₂ SO ₄	20
Sephacryl	40
DEAE	70
Hydroxyapatite	98

† Estimated by SDS-PAGE analysis.

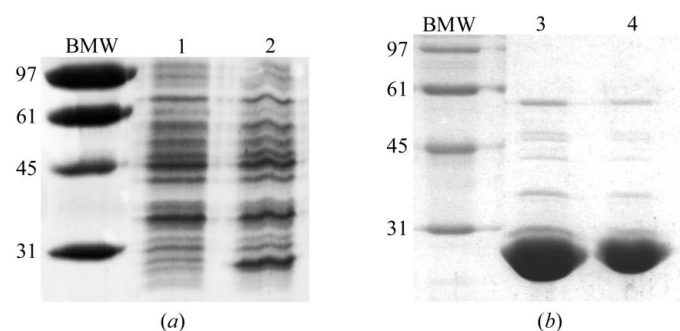


Figure 1
SDS-PAGE monitoring of the overexpression and purification of DehIVa. (a) Pre- and post-induction bacterial suspensions (lanes 1 and 2, respectively). DehIVa is clearly induced with the addition of IPTG (~25 kDa band). (b) Purified DehIVa; 15 and 7.5 µl sample loads of protein solution. The lane marked BMW is a broad molecular-weight ladder (kDa).

(~0.5 ml min⁻¹ over ~10 h). This sample was then desalted, the buffer changed to 30 mM Tris pH 7.4 and spun down in an ultracentrifuge (90 000 rev min⁻¹ for 30 min) to remove an unwanted contaminant. As a final step, the sample (~10 ml) was applied onto a hydroxyapatite (Biorad, Hercules, USA) column (five runs of 2 ml) and eluted off with a linear gradient to 70% 30 mM Tris pH 7.4, 90 mM Na₃PO₄ at 1 ml min⁻¹ for 40 min. The purified eluants were concentrated (6 ml Vivaspin concentrator tube with 3500 Da molecular-weight cutoff; Vivascience, Hanover, Germany) and pooled (5 ml at ~1.14 mg ml⁻¹; Fig. 1b). All chromatography except the hydroxyapatite runs was conducted at 277 K (hydroxyapatite at ~290 K). In preparation for crystallization trials, the hydroxyapatite-purified DehIVa was desalted and concentrated (6 ml Vivaspin with 3500 Da molecular-weight cutoff) to ~7.5 mg ml⁻¹ in a final volume of 800 µl in Tris buffer pH 7.4.

2.2. Crystallization

Crystallization screens were set up using JB Screens 2 and 3 from Jena Bioscience (Jena Biosciences, Jena, Germany). The hanging-

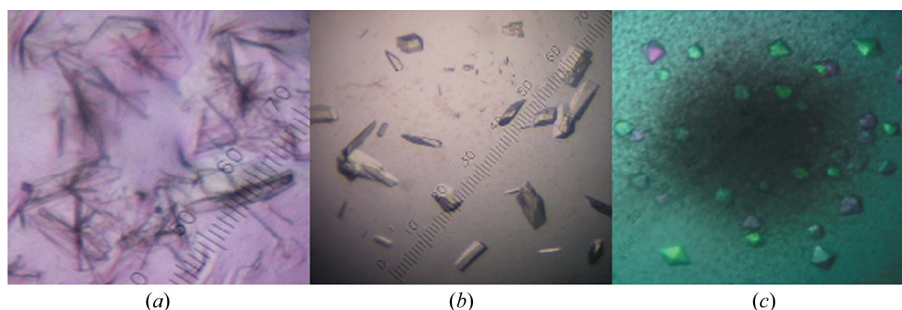


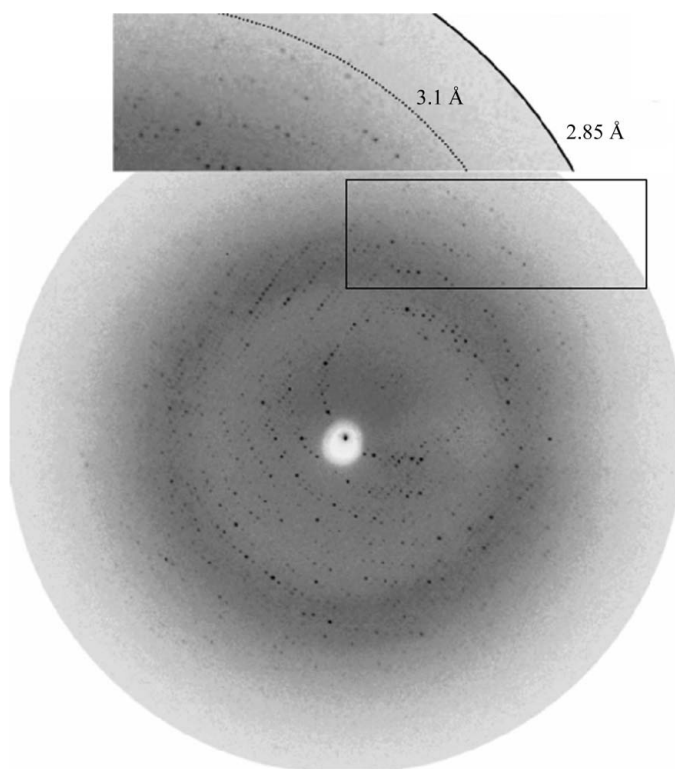
Figure 2
Microphotographs of three morphologically different crystal forms of DehIVa. Crystals form 1, 2 and 6 are shown in (a), (b) and (c), respectively. The largest crystal dimensions for each were ~0.3, 0.2 and 0.2 mm, respectively.

Table 2

Crystallographic diffraction data and processing statistics.

Values in parentheses are for the highest resolution shell.

Resolution range (Å)	30–3.10
Observed reflections	42428 (4645)
Unique reflections	15054 (1504)
Completeness (%)	96.4 (96.2)
Redundancy	2.82 (3.08)
R_{merge} (%)	18.7 (65.5)
$I/\sigma(I)$	6.32 (1.77)
No. of images	270

**Figure 3**

Picture of the diffraction pattern collected in-house from flash-frozen bipyramidal crystals of form 6. The exposure time was 10 min, with an oscillation range of 0.25° and a crystal-to-film distance of 160 mm.

drop vapour-diffusion method was used with a drop composed of 1 µl protein solution mixed with 1 µl well solution suspended over 300 µl well solution at room temperature (~293 K). Six morphologically different crystal forms grew within 48 h. The crystallization conditions for each form were as follows. (1) 30% PEG 4000; (2) 20% PEG 4000, 0.2 M (NH₄)₂SO₄; (3) 30% PEG 4000, 100 mM Tris–HCl pH 8.5, 200 mM lithium sulfate; (4) 15% PEG 4000, 100 mM sodium citrate pH 5.6, 200 mM ammonium sulfate; (5) 20% PEG 4000, 100 mM Na MES pH 6.5, 600 mM sodium chloride; (6) fine screening of these conditions provided useful diffraction data from crystals of a different morphology grown from 22% PEG 4000 and 0.65 M (NH₄)₂SO₄ in Tris buffer pH 7.4. Photographic images of crystal forms 1, 2 and 6 are presented in Fig. 2. Crystal forms 3, 4 and 5 were very small and were not amenable to microphotography.

2.3. X-ray diffraction analysis

Of the various crystal morphologies, only the hexagonal bipyramidal crystal form 6 (Fig. 2c) provided measurable diffraction data.

The crystals typically grew to ~0.2 × 0.2 × 0.2 mm over 10–24 h. The crystals did not grow any larger beyond this time. Preliminary data were collected in-house using a Rigaku RUH2R X-ray source with a rotating copper anode equipped with Osmic confocal optics, a MAR Research MAR345 detector and an Oxford Cryosystems 600 Series Cryostream. Crystals were transferred to a 10 µl drop composed of mother liquor modified to include the cryoprotectant glycerol at 20%(v/v) for approximately 5 s prior to flash-freezing to 100 K using the Cryostream. An X-ray diffraction image collected in-house is shown in Fig. 3.

Data processing was conducted using the *HKL* suite of programs (Otwinowski & Minor, 1997) and a summary of the data-processing statistics are shown in Table 2. Crystals were found to belong to the primitive hexagonal space group *P*3₁21, with unit-cell parameters $a = b = 104.2$, $c = 135.8$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$ and a maximum diffraction resolution of ~3.1 Å. Based upon the Matthews coefficient of 4.3 Å³ Da⁻¹, we anticipate that the asymmetric unit will contain between one and four monomers of DehIVa. A full structural determination using molecular replacement (with the DhIB structure, PDB code 1qq5) is currently under way.

Crystals were taken to beamline 14-ID-B, BioCARS Facility, Advanced Photon Source (APS) in Argonne for analysis with synchrotron radiation; however, owing to the deterioration of the crystals during transport or handling no usable X-ray diffraction data could be measured.

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