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Crystallization and preliminary X-ray analysis of a novel haloalkane dehalogenase DbeA from *Bradyrhizobium elkani* USDA94

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A novel enzyme, DbeA, belonging to the haloalkane dehalogenase family (EC 3.8.1.5) was isolated from *Bradyrhizobium elkani* USDA94. This haloalkane dehalogenase is closely related to the DbjA enzyme from *B. japonicum* USDA110 (71% sequence identity), but has different biochemical properties. DbeA is generally less active and has a higher specificity towards brominated and iodinated compounds than DbjA. In order to understand the altered activity and specificity of DbeA, its mutant variant DbeA1, which carries the unique fragment of DbjA, was also constructed. Both wild-type DbeA and DbeA1 were crystallized using the sitting-drop vapour-diffusion method. The crystals of DbeA belonged to the primitive orthorhombic space group $P2_12_12_1$, while the crystals of DbeA1 belonged to the monoclinic space group $C2$. Diffraction data were collected to 2.2 Å resolution for both DbeA and DbeA1 crystals.

1. Introduction

Haloalkane dehalogenases (HLDs; EC 3.8.1.5) are microbial enzymes that catalyse the hydrolytic conversion of halogenated aliphatic compounds to the corresponding alcohols. These enzymes play a key role in the aerobic mineralization of many halogenated compounds that have been recognized as environmental pollutants (Janssen *et al.*, 2005). HLDs are also applicable for bioremediation, decontamination and industrial biocatalysis (Swanson, 1999; Prokop *et al.*, 2004, 2005). Phylogenetic analysis revealed that the haloalkane dehalogenase family can be divided into three subfamilies denoted HLD-I, HLD-II and HLD-III (Chovancová *et al.*, 2007). To date, the crystal structures of three HLDs from subfamily HLD-II have been determined: DhaA from *Rhodococcus rhodochrous* NCIMB 13064 (Newman *et al.*, 1999), LinB from *Sphingobium japonicum* UT26 (Marek *et al.*, 2000) and Rv2579 from *Mycobacterium tuberculosis* H37Rv (Mazumdar *et al.*, 2008). These crystal structures have shown that the enzymes of subfamily HLD-II adopt essentially the same overall fold and are composed of two domains: the main domain and the cap domain containing the conserved catalytic pentad Asp-His-Glu+Asn-Trp. DbjA isolated from *Bradyrhizobium japonicum* USDA110 and three DhaA mutants have recently been crystallized (Sato *et al.*, 2007; Stsiapanava *et al.*, 2008); however, their structures have not yet been published.

A novel HLD named DbeA was recently isolated from *B. elkani* USDA94 (Ikeda-Ohtsubo *et al.*, 2009) and biochemically characterized by Mozga *et al.* (2009). This new member of the HLD-II subfamily is closely related to DbjA (Sato *et al.*, 2005), which exhibits a unique insertion in the N-terminus of the cap domain that is not present in other HLDs. DbeA shares the highest sequence identity (71%) to DbjA (Sato *et al.*, 2005). Its sequence identities with other HLD-II members are 60% for DmlA (Sato *et al.*, 2005), 47% for DhaA (Kulakova *et al.*, 1997), 41% for LinB (Nagata *et al.*, 1997) and 39% for DmbA (Jesenská *et al.*, 2005). DbeA is less active and has a higher specificity towards brominated and iodinated compounds compared with DbjA. These two enzymes also differ in temperature

Table 1
X-ray data-collection statistics for the DbeA and DbeA1 crystals.

Values in parentheses are for the highest resolution shell.

	DbeA	DbeA1
Beamline	BESSY MX14.1	DESY X11
Detector	MAR Mosaic 225 mm	MAR 555 flat panel
Resolution range (Å)	50–2.2 (2.28–2.2)	50–2.2 (3.0–2.2)
Unit-cell parameters (Å, °)	a = 62.7, b = 121.9, c = 161.9, $\alpha = \beta = \gamma = 90$	a = 133.8, b = 75.1, c = 77.6, $\alpha = \gamma = 90$, $\beta = 92$
Space group	$P2_12_12_1$	$C2$
Unique reflections	63890	38810
Redundancy	6.0 (3.4)	3.7 (3.7)
Completeness (%)	92.0 (62.8)	98.9 (98.9)
R_{merge}† (%)	6.6 (24.8)	12.9 (27.8)
$\langle I/\sigma(I)\rangle$	25.0 (4.3)	12.6 (6.6)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is an individual intensity of the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity of reflection hkl with summation over all data.

and pH profile in reaction with 1-iodohexane (Mozga *et al.*, 2009, in preparation).

A mutant of DbeA, designated DbeA1, was constructed using inverse PCR in order to study the importance of the insertion in the N-terminus of the cap domain to the activity and specificity of these enzymes. The fragment $_{143}$VAEEQDHAE$_{151}$ equivalent to the unique sequence of DbjA that is not present in DbeA and other HLDs (Ikeda-Ohtsubo *et al.*, 2009, in preparation) was inserted between Asp142 and Ala143 of DbeA. Crystallographic analysis of DbeA and DbeA1 was initiated in order to understand the structure–function relationships of the wild type and the insertion mutant. Here, we report the crystallization and diffraction data analysis of DbeA and DbeA1.

2. Materials and methods

2.1. Construction of the mutant, protein expression and purification

To overproduce wild-type DbeA and the mutant DbeA1, the corresponding genes were cloned into vector pET21b using *Nde*I and *Xho*I and transcribed from the T7 phage promoter under the control of *lac*UV5. The DbeA wild-type (accession No. AB478942) construct codes for residues 1–302 of DbeA with its C-terminus fused to ATRHHHHHH (where the A is residue 302 of the DbeA sequence). The DbeA1 mutant was constructed using inverse PCR. The fragment $_{143}$VAEEQDHAE$_{151}$, equivalent to the unique sequence of DbjA, was inserted between Asp142 and Ala143 of DbeA. *Escherichia coli* BL21 (DE3) cells containing the plasmid were cultured in LB medium at 310 K. Protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.2 m*M* when the optical density (at 600 nm) of the culture reached 0.6. After induction, the culture was incubated at 291 K for 5 h and then harvested. The cells were disrupted by sonication using an Ultrasonic Processor UP200S (Hielscher, Teltow, Germany). The supernatant was used after centrifugation at 100 000*g* for 1 h.

Both enzymes were purified using an Ni–NTA Sepharose HR 16/10 column (Qiagen, Hilden, Germany) as described previously (Nagata *et al.*, 1999). The enzymes were bound to the resin in equilibration buffer (20 m*M* potassium phosphate buffer pH 7.5, 0.5 *M* sodium chloride and 10 m*M* imidazole). Unbound and nonspecifically bound proteins were washed out with buffer containing 37.5 m*M* imidazole. The target enzymes were eluted using buffer containing 300 m*M* imidazole. The active fractions were pooled and dialysed against 100 m*M* Tris–HCl buffer pH 7.5 overnight. The enzymes were stored at 277 K in 100 m*M* Tris–HCl buffer pH 7.5 without additives.

2.2. Crystallization

The purified sample of DbeA (Fig. 1) stored at 277 K was used for crystallization experiments at a concentration of 4–6 mg ml^{-1} in 100 m*M* Tris–HCl buffer pH 7.5. Initial crystallization trials for wild-type DbeA were performed using the sitting-drop vapour-diffusion method and were carried out in CombiClover crystallization plates (Emerald Biosystems, Bainbridge Island, USA) at 292 K. The commercial MDL crystal screen kit (Molecular Dimensions Ltd, Suffolk, UK), Hampton Research Crystal Screen kit (Hampton Research, Aliso Viejo, USA) and JBScreen Classic Kits 1–10 cover 240 (Jena Bioscience GmbH, Jena, Germany) were tested to find preliminary crystallization conditions. The sitting-drop procedure (Ducruix & Giegé, 1999) was subsequently used for DbeA protein crystallization in Cryschem 24-well plates (Hampton Research, Aliso Viejo, USA). The reservoir contained 300–1000 µl of the precipitant reagent. Experiments were carried out at 277 and 292 K.

Crystallization of DbeA1 was performed using the same crystallization method with protein concentrations of 6.5–9 mg ml^{-1} in 100 m*M* Tris–HCl buffer pH 7.5. Drops consisting of 3 µl protein solution and 1 µl reservoir solution were equilibrated over 800 ml reservoir solution.

For inspection of crystals under UV light of wavelength 280 nm, a Dynamic Light Scatter 201 with a DUVI 204 dual light source (RiNA GmbH, Berlin, Germany) was used. Evaluation was based on the fluorescence of tryptophan in the protein crystals.

2.3. Data collection and processing

The diffraction data for DbeA were obtained on BESSY beamline MX 14.1 for macromolecular crystallography (Berlin, Germany) with a 0.918 Å monochromatic fixed wavelength. Freshly grown crystals (2–5 d old) were mounted in nylon loops (Hampton Research, Aliso Viejo, USA) directly from the crystallization drop and flash-cooled in a 100 K liquid-nitrogen stream without any additional cryoprotection. The size of the loop used to harvest the crystal was optimized to match the crystal size in order to minimize the amount of mother liquor around the crystal. A complete data set of 360 images was collected with 1° oscillation angle and a crystal-to-detector distance of 300 mm. Finally, 180 images were used for data processing using the *HKL*-3000 package (Minor *et al.*, 2006). Diffraction data for DbeA1 were collected on beamline X11 of the DORIS storage ring

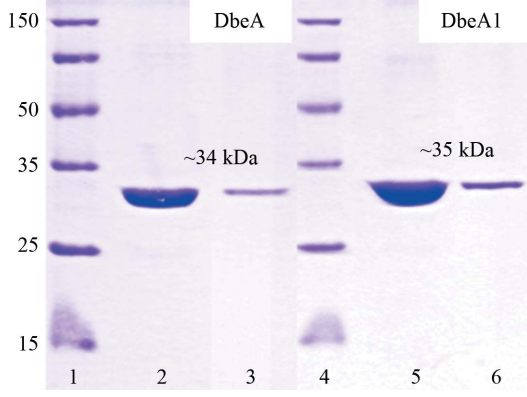

Figure 1
SDS–PAGE (12% gel under reduced conditions) stained with Coomassie Brilliant Blue R-250 monitoring the purity of the protein samples and dissolved crystals. Lanes 1 and 4, SDS–PAGE standards, high-range molecular-weight markers (Bio-Rad Laboratories); lanes 2 and 5, 3.0 ng DbeA sample and 4.0 ng DbeA1 sample used in crystallization experiments, respectively; lanes 3 and 6, dissolved crystals of DbeA and DbeA1, respectively.

with a monochromatic fixed wavelength of 0.8148 Å (DESY/EMBL Hamburg, Germany). Crystals were mounted in nylon loops (Hampton Research, Aliso Viejo, USA) and flash-cooled without additional cryoprotection in a liquid-nitrogen stream. A complete data set of 360 diffraction images with 0.5° oscillation angle and a crystal-to-detector distance of 300 mm was collected and processed using the *XDS* program package (Kabsch, 1993). Crystal parameters and X-ray data-collection statistics are summarized in Table 1.

3. Results and discussion

A freshly isolated sample of recombinant haloalkane dehalogenase DbeA from *B. elkani* USDA94 with high purity as shown by SDS–PAGE analysis (Fig. 1) was used for screening experiments. Thin needle-shaped crystals of DbeA were obtained within 2 d in crystallization solution containing 10–25%(*w*/*v*) PEG 3350 and 50–150 m*M* $MgCl_2$ (Fig. 2*a*). The crystal size and shape were further optimized by changing the crystallization temperature to 277 K and by the substitution of magnesium chloride by calcium acetate. The optimal crystallization conditions yielded colourless single needle-shaped crystals with dimensions of approximately 0.05 × 0.05 × 0.30 mm that grew within two weeks from a solution consisting of 100 m*M* Tris–HCl pH 7.5, 20%(*w*/*v*) PEG 3350 or 4000 and 150 m*M* calcium acetate (Fig. 3*a*).

The optimal crystallization conditions used for the wild-type enzyme were applied to the crystallization of DbeA1 together with screening using JBScreen Classic Kits 1–10 (Jena Bioscience GmbH,

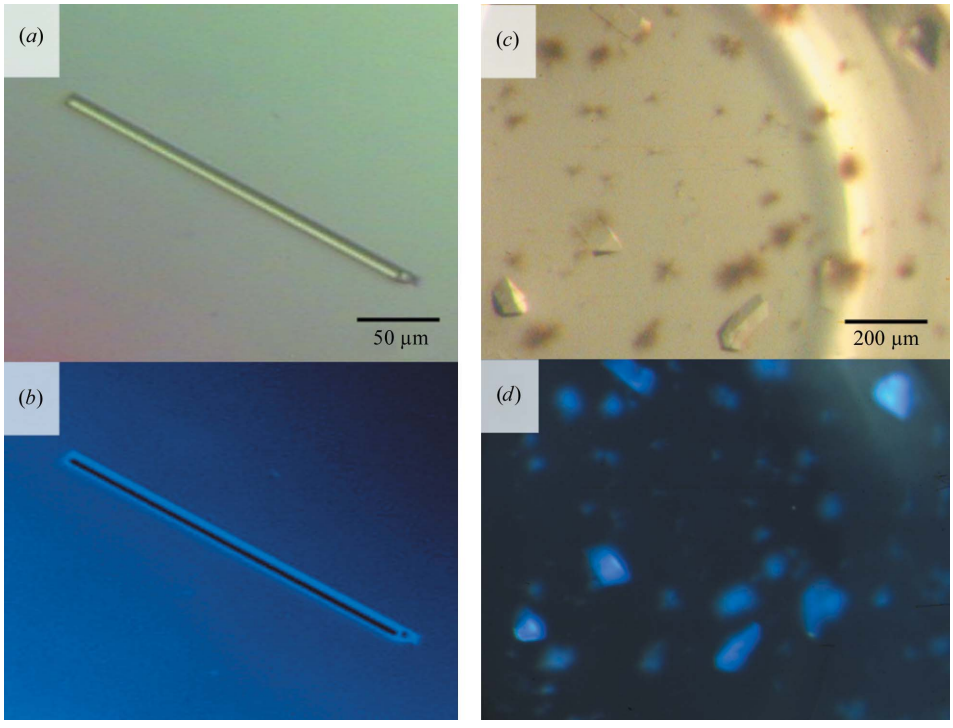

Figure 2
Microphotographs of initial crystals of DbeA and DbeA1. (*a*) and (*b*) show crystals of DbeA taken under visible and UV light, respectively. (*c*) and (*d*) show crystals of DbeA1 taken under visible and UV light, respectively.

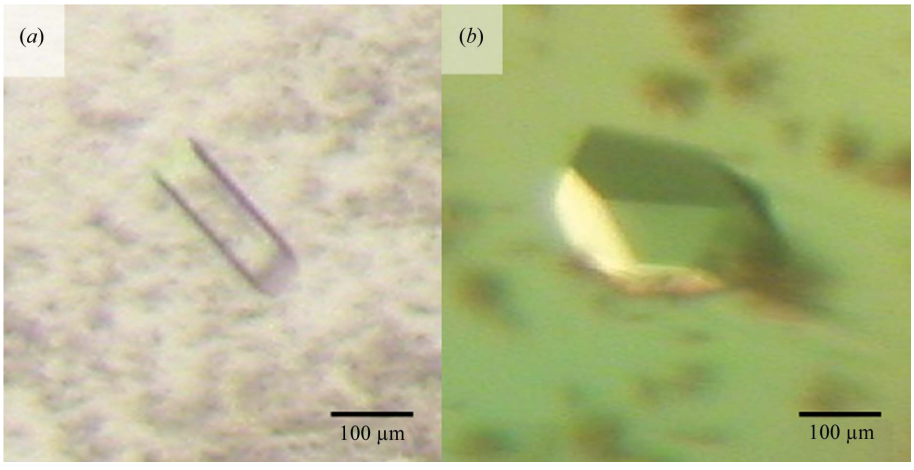

Figure 3
Optimized crystals of (*a*) DbeA and (*b*) DbeA1 derived from *B. elkani* used for diffraction analysis.

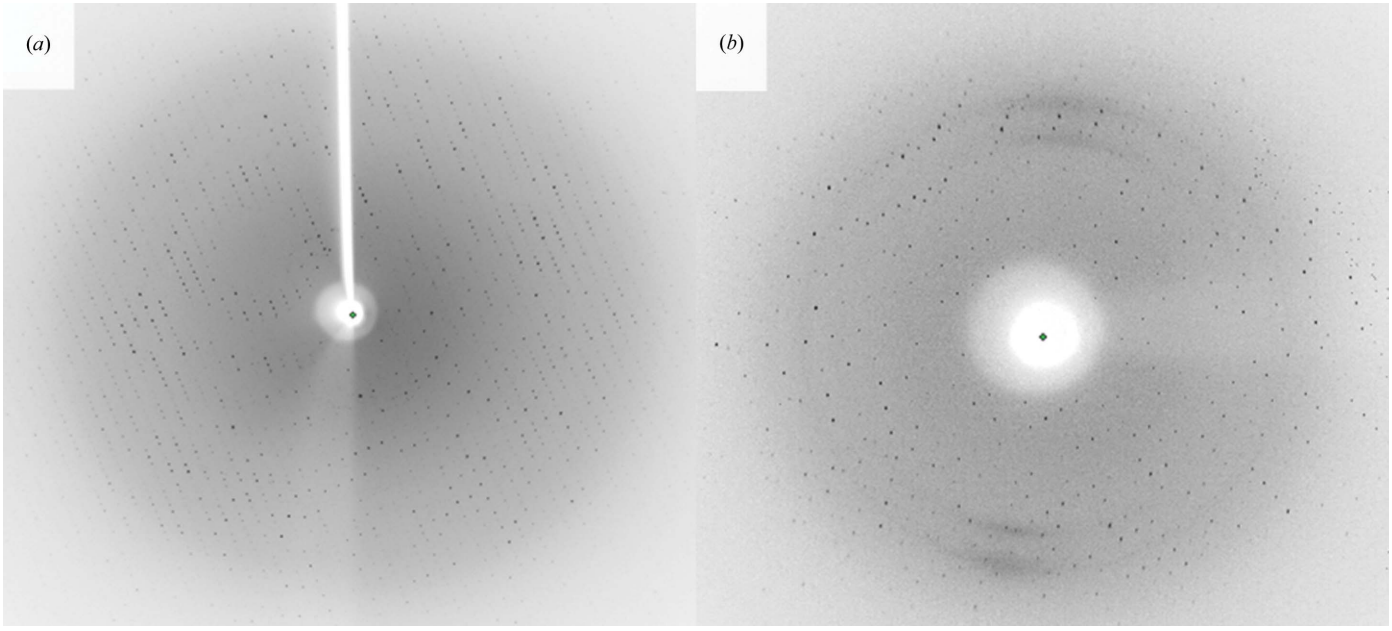

Figure 4
Diffraction images of (*a*) DbeA and (*b*) DbeA1 crystals. Diffraction images were produced using the *ipmosflm* GUI 1.0.0 program.

Jena, Germany) at 277 and 292 K. Colourless well shaped crystals of DbeA1 with dimensions of 0.15 × 0.07 × 0.12 mm (Fig. 3*b*) were grown within 5–8 d from reservoir solution composed of 100 m*M* Tris–HCl buffer pH 7.5, 25%(*w*/*v*) PEG 4000 and 130 m*M* calcium acetate.

To confirm the protein nature of the crystals of DbeA and DbeA1, crystals were inspected under UV light (Fig. 2) and dissolved crystals were analysed by SDS–PAGE electrophoresis (Fig. 1). Both methods confirmed the protein nature of the crystals and the presence of full-length protein.

Freshly prepared single crystals were used to collect diffraction data using synchrotron-radiation sources. Diffraction data sets were collected to 2.2 Å resolution for both DbeA and DbeA1 (Fig. 4).

DbeA protein crystallized in the primitive orthorhombic space group $P2_12_12_1$ and DbeA1 crystallized in the monoclinic space group *C*2. Evaluation of the crystal-packing parameters indicated that the lattice could accommodate four DbeA proteins in one asymmetric unit with a solvent content of approximately 46% and two molecules of DbeA1 in the asymmetric unit with about 45% solvent content. The complete data-collection statistics are summarized in Table 1.

The diffraction data will be used to determine the structure of DbeA by molecular replacement using the available structures of haloalkane dehalogenases of the HLD-II family. Structure determination will provide important clues to the architecture and the structure–function basis of substrate and inhibitor recognition of DbeA and DbeA1 proteins.

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