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Cloning, expression, purification and crystallization of dihydrodipicolinate synthase from the psychrophile *Shewanella benthica*

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Dihydrodipicolinate synthase (DHDPS) is an oligomeric enzyme that catalyzes the first committed step of the lysine-biosynthesis pathway in plants and bacteria, which yields essential building blocks for cell-wall and protein synthesis. DHDPS is therefore of interest to drug-discovery research as well as to studies that probe the importance of quaternary structure to protein function, stability and dynamics. Accordingly, DHDPS from the psychrophilic (cold-dwelling) organism *Shewanella benthica* (*Sb*-DHDPS) was cloned, expressed, purified and crystallized. The best crystals of *Sb*-DHDPS were grown in 200 m*M* ammonium sulfate, 100 m*M* bis-tris pH 5.0–6.0, 23–26%(*w*/*v*) PEG 3350, 0.02%(*w*/*v*) sodium azide and diffracted to beyond 2.5 Å resolution. Processing of diffraction data to 2.5 Å resolution resulted in a unit cell with space group $P2_12_12_1$ and dimensions $a = 73.1$, $b = 84.0$, $c = 143.7$ Å. These studies of the first DHDPS enzyme to be characterized from a bacterial psychrophile will provide insight into the molecular evolution of enzyme structure and dynamics.

1. Introduction

The enzyme dihydrodipicolinate synthase (DHDPS) catalyzes the first committed step of the lysine-biosynthesis pathway in plants and bacteria, namely the conversion of pyruvate and (*S*)-aspartate semialdehyde [(*S*)-ASA] to form hydroxytetrahydrodipicolinic acid (HTPA; Blickling, Renner *et al.*, 1997; Hutton *et al.*, 2007; Dogovski *et al.*, 2009). Given that DHDPS is not expressed in mammals and that the gene encoding this enzyme (*dapA*) has been shown to be essential to bacteria (Kobayashi *et al.*, 2003; Dogovski *et al.*, 2009; Sibarani, Gorman, Dogovski, Praszkier *et al.*, 2010), DHDPS is of particular interest to researchers as a potential antimicrobial drug target (Hutton *et al.*, 2007; Dogovski *et al.*, 2009). Consequently, DHDPS has been well characterized in a range of organisms, including the bacteria *Bacillus anthracis* (Blagova *et al.*, 2006; Voss *et al.*, 2009, 2010; 39% identity to *Sb*-DHDPS), *Corynebacterium glutamicum* (Rice *et al.*, 2008; 32% identity to *Sb*-DHDPS), *Escherichia coli* (Yugari & Gilvarg, 1965; Laber *et al.*, 1992; Mirwaldt *et al.*, 1995; Blickling, Beisel *et al.*, 1997; Blickling, Renner *et al.*, 1997; Blickling & Knablein, 1997; Dobson *et al.*, 2004, 2005; Griffin *et al.*, 2008, 2010; 50% identity to *Sb*-DHDPS), *Mycobacterium tuberculosis* (Kefala & Weiss, 2006; Kefala *et al.*, 2008; 34% identity to *Sb*-DHDPS), *Neisseria meningitidis* (Devenish *et al.*, 2009; 47% identity to *Sb*-DHDPS), *Sinorhizobium meliloti* (Phenix *et al.*, 2008; 44% identity to *Sb*-DHDPS), *Staphylococcus aureus* (Burgess, Dobson, Bailey *et al.*, 2008; Burgess, Dobson, Dogovski *et al.*, 2008; 36% identity to *Sb*-DHDPS), *Streptococcus pneumoniae* (Sibarani, Gorman, Dogovski, Parker *et al.*, 2010; Sibarani, Gorman, Dogovski, Praszkier *et al.*, 2010; 38% identity to *Sb*-DHDPS) and *Thermotoga maritima* (Pearce *et al.*, 2006; 38% identity to *Sb*-DHDPS) as well as the plant *Nicotiana sylvestris* (Ghislain *et al.*, 1990; Blickling, Beisel *et al.*, 1997; 36% identity to *Sb*-DHDPS). In addition, there has recently been heightened interest in the structure of DHDPS (Dogovski *et al.*, 2009) given that this oligomeric enzyme serves as an excellent model protein to probe the importance of quaternary structure to enzyme function, stability and

dynamics (Mirwaldt *et al.*, 1995; Blickling, Beisel *et al.*, 1997; Dobson *et al.*, 2005; Griffin *et al.*, 2008, 2010; Burgess, Dobson, Bailey *et al.*, 2008; Voss *et al.*, 2010). Moreover, a number of different quaternary architectures have been discovered across the range of organisms studied to date. All plant and most bacterial DHDPS enzymes form tetramers, best described as a dimer of dimers, although the orientation of the dimeric units in the tetramer varies between plant and bacterial species (Mirwaldt *et al.*, 1995; Dobson *et al.*, 2005; Kefala & Weiss, 2006; Pearce *et al.*, 2006; Blagova *et al.*, 2006; Kefala *et al.*, 2008; Phenix *et al.*, 2008; Rice *et al.*, 2008; Griffin *et al.*, 2008, 2010; Devenish *et al.*, 2009; Voss *et al.*, 2010). Recently, the crystal structure of a native dimeric form of DHDPS has also been characterized from the Gram-positive pathogen *Staphylococcus aureus* (Burgess, Dobson, Bailey *et al.*, 2008; Burgess, Dobson, Dogovski *et al.*, 2008; Girish *et al.*, 2008). The *S. aureus* enzyme has a significant increase in the buried surface area at the dimer interface compared with the equivalent interface in tetrameric DHDPS structures (Burgess, Dobson, Bailey *et al.*, 2008). The *S. aureus* DHDPS dimer also contains two electrostatic contacts at the dimer interface that are absent in all other DHDPS structures characterized to date (Burgess, Dobson, Bailey *et al.*, 2008). Consequently, it has been hypothesized that DHDPS has evolved from an ancestral dimeric unit to form either a tetramer (as in most DHDPS structures characterized to date) or a tight dimer (as in *S. aureus* DHDPS) as a means of reducing conformational flexibility at the dimer interface (Burgess, Dobson, Bailey *et al.*, 2008; Griffin *et al.*, 2008, 2010). The quaternary structure of DHDPS is therefore important in reducing protein dynamics and in turn enhancing catalytic function (Griffin *et al.*, 2008; Voss *et al.*, 2010). Accordingly, the aim of this study is to clone, express, purify and crystallize DHDPS from the cold-dwelling γ-proteobacterium *Shewanella benthica* (*Sb*) (Deming *et al.*, 1984; MacDonell & Colwell, 1985) with the view of determining a high-resolution crystal structure of the first DHDPS enzyme from a bacterial psychrophile. By studying oligomeric enzymes from bacterial extremophiles that inhabit different environments, including enzymes from bacteria that grow at low (*i.e.* psychrophilic) temperatures, the synergy between enzyme dynamics and quaternary structure can be examined. In turn, such studies will provide general insight into the molecular evolution of protein structure and function.

2. Materials and methods

2.1. Gene cloning

The *dapA* gene from *S. benthica* ATCC3392 was amplified by PCR from genomic DNA (kindly provided by Dr Rachel Codd, University of Sydney) using the forward and reverse primers SBF1 (CATATGGACAGCATCAGTACCA) and SBR1 (GGATCCTTATTTAACCTCAATTCGTGC), respectively. The primers were designed to incorporate *Nde*I and *Bam*HI restriction sites at the 5′ and 3′ ends of the *dapA* gene. The resultant PCR product was ligated into the pCRBlunt II-TOPO (Invitrogen) vector, producing the plasmid pJW1. The pJW1 plasmid was cut with restriction enzymes *Nde*I and *Bam*HI and the *dapA* gene was cloned into the corresponding sites of the pET28a expression vector, resulting in the plasmid pET28a-JW1. The integrity of the vectors described in this study was confirmed by restriction-enzyme analysis and dideoxynucleotide sequencing. Plasmid pET28a-JW1 was transformed into *E. coli* BL21 (DE3) for overexpression and purification of recombinant protein. The pET28a-JW1 plasmid is designed to produce N-terminal hexahistidine-tagged DHDPS from *S. benthica* (*Sb*-DHDPS).

2.2. Expression and purification

An overnight culture of *E. coli* BL21 (DE3) containing pET28a-JW1 was used to inoculate (1 in 100 dilution) 1 l Luria broth containing 100 µg ml^{-1} ampicillin. Cells were cultured at 303 K with shaking (180 rev min^{-1}) to an OD_{600} of 0.5. Recombinant protein expression was induced by addition of IPTG to a final concentration of 1.0 m*M*. Cultures were then placed at 289 K and incubated overnight (16 h) with shaking (180 rev min^{-1}). The IPTG-treated cells were harvested by centrifugation at 4400*g* and resuspended in 30 ml 20 m*M* Tris, 150 m*M* NaCl, 20 m*M* imidazole pH 8.0 (buffer *A*) containing Complete Protease Inhibitor Cocktail (Sigma–Aldrich). The cell suspension was lysed by sonication (MSE Soniprep 150 sonicator) by applying 20 s pulses at a power output of 14 µm with 1 min rest intervals for a total of nine cycles. The lysate was clarified by centrifugation (17 210*g* for 30 min at 277 K) and passing the supernatant through a combined 5 µm and 0.45 µm syringe filter (Millipore). Purification of the recombinant His-tagged product was achieved by loading the cell lysate onto three 5 ml HisTrap HP (GE Healthcare) columns attached in series. Once recombinant protein had bound, the columns were washed in buffer *A* until a steady baseline was achieved. Recombinant *Sb*-DHDPS was subsequently eluted by employing a 20–500 m*M* imidazole gradient over a volume of 120 ml, with 3 ml fractions collected across the entire gradient. Fractions that contained DHDPS activity were identified using the *o*-aminobenzaldehyde assay as described previously (Yugari & Gilvarg, 1965). DHDPS-active fractions were further characterized by SDS–PAGE. Fractions containing >95% pure *Sb*-DHDPS were pooled and dialyzed overnight into 20 m*M* Tris, 150 m*M* NaCl pH 8.0 (buffer *B*) before being aliquoted into 1 ml fractions and stored at 193 K. The DHDPS–DHDPR coupled assay (Coulter *et al.*, 1999; Dobson *et al.*, 2004) was employed to quantitatively determine the activity of purified *Sb*-DHDPS. In studies involving crystallization screens, *Sb*-DHDPS aliquots were thawed, concentrated to 10 mg ml^{-1} with an Amicon concentrator (molecular weight cut-off 10 000 Da) and buffer-exchanged into buffer *B* using a Sephacryl S-200 column (GE Healthcare) pre-equilibrated with buffer *B*.

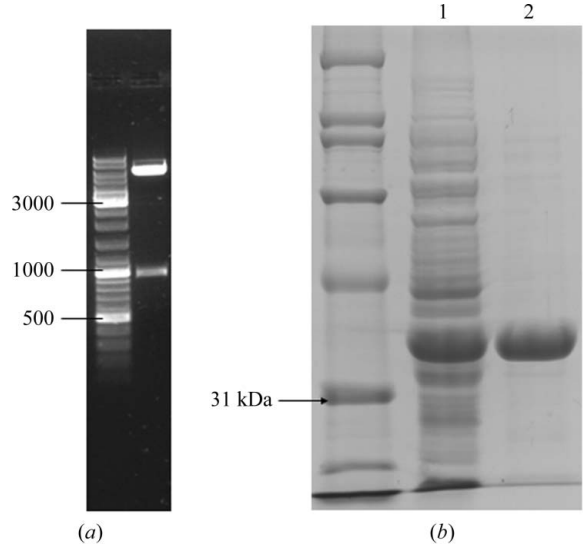


Figure 1
Cloning and purification of recombinant *S. benthica* DHDPS. (*a*) Agarose gel electrophoresis displaying a restriction-enzyme digest of pET28a-JW1 using *Nde*I and *Bam*HI. (*b*) SDS–PAGE gel showing purification of *Sb*-DHDPS. Lane 1, filtered cell lysate IPTG; lane 2, IMAC chromatography.

2.3. Electrospray ionization mass spectrometry

Prior to electrospray ionization time-of-flight (ESI–TOF) mass spectrometry, purified recombinant *Sb*-DHDPS was buffer-exchanged into Milli-Q deionized water using a 5 ml HiTrap HP column. The exact mass of *Sb*-DHDPS was obtained by directly injecting the protein sample onto an Accurate TOF LC/MS 6220 (Agilent Technologies) *via* an ESI source, followed by deconvolution of the primary mass-to-charge data using the program *MassHunter* (Agilent Technologies).

Trypsin digestion of *Sb*-DHDPS was also performed following excision of *Sb*-DHDPS from an SDS–PAGE gel. The excised band was incubated overnight with trypsin (Promega) at 310 K. Tandem mass spectrometry (MS/MS) was then performed on the trypsinized sample using an LC MSD Trap XCT (Agilent Technologies) equipped with an ESI source. Protein identification from MS/MS data was achieved using *MASCOT* (available at http//www.matrixscience.com; Matrix Science), searching for proteins within the NCBI database.

2.4. Protein crystallization

The crystallization of *Sb*-DHDPS was performed using previously described screening procedures (Burgess, Dobson, Dogovski *et al.*, 2008; Dobson *et al.*, 2008; Atkinson *et al.*, 2009; Voss *et al.*, 2009; Dommaraju *et al.*, 2010; Hor *et al.*, 2010; Sibarani, Gorman, Dogovski, Parker *et al.*, 2010). Recombinant *Sb*-DHDPS at 7.6 mg ml^{-1} was subjected to the JCSG+ Suite crystal screen at the CSIRO-Bio21 Collaborative Crystallization Centre (Bio21-C[3]; http://www.csiro.au/c3/) at 281 K (Newman *et al.*, 2005, 2008). Crystallization was performed using the sitting-drop vapour-diffusion method in 96-well plates with a protein volume of 150 nl and a reservoir volume of 150 nl in each droplet. Crystals of *Sb*-DHDPS were obtained from 200 m*M* ammonium sulfate, 100 m*M* bis-tris pH 5.5, 25%(*w*/*v*) PEG 3350, 0.02%(*w*/*v*) sodium azide mother liquor following 7–9 d incubation at 281 K. In-house screens were thus conducted around this condition in 24-well Linbro plates (Hampton Research) at 281 K using the hanging-drop vapour-diffusion method. The in-house screens varied the drop size used (from 1 to 4 µl, with *Sb*-DHDPS

Table 1
Purification of recombinant *Sb*-DHDPS.

Purification step	Total protein† (mg)	Total activity‡ (units)	Specific activity (units mg^{-1})	Yield (%)	Purification factor (fold)
Crude lysate	335	3570	10.7	—	—
Nickel-affinity chromatography	13.7	1420	104	39.9	9.70

† Values refer to expression from a 1 l culture. ‡ 1 unit = 1 µmol of NADPH per minute.

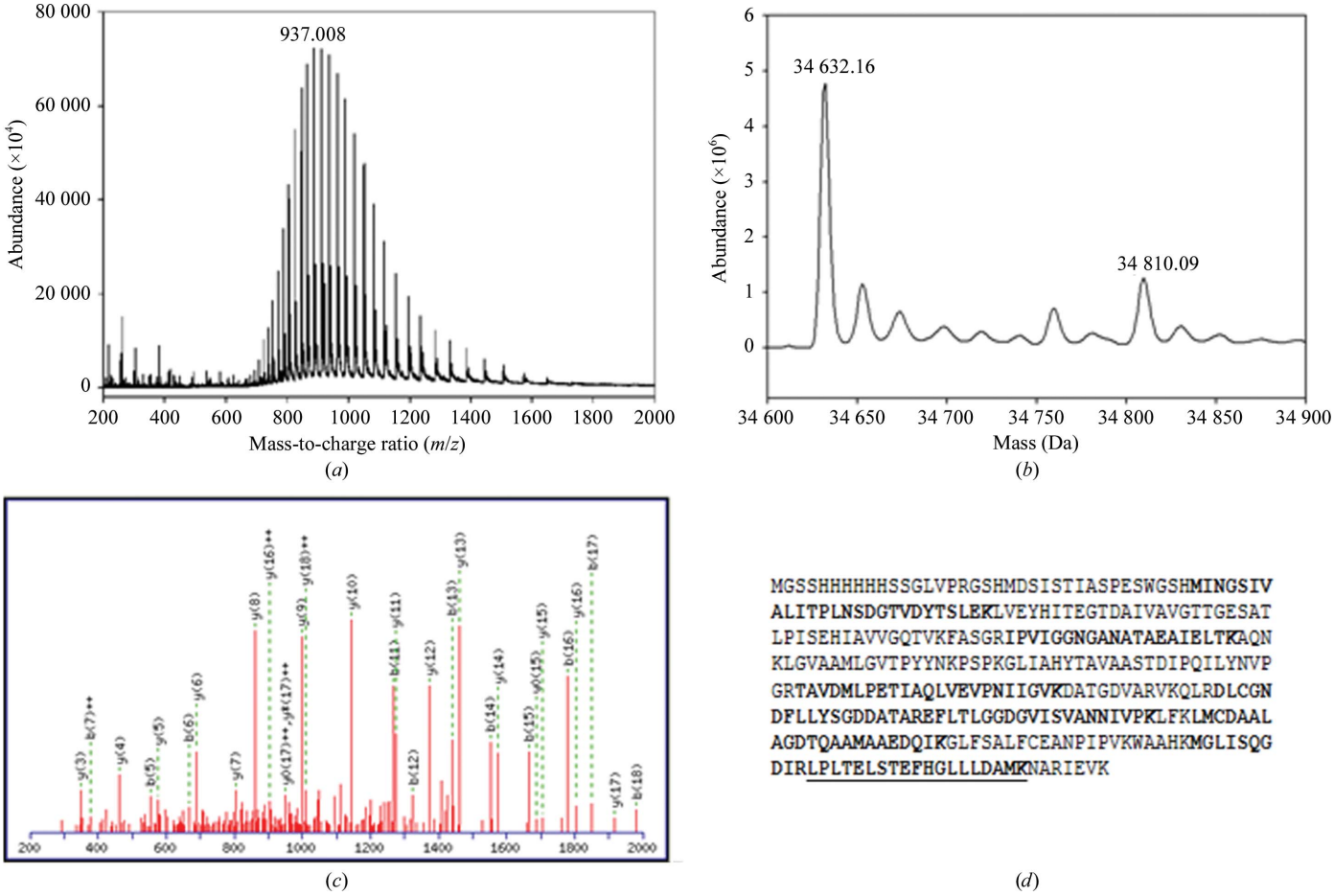


Figure 2
Mass-spectrometric analysis of recombinant *Sb*-DHDPS. (*a*) Raw mass-to-charge spectrum of *Sb*-DHDPS. (*b*) Deconvoluted mass spectrum of *Sb*-DHDPS, with the mass of the highest peak (34 632.16 Da) corresponding to the relative molecular weight of *Sb*-DHDPS with its N-terminal methionine cleaved (M_r = 34 632.6 Da). The mass annotated as 34 810.09 represents the tri-Na$^+$ adduct of *Sb*-DHDPS. (*c*) b-ion and y-ion series of the peptide corresponding to residues 305–323 of *Sb*-DHDPS obtained from *MASCOT*. (*d*) The sequence of *Sb*-DHDPS, with the residues in bold indicating the sequence coverage of the MS/MS analysis conducted by *MASCOT*. The underlined sequence represents the peptide shown in (*c*).

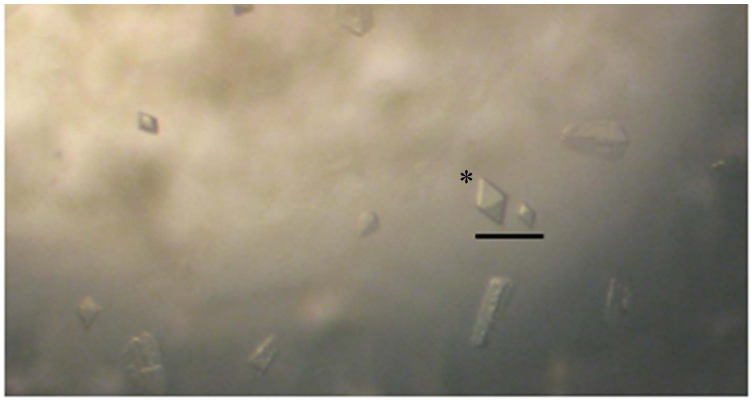
constituting 50% of the droplet), the pH of the buffer (pH 5.0–6.0) and the concentration of the precipitant [23–26%(*w*/*v*) PEG 3350], while keeping the concentration of ammonium sulfate constant.

2.5. Data collection and processing

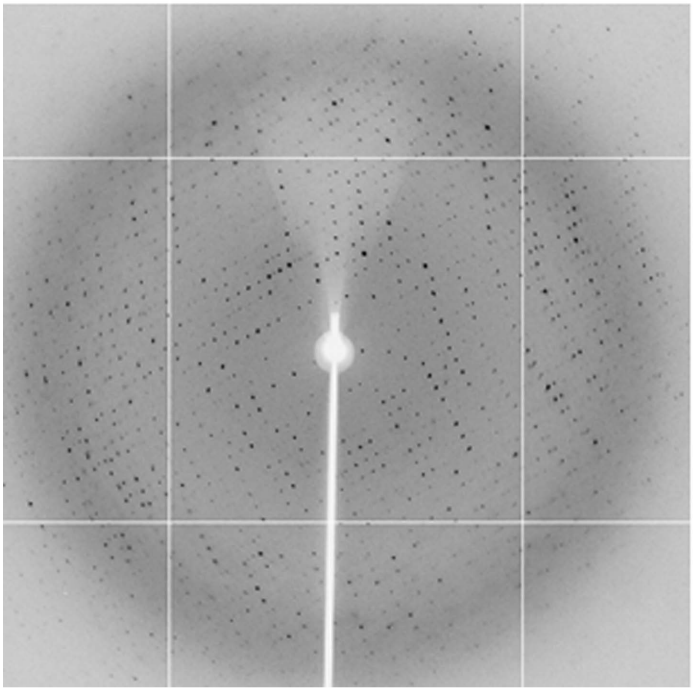
Prior to the collection of X-ray diffraction data, the crystals of *Sb*-DHDPS were cryoprotected in mother liquor containing 20%(*v*/*v*) glycerol and then flash-frozen in liquid nitrogen. Intensity data at 100 K were collected on the MX2 beamline at the Australian Synchrotron, Clayton using the program *Blu-Ice* (McPhillips *et al.*, 2002). Data were collected in 0.5° steps for 180° of rotation using a ADSC Q315r detector with an exposure time of 2 s. Diffraction data were processed and scaled using the programs *iMOSFLM* (Leslie, 1992) and *SCALA* (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

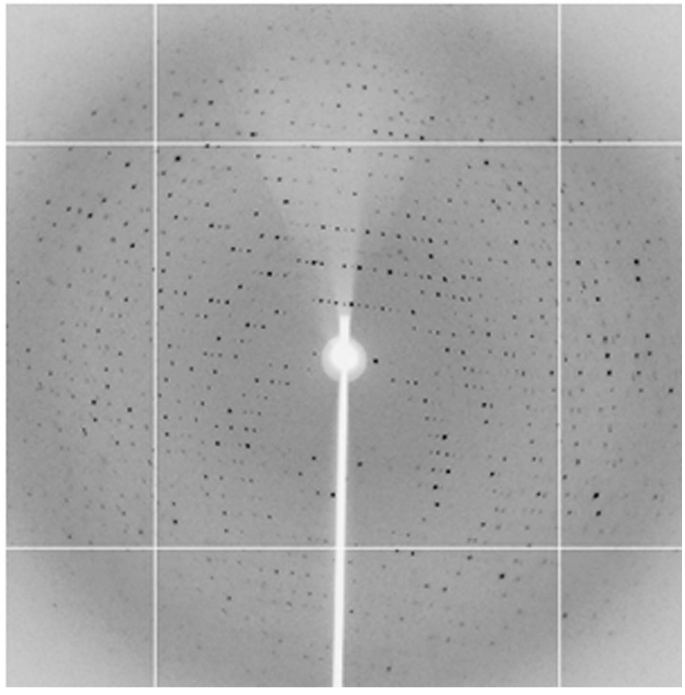
The *dapA* gene from *S. benthica* was amplified by PCR from genomic DNA and cloned into the expression vector pET28a to produce the plasmid pET28a-JW1 (Fig. 1*a*). This plasmid was transformed into *E. coli* BL21 (DE3) cells and the expression of soluble recombinant *Sb*-DHDPS was achieved by culturing cells at a temperature of 289 K (see §2.2). Expression temperatures above 293 K resulted in the production of insoluble recombinant protein (data not shown). A one-step purification protocol involving immobilized metal-affinity chromatography (IMAC) was employed to isolate recombinant *Sb*-DHDPS using an increasing imidazole gradient (see §2). Recombinant *Sb*-DHDPS eluted at imidazole concentrations of 225–290 m*M*. The single IMAC step produced >95% pure recombinant *Sb*-DHDPS (Fig. 1*b*) with a 9.7-fold increase in purity from the induced cell lysate and a specific activity of 104 U mg^{-1} (Table 1). The final yield of recombinant enzyme was 13.7 mg per litre of bacterial



(*a*)



(*b*)



(*c*)

Figure 3
Crystallization and X-ray diffraction of *Sb*-DHDPS. (*a*) Crystals of *Sb*-DHDPS. The scale bar indicates 50 µm. While different morphologies were present within the drop, diffraction data were collected from crystals of bipyramidal morphology. (*b*) Diffraction pattern to beyond 2.5 Å resolution (maximum resolution of approximately 2.35 Å) obtained from the crystal of *Sb*-DHDPS marked with an asterisk in (*a*). (*c*) Diffraction pattern of the same crystal as that shown in (*b*) but rotated 90°.

Table 2
X-ray data-collection statistics.

Values in parentheses are for the highest resolution bin.

Wavelength (Å)	0.95369
No. of images	360
Oscillation angle per frame (°)	0.5
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 73.1, b = 84.0, c = 143.7
Resolution (Å)	40.33–2.50 (2.64–2.50)
Observed reflections	115926
Unique reflections	31280
Completeness (%)	99.7 (99.7)
$R_{\rm merge}$†	0.076 (0.343)
$R_{\rm r.i.m.}$‡	0.089 (0.399)
$R_{\rm p.i.m.}$§	0.045 (0.201)
Mean $I/\sigma(I)$	9.5 (3.0)
Redundancy	3.7 (3.8)
Molecules per asymmetric unit	2
$V_{\rm M}$ (Å^3 Da^{-1})	2.12

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. ‡ $R_{\rm p.i.m.} = \sum_{hkl}[1/(N-1)]^{1/2} \times \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$. § $R_{\rm r.i.m.} = \sum_{hkl}[N/(N-1)]^{1/2}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

culture and the recombinant protein was shown to be enzymatically active using both the *o*-aminobenzaldehyde assay (Yugari & Gilvarg, 1965) and the DHDPS–DHDPR coupled assay (Coulter *et al.*, 1999; Dobson *et al.*, 2004). ESI–TOF mass-spectrometric analysis (Fig. 2) demonstrated that the recombinant protein has a calculated mass of 34 632.16 Da (Figs. 2*a* and 2*b*), which is consistent with the theoretical mass of the His-tagged construct ($M_{\rm r}$ = 34 632.6 Da). MS/MS data of a trypsin-treated *Sb*-DHDPS sample were also generated, resulting in 50% sequence coverage, which provided sufficient data to demonstrate that the sequence of the recombinant enzyme corresponded to *Sb*-DHDPS (Figs. 2*c* and 2*d*).

Sb-DHDPS was subsequently subjected to crystallization trials using commercial and in-house screens available at Bio21-C^3. Small crystals of *Sb*-DHDPS were successfully grown from mother liquor consisting of 200 m*M* ammonium sulfate, 100 m*M* bis-tris pH 5.5, 25%(*w*/*v*) PEG 3350, 0.02%(*w*/*v*) sodium azide. In-house screens were then conducted to generate larger crystals for X-ray diffraction analyses. These screens resulted in the crystals shown in Fig. 3(*a*). One of the resulting crystals (indicated with an asterisk in Fig. 3*a*) was used to collect X-ray diffraction data over 180° of rotation (0.5° oscillation step; 360 frames in total). Two of the resulting frames are shown in Figs. 3(*b*) and 3(*c*). Significant radiation damage occurred to the crystal caused by the intense beam of the MX2 microfocus beamline, as judged by the deterioration of the diffraction patterns with time. Data processed from the first 200 frames were therefore chosen for structure determination, as latter batches showed a marked increase in the *R* factors between batches and a significant rise in the scaling *B* factor or slope between batches. The processed data consequently resulted in a unit cell with space group $P2_12_12_1$ and unit-cell parameters a = 73.1, b = 84.0, c = 143.7 Å (Table 2). Systematic absences also strongly suggested the presence of a screw axis in the *k* and *l* planes and the possibility of a screw axis in the *h* plane, correlating with the predicted space group. The structure was solved by molecular replacement employing the program *Phaser* (McCoy *et al.*, 2005) using the structure of DHDPS from *Salmonella typhimurium* LT2 (PDB code 3g0s; J. Osipiuk, N. Maltseva, J. Stam, W. F. Anderson & A. Joachimiak, unpublished work; 50% identity to *Sb*-DHDPS). Both the LL gain (1407) and the rotation and translation function *Z* scores (27.0 and 36.3, respectively) from *Phaser*, along with the initial change in $R_{\rm free}$ values (from 0.4195 to 0.3245) in the first cycle of refinement conducted using the program *PHENIX* (Adams *et al.*, 2010), provide indications that the structure will be successfully determined by molecular replacement. Interestingly, *Sb*-DHDPS crystallized as a dimer with two molecules present within the asymmetric unit and a $V_{\rm M}$ (Matthews, 1968) calculated as 2.12 Å^3 Da^{-1}. No symmetry mates were generated that could be interpreted as a potential tetrameric form of the enzyme. Further analysis and refinement of the structure is in progress.

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