STRUCTURAL BIOLOGY
COMMUNICATIONS

ISSN: 2053-230X
journals.iucr.org/f

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Acta Cryst. (2015). F**71**, 1027–1032

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Overproduction, crystallization and X-ray diffraction data analysis of ectoine synthase from the cold-adapted marine bacterium *Sphingopyxis alaskensis*

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Received 13 April 2015
Accepted 8 June 2015

Edited by M. S. Weiss, Helmholtz-Zentrum Berlin für Materialien und Energie, Germany

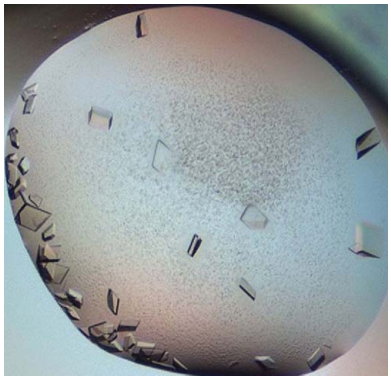
Keywords: compatible solute; osmostress protectant; chemical chaperone; enzyme; ectoine synthesis; cupin; X-ray analysis.

Supporting information: this article has supporting information at journals.iucr.org/f

Ectoine biosynthetic genes (*ectABC*) are widely distributed in bacteria. Microorganisms that carry them make copious amounts of ectoine as a cell protectant in response to high-osmolarity challenges. Ectoine synthase (EctC; EC 4.2.1.108) is the key enzyme for the production of this compatible solute and mediates the last step of ectoine biosynthesis. It catalyzes the ring closure of the cyclic ectoine molecule. A codon-optimized version of *ectC* from *Sphingopyxis alaskensis* (*Sa*) was used for overproduction of *Sa*EctC protein carrying a *Strep*-tag II peptide at its carboxy-terminus. The recombinant *Sa*EctC-*Strep*-tag II protein was purified to near-homogeneity from *Escherichia coli* cell extracts by affinity chromatography. Size-exclusion chromatography revealed that it is a dimer in solution. The *Sa*EctC-*Strep*-tag II protein was crystallized using the sitting-drop vapour-diffusion method and crystals that diffracted to 1.0 Å resolution were obtained.

1. Introduction

Compatible solutes are highly water-soluble organic osmolytes that are compliant with cellular biochemistry and physiology and can therefore be accumulated to exceedingly high intracellular levels. Microorganisms amass them, either through synthesis or uptake, to offset the detrimental consequences of a rise in the extracellular osmolarity on cellular hydration, turgor and growth (Kempf & Bremer, 1998). Ectoine [(4*S*)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid; Fig. 1*a*; Galinski *et al.*, 1985] is such a compatible solute, and the genes (*ectABC*) encoding its three biosynthetic enzymes (Louis & Galinski, 1997; Ono *et al.*, 1999; Supplementary Fig. S1) are widely distributed among members of the Bacteria (Widderich *et al.*, 2014). In addition to their function as physiologically well tolerated osmotic and temperature stress protectants (Bursy *et al.*, 2008; García-Estepa *et al.*, 2006; Kuhlmann *et al.*, 2011), ectoine and its derivative 5-hydroxyectoine [(4*S*,5*S*)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid; Höppner *et al.*, 2014; Supplementary Fig. S1] also serve as chemical chaperones since they can effectively preserve the functionality of biological macromolecules (Lippert & Galinski, 1992; Tanne *et al.*, 2014; Manzanera *et al.*, 2002). This has fostered a substantial interest in these compounds for their biotechnological exploitation as stabilizers of proteins, membranes and cells, for use in cosmetics and for potential medical applications (Pastor *et al.*, 2010; Graf *et al.*, 2008).



The biosynthesis of ectoine proceeds from L-aspartate-β-semialdehyde, a central hub in microbial amino-acid metabolism, and is mediated by the sequential reactions of L-2,4-diaminobutyrate transaminase (EctB; EC 2.6.1.76), 2,4-diaminobutyrate acetyltransferase (EctA; EC 2.3.1.178) and ectoine synthase (EctC; EC 4.2.1.108; Supplementary Fig. S1; Louis & Galinski, 1997; Ono *et al.*, 1999). The corresponding three enzymes from the highly salt-tolerant bacterium *Halomonas elongata* have already been biochemically characterized to some extent (Ono *et al.*, 1999). The crystal structure of EctA from *Bordetella parapertussis* in complex with its substrate L-2,4-diaminobutyrate (PDB entry 3d3s) has been determined within the framework of a structural genomics project. Likewise, the crystal structure of ectoine hydroxylase (EctD; EC 1.14.11) from *Sphingopyxis alaskensis* with all of its ligands and the reaction product 5-hydroxyectoine (PDB entry 4q5o) has also been reported (Höppner *et al.*, 2014).

Here, we focus on ectoine synthase, the key enzyme of the ectoine biosynthetic route (Ono *et al.*, 1999; Louis & Galinski, 1997) and a protein that can also be used as a diagnostic tool in database searches of microbial genome sequences to scan for potential microbial ectoine producers (Widderich *et al.*, 2014). A *BLAST* search of microbial genomes deposited in the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) suggests that EctC belongs to the cupin superfamily, a large group of proteins with a conserved β-barrel structural scaffold that perform diverse enzymatic and non-enzymatic functions (Uberto & Moomaw, 2013). An initial biochemical characterization of the ectoine synthases from the extremophiles *H. elongata*, *Methylomicrobium alcaliphilum* and *Acidiphilium cryptum* has already been carried out (Ono *et al.*, 1999; Witt *et al.*, 2011; Moritz *et al.*, 2015; Reshetnikov *et al.*, 2006). Each of these enzymes catalyzes the cyclization of N_γ-acetyl-L-2,4-diaminobutyrate (Fig. 1*a*), the reaction product of EctA (Supplementary Fig. S1), to ectoine with the concomitant release of a water molecule (Fig. 1*a*). In a side reaction, EctC can also hydrolyze ectoine and some synthetic ectoine derivatives with altered ring sizes (Witt *et al.*, 2011; Moritz *et al.*, 2015). A further minor side reaction of ectoine synthase is the formation of the synthetic compatible solute 5-amino-3,4-dihydro-2*H*-pyrrole-2-carboxylate (ADPC) through the cyclic condensation of glutamine (Witt *et al.*, 2011).

Although progress has been made with respect to the biochemical characterization of the main activity of ectoine synthase, the cyclization of N_γ-acetyl-L-2,4-diaminobutyrate (Ono *et al.*, 1999; Reshetnikov *et al.*, 2006), and its various interesting side reactions (Witt *et al.*, 2011; Moritz *et al.*, 2015), a full understanding of the enzyme activities of this ubiquitously distributed protein (Widderich *et al.*, 2014) is still lacking. Also, no structural information is currently available for proteins belonging to this family. To further the knowledge of ectoine synthase, we developed an overproduction and purification protocol for EctC based on a codon-optimized version of the *ectC* gene from *S. alaskensis* (*Sa*; Ting *et al.*, 2010) for its heterologous synthesis in *Escherichia coli* as a fusion protein modified with a *Strep*-tag II affinity tag at its carboxy-terminus. We demonstrate here that the recombinant *Sa*EctC-*Strep*-tag II protein can be efficiently overproduced and purified by affinity chromatography; it yields well diffracting crystals (to 1.0 Å resolution) that should allow the elucidation of the macromolecular structure of ectoine synthase.

2. Materials and methods

2.1. Macromolecule production

All recombinant DNA techniques followed routine procedures. The DNA sequence encoding the EctC protein (accession No. ABF54656) was retrieved from the genome

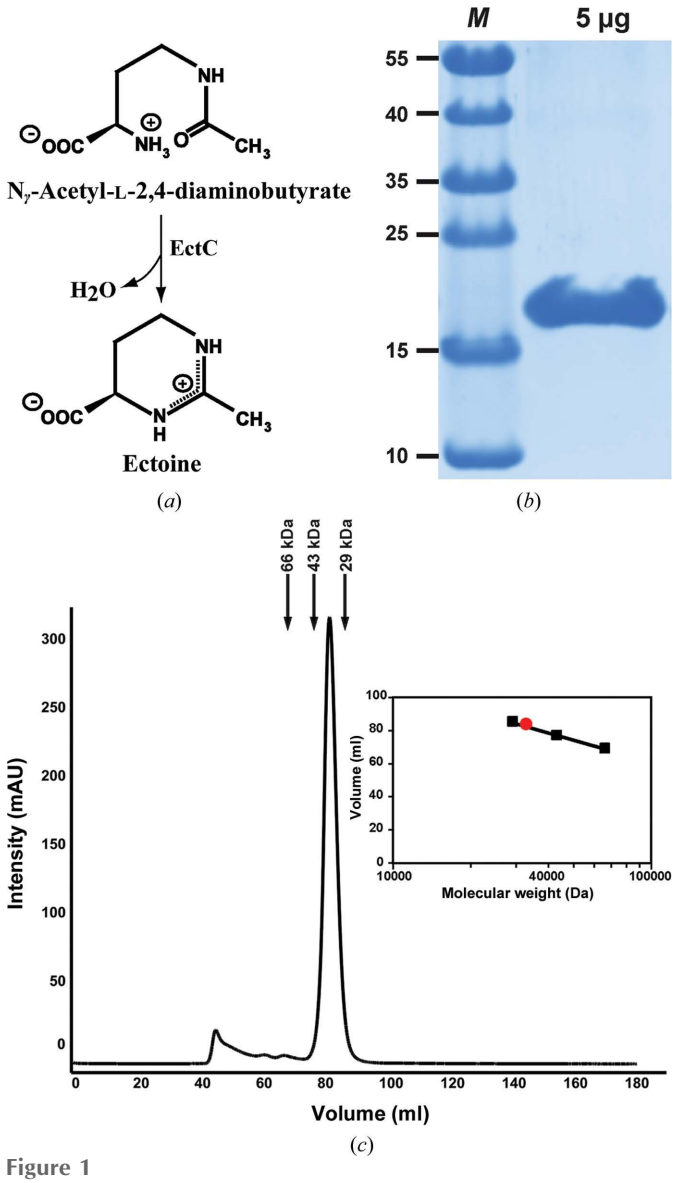


Figure 1
(*a*) Scheme of the EctC-catalyzed enzyme reaction. (*b*) SDS–PAGE analysis of the *Sa*EctC-*Strep*-tag II protein. Lane *M*, molecular-mass marker. (*c*) Gel-filtration analysis of the *Sa*EctC-*Strep*-tag II protein. A solution (3 mg ml^{-1}) of albumin (66 kDa), ovalbumin (43 kDa) and carboanhydrase (29 kDa) was used to standardize the HiLoad 16/600 Superdex 75 pg gel-filtration column. The inset shows the calculation of the molecular mass of the *Sa*EctC-*Strep*-tag II protein (closed red circle) with reference to the three marker proteins (closed black squares).

sequence of *S. alaskensis* strain RB2256 (Genome RefSeq No. NC_008048; Ting *et al.*, 2010). This template was used for the synthesis of a version of *ectC* that was codon-optimized for expression in *E. coli* (by Life Technologies, Darmstadt, Germany); the corresponding nucleotide sequence was deposited in the NCBI database under accession No. KR002036. The synthetic *ectC* DNA sequence present on the plasmid [pSynth_*ectC*(Sa)] provided by the supplier (Life Technologies) has been extended, at both its 5′- and 3′-ends, with a short DNA sequence containing LguI restriction sites to allow cloning into the pENTRY-IBA20 acceptor vector (IBA GmbH, Göttingen, Germany); this construction yielded plasmid pWN8. The DNA fragment carrying *ectC* was retrieved from pWN8 and inserted into the Esp3I site in the expression vector pASK-IBA3 (IBA GmbH) in such a way that the stop codon of *ectC* was removed and the 3′-end of the coding region was fused to a short DNA sequence encoding a nine-amino-acid (NWSHPQFEK) *Strep*-tag II affinity peptide. The resulting plasmid was named pWN12 and its nucleotide sequence was verified by DNA sequence analysis (carried out by Eurofins MWG, Ebersberg, Germany). Transcription of the *ectC* gene carried by pWN12 is driven by the *tet* promoter, the activity of which in turn is controlled by the TetR repressor, a genetic expression system that can be induced by adding anhydrotetracycline (AHT) to the growth medium.

2.1.1. Overexpression and purification of the *Sa*EctC-*Strep*-tag II protein. *E. coli* BL21 cells carrying the pWN12 expression plasmid were grown at 310 K in Minimal Medium A (MMA) with 0.5%(*w*/*v*) glucose as the carbon source, 0.5%(*w*/*v*) casamino acids, 1 m*M* $MgSO_4$ and 3 m*M* thiamine (Höppner *et al.*, 2014; Hoeppner *et al.*, 2014). The medium was supplemented with ampicillin (100 µg ml^{-1}) to select for the presence of plasmid pWN12 in the *E. coli* cells. The cells were grown in a 2 l Erlenmeyer flask (filled with 1 l medium) in an aerial shaker set to 180 rev min^{-1}. Overexpression of the plasmid-encoded *ectC* gene was initiated by adding the inducer AHT (final concentration of 0.2 mg per millilitre of culture) to the growth medium when the bacterial culture had reached an optical density (OD_{578}) of about 0.7. Subsequently, the growth temperature of the culture was reduced to 303 K, the speed of the aerial shaker was reduced to 100 rev min^{-1} and the cells were propagated for an additional 2 h to allow overproduction of the *Sa*EctC-*Strep*-tag II protein by the recombinant cells. Harvesting of the cells by centrifugation and their disruption by passage through a French Pressure Cell Press (SLM Aminco) followed the previously described procedures for the preparation of cell extracts containing the overproduced *Sa*EctD-*Strep*-tag II (Hoeppner *et al.*, 2014; Höppner *et al.*, 2014), except that the cells producing the *Sa*EctC-*Strep*-tag II protein were resuspended in a buffer consisting of 200 m*M* NaCl, 20 m*M* Tris pH 8 (buffer *A*). Cellular debris was removed by ultracentrifugation (60 min at 100 000*g* at 277 K) and the cleared supernatant was loaded onto a Strep-Tactin Superflow column (bed volume of 5 ml) that had been equilibrated with a solution of five bed volumes of buffer *A*. The column was then washed with ten column volumes of buffer *A*, and the *Sa*EctC-*Strep*-tag II protein was eluted from the affinity chromatography material with three column volumes of buffer *A* containing 2.5 m*M* desthiobiotin. Desthiobiotin and AHT were purchased from IBA GmbH, Göttingen, Germany. The concentration of the *Sa*EctC-*Strep*-tag II protein in the individual fractions eluted from the Strep-Tactin Superflow affinity column was measured with the Pierce BCA Protein Assay Kit (Thermo Scientific, Schwerte, Germany) and by using an extinction coefficient of 15 470 M^{-1} cm^{-1} at 280 nm and the molecular mass (16.3 kDa) of the full-length *Sa*EctC protein attached to the *Strep*-tag II affinity peptide. The purity and molecular mass of the *Sa*EctC-*Strep*-tag II protein was assessed by SDS–PAGE (15% polyacrylamide; Fig. 1*b*) using PageRuler Prestained Protein Ladder (Fischer Scientific GmbH, Schwerte, Germany) as a reference. The affinity-purified *Sa*EctC-*Strep*-tag II protein was routinely kept at 277 K. Prior to crystallization trials, the *Sa*EctC-*Strep*-tag II protein was concentrated to approximately 11 mg ml^{-1} (in 20 m*M* Tris, 200 m*M* NaCl pH 8) with Vivaspin 6 columns (Sartorius Stedim Biotech GmbH, Göttingen, Germany) with a 10 kDa molecular-weight cutoff. Macromolecule-production information is summarized in Table 1.

Table 1
Macromolecule-production information.

Source organism	*S. alaskensis* strain RB2256
DNA source	Synthetically made
Forward primer	GTGAAATGAATAGTTCGAC
Reverse primer	CGCAGTAGCGGTAAACGGC
Cloning vector	pSynth_*ectC*
Expression vector	pASK_IBA
Expression host	*E. coli*
Complete amino-acid sequence of the construct produced	MIVRNLGDIRKTDRNVRSDGWASARMLLKDDGMG-FSFHVTTLFAGSELRMHYQNHLEAVLVLKGTG-TIEDLATGEVHALRPGVMYALDDHDRHIVRPE-TDILTACVFNPPVTGREVHDESGAYPADPELA-REPVAADNWSHPQFEK

2.1.2. Size-exclusion chromatography of *Sa*EctC. Size-exclusion chromatography of the affinity-purified *Sa*EctC-*Strep*-tag II protein was performed on a HiLoad 16/600 Superdex 75 pg column (GE Healthcare, München, Germany) equilibrated and run in 200 m*M* NaCl, 20 m*M* Tris pH 8 under previously described conditions (Widderich *et al.*, 2014). 5 mg of the *Sa*EctC-*Strep*-tag II protein (in a volume of 1 ml) was loaded onto the size-exclusion column, which was standardized with albumin (66 kDa), ovalbumin (43 kDa) and carboanhydrase (29 kDa). These marker proteins were purchased from Sigma–Aldrich (Steinheim, Germany) and GE Healthcare (München, Germany).

2.2. Crystallization and preliminary X-ray analysis of *Sa*EctC

Several crystal hits for the *Sa*EctC-*Strep*-tag II protein were obtained using commercial screens from NeXtal (Qiagen, Hilden, Germany) and Molecular Dimensions (Suffolk, England) in Corning 3553 sitting-drop plates at 285 K. Homogeneous *Sa*EctC-*Strep*-tag II protein (0.1 µl of a solution at 11 mg ml^{-1}) was mixed with 0.1 µl reservoir solution and equilibrated against 50 µl reservoir solution. The most promising hit was found using a condition consisting of 0.05 *M*

calcium acetate, 0.1 *M* sodium acetate pH 4.5, 40%(*v*/*v*) 1,2-propanediol from the NeXtal Core IV suite (Qiagen, Hilden, Germany). This condition was optimized by grid screens around the initial condition and by the addition of several divalent cations. Large crystals were obtained either without any additive or on the addition of Ca^{2+} to the protein solution 30 min before the drops were placed. The crystals reached their maximum dimensions of about 50 × 50 × 70 µm after ten weeks. The crystals were harvested after overlaying the drop with 2 µl mineral oil and were flash-cooled in liquid nitrogen. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

Data sets were collected from a single crystal of the *Sa*EctC-*Strep*-tag II protein on beamline ID29 at the ERSF, Grenoble, France at 100 K using a Pilatus detector. After the initial diffraction tests, the data-collection strategy was first calculated using the *EDNA* software available at the beamline (Incardona *et al.*, 2009) and the subsequently collected data sets were processed using the *XDS* package (Kabsch, 2010*a*) and scaled with *XSCALE* (Kabsch, 2010*b*). Data-collection and processing statistics are summarized in Table 3.

3. Results and discussion

To provide the large amounts of ectoine synthase (EctC) required for structural analysis, we obtained a codon-optimized version of the *ectC* gene from the cold-adapted marine bacterium *S. alaskensis* (*Sa*; Ting *et al.*, 2010) for expression in *E. coli*. We chose the *Sa*EctC protein for our studies since we recently successfully determined the crystal structures of the apo and liganded forms of ectoine hydroxylase (EctD; Höppner *et al.*, 2014; Hoeppner *et al.*, 2014) from this microorganism. Overproduction of the *Sa*EctC-*Strep*-tag II protein in *E. coli* strain BL21 (pWN12) typically yielded approximately 40–50 mg of protein per litre of bacterial cell culture. As evaluated by SDS–PAGE, the *Sa*EctC-*Strep*-tag II protein was at least 98% pure as judged by optical inspection of a 15% SDS–polyacrylamide gel (Fig. 1*b*). Its electrophoretic mobility on the SDS–polyacrylamide gel was consistent with the theoretically calculated molecular mass (16.3 kDa, including the attached *Strep*-tag II peptide) of the recombinant protein (Fig. 1*b*).

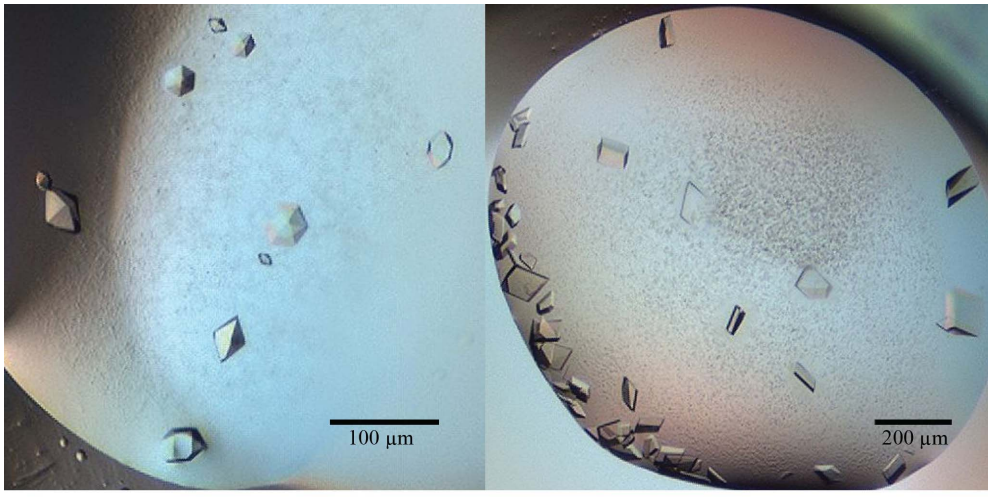
Table 2
Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	Corning 3553
Temperature (K)	285
Protein concentration (mg ml^{-1})	11
Buffer composition of protein solution	200 m*M* NaCl, 20 m*M* Tris pH 8 plus 5 m*M* Ca^{2+} prior to crystallization
Composition of reservoir solution	0.05 *M* calcium acetate, 0.1 *M* sodium acetate pH 4.5, 40%(*v*/*v*) 1,2-propanediol
Volume and ratio of drop	1:1
Volume of reservoir (µl)	300

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	ID29, ESRF, Grenoble, France
Wavelength (Å)	0.97625
Temperature (K)	100
Detector	Pilatus-6M
Crystal-to-detector distance (mm)	164.35
Rotation range per image (°)	0.05
Total rotation range (°)	360
Exposure time per image (s)	0.02
Space group	$P3_221$
a, *b*, *c* (Å)	72.715, 72.715, 52.330
α, β, γ (°)	90.0, 90.0, 120.0
Mosaicity (°)	0.06
Resolution range (Å)	30–1.0
Total No. of reflections	1364845 (46924)
No. of unique reflections	882245 (7645)
Completeness (%)	98.0 (83.7)
Multiplicity	16.5 (6.1)
$\langle I/\sigma(I)\rangle$	16.2 (2.0)
$R_{\rm r.i.m.}$ (%)	8.9 (115.4)
$CC_{1/2}$	99.9 (43.7)
Overall *B* factor from Wilson plot (Å^2)	10.2



(*a*) (*b*)

Figure 2
Crystals of *Sa*EctC. (*a*) Initial and (*b*) optimized crystals

The homogeneity of the affinity-purified *Sa*EctC-*Strep*-tag II protein was assessed analytically by size-exclusion chromatography. The vast majority of the protein eluted from a HiLoad 16/600 Superdex 75 pg column in a single symmetrical peak, and only a very minor portion of the *Sa*EctC-*Strep*-tag II preparation was present as aggregates (Fig. 1*c*). It eluted from the size-exclusion chromatography column as a species with a calculated molecular mass of 32.7 kDa, indicating that the *Sa*EctC-*Strep*-tag II protein forms a dimer in solution (Fig. 1*c*). This finding is in line with the purified native EctC protein from *H. elongata*, which is probably also a dimer in solution (Ono *et al.*, 1999). Taken together, we conclude from these experiments that the recombinant *Sa*EctC-*Strep*-tag II protein can be effectively overproduced in *E. coli* and purified with good yields and that these protein preparations are by and large homogeneous.

Well diffracting crystals of the *Sa*EctC-*Strep*-tag II protein were obtained with 0.06 *M* calcium acetate, 0.08 *M* sodium acetate pH 4.5, 38–42%(*v*/*v*) 1,2-propanediol using the sitting-drop vapour-diffusion method (Fig. 2). Native data sets for the *Sa*EctC-*Strep*-tag II crystal species were collected at 100 K to a maximum resolution of 1.0 Å (Fig. 3). Preliminary data processing using the *XDS* package displayed $P3_221$ symmetry (Table 1). The V_M value was calculated to be 2.32 Å^3 Da^{-1} with a monomer in the asymmetric unit, with a solvent content of 47% (Matthews, 1968).

Members of the cupin superfamily of proteins possess a rather well conserved common β-barrel core structural scaffold (Uberto & Moomaw, 2013), but our attempts to solve the *Sa*EctC crystal structure by molecular replacement using the crystal structure of the Zn^{2+}-containing cupin RemF from *Streptomyces resistomycificus* (PDB entry 3ht1; Silvennoinen *et al.*, 2009), a polyketide cyclase involved in the biosynthesis of the metabolite resistomycin, were unsuccessful. Notably, the *Sa*EctC protein possesses a single Cys residue that should be ideally suited for heavy-atom binding to obtain crystals of the *Sa*EctC-*Strep*-tag II protein that can be used to obtain initial phases *via* MAD experiments in order to solve the crystal structure of ectoine synthase.

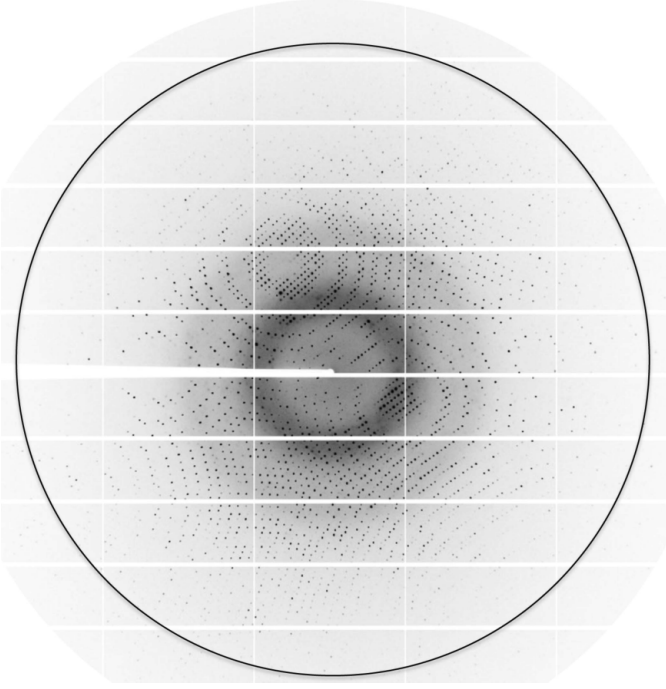


Figure 3
Diffraction images of *Sa*EctC (oscillation width 0.5°). The images were used to calculate the data-collection strategy using the *EDNA* software (Incardona *et al.*, 2009). The circle represents a resolution of 1.2 Å

Acknowledgements

We acknowledge the European Synchrotron Radiation Facility for the provision of synchrotron-radiation resources, especially the staff of the ID29 beamline at the ESRF, Grenoble, France. We gratefully acknowledge the financial support from the Heinrich Heine University, the Institute for Biochemistry at the University of Düsseldorf, the DFG-funded SFB-987 (University of Marburg) and the LOEWE Center for Synthetic Microbiology (Marburg) for this study. We thank Lutz Schmitt for his interest in and support of this collaborative project. NW is the recipient of a PhD fellowship from the International Max Planck Research School for Environmental, Cellular and Molecular Microbiology (IMPRS-Mic Marburg). We greatly appreciate the kind help of Vickie Koogle in the language editing of our manuscript and thank Jochen Sohn for his expert technical assistance.

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