CRYSTALLIZATION AND PRELIMINARY X-RAY DIFFRACTION ANALYSIS OF COLD-ACTIVE β-GALACTOSIDASE FROM *Arthrobacter* SP. C2-2

Hana PETROKOVÁ[a1], Eva VONDRÁČKOVÁ[a2], Tereza SKÁLOVÁ[a3], Jan DOHNÁLEK[a4], Petra LIPOVOVÁ[b1], Vojtěch SPIWOK[b2], Hynek STRNAD[b3], Blanka KRÁLOVÁ[b4] and Jindřich HAŠEK[a5,*]

[a] *Institute of Macromolecular Chemistry, Academy of Sciences of the Czech Republic, Heyrovského nám. 2, 162 06 Prague 6, Czech Republic; e-mail:* [1] *petrokova@imc.cas.cz,* [2] *vondrackova@imc.cas.cz,* [3] *skalova@imc.cas.cz,* [4] *dohnalek@imc.cas.cz,* [5] *hasek@imc.cas.cz*

[b] *Department of Biochemistry, Institute of Chemical Technology, Prague, Technická 5, 166 28 Prague 6, Czech Republic; e-mail:* [1] *petra.lipovova@vscht.cz,* [2] *vojtech.spiwok@vscht.cz,* [3] *hynek.strnad@vscht.cz,* [4] *blanka.kralova@vscht.cz*

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β-Galactosidase from psychrotrophic bacteria strain *Arthrobacter* sp. C2-2 catalyzes cleavage of β-D-galactosyl moieties from β-D-galactosides and is interesting for its activity at low temperatures. Various types of crystals with dimensions of up to 0.8 mm were obtained and X-ray diffraction data up to 1.9 Å were collected. The crystals belong to the monoclinic space group $P2_1$ with unit-cell parameters a = 140.1 Å, b = 205.7 Å, c = 140.5 Å and β = 102.3°. The enzyme (molecular weight of a monomer is 111 kDa) forms hexamers in the crystal structure (one hexamer per asymmetric unit). The phase problem was solved by molecular replacement. Structure refinement is in progress.

Keywords: Galactosidases; Glycosidases; Psychrotrophic; X-ray diffraction; Crystallization; Crystal growth; Crystal structure determination; Enzymes.

Enzymes from cold-adapted microorganisms (cold-active enzymes) are becoming attractive catalysts for a wide range of biotechnologies. Application of these enzymes allows to perform processes at low temperature and, therefore, it is possible to reduce costs of heating, to work with thermolabile compounds or to reduce microbial contamination. These enzymes are also important from the point of view of general aspects of biochemistry such as protein stability, folding and flexibility – activity trade-off[1]. Potential biotechnological applications of β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) range from production of lactose-free milk to biotransformations and oligosaccharide production. β-Galactosidases from different sources belong to four families of classification of

glycosidases (families 1, 2, 35 and 42)[2]. These families are proposed to share a common fold; however, their overall architecture is different. X-ray structures are known for β-galactosidase from *Escherichia coli* (family 2)[3,4], *Thermus thermophilus* A4 (family 42)[5] and *Sulfolobus solfataricus* P2 (family 1, specificity of this enzyme is not restricted to β-galactosides)[6].

Psychrotrophic strain *Arthrobacter* sp. C2-2 was isolated in the Antarctic area[7]. Two isoenzymes of β-galactosidase are produced by this strain, belonging to family 2 (isoenzyme C2-2-1) and family 35 (isoenzyme C2-2-2). Similar production of multiple isoenzymes from different families of β-galactosidases by one strain of the *Arthrobacter* genus has already been observed[8]. The gene of β-galactosidase isoenzyme C2-2-1 was isolated, sequenced and characterized as described elsewhere[9,10]. This enzyme is capable of cleaving lactose as well as a number of synthetic β-galactosides. Its optimal temperature is significantly shifted towards lower temperatures in comparison with enzymes from mesophilic and thermophilic species. It is also capable of catalyzing transglycosylation reactions, i.e. it can synthesize galactooligosaccharides during hydrolysis of lactose[10]. The galactose moiety can also be transferred to non-saccharide acceptors (unpublished results). This isoenzyme belongs to family 2 with 30% sequence identity to β-galactosidase from *E. coli*. The reaction cycle of *E. coli* β-galactosidase was explored by crystallographic studies that led to structures of complexes of β-galactosidase with substrates, products, transition-state analogs and trapped covalent reaction intermediates[4]. The enzyme from *E. coli* forms tetramers and its oligomeric state is known to be critical for its full activity. This oligomerization-state-dependent allosteric activation has been exploited in several applications such as α-complementation-based blue/white screening and protein–protein interaction monitoring[11] and as a molecular probe for detection of specific antibodies[12].

RESULTS AND DISCUSSION

Expression and Purification

Methods of gene isolation, protein expression and purification were previously described[9,10]. The gene of β-galactosidase from *Arthrobacter* sp. C2-2 (isoenzyme C2-2-1) was isolated by activity-based screening of the genome library of *Arthrobacter* sp. C2-2 and sequenced. The gene was cloned into expression vector pET16b in fusion with N-terminal histidine tag and factor Xa cleavage site. The vector containing insert was then transformed into *E. coli* BL21(DE3) strain. In order to avoid formation of inclusion bodies

cells were grown in minimal medium containing 100 μg ml^{-1} ampicillin with glycerol as a sole source of carbon at 20 °C. After 12 h of cultivation the expression was induced by addition of isopropyl β-D-thiogalactopyranoside (IPTG) to final concentration of 0.08 mmol l^{-1}. After another 5 h of expression, cells were harvested by centrifugation. The pellet was then resuspended in 50 mM phosphate buffer (pH 7.0) containing 300 mM KCl and 10 mM imidazole. Cells were lysed using a combination of lysozyme, sodium deoxycholate, Triton X-114 and sonication and cell debris were removed by centrifugation. Purification was performed by metalloaffinity chromatography in combination with gel permeation chromatography. In the first step the cell lysate was loaded onto a Ni^{2+}–nitrilotriacetic acid–agarose column. Enzyme was eluted with 250 mM imidazole in 50 mM phosphate buffer (pH 7.5) containing 300 mM KCl. Eluted fractions were pooled and concentrated using a Centriplus device (100 kDa cut-off). The N-terminal histidine tag was cleaved by factor Xa protease (80 U per 1 mg of β-galactosidase). The histidine tag fragment was separated by metalloaffinity chromatography on Ni^{2+}–nitrilotriacetic acid–agarose column. Pooled fractions containing the enzyme were concentrated using Centriplus and loaded on Sephacryl S-300 HR. The enzyme was eluted with 50 mM phosphate buffer (pH 7.5) containing 300 mM KCl with elution volume corresponding to a molecular weight of ca 600 kDa. Pooled fractions were concentrated on Centriplus prior to crystallization experiments. Protein purity was monitored using SDS-PAGE electrophoresis and protein concentration was determined by Bradford reagent assay.

Crystallization

The screening for crystallization conditions started using JBScreening kits (Jena Biosciences) Nos 5, 4 and 3 based on poly(ethylene glycol) (PEG) 8000–20000, PEG MME 5000–8000 and PEG 4000 plus, respectively. Crystallization was performed by the hanging-drop vapour-diffusion method at 291 K by mixing 2 μl of protein solution and 2 μl of precipitating solution. Typical reservoir contained 750 or 1000 μl of precipitating solution.

Crystallization of C2-2-1 is influenced by composition of the storage buffer. Two types of buffer solutions were tested: 50 mM Tris-HCl (pH 7.5) and 200 mM NaCl and 50 mM KH_2PO_4/K_2HPO_4 (pH 7.5). Tris buffer conditions lead to crystals more easily; however, Tris strongly decreases the activity of β-galactosidase. Therefore, further crystallization experiments were carried out with the protein in the phosphate buffer. Occasional microbial

contamination of the enzyme was prevented by addition of 1 mM NaN_3 to the protein solution.

It was found that PEG of molecular weight 8000 and higher gives crystals under a variety of conditions, mainly at pH higher than 6.0. Needle-like and occasionally plate-like crystals grew within several days in drops without any protein precipitation (Fig. 1a). Crystallization was observed even at low protein concentrations (2.5 mg ml^{-1}) probably due to a lower diffusion rate in PEG 8000.

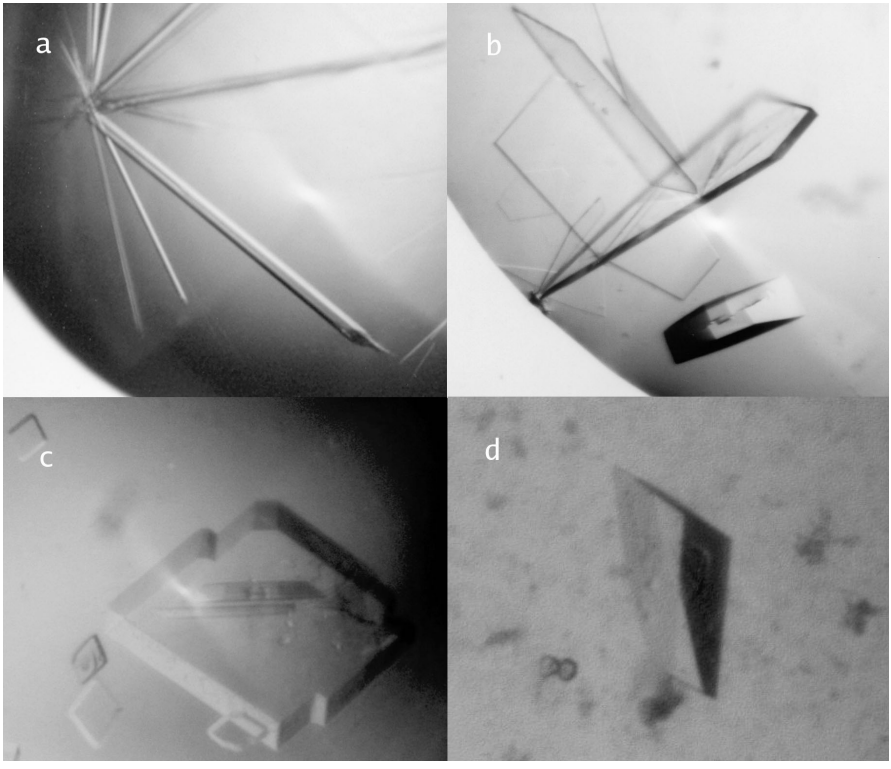

FIG. 1

Various types of crystals were obtained under various conditions. Reservoir composition: 10% (w/v) PEG 8000, 200 mM Ca acetate and 100 mM HEPES pH 7.5 (rods up to 0.05 × 0.2 mm) (a); 22% (w/v) PEG 4000, 150 mM NaCl, 150 mM $(NH_4)_2SO_4$ and 100 mM MES pH 6.5 (plates dimensions up to 0.75 × 0.01 mm) (b); 20% (w/v) PEG 4000, 200 mM NaCl, 200 mM $(NH_4)_2SO_4$ and 100 mM Na citrate pH 5.6 (rhombic crystals, the largest with diagonal size 0.8 mm) (c); the same conditions as in c, after microseeding (a wedge-like crystal used in the synchrotron data collection is shown, dimensions 0.3 × 0.2 × 0.1 mm) (d)

In PEG 4000 microcrystalline precipitate appeared under most conditions. Crystals grow in 15–20% (w/v) PEG 4000, 150–200 mM $(NH_4)_2SO_4$ and 100 mM Na citrate (pH 5.6–6.5). The protein concentration of 4 mg ml^{-1} was required. Crystals of various shapes were observed – horn, four-pointed star, wedge or rhomb (Figs 1b–1d). They appeared usually after 14 days and needed further 14 days for their growth.

For X-ray diffraction experiments, plate-like crystals (dimensions ca. 0.25 × 0.1 × 0.02 mm) grown by microseeding and with reservoir composition: 20% (w/v) PEG 4000, 200 mM NaCl, 200 mM $(NH_4)_2SO_4$ and 100 mM Na citrate buffer (pH 5.6) were selected. These crystals were produced with protein solution containing 10 mM KH_2PO_4/K_2HPO_4 (pH 7.5), 1 mM $MgSO_4$, 1 mM NaN_3 and 4 mg ml^{-1} of protein. A crystal mounted in a capillary at room temperature diffracted to the 3 Å diffraction limit using a rotation-anode X-ray radiation source. The diffraction quality decreased rapidly during data collection (after several hours of exposure).

Therefore the final diffraction data collection was performed with a flash-cooled crystal (obtained under the same crystallization conditions, Fig. 1d) at the ID29 beamline of the ESRF synchrotron radiation source. Several cryo-protectants were examined including PEG 400, PEG 4000, dimethyl sulfoxide, ethylene glycol and glycerol in order to optimize the cryo-conditions. The best results were obtained with 20% (v/v) ethylene glycol in mother liquor.

Synchrotron Data Collection

X-ray diffraction data were collected at 100 K on beam-line ID 29 of the source of synchrotron radiation ESRF in Grenoble, with an ADSC CCD Q210 detector in the bined mode, radiation wavelength 0.9168 Å, exposure time 0.1 s and crystal-to-detector distance 185 mm. 1800 images with an oscillation angle of 0.1° were collected. The crystal diffracted up to 1.9 Å (Fig. 2). Data were processed with HKL package[13] (programs Denzo and Scalepackvirus). Results of data processing are summarized in Table I.

Self-Rotation Function and Non-Crystallographic Symmetry

Self-rotation function was calculated with program MOLREP[14]. Figure 3 shows stereographic projections of the $\kappa = 120$ and 180° sections of the self-rotation function. The crystallographic $P2_1$ symmetry corresponds to peaks on y axis of the section $\kappa = 180°$. Left-right symmetry of the figures also results from crystallographic symmetry. The non-crystallographic

TABLE I
Crystal parameters and results of data processing. Values for the last resolution shell are given in parentheses

Parameter	β-Galactosidase from *Asthrobacter* sp. C2-2
Space group	$P2_1$
Unit-cell parameters, Å, °	a = 140.1, b = 205.7, c = 140.5, β = 102.3
Mosaicity, °	0.6
Resolution, Å	40.0–1.9 (1.93–1.90)
No. of observed partial intensities	19 863 409
No. of unique reflections	577 572 (24 388)
Average redundancy	2.6 (1.9)
R_{merge}, %	8.5 (45.1)
Completeness, %	95 (81)
$\langle I \rangle / \langle \sigma(I) \rangle$	10.3 (2.1)

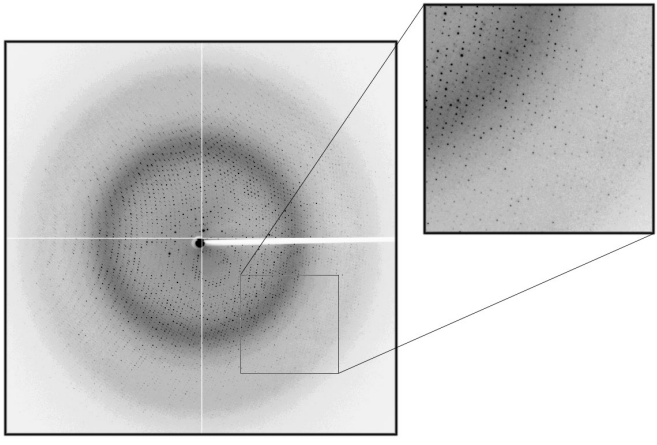

FIG. 2
X-ray diffraction pattern of cold-active β-galactosidase from *Arthrobacter* sp. C2-2. The edge of the image corresponds to resolution 1.9 Å

threefold symmetry follows from two equivalent peaks on the section κ = 120°. Three twofold axes separated by 120° and perpendicular to the threefold axis (peaks on the section κ = 180°) correspond to the internal symmetry of the hexamer formed by two trimers related by twofold symmetry.

Molecular Replacement

The calculation of the Mathews coefficient and solvent content[15] of the crystal suggested several monomers of the enzyme per asymmetric unit. Following the self-rotation function results, one, two or three trimers (solvent content 79, 59 or 38%) were expected in the crystal structure. Molecular replacement was solved in program AMORE [16] of the CCP4 package (version 5-beta)[17]. A monomer of β-galactosidase from *E. coli* (PDB code 1DP0)[18] was used as a model. At first dimers were composed of monomer solutions (the best dimer solution: R = 54.3%, CC = 27.5%). Different dimer solutions were manually assembled into a hexamer in program XFIT [19]. Retrospectively it was found that all correct monomer solutions appeared in the first seven translation search results for one molecule (CC/R for the first seven solutions 9.4–8.8/57.0–57.1, for the first incorrect solution 8.7/57.1). R factor calculated after rigid body refinement in program REFMAC5 [20] with the sequence of the model structure (R = 51.8%, 30% similarity) dropped to R = 41.7, R_{free} = 44.7% in the first steps of refinement and rebuilding. The asymmetric part of the unit cell contains 6 chemically identical molecules (molecular weight 110 853) with 1023 residues each. More than 50 000

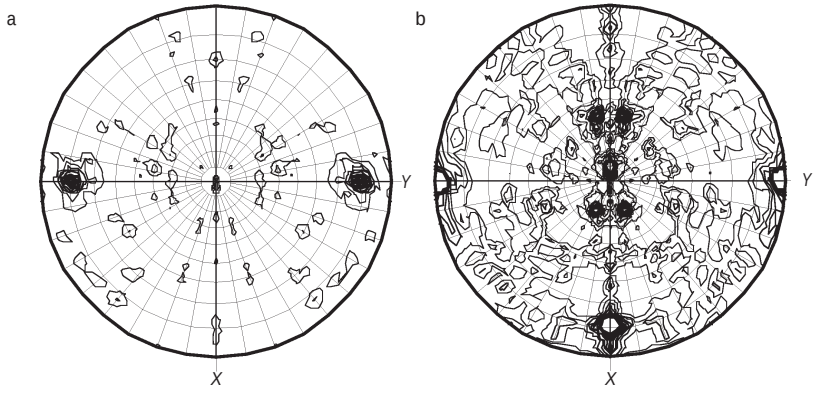

FIG. 3
Stereographic projection of the κ = 120° (a) and κ = 180° (b) sections of the self-rotation function calculated using data in the range 40.0–3.0 Å and an integration radius of 20 Å

independently refined non-hydrogen atoms represent about 200 thousand parameters against 580 thousand independent observations. The maps of electron density confirm correctness of the structure and the refinement is under progress.

CONCLUSIONS

The cold-active β-galactosidase from *Arthrobacter* sp. C2-2 (isoenzyme C2-2-1) was crystallized and X-ray diffraction data were collected up to 1.9 Å. Results of data processing are summarized in Table I. The phase problem was solved by molecular replacement. The space packing of the cold-active C2-2-1 β-galactosidase presented here differs significantly from the homologous *E. coli* β-galactosidase (30% sequence identity, PDB code 1BGL, a = 107.90 Å, b = 207.50 Å, c = 509.90 Å, β = 94.70°, $P2_1$ with two tetramers in asymmetric unit)[3].

The molecules of cold-active C2-2-1 β-galactosidase form hexamers in the crystal structure, one hexamer per asymmetric unit. The hexamer is a closed globular structure with a large central cavity, formed in between two face-to-face oriented trimers. Intermolecular adhesion leading to such a highly symmetrical clustering of molecules and its role in solution will be discussed later, after the final coordinates are available.

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