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Crystallization and preliminary X-ray diffraction analysis of levansucrase (LsdA) from *Gluconacetobacter diazotrophicus* SRT4

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The endophytic bacterium *Gluconacetobacter diazotrophicus* SRT4 secretes a constitutively expressed levansucrase (LsdA; EC 2.4.1.10), which converts sucrose to fructo-oligosaccharides and levan. Fully active LsdA was purified to high homogeneity by non-denaturing reversed-phase HPLC and was crystallized at room temperature by the hanging-drop vapour-diffusion method using ammonium sulfate and ethanol as precipitants. The crystals are extremely sensitive, but native data have been collected to 2.5 Å under cryogenic conditions using synchrotron radiation. LsdA crystals belong to the orthorhombic space group $P22_12_1$ or $P2_12_12$, with unit-cell parameters $a = 53.80$, $b = 119.39$, $c = 215.10$ Å.

1. Introduction

Levansucrase (EC 2.4.1.10; sucrose:2,6-β-D-fructan 6-β-D-fructosyltransferase) catalyses a transglycosylation reaction in which fructose, derived from sucrose, may be transferred to a variety of acceptors including water (hydrolysis), glucose (exchange), sucrose (oligofructoside synthesis) and fructan (polymerization) (Cote & Ahlgran, 1993). These reactions all occur *via* a 'ping-pong' kinetic mechanism involving the formation of a fructosyl-enzyme intermediate and its subsequent interception by the acceptor (Chambert *et al.*, 1974; Hernández *et al.*, 1995; Song & Jacques, 1999).

Levansucrases are produced by a wide range of sucrose-utilizing bacteria. The endophytic species *Gluconacetobacter diazotrophicus* secretes a constitutively expressed levansucrase which yields high levels of β-2,1-linked oligofructosides and levan from the natural substrate sucrose (Hernández *et al.*, 2000). Levansucrase produced by the Cuban strain SRT4 has been extensively characterized (Arrieta *et al.*, 1996; Hernández *et al.*, 1999; Betancourt *et al.*, 1999). The mature enzyme consists of a single polypeptide of molecular weight 60.4 kDa and isoelectric point 5.5 which contains an intramolecular disulfide bridge and an N-terminal blocking pyroglutamic acid residue.

Glycoside hydrolases have now been classified into over 90 families on the basis of amino-acid sequence similarities (CAZy database; http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html). Bacterial fructosyltransferases are included in family GH68. These enzymes, together with sucrose-6-phosphate hydrolases, invertases, fructanases and eukaryotic fructosyltransferases (GH family 32), comprise the β-fructosidase superfamily (reviewed in Pons *et al.*, 2000; Naumoff, 2001). Here, we present the purification, crystallization and preliminary diffraction analysis of the *G. diazotrophicus* SRT4 levansucrase from family GH68.

2. Materials and methods

2.1. Purification of LsdA and protein assays

G. diazotrophicus SRT4 was cultured to stationary phase (96 h) in LGIT medium (2% glycerol, 0.2% tryptone, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.002% $CaCl_2.2H_2O$, 0.0002% Na_2MoO_4, initial pH 7.0) at 303 K with shaking at 250 rev min^{-1}. Cells were removed by centrifugation, the culture supernatant was concentrated fivefold at 310 K using a rotatory evaporator and ethanol was added to 60% (final concentration) to precipitate high-molecular-mass exopolysaccharides. After centrifugation, LsdA remained in the supernatant fraction, from which ethanol was removed by rotary evaporation at 310 K. The enzyme solution was dialyzed against 10 m*M* Tris–HCl pH 7.0, filtered through 0.2 µm disposable filters and applied onto a semi-preparative butyl HPLC column (Vydac, USA). The column was washed with 0.1 *M* ammonium acetate pH 5.0 and proteins were eluted using an increasing gradient of 1-propanol (0–60%) in 0.1 *M* ammonium acetate pH 5.0. The peak fraction with LsdA activity was dialyzed against 10 m*M* Tris–HCl pH 7.0, concentrated to 10 mg ml^{-1} by ultrafiltration using a 10 kDa molecular-weight cutoff membrane (Millipore) and used for crystallization experiments.

LsdA concentration was determined by absorbance at 280 nm considering the theoretical extinction coefficient of 1.4 AU

(1 mg ml^{-1}). LsdA activity was measured as the glucose released from sucrose hydrolysis using a glucose oxidase–peroxidase-coupled colorimetric kit (Sigma). SDS–PAGE was performed as described by Laemmli (1970). Proteins were revealed by Coomassie Blue staining.

2.2. Crystallization, data collection and processing

The crystallization experiments were performed using the hanging-drop vapour-diffusion method in 24-well tissue-culture plates at room temperature. LsdA concentrated to 10 mg ml^{-1} in 10 mM Tris–HCl pH 7.0 was initially screened for crystallization conditions, varying the ammonium sulfate concentration (0.2–1.5 M) in the pH range 5.0–7.0. In each trial, 2 µl of purified enzyme and 2 µl of mother liquor were mixed over wells that contained 1 ml of the screening solution. Long needles grew over two weeks using ammonium sulfate in the concentration range 0.35–0.6 M in 0.1 M sodium citrate pH 5.6. Ethanol (30–60%) and the detergents SDS (1.5–5.0 mM) and β-octylglucoside (10–40 mM) were used to optimize the crystallization conditions. Crystal formation was accelerated twofold by adding 50 mM guanidinium chloride and 50 mM $MgCl_2$ to the crystallization solution. Crystals with improved morphology and size were transferred with difficulty to the cryo-protection solution (15% glycerol, 30% ethanol, 0.6 M ammonium sulfate, 0.1 M sodium citrate pH 5.6). A single crystal was mounted in a rayon-fibre loop and stored in liquid nitrogen. For data collection, the crystal was placed in a stream of N_2 gas at 100 K and 105° of data were collected with an oscillation angle of 0.5° per image at the ESRF (Grenoble, France) on beamline ID14-EH1 (λ = 0.934 Å) with an ADSC Quantum-4 detector. Data were processed with *MOSFLM* (Leslie, 1992) and scaled and reduced with *SCALA* from the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994).

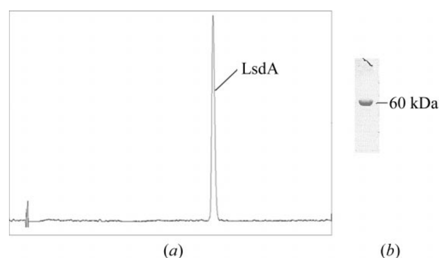

Figure 1
Analysis by rp-HPLC (*a*) and SDS–PAGE (*b*) of LsdA purified from the culture supernatant of *G. diazotrophicus* SRT4 and used for crystallization experiments. LsdA eluted from the HPLC column at 40% 1-propanol.

Table 1
Diffraction data statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.934
Crystal system	Primitive orthorhombic
Space group	$P22_12_1$ or $P2_12_12_1$
Unit-cell parameters (Å, °)	a = 53.8, b = 119.4, c = 215.1, $\alpha = \beta = \gamma = 90$
Unique reflections collected	48568
Redundancy	3.3
Resolution range (Å)	52–2.50 (2.64–2.50)
Completeness (observed) (%)	99.2 (98.5)
$R_{\rm merge}$	0.078 (0.32)
$\langle I/\sigma(I)\rangle$	11.6 (4.6)
Unit-cell volume (Å^3)	1381708
Matthews coefficient (Å^3 Da^{-1})	3.0 (2 molecules) or 2.0 (3 molecules)
Solvent content (%)	58 or 39

3. Results and discussion

The yield of LsdA was 58 mg per litre of culture with an initial purity of 70% from the culture supernatant of *G. diazotrophicus* SRT4 grown under shaking batch conditions. Conventional reversed-phase HPLC allowed LsdA purification to homogeneity, but the enzyme was irreversibly inactivated under the extremely acidic conditions (pH $\simeq$ 2.0) of the solvent system water/acetonitrile/TFA. A second purification protocol using as the main step a non-denaturing reversed-phase HPLC with the solvent system water/1-propanol/ammonium acetate pH 5.0 rendered a fully active enzyme with high homogeneity and purity (Fig. 1).

Initial attempts to crystallize LsdA using ammonium sulfate as the sole precipitant agent in 0.1 M sodium citrate pH 5.6 resulted in long thin needles that were not suitable for X-ray analysis. The addition of ethanol or the detergents SDS and β-octylglucoside to the crystallization solution allowed a significant improvement in crystal morphology and size. More highly reproducible crystals with prismatic shape reaching up to 180 µm in their largest dimension were achieved in the presence of 30% ethanol as a second precipitant agent (Fig. 2), although the high ethanol concentrations make subsequent handling extremely challenging.

LsdA crystal diffraction statistics are presented in Table 1. The crystals belong to the primitive orthorhombic crystal system. Because of the elongated shape of the crystals and the difficulties in handling the crystals, the reflections along the h00 axis (corresponding to the long axis of the crystal) were not measured during data collection. The exact space group could thus be either $P22_12_1$ or $P2_12_12_1$. Preliminary molecular-replacement attempts using *Bacillus subtilis* levansucrase (kindly provided prior to publication by Klaus Fütterer, University of Birmingham, England) gives better statistics for a two-molecule solution in space group $P2_12_12_1$. Levansucrases have been the target of structural studies since the preliminary work of LeBrun & van Rapenbusch (1980). We hope that a number of levansucrase crystal structures will soon be available to shed light on this fascinating family of enzymes.

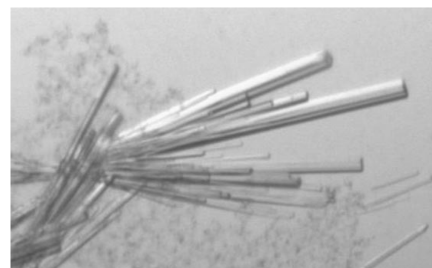

Figure 2
Typical crystals of LsdA obtained by equilibration against a solution of 0.5 M ammonium sulfate, 30% ethanol, 50 mM guanidinium chloride and 50 mM $MgCl_2$ in 0.1 M sodium citrate buffer pH 5.6. The maximum dimension of the crystals is about 0.18 mm.

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