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Overexpression, purification, crystallization and preliminary diffraction studies of the *Protaminobacter rubrum* sucrose isomerase SmuA

Palatinose (isomaltulose, α -D-glucosylpyranosyl-1,6-D-fructofuranose), a nutritional and acariogenic reducing sugar, is industrially obtained from sucrose by using immobilized cells of *Protaminobacter rubrum* that produce the sucrose isomerase SmuA. The isomerization of sucrose catalyzed by this enzyme also results in the formation of trehalulose (α -D-glucosylpyranosyl-1,1-D-fructofuranose) in smaller amounts and glucose, fructose and eventually isomaltose as by-products, which lower the yield of the reaction and complicate the recovery of palatinose. The determination of the three-dimensional structure of SmuA will provide a basis for rational protein-engineering studies in order to optimize the industrial production of palatinose. A recombinant form of the 67.3 kDa SmuA enzyme has been crystallized in the native state by the vapour-diffusion method. Crystals belong to the orthorhombic space group $P2_12_12_1$, with unit-cell parameters $a = 61.6$, $b = 81.4$, $c = 135.6$ Å, and diffract to 1.95 Å resolution on a synchrotron-radiation source.

1. Introduction

Palatinose, also known under the generic name isomaltulose (α -D-glucosylpyranosyl-1,6-D-fructofuranose), is a functional isomer of sucrose (α -D-glucosylpyranosyl-1,2- β -D-fructofuranoside) that has attracted worldwide attention for use as a food ingredient. Unlike sucrose, palatinose is 'tooth-friendly' and is digested much more slowly, leading not only to a low glycaemic and a low insulinaemic response, but also to a prolonged glucose supply (Lina *et al.*, 2002; Takazoe, 1989). Moreover, owing to its reducing properties, it represents an attractive renewable starting material in the chemical industry (Lichtenthaler & Peters, 2004). Naturally found in small quantities in sugar cane and honey (Takazoe, 1989), palatinose is industrially produced by enzyme conversion from sucrose. The enzyme responsible for this conversion is a sucrose isomerase (SI; EC 5.4.99.11), which was first isolated from *Protaminobacter rubrum* by Weidenhagen and Lorenz in 1957 (Weidenhagen & Lorenz, 1957*a,b*). The current large-scale production of isomaltulose is still carried out using immobilized cells of this organism (Hamada, 2002; Nakakuki, 2002).

SIs have also been found in several other strains, including *Serratia plymuthica* NCIB 8285 (Fujii *et al.*, 1983; Veronese & Perlot, 1998), *Erwinia rhapontici* NCPPB 1579 (Cheetham, 1984), *Klebsiella* sp. LX3 (Zhang *et al.*, 2002), *Klebsiella* sp. (Park *et al.*, 1992), *K. planticola* CCRC 19112 (Huang *et al.*, 1998; Park *et al.*, 1992), *Pantoea dispersa* (Wu & Birch, 2004, 2005), *Agrobacterium radiobacter* MX-332 (Nagai-Miyata *et al.*, 1993) and *Pseudomonas mesoacidophilus* MX-45 (Miyata *et al.*, 1992; Nagai *et al.*, 1994). The crystal structures of two SIs have been determined so far: the structure of PalI cloned from *Klebsiella* sp. strain LX3 (Zhang *et al.*, 2003) and solved to 2.2 Å resolution and the structure of MutB from *Pseudomonas mesoacidophilus* MX-45 determined to 1.6 Å resolution (Ravaud, Watzlawick, Haser *et al.*, 2005; Ravaud, Watzlawick, Mattes *et al.*, 2005; Ravaud *et al.*, in preparation). These structural data confirmed that the catalytic core domain of SI is a TIM-like (triose phosphate isomerase-like) (β/α)₈-barrel with the active-site architecture of glycoside hydrolase family 13 enzymes.

In addition to isomaltulose, the isomerization reaction of sucrose catalyzed by SIs also leads to the production of various proportions of

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trehalulose (α -D-glucosylpyranosyl-1,1-D-fructofuranose), glucose and fructose as by-products. The product ratio depends mainly on the bacterial strain, but also on the temperature, pH and reaction conditions. SI from *P. rubrum* produces mainly isomaltulose (~85%), with a minor content of trehalulose, monosaccharides and eventually isomaltose. However, the presence of trehalulose, isomaltose and monosaccharides remains a considerable industrial problem in that it limits the efficiency of isomaltulose production. Therefore, purification procedures leading to the removal of these side products need to be elaborated (Mattes *et al.*, 1998).

The recent cloning of the *smuA* gene of *P. rubrum* SI in *Escherichia coli* (Mattes *et al.*, 1998) has allowed us to prepare sufficient amounts of the highly purified enzyme for crystallization experiments. In this report, we describe the overexpression and the purification of the mature protein, which is composed of 573 amino-acid residues, and X-ray diffraction data analysis. We aim at contributing to an increased understanding of the mechanism and specificity of the isomerization reaction. Moreover, the idea is to provide a basis for the rational design of a more specific and efficient enzyme for industrial purposes.

2. Materials and methods

2.1. Expression of the sucrose isomerase SmuA

The *smuA* gene from *P. rubrum* CBS 547.77 was cloned into the L-rhamnose-inducible expression vector pJOE2702 (Volf *et al.*, 1996), resulting in plasmid pHWG314.

E. coli JM109 transformed with pHWG314 was used for expression of SmuA. Cells were grown at 310 K to an OD₆₀₀ of 0.3 in 200 ml 2YT medium containing 100 μ g ml⁻¹ ampicillin. SmuA production was induced upon addition of 0.1% (w/v) rhamnose and cultivation continued for 4 h at 303 K. Cells were harvested by centrifugation, washed and suspended in 10 mM calcium acetate buffer pH 5.5 and stored at 253 K.

2.2. Purification

All purification steps were performed at room temperature on an automated FPLC system (Amersham Biosciences).

Frozen recombinant *E. coli* cells were thawed and consecutively lysed by passing them twice through a French press cell (Aminco, SLM Instruments Inc.) at 6.9 MPa. The crude cell-free extract was obtained from the supernatant following centrifugation at 11 950g for 30 min at 277 K. Proteins in the supernatant were fractionated by cation-exchange chromatography. Crude cell-free extract (50 mg)

was applied onto a Superformance Fractogel EMD-SO₃⁻ 150-10 column (Merck) pre-equilibrated with 10 mM calcium acetate buffer pH 5.5 and bound proteins were eluted with an NaCl gradient (0–1 M) in the same buffer. 1 ml fractions were collected and tested for isomaltulose synthase activity. Active fractions were combined and further purified on a Mono-S HR 5/5 column (Amersham Biosciences) applying the same buffer gradient and tested further for purity by SDS-PAGE. The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard.

Fractions containing the purified protein were combined, dialyzed against 10 mM calcium acetate buffer pH 5.5 to eliminate NaCl and concentrated to 10 mg ml⁻¹ by ultrafiltration on Centricon YM-10 devices (Millipore) prior to crystallization trials.

2.3. Enzyme-activity assay

The isomaltulose synthase assay was carried out at 298 K in 10 mM calcium acetate buffer pH 5.5 in the presence of 100 mM sucrose. Enzyme activities are expressed as micromoles of isomaltulose formed per minute and millilitre. The concentration of isomaltulose was measured by high-performance anion-exchange chromatography (HPLC) analysis.

SmuA activity was tested qualitatively for glucose formation during sucrose conversion during the purification procedure by performing a 96-well microplate-adapted GOD/perit-test (Roche).

2.4. Product composition

The product composition resulting from SmuA reacting with 100 mM sucrose was determined at 298 K after 90% sucrose conversion and analysed by HPLC. The HPLC apparatus consisted of a pump (220, Bischoff, Leonberg, Germany) and an ESA Coulochem II electrochemical detector (Bischoff). The samples were injected into HPLC and separated on a CarboPac PA1 column (Dionex) with 0.22 M NaOH/0.02 M sodium acetate at a flow rate of 0.75 ml min⁻¹. The eluted sugars were detected by pulsed amperometry with an analytical cell (5040, ESA Coulochem II, Bischoff). Individual sugars were identified by comparison of retention times with those of standards. The relative percentage of each sugar was calculated based on its peak area. The standard sugars isomaltulose, trehalulose and isomaltose were obtained from Südzucker.

2.5. Crystallization

Automated crystallization screening was carried out using sitting-drop vapour diffusion in 96-well plates (Greiner) using a Mosquito nanolitre pipetting robot (TTP Labtech) and The Classics and The PEGs Screening Suites from Nextal (Nextal Biotechnologies, Montreal, Quebec, Canada). Drops of 200 nl protein solution and 200 nl reservoir solution were equilibrated over 100 μ l reservoir solution. Crystals of SmuA appeared within two weeks at 290 K from a reservoir solution containing 0.2 M lithium citrate and 20% (w/v) PEG 3350. These crystals grew to dimensions of 400 $\times$ 100 $\times$ 50 μ m (Fig. 1) and were cryoprotected prior to data collection by a 1 min soak in mother liquor containing 20% (v/v) ethylene glycol.

2.6. X-ray data collection and processing

Data were collected to 1.95 Å resolution from a single crystal at 100 K using a MAR CCD detector on ESRF (European Synchrotron Radiation Facility, Grenoble, France) beamline BM30A.

Diffraction data were processed and scaled using programs from the XDS package (Kabsch, 1993).

Figure 1
Typical native crystals of SmuA from *P. rubrum*.

Table 1

Data-collection statistics for native SmuA.

Values in parentheses are for the highest resolution shell (2.05–1.95 Å).

Synchrotron-radiation source	BM30A, ESRF, Grenoble
Detector	MAR CCD
Wavelength (Å)	0.9803
Data-collection temperature (K)	100
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	$a = 61.6$, $b = 81.4$, $c = 135.6$
Resolution range (Å)	19.9–1.95
Completeness (%)	95.9 (96.2)
Redundancy	3.9
Total No. of reflections	190217
No. of unique reflections	48471
$R_{\text{sym}}^{\dagger}$ (%)	7.2 (31.5)
$\langle I/\sigma(I) \rangle$	13.2 (4.7)

$\dagger R_{\text{sym}} = 100 \sum_{hkl} \sum_i |I(hkl)_i - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I(hkl)$, where $I(hkl)$ are the intensities of symmetry-related reflections and $\langle I(hkl) \rangle$ is the average intensity over all observations.

3. Results

The crude cell-free extract containing the 67.3 kDa enzyme SmuA exhibits a specific activity of 40 U mg⁻¹. Cation-exchange chromatography was used to purify SmuA owing to its elevated isoelectric point being higher than 9 (Watzlawick *et al.*, in preparation). Once purified (by more than tenfold) and homogeneous, SmuA showed a specific activity of 500 U mg⁻¹ and a K_m for sucrose of 32 mM. The main product of the reaction with 100 mM sucrose at 298 K was identified to be isomaltulose (82%); minor products were trehalulose (8%) and monosaccharides (5% glucose, 5% fructose).

Variation in the temperature as well as in the sucrose concentration alters the product composition. When using a 1.16 M sucrose solution, the products obtained were 2% glucose, 3% fructose, 1% isomaltose, 9% trehalulose and 85% isomaltulose. Hence, for industrial isomaltulose production 1.16 M sucrose is used and the conversion temperature is 293 K.

Extensive automated crystallization trials have resulted in crystals suitable for X-ray diffraction experiments. Data-collection statistics for SmuA crystals are given in Table 1.

When assuming the presence of one SmuA molecule in the asymmetric unit, the Matthews coefficient V_M is 2.5 Å³ Da⁻¹ and the solvent content is 51% (Matthews, 1968).

The native structure of SmuA was solved by molecular replacement using the program *AMoRe* (Navaza, 2001) and the full refined structure without water molecules of the isomaltulose synthase PalI from *Klebsiella* sp. LX3 (PDB code 1m53), with which SmuA displays 76.3% sequence identity, as a search model (Zhang *et al.*, 2003).

A unique solution which located the SmuA molecule in the asymmetric unit was obtained with a correlation coefficient of 53.9%

and an R factor of 40.0% using diffraction data in the resolution range 15–3.5 Å. Refinement of the structure is currently in progress.

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