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Expression, purification, crystallization and preliminary X-ray crystallographic studies of the trehalulose synthase MutB from *Pseudomonas mesoacidophila* MX-45

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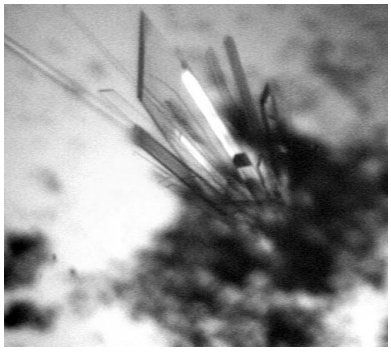


The trehalulose synthase (MutB) from *Pseudomonas mesoacidophila* MX-45, belonging to glycoside hydrolase family 13, catalyses the isomerization of sucrose to trehalulose (α-D-glucosylpyranosyl-1,1-D-fructofuranose) and isomaltulose (α-D-glucosylpyranosyl-1,6-D-fructofuranose) as main products and glucose and fructose in residual amounts from the hydrolytic reaction. To date, a three-dimensional structure of a sucrose isomerase that produces mainly trehalulose, as is the case for MutB, has been lacking. Crystallographic studies of this 64 kDa enzyme have therefore been initiated in order to contribute to the understanding of the molecular basis of sucrose decomposition, isomerization and of the selectivity of this enzyme that leads to the formation of different products. The MutB protein has been overexpressed, purified and crystallized using the hanging-drop vapour-diffusion method. Two different crystal forms have been obtained: one diffracts X-rays to 1.6 Å resolution using synchrotron radiation and belongs to space group $P1$, with unit-cell parameters a = 63.8, b = 72.0, c = 82.2 Å, α = 67.5, β = 73.1, γ = 70.8°, while the other form diffracts to 1.8 Å resolution using synchrotron radiation and belongs to space group $P2_1$, with unit-cell parameters a = 63.7, b = 85.9, c = 119.7 Å, β = 97.7°. A molecular-replacement solution has been found using the structure of the isomaltulose synthase (PalI) from *Klebsiella* sp. LX3 as a search model.

1. Introduction

MutB is a sucrose isomerase (EC 5.4.99.11) found in *Pseudomonas mesoacidophila* MX-45. It catalyses the isomerization of sucrose (α-D-glucosylpyranosyl-1,2-β-D-fructofuranoside) to trehalulose (α-D-glucosylpyranosyl-1,1-D-fructofuranose) and isomaltulose (α-D-glucosylpyranosyl-1,6-D-fructofuranose) as main products and glucose and fructose in residual amounts from the hydrolytic reaction (Miyata *et al.*, 1992). Sucrose is the most common sugar produced/accumulated by plants. Large quantities of this disaccharide accumulate in the edible parts of some plants, making it the most abundant natural sweetener in foods. The high available caloric content of sucrose and the ease by which it is metabolized by the microbes that cause dental caries has stimulated interest in other naturally occurring sweeteners (Hamada, 2002). Two of the most promising compounds for reducing both caloric uptake and dental caries are the isomers of sucrose: isomaltulose (also commonly named palatinose) and trehalulose. Isomaltulose is 30% as sweet as sucrose and can readily be crystallized and is used as a solid additive (Cheetham, 1982). Trehalulose is 70% as sweet as sucrose and is highly water-soluble (Ooshima *et al.*, 1991), making it particularly useful in highly sweetened foods such as jellies and jams. Furthermore, they exhibit very similar physical and organoleptic properties to sucrose, but are believed to be non-cariogenic, with lower rates of monosaccharides being released into the blood. These properties have aroused much industrial interest for their application as food ingredients.

Several microorganisms have been found to produce these two functional isomers of sucrose. It has been shown that the product composition varies depending on the bacterial strain used: *Protaminobacter rubrum* (Mattes *et al.*, 1998), *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) and *K. planticola* CCRC 19112 (Huang *et al.*, 1998) produce mainly isomaltulose (75–85%), whereas

Agrobacterium radiobacter MX-332 (Nagai-Miyata *et al.*, 1993) and *Pseudomonas mesoacidophila* MX-45 (Miyata *et al.*, 1992; Nagai *et al.*, 1994) cells are known to convert sucrose mainly to the isomer trehalulose (~90%). The reactions of these enzymes are strongly influenced by temperature.

Structural information on these enzymes is quite limited. In fact, only one three-dimensional structure of a sucrose isomerase has been reported, namely that of the isomaltulose synthase PalI from *Klebsiella* sp. LX3, recently solved to 2.2 Å resolution. This structure suggested that a unique RLDRD motif in the proximity of the active site is responsible for sucrose isomerization and influences product specificity (Li *et al.*, 2003; Zhang *et al.*, 2003).

The mature MutB protein consists of a single polypeptide chain with 557 amino-acid residues and a molecular weight of 64 kDa. Based on amino-acid sequence similarities, the enzyme has been classified into glycoside hydrolase family 13 (Mattes *et al.*, 1998) together with PalI, with which it displays 65.4% sequence identity. This family mainly contains polysaccharide-degrading enzymes such as α-amylases.

The MutB enzyme is stable at pH values between 5.1 and 6.7 and below temperatures of 313 K. The optimal pH and temperature for enzymatic activity based on sucrose decomposition are pH 5.8 and 313 K, respectively. Optimum conditions for trehalulose production are pH 5.5–6.5 at 293 K, with a yield of 91% for the production of trehalulose from a 20–40% sucrose solution (Nagai *et al.*, 1994). As a consequence of this product specificity and by analogy to PalI, MutB is henceforth referred to as a trehalulose synthase. We have cloned and expressed the trehalulose synthase *mut*B gene from *P. mesoacidophila* MX-45 in *Escherichia coli* (Watzlawick *et al.*, in preparation). The recombinant enzyme was overexpressed in *E. coli* and purified.

This is the first trehalulose synthase to be crystallized and the determination of the three-dimensional structure of this enzyme, which produces mainly trehalulose, will allow detailed analysis and comparative studies with the three-dimensional structure of PalI, which predominantly forms isomaltulose. These observations should contribute to a better understanding of the reaction mechanism and to determining the features involved in product specificity. It will be particularly interesting to examine the enzyme active centre in which the corresponding isomerization motif, as referred to in the PalI structure, is not conserved between the two enzymes.

2. Materials and methods

2.1. Purification of the trehalulose synthase MutB

2.1.1. MutB protein expression and purification. The *mut*B gene from *P. mesoacidophila* MX-45 was cloned into the L-rhamnose-inducible expression vector pJOE2702 (Volff *et al.*, 1996) to create pHWG 315 (Watzlawick *et al.*, in preparation) and transformed into *E. coli* JM109.

E. coli JM109 cells harbouring pHWG 315 were grown at 310 K to an OD_{600} of 0.3 in 200 ml 2×YT medium containing 100 µg ml^{-1} ampicillin. MutB production was induced upon addition of rhamnose [to a final concentration of 0.1%(*w*/*v*)] and cultivation continued for 4 h at 303 K. Cells were harvested by centrifugation (Heraeus Labofuge, 2780*g*, 10 min), washed and resuspended in 20 ml 10 m*M* calcium acetate buffer pH 7.5. Cells were lysed by passing them 2–3 times through a French press cell (Aminco, SLM Instruments Inc.) at 6.9 MPa and cell debris was removed by centrifugation (Sorvall RC5B Plus, 11 950*g*, 15 min, 277 K). The proteins in the supernatant were fractionated by fast protein liquid chromatography (FPLC, Amersham Biosciences) with an anion-exchange column. 50 mg was loaded onto a Superformance Fractogel EMD-DMAE 150-10 column (Merck) equilibrated with 10 m*M* calcium acetate buffer pH 7.5 and eluted with a NaCl gradient (0–1 *M*) in the same buffer. 1 ml fractions were collected and tested for MutB activity. Active fractions were combined and further purified on a Mono-Q HR 5/5 column (Amersham Biosciences) applying the same buffer gradient. Active fractions were tested further for purity by SDS–PAGE.

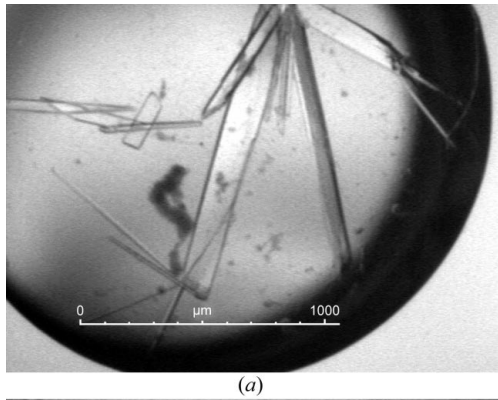
The fractions that contained the purified protein were combined, dialyzed against 10 m*M* calcium acetate buffer pH 6.5 and concentrated to 7 mg ml^{-1} by ultrafiltration on Centricon YM-10 devices (Millipore).

2.1.2. Enzyme assay. Trehalulose synthase assays were carried out at 298 K in 10 m*M* calcium acetate buffer pH 5.5 in the presence of 100 m*M* sucrose. Enzyme activities are expressed as micromoles of trehalulose formed per minute and millilitre. The concentration of trehalulose was measured by HPLC analysis.

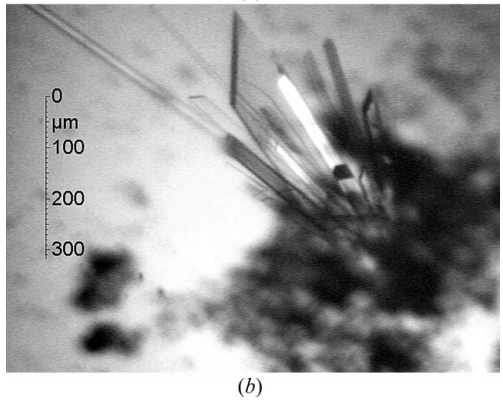
During the purification procedure, MutB activity was tested qualitatively for glucose formation during sucrose conversion, performing a 96-well microplate-adapted GOD/perit-test® (Roche). The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin as a standard.

2.2. Crystallization

Initial crystallization trials were performed using the sparse-matrix sampling method (Jancarik & Kim, 1991). The screening was set up using the hanging-drop vapour-diffusion technique in 24-well DropGuard™ crystallization plates and crystallization conditions were derived employing the *PEGs*™ Screening Suite (Nextal Biotechnologies, Montreal, Quebec, Canada). 2 µl droplets were



(*a*)



(*b*)

Figure 1
Typical crystals of MutB from *P. mesoacidophila* MX-45 grown under (*a*) crystallization condition 1 and (*b*) crystallization condition 2.

equilibrated against 500 µl reservoir solution at 277, 288, 292 and 298 K.

Initial MutB crystallization conditions in 15%(*w*/*v*) PEG 20 000 and 0.1 *M* Tris–HCl buffer pH 8.5 gave rise to a shower of small needle-shaped crystals at each temperature, which grew overnight.

These conditions were further optimized in order to reduce nucleation in the drops and to improve the crystal size and quality.

Crystals suitable for X-ray diffraction studies (Fig. 1*a*) were obtained after approximately one week in 12.5%(*w*/*v*) PEG 20 000 and 0.1 *M* Tris–HCl buffer pH 8.5 at 290 K. The protein:mother liquor ratio was 1:1 in the 2 µl drops and the initial protein concentration was 7 mg ml^{-1}.

Another set of conditions was found which led to crystals with higher diffraction power (Fig. 1*b*). The hanging-drop vapour-diffusion technique was again used at 290 K and crystals appeared after 3 d in drops in which 1 µl protein solution (7 mg ml^{-1}) was mixed with 0.8 µl well solution containing 0.1 *M* Tris–HCl pH 8.5 and 13%(*w*/*v*) PEG 20 000 with 0.2 µl 0.1 *M* L-cysteine as an additive employing Additive Screen 2 from Hampton Research (Laguna, CA, USA).

These two optimized conditions will henceforth be referred to as conditions 1 and 2, respectively.

Crystals grew to typical dimensions of 1.0 × 0.2 × 0.03 mm within 2 d but were unstable, tending to disappear after approximately one month in the drop.

The crystals were cryoprotected prior to the diffraction experiments by rapid soaking in mother liquor supplemented with 20% glycerol.

2.3. X-ray data collection and processing

Diffraction data were collected under cryoconditions (100 K) using two crystals (one from each of the crystallization conditions) at the ID14-EH4 beamline at the ESRF (European Synchrotron Radiation Facility, Grenoble, France) on an ADSC Quantum 4 detector.

The first data set (corresponding to a crystal grown under condition 1) was collected at a wavelength of 0.95 Å; the crystal-to-detector distance was 150 mm, the oscillation range per image angle was 1°, the total oscillation angle was 180° and the exposure time per image was 3 s (Fig. 2*a*).

The second data set (corresponding to a crystal grown under condition 2) was collected at a wavelength of 1.07 Å; the crystal-to-detector distance was 115 mm, the oscillation range per image angle was 1°, the total oscillation angle was 180° and the exposure time per image was 10 s (Fig. 2*b*).

Determination of unit-cell parameters and the integration of reflections were performed with the program *MOSFLM* (Leslie, 1991), whereas scaling was performed with the program *SCALA* from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

3. Results

E. coli JM 109 harbouring pHWG315 was used as a source of recombinant enzyme.

Expression of the recombinant MutB enzyme was carried out by induction with rhamnose. Harvesting and disruption of the cells yielded active enzyme in the supernatant with a specific activity of 80 U mg^{-1}. After purification by anion-exchange chromatography, a fivefold enrichment of MutB was achieved. SDS–PAGE analysis displayed a predominant MutB protein band and some minor bands. After a second purification step on MonoQ, the final product was purified more than tenfold, with a 30% final yield. Purified MutB exhibited a specific activity of 900 U mg^{-1} and its homogeneity was confirmed by SDS–PAGE.

The MutB enzyme was subjected to crystallization trials and after optimization of the crystallization parameters two different conditions were found. Diffraction data were collected on crystals from both conditions and interestingly the two crystals tested belonged to distinct crystal systems, being triclinic and monoclinic. Condition 1

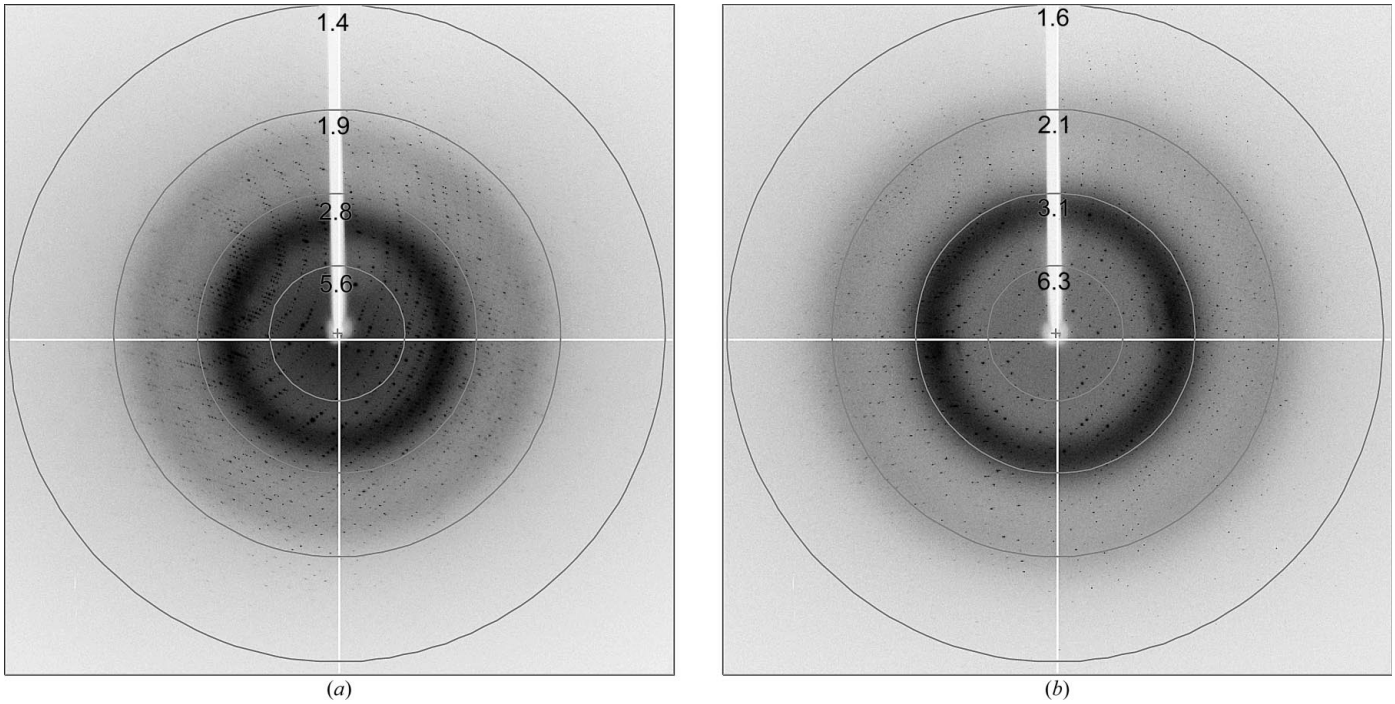


Figure 2
A 1° oscillation image collected at ESRF at 100 K on crystals from conditions 1 (*a*) and 2 (*b*).

Table 1
X-ray diffraction data.

Values in parentheses are for the highest resolution shell (1.9–1.8 Å for crystals from condition 1 and 1.7–1.6 Å for those from condition 2).

	Condition 1	Condition 2
Crystallization condition	0.1 *M* Tris–HCl pH 8.5, 12.5%(*w*/*v*) PEG 20 000	0.1 *M* Tris–HCl pH 8.5, 13%(*w*/*v*) PEG 20 000, 0.01 *M* L-cysteine
Synchrotron-radiation source	ID14-EH4 ESRF (Grenoble)	ID14-EH4 ESRF (Grenoble)
Detector	ADSC Quantum 4 CCD	ADSC Quantum 4 CCD
Wavelength (Å)	0.95	1.072
Data-collection temperature (K)	100	100
Space group	$P2_1$	$P1$
Unit-cell parameters (Å, °)	a = 63.7, b = 85.9, c = 119.7, $\alpha = \gamma = 90$, β = 97.7	a = 63.8, b = 72.0, c = 82.2, α = 67.4, β = 73.1, γ = 70.8
Resolution range (Å)	33.0–1.8	38.9–1.6
Completeness (%)	99.1 (99.1)	95.0 (95.0)
Multiplicity	3.4 (3.2)	1.9 (2.0)
Total No. reflections	389148	294913
No. unique reflections	112413	147205
$R_{\rm sym}$† (%)	7.6 (25.0)	8.6 (27.7)
$I/\sigma(I)$	6.1 (2.9)	4.7 (2.2)

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

crystals diffract X-rays to a resolution limit of 1.8 Å. Analysis of the diffraction pattern showed that these crystals belong to space group $P2_1$, with unit-cell parameters a = 63.7, b = 85.9, c = 119.7 Å, β = 97.7°. Condition 2 crystals diffract to a resolution limit of 1.6 Å. In this case, the space group was unambiguously determined to be $P1$ and the refined unit-cell parameters are a = 63.8, b = 72.0, c = 82.2 Å, α = 67.5, β = 73.1, γ = 70.8°.

Additional diffraction experiments have shown that the respective crystal forms are independent of the crystallization conditions, since the two forms coexist in the drops from each condition.

Assuming a molecular weight of 64 kDa (as deduced from the amino-acid sequence) and two molecules in the asymmetric unit gives a solvent content of 51% and a volume-to-weight ratio $V_{\rm M}$ of 2.5 Å^3 Da^{-1} for both crystal forms (Matthews, 1968).

Data-collection statistics for the two sets of data are reported in Table 1.

The molecular-replacement method using the *AMoRe* program (Navaza, 2001) was employed to solve the phase problem. For both crystal forms of MutB, the refined coordinates of the isomaltulose synthase PalI from *Klebsiella* sp. LX 3 (PDB code 1m53), which displays 65.4% sequence identity to MutB, were used as a search model (Li *et al.*, 2003; Zhang *et al.*, 2003).

Diffraction data from the first crystal form (monoclinic) in the resolution range 15–3.5 Å were used in the molecular-replacement search and a unique solution which located the two molecules in the asymmetric unit was obtained with a correlation coefficient of 59.3% and an R factor of 46.4%.

The same procedure was performed with the data from the second crystal form (triclinic) and in this case a correlation coefficient of 66.6% and an R factor of 32.0% were found.

Refinement of the two structures is currently in progress.

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