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Crystallization and preliminary X-ray diffraction analysis of the invertase from *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae invertase (ScInv) is an enzyme encoded by the *SUC2* gene that releases β-fructose from the nonreducing termini of various β-D-fructofuranoside substrates. Its ability to produce 6-kestose by transglycosylation makes this enzyme an interesting research target for applications in industrial biotechnology. The native enzyme, which presents a high degree of oligomerization, was crystallized by vapour-diffusion methods. The crystals belonged to space group $P3_121$, with unit-cell parameters a = 268.6, b = 268.6, c = 224.4 Å. The crystals diffracted to 3.3 Å resolution and gave complete data sets using a synchrotron X-ray source.

1. Introduction

Fructooligosaccharides (FOS) have been increasingly used in functional foods and pharmaceutical formulations owing to their prebiotic properties and other health-enhancing roles. FOS are composed of several fructose residues connected through β-glycosidic linkages and carry a terminal glucose residue at the reducing end. They are synthesized by 15–20% of flowering plants and also by a number of bacteria and fungi. These compounds have been related to carbohydrate storage and processes of drought and freezing tolerance in plants. In addition, FOS act as prebiotics, which are nondigestible food ingredients that improve the health of consumers by the stimulation of the growth of beneficial bifid bacteria in the digestive tract (Gibson & Roberfroid, 1995).

Enzymes that hydrolyse sucrose, referred to as invertases or β-fructofuranosidases (EC 3.2.1.26), catalyse the release of β-fructose from the nonreducing termini of various β-D-fructofuranoside substrates. Although the main function of these enzymes is hydrolytic, they also have transglycosylation activity that results in FOS biosynthesis. A wide chemical spectrum of FOS can be obtained by means of phylogenetically related microbial fructosyltransferases. Invertases contain two acidic residues (Asp or Glu) that act as nucleophile and acid/base catalysts and are responsible for the cleavage of glycosidic bonds. According to their amino-acid sequence (Coutinho & Henrissat, 1999), they are classified into family 32 of the glycosyl hydrolases (GHs), which is included in the GH-J clan together with the GH68 (inulosucrase) family. To date, three-dimensional structures of β-fructofuranosidases from the bacterium *Thermotoga maritima* (Alberto *et al.*, 2004) and the yeast *Schwanniomyces occidentalis* (Polo *et al.*, 2009; Alvaro-Benito *et al.*, 2010), as well as of exoinulinase from the fungus *Aspergillus niger* (Nagem *et al.*, 2004) and of the fructan exohydrolase together with the cell-wall invertase from the plants *Chicorium intybus* (Verhaest *et al.*, 2005) and *Arabidopsis thaliana* (Lammens *et al.*, 2008) have been solved. These enzymes are all members of the GH32 family. Furthermore, the structures of fructosyltransferases (FTs) from *Aspergillus japonicus* and *Pachysandra terminalis* have also been reported (Chuankhayan *et al.*, 2010; Lammens *et al.*, 2012). These studies have shown a common bimodular folding for the GH32 family, with an N-terminal fivefold β-propeller catalytic domain and a C-terminal β-sandwich domain with a still unknown function. Interestingly, this β-sandwich

domain has been shown to be involved in the dimerization of *S. occidentalis* β-fructofuranosidase, being directly implicated in shaping its active site (Alvaro-Benito *et al.*, 2010).

Currently, *Aspergillus* enzymes are the main industrial producers of FOS (Ghazi *et al.*, 2007), giving a mixture of FOS with an inulin-type structure containing β-(2→1)-linked fructose oligomers (^{1}F-FOS; 1-kestose, nystose or ^{1}F-fructofuranosylnystose). However, there is an interest in the development of novel molecules with improved prebiotic and physiological properties. In this context, β-(2→6)-linked FOS, in which the link exists between two fructose units (^{6}F-FOS; 6-kestose) or between a fructose and a glucosyl moiety (^{6}G-FOS; neokestose, neonystose and neofructofuranosylnystose), have enhanced prebiotic properties compared with commercial FOS (Marx *et al.*, 2000; Kilian *et al.*, 2002).

The β-fructofuranosidase (also called invertase) from *Saccharomyces cerevisiae* (ScInv) has been characterized and engineered to improve its transferase activity and to identify the enzymatic determinants for product specificity (Lafraya *et al.*, 2011). The enzyme shows a marked specificity for sucrose and also produces several FOS by transfructosylation, with the major product being the prebiotic trisaccharide 6-kestose. Structural analysis of ScInv is necessary to understand and improve its biological function and biotechnological potential. In this study, the purification, crystallization and preliminary X-ray crystallographic analysis of ScInv are reported.

2. Experimental procedures

2.1. Protein expression and purification

The gene encoding ScInv was cloned in vector pQE80L to generate a plasmid designated *SUC2*-pQE and expressed in *Escherichia coli* Rosetta2 cells as described in a previous publication (Lafraya *et al.*, 2011; Fig. 1*a*). The resulting His-tagged protein was purified by nickel-affinity chromatography (Figs. 1*b* and 1*c*). The eluted fractions containing ScInv (>95% purity according to SDS–PAGE analysis and Coomassie Blue staining) were dialysed (1/10 000) against 0.05 *M* Tris–HCl buffer pH 7 and were concentrated by ultracentrifugation through a membrane with a 20 000 cutoff (Pierce).

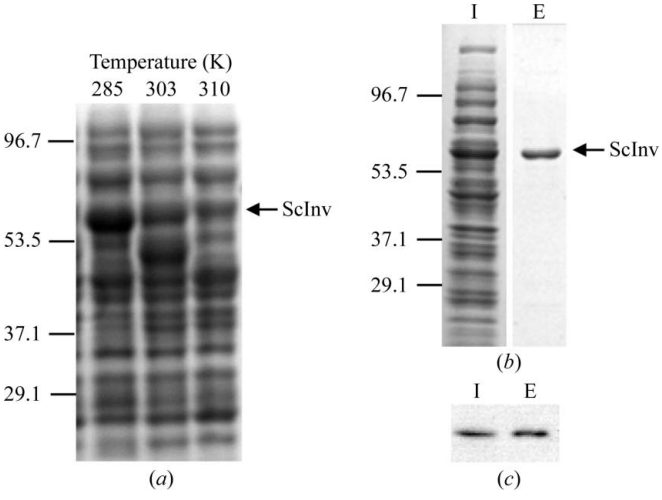

Figure 1
Analysis of the expression and purification of ScInv by SDS–PAGE. (*a*) Optimum conditions for the expression of the *SUC2* gene were studied after induction with 5 m*M* IPTG at 285, 303 or 310 K for 14, 5 or 2 h. The mobilities of molecular-mass standards and their sizes in kDa are indicated on the left. (*b*) ScInv purification using a nickel-affinity column. The degree of purification of the dialysed eluted protein (E) compared with the soluble extract injected into the column (I) is shown. (*c*) Identification of the heterologous protein ScInv from the injected (I) and eluted (E) samples by Western-blot analysis with an anti-His antibody (sc803; Santa Cruz Biotech).

2.2. Crystallization

Initial crystallization conditions for the purified protein samples were investigated by high-throughput techniques using a NanoDrop robot (Innovadyne Technologies Inc.) with the commercially available Crystal Screen, Crystal Screen 2, Index and SaltRx kits from Hampton Research and The PACT Suite and The JCSG+ Suite from Qiagen. The assays were carried out using the sitting-drop vapour-diffusion method at 291 K in Innovaplate SD-2 microplates (Innovadyne Technologies Inc.) by mixing 0.25 µl protein solution with 0.25 µl precipitant solution and equilibrating against 60 µl well solution. Several magnesium formate-containing solutions gave flake-like crystals and these conditions were optimized through further sitting-drop and hanging-drop experiments by mixing 1 µl protein solution with 1 µl precipitant solution (and changing these proportions) and equilibrating against 500 µl well solution in Cryschem plates (Hampton Research).

2.3. Data collection and processing

All crystals were soaked in precipitant solution containing an additional 30% glycerol before being flash-cooled to 100 K. Crystals were tested using in-house facilities (a rotating-anode generator with Cu *Kα* radiation) and synchrotron radiation. The best diffraction data set was collected at the ESRF, Grenoble, France. A total of 240 images were collected (1° rotation) with 1 s exposure time. The data sets were processed using the programs *iMOSFLM* (Battye *et al.*, 2011) and *SCALA* (Evans, 2006) as distributed in the *CCP*4 suite (Winn *et al.*, 2011).

3. Results and discussion

In the preliminary crystallization screening test, flake-like crystals were only obtained using magnesium formate solutions and were checked with Izit dye (Fig. 2*a*). The crystallization conditions were refined by varying several parameters such as the pH, temperature, sample size and the type/concentration of precipitant agent and by the use of additives. The addition of 2–4% PEG 3350 to 0.25–0.9 *M* magnesium formate and pH values from 5.5 to 6.5 yielded microcrystals (Fig. 2*b*) that failed to diffract on various synchrotron beamlines. Different crystallization methods were then tested, such as agarose-containing sitting drops (0.1–0.3%), microbatch-under-oil, streak-seeding and microseeding. Only the use of the microseeding technique increased the crystal size and allowed a preliminary diffraction pattern to be collected to 5 Å resolution using synchrotron radiation. Following this strategy, a new round of additives were checked. Rod-shaped crystals were obtained from precipitant containing 4–5% MPD (2-methyl-2,4-pentanediol; Fig. 2*c*). The quality of these crystals was further improved by incubating the protein sample with guanidinium chloride before setting up the crystallization drops. This produced large and regular crystals (Fig. 2*d*), but all attempts to increase the resolution of their diffraction pattern were unsuccessful.

The best invertase crystals were grown by mixing 11 mg ml^{-1} protein solution with 0.6 *M* magnesium formate, 3% PEG, 0.1 *M* bis-tris pH 6.5, 5% MPD, 1 m*M* tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 0.33 *M* guanidinium chloride (Fig. 2*d*). These crystals finally allowed a data set to be collected to 3.3 Å resolution on the ID23-1 beamline at the ESRF, Grenoble, France (Fig. 3). The data-collection statistics are given in Table 1. The unit-cell parameters

were a = 268.6, b = 268.6, c = 224.4 Å in space group $P3_121$. As calculated from its sequence analysis, the molecular weight of the monomer was 58.545 kDa (Fig. 1); therefore, assuming a reasonable Matthews coefficient value within the range 4.87–2.44 Å^3 Da^{-1} (Matthews, 1968), corresponding to 75–50% solvent content, the presence of eight to 16 molecules in the asymmetric unit would be expected. We investigated the local symmetry relating the subunits in the asymmetric unit using *POLARRFN* (Kabsch, 1976) from the *CCP*4 package. Several self-rotation functions were computed in the resolution range 15–3.5 Å, with Patterson vectors with a radius of integration of 25–40 Å. A stereographic projection of the κ = 180° section, shown in Fig. 4, revealed the presence of noncrystallographic twofold symmetry parallel to the c axis, which is compatible with the presence of one or two functional octamers in the asymmetric unit of the crystals, which would therefore present internal NCS 222 point symmetry.

Structure determination is in progress using the fructosyltransferase from *S. occidentalis* (PDB code 3f3k; Kuznetsova *et al.*, 2010), which shows 46% sequence identity. This model was used as

Table 1
Data-collection statistics for the ScInv crystal.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.067
Source	ESRF
Beamline	ID23-1
Temperature (K)	100
Detector	ADSC Q315R
Space group	$P3_121$
Unit-cell parameters (Å)	$a = b$ = 268.6, c = 224.4
Resolution limits (Å)	56.31–3.30 (3.48–3.30)
Total No. of reflections	873783 (134865)
No. of unique reflections	137870 (17424)
Multiplicity	6.4 (5.0)
$R_{\rm merge}$†	0.097 (0.377)
Mean $I/\sigma(I)$	8.1 (2.0)
Wilson B factor (Å^2)	51
Matthews coefficient (Å^3 Da^{-1})	4.87
Solvent content (%)	74.8
Monomers in asymmetric unit	8

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observed amplitude of reflection hkl and $\langle I(hkl)\rangle$ is the mean amplitude of measurements of reflection hkl.

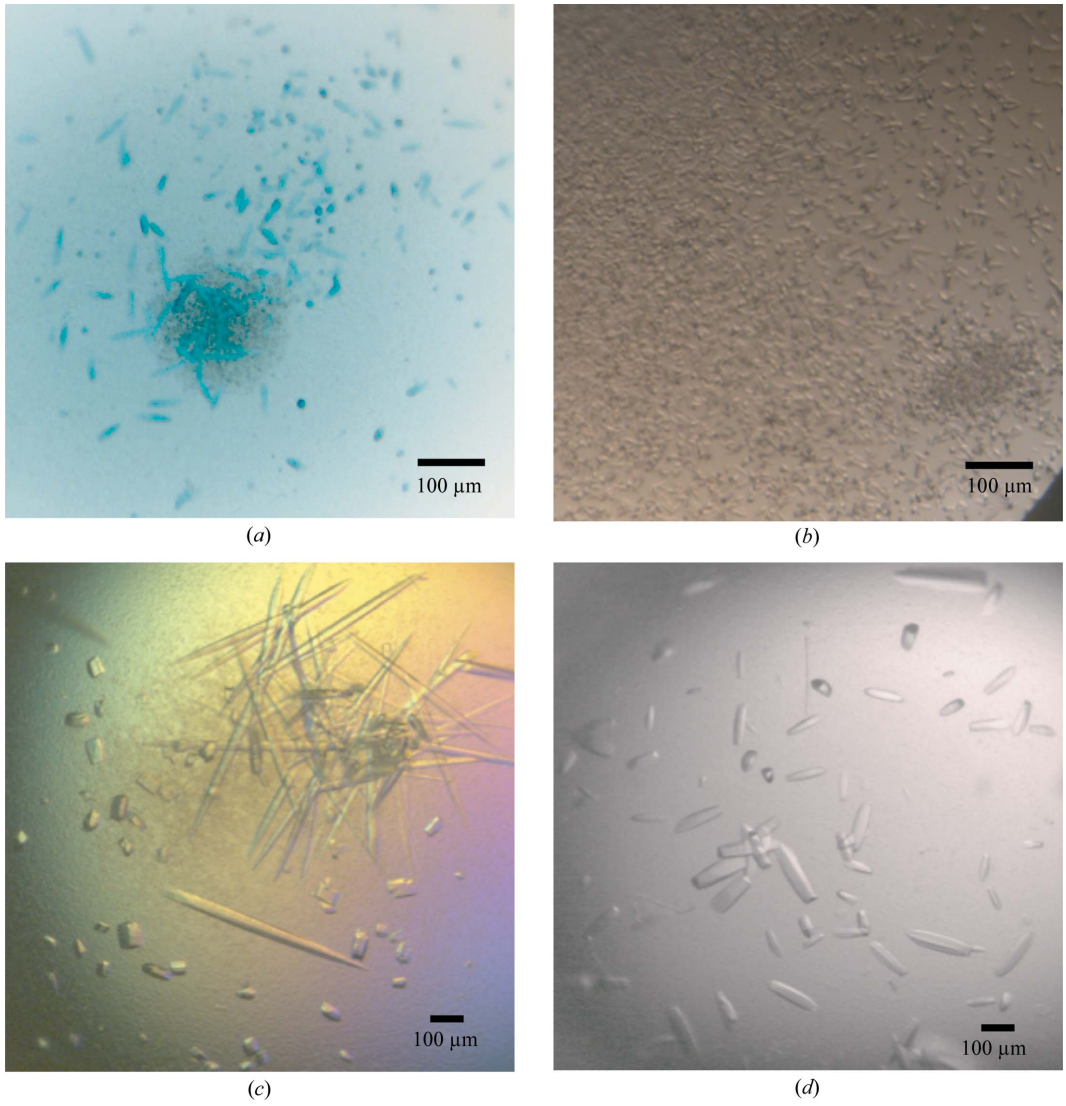

Figure 2
ScInv crystals obtained from (*a*) 0.5 *M* magnesium formate, 0.1 *M* bis-tris pH 5.5 (dyed with Izit), (*b*) 0.9 *M* magnesium formate, 3% PEG 3350, 0.1 *M* bis-tris pH 6.5, (*c*) 0.6 *M* magnesium formate, 5% PEG 3350, 0.1 *M* bis-tris pH 6.5, 5% MPD, 1 m*M* TCEP and (*d*) 0.6 *M* magnesium formate, 3% PEG 3350, 0.1 *M* bis-tris pH 6.5, 5% MPD, 1 m*M* TCEP, 0.33 *M* guanidinium chloride (drop ratio 2:1); these were the best crystals and were used for data collection.

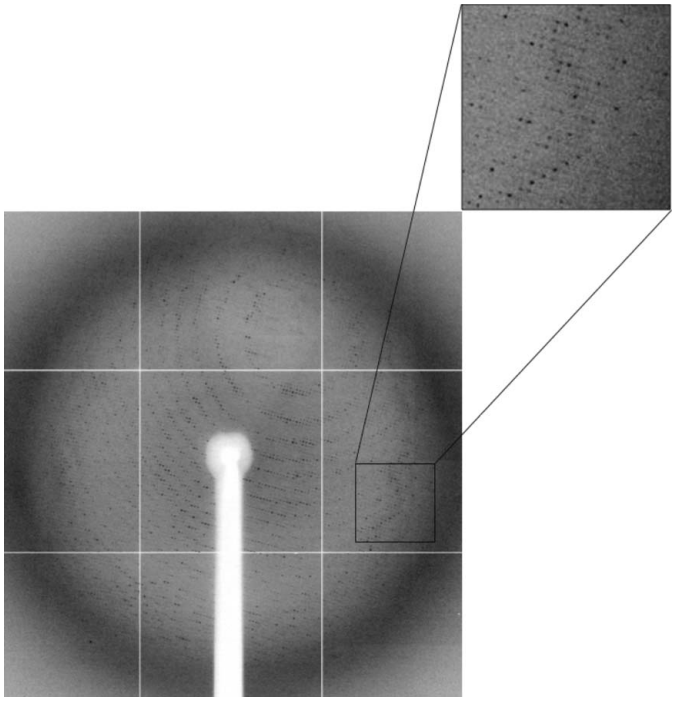

Figure 3
X-ray diffraction pattern of ScInv obtained using a synchrotron source. The maximum observed resolution is 3.3 Å.

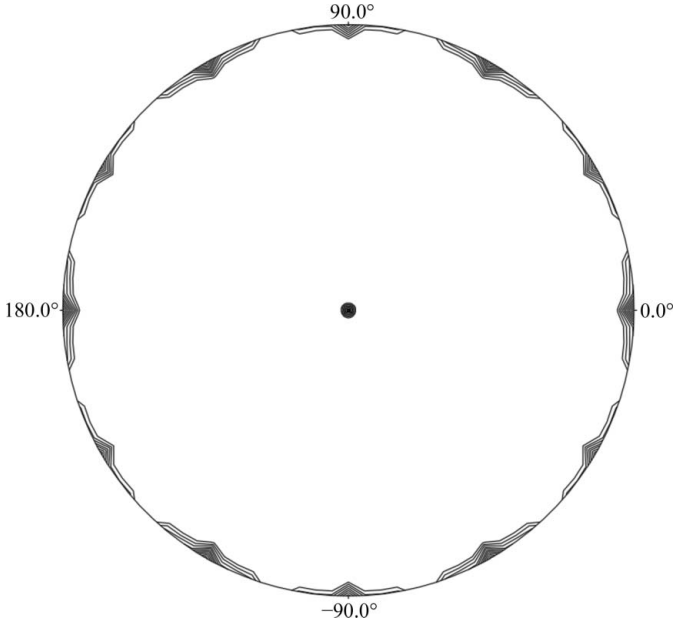

Figure 4
Plot of the $\kappa = 180^\circ$ section of the self-rotation function of ScInv crystals. The view is down the *c* axis; $\varphi = 0$ and $\varphi = 90^\circ$ correspond to the *a* and *b** axes, respectively.

a template in the molecular-replacement method with data to 3.5 Å resolution using *MOLREP* (Winn *et al.*, 2011). A solution containing eight molecules in the asymmetric unit has been found and preliminary structural refinement of this octamer with *REFMAC* (Murshudov *et al.*, 2011) decreased the *R* factor to 0.32 (R_{free} = 0.34). Model building and further refinement are ongoing.

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