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Expression, crystallization and preliminary X-ray crystallographic studies of *Arthrobacter globiformis* inulin fructotransferase

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A recombinant form of *Arthrobacter globiformis* inulin fructotransferase (DFAIII-producing) has been overexpressed in *Escherichia coli* and purified to homogeneity. Crystals were obtained at 293 K by the hanging-drop vapour-diffusion technique using 0.1 M Na HEPES pH 7.5 buffer containing 1.5 M lithium sulfate as a precipitant. Crystals of the recombinant wild-type enzyme diffracted to better than 1.5 Å at 100 K using a synchrotron-radiation source at the Photon Factory. The crystal belonged to space group $R32$, with unit-cell parameters $a = b = 92.02$, $c = 229.82$ Å in the hexagonal axes. Assuming the presence of one molecule in the asymmetric unit, the V_M value for the crystal was 2.15 Å^3 Da^{-1}, indicating a solvent content of 42.8%. Selenomethionine-derivative crystals belonged to a different space group, $C2$, with unit-cell parameters $a = 159.32$, $b = 91.92$, $c = 92.58$ Å, $\beta = 125.06$. Matthews coefficient calculations suggested that the $C2$ selenomethionine-derivative crystal contained three molecules per asymmetric unit.

1. Introduction

Inulin is a polyfructan consisting of linearly β-2,1-linked fructose with a terminal sucrose residue. Inulin is abundantly found in plants such as chicory (*Cichorium intybus* L.) and Jerusalem artichoke (*Helianthus tuberosus* L.), where it serves as a storage polysaccharide. These inulo-oligosacchrides, produced by chemical or enzymic degradation, are of great interest to the food industry as a source of new sweeteners and dietary fibres. As representatives of typical inulin-degrading enzymes which can degrade inulin into fructose and linear inulo-oligosacchides, inulinases (EC 3.2.1.7) have been reported from *Aspergillus awamori* (Arand *et al.*, 2002) and some fungal species (Ettalibi & Baratti, 1987; Pessoni *et al.*, 1999). A different type of inulin-decomposing enzyme, namely inulin fructotransferase (DFAIII-producing; IFTaseIII; EC 2.4.1.93), produced by *Arthrobacter ureafaciens* has been reported (Uchiyama *et al.*, 1973, 1975). IFTaseIII catalyzes the degradation of inulin into di-D-fructofuranose 1,2′:2,3′-dianhydride (DFAIII) and a small amount of oligosaccharides. We have previously reported the enzymatic properties and gene structure of IFTaseIII from *Arthrobacter globiformis* C11-1 (Haraguchi, Kishimoto *et al.*, 1988). IFTaseIIIs have also been reported from different *Arthrobacter* species, *Arthrobacter* sp. H65-7 (Yokota *et al.*, 1991) and *A. ilicis* (Kawamura *et al.*, 1991), and the gene structure of one of

them has been reported (Sakurai *et al.*, 1997). The gene structure of IFTaseIII from *A. globiformis* C11-1 suggested that the mature enzyme has a molecular weight of 43.5 kDa, containing 410 amino acids after processing an N-terminal 40 amino-acid signal peptide (Haraguchi *et al.*, 2000). As an example of another type of inulin fructotransferase, we have also reported on the inulin fructotransferase (DFAI-producing; IFTaseI; EC 3.2.1.200) from *A. globiformis* S14-3 which produces di-D-fructofuranose 1,2′:2,1′-dianhydride (DFAI; Haraguchi, Seki *et al.*, 1988). Although IFTases have recently been classified into a new glycoside hydrolase family GH91 in the CAZy database (Coutinho & Henrissat, 1999; http://afmb.cnrs-mrs.fr/~cazy/CAZY/index.html), the three-dimensional structures of the IFTases and other fructan-hydrolyzing enzymes have not been determined. Such structural information is useful for understanding the reaction mechanism as well as designing mutant enzymes with high activities and stabilities. Except for one preliminary crystallization paper (Arand *et al.*, 2002), there has been no report of the three-dimensional structure of an IFTase or an inulin-decomposing enzyme. Since IFTases have only negligible similarity in amino-acid sequence with not only other carbohydrate-acting enzymes but also many other enzymes, an exhaustive database homology search has not revealed a suitable model for molecular replacement. Here, we report on the over-

expression, purification, crystallization and preliminary X-ray studies of *A. globiformis* IFTIaseIII.

2. Experimental and results

2.1. Expression and purification

A DNA fragment of mature *A. globiformis* IFTaseIII, without the signal sequence and flanked by 5′-*Nde*I and 3′-*Hin*dIII cleavage sequences, was amplified by PCR with *Pfu* Turbo DNA polymerase (Stratagene) from the plasmid pIF3-1 (Haraguchi *et al.*, 2000) containing the complete *A. globiformis* IFTaseIII gene (DDBJ accession No. AB009696). The amplification primers were 5′-ACTCATAT-GGCAGACGGCCAGCAAGGC-3′ and 5′-ACGAACGTTGGTCCGTCAGGGGGT-CGG-3′. The fragment was ligated into the *Nde*I/*Hin*dIII-digested pET25b expression vector (Novagen). The resulting vector contained the initiation methionine ATG codon for mature protein initiation, but did not contain a polyhistidine or other tag for purification. The native form of IFTaseIII was overexpressed in *Escherichia coli* strain BL21(DE3) grown in LB medium containing 100 µg ml^{-1} ampicillin at 310 K with shaking (200 rev min^{-1}). At an OD_{600} of 0.6, protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside

(IPTG) to a final concentration of 1 m*M*. The cells were harvested by centrifugation at 5000*g* and lysed using 1/100 volume of 10 mg ml^{-1} lysozyme and 1/10 volume of 1% Triton X-100, followed by sonication. The supernatant (140 ml) of the lysate was resuspended in 150 ml of 10 m*M* Tris–HCl pH 8.0 containing 1 m*M* EDTA. Purification of the expressed IFTaseIII was carried out by column chromatography using DEAE-Toyopearl (Tosoh; column dimensions 2.5 × 10 cm) and by gel filtration using Toyopearl HW55 (Tosoh; column dimensions 2.5 × 100 cm). The selenomethionine derivative of IFTaseIII was overexpressed using the selenomethionine autotroph *E. coli* strain B834plysS(DE3) (Novagen) in leMaster medium containing 25 mg l^{-1} seleno-methionine. The column purification was performed using an ÄKTA system (Amersham Biosciences). The supernatant of the cell lysate was chromatographed twice using a Hi-Trap Q column (5 ml) and further purified using a Mono Q HR 5/5 column and applying a linear NaCl gradient (0–0.5 *M*) in 10 m*M* Tris–HCl pH 8.0 containing 1 m*M* EDTA and 5 m*M* DTT. About 25 mg of the soluble wild-type form of IFTaseIII and 10 mg of selenomethionine derivative were purified, respectively, from 1 l of culture medium. The active fractions from each chromatographic procedure were combined and concentrated to an OD_{280} of 10 (∼20 mg ml^{-1}) using a Centricon YM-10 centrifugal filter (Millipore). Protein purity was checked by SDS–PAGE and the gel was stained using Coomassie brilliant blue (Fig. 1). The apparent molecular weight of the recombinant IFTaseIII was 43 kDa by SDS–PAGE, which agreed well with the calculated value of 43.5 kDa deduced from the nucleotide sequence. The molecular weight estimated by Superose 12(10/30) gel-filtration column chromatography (ÄKTA system, Amersham Biosciences) showed a molecular weight corresponding to a trimer (data not shown).

2.2. Crystallization

Screening for conditions suitable for crystallization was initially carried out using Crystal Screens I and II (Hampton Research). All crystallization trials were conducted using the hanging-drop vapour-diffusion technique by mixing 3 µl of protein solution (∼10 mg ml^{-1}) with 3 µl of precipitating solution on a siliconized cover glass and equilibrating against 0.7 ml of reservoir solution. Small crystals in the form of square plates grew in less than one month using a buffer system containing 1.5 *M* lithium

sulfate. After refinement of crystallization conditions, the optimal condition for crystallization was found to be a mixture consisting of 0.1 *M* Na HEPES pH 7.5 buffer containing 1.5 *M* lithium sulfate as a precipitant at 293 K for both the native IFTaseIII and its selenomethionine derivative. Wedge-shaped crystals (0.3 × 0.3 × 0.2 mm) were obtained within two weeks at 293 K for both enzymes as a unique form (Fig. 2).

2.3. X-ray data collection and analysis

Diffraction data were collected at beam-lines BL6A and BL6B of the Photon Factory, Tsukuba, Japan. Crystals were soaked in a cryoprotectant solution consisting of a mixture of 30%(*w/v*) treha-lose and a solution containing 1.5 *M* lithium sulfate in 0.1 *M* Na HEPES pH 7.5. The crystals were picked up using a fibre loop and flash-frozen in a stream of nitrogen gas at 100 K. Initial data were collected using a Quantum R4 CCD detector (ADSC) in 1.0° oscillation steps over a range of 180° (BL6A). Multi-wavelength anomalous dispersion (MAD) data were collected on BL6B using an R-AXIS IV^{++} imaging-plate X-ray detector (Rigaku) in 2.0° steps over a range of 360°. All data sets were processed and scaled using *DPS/MOSFLM* (Ross-mann & van Beek, 1999). The crystals of the recombinant wild-type form of *A. globiformis* IFTaseIII belonged to space group *R*32, with unit-cell parameters *a* = *b* = 92.02, *c* = 229.82 Å, and diffracted to beyond 1.5 Å resolution. Assuming the presence of one inulin fructotransferase molecule in the asymmetric unit gave a V_M value of 2.15 Å^3 Da^{-1}, indicating a solvent content of approximately 42.8%. Crystals of the sele-nomethionine derivative belonged to a different space group, *C*2, with unit-cell parameters *a* = 159.32, *b* = 91.92, *c* = 92.58 Å,
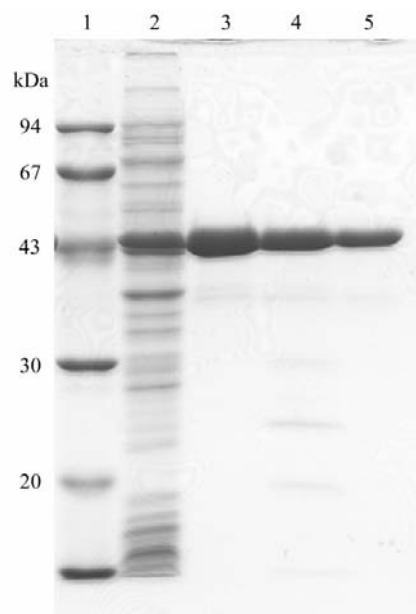

Figure 1
SDS–PAGE of overexpressed, recombinant *A. globiformis* IFTaseIII. Lane 1, molecular-weight markers; lane 2, cell lysate supernatant of wild-type IFTaseIII; lane 3, purified wild-type IFTaseIII; lane 4, purified selenomethionine derivative of IFTaseIII; lane 5, IFTaseIII purified from *A. globiformis* C11-1 culture broth.
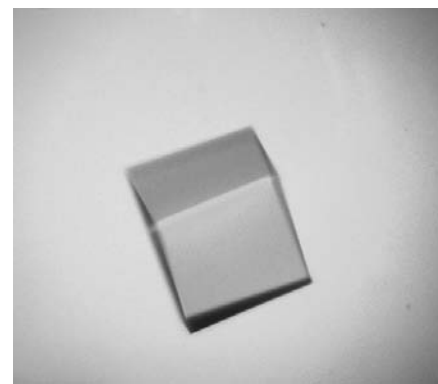

Figure 2
A crystal of recombinant *A. globiformis* IFTaseIII obtained by the hanging-drop vapour-diffusion method.

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Table 1
Crystal parameters and data-collection statistics.

Values in parentheses refer to the highest resolution shell. The unit-cell parameters for the SeMet derivative are average values from three-wavelength data.

Data	Native	SeMet (peak)	SeMet (edge)	SeMet (remote)
Source	PF BL6A	PF BL6B		
Space group	*R*32	*C*2		
Unit-cell parameters				
a (Å)	92.02	159.32		
b (Å)	92.02	91.92		
c (Å)	229.82	92.58		
β (°)		125.06		
Wavelength(Å)	1.0000	0.9789	0.9795	0.9600
Resolution (Å)	20–1.5 (1.58–1.50)	46–2.3 (2.42–2.30)	46–2.3 (2.42–2.30)	46–2.3 (2.42–2.30)
No. of reflections	115516 (16811)	48483 (7024)	48284 (7018)	48161 (6948)
Completeness (%)	99.6 (99.2)	99.5 (98.9)	98.4 (98.8)	99.2 (98.3)
R_{merge}	0.05 (0.240)	0.067 (0.127)	0.070 (0.140)	0.073 (0.158)
$I/\sigma(I)$	10.4 (3.3)	9.4 (5.8)	8.6 (5.4)	8.6 (4.7)
Multiplicity	4.4 (4.1)	7.1 (7.1)	6.1 (6.2)	7.3 (7.3)

$\beta = 125.06°$. They were estimated to contain three molecules in the asymmetric unit and the V_M value was calculated to be 2.15 Å^3 Da^{-1}, with a solvent content of approximately 42.8%, comparable values to those of the *R*32 crystals. Introduction of selenomethionine may have disturbed the crystallographic threefold symmetry existing in *R*32. These results indicated that the trimeric quaternary structure in solution might be related by a threefold axis coincident with the crystallographic threefold axis in the *R*32 crystal, while the trimeric structure might correspond to the three molecules in the asymmetric unit of the *C*2 crystal. The data sets for the MAD method were processed at 2.3 Å. The data-collection statistics are shown in Table 1. MAD phasing is currently under way.

References

Arand, M., Golubev, A. M., Neto, J. R., Polikarpov, I., Wattiez, R., Korneeva, O. S., Eneyskaya, E. V., Kulminskaya, A. A., Shabalin, K. A., Shishliannikov, S. M., Chepurnaya, O. V. & Neustroev, K. N. (2002). *Biochem. J.* **362**, 131–135.

Coutinho, P. M. & Henrissat, B. (1999). *Recent Advances in Carbohydrate Bioengineering*, edited by H. J. Gilbert, G. Davies, B. Henrissat & B. Svensson, pp. 3–12. Cambridge: The Royal Society of Chemistry.

Ettalibi, B. & Baratti, J. C. (1987). *Appl. Microbiol. Biotechnol.* **26**, 13–20.

Haraguchi, K., Kishimoto, M., Seki, K., Hayashi, K., Kobayashi, S. & Kainuma, K. (1988). *Biosci. Biotech. Bioeng.* **52**, 291–295.

Haraguchi, K., Mori, S. & Hayashi, K. (2000). *J. Biosci. Bioeng.* **89**, 590–595.

Haraguchi, K., Seki, K., Kishimoto, M., Nagata, T., Kasumi, T., Kainuma, K. & Kobayashi, S. (1988). *Biosci. Biotech. Bioeng.* **59**, 1809–1812.

Kawamura, M., Enomoto, K. & Tomita, F. (1991). *J. Ferment. Bioeng.* **72**, 262–265.

Pessoni, R. A. B., Figueredo-Ribeiro, R. C. L. & Braga, M. R. (1999). *J. Appl. Microbiol.* **87**, 141–147.

Rossmann, M. G. & van Beek, C. G. (1999). *Acta Cryst.* D**55**, 1631–1640.

Sakurai, H., Yokota, A. & Tomita, F. (1997). *Biosci. Biotech. Biochem.* **61**, 87–92.

Uchiyama, T., Niwa, S. & Tanaka, K. (1973). *Biochim. Biophys. Acta*, **315**, 412–420.

Uchiyama, T., Niwa, S. & Tanaka, K. (1975). *Biochim. Biophys. Acta*, **397**, 153–163.

Yokota, A., Enomoto, K. & Tomita, F. (1991). *J. Ferment. Bioeng.* **72**, 262–265.