Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray study of isomaltodextranase from *Arthrobacter globiformis*

Masatake Akita,[a]* Masahiro Mizuno,[b] Takashi Tonozuka,[b] Yoshiyuki Sakano,[b] Hirokazu Matsui,[c] Yuko Hidaka,[a] Yuji Hatada,[a] Susumu Ito[a] and Koki Horikoshi[a]

[a]Japan Marine Science and Technology Center (JAMSTEC), 2-15 Natsushima, Yokosuka 237-0061, Japan, [b]Department of Applied Biological Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan, and [c]Division of Applied Science, Hokkaido University Graduate School of Agriculture, Kita-9, Nishi-9, Kita-ku, Sapporo 060-8589, Japan

Correspondence e-mail: akitam@jamstec.go.jp

Received 16 November 2003
Accepted 5 January 2004

A recombinant isomaltodextranase (1,6-α-D-glucan isomaltohydrolase; EC 3.2.1.94) from an *Arthrobacter* sp. that hydrolyzes dextrans to generate isomaltose was purified and crystallized using the sitting-drop vapour-diffusion method at 293 K. X-ray diffraction data were collected to 1.8 Å. The crystals belong to space group *C*2, with unit-cell parameters $a = 199.1$, $b = 62.7$, $c = 57.4$, $\beta = 101.4^\circ$. Analysis of the Patterson self-rotation function suggests that the crystal contains one protein molecule in the asymmetric unit.

1. Introduction

Arthrobacter globiformis T6 produces an isomaltodextranase (IMD; EC 3.2.1.94) that hydrolyzes the α-1,6-glucosidic linkages of dextran and isomaltooligosaccharides from the non-reducing end to generate isomaltose (Sawai *et al.*, 1974, 1976). IMD also catalyzes transglycosylation, yielding various oligosaccharides (Kim *et al.*, 1995; Kim & Sakano, 1996). Isomaltose and isomaltooligosaccharides have potential applications in the food, cosmetic and medical industries (Keyes *et al.*, 1971; Ebisu *et al.*, 1974; Kim *et al.*, 1995).

IMD is classified as belonging to glycoside hydrolase family 27 based on its amino-acid sequence (http://afmb.cnrsmrs.fr/CAZY/index.html; Henrissat, 1991). The known structures in this family are α-galactosidase from *Oryza sativa* (Fujimoto *et al.*, 2003) and α-*N*-acetylgalactosaminidase from *Gallus gallus* (Garman *et al.*, 2002). Those proteins have $(\alpha/\beta)_8$-barrel domains and carbohydrate-binding domains. It is expected that IMD will have a similar domain structure.

The determination of the three-dimensional structure of IMD is important for clarifying the mechanisms of hydrolysis of dextran and substrate recognition of oligosaccharides having both α-1,4- and α-1,6-glucosidic linkages. The structure of IMD is also expected to be useful for the design of enzymes that can produce various oligosaccharides from dextran.

We have succeeded in high-level exo-expression of the gene for IMD in *Bacillus subtilis* cells. The IMD gene encodes a protein of 606 amino acids; the calculated molecular weight of IMD is 66 kDa. The pH optimum for activity was observed at pH $\simeq$ 3.0–3.5 and the optimal temperature for activity was 333 K. Here, we describe the crystallization and preliminary X-ray diffraction analysis of IMD.

2. Materials and methods

A. globiformis T6 was originally isolated by Sawai *et al.* (1974). The cloning of the IMD gene from the organism was performed by Iwai *et al.* (1994). To produce the IMD extracellularly, the cloned gene in plasmid pIMD1 (Iwai *et al.*, 1994) was amplified by PCR (polymerase chain reaction) using LA *Taq* DNA polymerase (TaKaRa Bio, Kyoto, Japan) and then inserted into the cloning site of the expression vector pHSP64 (Sumitomo *et al.*, 1995). The recombinant plasmid was introduced into *B. subtilis* ISW1214 cells. The *B. subtilis* cells harbouring the plasmid were cultured at 303 K for 48 h with shaking in a liquid medium composed of 12%(*w*/*v*) corn steep liquor (Nihon Syokuhin Kako, Shizuoka, Japan), 0.2%(*w*/*v*) Lab-Lemco powder (Oxoid, Hampshire, UK), 0.1%(*w*/*v*) yeast extract (Difco, MD, USA), 0.1%(*w*/*v*) KH_2PO_4, 0.02%(*w*/*v*) $MgSO_4{\cdot}7H_2O$, 0.05%(*w*/*v*) $CaCl_2$, 6%(*w*/*v*) maltose and 15 μg ml^{-1} tetracycline. The supernatant of the culture was obtained by centrifugation at 12 000 *g* for 10 min. IMD was purified to homogeneity by single-step column chromatography on DEAE-Toyopearl 650M (Tosoh, Tokyo, Japan). The purified enzyme was dialyzed against 10 m*M* Tris–HCl buffer pH 7.0 and then concentrated to 6 mg ml^{-1}. The protein concentration was measured using a Bio-Rad (Hercules, CA, USA) protein-assay kit with bovine serum albumin as a standard.

Crystallization of IMD was performed at 293 K using the sitting-drop vapour-diffusion method. The initial crystal screening of IMD was carried out using Hampton Research (Aliso Viejo, CA, USA) Crystal Screens I, II and Lite. 2 μl protein solution was mixed with an equal volume of the reservoir solution and equilibrated against 800 μl reservoir solution. To obtain a larger crystal, the conditions were varied based on the initial screenings.

Figure 1
A rod-shaped crystal of IMD grown in 20%(*w*/*v*) PEG 6000 and 0.3 *M* ammonium sulfate.

Prior to X-ray analysis, the crystals were soaked in a cryoprotectant consisting of the crystallization solution and 15%(*v*/*v*) glycerol. The crystals were then mounted on cryoloops and flash-frozen at 100 K using nitrogen gas. X-ray diffraction data were collected using an R-AXIS VII imaging plate (Rigaku, Tokyo, Japan) on an FR-E rotating-anode X-ray generator (Rigaku) operating at 45 kV and 45 mA with a copper target. The generated X-rays were focused using a confocal mirror. The X-ray wavelength was 1.54 Å, the crystal-to-film distance was 122 mm, the oscillation range was 1°, the exposure time was 180 s per frame and the total oscillation range was 180°. The collected data set was processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). The native data were analyzed by a self-rotation function search using the program *POLARRFN* from the *CCP*4 program suite (Collaborative Computational Project, Number 4, 1994). Initial phase-determination attempts were performed by the molecular-replacement method using *MOLREP* from the *CCP*4 program suite. The search models were α-galactosidase from *O. sativa* (Fujimoto *et al.*, 2003; PDB code 1uas) and α-*N*-acetylgalactosaminidase from *G. gallus* (Garman *et al.*, 2002; PDB code 1ktb).

3. Results and discussion

The purity of the protein solution was estimated by SDS–PAGE to be ∼95%. The yield was estimated by activity to be ∼70%. The initial attempt to crystallize IMD was performed using Hampton Research Crystal Screens. Hexagonal-shaped crystals with maximum dimensions of 25 × 25 × 10 µm were obtained from condition Nos. 30 and 31 of Crystal Screen Lite. The reagent compositions were 15%(*w*/*v*) PEG 8000/0.2 *M* ammonium sulfate for condition No. 30 and 15%(*w*/*v*) PEG 4000/0.2 *M* ammonium sulfate for condition No. 31. These regents did not contain a buffer (*e.g.* sodium acetate). The pH values of the reagents were ∼3.6 for condition No. 30 and ∼3.7 for condition No. 31 at 296 K.

After refinement of the crystallization conditions, we obtained two types of crystals. One was a hexagonal-shaped crystal, as obtained with the initial screen, and the other was a rod-shaped crystal. Both crystallization solutions contained 20%(*w*/*v*) PEG 6000 and 0.3 *M* ammonium sulfate (Wako Pure Chemical Ltd, Osaka, Japan). Hexagonal-shaped crystals were obtained when 0.1 *M* sodium acetate buffer pH 4.0 was added to the crystallization solution. The crystals were too small to use to collect X-ray diffraction data. In contrast, without sodium acetate, rod-shaped crystals grew to maximum dimensions of 100 × 100 × 500 µm (Fig. 1). The crystals were stable for at least one month at 293 K. Without sodium acetate, the pH of the solution was ∼5.4 at 296 K. Although we attempted to use 0.1 *M* sodium acetate and varied the pH from 4.0 to 5.5, no rod-shaped crystals were obtained. It thus appears that sodium acetate affects the solubility of IMD.

X-ray diffraction data were collected from the rod-shaped crystals. Although X-ray diffraction in the first image was observed to beyond 1.8 Å, that in the last image (180° rotated) was only observed to 1.8 Å. Moreover, the completeness beyond 1.8 Å had decreased drastically (below 70%); it seems that the crystals decayed. Therefore, we used the data to 1.8 Å. From the diffraction data processing, the crystals were found to be monoclinic and the space group was determined to be *C*2. The unit-cell parameters are $a = 199.1$, $b = 62.7$, $c = 57.4$ Å, $\beta = 101.4°$. A summary of the data-processing statistics is shown in Table 1. NCS-related peaks were not observed in the Patterson self-rotation function. Therefore, the asymmetric unit of the crystal probably contains one molecule (66 kDa), with an expected V_M of 2.7 Å^3 Da^{-1}, corresponding to a solvent content of 53.8% (Matthews, 1968). The initial attempts at phase determination using the molecular-replacement method failed. We are now preparing heavy-atom derivatives for MIR (multiple isomorphous replacement) or MAD (multiple anomalous scattering) phasing.

Table 1
Crystallographic parameters and data-collection statistics.

Values in parentheses refer to the outer shell.

Space group	*C*2
Unit-cell parameters	
a (Å)	199.1
b (Å)	62.7
c (Å)	57.4
β (°)	101.4
Resolution limits (Å)	70.0–1.8 (1.86–1.80)
No. observed reflections	464240
No. unique reflections	64332
Average $I/\sigma(I)$	35.6 (27.0)
Completeness (%)	99.6 (98.6)
$I > 3\sigma(I)$ (%)	98.4 (94.8)
R_{merge} (%)	2.9 (6.2)

The authors thank Dr T. Yamane and Mr T. Hikage for help with data collection. This study was supported in part by the National Project on Protein Structural and Functional Analyses from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Ebisu, S., Misaki, A., Kato, K. & Kotani, S. (1974). *Carbohydr. Res.* **38**, 374–381.
Fujimoto, Z., Kaneko, S., Momma, M., Kobayashi, H. & Mizuno, H. (2003). *J. Biol. Chem.* **278**, 20313–20318.
Garman, S. C., Hannick, L., Zhu, A. & Garboczi, D. N. (2002). *Structure*, **10**, 425–434.
Henrissat, B. (1991). *Biochem. J.* **280**, 309–316.
Iwai, A., Ito, H., Mizuno, T., Mori, H., Matsui, H., Honma, M., Okada, G. & Chiba, S. (1994). *J. Bacteriol.* **176**, 7730–7734.
Keyes, P. H., Hicks, M. A., Coldman, M., McCade, R. M. & Fitzgerald, R. J. (1971). *J. Am. Dent. Assoc.* **82**, 136–141.
Kim, Y. K. & Sakano, Y. (1996). *J. Appl. Glycosci.* **43**, 35–41.
Kim, Y. K., Tsumura, Y. & Sakano, Y. (1995). *Biosci. Biotechnol. Biochem.* **59**, 1367–1369.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Sawai, T., Toriyama, K. & Yano, K. (1974). *J. Biochem.* **75**, 105–112.
Sawai, T., Ukigai, Y. & Nawa, A. (1976). *Agric. Biol. Chem.* **40**, 1249–1250.
Sumitomo, N., Ozaki, K., Hitomi, J., Kawaminami, S., Kobayashi, T., Kawai, S. & Ito, S. (1995). *Biosci. Biotechnol. Biochem.* **59**, 2172–2175.