Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Purification, crystallization and preliminary diffraction study of β-galactosidase from *Penicillium* sp.

K. N. Neustroev,[a] E. A. de Sousa,[b] A. M. Golubev,[a] J. R. Brandão Neto,[b] E. V. Eneyskaya,[a] A. A. Kulminskaya[a] and I. Polikarpov[b]*

[a]Petersburg Nuclear Physics Institute, Gatchina, St Petersburg, 188350, Russia, and [b]Laboratório Nacional de Luz Síncrotron (LNLS), Caixa Postal 6192, CEP 13083-970, Campinas, SP, Brazil

Correspondence e-mail: igor@lnls.br

Received 21 July 2000
Accepted 23 August 2000

Crystals of an extracellular β-galactosidase from *Penicillium* sp. (MW = 120 ± 5 kDa) have been obtained from a sodium phosphate buffer using PEG as precipitant. The crystals belong to the tetragonal space group $P4_1$or $P4_3$, with unit-cell parameters $a = b = 110.82$, $c = 161.28$ Å, and diffract to 1.85 Å resolution at a synchrotron source.

1. Introduction

β-Galactosidase (E.C. 3.2.1.23) is an enzyme that hydrolyzes β(1–3)–β(1–4) galactosyl bonds in poly- and oligosaccharides. Many carbohydrases have been found to catalyse not only the hydrolysis reaction but also the reverse reaction of condensation or transglycosylation. This property, found in β-galactosidases from various sources, has been used for the enzymatic synthesis of galactose-containing oligosaccharides. The high degree of stereospecificity enables β-galactosidases to be used for chemo-enzymatic synthesis of galactooligosaccharides with β(1–3) and β(1–4) bond configurations (Usui *et al.*, 1993; Mori *et al.*, 1997). Thus, the enzymes from *Bacillus circulans* and from bovine testes have been successfully applied to the synthesis of Gal-β-1,3-Glc-NAc, a key oligosaccharide of sialyl-Lewis antigen (sLe[a]; Fujimoto *et al.*, 1998; Hedbys *et al.*, 1989). The same transglycosylation activity has been applied to the synthesis of Gal-β-1,3-Gal-NAC, which is an important constituent of the mucin-type glycoproteins, and for the synthesis of *N*-acetylallolactosamine (D-Gal-β-1,6-Glc-NAc; Vetere & Paoletti, 1996). To synthesize sialyl *N*-acetyllactoseamine, β-galactosidase from *Diplococcus pneumoniae* has been used together with sialidases (Ajisaka *et al.*, 1994). The transglycosylation ability of β-galactosidase has been used to obtain β-galactosyl-serine derivatives (Cantacuzene & Attal, 1991). The extracellular β-galactosidase from *Penicillium* sp. is found to have a high transglycosylation activity toward *p*-nitrophenyl β-D-galactopyranoside, lactose and methyl β-D-galactopyranoside, and appears to be a promising tool for many enzymatic syntheses. The structure and the mode of action of β-galactosidases are not well known as yet. The current work represents a first step toward the crystallographic structure elucidation of this enzyme.

2. Materials and methods

2.1. Protein preparation

Penicillium was incubated for 48 h as described previously for *Trichoderma* sp. (Savel'ev *et al.*, 1997) and the extracellular β-galactosidase was purified from 5 l of culture liquid. The mycelium was removed by centrifugation (3000*g*, 40 min) and the resulting supernatant was concentrated tenfold and transferred in 20 m*M* sodium acetate buffer pH 4.2 (buffer *A*) using hollow fibres. This crude solution was loaded on an SP-Sephadex column equilibrated with buffer *A* and eluted with 1 *M* NaCl in the same buffer. The resulting protein solution was concentrated to 20 ml using an Amicon PM-30 membrane, dialysed against 20 m*M* Tris–HCl buffer pH 7.2 (buffer *B*) and loaded onto a DEAE 5PW column (21.5 × 150 mm) equilibrated with the same buffer. The β-galactosidase was eluted with a linear gradient (0–0.5 *M*) of sodium chloride in buffer *B*. The fractions containing β-galactosidase were pooled, concentrated to 5 ml on an Amicon PM30 membrane and dialysed against buffer *B*. The resulting protein solution was passed through a MonoQ HR5/5 chromatography column equilibrated with buffer *B* in the same gradient of sodium chloride. Finally, the β-galactosidase fraction obtained was saturated with solid ammonium sulfate to a concentration of 1.7 *M* and applied to a phenyl Superose HR5/5 column equilibrated with 1.7 *M* ammonium sulfate in buffer *A* and eluted with a decreasing concentration (1.7–0 *M*) of ammonium sulfate. The single peak corresponding to the β-galactosidase activity was collected and the purified enzyme was dialysed against water and then lyophilized.

2.2. Biochemical analysis

The activity of the enzyme was monitored during purification steps using *p*-nitrophenyl-

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Number of frames	107
Space group	$P4_1$ ($P4_3$)
Unit-cell parameters (Å)	$a = b = 110.82$, $c = 161.28$
Resolution range (Å)	30.0–1.85 (1.93–1.85)
Total No. of reflections	695824 (44634)
No. of unique reflections	163512 (12364)
Redundancy	4.3 (3.6)
R_{sym}† (%)	11.0 (44.0)
Completeness (%)	99.8 (99.6)
$\langle I/\sigma(I)\rangle$	14.2 (2.1)

† $R_{sym} = \sum(I - \langle I\rangle)/\sum I$.

β-D-galactopyranoside as a substrate (Bahl & Agrawal, 1969). The unit of enzyme activity was defined as the amount of enzyme that hydrolyses 1 µmol of *p*-nitrophenyl-β-D-galactopyranoside per min at 310 K and pH 6.

All measurements of transglycosylation activity were carried out in 20 m*M* sodium acetate buffer pH 4.2 at 310 K. The reactions were terminated by boiling for 5 min. The transglycosylation activity was measured by analysing the reaction products using reversed-phase HPLC when *p*-nitrophenyl-β-D-galactopyranoside was used as the substrate (Eneyskaya *et al.*, 1998) or by HPLC on a Lichrosorb-NH_2 column when methyl-β-galactopyranoside and lactose were used as substrates (Savel'ev *et al.*, 1996).

2.3. X-ray diffraction

X-ray data were collected on a MAR Research 345 image-plate detector at the Laboratório Nacional de Luz Síncrotron (LNLS) protein crystallography beamline (Polikarpov, Oliva *et al.*, 1998; Polikarpov, Perles *et al.*, 1998) using the oscillation method. The X-ray wavelength was set to 1.38 Å to maximize the signal-to-noise ratio and to optimize the rate of data collection (Polikarpov *et al.*, 1997; Teplyakov *et al.*, 1998). The crystal-to-detector distance was set to 200 mm and the oscillation range was 1°. The crystals were quickly frozen in a gaseous nitrogen stream at 100 K (Oxford Cryosystems). The X-ray diffraction data (Table 1) were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997).

3. Results and discussion

3.1. β-Galactosidase properties and transglycosylation activity

The isolated β-galactosidase had a specific activity of 58 U per milligram of protein. The purified enzyme contained less than 0.1% admixture of carbohydrase activities. The estimation of molecular mass by SDS–PAGE yielded a value of 120 ± 5 kDa. This value coincided with the mass calculated from the elution profile on a Sephacryl S-300 column assuming that the enzyme exists as a monomer in solution.

β-Galactosidase revealed a high transglycosylation activity in the hydrolysis of *p*-nitrophenyl-β-D-galactopyranoside, methyl-β-D-galactopyranoside and lactose (donors) and galactose as the acceptor. HPLC analysis showed a high yield of products, up to 40–45%, in all reactions.

3.2. Crystallization and data collection

The lyophilized protein was dissolved in water to a final concentration of 5–10 mg ml^{-1} and 5–10 µl drops of the protein solution were mixed with equal volumes of 15% PEG 8000 in 50 m*M* sodium phosphate buffer at pH $\simeq$ 4.0 in crystallization trials. Hanging drops were equilibrated against 1 ml of the same PEG solution at room temperature. An amorphous precipitant was formed after 1–2 d and bipyramidal crystals started to grow after 4–5 d, reaching a maximal size of about 0.2 mm in each dimension.

The crystals belonged to the tetragonal space group $P4_1$ or $P4_3$, with unit-cell parameters $a = b = 110.82$, $c = 161.28$ Å, and diffracted to around 1.8 Å resolution. Calculation of the Matthews coefficient (Matthews, 1968) suggests the presence of one dimer per asymmetric unit (V_M = 2.06 $Å^3$ Da^{-1}) with a solvent content of 40.3%.

The freezing of the crystals significantly improved their stability in the X-ray beam, allowing data to be collected to higher resolution. The cryoprotection proved to be important in the process of data acquisition. A high-resolution native data set was collected (see Table 1). Screening for heavy-metal derivatives is under way.

The present work was supported by grant 00-04-48878 from the Russian Foundation for Basic Research (RFFI) and by grants 98/06761-1 and 99/03387-4 from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Brazil. AMG, EAS, JRBN and IP gratefully acknowledge the financial support of FAPESP and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

References

Ajisaka, K., Fujimoto, H. & Isomura, M. (1994). *Carbohydr. Res.* **259**, 103–115.
Bahl, O. P. & Agrawal, K. M. (1969). *J. Biol. Chem.* **244**, 2970–2978.
Cantacuzene, D. & Attal, S. (1991). *Carbohydr. Res.* **211**, 327–331.
Eneyskaya, E. V., Golubev, A. M., Kachurin, A. M., Savel'ev, A. N. & Neustroev, K. N. (1998). *Carbohydr. Res.* **305**, 83–91.
Fujimoto, H., Miyasato, M., Ito, Y., Sasaki, T. & Ajisaka, K. (1998). *Glycoconj. J.* **15**, 155–160.
Hedbys, L., Johansson, E., Mosbach, K., Larsson, P. O., Gunnarsson, A., Svensson, S. & Lonn, H. (1989). *Glycoconj. J.* **6**, 161–168.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mori, T., Fujita, S. & Okahata, Y. (1997). *Carbohydr. Res.* **298**, 65–73.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Polikarpov, I., Oliva, G., Castellano, E. E., Garratt, R. C., Arruda, P., Leite, A. & Graievich, A. (1998). *Nucl. Instrum. Methods A*, **405**, 159–164.
Polikarpov, I., Perles, L. A., de Oliveira, R. T., Oliva, G., Castellano, E. E., Garratt, R. C. & Graievich, A. (1998). *J. Synchrotron Rad.* **5**, 72–76.
Polikarpov, I., Teplyakov, A. & Oliva, G. (1997). *Acta Cryst.* D**53**, 734–737.
Savel'ev, A. N., Eneyskaya, E. V., Isaeva-Ivanova, L. S., Shabalin, K. A., Golubev, A. M. & Neustroev, K. N. (1997). *Glycoconj. J.* **14**, 897–905.
Savel'ev, A. N., Ibatullin, F. M, Eneyskaya, E. V., Kachurin, A. M. & Neustroev, K. N. (1996). *Carbohydr. Res.* **296**, 261–273.
Teplyakov, A., Oliva, G. & Polikarpov, I. (1998). *Acta Cryst.* D**54**, 610–614.
Usui, T., Kuboto, S. & Ohi, H. (1993). *Carbohydr. Res.* **244**, 315–323.
Vetere, A. & Paoletti, S. (1996). *Biochem. Biophys. Res. Commun.* **219**, 6–13.