Agustín Rico-Díaz,^{a,b} Ángel
Vizoso Vázquez,^a M. Esperanza
Cerdán,^a Manuel Becerra^a and
Julia Sanz-Aparicio^{b*}

^aGrupo EXPRELA, Departamento de Biología
Celular e Molecular, Facultade de Ciencias,
Universidade da Coruña, Campus da Zapateira,
s/n, 15071 A Coruña, Spain, and ^bGrupo de
Cristalografía Macromolecular y Biología
Estructural, Instituto de Química-Física
Rocasolano, CSIC, Serrano 119, 28006 Madrid,
Spain

Correspondence e-mail: xjulia@iqfr.csic.es

Received 12 June 2014

Accepted 2 September 2014

Crystallization and preliminary X-ray diffraction data of β -galactosidase from *Aspergillus niger*

β -Galactosidase from *Aspergillus niger* (An- β -Gal), belonging to the family 35 glycoside hydrolases, hydrolyzes the β -galactosidase linkages in lactose and other galactosides. It is extensively used in industry owing to its high hydrolytic activity and safety. The enzyme has been expressed in yeasts and purified by immobilized metal-ion affinity chromatography for crystallization experiments. The recombinant An- β -Gal, deglycosylated to avoid heterogeneity of the sample, has a molecular mass of 109 kDa. Rod-shaped crystals grew using PEG 3350 as the main precipitant agent. A diffraction data set was collected to 1.8 Å resolution.

1. Introduction

The enzyme β -D-galactosidase (EC 3.2.1.23) catalyzes the hydrolysis of terminal nonreducing β -D-galactose residues in β -D-galactosides. It has mainly been used for the hydrolysis of lactose in milk and other dairy products. Furthermore, β -galactosidases have transgalactosylation activities that make them very attractive for the production of prebiotic galactooligosaccharides (GOS). The β -D-galactosidase from the fungus *Aspergillus niger* (An- β -Gal) is one of the most used enzymes in the food industry. Its high optimal temperature allows its use at up to 323 K (Panesar *et al.*, 2006), which makes it very valuable for industrial applications such as the synthesis of GOS. Moreover, reported work (Dragosits *et al.*, 2014) has showed that its enzymatic activity is higher than that corresponding to other β -galactosidases from the *Aspergillus* genus. It also seems to tolerate fairly high amounts of organic solvents, which is an important trait in some transglycosylation-based applications using antibiotics or PEG (Dragosits *et al.*, 2014). Furthermore, *A. niger* is an organism that has been designated GRAS (generally recognized as safe) by the American Food and Drug Administration (Schuster *et al.*, 2002). Unfortunately, the too acidic optimum pH of An- β -Gal largely limits its applications in milk and neutral sweet cheese whey derived from hard cheese manufacturing (Rubio-Teixeira, 2006).

Despite the wide use of An- β -Gal in the dairy industry, its three-dimensional structure has not been solved. An- β -Gal encoded by the *lacA* gene belongs to family 35 of glycoside hydrolases (GH35), which contains most of the eukaryotic β -galactosidases. The three-dimensional structures of those from *Penicillium* sp., *Trichoderma reesei*, *A. oryzae* and *Homo sapiens* have previously been reported (Rojas *et al.*, 2004; Maksimainen *et al.*, 2011, 2013; Ohto *et al.*, 2012).

Knowledge of the molecular structure of An- β -Gal is necessary to fully understand its particular enzymatic activities and to improve its biotechnological potential. In this study, we describe the purification protocol used to overproduce the enzyme in *Saccharomyces cerevisiae*, the crystallization of its deglycosylated form and a preliminary X-ray crystallographic analysis.

2. Materials and methods

2.1. Macromolecule production

The *lacA* gene was amplified by PCR and cloned by homologous recombination in *S. cerevisiae*. The 30 first nucleotides of the sequence (signal peptide) were not cloned. The final construct was expressed grown in 400 ml YPHSM medium [1.5% (w/v) glucose,

Primers carry several nucleotides (underlined) of the specific sequence required for homologous recombination with the vector YEpFLAG and a 6×His sequence for introduction of a C-terminal tag (shown in *italics*).

3% (v/v) glycerol, 1% (w/v) yeast extract and 8% (w/v) peptone] at 303 K and 250 rev min⁻¹ for 72 h in a 2 l Erlenmeyer flask. Cells were collected by centrifugation (11 800g for 10 min at 277 K) and the extracellular medium was filtered through a 0.45 µm disposable syringe filter (Macherey-Nagel). The collected supernatants were applied onto an affinity chromatography system using a HisTrap HP 5 ml column coupled to an ÄKTAprime (GE Healthcare) that had been equilibrated with buffer *A* (100 mM sodium phosphate buffer, 500 mM NaCl, 25 mM imidazole pH 7). The column was equilibrated in buffer *A* and after sample injection it was washed with ten column volumes of the same buffer. The protein was then eluted with buffer *B* (100 mM sodium phosphate buffer, 500 mM NaCl, 500 mM imidazole pH 7). The protein solution was concentrated to 2 ml by ultrafiltration with Amicon Ultra-4 (Millipore). The homogeneity of the purified protein was evaluated by SDS-PAGE (Laemmli, 1970). Macromolecule-production details are given in Table 1.

Prior to crystallization experiments, glycan chains were removed using endoglycosidase H (Endo H; New England BioLabs) under native conditions. Endo H treatment was carried out for 3 h following the manufacturer's instructions. An extra purification step using a gel-filtration column (HiLoad 16/60 Superdex 200 prep-grade column, GE Healthcare) was performed after deglycosylation in order to eliminate Endo H contamination from the protein sample. The deglycosylated protein was concentrated to 1.5 mg ml^{-1} by ultrafiltration using Amicon Ultra-4 (Millipore).

Method	Vapour diffusion, sitting drop
Plate type, screening plate type, optimization	Innovaplate SD-2 Maxi 48-well Crystallization Plate
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	1.5
Buffer composition of protein solution	150 mM NaCl, 50 mM Tris-HCl pH 7.5
Composition of reservoir solution	21–24% (w/v) PEG 3350, 200 mM Li ₂ SO ₄ , 100 mM bis-tris pH 5.5–6.0
Volume and ratio of drop	2 µl, 1:1 ratio
Volume of reservoir (µl)	200

α -N-Gal is an extracellular highly glycosylated enzyme, its molecular weight decreasing more than 50% after treatment with endoglycosidase H (Endo H; Fig. 1). Moreover, these glycosylations make the protein very heterogeneous, which usually hinders the crystallization process. To remove this obstacle, the protein sample was treated with Endo H prior to the crystallization step (Lehle *et al.*, 2006). Endo H cleaves asparagine-linked oligomannoses, generating

a truncated sugar moiety with one *N*-acetylglucosamine residue remaining on the glycosylation site.

Initial screenings using the PACT and JCSG+ Suites with the purified deglycosylated An- β -Gal gave needles in some conditions of the JCSG+ Suite, which contain PEG 3350 as the main precipitant agent. Optimization was assayed by sampling protein and precipitant concentration and by varying the pH. The best rod-shaped crystals grew in 21 d from 21–24% (w/v) PEG 3350, 200 mM Li₂SO₄, 100 mM bis-tris pH 5.5–6.0 (Fig. 2a).

A full data set was collected at 100 K on the XALOC beamline at ALBA, Cerdanyola del Vallès, Spain. The crystals belonged to space group *P*₂₁₂₁₂₁, as observed from the systematic absences, with unit-cell parameters *a* = 85.57, *b* = 111.42, *c* = 126.73 Å, and diffracted to 1.8 Å resolution (Fig. 2b, Table 3). As calculated from its sequence analysis, the molecular mass of the monomer is 109 kDa. The Matthews coefficient value of 2.77 Å³ Da⁻¹ (Matthews, 1968), corresponding to 56% solvent content, reveals the presence of one molecule in the asymmetric unit. Structure determination by molecular replacement is in progress using the coordinates from *A. oryzae*

(a)

(b)

Figure 2

(a) Crystals of An- β -Gal grown in 21–24% (w/v) PEG 3350, 200 mM Li₂SO₄, 100 mM bis-tris pH 5.5. (b) X-ray diffraction pattern obtained using a synchrotron source. The outer circle corresponds to 1.8 Å resolution.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	ALBA beamline, XALOC
Wavelength (Å)	0.97947
Temperature (K)	100
Detector	PILATUS
Crystal-to-detector distance (mm)	293.12
Rotation range per image (°)	0.2
Total rotation range (°)	200
Exposure time per image (s)	0.2
Space group	<i>P</i> ₂ ₁ ₂ ₁ ₂ ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	85.57, 111.42, 126.73
Mosaicity (°)	0.52
Resolution range (Å)	42.79–1.80 (1.84–1.80)
Total No. of reflections	1160311 (49134)
No. of unique reflections	173058 (7448)
Completeness (%)	99.9 (99.9)
Multiplicity	6.7 (6.6)
$\langle I/\sigma(I) \rangle$	8.8 (4.4)
CC _{1/2} (%)	99.1 (68.5)
<i>R</i> _{merge} [†] (%)	10.6 (46.5)
Overall <i>B</i> factor from Wilson plot (Å ²)	8.3

[†] $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle|}{\sum_{hkl} \sum_i I_i(hkl)}$, where $I_i(hkl)$ is the *i*th measurement of reflection *hkl* and $\langle I(hkl) \rangle$ is the weighted mean of all measurements.

β -galactosidase (PDB entry 4iug; Maksimainen *et al.*, 2013), which shows 75% sequence identity. It is hoped that analysis of the structure will give insights into the mechanism and specificity of An- β -Gal, which is most useful for biotechnological purposes.

ARD and AVV received a Plan I2C fellowship from Xunta de Galicia. Research at Universidade da Coruña was supported by grant 10TAL103006PR from Xunta de Galicia and FEDER funds. General support to the laboratory during 2012–2013 was funded by ‘Programa de axudas para a consolidación e a estruturación de unidades de investigación competitivas do sistema galego de I+D+I’ (Xunta de Galicia) and by grant BIO2010-20508-C04-03 from Dirección General de Investigación-MINECO (Spanish Ministry of Innovation). This is a product of the Project ‘Factoría Española de Cristalización’ Ingenio/Consolider 2010.

References

- Battye, T. G. G., Kontogiannis, L., Johnson, O., Powell, H. R. & Leslie, A. G. W. (2011). *Acta Cryst.* **D67**, 271–281.
- Dragosits, M., Pflügl, S., Kurz, S., Razzazi-Fazeli, E., Wilson, I. B. & Rendic, D. (2014). *Appl. Microbiol. Biotechnol.* **98**, 3553–3567.
- Evans, P. (2006). *Acta Cryst.* **D62**, 72–82.
- Garman, E. F. & Mitchell, E. P. (1996). *J. Appl. Cryst.* **29**, 584–587.
- Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
- Lehle, L., Strahl, S. & Tanner, W. (2006). *Angew. Chem. Int. Ed. Engl.* **45**, 6802–6818.
- Maksimainen, M., Hakulinen, N., Kallio, J. M., Timoharju, T., Turunen, O. & Rouvinen, J. (2011). *J. Struct. Biol.* **174**, 156–163.
- Maksimainen, M. M., Lampio, A., Mertanen, M., Turunen, O. & Rouvinen, J. (2013). *Int. J. Biol. Macromol.* **60**, 109–115.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Ohto, U., Usui, K., Ochi, T., Yuki, K., Satow, Y. & Shimizu, T. (2012). *J. Biol. Chem.* **287**, 1801–1812.
- Panesar, P. S., Panesar, R., Singh, R. S., Kennedy, J. F. & Kumar, H. (2006). *J. Chem. Technol. Biotechnol.* **81**, 530–543.
- Rojas, A. L., Nagem, R. A. P., Neustroev, K. N., Arand, M., Adamska, M., Eneyskaya, E. V., Kulminskaya, A. A., Garratt, R. C., Golubev, A. M. & Polikarpov, I. (2004). *J. Mol. Biol.* **343**, 1281–1292.
- Rubio-Teixeira, M. (2006). *Biotechnol. Adv.* **24**, 212–225.
- Schuster, E., Dunn-Coleman, N., Frisvad, J. C. & Van Dijck, P. W. (2002). *Appl. Microbiol. Biotechnol.* **59**, 426–435.
- Winn, M. D. *et al.* (2011). *Acta Cryst.* **D67**, 235–242.