Journal of Structural Biology 174 (2011) 156–163

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Crystal structures of *Trichoderma reesei* β-galactosidase reveal conformational changes in the active site

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ARTICLE INFO

Article history:
Received 10 September 2010
Received in revised form 12 November 2010
Accepted 29 November 2010
Available online 3 December 2010

Keywords:
Glycosyl hydrolase
β-Galactosidase
Crystal structure
Conformational changes
Octaserine

ABSTRACT

We have determined the crystal structure of *Trichoderma reesei* (*Hypocrea jecorina*) β-galactosidase (Tr-β-gal) at a 1.2 Å resolution and its complex structures with galactose, IPTG and PETG at 1.5, 1.75 and 1.4 Å resolutions, respectively. Tr-β-gal is a potential enzyme for lactose hydrolysis in the dairy industry and belongs to family 35 of the glycoside hydrolases (GH-35). The high resolution crystal structures of this six-domain enzyme revealed interesting features about the structure of Tr-β-gal. We discovered conformational changes in the two loop regions in the active site, implicating a conformational selection-mechanism for the enzyme. In addition, the Glu200, an acid/base catalyst showed two different conformations which undoubtedly affect the pK_a value of this residue and the catalytic mechanism. The electron density showed extensive glycosylation, suggesting a structure stabilizing role for glycans. The longest glycan showed an electron density that extends to the eighth monosaccharide unit in the extended chain. The Tr-β-gal structure also showed a well-ordered structure for a unique octaserine motif on the surface loop of the fifth domain.

1. Introduction

β-Galactosidase is a hydrolase enzyme (E.C. 3.2.1.23) that catalyzes the hydrolysis of β-galactosides into monosaccharides. The enzyme hydrolyzes β(1–3)–β(1–4) galactosyl bonds in oligo- and disaccharides, but it also has an ability to catalyze the reverse reaction of the hydrolysis called transglycosylation. β-Galactosidases have been isolated from various sources, such as animals, plants, bacteria, yeasts and fungi and they have many important applications in the industrial and biotechnological fields (Wang et al., 2000; Kestwal and Bhide, 2007; Shaikh et al., 1999; Griffith and Wolf, 2002; Dickson et al., 1979). Perhaps the best known substrate for β-galactosidases is lactose that is especially found in milk. Lactose is a disaccharide that consists of galactose and glucose. The β-galactosidases which are able to hydrolyze lactose are also called lactases. A lactase deficiency leads to an inefficient lactose digestion. After weaning, most people lose 75–90% of their birth lactase level. Clinically, if lactose maldigestion causes physiological symptoms, the person is said to have lactose intolerance (Harrington and Mayberry, 2008). Lactases have been successfully used for the hydrolysis of lactose in dairy products, thus increasing the usability of these foodstuffs (Sener et al., 2008) and (Zhou et al., 2002). The transglycosylation ability makes the enzyme a useful tool for production of different oligosaccharides that are used, for example, in molecular biology (Higashiyama et al., 2004) and (Splechtna et al., 2006).

β-Galactosidases and other glycoside hydrolases utilize general acid hydrolysis mechanisms in order to cleave the *O*-glycosidic bond. Two glutamic or aspartic acid residues participate in the catalysis. One is functioning as an acid/base catalyst, the second as a nucleophile. The pK_a values of these residues contribute to the pH range in which the enzyme is active. According to the CAZy database (Cantarel et al., 2009), β-galactosidase belongs to sub-families 1, 2, 35 and 42 of the GH-A superfamily. All the members of GH-A usually have an $(\alpha/\beta)_8$ barrel as a catalytic domain, in which two glutamic acid residues act as an acid/base catalyst and a nucleophile (Davies and Henrissat, 1995). To date, there are eight native structures of β-galactosidase available from seven different organisms in the PDB. These structures are shown in Table 1. The tetrameric crystal structure of β-galactosidase from *Escherichia coli* was the first reported

Abbreviations: Tr-βgal, *Trichoderma reesei* β-galactosidase; Psp-β-gal, *Penicillium* sp. β-galactosidase; Btm-β-gal, *Bacteroides thetaiotaomicron VPI-5482* β-galactosidase; IPTG, isopropyl β-D-1-thiogalactopyranoside; PETG, 2-phenylethylβ-D-thiogalactoside; PDB, Protein Data Bank; NYSGXRC, New York Structural GenomiX Research Consortium; MPD, 2-methyl-2,4-pentanediol; GlcNac, *N*-acetylglucosamine; Man, mannose; Glc, glucose.

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doi:10.1016/j.jsb.2010.11.024

Table 1
Available structures of native β-galactosidases from different organisms in the PDB.

Organism	GH-family	Quaternary structure	Resolution (Å)	PDB-code	References
Sulfolobus solfataricus	1	Tetramer	2.02	1uwq	Gloster et al. (2004)
Arthrobacter sp. C2–2	2	Hexamer	1.90	1yq2	Skalova et al. (2005)
B. fragilis	2	Tetramer	1.90	3cmg	Unpublished results
B. thetaiotaomicron					
VPI-5482	2	Dimer	2.10	3bga	Unpublished results
E. coli	2	Tetramer	1.70	1dp0	Juers et al. (2000)
B. thetaiotaomicron					
VPI-5482	35	Monomer	2.15	3d3a	Unpublished results
Penicillium sp.	35	Monomer	1.90	1tg7	Rojas et al. (2004)
Thermus thermophilus A4	42	Trimer	1.60	1kwg	Hidaka et al. (2002)

β-galactosidase structure (Jacobson et al., 1994) and it is one of the most studied β-galactosidase, with several mutant and complex structures (Juers et al., 2001, 2000, 2009). The latest β-galactosidase structures from *Bacteroides fragilis* and *Bacteroides thetaiotaomicron* have been reported by the NYSGXRC in 2007–2008. The quaternary structures of β-galactosidases in the PDB vary greatly. The largest crystal structure of β-galactosidase has been reported for the *Arthrobacter* sp. *C2–2* enzyme which forms a 660 kDa hexameric structure (Skalova et al., 2005) whereas β-galactosidases from *Thermus thermophilus A4*, *Sulfolobus solfataricus* and *Penicillium* sp. form a trimer, tetramer and monomer, respectively (Hidaka et al., 2002), (Gloster et al., 2004) and (Rojas et al., 2004).

The extracellular Tr-β-gal belongs to the GH-35 family. The sequence and the enzymatic properties of this industrially potent enzyme have previously been reported (Seiboth et al., 2005) and (Gamauf et al., 2007). Tr-β-gal is active with several disaccharides, like lactose, lactulose, galactobiose, aryl- and alkyl-β-D-galactosides. In addition, Tr-β-gal is able to act on polymeric β-1,3- and β-1,4-galactans. In this paper, we report its three-dimensional monomeric structure at a 1.2 Å resolution and the complex structures with galactose, IPTG, and PETG at 1.5, 1.75 and 1.4 Å resolutions, respectively. Tr-β-gal is active at the pH range 3–7 (maximum at pH 5). There is a substantial decrease in the activity at temperatures higher than 60 °C (Gamauf et al., 2007). The high-resolution structures that have been determined create an excellent starting point for an analysis of the function Tr-β-gal and the design of mutants in order to improve the enzymatic properties for industrial applications.

2. Materials and methods

2.1. Protein purification, crystallization and data collection

The purification and crystallization of Tr-β-gal was performed as previously described (Maksimainen et al., 2009). In addition, the data collection of the native Tr-β-gal at 1.2 Å resolution has previously been reported (Maksimainen et al., 2009). All the data for the Tr-β-gal structures were collected on beamlines ID29 and ID14-2 at ESRF (Grenoble, France), except the data for the galactose complex, which was collected on beamline X12 at EMBL (Hamburg, Germany). The crystals were quickly vitrified to 100 K in a cold nitrogen stream. The data were processed with the XDS program (Kabsch, 1993). The data collection statistics are presented in Table 2.

2.2. Structure determination and model refinement

The three-dimensional structure of Tr-β-gal was determined by molecular replacement with the MOLREP program (Vagin and Teplyakov, 1997). Psp-β-gal (Rojas et al., 2004) (PDB code 1tg7) was used as a search model (56% identity by sequence comparison). The refinement of the native structure of Tr-β-gal was started by the program REFMAC5 (Murshudov et al., 1999) from CCP4 (The CCP4, 1994; Potterton et al., 2003). Due to the atomic resolution, the native structure was finally refined by the SHELXL program (Sheldrick, 2008). All Tr-β-gal complex structures were refined with REFMAC5 (Murshudov et al., 1999). The manual building was performed using the O (Jones et al., 1991) and COOT (Emsley

Table 2
Data collection statistics for the Tr-β-gal structures.

Item	Native	Galactose	IPTG	PETG
Resolution (Å) (Outer shell)	50–1.2 (1.30–1.20)	20–1.5 (1.60–1.50)	50–1.75 (1.80–1.75)	50–1.4 (1.50–1.40)
Wavelength (Å)	0.979	1.000	0.933	0.933
Beamline	ID29 (ESRF)	X12 (EMBL)	ID14–2 (ESRF)	ID14–2 (ESRF)
Temperature (K)	100	100	100	100
Space Group	P1	P1	P1	P1
Unit cell parameters (Å)	a = 67.4	a = 67.4	a = 69.5	a = 67.6
	b = 69.2	b = 69.3	b = 70.3	b = 68.7
	c = 81.5	c = 81.5	c = 82.4	c = 81.7
(deg.)	α = 109.1	α = 109.0	α = 108.5	α = 108.5
	β = 97.3	β = 97.3	β = 97.8	β = 97.7
	γ = 114.5	γ = 114.4	γ = 114.4	γ = 114.5
Total No. of reflections	719,611 (142,034)	358,026 (62,381)	230,614 (18,198)	533,122 (83,722)
No. of unique reflections	341,073 (71,839)	179,614 (31,300)	117,872 (9488)	220,582 (41,108)
I/δ	11.97 (3.28)	14.8 (3.4)	15.9 (3.1)	9.9 (3.6)
R-merge	5.5 (44.3)	3.9 (23.9)	3.4 (31.9)	6.2 (20.0)
Completeness (%)	90.6 (89.4)	92.6 (91.7)	91.0 (90.3)	92.6 (92.3)
Cryoprotectant	25 % (v/v) glycerol, 0.1 M Na-cacodylate pH 6.0	8 % (w/v) galactose, 20 % (v/v) MPD	1 M IPTG, 20 % (v/v) MPD	30 mM PETG, 20% methanol, 30 % (v/v) MPD
Soaking time (s)	5	5	5	5