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Structural basis of specificity in tetrameric *Kluyveromyces lactis* β-galactosidase

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A B S T R A C T

β-Galactosidase or lactase is a very important enzyme in the food industry, being that from the yeast *Kluyveromyces lactis* the most widely used. Here we report its three-dimensional structure both in the free state and complexed with the product galactose. The monomer folds into five domains in a pattern conserved with the prokaryote enzymes of the GH2 family, although two long insertions in domains 2 and 3 are unique and related to oligomerization and specificity. The tetrameric enzyme is a dimer of dimers, with higher dissociation energy for the dimers than for its assembly. Two active centers are located at the interface within each dimer in a narrow channel. The insertion at domain 3 protrudes into this channel and makes putative links with the aglycone moiety of docked lactose. In spite of common structural features related to function, the determinants of the reaction mechanism proposed for *Escherichia coli* β-galactosidase are not found in the active site of the *K. lactis* enzyme. This is the first X-ray crystal structure for a β-galactosidase used in food processing.

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1. Introduction

β-D-Galactosidase (β-D-galactoside galactohydrolase, E.C. 3.2.1.23), most commonly known as lactase, is one of the most important enzymes used in food processing that catalyzes the hydrolysis of terminal non-reducing β-D-galactose residues in β-D-galactosides. Conventionally, its main application has been in the hydrolysis of lactose in milk or derived products, particularly cheese whey. Lactose is a disaccharide formed by glucose and galactose that is found in milk. In humans, lactose intolerance or unabsorbed lactose is a common problem. In fact, it is estimated that lactose intolerance occurs in 70% of the world's adult population, and Eastern Asia has the highest number of lactose malabsorbers with more than 90% of its population (Husain, 2010). Lactose maldigestion and intolerance are caused by lactase insufficiency or non-persistence, which results from a decrease in the activity of the β-galactosidase, in the brush border membrane of the mucosa of the small intestine of adults (Juajun et al., 2011). Consequently, there is a considerable market for lactose-free milk and dairy products, which can be obtained by enzymatic hydrolysis using β-galactosidases (Oliveira et al., 2011).

Besides lactose maldigestion, crystallization of lactose can be a problem in dairy products such as ice cream and sweetened condensed milk. β-Galactosidases derived from food grade organisms can be successfully employed for these problems related to the milk sugar lactose (Juajun et al., 2011). The products of lactose hydrolysis, i.e. glucose and galactose, are sweeter and also much more soluble than lactose (Ganzle and Haase, 2008). Furthermore, disposal of large quantities of the lactose-containing by-products from cheese manufacturing, whey and whey permeates, causes serious environmental problems. It is estimated that approximately 160 million tons of whey are producing worldwide each year (Guimarães et al., 2010). However, whey can be used as a source of cheap, renewable and fermentable sugars after β-galactosidase-catalyzed hydrolysis for the production of added-value molecules or bulk commodities by lactose-negative microbes (Oliveira et al., 2011).

Apart from lactose hydrolysis, β-galactosidases with transgalactosylation activities are highly attractive for the production of added-value lactose derivatives. In particular, galacto-oligosaccharides (GOS), prebiotics that can stimulate the growth of beneficial bacteria such as bifidobacteria and lactobacilli, are increasingly finding application in functional foods, namely as low calorie sweeteners in fermented milk products, confectioneries, breads and beverages (Ganzle and Haase, 2008; Gosling et al., 2010; Park and Oh, 2010).

Abbreviations: AR-β-Gal, *Arthrobacter* sp. β-galactosidase; EC-β-Gal, *Escherichia coli* β-galactosidase; GH, Glycosyl Hydrolase; KL-β-Gal, *Kluyveromyces lactis* β-galactosidase; NCS, non-crystallographic symmetry; RMS, root mean square.

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Many organisms naturally synthesize β-galactosidase, including animals, plants and microorganisms; however, the easier manipulation and better yields from microorganisms have favoured their establishment as a main source for industrial production of β-galactosidases. Although bacteria could offer more versatility, the corroborated GRAS (Generally Recognized As Safe) status of yeasts like *Kluyveromyces lactis* and *Kluyveromyces marxianus*, and of fungi like *Aspergillus niger* and *Aspergillus oryzae*, still places them among the favorite sources of β-galactosidase for food biotechnology and pharmaceutical industry (Rubio-Texeira, 2006).

β-Galactosidase sequences can be deduced from various databases, and these can be classified into four different Glycosyl Hydrolase (GH) families 1, 2, 35 and 42, based on functional similarities (Cantarel et al., 2009). Those from eukaryotic organisms are grouped into family 35 with the exceptions of *K. lactis* and *K. marxianus* β-galactosidases (99% identity), which belong to the family 2 together with the prokaryotic β-galactosidases from *Escherichia coli* and *Arthrobacter* sp. Whereas the structures of these last two prokaryotic enzymes have been determined (Juers et al., 2000; Skálová et al., 2005), none of the eukaryotic β-galactosidase structures has been reported. In fact, to date, the X-ray crystal structures of eight different microbial β-galactosidases are available in the PDB, although none of the enzymes with solved structures is known to be used in food processing.

In this paper, we report the three-dimensional structure at 2.75 Å resolution and the complex structure with galactose at 2.8 Å resolution of the β-galactosidase from *K. lactis*, one of the most important and widely used enzymes of the food industry.

2. Materials and methods

2.1. Cloning, expression and purification

Cloning, expression and purification of *K. lactis* β-galactosidase (KL-β-Gal) was performed as described previously (Pereira-Rodríguez et al., 2010).

2.2. Crystallization and data collection

Crystallization of KL-β-Gal (3.5 mg mL^{-1} in 0.05 M Tris–HCl, 0.150 M NaCl and 0.002 M DTT, 7% glycerol) was performed on Cryschem (Hampton Research) sitting drop plates at 291 K as described previously (Pereira-Rodríguez et al., 2010). Small plate-shaped crystals grew in 23–27% (w/v) Polyethylene Glycol (PEG) 3350, 0.1 M Bis–Tris pH 7.5–7.0, 0.2 M sodium tartrate. Streak seeding (Stura and Wilson, 1991) performed under these conditions gave improved quality crystals that were suitable for X-ray diffraction experiments. Crystals of KL-β-Gal belonged to $P2_12_12_1$ space-group with four molecules in the asymmetric unit and 51% solvent content within the unit cell. For data collection, native crystals were transferred to cryoprotectant solutions consisting of mother liquor plus 20% (v/v) glycerol before being cooled to 100 K in liquid nitrogen. The complex with the product galactose was obtained by crystal soaking with the substrate lactose (Hassell et al., 2007). In order to minimize crystal damage, mother-liquor was substituted by the soaking solution (35% PEG 3350, 0.1 M Bis–Tris pH 7.0, 0.2 M sodium tartrate, 2 mM $MgCl_2$) saturated with lactose, incubated for 6 min and then cryocooled in liquid nitrogen.

Diffraction data were collected using synchrotron radiation at the European Synchrotron Radiation Facility (ESRF, Grenoble) on ID23.1 and ID14.4 beamlines. Diffraction images were processed with MOSFLM (Leslie, 1992) and merged using the CCP4 package (Collaborative Computational Project, 1994). A summary of data collection and data reduction statistics is shown in Table 1.

Table 1
Crystallographic statistics Values in parentheses are for the high resolution shell.

Crystal data	KL-β-Gal	KL-β-Gal – galactose
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell parameters		
a (Å)	140.030	140.381
b (Å)	153.340	153.454
c (Å)	216.160	217.166
Data collection		
Beamline	ID23.1 (ESRF)	ID14.4 (ESRF)
Temperature (K)	100	100
Wavelength (Å)	0.979	0.939
Resolution (Å)	62.53–2.75 (2.90–2.75)	49.30–2.80 (2.95–2.80)
Data processing		
Total reflections	874,614 (123,972)	1,379,068 (193,533)
Unique reflections	121,272 (17,499)	115,849 (16,726)
Multiplicity	7.2 (7.1)	11.9 (11.6)
Completeness (%)	100.0 (100.0)	100.0 (100.0)
I/σ (I)	4.3 (1.4)	7.7 (1.9)
Mean I/σ (I)	10.7 (3.6)	24.5 (6.7)
R_{merge}[a] (%)	17.2 (53.6)	9.9 (43.1)
R_{pim}[b] (%)	6.8 (21.5)	3.0 (13.2)
Molecules per ASU	4	4
Matthews coef. ($Å^3$ Da^{-1})	2.5	2.5
Solvent content (%)	51%	51%
Refinement		
R_{work}/R_{free}[c] (%)	20.7 / 24.4	21.4 / 24.6
Mean B-factors		
Peptide	19.6	35.1
Water	17.3	31.5
Ligands	22.8	34.6
No. of atoms		
Protein	33,300	33,300
Carbohydrate	60	48
Water molecules	1666	1047
Ramachandran (Chen et al., 2010)		
Favoured (%)	95.7	95.5
Outliers (%)	0.10	0.00
RMS deviations		
Bonds (Å)	0.008	0.009
Angles (°)	1.108	1.131
Protein Data Bank codes	3OBA	3OB8

[a] $R_{merge} = \sum_{hkl} \sum_i |I_i(hkl) - [I(hkl)]| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith measurement of reflection hkl and [I(hkl)] is the weighted mean of all measurements.
[b] $R_{pim} = \sum_{hkl} [1/(N-1)]\ 1/2 \sum_i |I_i(hkl) - [I(hkl)]| / \sum_{hkl} \sum_i I_i(hkl)$, where N is the redundancy for the hkl reflection.
[c] $R_{work}/R_{free} = \sum_{hkl} |Fo - Fc| / \sum_{hkl} |Fo|$, where Fc is the calculated and Fo is the observed structure factor amplitude of reflection hkl for the working/free (5%) set, respectively.

2.3. Structure solution and refinement

The structure of KL-β-Gal was solved by molecular replacement using the MOLREP program (Vagin and Teplyakov, 1997). The structure of *Arthrobacter* sp. β-galactosidase (PDB code 1YQ2) (Skálová et al., 2005) was used to prepare the search model using the program Chainsaw (Stein, 2008) and a protein sequence alignment of KL-β-Gal onto *Arthrobacter* β-galactosidase. A single solution containing four molecules in the asymmetric unit was found using reflections within 125–3.43 Å resolution range and a Patterson radius of 31 Å, which after rigid body fitting led to an *R* factor of 51%. Crystallographic refinement was performed using the program Refmac5 (Murshudov et al., 1997) within the CCP4 suite with flat bulk-solvent correction, and using maximum likelihood target features. Tight non-crystallographic symmetry restrictions were applied during first steps of refinement. Loop 246–274, which is ordered in molecules A and C and disordered in molecules B and C (more details in Section 3), and other small regions (as the last portion of the linker between domain 4 and 5), were excluded from the NCS restraints during model building, but best results were

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