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Cloning, production, purification and preliminary crystallographic analysis of a glycosidase from the food lactic acid bacterium *Lactobacillus plantarum* CECT 748^T

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ABSTRACT

In recent years, the exquisite stereoselectivity and high efficiency of carbohydrate-processing enzymes have been exploited for many biotechnological applications, including flavor enhancement in foods. In particular, much attention has been focused on the use of β-glucosidases for the enzymatic hydrolysis of flavorless glycoconjugates present in juices and wine beverages for the release aroma volatiles. With the aim to analyze a novel glycosidase with potential applications food industry we have produced and structurally characterized the Bgl glycosidase from the food lactic acid bacterium *Lactobacillus plantarum*. For that purpose, we have cloned and heterologously expressed the *bgl* gene (lp_3629) in *Escherichia coli*. The recombinant protein containing an amino terminal His_6 tag (Bgl) has been produced in a soluble form. Purified recombinant enzyme shows galactosidase activity against 4-nitrophenyl β-D-galactopyranoside but not glucosidase activity. Analytical size-exclusion gel filtration chromatography reveals that Bgl behaves in solution as a mixture of monomeric and a high-molecular weight assembly. Purified Bgl has been crystallized by the hanging-drop vapor-diffusion method at 18 °C. Diffraction data have been collected at ESRF to a resolution of 2.4 Å. The crystals belong to the space group *C*2 with unit-cell parameters $a = 196.7$, $b = 191.7$, $c = 105.9$, $\beta = 102.7°$. The structure refinement is in progress.

Introduction

In the past decades, the need for new carbohydrate materials and the development of glycomics have provided a boost to carbohydrate chemistry. Oligosaccharides can be prepared by classical organic chemistry using expensive and tedious methods. However, as an alternative, enzymatic synthesis, ideally in a stereo- and regio-specific manner, is a valued option. Elegant glycosidic bond formation can be accomplished by using glycosidases. In recent years, the recent advances in carbohydrate synthesis by glycosidases are based on two complementary approaches: the use of wild-type enzymes with engineered substrates, and mutant glycosidases [1].

Among the glycosidases, β-glucosidases have been subject of much work because of their importance in numerous biological processes and in biotechnological applications [2], such as food detoxification [3], biomass conversion [4] and over the past decade, flavor enhancement in beverages [5]. Indeed the intensive research carried out over the past two decades has demonstrated that, in a great number of fruit and other plant tissues, important flavor compounds accumulate as non-volatile and flavorless glycoconjugates, which make up a reserve of aroma to be exploited [6,7]. Therefore, hydrolysis of these glycosides leads to the liberation of volatiles. Wine aroma and flavor are influenced by grape-derived compounds and by the microorganisms which are present during winemaking. Due to the limited effect of glycosidases from grape and *Saccharomyces cerevisiae* in winemaking, a large part of glycosides is still present in young wines. Initially, attention had been focused on the use of exogenous glycosidases from yeast and from filamentous fungi to enhance wine aroma [8]. Nonetheless, many of the glycosidic activities from the various source organisms examined to date, were limited by their sensitivity to one or more of the key wine parameters: low pH, ethanol content, or residual sugar content [9]. Interestingly, the lactic acid bacteria which can thrive under these conditions, have received little attention as a potential source of glycosidic enzymes [10]. Recent reports have shown that lactic acid bacteria strains involved in wine malolactic fermentation possess β-glucosidase activity [11,12]. Likewise, it has been described that *Lactobacillus plantarum*, a wine-related lactic acid bacteria species, possesses a putative β-glucosidase (Bgl)[1]

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[1] *Abbreviations used:* Bgl, His_6-tagged β-galactosidase from *L. plantarum*; CTAB, cetyltrimethylammonium bromide.

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whose expression is regulated by abiotic stresses such as temperature, ethanol, and pH [13].

As glycosidases, and specifically β-glucosidases, are key enzymes in the enzymatic release of aromatic compounds from glycoside precursors present in fruits and fermenting products, and the application of these enzymes in a well-defined manner requires a large-scale production of the enzymes and a detailed knowledge of their structure, we decided to produce and physically characterize the putative β-glucosidase (Bgl) previously described in the food *L. plantarum* species. To our knowledge, this is the first time where the protein encoded by the bgl gene has been studied both functionally and structurally.

Materials and methods

Gene cloning and Bgl protein production

The *bgl* gene coding for a putative β-glucosidase (lp_3629) from *L. plantarum* CECT 748^T (ATCC 14917^T) was PCR-amplified by Hotstart Turbo *Pfu* DNA polymerase by using the primers 371 (5′- *CAT CATGGTGACGATGACGATAAGat*ggtagagtttccggaaggctttg) and 372 (5′-*AAGCTTAGTTAGCTATTATGCGTA*tcaaaacccattccgttccccaagc) (the nucleotides pairing the expression vector sequence are indicated in italics, and the nucleotides pairing the *bgl* gene sequence are written in lowercase letters). The 1.4-kb purified PCR product was inserted into the pURI3 vector by using the restriction enzyme- and ligation-free cloning strategy described previously [14,15]. Expression vector pURI3 was constructed based on the commercial expression vector pT7-7 (USB) but containing the following leader sequence MGGSHHHHHHGDDDDKM consisting of an N-terminal methionine followed by three spacer amino acids, a six-histidine affinity tag, a spacer glycine residue, and the five-amino acid enterokinase recognition site. Thus, the final recombinant Bgl would possess 477 amino acid residues with a molecular weight of 54.5 kDa. *E. coli* DH5α cells were transformed, recombinant plasmids were isolated and those containing the correct insert were identified by restriction-enzyme analysis, verified by DNA sequencing and then transformed into *E. coli* JM109 (DE3) cells for expression.

Cells carrying the recombinant plasmid, pURI3-Bgl, were grown at 37 °C in Luria–Bertani media containing ampicillin (100 μg ml^{-1}), until they reach an optical density at 600 nm of 0.4 and induced by adding IPTG (0.4 mM final concentration). After induction, the cells were grown at 22 °C during 20 h and collected by centrifugation.

Protein purification

The bacterial cell pellet was resuspended in Tris buffer (Tris–HCl 20 mM, pH 8.0, containing NaCl 100 mM) and homogenized by French Press. The lysate was centrifuged in an SS34 rotor at 20,000 rpm using a Sorvall centrifuge for 30 min. at 4 °C to remove the cell debris. The supernatant was then applied to a His-Trap FF Ni-affinity column (GE Healthcare). Non-specific adsorbed materials were washed off with Tris buffer containing 10 mM imidazole. Bgl was purified in a linear gradient of imidazole (10–500 mM) prepared in Tris buffer with an AKTA-prime (Pharmacia). Major peak fractions containing Bgl were pooled and dialyzed against 10 mM NaCl in 20 mM Tris–HCl buffer, pH 8.0. The protein was further purified by ion exchange chromatography on a HiTrap Q HP column (GE Healthcare) in a linear gradient of 10–500 mM NaCl in 20 mM Tris–HCl buffer, pH 8.0. Fractions containing Bgl were pooled and dialyzed against 100 mM NaCl, 2 mM DTT in 20 mM Tris–HCl buffer, pH 8.0. The sample was then concentrated and applied on a Superdex 200 prep grade column (GE Healthcare). The purified recombinant material thus obtained was concentrated with YM-10 Centricon filters (Millipore) to 9 mg/ml in 20 mM Tris–HCl, pH 8.0, containing 100 mM NaCl, 2 mM DTT, and 0.04% (w/v) sodium azide. Protein concentration was determined by UV–vis absorbance measurements with a Nanodrop® ND-1000 spectrophotometer, using an extinction coefficient of 2.15 (1 mg ml^{-1}, 1 cm, 280 nm) as estimated using the ExPASy sever [16].

Enzyme assay

Glycosidase activity was measured by determining the hydrolysis of *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-galactopyranoside as previously described [17]. The reaction mixture (500 μl) consisting of 20 mM Tris–HCl, pH 8.0, containing 100 mM NaCl and 2 mM DTT was incubated at 37 °C during regular time intervals. The reaction was stopped by adding an equal volume of 0.5 M glycine/NaOH buffer, pH 9.0, containing 2 mM EDTA. The color formation was measured at 420 nm in a UVmini-1240 UV–vis spectrophotometer (Shimadzu). Enzyme activity was measured as a function of the liberated *p*-nitrophenol, determined by the absorbance at 420 nm. One unit of β-galactosidase activity was defined as the amount of enzyme liberating 1 μmol of *p*-nitrophenol per minute under the above specified conditions. Control samples without added protein were considered for background correction.

Analytical size-exclusion chromatography

Analytical size-exclusion chromatography was performed on a Superdex 200 10/300 GL Tricorn column (GE Healthcare) equilibrated in 20 mM Tris–HCl, pH 8.0, 0.1 M NaCl, 2 mM DTT and 0.04% (w/v) sodium azide. The column was calibrated with thyroglobulin (667 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol deshydrogenase (150 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa), sperm whale myoglobin (17 kDa) and vitamin B12 (1.3 kDa) in the same buffer. The size of Bgl was determined from its K_{av} value ($K_{av} = (V_e - V_0)/(V_t - V_0)$; V_e: elution volume; V_0: void volume; V_t: total volume of the column) by interpolation in a calibration semi-log plot of the molecular mass of the standard proteins *versus* their K_{av} values.

Mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) on a Finnigan LCQ Deca ion trap Mass Spectrometer (Thermo Electron, San José, CA, USA) was used to determine the molecular mass of Bgl. Mass spectra were recorded in full scan mode (m/z = 450–2000) and protein peaks detected were deconvoluted using the BIOMASS deconvolution tool from BioWorks 3.1 software (Thermo Fisher Scientific).

Crystallization and preliminary X-ray diffraction studies

Initial crystallization conditions of Bgl at 291 K were determined using the sparse matrix method [18] with commercial screens from Hampton Research (Riverside, CA) and Qiagen in crystallization trials by the sitting-drop vapor-diffusion method in Innovaplate SD-2 96-well plates. Crystals of Bgl appeared in several conditions containing PEG 3350. Optimization of the crystallization conditions with additive and detergents screens from Hampton Research (Riverside, CA) rendered high-quality diffraction crystals in hanging drops containing 1 μl of the protein solution (9 mg/ml in 20 mM Tris–HCl, pH 8.0 with 0.1 M NaCl, 2 mM DTT and 0.04% (w/v) sodium azide), 1 μl of reservoir solution (15% (w/v) PEG 3350, 0.2 M di-ammonium phosphate, 0.1 M sodium cacodylate, pH 6.4, 2 mM DTT) and 1 μl of detergent CTAB