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Cloning, expression and structural stability of a cold-adapted β-galactosidase from *Rahnella* sp. R3

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ABSTRACT

A novel gene was isolated for the first time from a psychrophilic gram-negative bacterium *Rahnella* sp. R3. The gene encoded a cold-adapted β-galactosidase (R-β-Gal). Recombinant R-β-Gal was expressed in *Escherichia coli* BL21 (DE3), purified and characterized. R-β-gal belongs to the glycosyl hydrolase family 42. Circular dichroism spectrometry of the structural stability of R-β-Gal with respect to temperature indicated that the secondary structures of the enzyme were stable to 45 °C. In solution, the enzyme was a homo-trimer and was active at temperatures as low as 4 °C. The enzyme did not require the presence of metal ions to be active, but Mg^{2+}, Mn^{2+}, and Ca^{2+} enhanced its activity slightly, whereas Fe^{3+}, Zn^{2+} and Al^{3+} appeared to inactive it. The purified enzyme displayed K_m values of 6.5 mM for ONPG and 2.2 mM for lactose at 4 °C. These values were lower than the corresponding K_ms reported for other cold-adapted β-Gals.

1. Introduction

Cold-adapted enzymes isolated from psychrophilic microorganisms are capable of functioning at low temperatures ranging from 0 to 30 °C. These enzymes are extremely powerful tools for not only biotechnological purposes but also for industrial applications [1]. Because of their highly flexible structures, cold-adapted enzymes unfold at low to moderate temperatures [2], and unfolding can result in enzyme inactivation. The inactivation of enzymes at moderate temperatures is convenient in practice but also restricts reactions to low temperatures. Enzymes catalyze specific reactions at their catalytic sites, whose geometry is usually maintained under certain conditions (temperature, pH, presence of salts, etc.). As the ambient temperature increases, structural variations (of the secondary and tertiary structures) in cold-adapted enzymes distort the active site and decrease the enzyme's activity. Thus, understanding the mechanism of conformational variations with environmental changes for cold-adapted enzymes is undoubtedly instructive to engineering enzymes with specific purposes.

β-Galactosidases (EC 3.2.1.23) (β-Gals) from many organisms have been widely used in food manufacturing, such as in dairy processing (to hydrolyze lactose) and the synthesis of functional oligosaccharides. These enzymes play a crucial role in overcoming lactose intolerance, which is a global health issue, especially in Asia because of the lack of β-Gal in the human intestine. Cold-adapted β-Gals isolated from psychrophilic and psychrotrophic microorganisms living in cold environments (below 5 °C) [3] can be used to process dairy products at 4 °C to avoid spoilage and flavor changes [4]. Currently, the major commercial β-Gals used in lactose hydrolysis, such as Lactozym Pure® (Novozymes) and Maxilact® (DSM Food Specialties), are usually optimally active at moderate temperatures (around 37 °C) but display low activity at 4 °C. Thus, the dairy industry is in great need of cold-adapted β-Gals that are optimally active at conditions compatible with dairy processing (pH 6.5, and not inhibited by sodium, calcium or glucose) [5].

In recent years, several cold-adapted β-Gals have been isolated from different sources including *Arthrobacter* sp. C2-2 [6], *Lactococcus lactis* IL1403 [7] and *Halomonas* sp. S62 [8]. Recently, we discovered a cold-adapted β-Gal from *Rahnella* sp. R3

Abbreviations: β-Gal, β-galactosidase; R-β-Gal, cold-adapted β-galactosidase from *Rahnella* sp. R3; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ONPG, *ortho*-nitrophenyl-β-galactoside; ONP, *o*-nitrophenol; CD, circular dichroism spectroscopy.

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(R-β-Gal), a psychrophilic microorganism isolated from the No. 1 Glacier in the Tianshan Mountains (Xinjiang, China). The *Rahnella* species, first isolated from water samples in France [9], are gram-negative and facultative anaerobes [10,11]. To date, three *Rahnella* strains, namely *Rahnella* sp. BS 1, *Rahnella* sp. HS-39 [12] and *Rahnella aquatilis* 14-1 [13], have been reported to produce β-Gals with different enzymatic properties. In comparison to these β-Gals, R-β-Gal from *Rahnella* sp. R3 exhibited much higher catalytic activity (27 U/mg) at low temperatures.

In the present work, R-β-Gal was cloned and expressed in *Escherichia coli* BL21 (DE3). The variations in enzyme activity with temperature, pH and metal ions, were investigated with substrates of ONPG and lactose. The kinetic parameters (K_m, V_{max} and K_{cat}) were calculated by nonlinear regression. The structural stability was investigated by circular dichroism (CD) spectroscopy to monitor the variations in conformation (secondary structure) when the enzyme was incubated under different conditions. Furthermore, the relationship between enzyme structure and activity is discussed; future research will attempt to elucidate the evolution of this enzyme.

2. Materials and methods

2.1. Materials

5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) was purchased from Sangon Biotech (Shanghai, China). *ortho*-Nitrophenyl-β-galactoside (ONPG) and *o*-nitrophenol (ONP) were purchased from Sigma–Aldrich (St. Louis, MO, USA). All the restriction enzymes were obtained from Takara Biotechnology Co., Ltd. (Dalian, China) and were used according to the manufacturer's instructions. Nutrient media were supplied by Oxoid (Basingstoke, UK). All other reagents were analytical grade and were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Bacterial strains, plasmids, growth conditions

The *Rahnella* sp. R3 strain (CCTCC NO. M2012250) was isolated from frozen soil samples obtained by our group from Glacier No. 1, Tianshan Mountains. The strain was cultivated in Luria Broth (LB, 10 g/L tryptone, 5 g/L yeast extract and 10 g/L NaCl) and was grown at 15 °C with rotary shaking at 200 r/min for 24 h. *E. coli* DH5α and BL21 (DE3) (stored in our lab) were cultured in LB medium supplemented, as necessary, with ampicillin (100 μg/mL), X-Gal (40 μg/mL), and IPTG (24 μg/mL). The cloning vector pMD19-T and expression vector pCold I (Takara, Dalian, China) were used following the manufacturer's instructions.

2.3. R-β-Gal gene cloning

The *Rahnella* sp. R3 strain was grown at 15 °C in LB to the mid-log phase, and the chromosomal DNA from the cells was isolated using a Genomic DNA Prep Kit (Sangon Biotech, Shanghai, China) according to the protocol for gram-negative bacteria. A partial gene sequence was amplified using a pair of primers RQ-F1 and RQ-R1 (Table 1) designed from highly conserved regions, which were identified by multiple sequence alignment of amino acid sequences of β-Gals from the *Rahnella* genus. PCR amplification was performed in an Applied Biosystem ProFlex™ 3 × 32-well PCR System (Life technologies, NY, USA), and the amplified fragment of expected size was gel purified and was cloned into a pMD19-T vector for sequencing. The sequence of the amplified fragment was analyzed by the BLAST (National Center of Biotechnology Information, USA), and nested sequence-specific primers (sp1, sp2, sp3) for thermal asymmetric interlaced PCR (TAIL PCR) were designed and were used to isolate the complete sequence. Arbitrary degenerate primers (AC, LAD1-1, LAD1-2, LAD1-3 and LAD1-4) were also designed and synthesized (Table 1). The reaction parameters and arbitrary degenerate primers for TAIL PCR were modified as described in the report by Liu and Chen [14]. Nucleotide and amino acid sequence homology searches were performed using the BLAST. The signal peptides and cleavage sites were predicted with the Signal program (http://www.cbs.dtu.dk/services/SignalP). Bioinformatics analysis was performed with Vector NIT (Life technologies, NY, USA).

Table 1
Primers used for R-β-Gal cloning.

Primer name	Primer sequence
RQ-F1	ATGACGAAATTTCCTCTTCTGAGC
RQ-R2	TSACCTGATCGGTGTTAAAACGAC
AC	ACGATGGACTCCAGAG
LAD1-1	ACGATGGACTCCAGAGCGGCCGCVNVNNNGGAA
LAD1-2	ACGATGGACTCCAGAGCGGCCGCBNBNNNGGTT
LAD1-3	ACGATGGACTCCAGAGCGGCCGCVVNVNNNCCAA
LAD1-4	ACGATGGACTCCAGAGCGGCCGCBDNBNNNCGGT
Sp1	AAAAGTGCAGCTGATGAACGGGC
Sp2	GGCGGCGAATGCCATTGCGACAC
Sp3	ACCTTCTGGAGCCACACTTACACC
Bgal-*Bam*H I-F	CGCGGATCCATGACGAAATTTCCTCTTCTGAGC
Bgal-*Eco*R I-R	CCGGAATTCTTATTGTGTGATTTTACGCGTCAG

2.4. Expression and purification of recombinant R-β-Gal

PCR was performed with primers Bgal-*Bam*HI-F (forward) and Bgal-*Eco*RI-R (reverse) (Table 1) to amplify the R-β-Gal encoding sequences with an upstream *Bam*HI site and a downstream *Eco*RI site incorporated in the forward and reverse primers, respectively. The PCR product was digested with *Bam*HI and *Eco*RI and was inserted into the expression vector pCold I. The recombinant plasmid pCold I-Bgal was transformed into *E. coli* BL21 (DE3). The transformants were cultured at 37 °C in LB medium containing 100 μg/mL ampicillin with shaking (200 r/min) to an optimal density of 1.2 at 600 nm and were then transferred to 20 °C and were induced with 0.2 mM IPTG for an additional 32 h. The cells were harvested by centrifugation at 5000*g* for 30 min. Approximately 15 g of wet weight cells were obtained from 1 L of culture, and then resuspended in 50 ml binding buffer (6 mM imidazole, 20 mM Tris–HCl, 500 mM NaCl, pH 7.4). After the cells were disrupted by sonication on ice, the total lysate was cleared by centrifugation at 12,000*g* for 40 min at 4 °C and was then loaded onto a Ni–NTA Sefinose column (1.0 × 1.0 cm) (Sangon Biotech, Shanghai, China). Non-specific adsorbed materials were removed with a wash buffer (20 mM imidazole, 20 mM Tris–HCl, 500 mM NaCl, pH 7.4). The column was then eluted with an elution buffer (250 mM imidazole, 20 mM Tris–HCl, 500 mM NaCl, pH 7.4). The Ni-column eluent containing R-β-Gal appeared as a single band on 12% SDS PAGE.

2.5. Molecular mass determination

The relative molecular weight of the purified R-β-Gal was estimated by size exclusion chromatography with HR 10/30 Superdex-200 column (GE Healthcare, MI, USA). Protein molecular mass standards (thyroglobumin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase 158 kDa; and ovalbumin, 43 kDa) were purchased from GE Healthcare.

2.6. Enzyme assay

The hydrolytic activity of the recombinant R-β-Gal was determined by measuring the released ONP from ONPG and was quantified from the absorbance at 420 nm. One unit is defined as 1 μmol

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