INSTITUT PASTEUR

Research in Microbiology 161 (2010) 515–525

www.elsevier.com/locate/resmic

Efficient bioconversion of lactose in milk and whey: immobilization and biochemical characterization of a β-galactosidase from the dairy *Streptococcus thermophilus* LMD9 strain

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Received 15 February 2010; accepted 29 April 2010
Available online 28 May 2010

Abstract

The gene encoding β-galactosidase from dairy *Streptococcus thermophilus* strain LMD9 was cloned, sequenced and expressed in *Escherichia coli*. The recombinant enzyme was purified and showed high specific activity of 464 U/mg. This protein displays a homotetrameric arrangement composed of four 118 kDa monomers. Monitoring of the activity showed that this enzyme was optimally active at a wide range of temperatures (25–40 °C) and at pH from 6.5 to 7.5. Immobilization of the recombinant *E. coli* in alginate beads clearly enhanced the enzyme activity at various temperatures, including 4 and 50 °C, and at pH values from 4.0 to 8.5. Stability studies indicated that this biocatalyst has high stability within a broad range of temperatures and pH. This stability was improved not only by addition of 1 mM of Mn^{2+} and 1.2 mM Mg^{2+}, but essentially through immobilization. The remarkable bioconversion rates of lactose in milk and whey at different temperatures revealed the attractive catalytic efficiency of this enzyme, thus promoting its use for lactose hydrolysis in milk and other dairy products.

Keywords: β-galactosidase; Probiotic; Immobilization; Milk; Whey; Lactose hydrolysis

1. Introduction

Strong industrial interests are ascribed to β-galactosidases due to their hydrolase and transferase activities (Mahoney, 1998). As a consequence of their transglycosylation activity, these enzymes are widely used for production of oligosaccharides, some of which are very promising prebiotic agents (Coulier et al., 2009; Rycroft et al., 2001). Furthermore, via their hydrolytic activity, β-galactosidases are widely employed in the agrofood industry, essentially for reducing the lactose concentration in milk products with the aim of overcoming lactose intolerance (Scrimshaw and Murray, 1988). Lactose cleavage in milk represents the first probiotic effect conferred upon *Streptococcus thermophilus* and *Lactobacillus bulgaricus*, used routinely for yoghurt production (Guarner et al., 2005). Several reports described the molecular and biochemical characterization of β-galactosidases from lactic acid bacteria (Barrett et al., 2004; Ramana Rao and Dutta, 1981; Schmidt et al., 1989). Thermo-active and cold-adapted β-galactosidases from various bacteria such as *Bacillus licheniformis*, *Lactobacillus acidophilus* R22, *Geobacillus stearothermophilus*, *Sulfolobus solfataricus*, *Enterobacter agglomerans* B1, *Thermotoga maritima*, *Alicyclobacillus acidocaldarius* subsp. *Rittmannii, S. thermophilus* and *Arthrobacter psychrolactophilus* strain F2 were described as suitable biocatalysts for many industrial applications (Kim et al., 2004; Lu et al., 2007; Nakagawa et al., 2006; Nguyen et al., 2007a; Pisani et al., 1990; Phan Tran et al., 1998;

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doi:10.1016/j.resmic.2010.04.011

Ramana Rao and Dutta, 1981). Immobilization of β-galactosidases using different methods, for industrial purposes has also been reported (Hinberg et al., 1974; Heng and Glatz, 1994). Indeed, immobilization improves enzyme thermoactivity and thermostability and reduces glucose inhibition (Mateo et al., 2004; Chen et al., 2009; Elnashar and Yassin, 2008). Use of immobilized β-galactosidases for lactose hydrolysis in milk/whey as an efficient bioconverter on an industrial scale was reported (Haider and Husain, 2007, 2009a,b,c). For hydrolysis of lactose in whey and many fermented milk products, it would be of interest to determine a β-galactosidase efficient within a broad temperature range and at acidic pH values (Robinson, 1994). This would allow lactose bioconversion not only during the milk pasteurization step and storage of the products, but also during fermentation of milk. Whey is an inexpensive raw material with a pH between 5.5 and 6.5 containing a high amount of lactose, an attractive source of low calorie sweetener production (Rhimi et al., 2007). Hence, for efficient hydrolysis of the extracted lactose, the use of a β-galactosidase active at acidic pH would be particularly suitable.

Here we report the cloning, expression, purification and biochemical characterization of a β-galactosidase from the probiotic and food-grade *S. thermophilus* LMD9 strain. We also investigate the effect of immobilization of the enzyme on biochemical properties. The efficiency of free and immobilized enzymes on lactose hydrolysis in milk and whey is reported.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

Growth of the *S. thermophilus* LMD9 (*S. thermophilus*) strain under aerobic conditions at 42 °C in M17 medium (Difco) with 1% (w/v) glucose was previously described (Makarova et al., 2006; Terzaghi and Sandine, 1975). *Escherichia coli* NovaBlue (DE3) was used as host strain. Culture of the *E. coli* strain was done in Luria Bertani (LB) medium (Sambrook et al., 1989) supplemented, when required, with ampicillin (100 μg/ml), X-gal (100 μg/ml) and IPTG (isopropyl-β-D-thiogalactopyranoside) at 160 μg/ml. The pGEMT-Easy Vector plasmid (Promega) was used following the manufacturer's instructions.

2.2. DNA manipulation and PCR

S. thermophilus LMD9 total DNA was prepared as described by Pospiech and Neumann (1995). Preparation of plasmid DNA was carried out using the Gene JET™ Plasmid Miniprep kit (Fermentas). DNA digestion with restriction endonucleases and separation of fragments by agarose gel electrophoresis were performed as described (Sambrook et al., 1989). Polymerase chain reactions were carried out in a Gene Amp® PCR System 9700 (Applied Biosystems). The amplification reaction mixtures (100 μl) contained Phusion™ High-Fidelity DNA polymerase amplification buffer, 10 pmol of each primer, 100 ng of DNA template, and 2 units of Phusion HF enzyme (Finnzymes). Cycling parameters were 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, at 55 °C for 60 s, and 72 °C for 180 s and finally 15 min at 72 °C.

2.3. Cloning, overexpression and sequence analysis of the S. thermophilus *LMD9* lacZ *gene*

Based on the DNA sequence of *S. thermophilus* LMD9 β-galactosidase reported in the NCBI database (accession number YP_820735), two oligonucleotides, BGLMD9-D (5′GTTGGAGAAAAGGTAGAAGCTTTGTCTGAAGTTAT-TACCTTC3′) and BGLMD9-R (5′ACTAGATTCTTAATAAC TACCTAATTTAGTGGTTCAATCATG3′), were conceived. Chromosomal DNA isolated from *S. thermophilus* LMD9 was used as template and Phusion HF DNA polymerase for amplification. The resulting PCR product, with the expected size of nearly 3 kb, was purified using the QIAquick® Gel Extraction kit (Qiagen), following the manufacturer's instructions. The obtained fragment was treated with Taq DNA polymerase in the presence of 0.2 mM of dATP as recommended by the suppliers (Promega). Then, the resulting fragment was cloned in the pGEMT-Easy Vector (Promega) and transformed into *E. coli* NovaBlue (DE3) competent cells with the genotype: *endA1 hsdR17*(*r*K122*m*K121) *supE44 thi-1 recA1 gyrA96 relA1lac* [F9 *proA1B1 lacI*qZD*M15*::Tn*10* (Tcr)] (Novagen). The recombinant strains were selected by enzymatic restriction. The strain (MRS35) harboring the *S. thermophilus lacZ* gene under the control of the PT7 promoter was used for activity assays and grew at 37 °C to an OD_{600} of 1.0 followed by induction with IPTG (160 μg/ml). Subsequently, protein expression was allowed to continue overnight in culture at 37 °C. Notably, the amplified fragment contains the *lacZ* gene encoding full-length *S. thermophilus* LMD9 β-galactosidase and its RBS (ribosome binding site) region. The *lacZ* gene sequence was determined using an automated 3100 Genetic Analyzer (Applied Biosystems). Protein multiple sequence alignments were done using the CLUSTAL W program (Thompson et al., 1994).

2.4. Crude cell lysate preparation and enzyme purification

MRS35 cells were harvested by centrifugation (7500 g, 10 min at 4 °C) and the pellets were suspended in 100 mM HEPES buffer pH 6.5. Then, cell suspensions were incubated for 2 h on ice in the presence of 5 mg/ml lysozyme supplemented with one tablet of Complete™ EDTA-free protease inhibitor (Roche Applied Science) per 50 ml. Cell disruption was achieved by sonication at 4 °C for 6 min (pulsation of 3 s, Amplify-90) using a Vibra-Cell™ 72405 sonicator and cell debris were removed by centrifugation (30,000×*g*, for 30 min at 4 °C). For purification, crude cell extract from the MRS35 *E. coli* strain was heat-treated (55 °C, 60 min) followed by centrifugation at 30,000×*g*, for 30 min at 4 °C. Proteins were precipitated by ammonium sulfate saturation (40–50%), suspended in 100 mM HEPES buffer pH 6.5, concentrated and