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Production and secretion of *Lactobacillus crispatus* β-galactosidase in *Pichia pastoris*

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ABSTRACT

Lactobacillus β-galactosidases are mostly heterodimeric proteins, which are encoded by the two overlapping genes, *lacL* and *lacM*, and produced in recombinant prokaryotic systems for higher yield. This is the first report on the expression of a heterodimeric β-galactosidase from *Lactobacillus crispatus* B470 in *Pichia pastoris*. The overlapping consecutive genes, *lacL* and *lacM*, that shared 17 nucleotides were cloned from the genomic DNA of *L. crispatus*. A recombinant plasmid harboring both expression cassettes of *lacL* and *lacM* was constructed and transformed into *P. pastoris* GS115 competent cells. Two recombinant *P. pastoris* strains (GSLac01 and GSLac02) showed the highest β-galactosidase activities of 24.5 and 31.0 U/ml in the culture supernatants, respectively. The recombinant β-galactosidase (LcLacLM) from GSLac02 was purified to electrphoretic homogeneity by ion-exchange chromatography and molecular sieve chromatography. Similar to most *Lactobacillus* β-galactosidases that operate at moderately thermophilic and weak acid to neutral conditions, LcLacLM showed optimal activity at 50 °C and pH 5.5–6.5. It's the first report on functional and secretory expression of LacLM-type β-galactosidase in eukaryotic system. This strategy might be applied to the expression of other overlapping genes.

Introduction

β-Galactosidases (EC 3.2.1.23) are known to catalyze both hydrolytic and transgalactosylation reactions, and have wide applications in food industry, such as the manufacture of low-lactose milk products [1] and the synthesis of galacto-oligosaccharides [2–4]. Sources of β-galactosidases include microorganisms, plants and animals, but only a few β-galactosidases from *Kluveromyces lactis*, *Kluveromyces fragilis*, *Aspergillus niger* and *Aspergillus oryzae* are used in food safely and can be used in milk products [5,6].

Lactobacilli are well-known lactic acid bacteria, and are generally considered beneficial to humans for their positive roles in human health and food preservation [7,8]. β-Galactosidases from *Lactobacilli* spp., including *Lactobacillus reuteri* [9], *Lactobacillus* [10], *Lactobacillus crispatus* [11] and *Lactobacillus fermentum* [12], have been characterized, and many of them have great potentials in production of functional food and galacto-oligosaccharides [12–14]. Interestingly, the β-galactosidases from *L. fermentum* [12,14] and *L. acidophilus* R22 [15] are heterodimeric, consisting of a large subunit (LacL) and a small subunit (LacM). These β-galactosidase genes have been heterologously expressed in prokaryotic expression systems including *Escherichia coli* [10,12,16], *Lactobacillus plantarum* [14] and *Lactococcus lactis* [17]. One of them, the β-galactosidase from *Lactobacillus delbrueckii subsp. bulgaricus* DSM 20081 has shown excellent ability to synthesize galacto-oligosaccharide [14].

Compared with *E. coli* and *Lactobacillus* expression systems, the *Pichia* expression system offers several advantages, including very high cell density fermentation and extracellular secretion of recombinant proteins [18–21]. In this study, we cloned a heterodimeric LacLM-type β-galactosidase gene from *L. crispatus* B470 and functionally expressed the gene product in *Pichia pastoris* GS115. To our knowledge, it is the first report on production of heterodimeric β-galactosidase in eukaryotic expression system.

Materials and methods

Strains, plasmids and media

L. crispatus B470 was isolated from the genital tract of a Chinese female, and was deposited in Agricultural Culture Collection of China under the registration number of ACCC 03956. The specific activity of the LacLM-type β-galactosidase from *L. crispatus* B470 was 178 U/mg. *E. coli* TOP10 (TransGen, Beijing, China) and *P. pastoris* GS115 (Invitrogen, Carlsbad, CA, USA) were used as

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the gene cloning and expression hosts, respectively. Plasmid pPIC9 (Invitrogen) was used for gene cloning in *E. coli* TOP10 and expression in *P. pastoris* GS115. All media, including minimal dextrose (MD)[2] medium, minimal methanol (MM) medium, yeast peptone dextrose (YPD) medium, buffered glycerol-complex (BMGY) medium and buffered methanol-complex (BMMY) medium were prepared according to the instructions in the *Pichia* expression kit (Invitrogen).

Cloning of the β-galactosidase gene from L. crispatus B470

The cultures and genomic DNA of *L. crispatus* B470 were prepared as described by Nguyen et al. [10]. The primer pair, P1 (5′-GGATCCGTACGTAATGAAAGCAAATATCAAATGGC-3′) and P2 (5′-ATTATAGCGGCCGCTTAATTTAGCGTAAATGAGAATTC-3′), were designed to amplify the β-galactosidase gene (*LclacLM*) of *L. crispatus* B470, according to the nucleotide sequence of its closest homolog from *L. crispatus* ST1 [7]. The PCR product was cloned into pEASY-T3 (TransGen) to generate the recombinant plasmid pEASY-*LclacLM*. The presence of the β-galactosidase gene was confirmed by DNA sequencing.

Construction of recombinant plasmid pPIC9-lacLM

Two consecutive genes, *lacL* and *lacM*, were, respectively amplified from pEASY-*LclacLM* with two pairs of primers: P1 and P3 (5′-ATTATACGCGGCCGCTTATTTGTGTAAGCCATAGTAGTATC-3′), and P4 (5′-GGATCCGTACGTAATGGCTTACACAAATAATTTACACGTCG-3′) and P2, respectively. The PCR products were digested with *Sna*BI and *Not*I, and inserted into the same sites of pPIC9 to generate plasmids pPIC9-*lacL* and pPIC9-*lacM*, respectively. Using pPIC9 as template, the DNA fragment containing the origin of replication derived from pBR322 and ampicillin resistance gene was amplified with primers P5 (5′-TGAAATTTATCTCAAGATCTCTGCCTCGCGCGT TT-3′) and P6 (5′-CGTCTTTGGATGTTAGATCTCGAATAATAACTGTT-3′). The expression cassette of *lacL* consisting of the *AOX1* promoter, the α-factor secretion signal sequence, the open reading frame (ORF) for *lacL*, and the 3′-*AOX1* transcription termination fragment, was cloned from pPIC9-*lacL* using primers P7 (5′-AACAGTTATTATTCGA GATCTAACATCCAAAGACG-3′) and P8 (5′-CGTCTTTGGATGTTATCAA GCTTGCACAAACGAACTT-3′). The DNA fragment containing the *lacM* expression cassette, the ORF of *his4* and the 3′-*AOX1* transcription termination fragment was cloned from pPIC9-*lacM* using primers P9 (5′-ATCTAACATCCAAAGACGAAAGGTTGAATG-3′) and P10 (5′-AGATCTTGAGATAAATTTCACGTTTAAAATCAG-3′). The resulted DNA fragments were ligated into a circular molecule by homologous recombination using the CloneEZ kit (GenScript, Nanjing, China), finally generating the recombinant plasmid pPIC9-*LclacLM* for functional and secretory expression of the β-galactosidase gene from *L. crispatus* B470 in *P. pastoris*.

Expression of the L. crispatus β-galactosidase in P. pastoris

The recombinant plasmid pPIC9-*LclacLM* was linearized with *Bgl*II, and then transformed into *P. pastoris* GS115 competent cells via electroporation. The cells were then grown on MD plates at 28 °C for 72 h, followed by incubation on MM plates with 40 μl of 40 mg/ml X-gal spread for another 48 h. Transformants with blue plaque were considered as positive recombinant strains producing β-galactosidases. Positive strains were then inoculated in 50 ml BMGY at 28 °C for 48 h in shaking flasks. The cells were harvested by centrifugation (10,000*g* for 5 min) and then grown in 25-ml BMMY with methanol induction (1.0% [v/v]) at 28 °C for 72 h. The supernatants were collected by centrifugation (10,000*g* for 5 min) and subjected to enzyme activity assay. High-cell-density fermentation in a 2-l bioreactor was performed according to the *Pichia* fermentation guidelines (Invitrogen).

Purification of recombinant β-galactosidase

The culture supernatant was concentrated using a membrane with a 10-kDa molecular weight cut-off, and dialyzed in 50 ml buffer A (20 mM phosphate–citrate buffer, pH 7.5). The retentate was applied onto a HiTrap Capto Q column (1 ml) that was pre-equilibrated with buffer A on an automated FPLC system (Äkta Purifier, GE Healthcare, Uppsala, Sweden). The recombinant β-galactosidase was eluted using a linear gradient of 0–0.5 M NaCl in buffer A. The fractions showing β-galactosidase activity were further purified via molecular sieve chromatography (GE Healthcare) with buffer A as the mobile phase. The protein purity was evaluated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Enzyme assays

The β-galactosidase activity was assayed using *o*NPG as the substrate. The reaction system containing 200 μl of enzyme solution and 800 μl of 0.25% (w/v) *o*NPG in pre-incubated (50 °C for 5 min) phosphate–citrate buffer (50 mM, pH 6.0) was incubated at 50 °C for 15 min, followed by addition of 1 ml of 10% trichloroacetic acid and 2 ml of 1 M Na_2CO_3 sequentially. The absorbance at 420 nm was measured. One unit of β-galactosidase was defined as the amount of enzyme that released 1 μmol of o-nitrophenol per minute under the standard conditions (pH 6.0, 50 °C, 15 min).

The β-galactosidase activity for lactose was determined in the reaction system containing 200 μl of enzyme solution and 800 μl of lactose solution (250 g/l in 50 mM Na_2HPO_4-citric acid buffer, pH 6.0). The mixtures were incubated at 50 °C for 15 min, and the reactions were terminated by incubation at 100 °C for 10 min. The amount of glucose released was determined by high performance liquid chromatography (HPLC, Waters, Boston, MA, USA). One unit of lactase activity referred to the amount of enzyme necessary for the formation of 1 μmol of D-glucose per minute under the under the standard conditions as described above.

Characterization of the recombinant β-galactosidase LcLacLM

Enzyme properties were characterized with *o*NPG and lactose as the substrates. The optimal temperature for LcLacLM activity was determined by measuring the β-galactosidase activity in the range of 20–75 °C in 50 mM phosphate–citrate buffer (pH 6.0). The pH profile versus enzyme activity was assessed in 50 mM phosphate–citrate buffer at pH 4.0–8.0. To determine the pH stability of LcLacLM activity, the enzyme was incubated in 50 mM phosphate–citrate buffer at pH 4.0–8.0, 30 °C for 120 min without substrate, and the residual activity was measured as described above. Enzyme thermostability was determined by measuring the residual β-galactosidase activity after incubation of the enzyme in 50 mM phosphate–citrate buffer (pH 6.0) without substrate at various temperatures for different durations.

Steady-state kinetic data were obtained at 50 °C in 50 mM phosphate–citrate buffer (pH 6.0) containing 0–25 mM *o*NPG. K_m and V_{max} were calculated by fitting the data to the Michaelis–Menten equation using the nonlinear regression function with the software GraphPad Prism (London, UK).

The effects of metal ions on the activity of recombinant LcLacLM were also determined in the presence of 1 or 5 mM of K^+, Ag^+, Mn^{2+},

[2] *Abbreviations used:* MD, minimal dextrose; MM, minimal methanol; YPD, yeast peptone dextrose; BMGY, buffered glycerol-complex; BMMY, buffered methanol-complex; ORF, open reading frame; SDS–PAGE, sodium dodecylsulfate–polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; CWW, cell wet weight; EA, enzyme activity.