Cloning, purification, and characterization of β-galactosidase from Bacillus licheniformis DSM 13

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Cloning, purification, and characterization of β-galactosidase from *Bacillus licheniformis* DSM 13

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Abstract The gene encoding homodimeric β-galactosidase (*lacA*) from *Bacillus licheniformis* DSM 13 was cloned and overexpressed in *Escherichia coli*, and the resulting recombinant enzyme was characterized in detail. The optimum temperature and pH of the enzyme, for both *o*-nitrophenyl-β-D-galactoside (*o*NPG) and lactose hydrolysis, were 50°C and 6.5, respectively. The recombinant enzyme is stable in the range of pH 5 to 9 at 37°C and over a wide range of temperatures (4–42°C) at pH 6.5 for up to 1 month. The K_m values of LacA for lactose and *o*NPG are 169 and 13.7 mM, respectively, and it is strongly inhibited by the hydrolysis products, i.e., glucose and galactose. The monovalent ions Na^+ and K^+ in the concentration range of 1–100 mM as well as the divalent metal cations Mg^{2+}, Mn^{2+}, and Ca^{2+} at a concentration of 1 mM slightly activate enzyme activity. This enzyme can be beneficial for application in lactose hydrolysis especially at elevated temperatures due to its pronounced temperature stability; however, the transgalactosylation potential of this enzyme for the production of galacto-oligosaccharides (GOS) from lactose was low, with only 12% GOS (*w*/*w*) of total sugars obtained when the initial lactose concentration was 200 g/L.

Keywords β-Galactosidase · *Bacillus licheniformis* · Lactose hydrolysis · Transglycosylation · Recombinant

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Introduction

β-Galactosidases (β-gal, lactase, EC 3.2.1.23) catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides (such as lactose) (Nakayama and Amachi 1999) and are found widespread in nature. β-Galactosidases have been isolated and characterized from many different sources including microorganisms, plants, and animals. At present, more than a hundred putative β-galactosidase sequences can be deduced from various databases, and these can be classified into four different glycoside hydrolase (GH) families GH-1, GH-2, GH-35, and GH-42, based on functional similarities (Cantarel et al. 2009). Microbial β-galactosidases have attracted considerable attention for biotechnological applications, and hence numerous reports (Halbmayr et al. 2008; Nguyen et al. 2006; Rahim and Lee 1991; Rajakala and Karthigai 2006) and reviews (Husain 2010; Nakayama and Amachi 1999; Park and Oh 2010a) on the characterization of these enzymes from various organisms have been published. The hydrolysis of lactose

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or related compounds by β-galactosidase can be used to improve digestibility, solubility, and sweetness of dairy products. Furthermore, β-galactosidases can be conveniently used to cleave lactose into glucose and galactose, which in turn serves as an easily metabolizable and renewable substrate for a number of different fermentations. In addition, β-galactosidases can also possess transgalactosylation activity, which has recently gained considerable interest for the production of galacto-oligosaccharides (GOS), prebiotics that can stimulate the growth of beneficial bacteria such as bifidobacteria and lactobacilli (Macfarlane et al. 2008; Rastall and Maitin 2002).

Lactose maldigestion and intolerance are caused by lactase insufficiency or nonpersistence, which results from a decrease in the activity of the lactose-cleaving enzyme, β-galactosidase (lactase), in the brush border membrane of the mucosa of the small intestine of adults. 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 (de Vrese et al. 2001). Besides lactose maldigestion, crystallization of lactose can be a problem in dairy products such as ice cream and sweetened condensed milk. At high lactose concentrations, crystallization of this disaccharide can occur especially at low temperatures, resulting in sandiness of the products (Ganzle et al. 2008). β-Galactosidases derived from food-grade organisms can be successfully employed for these food-related problems related to the milk sugar lactose. The products of lactose hydrolysis, i.e., glucose and galactose, are sweeter and better digestible than lactose, thus helping to alleviate lactose maldigestion. These monosaccharides are also much more soluble than lactose; hence, sandy defects in dairy products can be avoided (Ganzle et al. 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 177 million tons of whey, equaling approximately nine million tons of lactose, are accumulating worldwide, of which roughly one third is still disposed of in the environment or used as animal feed (Ganzle et al. 2008). This can be used as a source of cheap, renewable, and fermentable sugars after β-galactosidase-catalyzed hydrolysis for a number of different large-scale biotechnological processes (Ganzle et al. 2008).

Bacillus licheniformis is a soil-dwelling endospore-forming microorganism, which has been used extensively for the industrial-scale production of important enzymes including thermostable amylase, proteases, β-lactamase, and α-acetolactate decarboxylase, as well as for the production of smaller compounds such as the antibiotic bacitracin and various organic metabolites, e.g., 2,3-butanediol and glycerol (Schallmey et al. 2004). Recently, the complete genome of *B. licheniformis* strain DSM 13 was reported, and it was pointed out that its genome contains several genes encoding enzymes of significant interest for biotechnological applications (Veith et al. 2004). Thus, the elucidation of the genome opened the door for further research on industrially relevant enzymes, and for example, a recombinant *B. licheniformis* chitinase (Songsiriritthigul et al. 2010b), endo-β-mannanase (Songsiriritthigul et al. 2010a), arabinose isomerase (Prabhu et al. 2008), and α-amylase (Hmidet et al. 2008) were studied in detail after cloning and expression of the respective gene. In this paper, we describe the cloning of β-galactosidase from *B. licheniformis* DSM 13 and its expression in *E. coli.* Furthermore, the properties of the recombinant enzyme are reported.

Materials and methods

Chemicals and enzymes

All chemicals were purchased from Sigma (St. Louis, MO, USA) and were of the highest quality available unless otherwise stated. Glucose oxidase from *Aspergillus niger* (lyophilized, 211 U/mg) was purchased from Fluka (Buchs, Switzerland), and horseradish peroxidase (POD) (lyophilized, 181 U/mg) was from Sigma. All restriction enzymes were from New England Biolabs (Beverly, MA, USA). Pfu DNA polymerase was purchased from Promega (Madison, WI, USA), while Phusion High-Fidelity DNA polymerase was from Finnzymes OY (Espoo, Finland), and T4 DNA ligase was from Fermentas (Vilnius, Lithuania). Isopropyl-β-D-thiogalactopyranoside (IPTG) was from Roth (Karlsruhe, Germany), and the test kits for determination of D-glucose and D-galactose were from Megazyme (Bray, Ireland).

Bacterial strains and culture conditions

B. licheniformis DSM 13 (ATCC 14580) was obtained from DSMZ (German Collection of Microorganisms and Cell Cultures; Braunschweig, Germany). The strain was grown aerobically at 37°C in nutrient broth (NB) containing 5 g/L peptone and 3 g/L meat extract. *E. coli* TOP10 (Invitrogen, Carlsbad, CA, USA) was grown at 37°C in Luria–Bertani (LB) broth containing 100 μg/mL ampicillin for maintaining the plasmid.

Cloning of *B. licheniformis* β-galactosidase

The gene encoding β-galactosidase from *B. licheniformis* DSM 13 was cloned by a polymerase chain reaction (PCR)-based method as previously described (Yamabhai et al. 2008). The oligonucleotide primers for PCR amplification of the *B. licheniformis lacA* gene were designed from the published genome of *B. licheniformis* DSM 13 (GenBank

accession number AE017333). The primers lacA_F2 GCA AGC TTC GCT CCA TAT GCCA and lacA_R2 GTG GTC GAC AGA TCT CTC GAG CTC TTT TG containing *Nde*I and *Xho*I restriction sites, respectively, were used for cloning into the corresponding sites of pMY201 (Yamabhai 2008). The initial denaturation step at 95°C for 5 min was followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 30 s, and extension at 72°C for 4.5 min. The final cycle was followed by additional 10-min elongation at 72°C (Yamabhai 2008). The resulting expression plasmid pOJBlilacA2 encodes the recombinant β-galactosidase carrying a C-terminal 10× His-tag followed by the FLAG tag (Fig. 1). The integrity of the construct was confirmed by automated DNA sequence analysis (VBC-Biotech).

Sequence analysis

Assembly and analysis of DNA sequences were done by Vector NTI. The basic local alignment search tool from the National Center for Biotechnology Information website was used for database searches. The comparison of β-galactosidase from *B. licheniformis* DSM 13 with homologous proteins was carried out using the program ClustalW followed by Esprit (Gouet et al. 1999).

Expression of the β-galactosidase gene in *E. coli*

E. coli TOP10 carrying the pOJBlilacA2 plasmid was grown in LB medium containing 100 μg/mL ampicillin at 37°C overnight. The overnight cultures (2% inoculum) were used to inoculate 1,500 mL of TB medium containing 100 μg/mL ampicillin. The cultures were incubated at 37°C until the optical density at 600 nm reached 0.9. The cultures were then induced by adding 0.01 mM IPTG and further incubated at 18°C for 45 h. Subsequently, the induced cells were harvested and washed once with 50 mM sodium phosphate buffer, pH 6.5, and then 50 g of cell paste in 300 mL of 50 mM sodium phosphate buffer pH 6.5 was disrupted using a continuous homogenizer (APV-2000, Silkeborg, Denmark). Cell debris was removed by centrifugation (25,000×*g*, 4°C, 15 min) to obtain the crude extract.

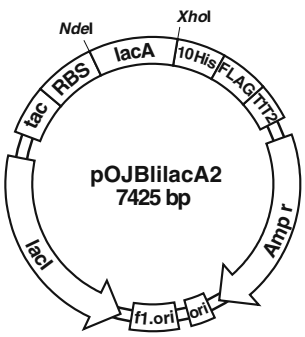

Fig. 1 Schematic overview of the pOJBlilacA2 plasmid. The plasmid is based on the pFLAG-CTS vector (Sigma, St. Louis, MO, USA): *tac* a hybrid of *trp* and *lac* promoter, *RBS* Shine-Dalgarno ribosome-binding site, *lacA* structural gene of β-galactosidase from *B. licheniformis* DSM 13, *10His* deca-histidine tag, *FLAG* octapeptide tag, T_1T_2 ribosomal RNA operon compound terminator, *Amp r* ampicillin resistance marker, *ori* (pBR322 ori) origin of double-strand replication of recombinant plasmid, *f1● ori* origin for single-strand replication of positive strand via M13 K07 Helper Phage, *lacI* repressor of tac promoter

Protein purification

The crude extract was loaded onto a 15-mL Ni Sepharose 6 fast-flow column (GE Healthcare, Uppsala, Sweden) that was pre-equilibrated with buffer A (50 mM sodium phosphate buffer, 0.2 M NaCl, 20 mM imidazole, pH 6.5). The protein was eluted at a rate of 1.5 mL/min with a 75-mL linear gradient from 0% to 100% buffer B (50 mM sodium phosphate buffer, 0.2 M NaCl, 1 M imidazole, pH 6.5). Active fractions were pooled, desalted, and concentrated by membrane ultrafiltration with a 10-kDa molecular weight cutoff (Amicon, Beverly, MA, USA). The purified enzyme was stored in 50 mM sodium phosphate buffer pH 6.5 containing 1 mM EDTA at 4°C until used.

Enzyme assays

β-Galactosidase activity was measured at 30°C and pH 6.5 by using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) and lactose as substrates according to previously published methods (Nguyen et al. 2006). The range of enzyme activity units used was between 2,000–10,000 and 40–250 U/mL for *o*NPG and lactose, respectively.

Protein determination

Protein concentrations were determined by the method of Bradford using bovine serum albumin as a standard.

Gel electrophoresis and active staining

Native polyacrylamide gel electrophoresis (PAGE) and denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were done using the PhastSystem unit (GE Healthcare, Uppsala, Sweden). Active staining using 4-methylumbelliferyl β-D-galactopyranoside as the substrate was carried out as previously described (Nguyen et al. 2006). Isoelectric focusing was performed on a PhastSystem unit according to the manufacture's instructions. The p*I* marker protein kit (pH 3–10, GE Healthcare) was used to estimate the p*I* value after the proteins were visualized by staining with Coomassie Brilliant Blue.

Steady-state kinetic measurements

All steady-state kinetic measurements were performed at 30°C using *o*NPG and lactose as the substrates in 50 mM sodium phosphate buffer (pH 6.5) with concentrations ranging from 0.1 to 22 mM for *o*NPG and 1 to 600 mM for lactose. Furthermore, the inhibition of *o*NPG hydrolysis by D-galactose and D-glucose as well as that of lactose hydrolysis by D-galactose was investigated. The kinetic parameters and inhibition constants were calculated by nonlinear regression, and the observed data were fitted to the Henri–Michaelis–Menten equation using Sigma Plot (SPSS Inc., Chicago, IL, USA).

pH and temperature dependence of activity and stability

The pH dependence of the enzymatic release of *o*NP from *o*NPG and D-glucose from lactose was measured for the range of pH 4 and 8, using Britton–Robinson buffer (containing 20 mM each of phosphoric, acetic, and boric acid) adjusted to the required pH values, under otherwise standard assay conditions. To determine the pH stability, the enzyme samples were incubated at various pH values and 37°C, and the remaining enzyme activity was determined at various time intervals using *o*NPG as the substrate under standard assay conditions.

The temperature dependence of enzyme activity (both *o*NPG and lactose activity) was determined by measuring the activity over the range of 20–70°C for 10 min. The temperature stability of the enzyme was studied by incubating enzyme samples in 50 mM sodium phosphate buffer, pH 6.5 at various temperatures in the range of 4–65°C. At certain time intervals, samples were withdrawn, and the residual activity was measured with *o*NPG as the substrate under standard assay conditions.

Effect of various cations

To study the effect of various cations on the release of *o*NP from *o*NPG, the enzyme samples were assayed with 22 mM *o*NPG (in 10 mM Bis-Tris buffer, pH 6.5) in the presence of 0.04 mM EDTA and various monovalent and divalent cations with final concentrations of 1.0, 10, and 100 mM (chloride or sulfate form) at 30°C for 10 min (Hmidet et al. 2008). The measured activities were compared with the activity of the enzyme solution without added cations under the same conditions.

Hydrolysis of lactose

Hydrolysis of lactose was carried out in discontinuous mode using eight lactase units per milliliter of reaction mixture. These reactions were carried out at two process temperatures (37°C, 60°C), using either 50 or 200 g/L initial lactose concentration in 50 mM sodium phosphate buffer (pH 6.5) and constant agitation by shaking at 300 rpm. Samples were withdrawn at various intervals, and the composition of the sugar mixtures was analyzed by capillary electrophoresis and high-performance anion exchange chromatography with pulsed amperometric detection together with authentic reference materials following methods described previously (Splechtna et al. 2006).

Results

Cloning and expression of β-galactosidase LacA from *B. licheniformis* DSM 13 in *E. coli*

The β-galactosidase LacA from *B. licheniformis* DSM 13, which is encoded by the *lacA* gene, was amplified, cloned, and expressed in *E. coli* TOP10. The *lacA* gene consists of an open reading frame of 2,055 bp, encoding 684 amino acid residues (and a stop codon) with a calculated molecular mass of 79 kDa (Yamabhai et al. 2008). The recombinant enzyme was fused with a C-terminal 10× His-tag followed by the FLAG tag to facilitate purification by one-step affinity chromatography using Ni-NTA agarose. Gene expression was optimized by varying the IPTG concentrations (0.01, 0.1, and 1 mM) and induction temperature (18°C and 25°C). The highest β-galactosidase activity produced by *E. coli* TOP10 carrying pOJBlilacA2 was obtained after 45 h of induction with 0.01 mM IPTG at 18°C. Using these conditions and cultivations in shaken flasks, approximately 74 kU of β-galactosidase activity per liter of fermentation broth with a specific activity of 51 U/mg was obtained; this corresponds to roughly 275 mg of recombinant protein formed per liter.

The His-tagged enzyme was purified to apparent homogeneity by a single-step purification protocol using a Ni Sepharose 6 fast-flow column (Fig. 2). The overall yield was 73%, and approximately 54 kU (200 mg) of purified recombinant enzyme was obtained per liter of fermentation broth with a specific activity of 270 U/mg (Table 1).

The recombinant β-galactosidase from *B. licheniformis* showed a molecular mass of ~75 kDa when analyzed by SDS-PAGE (Fig. 2a). This relates very well to the calculated mass of 78,851 Da for the LacA β-galactosidase (GenBank AAU43090). Native PAGE analysis (Fig. 2b) gave a molecular mass of approximately 160 kDa, indicating that the enzyme is a homodimer consisting of two identical 79-kDa subunits. Activity staining of the purified recombinant β-galactosidase on native PAGE using 4-methylumbelliferyl β-D-galactopyranoside as a substrate also yielded a single band of ~160 kDa corresponding to the intact homodimer (Fig. 2b). The isoelectric point of recombinant β-galactosidase overexpressed in *E. coli* was

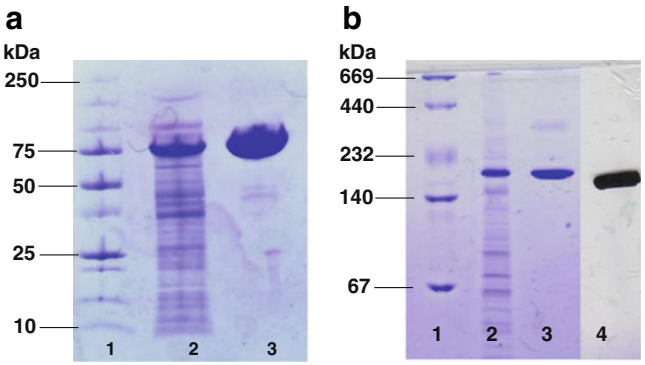

Fig. 2 SDS-PAGE (**a**) and native PAGE (**b**) analysis of β-galactosidase (lacA) from *B. licheniformis* DSM 13 overexpressed in *E. coli* TOP10. **a** *Lane 1*, recombinant molecular mass markers (Bio-Rad); *lanes 2* and *3*, Coomassie blue staining of crude extract and purified recombinant β-galactosidase, respectively. **b** *Lane 1*, high molecular weight markers (GE Healthcare); *lanes 2* and *3*, Coomassie blue staining of crude extract and purified recombinant β-galactosidase, respectively; *lane 4* activity staining with 4-methylumbelliferyl β-D-galactopyranoside of purified recombinant β-galactosidase. Approximately 5 μg of total protein was loaded onto each lane

found to be in the range of ~5.5 as analyzed by analytical isoelectric focusing (data not shown), which corresponds well to the theoretical value of 5.75.

β-Galactosidase from *B. licheniformis* can be classified as a member of GH-42, based on amino acid similarities and according to the Carbohydrate-Active Enzymes databank (http://www.cazy.org). The amino acid sequence alignment of this enzyme with other bacterial GH-42 members is shown in the Electronic Annex 1 in the online version of this article. Amino acid sequence similarities of β-galactosidase from *B. licheniformis* (GenBank accession number AAU43090) and those from other bacteria are 76.6% for *Bacillus subtilis* β-gal (GenBank accession number ABQM01000009), 47.9% for *Clostridium perfringens* β-gal (GenBank accession number BAB79873), 36.9% for *Bacillus circulans* β-gal (GenBank accession number AAA22258), 35.6% for *Bacillus stearothermophilus* β-gal (GenBank accession number P19668), 26.9% for *Thermotoga neapolitana* β-gal (GenBank accession number AAC24217), 26.8% for *Thermus thermophilus* β-gal (GenBank accession number BAA28362), 25.3% for *Thermotoga maritima* β-gal (GenBank accession number AAD36270), and 24.9% for *Haloferax* sp. (strain Aa 2.2) β-gal (GenBank accession number AAB40123).

Enzyme kinetics

The steady-state kinetic constants as well as the inhibition constants determined for the hydrolysis of lactose and *o*NPG are summarized in Table 2. The k_{cat} values were calculated on the basis of theoretical v_{max} values obtained by nonlinear regression using Sigma Plot. The catalytic efficiencies (k_{cat}/K_m) for the two substrates, lactose and *o*NPG, indicate that *o*NPG is clearly the preferred substrate, both because of more favorable K_m and k_{cat} values.

One of the end products of the β-gal-catalyzed hydrolysis of lactose, D-galactose, competitively inhibited both the hydrolysis of lactose and *o*NPG with inhibition constants of 0.93 and 0.95 mM, respectively. This competitive inhibition is surprisingly strong, also as judged on the basis of the ratios of the inhibition constants for D-galactose during hydrolysis of lactose and *o*NPG and the Michaelis constants for these substrates, $K_{i,Gal}/K_{m,Lac}$=0.0055 and $K_{i,Gal}/K_{m,oNPG}$=0.069, respectively. In addition, inhibition by the second end product, D-glucose, was studied with *o*NPG as the substrate. Again, glucose is a competitive inhibitor of β-gal from *B. licheniformis* (K_i of 83.2 mM, Table 2); however, its inhibiting effect is only moderate compared to D-galactose as is obvious from the ratio of K_i to K_m ($K_{i,Glu}/K_{m,oNPG}$=6.1).

Effects of pH and temperature

The pH optimum of β-galactosidase activity is 6.5 for both lactose and *o*NPG hydrolysis (Fig. 3a). The enzyme is stable in the pH range of 5 to 8, and it is most stable at pH 6.5, retaining more than 90% and 80% of its activity when incubated at pH 6.5 and 37°C for 5 days and 1 month, respectively (Fig. 3b).

The optimum temperature of β-galactosidase activity was 50°C when using both lactose and *o*NPG as substrates under standard 10-min assay conditions (Fig. 4a). The effect of temperature on the stability of the enzyme was also investigated. The enzyme was stable over a wide range of temperatures (4–42°C) and when kept at these temperatures for up to 1 month (Fig. 4b). The enzyme was most stable at 37°C, retaining ~90% of its activity after 1 month at this temperature. The enzyme had a half-life time of activity ($t_{1/2}$) of approximately 7 days, 5 h, and 30 min at 55°C, 60°C, and 65°C, respectively.

Table 1 Purification of recombinant, His-tagged β-galactosidase from *B. licheniformis*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)[a]	Purification fold	Recovery (%)
Crude enzyme extract	6,000±26	118±1.6	51±0.2	1.0	100
Affinity chromatography (Ni Sepharose fast flow)	4,400±15	16.2±0.6	270±0.9	5.3	73

[a] Activity was measured using *o*NPG as a substrate

Table 2 Kinetic parameters of recombinant β-galactosidase from *B. licheniformis* for the hydrolysis of lactose and *o*NPG

Substrate	v_{max} (μmol min^{-1} mg^{-1})	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})	$K_{i,Gal}$ (mM)	$K_{i,Glu}$ (mM)
Lactose	13±0.04	169±0.8	34.5±0.1	0.20±0.00	0.93±0.05	–
*o*NPG	299±2.3	13.7±0.1	785±6.1	57.3±0.05	0.95±0.02	83.2±1.1

Values are the average of duplicate experiments and represented as mean ± standard deviation

Effect of various cations on catalytic activity

The hydrolysis of *o*NPG by β-galactosidase from *B. licheniformis* was slightly activated by monovalent ions, i.e., Na^+ and K^+. Concentrations of these ions in the range of 1–100 mM exerted these stimulating effects (Table 3). The effects of K^+, Mg^{2+}, and Ca^{2+} in the presence of 10 mM Na^+ were also tested to determine whether a possible synergism exits with respect to the activation of the enzyme by cations (Table 4). However, these synergistic effects were found to be insignificant. The presence of 1 mM Mn^{2+} together with the presence of 10 mM Na^+ slightly stimulated the activity of the enzyme, while the presence of 10 mM Mn^{2+} inhibited the enzyme activity by ~40%.

Bioconversion of lactose

β-Galactosidase-catalyzed conversion of lactose is of interest both for the hydrolysis of this disaccharide as well as for the formation of GOS. Lactose hydrolysis with low levels of GOS formation could be observed for β-galactosidase from *B. licheniformis* when employing initial lactose concentrations of both 50 and 200 g/L. The time course of lactose conversion (50 g/L initial concentration and pH 6.5 employing 8 U_{Lac}/mL β-galactosidase activity, which corresponds to ~30 mg/L of recombinant β-gal) and formation of monosaccharides in discontinuous lactose hydrolysis processes is shown in Fig. 5a for two different temperatures, i.e., 37°C, at which the enzyme is most stable, and 60°C. Unconverted lactose and monosaccharides are the main components of the sugar mixtures at both process temperatures (37°C and 60°C), and low amounts of GOS were formed. Initially, the reaction proceeded rapidly, and lactose conversion was significantly faster at 60°C than at 37°C. Approximately 45% of lactose were hydrolyzed within the first 3 h of the reaction at 60°C, while ~25% were cleaved at 37°C. Thereafter, the reaction slowed down, most probably as a result of both the unfavorably high K_m and the inhibition by mainly D-galactose (Fig. 5a).

In addition, the formation of GOS was studied at various conditions as well for β-galactosidase from *B. licheniformis*. For initial lactose concentrations of 200 and 50 g/L and at a process temperature of 60°C, the maximum GOS yields were approximately 12% and 7%, respectively (Fig. 5b). The impact of different reaction temperatures (37°C and 60°C) was investigated at the initial lactose concentration of 200 g/L. GOS yields obtained at 60°C were significantly higher than at 37°C, with approximately 12% and 5% of total sugars, respectively (Fig. 5b).

Discussion

We have cloned, expressed in *E. coli*, and studied in detail the biochemical properties of GH-42 β-galactosidase (LacA) from *B. licheniformis*. The recombinant enzyme showed some properties that are appropriate for application in various processes requiring lactose hydrolysis, for example, its broad pH optimum of 5.5 to 8, its stability at

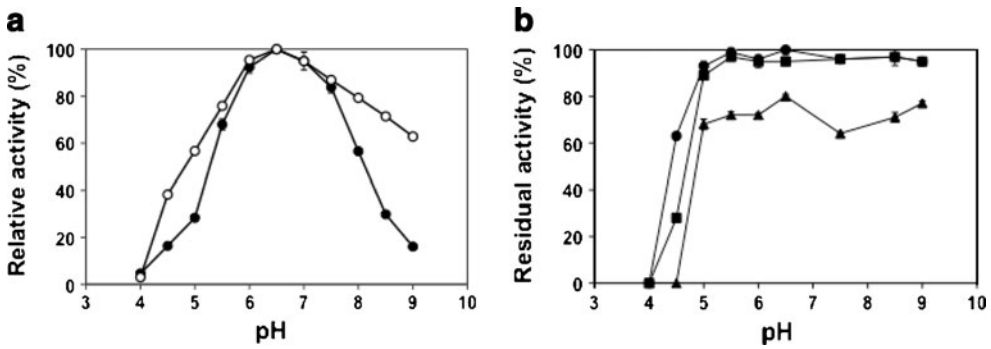

Fig. 3 Effect of the pH value on activity and stability of β-galactosidase (lacA) from *B. licheniformis*. **a** Optimum pH was determined under standard assay conditions using either *o*NPG (*filled circle*) or lactose (*empty circle*) as substrates. **b** pH stability using lactose as substrate is shown as residual activity after an incubation at 37°C for 24 h (*filled circle*), 5 days (*filled square*), and 1 month (*filled triangle*). The buffer system used was Britton–Robinson buffer. Values are the average of duplicate experiments with standard deviation shown as *error bars*

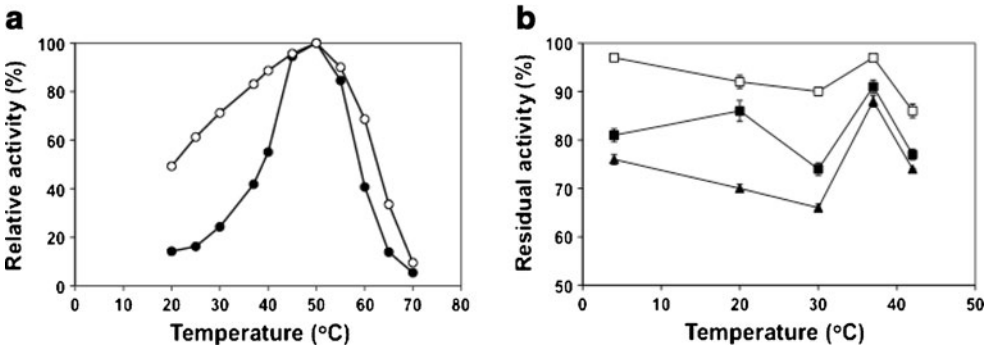

Fig. 4 Effect of temperature on activity and stability of β-galactosidase (lacA) from *B. licheniformis*. **a** Optimum temperature was determined under standard assay conditions (10-min incubation) and varying temperatures using either *o*NPG (*filled circle*) or lactose (*empty circle*) as substrates. **b** Temperature stability using lactose as substrate is shown as residual activity after an incubation at pH 6.5 for 24 h (*empty square*), 5 days (*filled square*), and 1 month (*filled triangle*) in 50 mM sodium phosphate buffer. Values are the average of duplicate experiments with standard deviation shown as *error bars*

process-relevant temperatures, or its insensitivity to Ca^{2+}. So far, there have been many reports dealing with different facets of the characterization of β-galactosidases from various microorganisms, i.e., *Kluyveromyces marxianus* (Rajakala and Karthigai 2006), *B. subtilis* (Rahim and Lee 1991), *Lactobacillus plantarum*, and *Lactobacillus sakei* (Halbmayr et al. 2008), *Lactobacillus reuteri* (Nguyen et al. 2006), and *Bacillus coagulans* (Navneet et al. 2002). Our results indicate that *B. licheniformis* LacA possesses unique characteristics that should be valuable for various aspects of research on β-galactosidase in the future.

The *lacA* gene of *B. licheniformis* when overexpressed in *E. coli* resulted in the encoded recombinant homodimeric β-galactosidase with a molecular mass of approximately 160 kDa consisting of two identical subunits of ~78 kDa as shown on native and SDS-PAGE. The amino acid sequence alignment of β-galactosidases from *B. licheniformis* DSM 13 with eight other bacterial enzymes (all from glycosyl hydrolase family 42, GH-42) revealed the conservation of Glu141 and Glu312, which are essential for the catalytic mechanism. Interestingly, *E. coli* β-galactosidase is a homotetramer (Matthews 2005), GH-42 β-galactosidase from *T. thermophilus* is a homotrimer (Hidaka et al. 2002), while the β-galactosidases from *L. reuteri* and *B. licheniformis* were proposed to be a heterodimer (Nguyen et al. 2007b) or homodimer (from this study), respectively.

Table 3 Effect of Na^+ and K^+ on the activity of β-galactosidase from *B. licheniformis* DSM13

Cation	Relative activity (%)[a]		
	1 mM	10 mM	100 mM
Na^+	128±0.9	126±0.1	131±0.2
K^+	118±0.7	122±0.2	130±0.2

Values are the average of duplicate experiments and represented as mean ± standard deviation

[a] The relative activity is based on standard conditions without added cations (100%)

The specific activity of *B. licheniformis* LacA compares well with values reported for other microbial β-galactosidases, most notably those isolated from various *Bacillus* spp.-specific activity of 5.1 and 60 U/mg for *B. circulans* and *Bacillus megaterium* β-gal, respectively (Park and Oh 2010a), indicating the high activity of the *B. licheniformis* enzyme (O'Connell and Walsh 2007). The pH optima are slightly different from those reports for purified β-galactosidase from *B. stearothermophilus* (pH 5.5) (Griffiths and Muir 1978) and *B. licheniformis* ATCC 9800 (pH 5.5) (Trân et al. 1998). Analysis of enzyme stability at various pH and temperature indicated that this enzyme is very stable when compared to other industrially used enzymes. While the recombinant *B. licheniformis* β-galactosidase is stable between pH 5 and 9 at 37°C and up to 42°C at pH 6.5 for up to 1 month, *Kluyveromyces fragilis* β-galactosidase has only 50% remaining activity when stored at 40°C at pH 6–7 for 20 h (Ladero et al. 2006). *A. niger* β-galactosidase is stable at pH 2.5–3 at temperature below 70°C in a 5-min assay (Widmer and Leuba 1979), and *B. circulans* β-galactosidase is stable between pH 5 and 9 and at temperatures below 50°C in a 30-min assay (Fujimoto et al. 1998). Therefore, this recombinant enzyme is highly suitable for various biotechnological applications.

The lower Michaelis constant for the chromogenic model substrate (*o*NPG) than that for the natural substrate (lactose) is in accordance with the properties of β-galactosidases from a number of different sources (Nguyen et al. 2006; Samoshina and Samoshin 2005). The K_m value of 169 mM determined for lactose was found to be quite high when compared to other β-galactosidase members of family GH-42, for instance, 3.7, 2.94, and 2.71 mM for three isoforms of β-gal from *B. circulans*, respectively (Vetere and Paoletti 1998); 6.18 mM for β-gal from *Bacillus* sp. MTCC 3088 (Chakraborti et al. 2000); or

Table 4 Synergistic effect of different cations on β-galactosidase activity from *B. licheniformis*

Cation	10 mM Na^+ 1 mM K^+	10 mM Na^+ 10 mM K^+	10 mM Na^+ 1 mM Mn^{2+}	10 mM Na^+ 10 mM Mn^{2+}	10 mM Na^+ 1 mM Mg^{2+}	10 mM Na^+ 10 mM Mg^{2+}	10 mM Na^+ 1 mM Ca^{2+}	10 mM Na^+ 10 mM Ca^{2+}
Relative activity (%)[a]	123±0.8	126±1.1	136±1.3	78±2.9	134±1.5	137±0.3	135±0.1	136±1.3

Values are the average of duplicate experiments and represented as mean± standard deviation

[a] The relative activity is based on standard conditions without added cations (100%)

42 mM for β-gal from *Thermus* sp. IB-21 (Kang et al. 2005). However, this K_m value is comparable to those reported for commercial fungal and yeast enzymes that are commonly employed in biotechnological applications (36–180 mM for β-gal from *Aspergillus oryzae* and 54–99 mM for β-gal from *A. niger*) (De Roos 2004).

Various degrees of inhibition as well as different inhibition types by both end products D-galactose and D-glucose are commonly found for β-galactosidases from different organisms, such as *Bacillus* (*Geobacillus*) *stearothermophilus* ($K_{i,Gal}$=2.5 mM) (Goodman and Pederson 1976), *L. reuteri* ($K_{i,Gal}$, $K_{i,Glu}$=115 and 683 mM, respectively) (Nguyen et al. 2006), *Arthrobacter* sp. ($K_{i,Gal}$=12 mM), *Kluyveromyces lactis* ($K_{i,Gal}$, $K_{i,Glu}$=45 and 758 mM, respectively), *Thermus* sp. ($K_{i,Gal}$, $K_{i,Glu}$=3 and 50 mM, respectively), and *Caldicellulosiruptor saccharolyticus* ($K_{i,Gal}$, $K_{i,Glu}$=12 and 1,170 mM, respectively). While a competitive inhibition is prevailing with D-galactose, which typically is a much stronger inhibitor, the inhibition by D-glucose can be of the competitive, noncompetitive, and uncompetitive type (Park and Oh 2010a). Interestingly, different GH-42 β-galactosidases can vary considerably in their inhibition by D-galactose. β-Gal from *B. licheniformis* shows, as reported in the result section, an unexpectedly high end-product inhibition ($K_{i,Gal}$=0.93 mM; $K_{i,Gal}/K_{m,Lac}$=0.0055), which was almost equally pronounced as that observed in *B. stearothermophilus* β-gal ($K_{i,Gal}$=2.5 mM; $K_{i,Gal}/K_{m,Lac}$=0.023) (Goodman and Pederson 1976). In contrast, the GH-42 β-gal from *C. saccharolyticus* is much less affected by its end product D-galactose ($K_{i,Gal}$=12 mM; $K_{i,Gal}/K_{m,Lac}$=0.4) (Park and Oh 2010b) as judged by the $K_{i,Gal}/K_{m,Lac}$ ratio. This inhibition of β-galactosidase activity by its end products can be a serious technological problem, as close to complete hydrolysis of lactose, either when aiming at producing the hydrolyzate as a renewable feedstock for further fermentations or at food applications, can only be achieved by a significantly prolonged conversion time or by adding disproportionate amounts of enzyme. Very few studies have addressed structure/function relationships of β-galactosidases with respect to this end product inhibition. Some recent preliminary data, however, indicate that some variants of the *C. saccharolyticus* β-gal, in which active site residues were exchanged, show reduced inhibition (Park and Oh 2010a). Because of this pronounced inhibition, β-gal from *B. licheniformis* could be an excellent model protein for future structure/function studies, e.g., in directed evolution approaches or by approaches of semi-rational design, in order to obtain a better fundamental understanding of end-product inhibition of β-galactosidases.

The observations of the effects of various cations are in agreement with the reports on the requirements for monovalent and divalent metal ions for optimal activity

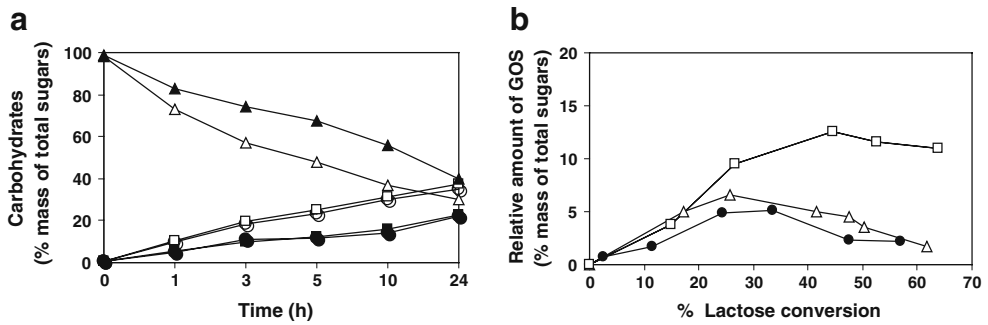

Fig. 5 Bioconversion of lactose. **a** Time course of lactose conversion in discontinuous batch processes. The reactions were carried out using an initial lactose concentration of 50 g/L in 50 mM sodium phosphate buffer (pH 6.5) and 8 U_{Lac}/mL β-galactosidase activity both at 37°C [lactose (*filled triangle*), glucose (*filled square*), galactose (*filled circle*)] and 60°C [lactose (*empty triangle*), glucose (*empty square*), galactose (*empty circle*)]. The amounts of sugars at various time intervals are reported as percentage of mass of total sugars. **b** Formation of GOS during lactose conversion at different temperatures and initial lactose concentrations. The reactions were performed at 60°C, using 50 g/L (*empty triangle*) and 200 g/L (*empty square*) of lactose in 50 mM sodium phosphate buffer (pH 6.5) as substrates, and at 37°C, using 200 g/L lactose in 50 mM sodium phosphate buffer (pH 6.5) (*filled circle*) as substrate. The relative amount of GOS (percent mass of total sugars) at different levels of lactose conversion is shown

and stability for a number of different β-galactosidases (Nakayama and Amachi 1999; Nguyen et al. 2007a; Nguyen et al. 2006). Ca^{2+} is a known inhibitor of some β-galactosidases (Garman et al. 1996; Smart et al. 1985), but interestingly, it could to some extent activate β-galactosidase from *B. licheniformis* (Table 4) when added in concentrations of 1–10 mM, which also correlates to concentrations of free calcium in milk or whey. This property can be advantageous for applications in lactose conversion processes directly in milk or when using lactose-rich substrates based on whey with high level of free Ca^{2+} in solution. In addition, we found that 10 mM of Cu^{2+}, Zn^{2+}, and Fe^{2+} inactivated β-galactosidase activity by 100%, 94%, and 66%, respectively (data not shown).

The results of transglycosylation reaction analysis are in accordance with the previous observations that an increase in the initial lactose concentration is one of the main factors for GOS formation in addition to enzyme source, temperature, and pH of the reaction condition (Gosling et al. 2010; Park and Oh 2010a; Zarate and Lopez-Leiva 1990). Furthermore, the effect of the reaction temperature on GOS yields is remarkable. Generally, higher temperatures in processes aiming at GOS formation are thought after since an increase in temperature improves the solubility of lactose, which is relatively low at ambient temperatures, and increased lactose concentrations are one of the main factors affecting GOS yields (Gosling et al. 2010). A positive effect of the reaction temperature on GOS yields has been reported in very few studies (Boon et al. 2000; Hsu et al. 2007), but it was concluded from a comparative study using four different β-galactosidases that the effect of temperature is small (Boon et al. 2000). An increase in the process temperature from 37°C to 60°C at a constant lactose concentration of 200 g/L (585 mM) could more than double the GOS yields attained when using β-galactosidase from *B. licheniformis*. The different maximal yields were however obtained at different levels of lactose conversion, as can be expected from a kinetically controlled reaction, indicating that the conversion process has to be investigated over the entire range of lactose conversion for conclusive results. The GOS yield observed in this work was not as high as expected. β-Galactosidases from family GH-42 are thought to have lesser activity for both hydrolysis and transfer than GH-2 β-galactosidases, yet no systematic study has been aimed at this comparison of the two GH families (Gosling et al. 2010). The rather low GOS yields obtained with β-galactosidase from *B. licheniformis*, however, seem to corroborate this observation.

One weakness of this enzyme is its strong inhibition by the end products, mainly D-galactose, which slows down the hydrolysis process considerably when accumulating during the hydrolysis process. Nevertheless, the hydrolytic property of β-galactosidase from *B. licheniformis*, an organism that has been widely used for the production of food-approved enzymes, could be beneficial for partial lactose removal in food products, or for improving the quality of dairy products by increasing their solubility and sweetness. It is generally accepted that 50–80% lactose-reduced milk will satisfy the physiological requirements of the majority of lactose-intolerant groups (Indyk et al. 1996). This strong inhibition by D-galactose together with the fact that the enzyme is encoded by a single gene should make it an attractive starting point for detailed structure/function studies on end product inhibition of β-galactosidases, which is not well understood at present and which hampers the efficient utilization of lactose as a renewable sugar, as well as for further improvements by various techniques in directed evolution (Arnold and Volkov 1999).

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