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Factors influencing synthesis and activity of β-galactosidase in *Lactobacillus acidophilus*

Tarun Bhowmik, M.C. Johnson and Bibek Ray

Food Microbiology Laboratory, University of Wyoming, Laramie, WY, U.S.A.

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SUMMARY

In the type-strain *Lactobacillus acidophilus* ATCC 4356 β-galactosidase (β-gal) was inducible; lactose, galactose, melibiose and probably maltose, but not glucose, fructose, mannose, sucrose and cellobiose, induced β-gal synthesis. Glucose partially inhibited β-gal-induction by lactose but not by isopropyl-β-D-thiogalactoside. β-gal synthesis during cell growth was maximal at 0.4% lactose, stimulated by Ca^{2+} but inhibited by Mg^{2+} and Mn^{2+}. β-gal in the cell-free extract had optimum activity at pH 6.5 and at 45°C. The enzyme activity was stimulated by Mg^{2+}, inhibited by Ca^{2+}, destroyed by oxidizing agents and protected by reducing agents.

INTRODUCTION

Consumption of foods containing *Lactobacillus acidophilus* has been credited with many health benefits in humans, including enhanced digestion of lactose in lactose-intolerant individuals [6–8, 16–18]. Dairy products either fermented with *L. acidophilus* or to which live *L. acidophilus* has been added have been reported to be tolerated by lactose intolerants without obvious complications [1,12]. In both types of products *L. acidophilus* supplies the β-galactosidase (EC 3.2.1.23; β-gal) necessary for the hydrolysis of lactose [2,12] with resultant reduction of the level of lactose in these fermented dairy products. Consumption of live *L. acidophilus* may provide β-gal for lactose hydrolysis in the small intestine.

Recent studies have shown that *L. acidophilus* hydrolyzes lactose principally by β-gal [3,5,19]. We reported that β-gal in *L. acidophilus* is inducible and that strains differ greatly in their level of activity [5]. We also observed that the level of β-gal activity was dependent on the growth conditions [5]. For optimum benefit, it is important that a dietary adjunct containing *L. acidophilus* should have cells with high β-gal activity. However, very little information is available on the influence of growth conditions on the synthesis and activity of β-gal in *L. acidophilus*. The objectives of this study were to evaluate the influence of different growth conditions and assay parameters on the activity of β-gal in *L. acidophilus*.

Correspondence: Dr. B. Ray, Food Microbiology Laboratory, University of Wyoming, Laramie, WY 82071, U.S.A.

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MATERIALS AND METHODS

Culture and culture conditions

L. acidophilus ATCC 4356, obtained from American Type Culture Collection (ATCC, Rockville, MD), was grown in a basal broth medium similar to Lactobacillus MRS broth (Difco No. 0881) except that beef extract was omitted and filter-sterilized (0.45 μm pore size membrane filter) carbohydrate solution was added as desired. All cultures, unless otherwise indicated, were incubated for 16 h at 37°C for the cells to reach late exponential phase.

Preparation of crude enzyme extract

Cells were harvested by centrifugation, washed twice with deionized water (dH_2O) and resuspended to the original volume in dH_2O. Ten milliliters of cell suspension and 10 ml of glass beads (0.1 mm) were homogenized in a Braun MSK homogenizer for 3 min at 4°C to break the cells. The glass beads were removed by filtration and the filtrate was centrifuged at 15000 $\times$ g for 15 min at 4°C. The supernatant was the cell-free enzyme extract used in these studies. The pellets did not have any β-gal activity.

β-Galactosidase assay

The standard reaction mixture consisted of: 0.2 ml cell-free extract, 1.6 ml Z buffer [13] (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM 2-mercaptoethanol, pH 7.0), and 0.2 ml 10 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG). The contents were incubated at 37°C for 5 min and the reaction was stopped by adding 1 ml of 1 M Na_2CO_3. The absorbance (OD) was read at 420 nm and the amount of *o*-nitrophenol (ONP) liberated was determined from a standard curve (μmol ONP vs. OD_{420}). The protein content of the cell-free extract was estimated with the Coomassie Brilliant Blue binding assay (Bio-Rad, Richmond, CA). A unit of activity was defined as μmol ONP/μg protein/min.

Influence of growth conditions on β-galactosidase synthesis

The influence in the culture media of several carbohydrates, different concentrations of lactose and salts of three divalent cations on β-gal activity was studied. Cells were grown in the presence of 2% (w/v) lactose, galactose, melibiose, glucose, sucrose, fructose, mannose, or cellobiose. The influence of lactose concentration was studied by growing the cells in the presence of 0.1–2.0% (w/v) lactose. The salts, $CaCl_2$, $MgSO_4$, or $MnSO_4$ in concentrations from 5 to 20 mM, were added to the growth medium containing 0.4% lactose. The basal medium contained 0.3 mM $MnSO_4$, 0.4 mM $MgSO_4$ and no $CaCl_2$. Cells were centrifuged after incubation overnight at 37°C and crude enzyme extracts were prepared and assayed.

Determination of pH and temperature optima of β-galactosidase

Enzyme extract was prepared from an overnight culture grown in the presence of 0.4% lactose. The optimum pH was determined by assaying β-gal in Z buffer which had the pH adjusted from 5.0 to 8.5. Effect of temperature on β-gal activity was also assayed in Z buffer from 25°C to 70°C.

Influence of salts, buffers and reducing agents on β-galactosidase activity

The effect of salts on β-gal activity was studied in X buffer (Z buffer minus $MgSO_4$). Higher concentrations (5–20 mM) of $CaCl_2$ were studied in HEPES buffer (pH 7.0) due to calcium phosphate precipitation in the original buffer. The influence of reducing agents on β-gal was studied in Y buffer (Z buffer minus 2-mercaptoethanol). Results are reported as percentages of the level of activity observed in complete Z buffer (Table 2). See Table 2 for specific agents tested.

Storage stability of β-galactosidase from L. acidophilus 4356

Crude enzyme extract was obtained from an

overnight culture grown in the presence of 0.4% lactose as described above. Samples were stored in three different buffers: 0.1 M PO_4 (pH 7.0), 0.1 M PO_4 + 20 mM *p*-chloromercuribenzoate (PCMB) (pH 7.0), and Z buffer at 4°C for up to 6 days. A 0.2 ml sample was taken and assayed each day in 1.6 ml of Z buffer.

Induction of β-galactosidase in L. acidophilus 4356

The culture was transferred four times in broth containing glucose. The cells were harvested in late exponential phase (12 h), washed and then resuspended in sterile phosphate-buffered saline (PBS), pH 7.0, and incubated at 37°C for 2 h, to deplete intracellular reserves. The microorganisms were then centrifuged and resuspended in basal broth containing one of the following: 10 mM lactose; 10 mM lactose + 10 mM isopropyl-β-D-thiogalactoside (IPTC); 10 mM glucose + 10 mM lactose; 10 mM glucose + 10 mM IPTG; 10 mM glucose. The cell suspensions were incubated at 37°C and samples were withdrawn at selected intervals up to 2 h and assayed for β-gal activity using chloroform permeabilized cells.

RESULTS

Influence of growth conditions on β-galactosidase activity

Growth of *L. acidophilus* 4356 in the presence of lactose, galactose and melibiose produced β-gal activities of 4.2, 3.8 and 2.7 μmol ONP/μg crude enzyme protein/min, respectively (Table 1). Maltose induced a low level of β-gal, while glucose, sucrose, fructose, mannose and cellobiose did not induce β-gal activity.

The level of β-gal activity produced by *L. acidophilus* 4356 was dependent on the lactose concentration in the growth medium (Fig. 1). The highest activity was found in cells grown in the presence of 0.4% lactose and activity decreased at both lower and higher concentrations. At 0.4% and higher lactose concentrations, cell growth was also higher (data not presented).

The addition of $CaCl_2$ to the growth medium stimulated higher levels of β-gal activity than in the control (Fig. 2). The presence of additional $MgSO_4$ or $MnSO_4$ decreased levels of β-gal activity, while in the presence of 20 mM $MnSO_4$ the cells failed to grow.

Since β-gal activity was determined in relation to total soluble cell protein, these figures could be considered indicative of β-gal synthesis. Therefore, it appeared that 0.4% lactose and 5 mM $CaCl_2$ stimulated maximum β-gal synthesis within the parameters studied.

Table 1

Effect of carbohydrates on the synthesis of β-galactosidase in *L. acidophilus* ATCC 4356

Carbohydrate (2%, w/v)	Activity[a]
Lactose	4.2 ± 0.4
Galactose	3.8 ± 0.7
Melibiose	2.7 ± 0.5
Maltose	0.1 ± 0.0
Glucose	0
Sucrose	0
Fructose	0
Mannose	0
Cellobiose	0

[a] Data based on two trials; triplicate samples were used in each trial. Activity in cell-free extract was defined as μmol ONP/μg protein/min at 37°C.

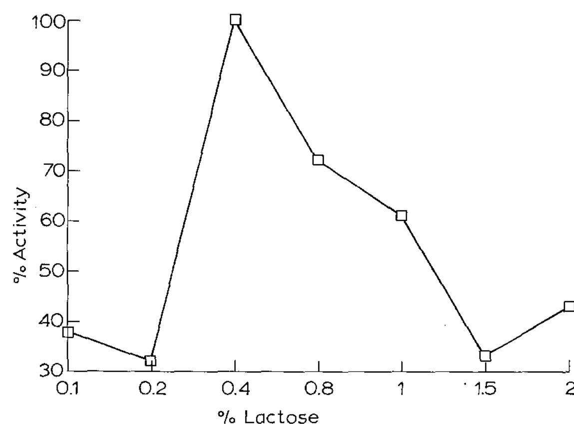


Fig. 1. Effect of lactose concentration in the growth medium on level of β-galactosidase activity in *L. acidophilus* 4356. 100% activity = 6.3 μmol ONP/μg protein/min.

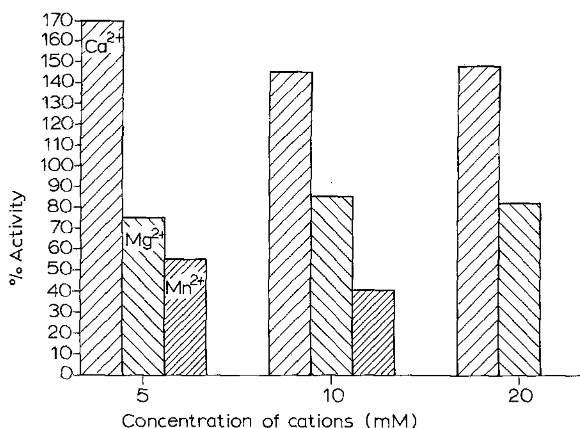
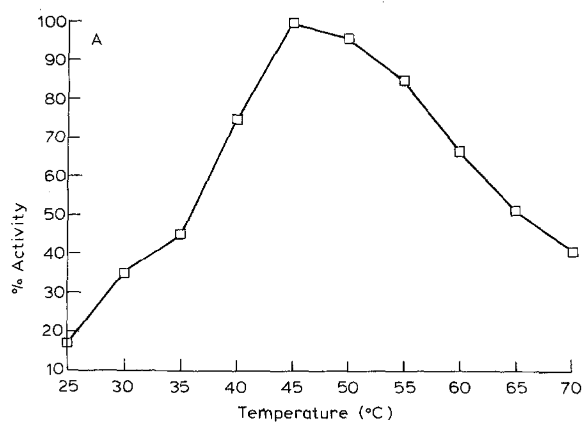


Fig. 2. Effect of divalent cations in the growth medium on β-galactosidase synthesis in *L. acidophilus* 4356. 100% activity = 3.2 μmol ONP/μg protein/min.



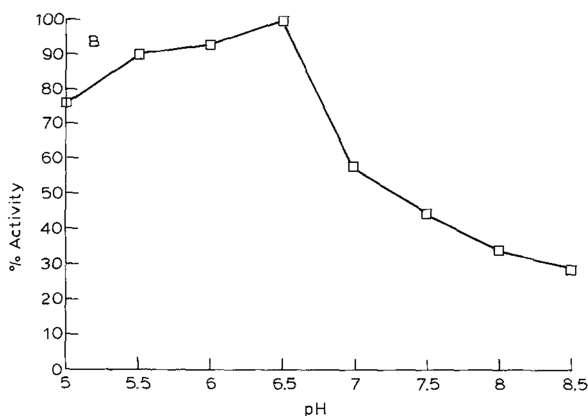


Fig. 3. The effect of temperature (A) and pH (B) of assay medium on the activity of crude enzyme extracts from *L. acidophilus* 4356. (A) 100% activity = 9.1 μmol ONP/μg protein/min. (B) 100% activity = 4.0 μmol ONP/μg protein/min.

Temperature and pH optima of β-galactosidase from L. acidophilus 4356

Crude enzyme preparations from cells grown in the presence of 0.4% lactose were assayed in Z buffer to determine temperature and pH optima (Fig. 3). The highest specific activity was found at pH 6.5 and at 45°C.

Influence of salts, buffers and reducing agents on β-galactosidase activity

The activity of crude enzyme preparations from *L. acidophilus* 4356 were determined in several buffers and salts (Table 2). Activity in Z buffer was set at 100% for comparison with other buffers and chemicals. Divalent cations seem to be necessary for activity, since 20 mM EDTA reduced the activity to 26.9%. However, calcium was not an effective cation as it inhibited β-gal activity. The presence of a sulfhydryl reducing compound caused about a 25% enhancement of β-gal activity.

Storage stability of β-galactosidase

The activity of crude β-gal extract was most stable in a phosphate buffer (Fig. 4). The presence of 20 mM PCMB caused total loss of activity by the sixth day of storage at 4°C.

Induction of β-galactosidase activity

The following compounds induced β-gal activity in starved *L. acidophilus* 4356: lactose, lactose + glucose, lactose + IPTG, and glucose + IPTG (Table 3). There was a lower level of induction when a combination of glucose and lactose was used. Glucose alone did not induce β-gal activity.

DISCUSSION

L. acidophilus 4356 has an inducible β-gal system which is activated by lactose, galactose or melibiose and under certain circumstances by IPTG. Thus the galactoside moiety was apparently important in the induction of β-gal in *L. acidophilus*. Other workers have also reported the presence of β-gal in different

Table 2

Influence of salts and buffers on β-galactosidase activity in *L. acidophilus* ATCC 4356

Agent	% activity[f]
Z buffer[a]	100
X buffer[b]	63.7 ± 7.2
Z buffer + EDTA (20 mM)	26.9 ± 6.3
Tris[c] (1 mM, pH 7.0)	75.3 ± 2.3
HEPES[d] (1 mM, pH 7.0)	82.8 ± 3.6
X buffer + 1mM $HgCl_2$	58.9 ± 4.2
X buffer + 1 mM $MgCl_2$	109 ± 1.0
X buffer + 1 mM $ZnSO_4$	79.1 ± 8.1
X buffer + 1 mM $AgNO_3$	78.2 ± 7.9
X buffer + 1 mM $CaCl_2$	48.9 ± 4.4
HEPES + 5 mM $CaCl_2$	9.3 ± 1.1
HEPES + 10 mM $CaCl_2$	0.0
HEPES + 20 mM $CaCl_2$	0.0
HEPES + 1 mM $MgSO_4$	123.9 ± 3.1
Y buffer[e]	74.5 ± 2.2
Y buffer + 50 mM L-cysteine	98.1 ± 1.1
Y buffer + 50 mM dithiothreitol	86.9 ± 2.4

[a] Z buffer: 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM 2-mercaptoethanol, pH 7.0.
[b] X buffer: Z buffer minus $MgSO_4$, pH 7.0.
[c] Tris: Tris-(hydroxymethyl)aminomethane.
[d] HEPES: *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.
[e] Y buffer: Z buffer minus 2-mercaptoethanol, pH 7.0.
[f] Data based on two trials; triplicate samples were used in each trial. 100% activity = 3.3 μmol ONP/μg protein/min.

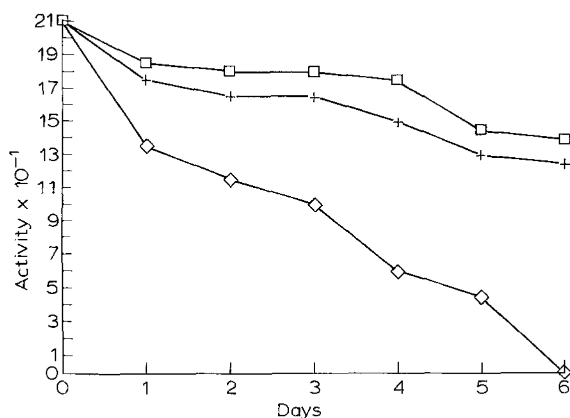


Fig. 4. Storage stability of β-galactosidase crude enzyme extract from *L. acidophilus* 4356. Samples stored at 4°C in (□), 0.1 M PO_4, pH 7.0; (+) Z buffer, pH 7.0; or (◇) 0.1 M PO_4 + 20 mM *p*-chloromercuribenzoate, pH 7.0. Activity × 10^{-1} = μmol ONP/μg protein/min.

Table 3

Induction of β-galactosidase activity in *L. acidophilus* 4356

Inducer[a]	Total activity[b] at:	
	0 min	60 min
Lactose	0	228
Lactose + Glucose	0	131
Lactose + IPTG[c]	0	232
Glucose + IPTG	0	280
Glucose	0	0

[a] Each inducer was present at a concentration of 10 mM.
[b] Micromoles ONP/cell turbidity unit/min, using permeabilized cells.
[c] Isopropyl-β-D-thiogalactoside.

L. acidophilus strains [3,5,19] and its induction by lactose and galactose [5]. Fisher et al. [5] and Cesca et al. [3] reported that although β-gal was the principal lactase enzyme in *L. acidophilus*, phospho-β-galactosidase (EC 3.2.1.85; P-β-gal) was also present at low levels in the strains they tested. Presence of both β-gal and P-β-gal in many *Lactobacillus* species has been reported by others [3,11,14].

Although lactose was an inducer of β-gal synthesis, the degree of induction was greatly affected by the lactose concentration in the growth medium. The maximum level of β-gal activity was found in cells grown in the presence of 0.4% lactose. At 2% lactose the β-gal level was reduced by 50%. Others have shown that *L. acidophilus* grown in 0.25% lactose has higher β-gal activity than when it is grown in 1% lactose [3,19]. This characteristic will be of particular interest when *L. acidophilus* is used to ferment milk, which has about 5% lactose.

Levels of calcium from 5 to 20 mM in the growth medium stimulated synthesis of β-gal while magnesium and manganese caused an apparent decrease in its production. The influence of cations on the fermentation of milk, which contains 3 mM Ca^{2+} and 0.3 mM Mg^{2+}, should be an important consideration in the production of fermented acidophilus products.

The optimum pH of β-gal from *L. acidophilus* ATCC 4356 was 6.5 in Z buffer. In the same strain, Cesca et al. [3], using acetate buffer, and Fisher et al. [5], using phosphate buffer, observed the pH optimum to be 5.8 and 7.0, respectively. The temperature optimum for this enzyme was 45°C, as reported by Cesca et al. [3]. At concentrations of 5 mM and higher, calcium completely inhibited β-gal activity of *L. acidophilus*. Inhibition of β-gal activity of several *Lactobacillus* species by ⩾5 mM calcium has also been observed by others [3,4,20]. Inhibition of β-gal activity in a cell-free enzyme extract of *L. acidophilus* by calcium needs close consideration. This is especially important for treating milk with microbial β-gal extract to reduce the lactose content and to make it suitable for consumption by lactose-intolerant individuals and production of fermentable products [9,15,20]. For the best results, the optimum pH, temperature and the influence of calcium and magnesium on β-gal activity from the microbial species should be considered.

As observed by Cesca et al. [3], 2-mercaptoethanol and other reducing compounds enhanced β-gal activity in several *Lactobacillus* species, suggesting the involvement of sulfhydryl groups in or near the active site of β-gal. Such a structure for the active site of β-gal in *Lactobacillus* species has been suggested by others [4,10]. The loss of β-gal activity in the presence of PCMB, an agent that oxidizes -SH groups, also supported the involvement of -SH groups in β-gal activity. In studies that require measurement of β-gal activity or in using β-gal for lactose hydrolysis in dairy products, the importance of maintaining a reduced state should be recognized.

Storage of cell-free enzyme extract at 4°C also resulted in a loss of activity by about 50% in 6 days. How this relates to the β-gal activity in either cultured products or products containing *L. acidophilus* and stored at 4°C needs to be determined.

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