Folia Microbiol. 45 (1), 29–34 (2000) http://www.biomed.cas.cz/mbu/folia/

Isolation and Characterization of β-Galactosidase from *Lactobacillus crispatus*

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Received 12 July 1999

ABSTRACT. β-Galactosidase was isolated from the cell-free extracts of *Lactobacillus crispatus* strain ATCC 33820 and the effects of temperature, pH, sugars and monovalent and divalent cations on the activity of the enzyme were examined. *L. crispatus* produced the maximum amount of enzyme when grown in MRS medium containing galactose (as carbon source) at 37 °C and pH 6.5 for 2 d, addition of glucose repressing enzyme production. Addition of lactose to the growth medium containing galactose inhibited the enzyme synthesis. The enzyme was active between 20 and 60 °C and in the pH range of 4–9. However, the optimum enzyme activity was at 45 °C and pH 6.5. The enzyme was stable up to 45 °C when incubated at various temperatures for 15 min at pH 6.5. When the enzyme was exposed to various pH values at 45 °C for 1 h, it retained the original activity over the pH range of 6.0–7.0. Presence of divalent cations, such as Fe^{2+} and Mn^{2+}, in the reaction mixture increased enzyme activity, whereas Zn^{2+} was inhibitory. The K_m was 1.16 mmol/L for 2-nitrophenyl-β-D-galactopyranose and 14.2 mmol/L for lactose.

Lactose makes up about 4.5–5.0 % of the total solids in fluid milk and about 4.8–4.9 % of the total solids in cheese whey. The low solubility of lactose presents problems in the concentration of whey for transportation, storage, and stock-food purposes. Lactose also can cause undesirable "sandiness" in frozen desserts. The comparatively low sweetness of lactose restricts its use and the use of whey concentrates in many food applications. Furthermore, the digestion of lactose is a problem faced by a large proportion of the world's population. These problems can be alleviated by hydrolyzing lactose to glucose and galactose. Glucose and galactose are sweeter, more soluble and are more readily digested than lactose (Greenberg and Mahoney 1982; Smart *et al.* 1985).

Lactose can be hydrolyzed either by acids or by enzymes. Enzymic hydrolysis of lactose into glucose and galactose with β-galactosidase (EC 3.2.1.23) is particularly suitable for milk and cheese whey because they also contain protein and other organic substances. Many microorganisms have been surveyed for the purpose of selecting organisms capable of producing high levels of β-galactosidase, that is highly active and stable (Gekas and López-Leiva 1985; Greenberg and Mahoney 1982; Saito *et al.* 1992; Sani *et al.* 1999). The enzyme has been isolated from numerous microorganisms, characterized extensively, and tested for commercial application (Gekas and López-Leiva 1985; Greenberg and Mahoney 1982). However, only a few β-galactosidase preparations are generally recognized as safe (GRAS) for use in foods and food systems (Gekas and López-Leiva 1985; Linko *et al.* 1992). Most of the lactose-hydrolyzing enzymes currently available on the market are heat-labile and have low activity at refrigeration temperature (Harju 1987; Rahim and Lee 1991). Therefore, there is a definite need for β-galactosidase that is stable at high and low temperatures and could be approved as GRAS for hydrolysis of lactose in milk and other dairy products.

The objectives of this study were to isolate and study the properties of β-galactosidase produced by the homofermentative thermophilic lactic acid bacterium *L. crispatus*. *L. crispatus* is isolated from the intestinal tract, buccal cavity, feces, and vagina of humans, as well as from the crops and ceca of chickens (Kandler and Weiss 1986). The literature regarding biochemical characteristics of *L. crispatus* is sparse. The present study examines the effects of sugars (glucose, galactose, lactose, maltose) on the production of β-galactosidase. The activity and stability of the crude extract of the enzyme with regard to its optimum temperature and pH are also examined.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Lactobacillus crispatus strain ATCC 33820 was obtained from the *American Type Culture Collection* (Rockville, MD). The culture was maintained in lactobacilli

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MRS broth (*Difco Laboratories*, Detroit, MI). The organism was subcultured at 37 °C once a week and stored in a refrigerator (4 °C) or as frozen stock at 80 °C in 10 % glycerol. For growth determination and enzyme assays, MRS broth containing 2 % (*W/V*) galactose, glucose, lactose, or maltose was used.

Determination of bacterial growth. Growth of *L. crispatus* in media containing various sugars was determined by inoculating a 1 d culture (1 %, *V/V*) into MRS media containing either 2 % (*W/V*) galactose, glucose, lactose, or maltose (modified MRS medium) and incubating at 37 °C for 2 d. Cell concentration was determined by surface-plating duplicate 0.1-mL portions of a series of 10-fold dilutions on modified MRS plates. Plates were incubated in anaerobic jars (*Difco*) for 1 d at 37 °C and CFU/mL were recorded.

Preparation of cell free crude extracts. Cells grown in 200 mL of modified MRS broth were harvested by centrifugation (RC-SB refrigerated centrifuge *Sorvall*, Newton, CT) at 10000 ***g*** for 15 min. The supernatant was assayed for extracellular β-galactosidase. The pellet obtained from the centrifugation was washed three times with sodium phosphate buffer (50 mmol/L, pH 7.0), suspended in the same buffer, and cells were lyzed either by sonication or by lysozyme. For sonication, the cells were suspended in 10 mL sodium phosphate buffer (50 mmol/L, pH 7.0) and lyzed in a Sonicator W-225R (*Heat System-Ultrasonics*, New York; 20 kHz) according to Toba *et al.* (1981). For lysozyme treatment, the cells were suspended in 200 mL sodium phosphate buffer (50 mmol/L, pH 7.0) containing 5 mmol/L benzamidine (*Sigma*, St. Louis, MO) and 30 g/mL lysozyme (*Sigma*), and incubated for 16 h at 37 °C (Greenberg and Mahoney 1982). The cell debris from each treatment was removed by centrifugation (15000 ***g***, 30 min), and the supernatant, considered as the cell-free extract, was assayed for enzyme activity. The supernatant was stored at 80 °C until further use.

Enzyme assays

2-Nitrophenyl β-D-galactopyranoside (ONPG) as substrate. The enzyme activity was assayed by an initial-rate assay procedure of Goodman and Pederson (1976). The reaction mixture was composed of 1.9 mL of an appropriate buffer [50 mmol/L citrate phosphate buffer (pH 4.0–6.0), 50 mmol/L sodium phosphate buffer (pH 6.0–8.0), or 50 mmol/L glycine-KOH buffer (pH 8.0–9.0)] containing 3 mmol/L ONPG (*Sigma*) and 0.1 mL cell-free extract. Samples containing buffer and ONPG were tempered for 5 min in a water bath at the desired temperature. The reaction was initiated by the addition of 0.1 mL of cell-free extract. When the mixture was visibly yellow, the reaction was terminated by the addition of 0.5 mL of 1 mol/L disodium carbonate and the time was recorded. The absorbance was measured spectrophotometrically (Spectronic 601, *Milton Roy Co.*) at 420 nm, and the molar concentration was determined from a 2-nitrophenol standard curve. One unit of β-galactosidase activity is 1 kat or 1 nkat (the amount of the enzyme required to release 1 mol or 1 nmol of 2-nitrophenol per s under specified conditions).

To determine the enzyme stability, all reagents except the substrate were placed in a test tube at the reaction temperature. After appropriate incubation, the initial-rate assay was performed (Goodman and Pederson 1976).

Lactose as substrate. The enzyme activity was determined by using galactose and glucose UV-test kits (*Boehringer*, Mannheim, Germany) as per manufacturer's instructions. The reaction mixture was composed of 30 mmol/L lactose in 1.9 mL buffer of appropriate pH and 0.1 mL cell-free extract. After incubation at the desired temperature, the molar concentration of D-galactose or D-glucose in the reaction mixture was determined. One unit of β-galactosidase activity is 1 nkat (the amount of the enzyme required to release 1 nmol of D-galactose or D-glucose per s under specified conditions).

Protein assay. Protein content of cell-free crude extract was determined using bicinchoninic acid (*Pierce Chemical Co.*, Rockford, IL) (Smith *et al.* 1985). Bovine serum albumin (*Sigma*) was used as standard. The assay procedure consisted of mixing 0.1 mL sample of extract with 2 mL of the assay mixture in a test tube. After incubation for 30 min at 37 °C, the absorbance was measured at 562 nm against a reagent blank.

Enzyme production. Effect of sugars on the enzyme production was examined by measuring the enzyme activity of crude extracts from media containing one of the following: 2 % (*W/V*) galactose, glucose, lactose, or maltose.

The effect of sugars on the induction and repression of β-galactosidase was studied during cell growth at 37 °C in modified MRS broth containing 1 % (*W/V*) galactose, glucose, or lactose. The organism was inoculated (a 1-d culture at 1 % *V/V*) into the broth containing the required sugar. After about 1 d of incubation an additional 1 % (*W/V*) sugar was aseptically added and the enzyme activity of crude extracts from each medium was determined.

Enzyme properties. The effect of pH on the *enzyme activity* was determined at 45 °C over a pH range of 4.0–9.0 at increments of 0.5 pH units. Citrate–phosphate buffer was used for lower pH values,

sodium phosphate buffer for neutral pH values, and glycine-KOH buffer for alkaline pH values. The effect of temperature was determined at pH 6.5 over the range from 20 to 60 °C at 5 K increments. The effect of mono- and divalent cations was determined at 45 °C by using sodium phosphate buffer (50 mmol/L, pH 6.5) containing KCl, NaCl, $CaCl_2$, $CoCl_2$, $FeCl_2$, $MgCl_2$, $MnCl_2$, or $ZnCl_2$.

The effect of pH on the *stability of the enzyme* was examined by measuring the residual activity of the enzyme after keeping the enzyme for 1 h at 45 °C over a pH range of 4.0–9.0 at increments of 0.5 pH units (Ramana Rao and Dutta 1981). Citrate–phosphate, sodium phosphate, or glycine-KOH buffers of appropriate pH were used. The effect of temperature was determined by measuring the residual activity of the enzyme at 45 °C and pH 6.5 after holding the enzyme for 4 h at 4, 10, 25, 37, 45, 50, 55, and 60 °C (Toba *et al.* 1981).

Values for the Michaelis–Menten constant (K_m) and dissociation constant of the enzyme inhibitor complex (K_i) were determined from reciprocal plots of the enzyme velocity data at different concentrations of substrate (ONPG and lactose) and inhibitor (galactose and glucose) (Lineweaver and Burk 1934).

All trials were replicated at least three times. The results are the mean values.

RESULTS

Conditions of growth and production of β-galactosidase. L. crispatus grew more rapidly in MRS broth containing 2 % (*W/V*) glucose reaching stationary phase after 16 h. The growth was moderate in media containing lactose or maltose and slowest, reaching stationary phase after 44 h, in the presence of galactose (Fig. 1). Egg-white lysozyme and sonication were compared for the extraction of intracellular β-galactosidase. Lysozyme was not very effective in lyzing the cells. However, sonication was an effective treatment for the extraction of the enzyme.

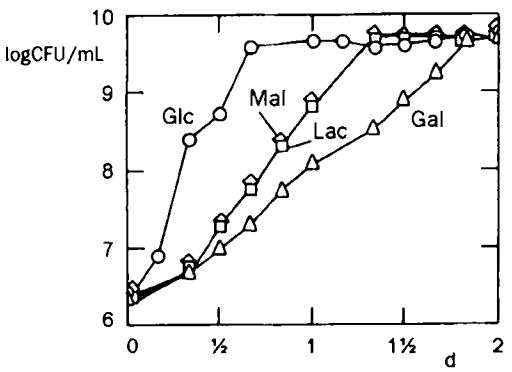

Fig. 1. Effect of sugars on the growth (CFU/mL) of *L. crispatus*; Gal – galactose, Glc – glucose, Lac – lactose, Mal – maltose.

L. crispatus grown in MRS broth containing galactose showed high β-galactosidase activity, whereas the enzyme activity in extracts from the broth containing glucose or maltose was insignificant. Moderate levels of enzyme production were observed during the growth of *L. crispatus* on lactose (Table I).

The rates of β-galactosidase synthesis by *L. crispatus* in MRS broth containing glucose increased with the addition of galactose or lactose. The addition of galactose resulted in higher levels of the enzyme than did the addition of lactose (Fig. 2 *top*). The addition of glucose or lactose to cells growing on galactose repressed β-galactosidase production, the repression being much greater with glucose (approximately a 13-fold decrease) than with lactose (approximately a 2-fold decrease) (Fig. 2 *middle*). The enzyme production in cells grown on lactose was repressed (approximately a 19-fold decrease) after the addition of glucose. The rates of enzyme production increased (approximately a 1.3-fold increase) with the addition of galactose (Fig. 2 *bottom*).

Table I. Effect of sugars on the production of intracellular β-galactosidase in *L. crispatus*

Sugar	nkat/mL[a]
Galactose	2540
Lactose	1670
Maltose	59
Glucose	4.9

[a]Enzyme activity, per mL of cell-free extract.

Properties of β-galactosidase. The optimum pH for the enzyme activity on both ONPG and lactose was found to lie between 6.0 and 6.5 (Fig. 3). When the enzyme activity was determined at different temperatures in the presence of ONPG and lactose, a gradual increase in the activity of the enzyme up to 45 °C was observed, with a rapid decrease thereafter. Among the various mono- and divalent cations examined, Mn^{2+} was found to have a positive effect on enzyme activity. The presence of Zn^{2+} reduced the enzyme activity by 23 % (Table II).

When the enzyme was exposed to various pH values at 45 °C for 1 h, it retained its original activity over the pH range of 6.0–6.5 (Fig. 3), the enzyme being stable up to 45 °C when incubated at various temperatures for 15 min and pH 6.5.

The values for Michelis–Menten kinetic constants were determined by using replots of slopes and intercepts of double-reciprocal plots. The apparent K_m, of β-galactosidase was found to be 1.16 mmol/L for ONPG and 14.2 mmol/L for lactose.

DISCUSSION

The ineffectiveness of egg-white lysozyme treatment for the disruption *L. crispatus* cell walls suggests that the cell wall of *L. crispatus* could be resistant to lysozyme. Although cell walls of many *Lactobacillus* species are susceptible to lysozyme, some, such as *L. casei*, are reported to be resistant to lysozyme (Kang *et al.* 1987). Absence of β-galactosidase activity in the supernatant of *L. crispatus* indicates that this organism produces intracellular β-galactosidase, a characteristic observed in many members of the genus *Lactobacillus* (Fisher *et al.* 1985; Sasaki *et al.* 1993).

Synthesis of β-galactosidase in *L. crispatus* was dependent upon the composition of the growth medium. The increased synthesis of the enzyme observed when the cells were grown on galactose or lactose indicates that its synthesis in *L. crispatus* is inducible which is in accordance with the inducible synthesis of β-galactosidase by the other members of the genus *Lactobacillus* (Hickey *et al.* 1986; Premi *et al.* 1972; Sasaki *et al.* 1993).

The presence of glucose in the medium resulted in a rapid growth of the organism, but yielded the lowest amount of β-galactosidase. The enzyme production increased when glucose in the medium was substituted with maltose, lactose, or galactose. *L. crispatus* produced higher amounts of β-galactosidase when grown on galactose than on lactose. However, this is in marked contrast to the normally higher production of β-galactosidase in the presence of lactose than galactose by other members of the genus *Lactobacillus* (Hickey *et al.* 1986). It is possible that within the *L. crispatus* cell, either galactose itself is a very efficient inducer or glucose from the hydrolysis of lactose inhibits the synthesis of

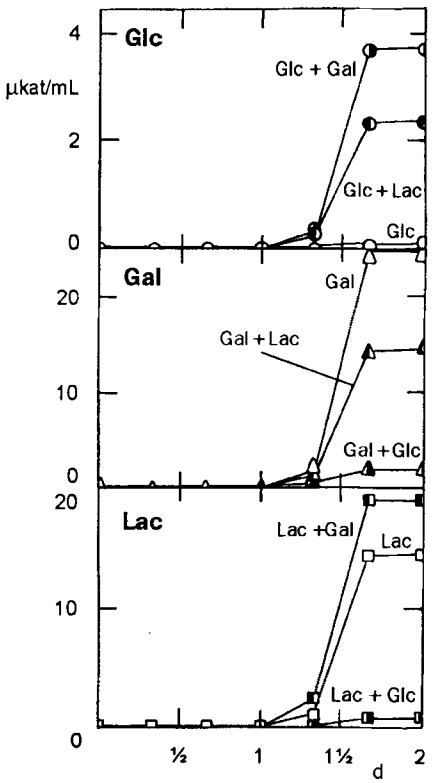

Fig. 2. Effect of sugars on the induction and repression of β-galactosidase synthesis (enzyme activity, kat/mL of cell-free extract) in *L. crispatus*. The organism was inoculated into modified MRS broth containing 1 % (*W/V*) glucose (*top*), galactose (*middle*), or lactose (*bottom*). After 1-d incubation an additional 1 % (*W/V*) sugar was added.

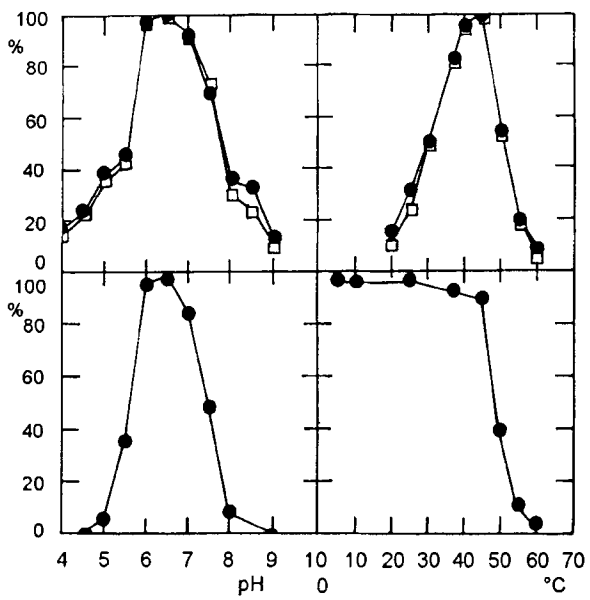

Fig. 3. Effect of pH (at 45 °C; *left*) and of temperature (at pH 6.5; °C; *right*) on the activity (rel. %; *top*) and on the stability (rel. %; *bottom*) of β-galactosidase from *L. crispatus* with ONPG (●) and lactose (□) as substrates.

β-galactosidase. The mechanism for the inhibitory effect of glucose on the expression of the *lac* operon in lactobacilli is not clear. However, in enteric bacteria and *Streptococcus thermophilus*, the induced synthesis of β-galactosidase is known to be negatively regulated by the intracellular level of glucose (Beckwith 1987; Magasanik and Neidhardt 1987; Poolman 1993; Poolman *et al.* 1990).

The addition of lactose to the growth medium containing galactose was found to inhibit the synthesis of β-galactosidase. Such an inhibition of the enzyme production by lactose

has not been noted for other microorganisms. The repression of β-galactosidase synthesis by the addition of lactose was perhaps due to catabolite repression. It is possible that as lactose is transported into the cells and hydrolyzed by β-galactosidase, the intracellular concentration of glucose would increase there by reducing the expression of the *lac* operon. The increased synthesis of the enzyme in the lactose-containing medium when galactose is added supports this hypothesis.

Table II. Effect of mono- and divalent cations (mmol/L) on the activity of β-galactosidase (rel. %) from *L. crispatus*

Metal ion	mmol/L	%	Metal ion	mmol/L	%
None[a]	–	100			
NaCl	10	100	$MgCl_2$	1	98
KCl	10	106	$CoCl_2$	1	109
$ZnCl_2$	1	77	$FeCl_2$	1	113
$CaCl_2$	1	92	$MnCl_2$	1	127

[a]Control (cell-free extract in sodium phosphate buffer, pH 6.5).

The weakly acidic optimum pH (6.0–6.5) and the stability of β-galactosidase at weakly acidic and neutral pH (6.0–7.0) makes this enzyme from *L. crispatus* suitable for the hydrolysis of lactose in fluid milk, sweet whey, and other dairy products. This compares with pH optima of 7.0 reported for the enzyme isolated from *L. bulgaricus* and *L. plantarum*, and pH between 6.0 and 6.5 for the enzyme from *Lactococcus lactis* and *S. thermophilus* (Premi *et al.* 1972; Toba *et al.* 1981). The enzyme from *Kluyveromyces fragilis*, the most widely used source of enzyme for food applications, and various other bacterial sources are reported to have a near neutral optimum pH (Gekas and López-Leiva 1985; Greenberg and Mahoney 1981; Rahim and Lee 1991). However, enzyme preparations from molds, such as *Aspergillus niger* and *A. oryzae*, have pH optima in the acidic range (Gekas and López-Leiva 1985; Greenberg and Mahoney 1981; Riou *et al.* 1992).

The stability of β-galactosidase from *L. crispatus* in the temperature range of 4–45 °C suggests that the enzyme could be more useful in commercial processes than that obtained from the yeast, *K. fragilis*. β-Galactosidase of *K. fragilis* was reported to be heat-labile and unstable at the temperatures milk and milk products are generally stored (Blankenship and Wells 1974; Harju 1987; Ramana Rao and Dutta 1981).

The temperature optimum of 45 °C for β-galactosidase from *L. crispatus* was shown to be close to the optimum temperature for the enzyme from *K. fragilis* (Ramana Rao and Dutta 1979) and to be slightly lower than the optima reported for the enzyme from other thermophilic lactobacilli (Premi *et al.* 1972; Toba *et al.* 1981). Enzyme preparations from *L. bugaricus*, *L. lactis*, and *L. plantarum* are reported to have an optimum temperature of 50 °C and those from *L. helveticus* and *S. thermophilus* between 55 and 60 °C (Premi *et al.* 1972; Toba *et al.* 1981). However, Wierzbicki and Kosikowski (1973) reported that *L. bulgaricus* and *L. helveticus* are relatively poor producers of β-galactosidase.

β-Galactosidase from *L. crispatus* was activated by divalent metal ions such as Mn^{2+}, Co^{2+}, and Fe^{2+}. Monovalent cations had little effect on the activity of the enzyme. However, Monod and Cohn (1952) and Iwasaki *et al.* (1971) have reported that monovalent, as well as divalent, cations activate β-galactosidase from *L. bulgaricus* and *L. bifidus*.

The results of our study suggest that *L. crisatus* produces an intracellular β-galactosidase with maximum activity at pH 6.5 and 45 °C. The enzyme is active between pH 4 and 9. The synthesis of β-galactosidase is induced by the presence of galactose in the medium. Sonication is superior to lysozyme treatment for the extraction of enzyme.

This research was partially supported by a grant from *The University of Wisconsin System Applied Research Program.*

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