CURRENT MICROBIOLOGY Vol. 40 (2000), pp. 181–184
DOI: 10.1007/s002849910036

Current Microbiology
An International Journal

Purification and Characterization of Invertase from *Lactobacillus reuteri* CRL 1100

Silvia Cuezzo de Ginés,[1,4] María C. Maldonado,[2] Graciela Font de Valdez[3,4]

[1]Cátedra de Bioquímica Clínica III, Universidad Nacional de Tucumán, Argentina
[2]Cátedra de Microbiología Industrial, Universidad Nacional de Tucumán, Argentina
[3]Cátedra de Microbiología Superior, Universidad Nacional de Tucumán, Argentina
[4]Centro de Referencia para Lactobacilos (CERELA), Chacabuco 145, 4000 S.M. de Tucumán, Argentina

Received: 13 August 1999 / Accepted: 15 September 1999

Abstract. The invertase of *Lactobacillus reuteri* CRL 1100 is a glycoprotein composed by a single subunit with a molecular weight of 58 kDa. The enzyme was stable below 45°C over a wide pH range (4.5–7.0) with maximum activity at pH 6.0 and 37°C. The invertase activity was significantly inhibited by bivalent metal ions (Ca^{++}, Cu^{++}, Cd^{++}, and Hg^{++}), β-mercaptoethanol, and dithiothreitol and partially improved by ethylenediaminetetraacetic acid. The enzyme was purified 32 times over the crude extract by gel filtration and ion-exchange chromatography with a recovery of 17%. The K_m and V_{max} values for sucrose were 6.66 mM and 0.028 μmol/min, respectively. An invertase is purified and characterized for the first time in *Lactobacillus*, and it proved to be a β-fructofuranosidase.

Most microbial species being used today as probiotics or starter cultures in the fermented foods industry belong to lactic acid bacteria. Among them, *Lactobacillus* (*Lb.*) *reuteri* is the only enterolactobacillus known to be indigenous to a broad spectrum of hosts [8]; it also plays an important role in sour dough fermentations. The maltose and fructose cofermentation in sour dough by heterofermentative lactobacilli increase the concentration in organic acids (acetate, lactate), which enhances the flavor and organoleptic quality of sourdough bread [6]. Sucrose, a disaccharide composed of glucose and fructose, represents 1% of the soluble carbohydrates in wheat flour. To metabolize this sugar, microorganisms should have invertase activity (β-D-fructofuranoside fructohydrolase), an enzyme that has been thoroughly investigated in eukaryotes, e.g., *Aspergillus niger* [11], *Fusarium oxisporum* [9], but scarcely in prokaryotes. In the present study, the enzyme invertase was purified and characterized for the first time in *Lactobacillus*; *Lb. reuteri* CRL 1100 β-fructofuranosidase.

Materials and Methods

Microorganisms, culture conditions, and preparations of cell-free crude extracts. *Lb. reuteri* CRL 1100 was obtained from the culture collection of CERELA. Microorganisms were grown in 1.5 L of MRS broth [3] supplemented with 1% sucrose as the sole energy and carbon source, at 37°C for 16 h. Cells were harvested, washed twice with sterile 0.05 M phosphate buffer (pH 7.0), resuspended at 30% (wt/vol) in 0.2 M acetate buffer (pH 5.0), and disrupted with a French pressure cell. Cell debris was removed by centrifugation at 10,000 *g* for 15 min, and the supernatant was used as crude extract. All operations were carried out at 4°C.

Enzyme purification. Purification of invertase was accomplished from cell-free extracts by using three successive steps, each carried out at 4°C. The crude enzyme preparation was brought to 45% ammonium sulfate saturation, and the resulting precipitate was removed by centrifugation at 10,000 *g* for 10 min. Ammonium sulfate (Sigma Chemical Co) was added to the supernatant up to 80% saturation. The precipitate was collected by centrifugation as above, and it was dissolved in 0.2 M acetate buffer (pH 5.0). Then, the solution was dialyzed overnight against the same buffer. The dialyzed enzyme solution was applied to a column (3 × 35 cm) of Sephadex G-150 (Sigma Chemical Co) pre-equilibrated with the same buffer. Elution was performed at a flow rate of 0.3 ml/min, and 2-ml fractions were collected. The active enzyme fractions containing β-fructofuranosidase were pooled and applied to an anion-exchange column (1.5 × 20.0 cm) on DEAE-Sephacel (Sigma Chemical Co) pre-equilibrated with 0.2 M acetate buffer (pH 5.0). A linear KCl gradient from 0 to 1 M in 150 ml of the same buffer was applied. Fractions of 2.5 ml were collected at a flow rate of 0.25 ml/min. The enzyme fractions of the activity peak were pooled (fractions 17 and 18) and used for the determination of the main characteristics of the enzyme.

Protein estimation and enzyme assay. Protein was determined by the method of Bradford [1] with bovine serum albumin as standard.

Correspondence to: G. Font de Valdez

Absorbance at 280 nm (A_{280}) was used for monitoring proteins in column eluates. Enzymatic activity was determined at 37°C, pH 5.0, by measuring the glucose released with an enzymatic kit (Wiener Lab, Argentina). The assay mixture to determine the enzyme activity consisted of 20 µl sucrose 1 M (dissolved in 0.2 M acetate buffer, pH 5.0) and 50 µl of purified enzyme extract in a final volume of 0.2 ml. The reaction was carried out at 37°C (pH 5.0) for 15 min and stopped by heating the mixture in boiling water for 10 min. One unit of invertase (U/ml) was defined as the amount of enzyme required to produce 1 µmol of glucose per ml per min at 37°C, pH 5.0; specific activity was expressed as units/mg of protein (U/mg prot).

Enzyme kinetics. Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were determined as a function of the sucrose concentration at 37°C and pH 5.0. K_m and V_{max} were calculated from the slope and intercept of the regression line of Lineweaver-Burk plots.

Molecular mass. The molecular weight of the native enzyme was estimated by gel filtration through Sephadex G-150 with 0.2 M acetate buffer (pH 5.0) as eluent. Individual runs of molecular-mass standards were performed to create a standard curve. Molecular markers (Sigma Chemical Co) were lysozyme (14.3 kDa), trypsinogen (24 kDa), ovalbumin (45 kDa), and bovine serum albumin (66 kDa).

Carbohydrate content. The amount of carbohydrate was measured by the phenol sulfuric acid method with D-glucose as standard [4].

Effect of temperature. The enzyme activity was measured in the range of 20–75°C. The reaction mixture (pH 5.0) was incubated for 15 min at different temperatures, and the residual activity was measured at 37°C. To estimate thermal stability, the enzyme was incubated at pH 5.0 for 30 min at different temperatures, and the reaction mixture was processed as usual.

Effect of pH. The range of pH from 2.0 to 8.0 was obtained by adding to the reaction mixture 0.2 M glycine-HCl buffer (pH 2–3), 0.2 M acetate buffer (pH 4–6), or 0.2 M phosphate buffer (pH 7–8). To estimate the enzyme stability, the pure extract was incubated at different pH values during 2 h at 4°C before determination.

Effects of chemicals. The effects of $MnCl_2$, $MgSO_4$, $CuSO_4$, $CdCl_2$, $HgCl_2$, and NaCl (1 mM each); $CaCl_2$ and ethylenediaminetetraacetic acid (EDTA) (1–10 mM); and common inhibitors like dithiothreitol (DTT), β-mercaptoethanol, and iodoacetamide (1.0 and 10.0 mM each) on invertase activity were tested. A mixture containing the purified enzyme solution, sucrose 0.1 M, and the selected inhibitor was incubated at 37°C for 15 min. The enzyme activity was assayed under standard conditions, and it was expressed as a percentage of the activity without effector (control).

Substrate specificity. The enzyme mixture containing 0.1 M of each substrate (melezitose, raffinose, stachyose, melibiose, lactose, maltose, and sucrose) was incubated at 37°C (pH 5.0) for 15 min, and the activity was determined as before. The enzyme activity was expressed as a percentage of the activity determined in sucrose.

Reproducibility. All results presented in this paper are the means of two independent assays. The variations were less than 10%.

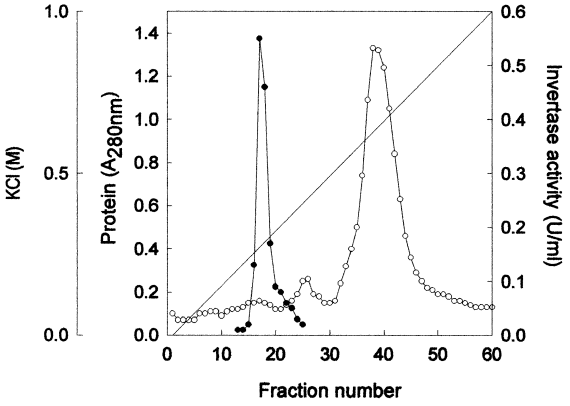

Fig. 1. *Lb. reuteri* β-fructofuranosidase purification step by DEAE-Sephacel chromatography. Symbols: ○ absorbance at 280 nm; ●, β-fructofuranosidase activity; ——, KCl concentration. Experimental conditions are described under Materials and Methods.

Table 1. Purification of invertase from *Lb. reuteri* CRL 1100

Purification step	Total units	Protein (mg/ml)	Specific activity (U/mg prot)	Purification factor	Yield (%)
Crude extract	18.3	3.2	0.3	1.0	100.0
Ammonium sulfate	9.8	3.0	0.4	1.4	60.2
Sephadex G-150	6.7	0.2	2.0	6.9	41.3
DEAE-Sephacel	2.7	0.1	9.2	31.2	16.9

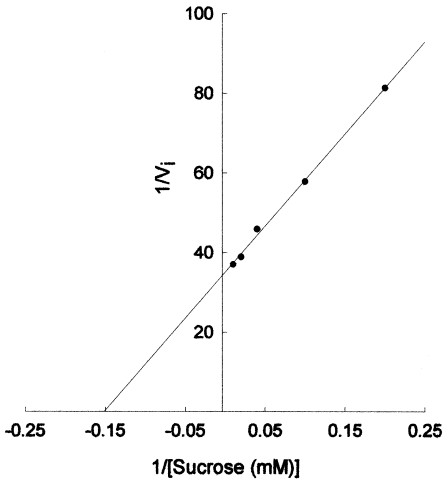

Fig. 2. *Lb. reuteri* β-fructufuranosidase, K_m and V_{max} determinations with different concentrations of sucrose as substrate (Lineweaver-Burk plot).

Results and Discussion

Purification of fructofuranosidase. The invertase from *Lb. reuteri* CRL 1100 was purified to homogeneity by $(NH_4)_2SO_4$ precipitation followed by gel filtration on Sephadex G-150 and ion-exchange chromatography on DEAE-Sephacel (Fig. 1). The activity peak was eluted between 0.27 M and 0.34 M KCl. The yield of the purification process (Table 1) was 17%, and the specific activity of the purified invertase was 0.17 U/mg prot. This value is about 32 times higher than that of the crude enzyme preparation.

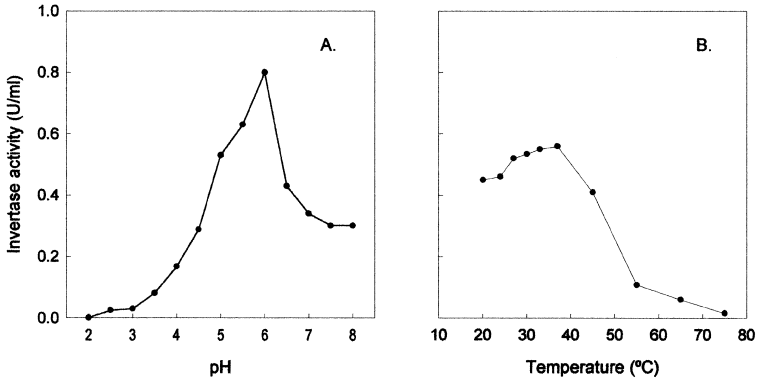

Fig. 3. Effect of pH (A) and temperature (B) on β-fructofuranosidase from *Lb. reuteri* CRL 1100. Experimental details are described in the text.

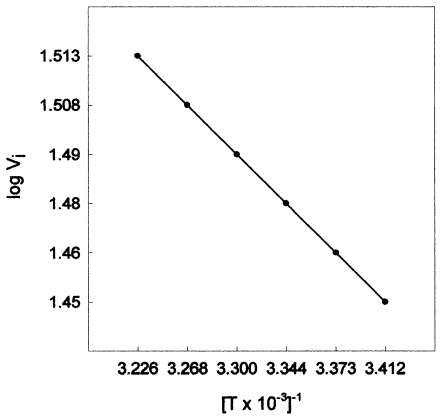

Fig. 4. Arrhenius plot of the invertase-catalyzed reaction. Assays were conducted at various temperatures as described in Materials and Methods.

Table 2. Effect of chemicals on the invertase activity of *Lb. reuteri* CRL 1100

Compound	Concentration (mM)	Relative activity (%)
Control	—	100
$MnCl_2$	1	98
$MgSO_4$	1	95
$CaCl_2$	1	82
	10	51
$CuSO_4$	1	10
$CdCl_2$	1	34
$HgCl_2$	1	33
NaCl	1	100
EDTA	1	106
	10	147
DTT	1	16
	10	0
β-Mercaptoethanol	1	27
	10	5
Iodoacetamide	1	113
	10	152

Properties and characteristics of the enzyme. The carbohydrate content of the purified invertase of *Lb. reuteri* CRL 1100 was 48% glucose. It gave a single band with a molecular weight of 58 kDa when subjected to SDS-PAGE, which was confirmed by gel filtration of the native enzyme. Accordingly, the enzyme of *Lb. reuteri* CRL 1100 appears to be composed of a single subunit, in agreement with the β-fructofuranosidase I of *Arthrobacter* sp. K-1 [5] and that of *Azotobacter chroococcum* [2]. In contrast, the invertase of *Aspergillus niger* [11] and *Ricinus communis* [10] has a dimeric and a heptamer structure, respectively.

The apparent K_m and V_{max} values of the pure enzyme for sucrose were estimated as being 6.66 mM and 0.028 μmol/min, respectively, at pH 5.0 and 37°C (Fig. 2). This K_m value was similar to that found for *R. communis* (8.7 mM) [10] and *A. chroococcum* (5 mM) [2], whereas it has a smaller affinity for sucrose compared with the invertase of *A. niger* (0.0625 mM) [11].

The optimum pH was found to be 6.0 (Fig. 3A). The enzyme was stable in the range of pH 5.0–6.0; it kept 60–50% of its optimal activity at pH 7.0–8.0, but almost no activity under pH 3.0. The optimal temperature for the purified *Lb. reuteri* β-fructofuranosidase was 37°C (Fig. 3B), with an activation energy of 2087 cal/mol calculated from an Arrhenius plot (Fig. 4). The enzyme remained stable at temperatures below 45°C, but retained only 15% of its original activity during incubation at 55°C. The effects of metal ions and some other organic chemicals on the enzyme activity are shown in Table 2. The enzyme of *Lb. reuteri* CRL 1100 was not affected by Mg^{++}, Mn^{++}, or Na^{+} but was markedly inhibited by Cu^{++} 1 mM (90%) and partially by Cd^{++} 1 mM (66%) and Hg^{++} 1 mM (67%). The addition of 10 mM EDTA (a metal-complexing reagent) caused about 47% increase in invertase activity, while Ca^{++} (at the same concentration) reduced the activity to 50%.

The invertase activity was enhanced by iodoacetamide (a special SH-blocking agent) and inhibited by DTT and β-mercaptoethanol, which are sulfhydryl reduc-

ing compounds. These results suggest that disulfur bonds are necessary to preserve the invertase of *Lb. reuteri* CRL 1100, in contrast to those found for other invertases [10, 11].

The enzyme was active on sucrose, raffinose, and stachyose (29% and 23% of sucrose activity respectively), but not on melezitose, melibiose, lactose, or maltose, which are saccharides without β-D-fructofuranosyl residue. These results suggest that the enzyme of *Lb. reuteri* is a β-fructofuranosidase, as it requires a β-D-fructofuranosyl residue to be active. In conclusion, the first *Lactobacillus* invertase has been purified to homogeneity and well characterized. In the particular case of *Lb. reuteri*, a probiotic microorganism, the characteristics of the enzyme may be of considerable significance in the ecology of lactic acid bacteria, both in natural habitats and current or envisaged applications.

ACKNOWLEDGMENTS

This work was supported by grants from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina, Centro Argentino-Brasileño de Biotecnología (CABBIO), and Universidad Nacional de Tucumán, Argentina.

Literature Cited

1. Bradford M (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
2. De la Vega M, Cejudo F, Paneque A (1991) Purification and properties of an extracellular invertase from *Azotobacter chroococcum*. Enzyme Microbiol Technol 13:267–271
3. De Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. J Appl Bacteriol 23:130–135
4. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for the determination of sugars and related substances. Anal Chem 28:350
5. Fujita K, Hara K, Hashimoto H, Kitahata S (1990) Purification and some properties of β-fructofuranosidase I from Arthrobacter sp. K-1. Agric Biol Chem 54:913–919
6. Gobetti M, Corsetti A, Rossi J (1995) Maltose-fructose cofermentation by *Lactobacillus brevis* subsp. *lindneri* CB 1 fructose-negative strain. Appl Microbiol Biotechnol 42:939–944
7. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685
8. Mitsuoka T (1992) The human gastrointestinal tract. In: The lactic acid bacteria, volume 1. The lactic acid bacteria in health and disease. BJB Wood (ed.). New York: Elsevier
9. Nishizawa M, Maruyama Y, Nakamura M (1980) Purification and characterization of invertase isozymes from *Fusarium oxysporum*. Agric Biol Chem 44:489–498
10. Prado F, Vattuone M, Fleischmacher O, Sampietro A (1985) Purification and characterization of *Ricinus communis* invertase. J Biol Chem 260:4952–495
11. Rubio M, Maldonado C (1995) Purification and characterization of invertase from *Aspergillus niger*. Curr Microbiol 31:80–83