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Purification and Characterization of an Intracellular β-Glucosidase from *Lactobacillus casei* ATCC 393

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ABSTRACT

The lactic acid bacterium, *Lactobacillus casei,* produces an intracellular β-glucosidase when grown on Man-Rogosa-Sharpe (MRS) medium with cellobiose as carbon source. The β-glucosidase activity is produced intracellulary, and no extracellulary activity was detected. The enzyme was purified by ion-exchange chromatography and gel filtration. The molecular mass of the purified intracellular β-glucosidase as estimated by gel filtration was 480 kDa, consisting of six probably identical subunits. The enzyme exhibited optimum activity at 35°C and pH 6.3 with citrate-phosphate buffer. The enzyme was active against soluble glycosides with (1→4)-β configuration and from Lineweaver Burk plots, K_m value of 16 mmol/L was found for β-pNPG. The β-glucosidase was competitively inhibited by glucose, and no glycosyl transferase activity was observed in the presence of ethanol.

Index entries: β-glucosidase; purification; *Lactobacillus casei*

INTRODUCTION

β-Glucosidases (β-D-glucoside glucohydrolase, EC 3.2.1.21) constitute a group of well-studied hydrolases that have been isolated from members of all three domains of life: eukarya, bacteria, and archaea *(1)*. The princi-

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pal reaction catalyzed by this class of enzyme is the hydrolytic cleavage of β-glycosidic linkages of low-molecular-mass glycosides. The physiological roles postulated for β-glucosidases are extremely diverse: glucoside ceramide catabolism in human tissue, cell wall, pigment, and cyanoglucoside metabolism, defense against pathogens in plants, and the utilization of oligosaccharide substrates by many fungi and bacteria *(2)*. Recently, this enzyme was also studied for its potential to liberate aroma rich terpenes: such aroma precursor compounds found in certain fruits (mango, passion fruit, grapes) and bonded to glucosides (terpenylglucosides) are more effectively liberated than by acid hydrolysis (3,4). β-Glucosidases were also studied for cassava detoxication (4,6). Cassava is detoxified during processing by the endogenous β-glucosidase, linamarase, present in the enlarged cassava root. The enzyme is released during the grating of the roots, but apparently, the quantity of enzyme released is not sufficient to break down the glucoside present in the root completely (7). Exogenous microbial β-glucosidase showing activity against linamarin could be used in combination with the endogenous linamarase of the tuber roots to ensure greater breakdown of the linamarin.

Lactic acid bacteria are widespread in nature, commonly found in milk, dairy products, fermented foods (meats, vegetables, breads), vegetable material, and intestinal mucous membranes of humans and animals. The lactic acid bacteria constitute a group that is beneficial to humans; several of its members are responsible, wholly or in part, for the production of organoleptic characteristics and the preservation of some food products.

In this article, we report the purification and detailed biochemical study of an intracellular β-glucosidase of *Lactobacillus casei* ATCC 393, that is expected to possess not only fundamental functions in the host organism, but also important physicochemical characteristics applicable to the food industry. To our knowledge, this study is the first purification and characterization of a β-glucosidase from *L. casei*.

MATERIALS AND METHODS

Organism and Culture Conditions

The strain used in this study was *L. casei* ATCC. Dicks et al. *(8)* have recently proposed reclassifing this strain as *Lactobacillus zeae* nom. rev. (type strain ATCC 15820) based on DNA profiles.

This microaerophilic strain was incubated at 37°C in Erlenmeyer flasks filled to the maximum of their capacity. The culture medium was (Man-Rogosa-Sharpe) MRS *(9)* adjusted to pH 6.3 with 1 mol/L HCl. The carbon source was cellobiose (10 g/L) and the medium was sterilized by autoclaving at 120°C for 20 min.

Enzyme Extraction

The bacterial cells were harvested by centrifugation (12,000*g*, 10 min). Following two rinses with phosphate buffer (20 mmol/L, pH 6), the pellet was redispersed in phosphate buffer containing Triton X-100 (1% w/v) and sonicated using a Sonifier 250 (Branson Co., Danbury, CT). The power was 60 W, and the cells were submitted to 0.5 s/s of sonification for 15 min. The resulting suspension was centrifuged (12,000*g*; 10 min) and the supernatant (S1) contained the soluble β-glucosidase.

Enzyme and Protein Assay

β-Glucosidase activity against *p*-nitrophenyl-β-D-glucopyranoside (pNPG) was determined by adding 0.1 mL of enzyme solution to 4.9 mL of citrate-phosphate buffer (0.1 mol/L, pH 6.3) containing pNPG (5 mmol/L final) *(10)*. One unit of β-glucosidase activity (U) was defined as the quantity of enzyme required for hydrolysis of one μm substrate (pNPG) per min (U/mL) under the above experimental conditions.

In the case that the substrates did not contain chromogenic aglycons, β-glucosidase activity was determined by assaying the liberated glucose. In that case, enzyme solution (0.1 mL) and the substrate (0.2 mol/L final) were added to 2.9 mL of citrate-phosphate buffer (0.1 mol/L; pH 6.3). Liberated glucose was determined using a hexokinase and glucose 6-phosphate-dehydrogenase procedure *(11)*.

Protein was determined by the method of Biuret on samples containing cell extract *(12)* and, in all the others cases, by the method of Lowry *(13)*.

β-Glucosidase Purification

The S1 supernatant fluid was ultracentrifuged (135,000*g*, 90 min, 4°C), and the resulting supernatant (S2) was applied to a Q-Sepharose ion-exchange column (Pharmacia HR, Uppsala, Sweden, 26 mm × 400 mm) previously equilibrated with Tris-HCl buffer (50 mmol/L; pH 7). The elution of enzyme activity was performed with a linear gradient of NaCl from 0.1 to 0.7 mol/L. The elution rate was 250 mL/h, and the eluate was collected in 10-mL fractions. Bulked fractions containing β-glucosidase activity were subjected to a gel-filtration chromatography (Sephacryl S-300, Pharmacia HR, 10 × 1000 mm). Enzyme activity was eluated with Tris-HCl (50 mmol/L, pH 7) containing glycerol 10% (w/v). The elution rate was 12.5 mL/h, and the eluate was collected in 1.5-mL fractions. The column was calibrated using the following molecular-mass standards: ferritin, 440 kDa; β-amylase, 200 kDa; alcohol dehydrogenase, 150 kDa; bovine serum albumin, 66 kDa; cytochrome-*c*, 12.4 kDa.

Polyacrylamide Gel Electrophoresis

For native and SDS polyacrylamide gel electrophoresis *(14)* precast slab gels from Touzart et Matignon (Paris, France) were used. Electrophoresis was performed at pH 8.3 with Tris (25 mmol/L) glycine (0.192 mol/L) buffer. For SDS-PAGE, enzyme and mol-wt standards (Bio-Rad Laboratories, Hercules, CA) were denatured in the presence of 5% (v/v) β-mercaptoethanol and 2% (w/v) SDS 100°C for 10 min. Electrophoresis was carried out at room temperature and at 25 mA constant current on vertical slabs (10 × 8 cm). The protein bands were distinguished with Coomassie brillant blue R-250. Isoelectric focusing (IEF) PAGE was performed on the Multiphor II Electrophoresis (Pharmacia) using a precast gel, Ampholine PAGE plate (245 × 110 × 1 mm, Pharmacia) for p*I* between 3.5 and 9.5.

RESULTS AND DISCUSSION

Location and Biosynthesis of the β-Glucosidase

Study of β-glucosidase activity in relation to the physiological stage of the culture during growth on MRS medium with cellobiose (10 g/L) as carbon source showed that enzyme activity was produced intracellulary: the enzyme activity was not detected in the culture medium at any stage of growth. Maximum enzyme activity was reached at the end of the exponential growth phase after 48 h of growth. After 72 h incubation, activity was completely lost, and no activity was found extracellularly. Some endoproteolysis activity could be responsible for that disappearance.

With cellobiose (10 g/L) as the growth carbon source, 7 mU/mg proteins of β-glucosidase activity was produced, but no β-glucosidase activity was detected when glucose was used as carbon source.

Purification of the β-Glucosidase

The S1 supernatant fluid was ultracentrifuged (135,000*g*, 90 min, 4°C), and the supernatant (S2) was fractionated on a Q-sepharose column (Fig. 1). The β-glucosidase activity was eluted as a single peak in fractions 49–56. Fractions 50–53 were pooled and concentrated in an Amicon cell with a PM 10 membrane and chromatographed on Sephacryl S-300 column (Fig. 1). The enzyme was found in fractions 26–40. Fractions 27–31 were pooled to give the purified β-glucosidase preparation. The data for the purification steps are summarized in Table 1. The specific activity of the purified enzyme is 43.9. The purity of the β-glucosidase preparation was confirmed by native and SDS-PAGE, and just a single band was detected in both cases. No enzyme isoforms were detected during any stage of the purification.

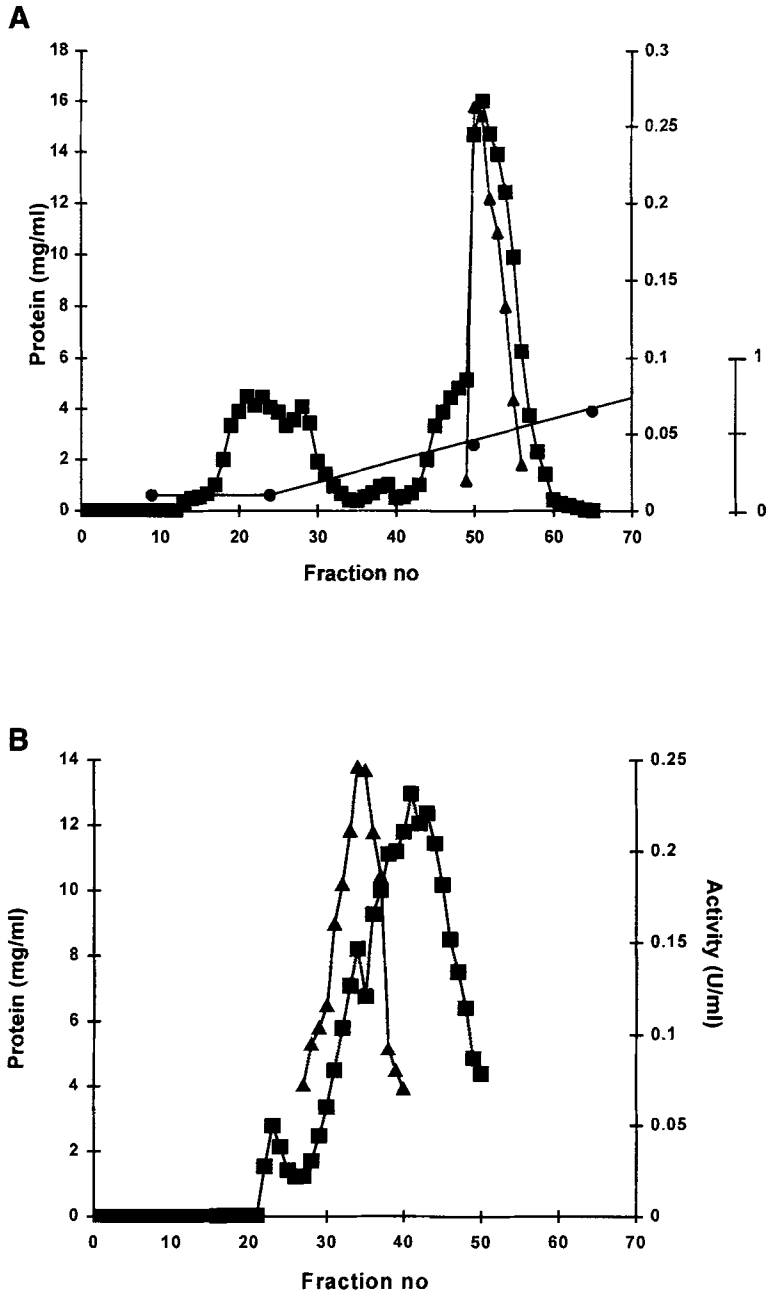

Fig. 1. Purification of the β-glucosidase of *L. casei*. Elution of the β-glucosidase activity from a Q-Sepharose fast-flow chromatography column **(A)** and a Sephacryl S-300 gel filtration column **(B)**. ■, protein; ▲, activity on pNPG; ●, NaCl concentration (0.1–0.7 mol/L).

Table 1
Summary of the Purification of *L. casei* β-Glucosidase

Purification step	Protein, mg	Specific activity, mU/mg protein	Yield, %	Purification factor
S1	5800	7	100	1.0
S2	3500	8	69	1.1
Q-Sepharose	595	16.5	10.2	2.4
Sephacryl S-300	18.8	43.9	5.1	6.3

Physical and Chemical Properties of the β-Glucosidase

The properties of the enzyme were determined using the purified extract. The molecular mass of the β-glucosidase was estimated to be 480 kDa by Sephacryl S-300 chromatography. This value is close to that previously obtained for other β-glucosidases, which were 360 kDa for *Leuconostoc mesenteroides (6)*, 400 kDa for *Candida entomophila (15)*, and 480 kDa for *Aspergillus fumigatus (16)*. The denatured enzyme showed one major band (molecular mass 80 kDa) on SDS-PAGE. Thus, the β-glucosidase seems to be composed of six subunits. It is interesting to note that most β-glucosidases purified from eubacteria and archaea, e.g., *Streptomyces reticuli* or *Clostridium thermocellum* were reported to be monomeric *(17,18)*. β-Glucosidases from eubacteria and archaea were also described with dimeric *(19)* and tetrameric structures *(20)*. However, the β-glucosidase of *L. casei* is the first β-glucosidase that has been described with a hexameric structure.

The p*I* was estimated to be 4.5. It is quite similar to those found for the β-glucosidase of *L. mesenteroides* (pL 4.2) *(6)* and for the β-glucosidase of *A. fumigatus* (p*I* 4.5) *(16)*. β-Glucosidases from bacteria have generally acidic p*I*s *(21)*.

The denaturation of the enzyme was monitored by measuring its activity against pNPG at 30°C, following incubation at several temperatures for various time periods. The calculated inactivation energy determined by the Arrhenius plots was 279.5 kJ/mol. The value is similar to those generally reported for enzymes, in which the inactivation energy lies between 150 and 400 kJ/mol. These values are high, but can be explained by the necessity of simultaneously breaking an important number of bindings. The enzyme was not heat-resistant with rapid inactivation at 45°C and above.

Kinetic Properties of the Purified β-Glucosidase

The influence of the pH on enzymatic activity has been studied (Fig. 2). The optimum pH was 6.3 in citrate-phosphate buffer (0.1 mol/L).

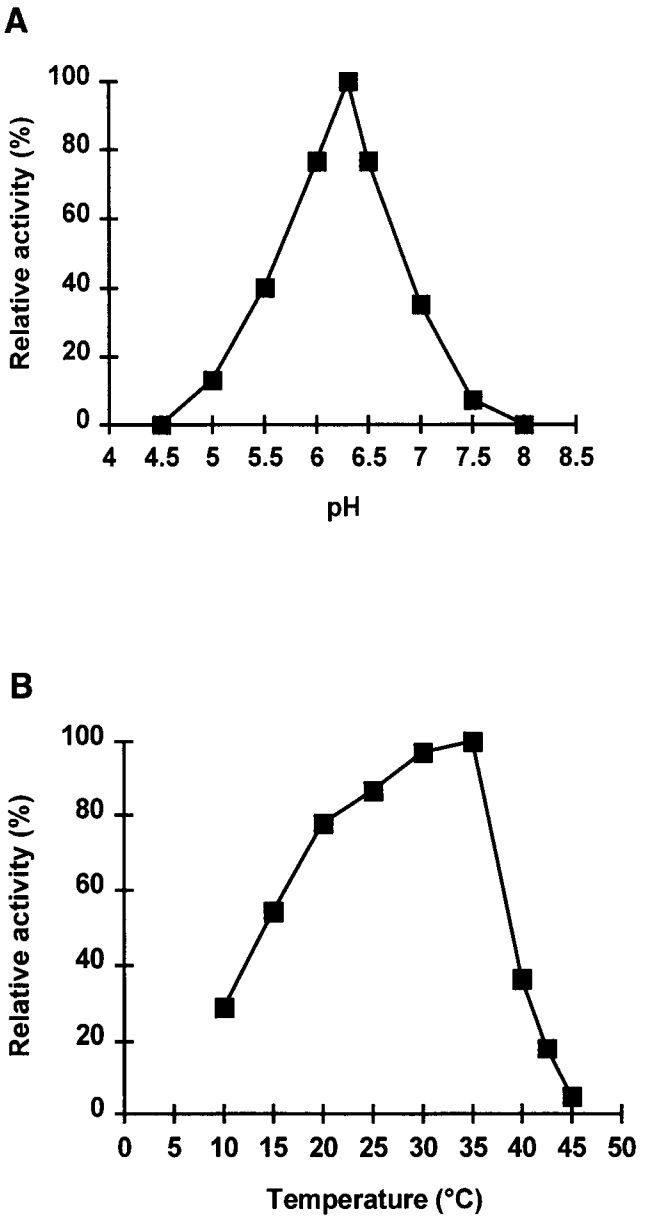

Fig. 2. Effect of pH **(A)** and temperature **(B)** on the β-glucosidase of *L. casei*. Activity was measured against pNPG in citrate-phosphate buffer (0.1 mol/L). The 100% activity corresponds to 11 mU/mg of protein.

The influence of temperature on activity has been studied with citrate-phosphate buffer (0.1 mol/L, pH 6.3) (Fig. 2). Activity measurements were performed at several temperatures. The activity plot for pNPG gives an optimal temperature of 35°C. The thermal activation energy determined from the Arrhenius plots is 32.5 kJ/mol. The enzyme has a low opti-

Table 2
Substrate Specificity of Purified β-Glucosidase of *L. casei*[a]

Substrates	Configuration of glycoside linkage	Concentration, mmol/L	Specific activity, mU/mg protein
Aryl and alkyl-D-glucosides			
p-Nitrophenyl-β-D-glucoside	β(1–4)	5	11
p-Nitrophenyl-α-D-glucoside	α(1–4)	10	1.1
o-Nitrophenyl-β-D-glucoside	β(1–4)	10	4.4
Methyl-β-D-glucoside	β(1–4)	10	0.3
Salicin	β(1–4)	20	0.3
Cyanoglucosides			
Linamarin	β(1–4)	5	0
Prunassin	β(1–4)	5	0.6
Diglucosides			
Cellobiose	β(1–4)	5	50
Maltose	α(1–4)	5	0
Others			
p-Nitrophenyl-β-D-cellobioside	β(1–4)	10	0
p-Nitrophenyl-β-D-galactoside	β(1–4)	10	0.5
p-Nitrophenyl-β-D-fucoside	β(1–4)	10	0
p-Nitrophenyl-β-D-rhamnoside	β(1–4)	5	0
p-Nitrophenyl-β-D-glucuronide	β(1–4)	10	0
p-Nitrophenyl-α-L-rhamnoside	α(1–4)	10	0
Saccharose	β(1–4)	5	0
Starch	α(1–4)	5	0

[a]Depending on the type of substrate, activity was determined by measuring the release of either *p*-nitrophenyl (400 nm) or glucose (glucose oxidase method), at 30°C, as described in Materials and Methods.

mal temperature and a low thermal activation energy compared with *L. mesenteroides (6)*, 50°C and 55.5 kJ/mol; and with *C. entomophila (15)*, 60°C and 68.9 kJ/mol.

The action of the purified β-glucosidase was tested over a large number of substrates with α and β configurations. The results, summarized in Table 2, show that the β-glucosidase is active against (1→4)-β and (1→4)-α linkage configuration (alkyl-glucosides, aryl-glucosides), and against cellobiose, which is a diglucoside with (1→4)-β configuration. From Lineweaver Burk plots, a K_m value of 16 mmol/L was found for β-pNPG. However, the β-glucosidase was not active against diglucoside with a (1→4)-α-configuration like maltose. The cyanoglucosides linamarin and prunassin were also tested. The β-glucosidase was able to hydrolyze prunassin, but not limamarin, the main cyanogenic glycoside found in cas-

sava roots. Thus, the β-glucosidase of *L. casei* is not suitable for a cassava detoxication process.

The effect of various cations at 10^{-2} mol/L was tested. The enzyme is inhibited by Hg^{2+}, Ag^{2+}, and more weakly by Zn^{2+}, Cu^{2+}, Ni^{2+}. The action of some effectors, such as EDTA (10 mmol/L), a chelating agent, allowed us to conclude that the intracellular β-glucosidase of *L. casei* is dependent on a metallic cofactor at its active site. The inhibition by *N*-bromosuccinimide at 10 mmol/L indicates that tryptophan residues of the enzyme are important in its catalytic action *(22)*. The *para*-chloromercuribenzoate, a well-known SH blocking agent, had a inhibitory effect on enzyme activity. This result, in combination with the observed high sensitivity toward Hg^{2+}, may indicate the presence of important sulfhydryl groups. Iode (5 mmol/L), an agent reacting with tyrosine, strongly inhibited the catalytic activity.

Glucose inhibition was studied using pNPG as substrate. A competitive-type inhibition was observed. The K_i obtained at the intersection of the lines on the Dixon plot was 16 mmol/L. This value is quite similar to those found for *L. mesenteroides (6)*. The effect of ethanol was tested on β-glucosidase activity using pNPG as substrate, and no glycosyl transferase activity was observed in the presence of ethanol.

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