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A Thermostable α-Galactosidase from *Lactobacillus fermentum* CRL722: Genetic Characterization and Main Properties

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Abstract. α-Galactosidase (α-Gal) enzyme, which is encoded by the *melA* gene hydrolyzes α-1,6 galactoside linkages found in sugars, such as raffinose and stachyose. These α-galacto-oligosaccharides (α-GOS), which are found in large quantities in vegetables, such as soy, can cause gastrointestinal disorders in sensitive individuals because monogastric animals (including humans) do not posses α-Gal in the gut. The use of microbial α-Gal is a promising alternative to eliminate α-GOS in soy-derived products. Using degenerate primers, the *melA* gene from *Lactobacillus (L.) fermentum* CRL722 was identified. The complete genomic sequence of *melA* (2223 bp), and of the genes flanking *melA*, were obtained using a combination of polymerase chain reaction–based techniques, and showed strong similarities with the α-Gal gene of thermophilic microorganisms. The α-Gal gene from *L. fermentum* CRL722 was cloned and the protein purified from cell-free extracts of the native and recombinant strains using various techniques (ion exchange chromatography, salt precipitation, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and ultra-filtration); Its main biochemical properties were determined. The enzyme was active at moderately high temperatures (55°C) and stable at wide ranges of temperatures and pH. The thermostable α-Gal from *L. fermentum* CRL722 could thus be used for technological applications, such as the removal of α-GOS found in soy products. The complete *melA* gene could also be inserted in other micro-organisms, that can survive in the harsh conditions of the gut to degrade α-GOS in situ. Both strategies would improve the overall acceptability of soy-derived products by improving their nutritional value.

The α-Galactosidase (α-Gal) enzyme hydrolyzes α-1,6 galactoside linkages found in sugars, such as raffinose and stachyose. These α-galacto-oligosaccharides (α-GOS), which are found in large quantities in some vegetables such as soy, can cause gastrointestinal disorders in sensitive individuals because monogastric mammals (such as humans) do not posses a pancreatic or brush border α-Gal in the small intestine, thus hampering the acceptability of soy-derived foods. In this context, exogenous α-Gal may be used to alleviate unwanted symptoms attributed to α-GOS consumption (abdominal pain, diarrhea and flatulence, for example) while also increasing the nutritional value of soy-containing products by releasing digestible monosaccharides from complex α-GOS. Although several genes for microbial α-Gals have been characterized, little is known about the α-Gal–encoding genes from potential probiotic bacteria or those that can be used in probiotic formulations. Recently, α-Gal–encoding genes from the

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lactic acid bacteria (LAB) *Lactobacillus plantarum* [1] and *Lactococcus raffinolactis* [2] have been studied.

Previous studies performed by our group have shown that *L. fermentum* CRL722: (1) exhibits the highest α-Gal production among our lactobacilli strain collection [3], (2) produces an α-Gal capable of degrading raffinose and stachyose from soy [4], and (3) is able to use α-GOS as energy sources for growth in soymilk [4], thus decreasing gastrointestinal disorders, such as the abnormal cecum growth associated with α-GOS consumption in conventional mice [5]. However, *L. fermentum* CRL722 cannot persist in the small intestine, and consequently the α-Gal delivered to this portion of the gastrointestinal tract is short-lived [6]. The goal of this work was to perform the molecular characterization of *L. fermentum* CRL722 α-Gal to better understand its biochemical properties and, further, to improve the control of enzyme supplementation to achieve α-GOS degradation in situ.

Materials and Methods

Bacterial strains and growth conditions. *L. fermentum* CRL722 used in this study was obtained from the Culture Collection (CRL) of the Centro de Referencia para Lactobacilos (CERELA, San Miguel de Tucumán, Argentina) and grown in deMan-Rogosa-Sharpe medium [7] at 37°C without shaking. *Escherichia coli* were grown at 37°C in Luria-Bertani (LB) medium at 37°C under aerobic conditions adding ampicillin (100 μg/ml) when required.

DNA technique. Chromosomal DNA was extracted from lactobacilli as previously described [8]. Restriction enzymes and alkaline phosphatase were purchased from New England Biolabs (Beverly, MA) or Promega (Madison, WI). T4 DNA ligase and DNA polymerases were purchased from Invitrogen Life Technologies (Carlsbad, CA) and were used as recommended by the suppliers. *E. coli* $CaCl_2$-competent cell preparation, heat shock transformation, and recombinant DNA techniques were performed according to conventional methods [9]. Amplified fragments were cloned in pBluescript II SK+(pBS) (Stratagene, La Jolla, CA) using *E. coli* DH5α (*supE*44, Δ*lac*U169 [ϕ*80d lac*ZΔM15], *hsd-17, rel*A1, *end*A1, *gyr*A96 *thi*-1, *rel*A1) as host or in the pET28b (+) expression vector (Novagen, Madison, WI) using *E. coli* BL21 (DE3) [F^-, *ompT, hsdS*$_B$ (r_B^- m_B^-) *gal dcm* (DE3)] as host.

Preparation of cell-free extracts. Late exponential phase cultures of *L. fermantum* CRL722 (500 ml) were harvested by centrifugation at 6000 × g for 10 minutes. This and all subsequent steps were carried out at 4°C. The cells were washed twice with McIlvaine buffer (Na_2HPO_4–citric acid at pH 5.8) [10] and resuspended in 40% (m/v) of the same buffer. Cells were disrupted by three passages through a French pressure cell press (Thermo Spectronic, Rochester, NY) at a pressure of 25,000 psi. The unbroken cells were removed by centrifugation at 10,000 × *g* for 10 minutes and the supernatant (crude extract) was used for subsequent analyses.

Purification of the enzyme. The crude extract was loaded on a Q Sepharose Fast Flow Column (3 × 10 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated in Tris buffer (40 mM at pH 7.0). Elution was performed at a flow rate of 2 ml/min with the same buffer containing 0.2, 0.3, 0.5, and 2.0 M NaCl, and fractions (2.5 ml) exhibiting α-Gal activity were collected and pooled. The pooled fractions from ion-exchange chromatography were concentrated using Centriprep Centrifugal Filter Device YM-100 (100-kDa cutoff; Millipore, Bedford, MA) as described by the manufacturer. A fractionated precipitation, by addition of solid ammonium sulfate, was performed on the high molecular–weight fraction (which showed α-Gal activity) obtained during the ultrafiltration step. $(NH_4)_2SO_4$ at 45% saturation was used; the mixture was agitated for 60 minutes, and the supernatant was collected by centrifugation at (10,000 × *g* for 15 minutes). $(NH_4)_2SO_4$ was added to achieve 75% saturation and processed in the same manner as the previous step. The precipitate from 75% saturation was resuspended in 10 ml McIlvaine buffer (5 mM at pH 5.8) and dialyzed for 12 hours against distilled/deionized water, which was changed every 4 hours. The pooled fraction from ammonium sulfate precipitation was concentrated using Millipore 25 Ultrafree-CL Polisulfone Filters (300-kDa cutoff, Millipore, Bedford, MA). The high molecular–weight fraction, which showed enzymatic activity, was collected and used in biochemical analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS·PAGE) was carried out using a 5% stacking gel and a 9% separation gel.

Enzymatic assays. α-Gal activity was determined using a modified version of the Church method [11]. Briefly, 27.5 μl McIlvaine buffer (4.5×) and 12.5 μl of the specific substrate *p*-nitrophenyl-α-D-galactopyranoside (30 mM) were added to a 85-μl sample, and incubated at 37°C for 15 minutes. The reaction was stopped by adding 125 μl sodium carbonate (0.5 M). Absorbance at 405 nm was measured using a VersaMax Tunable Microplate Reader (Molecular Devices). One unit of α-Gal activity was defined as the amount of enzyme that releases 1.0 μmol p-nitrophenyl per min. The protein concentration was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) based on Bradford's method [12] using bovine serum albumin as a standard.

Effect of pH, temperature, and metal ions on enzyme activity. The effect of pH on α-Gal activity was determined according to the previously described technique diluting the samples in McIlvaine buffer with a pH range of 3.0 to 8.0. The influence of temperature on enzymatic activity was determined by incubating the assay mixture for 15 minutes at temperatures from 4°C to 70°C. To determine the effect of different metal ions, stock solutions were prepared in McIlvaine buffer at pH 5.8 and added separately to the reaction mixture at a final concentration of 1 mM. The residual enzyme activity was assayed and expressed as a percentage of the activity determined in McIlvaine buffer alone, which was used as control (100% activity).

Results and Discussion

To identify the gene responsible of α-Gal activity in *L. fermentum* CRL722 degenerated primers (aga298: 5′, ATCCTTYCARACNCCNGA 3′ and aga482r: 5′ TTCA TRTCCCAYTTDATRTA 3′) were designed based on the conserved region of several genes for α-Gal. Using these primers, a 575-bp polymerase chain reaction PCR fragment corresponding to the gene was obtained and further designated *melA* according to the first gene for α-Gal described in a lactobacilli species [1]. Subsequently, a combination of uneven PCR [9], Southern hybridization, and reverse PCR techniques were used to obtain the sequence of a 3,000-bp region spanning the complete

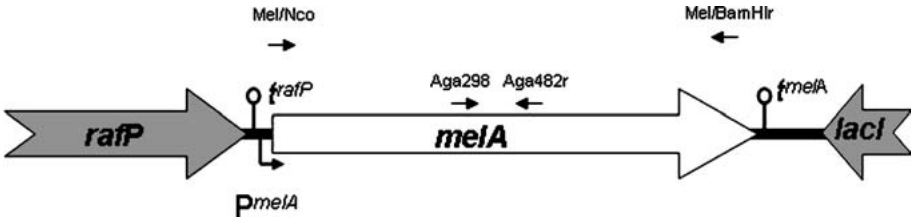

Fig. 1. Schematic representation of *melA* locus in *L. fermentum* CRL722. White arrow denotes complete ORF; grey arrows denote incomplete ORF. Transcriptional promoter (P), terminators (t), primers (small arrows) used are indicated.

melA gene as well as flanking sequences encoding for a putative sugar transporter, identified as *rafP* (5′ of *melA*, in the same orientation) and the partial sequence of an open reading frame with homology to a sugar-binding transcriptional regulator of the LacI family, downstream to *melA*, in opposite orientation (denoted *lacI)* (Fig. 1). The 2,223-bp *melA* is preceded by a promoter and is flanked by two potential transcriptional terminator elements, with 13.9 kcal/mol (upstream) and 14.2 kcal/mol (downstream) values of free-energy change (ΔG_0). These elements suggest that *melA* produces a monocistronic transcript, which encodes a 741-amino-acid (aa) polypeptide of approximately 84.2 kDa. The SDS-PAGE of purified fractions from *L. fermentum* CRL722 showed a major band of approximately 84 kDa, which confirms the predicted molecular mass of *MelA* protein (data not shown). The genetic organization of the *melA* locus resembles the one previously studied in *L. plantarum* [1], except for *LacI*, which is replaced by β-Gal–encoding genes in *L. plantarum.* BLASTx comparison revealed strong similarity of *melA* from *L. Fermentum* CRL722 with α-Gals from thermophilic micro-organisms, such as *Bacillus halodurans* (61%; gi:25312336), *Thermoanaerobacter ethanolicus* (60%; gi:2920686), *Geobacillus stearothermophilus* (59%; gi:7839139), and *Clostridium stercorarium* (59%; gi:28268728), and to a slightly lower extent, with α-Gals from same-genus organisms: *L. gasseri* (56%; gi:52857912), *L. acidophilus* (55%; gi:58337709), *L. johnsonii* (54%; gi:42518348), and *L. plantarum* (52%; gi: 15042935). The higher sequence similarity with enzymes from thermophilic microorganisms than with same-genus microorganism's enzymes would explain in part the thermostable properties of the enzyme described here (see later, temperature studies). Considering the aa sequence and catalytic domain, this α-Gal is included in glycosyl hydrolases family 36 according to Henrissat's classification [13]. The GenBank accession numbers of the described nucleotide sequences are as follows: AY612895 (complete *melA* gene), AY954917 *(rafP*, partial cds), and AY95498 (putative *lacI*, partial cds) (Table 1).

To clone the *melA* gene from *L. fermentum* CRL722 for heterologus expression in *E. coli*, a 2.2-kb PCR amplicon obtained using specific primers (Mel/Nco: 5′-CCCCATGGTCATGCCAGTCTTTATC AACGA-3′ and Mel/BamHIr: 5′-CGGGATCCTCTTA TGCGCCAACTGTTCGTC-3′), was inserted in the commercial expression vector pET28b(+) (Novagen, Madison, WI), and the new plasmid, designated pET-melAf, was introduced in *E. coli* BL21 (DE3). This modified strain (*E. coli* BL21 pET-melAf) produces active α-Gal, whereas the native strain (*E. coli* BL21) or the same strain containing the empty expression vector pET28b(+) (*E. coli* BL21-pET) either did not, or its activity was less than the detection levels of our system (data not shown).

To compare the enzymatic properties of the native and recombinant enzymes, we purified α-Gal from *L. fermentum* CRL722 and *E. coli* BL21 pET-melAf as described previously. The results from ultrafiltration with 300-kDa cutoff filters indicated that the enzyme has a molecular mass higher than 300 kDa. Consequently, the native enzyme is at least a 300-330-kDa homotetramer, given that the size of MelA monomer is approximately 84 kDa as deduced both from the *melA* gene sequence and SDS-PAGE of *E. coli* BL21 pET-melAf and *L. fermentum* CRL722 extracts. These results differ from those published by Garro et al. [14], in which α-Gal from *L. fermentum* was composed of four subunits of 45 kDa each.

To achieve a partial biochemical characterization of the α-Gal from *L. fermentum* CRL722, we compared the effect of pH, temperature, and metal ions on α-Gal activity using the native and recombinant purified enzymes. For pH studies, the enzymes were diluted in McIlvaine buffer in a pH range of 3.0 to 8.0, and both showed more than 50% activity between pH 4.3 and 6.5 (optimal pH value at 4.8). The activity was still 20% at pH 8 (Fig. 2A). This pH profile is unusual in that the slopes of the lower and upper limbs of the graph are very different. This suggests either that an essential ionizable group with pKa of approximately 4.5 is present or that a partial loss of activity caused by deprotonation of a group with pKa of approximately 6 occurred. Future studies will reveal the veracity of these hypotheses.

For temperature studies, the assay mixture was incubated for 15 minutes at temperatures from 4°C to 70°C. A 50% activity was observed between 35°C and

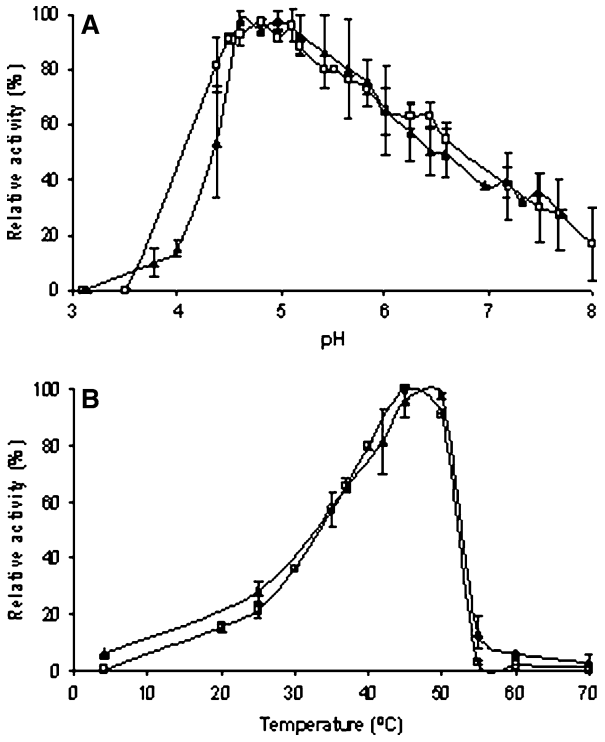

Fig. 2. Effect of pH (A) and temperature (B) on α-Galactosidase activity from purified native enzyme from *L. fermentum* CRL722 (open squares) and recombinant enzyme from *E. coli* BL21 pET-melAf (closed triangles). Results are expressed as means ± SD (n = 4).

52°C, with the optimal temperature being close to 50°C (Fig. 2B). At this relatively high temperature, the enzyme is quite stable because no activity loss was observed after 30 minutes of incubation at 50°C. At 25°C the enzyme still kept 20% activity; however, at temperatures above 55°C it drastically decreased. These results differ slightly compared with previous reports in which α-Gal of another *L. fermentum* strain had an optimum temperature of 45°C with enzyme inactivation starting at 50°C [15]. These results suggest that in *L. fermentum* α-Gal's properties (such as thermostability) could be a strain-dependent characteristic.

Table 2 shows the effect of different cations on enzyme activity. Mercury was the strongest inhibitor and caused a 70% to 80% decrease of α-Gal activity. Other metal ions-such as Cu^{++}, Zn^{++}, and Cd^{++} also affected α-Gal, albeit to a lower extent, ranging from 23% to 27%, 21% to 25%, and 14% to 18% decreases in enzymatic activity, respectively. All other ions tested in this study had negligible effects on this enzymatic activity. The data suggest that the enzyme may contain an essential sulfhydryl (or thiol) group that is chemically modified by mercuric ion, and also possibly by cupric ion. Previous studies [11, 15] have also shown that mercury is a strong inhibitor of several α-Gals, suggesting the presence of thiol groups at the enzymes' catalytic site.

Table 1. Purification of α-Galactosidase from *Lactobacillus fermentum* CRL722

Purification step	Total activity (U)	Amount of protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Cell-free extract	77.0	367.0	0.21	1.0	100
Q Sepharose	15.4	39.6	0.39	1.9	20
Centriplus (100 kDa)	15.1	16.2	0.94	4.5	20
$(NH_4)_2SO_4$ (45%)	12.8	10.0	1.28	6.1	17
$(NH_4)_2SO_4$ (75%)	11.3	3.0	3.78	18.0	17
Millipore (300 kDa)	6.1	1.4	4.37	20.8	8

Table 2. Effect of metal ions on α-Gal activity of *L. fermentum* CRL722 and *E. coli* BL21 pET-melAf

Compound	*L. fermentum* CRL722	*E. coli* BL21 pET-melAf
H_2O	100.0 ± 0.0	100.0 ± 0.0
$CaCl_2$	90.4 ± 1.1	91.0 ± 0.8
$HgCl_2$	20.9 ± 9.4	29.6 ± 5.3
$ZnSO_4$	78.3 ± 7.8	74.4 ± 5.5
$FeSO_4$	99.9 ± 0.3	98.6 ± 2.0
$CuSO_4$	76.8 ± 8.2	72.7 ± 5.8
$MnSO_4$	91.5 ± 4.7	93.8 ± 3.3
$MgSO_4$	93.8 ± 1.9	94.8 ± 1.3
$MnCl_2$	92.1 ± 2.7	93.4 ± 1.9
$MgCl_2$	92.6 ± 3.6	94.4 ± 2.5
LiCl	93.3 ± 2.9	94.7 ± 2.0
KC1	96.7 ± 1.5	96.0 ± 1.0
NaCl	98.6 ± 1.4	97.8 ± 1.0
CdCl	86.0 ± 9.0	81.5 ± 6.3

Results are expressed as means ± SD (n = 4).

In summary, native and recombinant enzymes showed similar activities under the studied conditions of pH temperature and in the presence of different metal ions.

Concerning the significance of α-Gal, two main uses have been proposed for LAB's α-Gals. The first one involves the use of *Lactococcus raffinolactis's* α-Gal as a food-grade selection marker in *Streptococcus thermophilus*, but the enzyme fails to be expressed at 42°C, the growth temperature for this microorganism [16], revealing that temperature resistance is important. The second one goes toward the improvement of soy-based foods as a result of α-Gal activity from probiotic preparations to relieve specific gastrointestinal discomforts associated with α-GOS consumption. In this direction, this study's purpose was to determine the biochemical and genetic properties of α-Gal from *L. fermentum* CRL722. It is active over a wide range of pH and

temperatures, which is in agreement with the fact that this enzyme shares high similarity with α-Gals from thermophilic organisms. Its thermostability could be relevant for its future use in industrial processes, mainly in fermented vegetable-product preparations. Also, this α-Gal activity is promising in the elaboration of novel soy-products with improved organoleptic and digestibility attributes, such as the ability to degrade α-GOS into its digestible galactose and glucose moieties, which can be absorbed directly in the small intestine. That would prevent α-GOS delivery in the large intestine and would both increase the nutritional value of the product and protect sensitive individuals from α-GOS-derived symptoms. This work is part of a wide set of studies on α-Gal from *L. fermenlum* CRL722 [3–6, 17] and provides supporting evidence for the use of this source of thermostable enzyme for technological applications.

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