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Extracellular α-Galactosidase from *Debaryomyces hansenii* UFV-1 and Its Use in the Hydrolysis of Raffinose Oligosaccharides

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Raffinose oligosaccharides (RO) are the factors primarily responsible for flatulence upon ingestion of soybean-derived products. ROs are hydrolyzed by α-galactosidases that cleave α-1,6-linkages of α-galactoside residues. The objectives of this study were the purification and characterization of extracellular α-galactosidase from *Debaryomyces hansenii* UFV-1. The enzyme purified by gel filtration and anion exchange chromatographies presented an M_r value of 60 kDa and the *N*-terminal amino acid sequence YENGLNLVPQMGWN. The K_m values for hydrolysis of *p*NPαGal, melibiose, stachyose, and raffinose were 0.30, 2.01, 9.66, and 16 mM, respectively. The α-galactosidase presented absolute specificity for galactose in the α-position, hydrolyzing *p*NPGal, stachyose, raffinose, melibiose, and polymers. The enzyme was noncompetitively inhibited by galactose (K_i = 2.7 mM) and melibiose (K_i = 1.2 mM). Enzyme treatments of soy milk for 4 h at 60 °C reduced the amounts of stachyose and raffinose by 100%.

KEYWORDS: α-Galactosidase; *Debaryomyces hansenii* UFV-1; raffinose oligosaccharides; characterization; flatulence

INTRODUCTION

The enzyme α-galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22) catalyzes hydrolysis of α-1,6-linked α-galactoside residues from simple oligosaccharides such as melibiose, raffinose, and stachyose and from polymeric galactomannans (*1*). α-Galactosidase is widely distributed in microorganisms, plants, and animals (*2*−*4*). Purification and characterization of α-galactosidases from several sources have been reported, and according to their source, their properties differ markedly (*5*−*8*).

This enzyme is of particular interest in view of its biotechnological applications. Microbial or plant α-galactosidases are added to soybean meal, soy milk, and molasses to hydrolyze the raffinose oligosaccharides (ROs) to digestible carbohydrates and thereby moderate the flatulence-causing property of soybean products (*9*, *10*). Enzymatic hydrolysis of hemicelluloses is of interest for the pulp and paper industry, and α-galactosidase-modified galactomannan has been used to improve the gelling properties of polysaccharide (*11*).

Several yeast strains are known to assimilate melibiose as a nutrient, so they possibly have significant α-galactosidase activity. In addition to melibiose, galactose has been shown to induce enzyme synthesis and results from our laboratory suggest that galactose could induce intra- and extracellular α-galactosidases in *Debaryomyces hansenii* UFV-1. It is the most frequent yeast species in protein-rich fermented products such as sausages and cheeses (*12*).

In the present study, we report the purification and characterization of the α-galactosidase secreted by *D. hansenii* UFV-1 when grown on galactose. The hydrolytic properties and substrate specificities of this α-galactosidase were studied to elucidate its possible applications.

MATERIALS AND METHODS

Microorganism. The yeast strain used in this study was isolated from a dairy environment in Minas Gerais, Brazil, and has been maintained in the culture collection of the Laboratory of Microorganism Physiology, BIOAGRO, UFV, Brazil. This yeast was identified by the Institute of Yeasts Identification, Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands, as *D. hansenii* (Zopf) Lodder & Kreger-van Rij var. *fabryi* Nakase & Suzuki. In this work, it is designated as *D. hansenii* UFV-1.

Conditions for Enzyme Production. *D. hansenii* UFV-1 stored at −80 °C in glycerol and YPD medium (1% yeast extract, 2% peptone, and 2% glucose) was streaked on a YPD agar surface (1.5% agar) and maintained in an incubation chamber at 30 °C for 36 h. The yeast was then activated in YPD liquid medium and incubated at 30 °C, 200 rpm,

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Table 1. Summary of *D. hansenii* UFV-1 Extracellular α-Galactosidase Purification

purification step	volume (mL)	protein (mg)	activity (U/mL)	total activity[a] (U)	specific activity (U/mg protein)	purification factor	recovery (%)
lyophilized extract	102.0	7.45	0.87	88.7	11.9	1.00	100.0
Sephadex G-150	190.0	0.57	0.31	58.9	103.0	8.65	66.4
DEAE-Sepharose	17.0	0.26	3.04	51.7	199.0	16.70	58.3

[a] One unit (U) of enzyme activity is defined as the amount of enzyme that released 1 μmol of *p*NP per minute.

for 12−15 h. The cells obtained after centrifugation (4000*g* for 5 min at 4 °C) were inoculated in mineral medium containing 0.62 g/L KH_2PO_4, 2.0 g/L K_2HPO_4, 1.0 g/L $(NH_4)_2SO_4$, 0.1 g/L $MgSO_4 \cdot 7H_2O$, and 5.0 g/L yeast extract with galactose as the carbon source. After incubation at 30 °C, 200 rpm for 31 h, the biomass was separated by centrifugation and the supernatant containing α-galactosidase was lyophilized.

α-Galactosidase Purification. The lyophilized enzymatic sample resuspended in 25 mM sodium acetate buffer was subjected to gel filtration chromatography in a Sephadex G-150 column (87.5 cm × 2.5 cm) equilibrated with 25 mM sodium acetate buffer, pH 5.5. The proteins were eluted at a flow rate of 20 mL/h, and 3.3 mL fractions were collected. Fractions containing α-galactosidase activity were pooled and subjected to ion exchange chromatography in a DEAE-Sepharose column (14.5 cm × 1.9 cm) equilibrated with 0.1 M sodium acetate buffer, pH 5.5. The proteins were eluted at a flow rate of 40 mL/h, with a linear increasing gradient of NaCl (0−1.0 M) in 0.1 M sodium acetate buffer. All purification procedures were performed at 4 °C. The active fraction was pooled and analyzed for purity by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

Enzyme Assay. α-Galactosidase was assayed in a reaction system containing 650 μL of 0.1 M sodium acetate buffer, pH 5.0, 100 μL of enzyme solution (0.75 μg protein/mL), and 250 μL of 2 mM *p*-nitrophenyl-α-D-galactopyranoside (*p*NPαGal) or other synthetic substrates. The reaction was run for 15 min at 60 or at 40 °C for the purification assays and ended with the addition of 1 mL of 0.5 M sodium carbonate. The amount of *p*-nitrophenol (*p*NP) released was determined at 410 nm. This procedure was defined as the standard assay.

The activities against melibiose, maltose, gentiobiose, and lactose were evaluated by the glucose−oxidase method (*13*). When sucrose, raffinose, and stachyose were used as the substrate, the production of reducing sugar was determined using the 3,5-dinitrosalicylate reagent (*14*). One enzyme unit (U) was defined as the amount of enzyme that released 1 μmol of product per min under assay conditions.

Determination of Protein Concentration. The protein concentration in the enzymatic extract was determined by the Coomassie Blue binding method with bovine serum albumin (BSA) as the standard (*15*).

Determination of Molecular Mass. The enzyme molecular mass was estimated by SDS−PAGE using a 12.5% polyacrylamide gel (*16*). After electrophoresis, the gel was silver-stained (*17*). The molecular mass standards (Pharmacia) were as follows: BSA, 66.0 kDa; ovalbumin, 45.0 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36.0 kDa; carbonic anhydrase, 29.0 kDa; trypsinogen, 24.0 kDa; trypsin inhibitor, 20.1 kDa; and α-lactalbumin, 14.2 kDa.

Effect of pH and Temperature. The pH effect of the α-galactosidase activity was investigated at different pH levels (from 3.0 to 8.0) by Mcllvaine buffer (citric acid/sodium phosphate) at 60 °C (*18*). The pH stability of α-galactosidase was also determined by incubating 15 μL of enzyme solution (15.0 μg protein/mL) with 285 μL of above-mentioned buffer at 60 °C for 30 min. After incubation, 100 μL of the mixture was used for determination of the residual activity, according to the standard assay, using *p*NPαGal as the substrate.

The optimum temperature was determined within a temperature range of 25−80 °C at pH 5.0. Thermal stability was investigated by incubating 100 μL of enzyme solution (0.75 μg protein/mL) and 650 μL of 0.1 M sodium acetate buffer, pH 5.0, at various temperatures (40, 50, 55, 60, 65, and 70 °C) for 0−48 h. After incubation, 250 μL of 2 mM *p*NPαGal was added, and the remaining activity was measured. Results of the analyses are presented as means ± SD for three measurements.

Determination of Kinetic Parameters. The Michaelis−Menten constant (K_m) and V_{max} for substrate hydrolysis were calculated by the Michaelis−Menten plot. The substrate concentrations expressed in mM were 0.05, 0.075, 0.1, 0.15, 0.2, 0.25, 0.3, 0.4, 0.5, 0.6, 0.75, 0.9, and 1.0 for *p*NPαGal; 1.25, 2.5, 5, 10, 20, 30, 40, 60, 70, and 80 for raffinose; 2.5, 5, 8, 10, 15, 20, 25, and 30 for stachyose; and 0.15, 0.3, 0.625, 1.25, 2.5, 5.0, 7.5, 10, 15, 20, 30, and 40 for melibiose. The inhibition constants (K_i) for galactose and melibiose were calculated by the Dixon plot. The *p*NPαGal concentrations were 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 mM. The concentrations of inhibitor galactose were 0.5, 1.0, and 2.0 mM, and the melibiose concentrations were 0.25, 0.50, and 1.0 mM.

Substrate Specificity. Enzymatic assays were performed with various synthetic, natural, and polymeric substrates. The reaction mixtures contained 650 μL of 0.1 M sodium acetate buffer, pH 5.0, 70 μL of enzyme solution (0.75 μg protein/mL), and 250 μL of synthetic substrates (2 mM), or lactose, maltose, gentiobiose, stachyose, and sucrose (10 mM), or raffinose (15 mM), or melibiose (2 mM), or locust bean gum and guar gum solutions (1%). The activities were measured under standard assay conditions at 60 °C. The data presented for all enzyme activity determinations are mean values ± SD of three measurements.

Effect of Ions, Simple Sugars, and Reducing Agents. The effect of ions, simple sugars, and reducing agents on the enzyme activity was assayed by the standard methods; enzyme samples were preincubated with each of the compounds (10 mM) in 0.1 M sodium acetate buffer, pH 5.0, for 20 min at 60 °C. The data presented for all enzyme activity determinations are mean values ± SD of triplicate assays.

N-Terminal Amino Acid Sequence Analysis. After blotting onto PVDF membrane, the N-terminal amino acid sequences of the α-galactosidase were determined by automated Edman degradation, using an automatic protein sequencer (PPSQ-21/23). Similarity searches were preformed using BLASTp software.

Enzymatic Hydrolysis of Oligosaccharides Present in Soy Milk. Soy milk was prepared from dry seeds (50 g). The seeds were chopped up, homogenized in 400 mL of water at 80 °C, incubated for 10 min at 85 °C, and filtered through cheesecloth. Soy milk samples (5 mL) were then incubated with either water or 10.5 U of purified α-galactosidase for 0, 2, 4, and 6 h under shaking (100 rpm) at 60 °C. The reaction mixtures were dried, and the soluble sugars from 20 to 30 mg of lyophilized samples were extracted with 80% aqueous ethanol (v/v) (*9*). The solvent was evaporated at 50 °C, and the sugars were resuspended in 1 mL of 80% ethanol and analyzed by high-performance liquid chromatography (HPLC) on a Shimadzu series 10A chromatograph. An analytical column [aminopropil (NH_2)] was used for this purpose, eluted with an acetonitrile−water isocratic mixture (80:20 v/v) at 35 °C at a flow rate of 1 mL/min. The individual sugars were automatically identified and quantified by comparison with the retention times and standard sugar concentrations. Gentiobiose was used as an internal standard as it does not interfere with the other sugars and is not found in soybean seeds.

RESULTS AND DISCUSSION

Galactose-grown *D. hansenii* UFV-1 produced high levels of intracellular and extracellular α-galactosidases, similar to other species such as *Debaryomyces castellii* IFO 1359, *Debaryomyces nepalensis* IFO 1428, and other yeasts (*19*). Results of the purification of extracellular α-galactosidase from *D. hansenii* UFV-1 are summarized in **Table 1**. The concentrated culture supernatant was subjected to gel filtration chromatography resulting in the separation of one protein fraction with

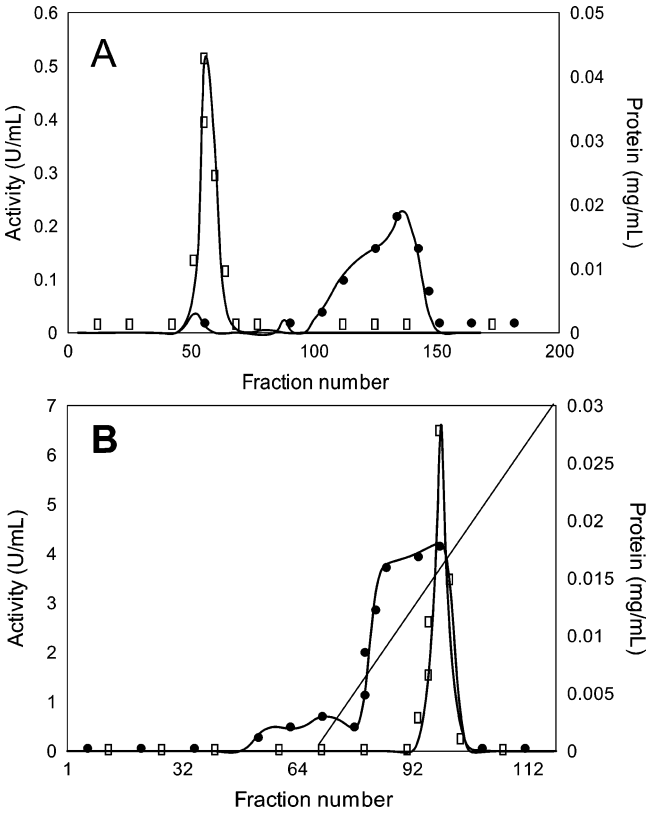

Figure 1. Elution profile of the α-galactosidase from *D. hansenii* UFV-1 on a (**A**) Sephadex G-150 column and (**B**) DEAE-Sepharose column. α-Galactosidase activity, □; protein, ●; and gradient of NaCl, –.

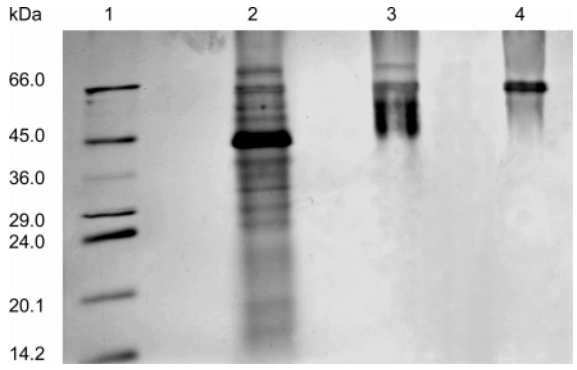

Figure 2. SDS–PAGE (12.5%) of *D. hansenii* UFV-1 α-galactosidase purification steps. Lane 1, molecular mass standards; lane 2, lyophilized extract fraction; lane 3, Sephadex G-150 fraction; and lane 4, DEAE-Sepharose fraction. Protein gel was stained with silver.

α-galactosidase activity (**Figure 1A**). This step resulted in considerable specific activity enrichment (**Table 1**). The final step was carried out by ion exchange chromatography. The active fractions were eluted from 0.45 to 0.6 M NaCl and appeared as a single activity peak (**Figure 1B**). This procedure resulted in a purification factor of 16.7 with a recovery level of the original α-galactosidase activity of 58.3% (**Table 1**). Similar results were reported for purification of extracellular *Saccharomyces carlsbergensis* α-galactosidase (*2*). No invertase activity was detected in the final enzyme preparation.

The electrophoretic profile of the enzyme in SDS−PAGE confirmed the presence of a single protein band, with an estimated molecular mass of 60 kDa (**Figure 2**). In previous reports, purified α-galactosidases from *Penicillium purpurogenum*, *Aspergillus fumigatus*, and *Thermomyces lanuginosus* IMI 158749 presented M_r values of 67 (*20*), 54.7 (*21*), and 57 kDa (*6*), respectively. The N-terminal amino acid sequence of *D. hansenii* UFV-1 α-galactosidase was determined as YENGLN-LVPQMGWN (**Table 2**). Although this region does not appear to be conserved among the known α-galactosidases, the N-terminal amino acid sequence of *D. hansenii* UFV-1 α-galactosidase shared a high similarity with other microbial α-galactosidases, which belong to the glycoside hydrolase family 27 in comparison with the sequences available in the protein database (**Table 2**). The alignment of the N-terminal amino acid sequence of *D. hansenii* UFV-1 α-galactosidase (14 amino acid residues) with the sequence of *D. hansenii* CBS767 α-galactosidase showed two nonconservative changes, a substitution of N for G at residue six and a substitution of V for T at residue eight. However, the N and V residues presented in *D. hansenii* UFV-1 were conserved in other microbial α-galactosidases, as observed in the sequence of the *Magnaporthe grisea* 70-15 α-galactosidase, which showed the perfect match to the last 12 amino acids of the protein under study (**Table 2**).

Substantial activity against *p*NPαGal was observed for enzyme preparation within a temperature range of 40−65 °C and pH range of 3.5−6.0 (**Figure 3**). The enzyme achieved maximal substrate hydrolysis at a temperature of 60 °C (**Figure 3B**). The optimum pH for the enzyme was 5.0 (**Figure 3A**). These optimum pH and temperature values are close to those determined for hydrolysis of *p*NPαGal by four α-galactosidases from *Aspergillus niger* ATCC 46890 (*1*), *Torulaspora delbrueckii* IFO 1255 (*31*), and *Thermomyces lanuginosus* IMI 158749 (*6*).

The K_m and V_{max} values were calculated by the Michaelis−Menten plot for *p*NPαGal, melibiose, raffinose, and stachyose (**Table 3**). The K_m value for *p*NPαGal is comparable to the one determined for hydrolysis of the same substrate by α-galactosidase purified from *Aspergillus fumigatus* (*21*) and *Ganoderma lucidum* (*32*). The K_m values for raffinose and stachyose were lower than those determined for hydrolysis of the same substrates by the enzyme of *T. delbrueckii* IFO 1255 (*31*). For the natural substrates in use, the lowest K_m and V_{max} values were calculated for melibiose. Results indicate that *D. hansenii* UFV-1 α-galactosidase presents a higher affinity for melibiose and that the complex ES formation is not the limiting step for the reaction. The ratio V_{max}/K_m showed that substrate *p*NPαGal was used more efficiently by the enzyme, followed by stachyose, raffinose, and melibiose. The purified α-galactosidase was thermostable. No loss in enzyme activity was observed after incubation for 24 h at 40 °C. The enzyme maintained about 91% of its original activity after 3 h at 60 °C, but 40% of the activity was lost following 15 min of incubation at 70 °C (**Figure 4**). The half-life of *D. hansenii* UFV-1 α-galactosidase at 50, 55, 60, 65, and 70 °C was 821, 647, 373, 180, and 34.6 min, respectively. These $t_{1/2}$ values were higher than those reported for soybean α-galactosidases (*9*, *10*) but lower than those reported for α-galactosidase from *Bacillus stearothermophilus* NCIM 5146 (*8*).

The *D. hansenii* UFV-1 α-galactosidase retained about 82% of its activity after incubation for 30 min at 60 °C in a pH range of 4.0−7.6, but 90% of the activity was lost after incubation in the same conditions at pH 3.0 (**Figure 5**). The enzyme showed significant stability for a wide range of pH and temperature levels, which is desirable for industrial applications.

The activity of α-galactosidase against several substrates is shown in **Table 4**. Under the experimental conditions, this enzyme was the most effective to hydrolyze *p*NPαGal, followed by stachyose, raffinose, and melibiose, as expressed in the V_{max}/K_m ratio (**Table 3**). Lactose and synthetic substrates containing

Table 2. Comparison of *D. hansenii* UFV-1 α-Galactosidase N-Terminal Sequence to Other Microbial α-Galactosidases[a]

Organism	Sequence	Reference
Debaryomyces hansenii UFV-1	**YENGLNLVPQMGWN**	This paper
Magnaporthe grisea 70-15	36 **NGLNLVPQMGWN** 47	22
Debaryomyces hansenii CBS767	21 **YENGL**GL**TPQMGWN** 34	23
Torulaspora delbrueckii	25 **NGL**GL**TPQMGWN** 36	24
Zygosaccharomyces mrakii	24 **NGL**GL**TPQMGWN** 35	25
Saccharomyces cerevisiae	24 **NGL**GL**TPQMGW**D 35	26
Saccharomyces mikatae	24 **NGL**GL**TPQMGW**D 35	27
Schizosaccharomyces pombe	27 **NGL**GL**KPQMGWN** 38	28
Phanerochaete chrysosporium	22 D**NGL**AI**TPQMGWN** 34	29
Mortierella vinacea	23 **NGL**AI**TPQMGWN** 34	30

[a] Amino acid residues conserved in all sequences are printed in bold.

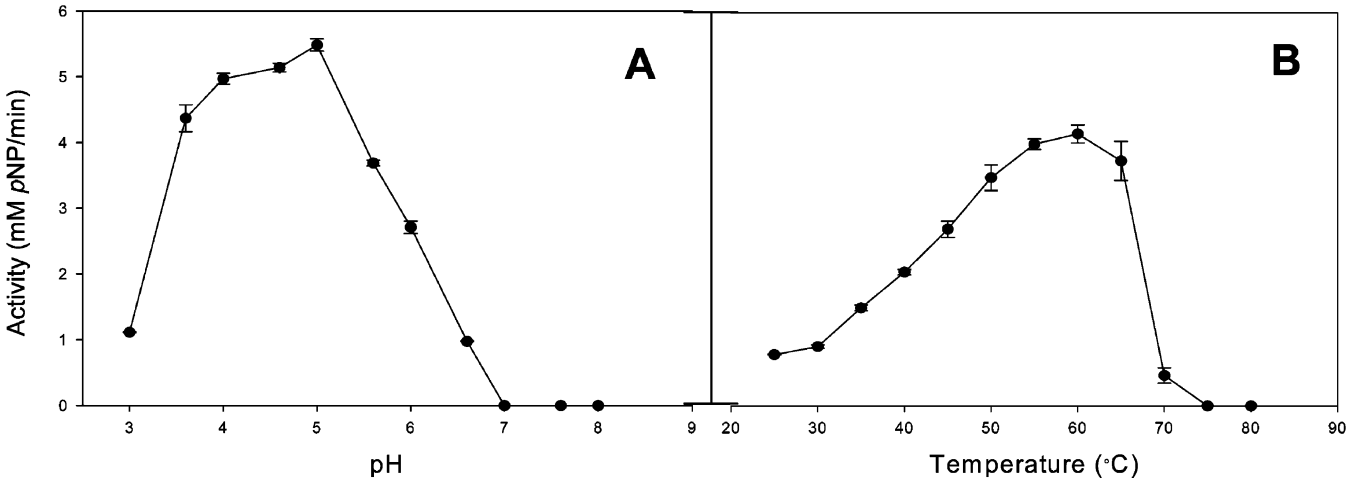

Figure 3. pH (**A**) and temperature (**B**) influences on the activity of α-galactosidase from *D. hansenii* UFV-1.

Table 3. K_m, V_{max}, and V_{max}/K_m Values Determined by Michaelis–Menten Plot

substrates	K_m (mM)	V_{max}[a]	V_{max}/K_m (min^{-1})
*p*NPαGal	0.30	6.09	20.3
melibiose	2.01	0.02	0.01
stachyose	9.66	8.18	0.85
raffinose	16.0	5.99	0.37

[a] V_{max} is expressed in mM *p*NP/min for *p*NPαGal, mM glucose/min for melibiose, and mM reducing sugar/min for stachyose and rafinose.

β-linkages or containing xylose, arabinose, mannose, and glucose residues were not hydrolyzed by the enzyme. Thus, the enzyme presents specificity not only for anomeric carbon but it also seems to be regiospecific for the galactoside configuration, in contradistinction to some promiscuous glycosidases reported in the literature (*33*). The enzyme exhibited the ability to hydrolyze polymers such as locust bean gum and guar gum, suggesting its potential industrial application for an improvement of the gelling properties of polysaccharide. On the other hand, *Trichoderma reesei* α-galactosidase and *A. niger* α-galactosidase showed high specificity for oligosaccharides and low activity for polymeric substrates (*34*). The aforementioned α-galactosidases from *Penicillium simplicissimum* seem to have different substrate specificities. One form of the enzyme is able to hydrolyze galactomannan, while the other acts more specifically on small oligosaccharides (*35*). Extracellular α-galactosidase from *D. castellii* IFO 1359 (*19*) and rice α-galactosidase (*36*) hydrolyzed galactomanno-oligosaccharides such as Gal^3Man_3 (6^3-α-D-galactopyranosyl-1,4-β-D-mannotriose).

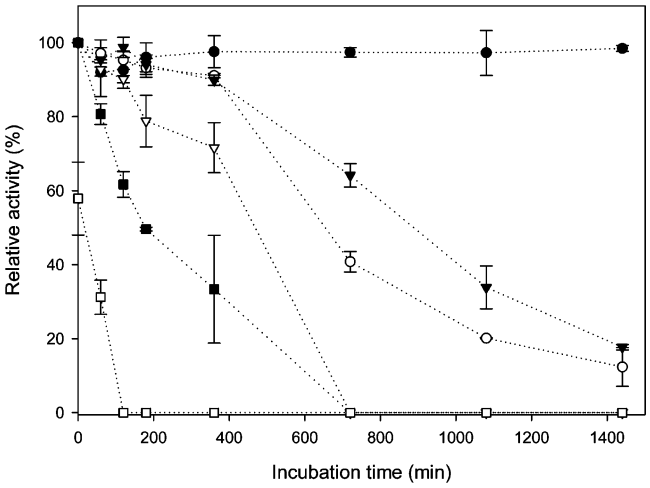

Figure 4. Temperature influences on the stability of the α-galactosidase from *D. hansenii* UFV-1. Enzyme preparations were preincubated for 48 h at temperatures of 40 (●), 50 (▼), 55 (○), 60 (▽), 65 (■), and 70 °C (□).

The *D. hansenii* UFV-1 α-galactosidase showed distinct sensitivities to simple sugars and mono- and bivalent ions (**Figure 6**). The enzyme presented very low or no inhibition by EDTA, Mg(II), iodoacetamide, Na(I), SDS, K(I), Ca(II), β-mercaptoethanol, raffinose, maltose, sucrose, D-glucose, lactose, gentiobiose, stachyose, and D-mannose. The enzyme was completely inhibited by Cu(II) and Ag(I) and was partially inhibited by D-galactose and melibiose. Reduction in the

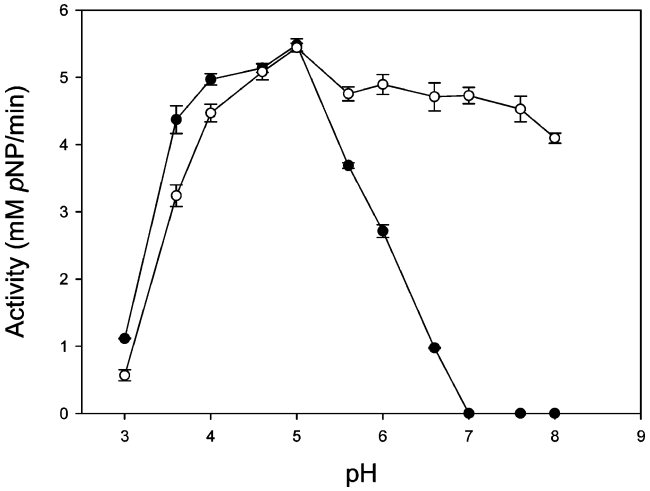

Figure 5. pH influences on the activity (●) and stability (○) of α-galactosidase from *D. hansenii* UFV-1.

Table 4. Hydrolysis of Several Substrates with α-Galactosidase from *D. hansenii* UFV-1

substrate	concentration[a]	activity (U/mL) ± SD
*p*NPαGal[b]	0.5	3.39 ± 0.15
*p*NPβGal[b]	0.5	0.0
*p*NPαGlc[b]	0.5	0.0
*p*NPβXil[b]	0.5	0.0
*p*NPαMan[b]	0.5	0.0
*p*NPαAra[b]	0.5	0.0
*o*NPβGal[b]	0.5	0.0
*o*NPβGlc[b]	0.5	0.0
sucrose	10	0.0
stachyose	10	3.84 ± 0.02
raffinose	15	2.78 ± 0.05
melibiose	2	0.002
gentiobiose	10	0.0
maltose	10	0.0
lactose	10	0.0
locust bean gum	1	0.64 ± 0.01
guar gum	1	0.56 ± 0.01

[a] Concentrations in mM, except the locust bean gum and guar gum substrates (%). [b] *p*NPβGal, *para*-nitrophenyl-β-D-galactopyranoside; *p*NPαGlc, *para*-nitrophenyl-α-D-glucopiranoside; *p*NPβX, *para*-nitrophenyl-β-D-xylopyranoside; *p*NPαA, *para*-nitrophenyl-α-D-arabinopyranoside; *p*NPαM, *para*-nitrophenyl-α-D-mannopyranoside; *o*NPβGlc, *ortho*-nitrophenyl-β-D-glucopyranoside; and *o*NPβGal, *ortho*-nitrophenyl-β-D-galactopyranoside.

α-galactosidase activity by Cu(II) and Ag(I) was reported for α-galactosidases purified from *T. delbrueckii* IFO 1255 (*31*) and *B. stearothermophilus* NCIM 5146 (*8*). Participation of carboxyl and/or histidine imidazolium groups in the catalytic action is supposed on the basis of the inhibitory effect (*37*). It has been suggested that α-galactosidase is not a metalloenzyme and that the sulfhydryl groups do not take part in catalysis, since there is no enzyme inhibition under EDTA or iodoacetamide treatment, respectively. This is in agreement with results reported for α-galactosidase isolated from *Penicillium* sp. 23 (*38*) and for soybean α-galactosidase (*10*).

Galactose and melibiose inhibition were found to be noncompetitive, and the K_i values were 2.7 and 1.2 mM, respectively, as determined by the Dixon plot, in opposition to data found in the literature for most α-galactosidases that are competitively inhibited by D-galactose (*9, 39*).

The data presented for all α-galactosidase activity determinations are mean values of triplicate assays in which the standard deviations values were always smaller than 10%.

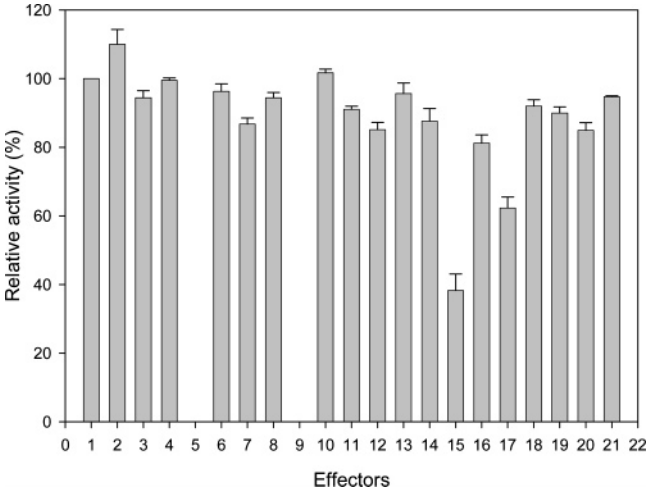

Figure 6. Effect of EDTA (2), $MgCl_2$ (3), iodoacetamide (4), $AgNO_3$ (5), NaCl (6), SDS (7), KCl (8), $CuSO_4$ (9), $CaCl_2$ (10), β-mercaptoethanol (11), raffinose (12), maltose (13), sucrose (14), melibiose (15), D-glucose (16), D-galactose (17), lactose (18), gentiobiose (19), stachyose (20), and mannose (21) on α-galactosidase from *D. hansenii* UFV-1 and without effectors (1). The final concentration of all effectors was 2 mM.

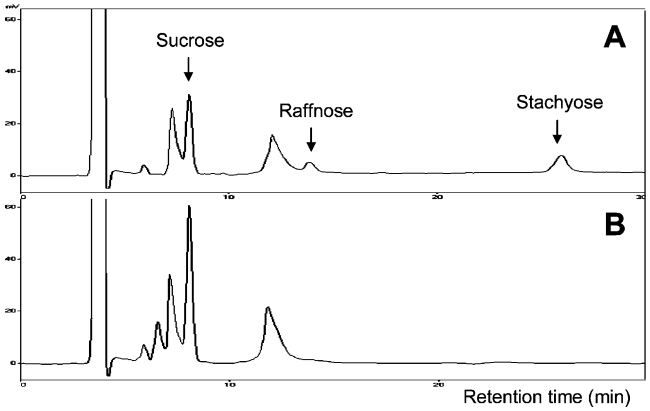

Figure 7. HPLC analysis of hydrolysis products of ROs present in soy milk by extracellular α-galactosidase from *D. hansenii* UFV-1. (**A**) Soy milk before enzyme treatment and (**B**) after 4 h of enzyme treatment.

The potential of *D. hansenii* UFV-1 α-galactosidase to hydrolyze the oligosaccharides present in soybean aqueous extract (soy milk) was demonstrated (**Figure 7**). Sucrose, raffinose, and stachyose were present in the reaction mixture at the concentrations 3.69, 1.33, and 2.70% (w/v), respectively (**Figure 7A**). After 2 h of incubation with the enzyme, a reduction of 24 and 100% was observed in the amount of raffinose and stachyose, respectively. The difference in hydrolysis may be due to accumulation of raffinose, which is formed after stachyose hydrolysis. However, when the enzyme was incubated with soy milk under the same conditions for a period of 4 h (**Figure 7B**), raffinose and stachyose were completely hydrolyzed and the sucrose concentration rose to 6.53% (w/v). No oligosaccharide hydrolysis was detected in control tubes where the enzyme extracts had been replaced by water. As the enzyme preparation showed no invertase activity, our results indicate that *D. hansenii* UFV-1 α-galactosidase acts on the oligosaccharides present in soy milk. The ability of the enzyme to hydrolyze stachyose and raffinose is of particular interest for biotechnological applications. Several food scientists have suggested the possibility of improving the nutritional value of soy milk and soybean flour by reducing the RO content. Microbial α-galactosidases were used to degrade RO in soy milk

(*40*−*42*). Nevertheless, no reliable, inexpensive, and efficient enzymic process with native or recombinant yeast enzymes is available so far. The potential enzymes suggested for this purpose are generally of microbial origin and do not have the GRAS (generally recognized as safe) status. *D. hansenii* is the most frequent yeast species found in protein-rich fermented products, such as sausages and cheeses (*43*). There should be no restriction regarding safeness for the use of this microorganism in food processing.

As the *D. hansenii* UFV-1 α-galactosidase shows stability at the pH range of soy milk (6.0−6.5) and at the temperature of 60 °C, the enzyme could be applied after the soy milk thermal treatment, during the cooling step, at approximately 60 °C. The properties of α-galactosidase described here, especially the broad pH and temperature stability and the specificity for ROs, suggest its application to remove flatulence-inducing compounds from soy milk.

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