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Debaryomyces hansenii UFV-1 Intracellular α-Galactosidase Characterization and Comparative Studies with the Extracellular Enzyme

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Debaryomyces hansenii cells cultivated on galactose produced extracellular and intracellular α-galactosidases, which showed 54.5 and 54.8 kDa molecular mass (MALDI-TOF), 60 and 61 kDa (SDS−PAGE) and 5.15 and 4.15 p*I* values, respectively. The extracellular and intracellular deglycosylated forms presented 36 and 40 kDa molecular mass, with 40 and 34% carbohydrate content, respectively. The *N*-terminal sequences of the α-galactosidases were identical. Intracellular α-galactosidase showed smaller thermostability when compared to the extracellular enzyme. *D. hansenii* UFV-1 extracellular α-galactosidase presented higher k_{cat} than the intracellular enzyme (7.16 vs 3.29 s^{-1}, respectively) for the *p*-nitrophenyl-α-D-galactopyranoside substrate. The K_m for hydrolysis of *p*NPαGal, melibiose, stachyose, and raffinose were 0.32, 2.12, 10.8, and 32.8 mM, respectively. The intracellular enzyme was acompetitively inhibited by galactose (K_i = 0.70 mM), and it was inactivated by Cu(II) and Ag(I). Enzyme incubation with soy milk for 6 h at 55 °C reduced stachyose and raffinose amounts by 100 and 73%, respectively.

KEYWORDS: α-Galactosidases; *Debaryomyces hansenii* UFV-1; characterization; deglycosylation; galacto-oligosaccharides

INTRODUCTION

α-Galactosidases (α-D-galactoside galactohydrolase EC 3.2.1.22) are known to occur widely in microorganisms, plants and animals, and some of them have been purified and characterized (*1, 2*). According to their source, mechanism and specificity, their properties differ markedly, and they are grouped into classes and clans (http://www.cazy.org/). Some of these enzymes catalyze the hydrolysis of α-1,6-linked galactosyl residues from galacto-oligosaccharides (GO) and polymeric galacto-(gluco)mannans. Several industrial applications of α-galactosidases are known, mainly in the sugar industry, where they improve the crystallization of sucrose by hydrolyzing raffinose in beet sugar syrups (*3*). Moreover, they can enhance the bleaching effect in the pulp and paper industry (*4*). They can also be used to improve the gelling properties of galactomannans used as food thickeners (*5*). α-Galactosidases are also of interest in biomedical applications, e.g., for the treatment of Fabry disease by enzyme replacement therapy (*6*) or for blood type conversion (*7*). The enzyme can be used for hydrolysis of raffinose, stachyose, and polysaccharides that induce gastrointestinal disturbances and flatulence in humans and which are present in soy milk and in other legume-derived foods (*8, 9*). Dietary supplementation with α-galactosidase can improve energy values and nutrient bioavailability, resulting in better growth performance and intestinal viscosity (*10*). Fungal α-galactosidases are the most suitable for technological applications mainly due to their acidic optima pH and broad stability profiles (*8*). However, the cost of production is one important factor which limits the use of this enzyme in industrial processes. It has been recently demonstrated that the yeast isolate *Debaryomyces hansenii* UFV-1 is very promising in the production of extracellular and intracellular α-galactosidases (*11*). *Debaryomyces hansenii* is a halophile yeast found in shallow seawaters

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(*12*) but also in salty food products like meat and dairy products (*13*). *D. hansenii* UFV-1 extracellular α-galactosidase was characterized, and its efficiency was demonstrated in the hydrolysis of GO present in soybean products (*11, 14*). In addition, *D. hansenii* UFV-1 permeabilized cells, containing intracellular α-galactosidase, were a good biocatalyzer for GO hydrolysis in soybean products (*14*). Therefore, the study of *D. hansenii* UFV-1 α-galactosidases is interesting, since both enzymes could be used in biotechnological processes. In this paper we characterized *D. hansenii* UFV-1 intracellular α-galactosidase and compared that with the extracellular enzyme.

MATERIALS AND METHODS

Yeast Strain and Culture Conditions. The fungal strain used was *Debaryomyces hansenii* UFV-1. It was isolated from a dairy plant and kept in the collection of the Departamento de Microbiologia, Universidade Federal de Viçosa (Viçosa, MG, Brasil). The taxonomic identification was carried out by the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands). *D. hansenii* UFV-1 stored at −80 °C in glycerol and YPD medium (1% yeast extract, 2% peptone and 2% glucose) was streaked on YPD agar surface (1.5% agar) and kept in an incubation chamber for 36 h at 30 °C. The yeast was then activated in YPD liquid medium and incubated for 12−15 h, 200 rpm at 30 °C. An aliquot of the cells obtained after centrifugation (4000*g* for 5 min at 4 °C) was transferred to one liter of mineral medium (*11*) toward OD_{600nm} 0.1 (0.053 mg/mL). After incubation for 31 h, 200 rpm, at 30 °C, the biomass was separated by centrifugation (4000*g* for 5 min at 4 °C) and frozen (−20 °C) for enzyme extraction.

Extraction of Intracellular Enzyme. *D. hansenii* UFV-1 cells (15 g) were ground with liquid nitrogen and resuspended in 40 mL of 0.1 M sodium acetate buffer, pH 5.0, containing 0.25% (by weight) of Triton X-100. This mixture was submitted to a series of nitrogen freezing and thawing at 40 °C in a water bath. It was then submitted to an ultrasonic bath (Branson, USA) for 10 min, and it was centrifuged (25900*g* for 30 min at 4 °C). The supernatant was used as the source of intracellular enzyme.

Purification of Intracellular α-Galactosidase. The enzymatic extract was kept at −20 °C for 24 h (cryoprecipitation) and then thawed, and the precipitate was removed by centrifugation (25900*g* for 30 min at 4 °C). After this procedure, the supernatant was loaded onto a Sephadex G-150 size exclusion column (87.5 cm × 2.5 cm), equilibrated with 25 mM sodium acetate buffer, pH 5.5 at 4 °C. The proteins were eluted at a flow rate of 20 mL/h, and 3.5 mL fractions were collected. Fractions containing α-galactosidase activity were pooled and loaded onto a DEAE-Sepharose anion exchange column (14.5 cm × 1.9 cm), equilibrated with 0.1 M sodium acetate buffer, pH 5.5 at 4 °C. Elution was performed at the flow rate of 40 mL/h, with a linear gradient formed with 150 mL of 0.1 M sodium acetate buffer and 150 mL of the same buffer containing 1.0 M NaCl. The active fractions were pooled and analyzed for purity by SDS−PAGE.

Enzyme Assay. The activity of α-galactosidase was assayed by measuring the amount of *p*-nitrophenol (*p*NP) released from the hydrolysis of *p*-nitrophenyl-α-D-galactopyranoside (*p*NPαGal) as substrate. The standard reaction mixture contained 2 mM *p*NPαGal, 0.1 M sodium acetate buffer, pH 5.0, and the enzyme preparation (0.35 μg protein/mL) in a final volume of 1.0 mL. After incubation at 55 °C or at 40 °C for the purification assays, for 15 min, 1.0 mL of 0.5 M sodium carbonate was added to the mixture to stop the reaction. The absorbance of the mixture was then measured at 410 nm. The amount of *p*NP released was calculated according to the standard curve (0−0.16 μmol of *p*NP).

The activities against maltose, gentiobiose, lactose, and melibiose were determined by the glucose-oxidase method (*15*). The production of reducing sugar was determined using the 3,5-dinitrosalicylate reagent (*16*), when sucrose, raffinose, and stachyose were used as substrates.

One unit of enzyme activity (U) was defined as the amount of enzyme that releases 1.0 μmol of product per min under the assay conditions.

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

Protein Determination. Protein concentration in the enzymatic extracts was determined by the BCA (bicinchoninic acid) method (*17*) with bovine serum albumin (BSA) as the standard.

Molecular Mass Determination. The molecular mass (M_r) of the purified enzyme was estimated by SDS−PAGE using a 12.5% polyacrylamide gel (*18*). Molecular mass standards from Sigma Chemical Co. (St. Louis, MO, USA) were bovine serum albumin (66 kDa), egg albumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhidrase (29 kDa), trypsinogen (24 kDa), and trypsin inhibitor (20.1 kDa). After electrophoresis, the protein was visualized by silver staining (*19*). The molecular mass of the native enzymes was determined using MALDI-TOF mass spectrometry where chromatographic fractions were dissolved in a saturated α-cyano-4-hydroxycinnamic acid matrix solution 1:3 (w:w), spotted onto a MALDI target plate and dried at room temperature for 15 min. The average molecular mass was obtained in an AUTOFLEX III (Bruker) in linear positive mode, with external calibration, using the Protein Calibration Standard II for Mass Spectrometry calibration molecules: trypsinogen $[M + H]^+ = 23{,}982$, protein A $[M + H]^+ = 44{,}613$, albumin-bovine $[M + H]^+ = 66{,}431$, protein A $[M + 2H]^{2+} = 22{,}306$, albumin-bovine $[M + 2H]^{2+} = 33{,}216$ (Bruker Daltonics). The Flex Analysis Software (Bruker Daltonics) was used to interpret mass spectra.

Amino Acids Analysis of *D. hansenii* UFV-1 α-Galactosidases. Triplicate samples of purified *D. hansenii* UFV-1 intracellular and extracellular α-galactosidases were hydrolyzed with 6 M HCl plus 0.1% (v:v) phenol in vapor phase hydrolysis during 22 h at 110 °C, and the amino acids were reacted with phenylisothiocyanate to produce phenylthiocarbamyl derivatives. Amino acid standard (Pierce, Co, Rockford, IL) and hydrolysis products were analyzed by RP-HPLC after derivatization (*20*).

Chromatofocusing. The α-galactosidase isoelectric point (p*I*) was estimated by chromatofocusing. Previously, the enzyme was dialyzed against start buffer, which contained 25 mM Bis-Tris/HCl, pH 6.0 (Amersham Pharmacia Biotech, Uppsala, Sweden). Around 100 μg of protein was applied onto a chromatofocusing chromatography Mono P HR 5/5 column (FPLC, Amersham Pharmacia Biotech) equilibrated with start buffer, pH 6.0. Elution was performed with 10% (v/v) Polybuffer 74, pH 4.0 (Amersham Pharmacia Biotech), using a flow rate of 30 mL/h. The pH measurement of eluted fractions was performed by a potentiometer coupled to FPLC apparatus and using an isolated pH electrode. Ovalbumin (200 μg/mL) was used as a standard (p*I* of 4.5).

Total Carbohydrate Determination. The carbohydrate content in the purified α-galactosidases was estimated using the phenol−sulfuric acid procedure (*21*). A glucose standard curve was plotted for each assay with glucose concentrations varying up to 70 μg/mL.

α-Galactosidase Deglycosylation. Deglycosylation of *D. hansenii* UFV-1 extracellular and intracellular α-galactosidases was performed using a N-Glycosidase F Deglycosylation Kit (New England Biolabs, Beverly, MA). Protein (200 μg) was incubated with 90 μL of 10× diluted denaturing buffer (5% SDS and 10% 2-mercaptoethanol) at 100 °C for 10 min. After denaturation, 10 μL of 10× concentrated 50 mM sodium phosphate buffer, pH 7.5, 10 μL of 10% Nonidet P40 and 2 μL of PNGase F (500,000 U/mL) purified of *Flavobacterium meningosepticu* were added. The reaction mixture was incubated at 37 °C for 31 h. Reaction products were separated by SDS−PAGE and visualized by silver staining.

N-Terminal Amino Acid Sequencing. The amino acid residue sequence of the intracellular α-galactosidase was determined by Edman degradation in a automated protein sequencer (Shimadzu PPSQ-21A, Shimadzu, Kyoto, Japan) coupled to a reverse-phase separation of PTH-amino acids in a WAKOSIL-PTH (4.6 × 250 mm) column (Wako, Osaka, Japan) at a flow rate 1.0 mL/min, with detection at 235 nm. Using the BLAST-P software, the obtained sequence was compared to known protein sequences.

Effect of pH and Temperature on Intracellular α-Galactosidase Activity. The influence of pH on α-galactosidase activity was determined within the pH range from 3.0 to 8.0 by using the McIlvaine

buffer (citric acid/sodium phosphate) at 55 °C (*22*). The effect of the temperature on the enzyme activity on *p*NPαGal was determined in 0.1 M sodium acetate buffer, pH 5.0, at a temperature range of 25−80 °C. For determination of α-galactosidase thermal stability, the enzyme was preincubated at various temperatures (40, 55 and 65 °C) in 0.1 M sodium acetate buffer, pH 5.0, for 0−48 h. After incubation, 2 mM *p*NPαGal was added, and the remaining activity was measured by the standard method. Results of the analyses are presented as the mean ± SD for three measurements.

Substrate Specificity. The enzyme was tested for its ability to hydrolyze 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 (0.35 μg protein/mL), and 250 μL of synthetic substrates (2 mM), or lactose, maltose, gentiobiose, stachyose, and sucrose (4 mM), or raffinose (30 mM), or melibiose (2 mM), or locust bean gum and guar gum solutions (1%). The following synthetic substrates were employed: *p*NPβGal, 4-nitrophenyl-β-D-galactopyranoside; *p*NPαGlc, 4-nitrophenyl-α-D-glucopiranoside; *p*NPβX, 4-nitrophenyl-β-D-xylopyranoside; *p*NPαA, 4-nitrophenyl-α-D-arabinopyranoside; *p*NPαM, 2-nitrophenyl-α-D-mannopyranoside; *o*NPβGlc, 2-nitrophenyl-β-D-glucopyranoside; *o*NPβGal, 2-nitrophenyl-β-D-galactopyranoside. The activities were measured under standard assay conditions at 55 °C. The data presented for all enzyme activity determinations are mean values ± SD of three measurements.

Effects of Several Compounds on Intracellular α-Galactosidase Activity. The effect of ions, simple sugars, and reducing agents on α-galactosidase activity was assayed by following the hydrolysis of *p*NPαGal. Enzyme samples were preincubated with each compound (10 mM) in 0.1 M sodium acetate buffer, pH 5.0, for 20 min at 55 °C. The data presented for all enzyme activity determinations are mean values ± SD of triplicate assays.

Kinetic Properties. The intracellular α-galactosidase Michaelis−Menten constant (K_m) for substrate hydrolysis was calculated by nonlinear curve fitting of the data to the Michaelis−Menten plot. The substrate concentrations were 0.05 to 1.0 mM for *p*NPαGal; 5.0 to 120 mM for raffinose; 2.5 to 30 mM for stachyose, and 0.15 to 40 mM for melibiose. The α-galactosidase catalytic constants (k_{cat}), using *p*NPαGal substrate concentrations ranging from 0.4 to 2.4 mM, were calculated by the direct linear method with Hanes−Woolf plots.

The inhibition constant (K_i) for galactose was calculated by the Dixon plot by using *p*NPαGal as substrate at the following concentrations: 0.05 to 1.0 mM. The concentrations of galactose were 0.5, 1.0, and 2.0 mM.

Treatment of Soy Milk with Intracellular α-Galactosidase. Soy milk prepared from dry seeds (5 mL) was incubated with either water or an aqueous solution of 10.5 U of purified α-galactosidase for 0, 2, 4, and 6 h under shaking (100 rpm) at 55 °C. Each reaction mixture was dried, and the soluble sugars were extracted from 20−30 mg of lyophilized samples with organic solvents (*23*). The solvent was evaporated at 50 °C and the sugars resuspended in 1.0 mL of 80% ethanol. The sugars were analyzed by HPLC system on a Shimadzu series 10A chromatograph using an analytical column [aminopropyl (−NH_2)] eluted with an acetonitrile−water isocratic mixture (80:20 v:v) at 35 °C, at a flow rate of 1.0 mL/min. Individual sugars were automatically identified and quantified by comparison with retention times and known concentrations of standard sugars. Gentiobiose was used as an internal standard as it does not interfere with the other sugars and it is not found in soybean seeds.

RESULTS AND DISCUSSION

Production and Purification of Intracellular α-Galactosidase. *Debaryomyces hansenii* UFV-1 grown in liquid medium containing galactose as a carbon source produced both extracellular (0.9 U/mL) and intracellular (1.5 U/mL) α-galactosidases. The highest specific activity of extracellular α-galactosidase was observed after 31 h of cultivation (11.9 U/mg protein). At this time, the dry cell mass was 3.4 mg/mL and the specific activity of intracellular α-galactosidase was 0.51 U/mg cell and 0.9 U/mg protein. Therefore, the bioprocess was terminated after 31 h of cultivation. On the other hand, β-galactosidase and invertase presented only intracellular activity (data not shown). Several strains of yeasts are described to produce both intra- and extracellular α-galactosidases (*24*). *Saccharomyces carlsbergensis* contains a soluble α-galactosidase localized inside the cell and an inducible form which is mainly secreted (*25*). *D. hansenii* UFV-1 extracellular α-galactosidase has been purified and partially characterized (*11*). To investigate possible differences between *D. hansenii* UFV-1 internal and external α-galactosidases, the intracellular enzyme was compared to the extracellular one. The purification procedure for intracellular α-galactosidase is summarized in the **Table 1**. The specific activity of the enzyme increased from an average of 0.91 mmol L^{-1} min^{-1} mg^{-1} of protein in the crude extract to 263 mmol L^{-1} min^{-1} mg^{-1} after DEAE-Sepharose anion exchange column. Thus, the resultant purification factor was 289 times with 45% overall yield.

Table 1. Summary of the Purification Steps of *D. hansenii* UFV-1 Intracellular α-Galactosidase

purification step	protein (mg)	total activity[a] (U)	specific activity (U/mg)	purification factor	recovery (%)
crude extract	64.5	59.0	0.91	1.00	100
cryoprecipitation	58.3	54.7	0.94	1.03	93
Sephadex G-150	1.24	46.7	37.7	41.4	79
DEAE-Sepharose	0.10	26.3	263.0	289.0	45

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

Molecular Mass and Isoelectric Point. The protein elution profile and the corresponding enzyme activities are shown in **Figure 1**. The electrophoretic profile of the purified intracellular α-galactosidase in SDS−PAGE confirmed the presence of a single protein band, with an estimated molecular mass of 61 kDa (**Figure 2**, lane 4). Using MALDI-TOF mass spectrometry, the intracellular enzyme showed molecular mass of 54.84 ± 0.05 kDa. Molecular mass of approximately 60 kDa was reported for extracellular α-galactosidase from the same yeast using SDS−PAGE (*11*) and 54.50 ± 0.05 kDa determined by MALDI-TOF mass spectrometry. The molecular mass values estimated by SDS−PAGE from deglycosylated extracellular and intracellular enzymes were 36 and 40 kDa, respectively (**Figure 2**, lanes 3 and 5).

The isoelectric point (p*I*) of *D. hansenii* UFV-1 intracellular α-galactosidase was smaller than the value obtained for the extracellular enzyme (4.15 vs 5.15). The p*I* values of *D. hansenii* UFV-1 α-galactosidases are in the common range (3.6−6.4), for α-galactosidases from widely different sources (*26*).

Carbohydrate Content, Amino Acid Composition and *N*-Terminal Sequence of α-Galactosidases. The carbohydrate content of the *D. hansenii* UFV-1 intracellular enzyme quantified by phenol−sulfuric acid procedure was smaller (34%) when compared to the value obtained for the extracellular α-galactosidase (40%) from the same yeast (*11*), from *Humicola* sp. (8.3%) (*8*), and from *Thermomyces lanuginosus* CBS 395.62/b (5.3%) (*27*).

Amino acid analysis from both *D. hansenii* UFV-1 intracellular and extracellular α-galactosidases showed few variations in their contents (**Table 2**). *D. hansenii* UFV-1 intracellular α-galactosidase had the *N*-terminal amino acid sequence YENGLNLVPQMGWNSWNKFGXHI where X is most probably a Cys residue. Comparison of its *N*-terminal amino acid sequence with amino acid sequences from other α-galactosidases is given in **Table 3**. The *N*-terminal amino acid sequence of *D. hansenii* UFV-1 intracellular α-galactosidase was identical to

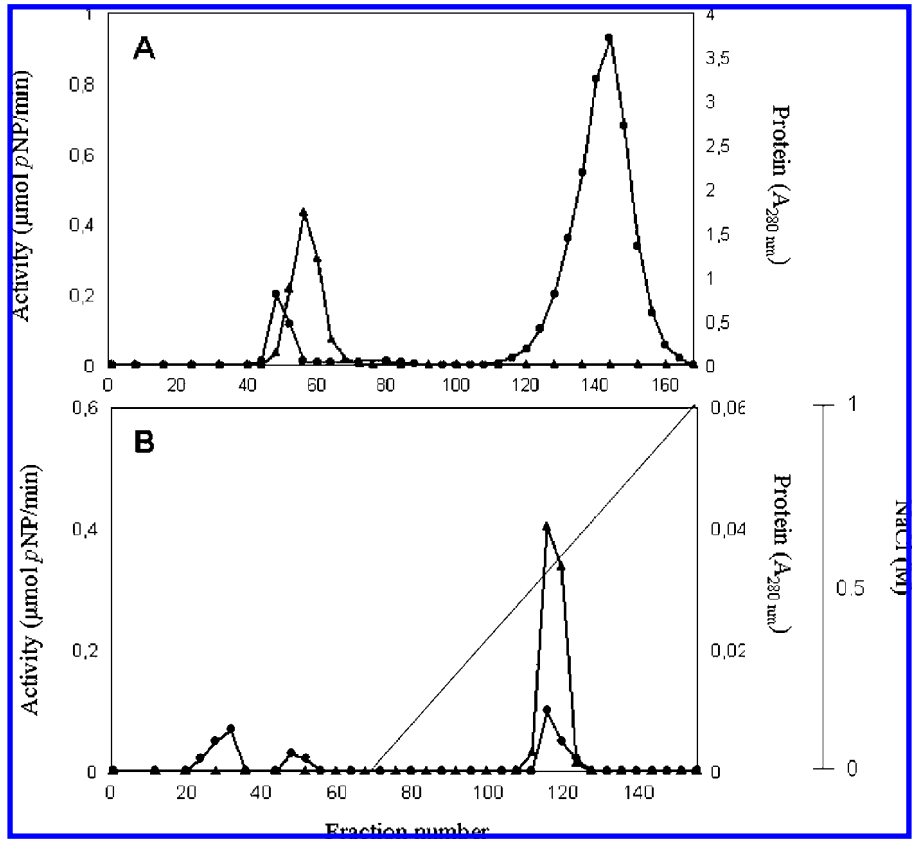

Figure 1. Elution profile of the intracellular α-galactosidase from *D. hansenii* UFV-1 on a Sephadex G-150 column (**A**) and DEAE-Sepharose column (**B**). α-Galactosidase activity (▲); protein (●) and NaCl gradient (—).

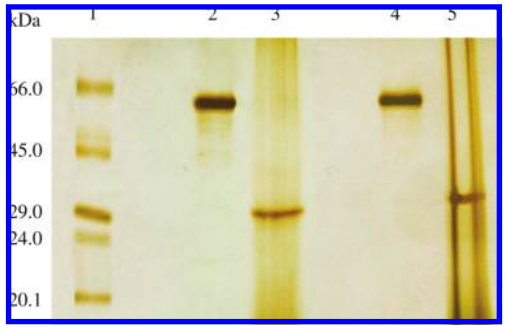

Figure 2. SDS−PAGE (12.5%) of purified and deglycosylated *D. hansenii* UFV-1 α-galactosidases. Lane 1: molecular mass standards. Lane 2: native extracellular enzyme. Lane 3: deglycosylated extracellular enzyme. Lane 4: native intracellular enzyme. Lane 5: deglycosylated intracellular enzyme. Protein gel was stained with silver nitrate (*19*).

the sequence of the extracellular enzyme (*11*). The alignment of the *N*-terminal amino acid sequence of *D. hansenii* UFV-1 α-galactosidase (23 amino acid residues) with the sequence of *D. hansenii* CBS767 α-galactosidase showed five nonconservative changes (*28*). However, the N and V residues presented in *D. hansenii* UFV-1 α-galactosidase were conserved in other microbial α-galactosidases, as observed in the sequence of the *Magnaporthe grisea* 70-15 α-galactosidase (*29*). The K and I residues were conserved in the sequence of the *Schizosaccharomyces pombe* (*30*) (**Table 3**). The high similarity shown by the α-galactosidases presented in **Table 3**, which are classified as glycosyl hydrolase family 27 members, suggests that *D. hansenii* UFV-1 α-galactosidases also belongs to this family.

There are reports of production and characterization of both intracellular and extracellular fungi α-galactosidases, such as those from *Aspergillus nidulans* (*37*) and *Aspergillus tamarii*

Table 2. Amino Acid Composition of *D. hansenii* UFV-1 α-Galactosidases[a]

amino acid	intracellular mol/mol	extracellular mol/mol
Asx	63.6	48.8
Glx	42.8	44.2
Ser	30.2	38.3
Gly	37.1	44.3
His	6.1	12.2
Arg	10.4	10.5
Thr	22.3	18.8
Ala	20.2	22.7
Pro	13.5	12.4
Tyr	15.3	9.9
Val	20.2	15.9
Met	4.1	3.2
Cys	nd	nd
Ile	21.9	16.2
Leu	23.7	17.5
Phe	12.9	9.5
Lys	22.1	15.4
total	366.5	339.7

[a] Cys was not detected (nd); Trp was not determined due to hydrolysis instability; mol/mol was calculated as 36 kDa for extracellular and 40 kDa for intracellular α-galactosidase; Asx and Glx correspond to sum of aspartic acid/asparagine and glutamic acid/glutamine; average standard deviation for triplicate analysis was less than 8%.

(*38*), showing similar or different enzymatic properties. Evidence suggests that intracellular and extracellular α-galactosidases from *D. hansenii* UFV-1 could be products of a single gene, because they have similar molecular mass and their *N*-terminal amino acid sequences are identical. However, there is a difference in the carbohydrate content between the intracellular (34% by mass) and extracellular (40% by mass) forms. These data suggest that *D. hansenii* UFV-1 α-galactosidases could exist as two glycoforms. The glycosylation level of a protein can

Table 3. Sequence Alignment of *D. hansenii* UFV-1 Intracellular α-Galactosidase with Other Microbial α-Galactosidases[a]

organism		sequence		reference
Debaryomyces hansenii UFV-1		**YENGLNLVPQMGWNSWNKFGXHI**		this study
Debaryomyces hansenii CBS767	21	**YENGL**GL**TPQMGWNSWN**IY**GC**DI	43	*28*
Magnaporthe grisea 70-15	36	**NGLNLVPQMGWNN**W**N**AYH**C**DV	56	*29*
Schizosaccharomyces pombe	27	**NGL**GLK**PQMGWNSWNK**YA**C**DI	47	*30*
Zygosaccharomyces mrakii	24	**NGL**GL**TPQMGWNN**W**NTFAC**NV	44	*31*
Saccharomyces cerevisiae	24	**NGL**GL**TPQMGW**D**SWNTFAC**DV	35	*32*
Saccharomyces mikcatae	24	**NGL**GL**TPQMGW**DN**WNTFACDV**	44	*33*
Emericella nidulans	26	DD**GL**ART**PQMGWN**TYN**Q**YN**C**	45	*34*
Aspergillus oryzae	24	D**G**VGRL**PAL**G**WNTWNAFGC**DI	44	*35*
Aspergillus fumigatus Af293	267	D**NGL**ART**PQMGWNS**YN**Y**YS**C**	286	*36*

[a] Conserved amino acid residues in all sequences are shown in bold. "X" is most probably a Cys residue.

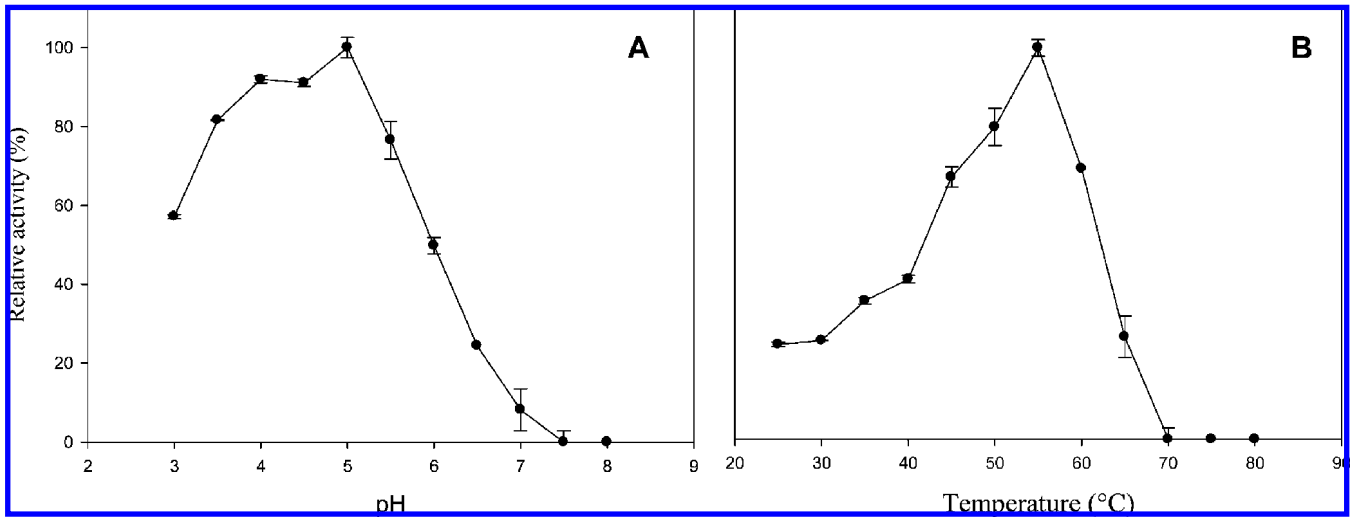

Figure 3. pH (**A**) and temperature (**B**) effects on the activity of the *D. hansenii* UFV-1 intracellular α-galactosidase.

affect its structure, stability and movement through the secretory pathways. Thermostability and degree of glycosylation are two closely linked factors in a protein (*39*). The *D. hansenii* UFV-1 intracellular α-galactosidase showed a smaller degree of glycosylation and smaller thermostability compared to the extracellular enzyme. The higher glycosylation level of extracellular enzyme could also be an important factor for its secretion.

Effect of pH and Temperature on Intracellular α-Galactosidase Activity. Substantial activity against *p*NPαGal was observed for intracellular enzyme preparation within a temperature range of 45−60 °C and pH range of 3.5−5.5 (**Figure 3**). The enzyme showed an optimum pH of 5.0 (**Figure 3A**) and an optimum temperature of 55 °C (**Figure 3B**). The slightly acidic pH optimum of α-galactosidase (4.6−6.2) is typical of some fungal glycosyl hydrolases. *D. hansenii* UFV-1 intracellular α-galactosidase kept about 80% of its original activity after incubation for 8 h at 40 °C, but only 30% of its activity remained after incubation for 41 h at that temperature (**Figure 4**). At 55 °C, the enzyme maintained 85 and 40% of its original activity after preincubation for 7 and 25 h, respectively. The half-life ($t_{1/2}$) values of *D. hansenii* UFV-1 intracellular α-galactosidase at 40, 55 and 65 °C were 39 h, 31 h, and 38 min, respectively. Similar results have been shown for *Humicola* sp. α-galactosidase (*8*), and higher $t_{1/2}$ values were determined for the *D. hansenii* UFV-1 extracellular α-galactosidase at 65 and 70 °C, which were 3 h and 35 min, respectively. The thermostability of the enzymes is an important parameter for biotechnological applications. The stability exhibited by *D. hansenii* UFV-1 α-galactosidases in a broad range of temperatures indicates that these enzymes could be used for industrial applications, especially for processing of GO in soybean products.

Substrate Specificity. The substrate specificities of *D. hansenii* UFV-1 intracellular α-galactosidase were investigated by using synthetic substrates, galactose-containing oligosaccharides, and polymers. Under the experimental conditions, the enzyme was highly selective, showing absolute specificity for α-galactosyl bond. This enzyme hydrolyzed *p*NPαGal (2.49 U/mL ± 0.03) but showed no activity for other synthetic substrates such as glucosidic derivatives or for β-linked glycosides. These results indicate that the configurations at C1 and C4 atoms of the substrates are very important for their interaction with the catalytic site. When assayed against natural substrates, the enzyme hydrolyzed α-galactosyl linkage in the oligosaccharides, and showed highest activity for stachyose (3.19 U/mL ± 0.01) followed by raffinose (2.28 U/mL ± 0.01). The enzyme was able to hydrolyze polymers, such as locust bean gum and guar gum, 0.55 U/mL ± 0.01 and 0.55 U/mL ± 0.02, respectively, suggesting its potential industrial application for modification of gelling and rheological properties of the polysaccharides. Our results demonstrated that *D. hansenii* UFV-1 α-galactosidases can hydrolyze exclusively α-D-galactosides in natural, synthetic, and polymeric substrates (*11*).

Effects of Several Compounds on Intracellular α-Galactosidase Activity. The effect of various mono- and divalent metal ions, monosaccharides, and reducing and nonreducing oligosaccharides on the activity of the *D. hansenii* UFV-1 intracellular α-galactosidase was examined under the standard enzyme assay conditions (**Figure 5**). The enzyme lost 70% of its activity in the presence of Ag(I) and became completely inactive in the presence of Cu(II). On the other hand, the activity of *Ganoderma lucidum* α-galactosidase (*40*) seemed to be slightly stimulated by Cu(II) and decreased to 32% of its original

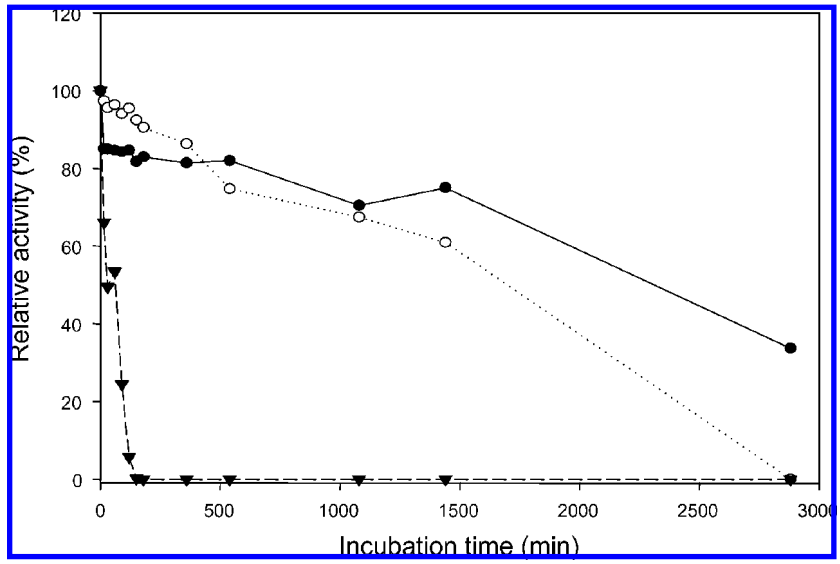

Figure 4. Effect of temperature on the stability of the *D. hansenii* UFV-1 intracellular α-galactosidase. Enzyme preparations were preincubated for 48 h at 40 °C (●); 55 °C (○) and 65 °C (▼).

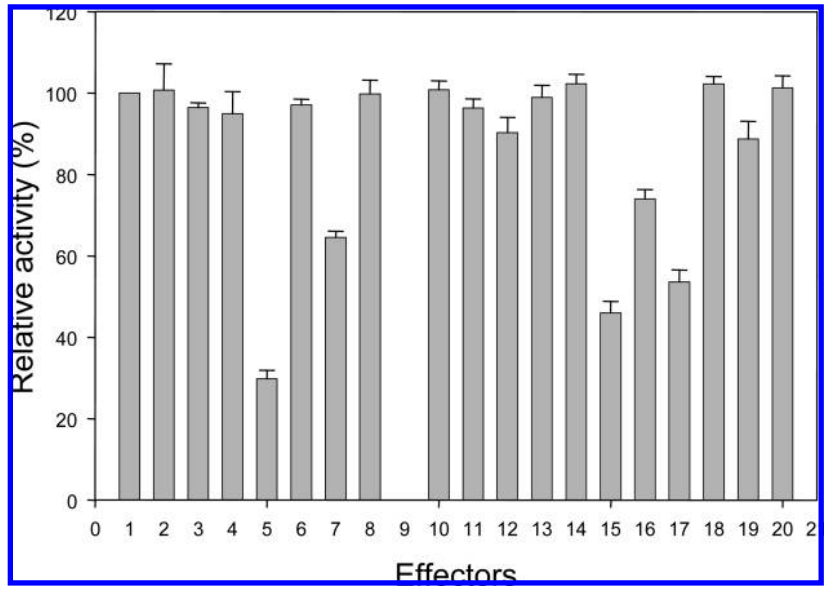

Figure 5. Effect of EDTA (2); $MgCl_2$ (3); iodoacetamide (4); $AgNO_3$ (5); NaCl (6); SDS (7); KCl (8); $CuSO_4$ (9); $CaCl_2$ (10); β-mercaptoethanol (11); raffinose (12); maltose (13); sucrose (14); melibiose (15); D-glucose (16); D-galactose (17); D-mannose (18); lactose (19); stachyose (20) on *D.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

activity by addition of Ag(I). The *D. hansenii* UFV-1 intracellular α-galactosidase activity presented very low or no inhibition by EDTA, Mg(II), iodoacetamide, Na(I), K(I), Ca(II), β-mercaptoethanol, raffinose, maltose, sucrose, D-glucose, lactose, stachyose, and D-mannose. The enzyme activity against *p*NPαGal was partially decreased by D-galactose and by melibiose (**Figure 5**). D-Galactose inhibited the *D. hansenii* UFV-1 intracellular enzyme acompetitively, and the K_i value determined by the Dixon plot was 0.70 mM. On the one hand, competitive inhibition by the same substrate has been reported for *Candida javanica* α-galactosidase (*41*). On the other hand, the *D. hansenii* UFV-1 extracellular enzyme showed noncompetitive inhibition by galactose (*11*).

Kinetic Properties. The lowest K_m value determined for all the substrates assayed was obtained for *p*NPαGal (0.32 mM). On the other hand, for the natural substrates, the lowest K_m value was determined for the hydrolysis of melibiose (2.12 mM). The smaller K_m value for stachyose (10.8 mM) compared to the value for raffinose (32.8 mM) indicates that the *D. hansenii* UFV-1 intracellular α-galactosidase has a relatively large substrate site (**Table 4**). This is very interesting for industrial purposes as the amount of stachyose in soybean products is higher than that of raffinose. Activities on aryl α-D-galactosides as well as on melibiose, raffinose, and stachyose are exhibited by several α-galactosidases, but most of these enzymes present a higher affinity for the trisaccharide raffinose than for the tetrasaccharide stachyose (*42*). Also, the K_m values calculated for the *D. hansenii* UFV-1 extracellular α-galactosidase (*11*) with the same substrates (melibiose, raffinose and stachyose) were close to

Table 4. K_m, k_{cat} and k_{cat}/K_m Values Determined for *D. hansenii* UFV-1 Intracellular α-Galactosidase

substrates	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM $s)^{-1}$
*p*NPαGal	0.32	3.29	10.28
melibiose	2.12	0.03	0.01
stachyose	10.8	6.05	0.56
raffinose	32.8	4.12	0.13

Table 5. Hydrolysis of Oligosaccharides Present in Soy Milk by Intracellular α-Galactosidase from *D. hansenii* UFV-1

	content (%) ± SD	
incubation time (h)	raffinose	stachyose
0	1.50 ± 0.02	3.30 ± 0.01
2	0.56 ± 0.03	0.0
4	1.08 ± 0.01	0.0
6	0.41 ± 0.02	0.0

the values determined in this work for the intracellular enzyme (**Table 4**). The K_m values of *Humicola* sp. intracellular α-galactosidase, determined for *p*NPαGal, raffinose, and stachyose were 0.28, 1.45 and 1.42 mM, respectively (*8*). The *D. hansenii* UFV-1 intracellular α-galactosidase presented smaller k_{cat} value for *p*NPαGal when compared to the extracellular enzyme (3.29 vs 7.16 s^{-1}) as calculated by Hanes−Woolf plot.

Hydrolysis of Oligosaccharides in Soy Milk by Intracellular α-Galactosidase. The hydrolysis of galacto-oligosaccharides (GO) by *D. hansenii* UFV-1 intracellular α-galactosidase in soy milk is displayed in **Table 5**. The reaction mixture initially contained sucrose, raffinose, and stachyose at the concentrations 3.35, 1.50, and 3.30% (w:v), respectively (**Table 5**). After 2 h of incubation with the enzyme, a reduction of 63 and 100% was observed in the amounts of raffinose and stachyose, respectively. After incubation for 4 and 6 h, the amount of raffinose was reduced by 28 and 73%, respectively, while the sucrose concentration rose to 6.74% (**Table 5**). No oligosaccharide hydrolysis was detected in control tubes without enzyme. The ability of the enzyme to hydrolyze stachyose and raffinose is of particular interest for biotechnological applications. The high ability of *D. hansenii* UFV-1 intracellular α-galactosidase to hydrolyze stachyose is a desirable feature for industrial purposes as the concentration of this sugar in soy derived products is higher than that of raffinose. As the enzyme preparation showed no invertase activity, our results indicate that *D. hansenii* UFV-1 intracellular α-galactosidase acts on GO present in soy milk. Many α-galactosidases from microorganisms have been used to degrade GO in soy milk (*8, 43*). However, the potential enzymes suggested for this purpose are generally produced by microorganisms that do not have the GRAS (generally recognized as safe) status. *Debaryomyces hansenii* is a yeast species frequently found in protein-rich fermented products, such as sausages and cheeses, where it contributes to the development of special flavors of those products (*44*). There should be no restriction regarding safeness for the use of this microorganism in food processing. The high yield of *D. hansenii* UFV-1 intracellular α-galactosidase, its acid optimum pH and its acceptable heat stability may prove useful in industrial applications for degradation of GO from soy milk and from other soy products. The possibility of a combined use of both *D. hansenii* UFV-1 intracellular and extracellular α-galactosidases could enhance the enzyme yield, making more economically viable the process for GO hydrolysis in soybean products.

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