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Purification and Characterization of *Aspergillus terreus* α-Galactosidases and Their Use for Hydrolysis of Soymilk Oligosaccharides

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Abstract α-Galactosidases has the potential to hydrolyze α-1-6 linkages in raffinose family oligosaccharides (RFO). *Aspergillus terreus* cells cultivated on wheat bran produced three extracellular forms of α-galactosidases (E1, E2, and E3). E1 and E2 α-galactosidases presented maximal activities at pH 5, while E3 α-galactosidase was more active at pH 5.5. The E1 and E2 enzymes showed stability for 6 h at pH 4–7. Maximal activities were determined at 60, 55, and 50°C, for E1, E2, and E3 α-galactosidase, respectively. E2 α-galactosidase retained 90% of its initial activity after 70 h at 50°C. The enzymes hydrolyzed ρNPGal, melibiose, raffinose and stachyose, and E1 and E2 enzymes were able to hydrolyze guar gum and locust bean gum substrates. E1 and E3 α-galactosidases were completely inhibited by Hg^{2+}, Ag^{+}, and Cu^{2+}. The treatment of RFO present in soy milk with the enzymes showed that E1 α-galactosidase reduced the stachyose content to zero after 12 h of reaction, while E2 promoted total hydrolysis of raffinose. The complete removal of the oligosaccharides in soy milk could be reached by synergistic action of both enzymes

Keywords α-Galactosidase · *Aspergillus terreus* · Raffinose · Stachyose · Soybean · Anti-nutritional factors

Introduction

α-Galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22) is an exo-acting enzyme that mainly cleaves α-1-6-linked D-galactosyl residues from a wide range of substrates, including linear and branched oligosaccharides, polysaccharides and synthetic substrates, such as ρ-nitrophenyl-α-D-galactopyranoside [1, 2]. A number of industrial applications of α-galactosidases are known, mainly in the sugar and food industry, pulp and paper industry

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or medicine. Despite their hydrolytic activity, α-galactosidases are also powerful tools for oligosaccharide synthesis by transglycosylation or reverse hydrolysis reactions [3].

Soybean and soy products contain raffinose family oligosaccharides (RFO), mainly raffinose, stachyose, and traces of verbascose. The consumption of soy derivatives is limited by the presence of RFO, which are considered anti-nutritional factors for human and monogastric animals. Since monogastric animals and human beings do not have the α-galactosidase enzyme that cleaves the α-1,6 galactosyl linkage in their digestive tract, the intact oligosaccharide is not absorbed. These oligosaccharides accumulate in the large intestine, where anaerobic microorganisms ferment them and lead to flatus formation [4].

Many attempts have been proposed to reduce the RFO contents present in soy products, such as dehulling, soaking, cooking, γ-irradiation, aqueous or alcoholic extraction, germination, and fermentation [5, 6]. However, the enzymatic processing of soy products using α-galactosidases seems to be the most rational and effective alternative to remove the RFO and improve the nutritional value of soy products [7].

Fungal α-galactosidases are the most suitable for technological applications mainly due to their acidic optima pH and broad stability profiles [7–9]. The most important industrial application of α-galactosidases is presently in the sugar-making industry [10]. Small amounts of raffinose and/or stachyose adversely affect the crystallization of sucrose. However, they can be easily converted into sucrose by treatment with α-galactosidases [11]. Fungi belonging to the genus *Aspergillus* are filamentous microorganisms that are able to secrete large amounts of various enzymes in their culture medium. Therefore, it is particularly used for the production of commercially important enzymes [12].

The aims of this work were to produce, purify and characterize three extracellular α-galactosidases from *Aspergillus terreus* and evaluate their biotechnological potential, especially to reduce the RFO present in soybean milk.

Materials and Methods

Materials

The substrates ρ-nitrophenyl-α-D-galactopyranoside (ρNPGal), m-nitrophenyl-α-D-galactopyranoside (mNP-α-Gal), ρ-nitrophenyl-β-D-galactopyranoside (ρNP-β-Gal), *o*-nitro phenyl-α-D-galactopyranoside (*o*NP-α-Gal), *o*-nitrophenyl-β-D-glucopyranoside (*o*NP-β-Glc), ρ-nitrophenyl-α-D-glucopyranoside (ρNP-α-Glc), ρ-nitrophenyl-β-D-glucopyranoside (ρNP-β-Glc), ρ-nitrophenyl-α-D-mannopyranoside (ρNP-α-Man), ρ-nitrophenyl-α-D-arabinopyranoside (ρNP-α-Ara), ρ-nitrophenyl-β-D-xylopyranoside (ρNP-β-Xyl), the carbohydrates D-galactose, D-manose, raffinose, stachyose, melibiose, locust bean gum, guar gum, ρ-nitrophenol (ρNP), and D(+)-Melibiose–Agarose Matrix resin were purchased from Sigma Chemical (St. Louis, MO, USA). The sugars sucrose, fructose, and D-glucose were purchased from Merck S.A. Indústrias Químicas (São Paulo, Brazil). The chromatography resins Sephacryl S-200, Sephacryl S-300, Phenyl Sepharose, DEAE-Sephacel, and Q-Sepharose were obtained from Amersham Biosciences (Uppsala, Sweden). All other chemicals used were of analytical grade.

Organism Growth and Enzyme Production

Aspergillus terreus CCT 4083 were obtained from André Toselo Tropical Research Foundation, Campinas, São Paulo, Brazil. The stock culture was maintained by serial

transfers on potato dextrose agar medium and kept at 4°C. Spores were transferred to liquid medium (final concentration of 10^7 mL^{-1}) containing (in g L^{-1}): KH_2PO_4, 7.0; K_2HPO_4, 2.0; $MgSO_4.7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; yeast extract, 0.6, and wheat bran, 10. The flasks were incubated at 28°C, 150 rpm for 10 days. Aliquots were collected in time intervals of 24 h. Mycelium was then removed by filtration using sterile Whatman filter paper and the filtrate containing α-galactosidase was kept at −20°C.

α-Galactosidase Purification

α-Galactosidases were purified from *A. terreus* grown in liquid media containing 1.0% (*w*/*v*) of wheat bran, according to the scheme shown in Fig. 1. Enzyme purification was carried out at 4°C and in all steps of the process the collected fractions were assayed for protein content (A280) and α-galactosidase activity. α-Galactosidases were purified by affinity chromatography on columns of Sephacryl-S-200, Q-Sepharose, Phenyl Sepharose, DEAE-Sephacel and D(+)-Melibiose–Agarose Matrix, besides preparative polyacrylamide gel electrophoresis (PAGE). The details of α-galactosidases purification are discussed under Results and Discussion.

Enzyme Assay and Protein Determination

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, and 250 μL of 2 mM ρNPGal or other synthetic substrates. The reaction was run for 15 min at 40°C and ended with the addition of 1 mL of 0.5 M sodium carbonate. The amount of ρNP released was determined at 410 nm. This procedure was defined as the standard assay. The activities over melibiose, maltose, and lactose were evaluated according to the glucose-oxidase method [13]. When sucrose, raffinose, stachyose, locust bean gum, and guar gum were used as substrate, the production of reducing sugars was determined using the 3,5-dinitrosalicylate reagent [14]. Except for the chromatographic experiment, the enzyme activity values presented are mean values of triplicate assays. Standard deviation values were always smaller than 10% of the

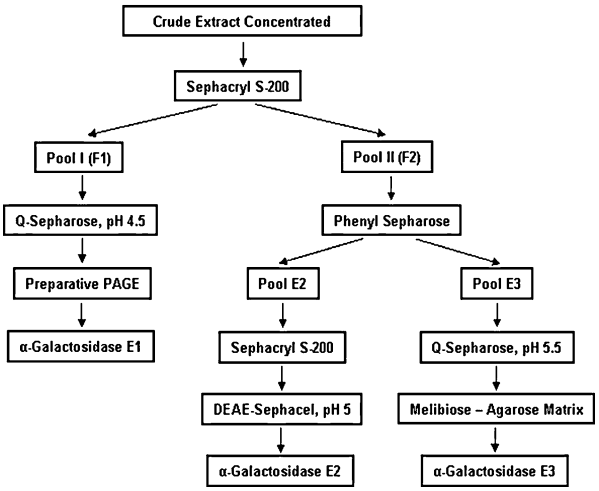

Fig. 1 Purification scheme of α-galactosidases from *Aspergillus terreus*

mean value. One enzyme unit (U) was defined as the amount of enzyme that releases 1 μmol of product per min under assay conditions.

The protein concentration in the enzymatic extracts was determined by the Coomassie blue binding method [15] with bovine serum albumin as a standard.

Determination of Molecular Mass

Enzyme preparations were analyzed by sodium dodecyl sulfate-PAGE (SDS-PAGE), 12.5% *w*/*v*, as described by Laemmli [16] and proteins were silver-stained according to Blum et al. [17]. The molecular mass standards (Amersham Biosciences, Uppsala, Sweden) were the following: 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). The molecular mass of native α-galactosidases from *A. terreus* was determined by elution from a Sephacryl S-300 column. The following proteins were used as molecular mass standards: ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), albumin (67 kDa), ovoalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa).

Effect of pH and Temperature

The effect of pH on α-galactosidase activities was determined within the pH range of 3.0–7.0 using McIlvaine buffer (citric acid/sodium phosphate) [18] at 40°C under otherwise standard enzyme assay conditions. The optimum temperature was determined within the temperature range of 35–70°C, at pH 5.0. Relative activities were calculated in relation to the maximal activity, which was considered to be 100%.

The pH stability of α-galactosidases was also determined by incubation of 300 μL of enzyme solution with 700 μL of the above mentioned buffer at 40°C for 6 h. After incubation, 100 μL of the mixture was used for the determination of the residual activity, according to the standard assay, using ρNPGal as substrate. The thermal stability was investigated by incubation of enzymatic aliquots in sodium acetate buffer 100 mM, pH 5.0 at several temperatures for different time periods. After incubation, 250 μL of 2.0 mM ρNPGal was added, and the remaining activity was measured.

Determination of Kinetic Parameters

Kinetic experiments were performed at pH 5.0 and 60, 55 and 50°C for α-galactosidases E1, E2, and E3, respectively. The parameters K_M and V_{max}, for ρNPGal, stachyose, raffinose, and melibiose hydrolysis were determined by using the Michaelis–Menten plot. The substrate concentrations ranged from 0.01 to 10.00 mM in the case of ρNPGal, from 1 to 250 mM for raffinose and stachyose and from 1 to 100 mM for melibiose. The inhibition constants (K_i) of the E2 and E3 α-galactosidases, using galactose as inhibitor were calculated by the Dixon plot. The ρNPGal concentrations ranged from 0.01 to 4.0 mM. The concentrations of galactose ranged from 0.75 to 3.0 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, 100 μL of enzyme solution, and 250 μL of solution containing the following substrates: ρNPGal,

mNP-α-Gal, ρNP-β-Gal, *o*NP-α-Gal, *o*NP-β-Glc, ρNP-α-Glc, ρNP-β-Glc, ρNP-α-Man, ρNP-α-Ara, ρNP-β-Xyl (at final concentration of 0.5 mM), raffinose, stachyose, melibiose, sucrose, maltose, lactose (at final concentration of 25 mM), locust bean gum, and guar gum (at final concentration of 0.5%). The activities were measured under standard assay conditions and the data presented for all enzyme activity determinations are mean values± SD of three measurements.

Effect of Ions, Simple Sugars, and Reducing Agents

The enzyme samples were pre-incubated with each of the compounds (2 mM) in 0.1 M sodium acetate buffer, pH 5.0, for 15 min at 40°C. After pre-incubation, the effect of ions, simple sugars, and reducing agents on the enzyme activity was determined according to the standard assay. Relative activities were calculated in relation to the control activity, which was considered to be 100%.

Treatment of Fat-Free Soybean Flour with α-Galactosidase

Commercial fat-free soybean flour (2 g) was mixed with water 1:10 (*w*/*v*). The suspension was added with 10 U of E1, E2, and E3 α-galactosidases purified from *A. terreus* (5 U of enzyme/g soybean flour). The mixtures were incubated for 2, 4, 6, 8, 10, and 12 h under agitation (100 rpm) at 40°C. The concentration of raffinose and stachyose were determined during hydrolysis reaction as described previously [19]. Each reaction mixture was lyophilized, and the soluble sugars were extracted from 30 mg of dried powder with 80% aqueous ethanol (*v*/*v*). The solvent was evaporated at 45°C, and the sugars were resuspended in 80% ethanol. The samples were analyzed by HPLC on a Shimadzu series 10A chromatograph (Kyoto, Japan), using the analytical column Supelcosil LC-NH_2 25 cm×4.6 mm (Supelco, Bellefonte, PA), eluted with an acetonitrile/water isocratic mixture (80:20 *v*/*v*) at 35°C, at a flow rate of 1 mL min^{-1}. The individual sugars were monitored by a refractive index detector model 6A from Shimadzu and automatically identified and quantified by comparison with the retention times and standard sugar concentrations.

Results and Discussion

The fungus *A. terreus* was cultivated in mineral media containing wheat bran as carbon source and the production of enzymes α-galactosidase was monitored for 240 h. Despite no α-galactosidase activity have been detected before 48 h of fermentation, wheat bran showed to be a good inducer for α-galactosidase production and a maximum activity of 0.875 U mL^{-1} was observed 168 h after inoculation and this time of fermentation was used to obtain of the enzymatic extract containing α-galactosidase activity. The purification scheme of the α-galactosidases from A. *terreus* is given in Fig. 1. The crude enzymatic extract was filtered to remove mycelia and others impurities and concentrated by an Amicon Ultrafiltration unit using YM-10 (cut-off Mr 10,000 Da) membrane filter. Samples of concentrated extract were loaded on a Sephacryl S-200 molecular exclusion column (87.5× 2.5 cm), previously equilibrated with 25 mM sodium acetate buffer, pH 5.5. The elution was carried out at 20 mL h^{-1} with the same buffer and the eluted fractions were assayed for α-galactosidase. It was observed two peaks containing enzymatic activity. The peaks were pooled and nominated F1 and F2, following the order of elution.

The first peak (F1) was applied to a Q-Sepharose column (10×2 cm) previously equilibrated with 50 mM sodium acetate buffer, pH 4.5. Elution was done at 40 mL h^{-1} with the same buffer until absorbance at 280 nm reached zero. The bound proteins were eluted with a gradient consisting of 200 mL 50 mM sodium acetate buffer, pH 4.5, and 200 mL of the same buffer containing 0.7 M NaCl. The fractions containing α-galactosidase activity were pooled, concentrated by ultrafiltration and submitted to a preparative polyacrylamide gel electrophoresis. In this step, 1 mL of enzymatic sample was mixed with 0.154 mL of Tris/HCl, 0.5 M pH 6.8 and 0.128 mL of glycerol. Bromophenol blue (0.025% w/v) was added as a tracking dye to trace the migration of samples on the gel. The electrophoresis was performed using 5% (*w*/*v*) stacking polyacrylamide gel and 12.5% (*w*/*v*) separating gel and run at 200 V for 8 h. At the end of electrophoresis, a piece of gel was gently cut, placed in a Petri dish and incubated in acetate buffer, 100 mM, pH 5, containing ρNPGal (4 mg mL^{-1}) until the appearance of yellow band which indicated the position of enzyme α-galactosidase in the gel. After detecting the position of enzyme in the gel, the fragment was realigned in original gel, and the correspondent region of the gel that contained the enzyme α-galactosidase was detached and the protein was eluted in sodium acetate buffer 100 mM, pH 5. The enzyme obtained after this process was denominated α-galactosidase E1, and it was purified at apparent homogeneity with a purification factor of 73.1-fold and a recovery of 7.01%.

Meanwhile, the second peak from Sephacryl S-200 chromatography (F2) was applied to a Phenyl Sepharose column (9.5×1.5 cm) previously equilibrated with 25 mM sodium acetate buffer, pH 5.5, containing 1 M ammonium sulfate. Elution was done at 50 mL h^{-1} with the same buffer until absorbance at 280 nm reached zero, followed by decreasing linear gradient of $(NH_4)_2SO_4$ (1–0 M) in the same buffer of equilibrium. Two different pools of fractions showing α-galactosidase activity were obtained after application of the gradient and they were pooled and nominated E2 and E3 following the order of elution.

The peak E2 was concentrated by ultrafiltration system (10 kDa mass cut-off membrane) and directly reloaded in a Sephacryl-S200 column (87.5×2.5 cm), at same conditions specified above. The fractions containing α-galactosidase activity was pooled and applied in a DEAE-Sephacel column (7.5×1.5 cm) previously equilibrated with 100 mM sodium acetate buffer, pH 5.0. Elution was done at 40 mL h^{-1} with the same buffer, followed by crescent linear gradient of NaCl (0–0.3 M) in the same buffer of equilibrium. A single peak of α-galactosidase activity eluted from DEAE-Sephacel column. This enzyme was denominated α-galactosidase E2 and it was purified at apparent homogeneity with a purification factor of 60.26-fold and a yield of 16.38%.

The peak E3 obtained after hydrophobic interaction chromatography was subjected in a Q-Sepharose column (10×2 cm) previously equilibrated with 50 mM sodium acetate buffer, pH 5.5. The column was washed with equilibrium buffer (flow of 40 mL h^{-1}) and the binding protein were eluted by a crescent linear gradient of NaCl (0–0.7 M) in the same buffer. The fractions containing α-galactosidase activity were pooled and then applied in an affinity column D(+)Melibiose–Agarose Matrix (8×1.5 cm) which was equilibrated with 50 mM sodium acetate buffer, pH 5.5. The column was thoroughly washed with equilibrium buffer and the enzyme α-galactosidase was eluted with the same buffer containing ρNPGal 4 mM. This procedure was done to obtain the enzyme α-galactosidase E3, and it allows a purification of 50.78-fold and a recovery of 7.31%.

A resume of all results obtained during purification process of α-galactosidases from *A. terreus* can be observed in Table 1. The α-galactosidases E1 and E2 were purified to apparent homogeneity (Fig. 2), however, enzyme E3 was partially purified once that a single protein band could not be observed in SDS-Page (data not shown). The molecular

Table 1 Summary of the results obtained during the purification process of the α-galactosidases E1, E3, and E3 from *Aspergillus terreus*

Purification step/Enzyme	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Recovery (%)	Purification factor
Crude extract	56.32	124.98	0.45	100.0	1.0
α-Galactosidase E1					
Sephacryl S-200	10.27	2.49	4.12	18.24	9.15
Q-Sepharose pH 4.5	7.19	0.83	8.46	12.76	18.80
Preparative Gel	3.95	0.12	32.92	7.01	73.15
α-Galactosidase E2					
Sephacryl S-200	33.77	13.18	2.56	59.96	5.69
Phenyl Sepharose	10.80	3.31	3.26	19.18	7.24
Sephacryl S-200	10.64	1.42	7.5	18.90	16.66
DEAE-Sephacel	9.22	0.34	7.12	16.38	60.26
α-Galactosidase E3					
Sephacryl S-200	33.77	13.18	2.56	59.96	5.69
Phenyl Sepharose	11.82	2.98	3.97	20.99	8.82
Q-Sepharose pH 5.5	7.16	0.54	13.25	12.71	29.44
Melibiose column	4.12	0.18	22.89	7.31	50.87

mass of the α-galactosidases E1 and E2, estimated by SDS-PAGE were 72.3 and 50 kDa, respectively (Fig. 2). The native molecular mass of E1 and E2 α-galactosidases, determined by gel filtration chromatography was 350.0 and 77.3 kDa, respectively. These results suggest that E2 α-galactosidase is a monomer in its native form, while E1 α-galactosidase could be a homopentamer. The native molecular mass of E3 α-galactosidase was 51.7 kDa.

Enzymes with α-galactosidase activity are produced by several organisms, often in multiple forms [20]. *Stretomyces griseoloalbus* produced three α-galactosidases, α-Gal I, α-Gal II and α-Gal III, presenting molecular masses of 72, 57 and 35 kDa, respectively [21]. The saprophytic fungi *Trichoderma reesei* has at least three genes that encode to three different enzymes α-galactosidases [22]. Multiples α-galactosidases are produced by *Aspergillus niger* [20, 23] and the expression of these enzymes can be directed by different carbon sources utilized in the culture media [24]. The molecular mass of α-galactosidases

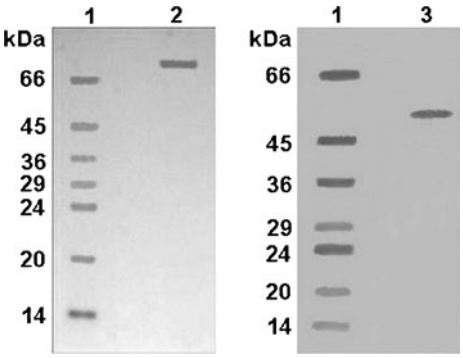

Fig. 2 SDS-PAGE (12.5%) of purified *Aspergillus terreus* α-galactosidases. MW marker (*line 1*); α-galactosidase E1 (*line 2*); α-galactosidase E2 (*line 3*)

produced by different microorganisms is a characteristic extremely diverse. It was demonstrated that extracellular α-galactosidases produced by *Thermomyces lanuginosus*, *Debaryomyces hansenii*, and *Pleorotus florida* are monomeric proteins with 57, 60, and 99 kDa, respectively [25–27]. On the other hand, α-galactosidases produced by *Penicillium* sp., *Umbelopsis vinacea* and *A. niger* were described as tetrameric proteins, composed by four identical subunits with 82, 54, and 94 kDa, respectively [20, 28, 29].

The E1, E2, and E3 α-galactosidase forms achieved maximal substrate hydrolysis at temperatures of 60, 55 and 50°C, respectively (Fig. 3).

The E1 and E2 α-galactosidases presented higher thermostability compared with E3 α-galactosidase. The E1 and E2 enzymes maintained 90% (Fig. 4a) and 80% (Fig. 4b) of their original activity after pre-incubation for 12 h at 55°C, respectively. However, E3 α-galactosidase retained only 5% of its activity when pre-incubated for 12 h under the same conditions (Fig. 4c). The thermoestability of enzymes is advantageous for industrial application, since most industrial processes are carried out at high temperatures, which lead to danaturation of thermolabile enzymes [21]. The E1 α-galactosidase was the most thermo tolerant enzyme from *A. terreus*, followed by E2 α-galactosidase.

A. terreus$_{GR}$ α-galactosidase presented optimum temperature at 65°C and stability for 40 min at this temperature [2]. α-Galactosidase from *Aspergillus fumigatus* [30], *Monascus pilosus* [31], and *Toluraspora delbrueckii* IFO 1255 [32] showed higher activity at 55°C. Differently, α-galactosidases from *Penicillium griseoroseum* and *Penicillium* sp. were more active at 45 and 40°C, respectively, and both enzymes were rapidly inactivated when incubated at temperatures above 50°C [7, 29].

A. terreus α-galactosidases showed to be acidic enzymes. The E1 and E2 α-galactosidases showed maximum activity at pH 5, while the optimum pH of the E3 enzyme was 5.5 (Fig. 5a). The E1 and E2 α-galactosidases were stable in a wide pH range and retained more than 90% of their original activity after 6 h of incubation at pH values from 4.0 to 7.0 (Fig. 5b). Contrariwise, the E3 enzyme maintained more than 90% of the residual activity only when incubated at pH range of 5.0–5.5. The acidic pH optimum of the *A. terreus* α-galactosidases is typical of some fungal glycosyl hydrolases, such as α-galactosidases from *A. niger* [20], *Rhizopus* sp. [33] and *Aspergillus oryzae* [34]. The α-galactosidase from white rot fungi *P. florida* was optimally active at pH 4.6–5.0 and the enzyme was stable at a pH range from 4.0 to 9.0 [26]. The α-galactosidase produced by the yeast *D. hansenii* had optimum pH of 5.0, and retained 90% of its residual activity after 30 min at pH range of 4.0–7.5 [27]. Bacterial α-galactosidases, such as α-galactosidases from

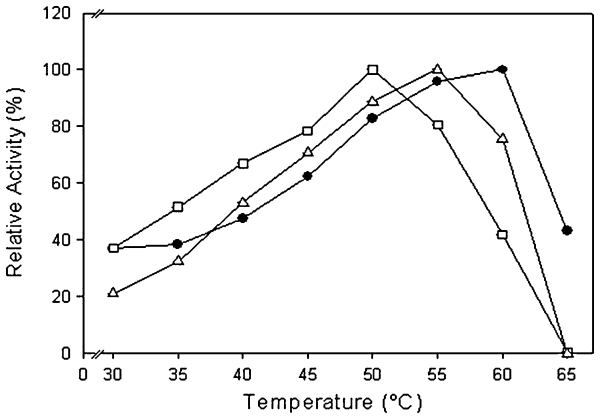

Fig. 3 Effect of temperature on the activity of α-galactosidases E1 (*black circles*). E2 (*white triangles*) and E3 (*white squares*) from *Aspergillus terreus*

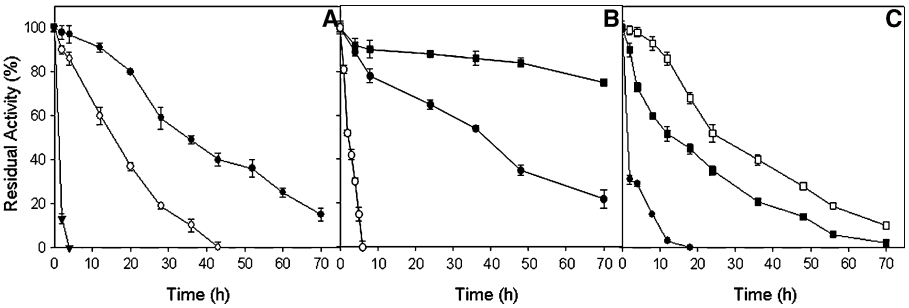

Fig. 4 Effects of different temperatures on the stability of the α-galactosidase E1 (**a**), E2 (**b**), and E3 (**c**) from *Aspergillus terreus*. Temperatures: 45°C (*white squares*); 50°C (*black squares*); 55°C (*black circles*); 60°C (*white circles*) and 65°C (*black down-pointing triangles*)

Thermoanaerobacterium polysaccharolyticum, *Escherichia coli*, *Bacillus stearothermophilus*, and *Thermotoga neapolitana* were optimally active at pH 8.0, 8.1, 7.0–7.5, and 7.0, respectively [35–38]. The stability exhibited by *A. terreus* E1 and E2 α-galactosidases in a broad range of temperatures and at pH 4.0–7.0 indicates that these enzymes could be used for industrial applications.

The substrate specificity of α-galactosidases from *A. terreus* was investigated by using oligosaccharides, polysaccharides and synthetic substrates (Table 2). The α-galactosidases efficiently hydrolyzed the synthetic substrate ρNPGal. The enzymes E1 and E3 scarcely hydrolyzed *o*NP-α-Gal, while mNP-α-Gal was slightly hydrolyzed by E2 α-galactosidase. The enzymes did not hydrolyze other synthetic substrates containing β-linkages or xilose, arabinose, mannose and glucose residues, suggesting that the enzymes are specific to hydrolyse residues of galactose α-linked. The E1, E2, and E3 enzymes hydrolyzed the natural substrates melibiose, raffinose and stachyose. Only the preparation containing E3 α-galactosidase showed ability to hydrolyze sucrose. This result indicated that this enzymatic preparation also contained invertase activity. Thus, the hydrolysis of raffinose and stachyose by E3 could be due to both α-galactosidase and invertase acitivities. All α-galactosidases from *A. terreus* were able to hydrolyze the polymeric substrates guar gum and locust bean gum (Table 2). Dey et al. [39] classified α-galactosidases

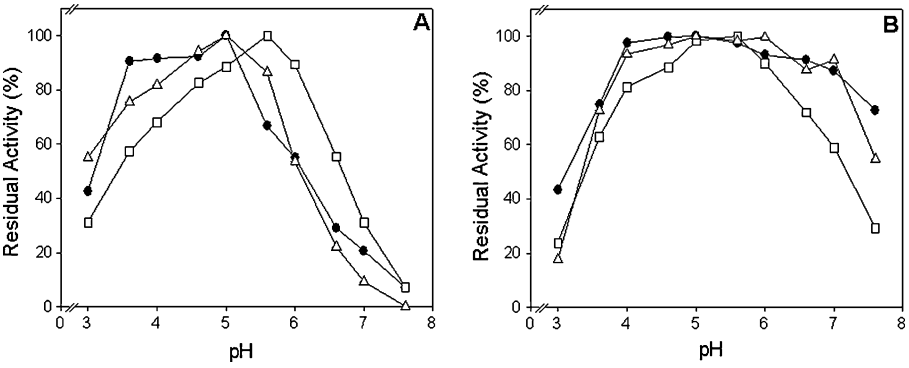

Fig. 5 Effect of pH on the activity (**a**) and stability (**b**) of α-galactosidases E1 (*black circles*). E2 (*white triangles*) and E3 (*white squares*) from *Aspergillus terreus*

Table 2 Hydrolysis of several substrates by α-galactosidases E1, E2, and E3 from *Aspergillus terreus*

		Activity (μM min^{-1})±SD		
Substrate	Concentration	E1	E2	E3
ρNPGal	0.5	13.10±0.51	14.2±0.03	13.70±1.33
m-NP-α-Gal	0.5	0,00	0.41±0.003	0
ρ-NP-β-Gal	0.5	0,00	0	0
o-NP-α-Gal	0.5	0.51±0.04	0	0.52±0.002
o-NP-β-Glc	0.5	0	0	0
ρ-NP-α-Glc	0.5	0	0	0
ρ-NP-β-Glc	0.5	0	0	0
ρ-NP-α-Man	0.5	0	0	0
ρ-NP-α-Ara	0.5	0	0	0
ρ-NP-β-Xil	0.5	0	0	0
Raffinose	25	4.15±0,44	1.44±0.03	20.27±1.12
Stachyose	25	5.96±3.72	1.14±0.01	18.06±1.41
Sucrose	25	0,00	0	19.34±0.75
Melibiose	25	1.90±0.07	0. 21±0.07	0.5±0.25
Maltose	25	0,00	0	0
Lactose	25	0,00	0	0
Guar gum	0.5	0.67±0.09	0.41±0.03	0.36±0.001
Locust bean gum	0.5	0.98±0.08	0.85±0.02	0.50±0.05

into two groups based on their substrate specificity. One group was specific for smaller α-galactosides, such as alkyl- and aryl-galactosides, melibiose and oligosaccharides and other group acted on polymeric galactomannan and also hydrolyzed smaller substrates to various extents. *A. terreus* α-galactosidases could be grouped in the second category, such as α-galactosidases from *D. hansenii* [27], AGL I and AGL III from *Penicillium simplicissimum* [40], and α-galactosidase from *Tachigali multijuga* seeds [41]. The ability to hydrolyze guar gum and locust bean gum also showed that *A. terreus* α-galactosidases may be useful industrially for improving the gelling properties of polysaccharides [42].

A. terreus α-galactosidases showed smaller K_M values to ρNPGal, compared with the natural substrates, indicating higher affinity to this synthetic substrate (Table 3). Usually, microbial α-galactosidases present higher affinity to synthetic substrate ρNPGal instead of natural substrates [2, 27, 34, 43, 44]. However, α-galactosidase from *T. lanuginosus* was preferentially active on its natural substrates raffinose and stachyose in comparison to the artificial substrate [45]. The E1 α-galactosidase presented higher affinity to the natural substrate melibiose, followed by stachyose and raffinose (Table 3). The higher affinity to stachyose compared with raffinose was described to α-galactosidases from *D. hansenii* [27], *B. stearothermophilus* [44] and *A. terreus*$_{GR}$ [2]. The E2 α-galactosidase was more active to raffinose compared with stachyose, similarlly to α-galactosidases I, II, and III from *Aspergillus tamarii* [43, 46]. The E3 α-galactosidase presented the smaller K_M value to ρNPGal, compared with E1 and E2 enzymes. On the other hand, the higher K_M value to melibiose was determined to E3 α-galactosidase. The E1, E2 and E3 α-galactosidases presented V_{max} values for ρNPGal of 0.14, 1.02, and 0.07 mM min^{-1}, respectively. The lowest K_M and V_{max} values were calculated for E3 enzyme. Results indicate that E3 α-galactosidase

Table 3 Comparison between kinetics proprieties of α-galactosidases E1, E2, and E3 from *Aspergillus terreus* and various microbial α-galactosidases

Organism	K_M (mM)				
	pNPGal	Raffinose	Stachyose	Melibiose	Rererence
A. terreus E1	0.66	27.93	10.94	1.92	This work
A. terreus E2	0.75	32.99	54.74	7.39	This work
A. terreus E3	0.20	–	–	20.14	This work
Aspergillus oryzae	0.83	5.50	–	–	[39]
A. terreus$_{GR}$	0.11	0.42	0.33	–	[2]
Aspergillus tamarii α-Gal I	2.30	27.70	35.5	3.7	[50]
A. tamarii α-Gal II	3.80	71.40	72.00	3.80	[50]
A. tamarii α-Gal II	1.30	26.30	33.30	–	[48]
Debaryomyces hansenii	0.30	16.00	9.66	2.01	[9]
Thermomyces lanuginosus	1.13	1.61	1.17	–	[49]
Bacillus stearothermophilus	0.62	6.66	3.33	13.33	[51]

presents higher affinity for ρNPGal, but the lowest hydrolysis rate, indicating that the complex ES formation is not the limiting step for the reaction. For the natural substrates melibiose, raffinose and stachyose, the V_{max} values calculated for E1 enzyme were 0.07, 0.26, and 0.30 mM min^{-1}, respectively; and 4.72, 0.33, and 0.06 mM min^{-1}, respectively, for E2 α-galactosidase. The V_{max} value of E3 enzyme against melibiose was 0.01 mM min^{-1}. The K_M and V_{max} values for raffinose and stachyose could not be determined for the E3 α-galactosidase due the invertase contamination of this enzymatic fraction. The E2 enzyme showed higher hydrolysis rate of the disaccharide melibiose, followed by raffinose and stachyose, tri- and tetrasaccharides, respectively. On the other hand, E1 enzyme hydrolyzed raffinose and stachyose at the same rate and showed lower V_{max} to melibiose.

The sensitivity of purified α-galactosidases to various metal ions, sugar, and inhibiting reagents was tested (Table 4). The Hg^{2+}, Ag^{+} completely inhibited E1, E2, and E3 α-galactosidases. Cu^{2+} promoted total inhibition of E1 and E3 α-galactosidase activities, but E2 enzyme maintained about 28% of its activity in the presence of this ion. The inhibition of enzyme activity by metal cations, such as Hg^{2+}, Ag^{+}, and Cu^{2+} usually suggests reaction with thiol groups and/or carboxyl, amino and imidazolium groups of histidine in the active site [47]. The inhibition of α-galactosidases by Hg^{2+}, Ag^{+}, and Cu^{2+} is a classical result, which was reported for several microbial [2, 7, 27, 35, 44, 48] and plant [19, 41, 49] α-galactosidases. The activity of *A. terreus* α-galactosidases was not affected by EDTA, indicating that the enzymes were not a metalloenzymes. The E1 and E3 α-galactosidases were totally inhibited by SDS 1%, while E2 enzyme kept around 85% of its activity in the presence of this denaturant agent. This result suggests good structural stability of E2 α-galactosidase. The activity of E1 α-galactosidase to ρNPGal was significantly reduced by the natural substrates, mainly melibiose, while the E2 α-galactosidase was slightly affected by these sugars. Differently, the activity of E3 α-galactosidase over synthetic substrate was not affected by melibiose at the concentration of 2 mM. These results are in accordance with the K_M values obtained for *A. terreus* α-galactosidases, which showed that E1 enzyme presented higher affinity to melibiose, followed by E2 and E3 enzymes.

Table 4 Effect of sugars, salts, sodium dodecyl sulfate, EDTA, and 2-mercaptoethanol on α-galactosidases E1, E2, and E3 from *Aspergillus terreus*

Effector	Relative activity (%)±SD		
	E1	E2	E3
Control	100±1.5	100±1.3	100±1.9
EDTA	94±2.7	89±3.5	88±1.1
$MgCl_2$	93±1.8	87±2.3	101±0.9
$CaCl_2$	96±1.0	84±1.4	102±1.3
$CuSO_4$	0	28±1.4	0
KCl	99±0.3	93±2.2	100±0.7
NaCl	98±0.7	92±2.7	98±1.8
$HgCl_2$	0	0	0
$AgNO_3$	0	0	0
β-Mercaptoethanol	97±3.2	94±1.5	94±2.1
SDS	0	85±2.6	0
Lactose	94±0.5	90±3.4	99±1.7
Maltose	91±0.4	85±4.2	94±2.4
Melibiose	38±0.7	77±2.4	94±0.8
Raffinose	65±1.5	87.5±4.3	98±1.7
D-Mannose	87±1.1	92±0.08	94±0.7
D-Galactose	96±1.4	51±0.6	52±0.6
Stachyose	72±0.8	93±5.7	95±0.9
Sucrose	92±1.0	87±8.2	99±1.5
Glucose	88±1.9	92±5.4	94±2.1
Fructose	98±1.3	96±1.6	98±1.7

D-Galactose was a competitive inhibitor of E2 and E3 α-galactosidase activities, with K_I values of 0.61 and 0.76 mM, respectively. D-Galactose is known as a potent inhibitor of microbial and vegetable α-galactosidases [19–21, 41, 50]. However, the activity of E1 α-galactosidase was not significantly affected by D-galactose. This is important from the industrial point of view, since galactose tolerance is an appreciable character, which

Table 5 Hydrolysis of oligosaccharides present in soy milk by α-galactosidases E1, E2 and E3 from *Aspergillus terreus*

Time (h)	Oligosaccharides content (%)±SD					
	E1		E2		E3	
	Raffinose	Stachyose	Raffinose	Stachyose	Raffinose	Stachyose
0	1.37±0.17	2.56±0.20	1.37±0.11	2.54±0.04	1.38±0.15	2.53±0.18
4	2.38±0.45	1.25±0.03	0.64±0.13	2.28±0.58	1.21±0.07	2.11±0.12
8	2.22±0.21	0.67±0.10	0.44±0.05	1.67±0.02	1.50±0.13	2.1±0.07
12	1.7±0.18	0.0	0.0	1.00±0.02	1.14±0.08	1.92±0.16

improves the efficiency of α-galactosidases in liberating galactose residues form oligomeric and polymeric α-galactosides [21]. α-Galactosidases from *T. reesei* [51], and *B. stearothermophilus* NCIM-5146 [50], were competitively inhibited by galactose. On the other hand, extracellular and intracellular α-galactosidases from *D. hansenii* UFV-1 were noncompetitively and competitively inhibited by galactose, respectively [9, 27].

The potential *A. terreus* α-galactosidases to hydrolyze the oligosaccharides present in soybean aqueous extract (soy milk) was evaluated and the results are shown in Table 5. E1 α-galactosidase promoted total hydrolysis of stachyose after 12 h of soy milk treatment. At the same time, the raffinose content increased 24.0%. This difference has been caused by raffinose accumulation, which is formed after stachyose hydrolysis. On the other hand, E2 enzyme completely hydrolyzed raffinose after soy milk treatment for 12 h, but 40.0% of initial stachyose content was maintained after this period. α-Galactosidase E3 showed the weakest performance to hydrolyze RFOs present in soy milk. This enzyme hydrolyzed 17.0 and 24% of raffinose and stachyose, respectively, after soy milk treatment for 12 h. These results suggest a close relation between the performance of enzymes and their substrate affinities. As described previously, E1 α-galactosidase had a higher affinity to stachyose compared to raffinose (K_M values to raffinose and stachyose were 27.93 and 10.97 mM, respectively) and it could explain the preferential hydrolysis of this tetrasaccharide in soybean aqueous extract. Similarly, an incomplete hydrolyzis of stachyose by E2 α-galactosidase could be justified by lower affinity of the enzyme to this substrate (K_M values to raffinose and stachyose were 32.94 and 54.74 mM, respectively).

Considering the preferential hydrolysis of stachyose and raffinose by E1 and E2 enzymes, respectively, it could be suggested that the total hydrolysis of RFOs present in soy milk will be reached by the synergistic action of both enzymes. In a previous work we reported a complete depletion of raffinose and stachyose present in soybean aqueous extract after 4 h of treatment with *A. terreus* partially purified enzymatic extract containing E1, E2 and E3 α-galactosidases [7].

Conclusion

In the present work, we purified and compared the properties of three α-galactosidases secreted by *A. terreus* grown in liquid medium containing wheat bran as carbon source. The α-galactosidases were effective for RFO reduction in soymilk.

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