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A thermostable α-galactosidase from *Lenzites elegans* (Spreng.) *ex* Pat. MB445947: purification and properties

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Abstract An α-galactosidase was isolated from a culture filtrate of *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 grown on citric pectin as carbon source. It was purified to electrophoretic homogeneity by ammonium sulfate precipitation, gel filtration chromatography and anion-exchange chromatography. The relative molecular mass of the native purified enzyme was 158 kDa determined by gel filtration and it is a homodimer (Mr subunits = 61 kDa). The optimal temperature for enzyme activity was in the range 60–80 °C. This α-galactosidase showed a high thermostability, retaining 94 % of its activity after preincubation at 60 °C for 2 h. The optimal pH for the enzyme was 4.5 and it was stable from pH 3 to 7.5 when the preincubation took place at 60 °C for 2 h. It was active against several α-galactosides such as *p*-nitrophenyl-α-D-galactopyranoside, α-D-melibiose, raffinose and stachyose. The α-galactosidase is a glycoprotein with 26 % of structural sugars. Galactose was a non-competitive inhibitor with a Ki = 22 mM versus *p*-nitrophenyl-α-D-galactoside and 12 mM versus α-D-melibiose as substrates. Glucose was a simple competitive inhibitor with a Ki = 10 mM. Cations such as Hg^{2+} and *p*-chloromercuribenzoate were also inhibitors of this activity, suggesting the presence of –SH groups in the active site of the enzyme. On the basis of the sequence of the N-terminus (SPDTIVLDGTNFALN) the studied α-galactosidase would be a member of glycosyl hydrolase family 36 (GH 36). Given the high optimum temperature and heat stability of *L. elegans* α-galactosidase, this fungus may become a useful source of α-galactosidase production for multiple applications.

Keywords α-Galactosidase · Enzyme purification · *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 · Mycology

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Introduction

Fungi are important in forest ecosystems because of their unique capacity to break down woody plant components. Among them, white rot fungi degrade lignin along with the major constituents of

hemicelluloses (hetero-1,4-β-D-xylans, hetero-1,4-β-D-mannans, galacto-glucomannans, galactomannans and glucomannans) leaving wood spongy and bleached. Consequently, wood decay fungi produce a complex mixture of fungal polysaccharases that have the potential to dissolve plant cell walls. These enzymatic systems were applied to protoplast production, in the elaboration of baby and geriatric foods, nectars, concentrates from fruits and vegetables, hydrolysis of raffinose in beet sugar molasses and the raffinose family of oligosaccharides (RFOs) in soybean milk, (Leal et al. 1994; Whitaker 1984; Manonmani and Sreekantiah 1987; Gilbert and Hazlewood 1993), for pulp and paper production and the feedstock industry among others.

The degradation of galactomannans (the most abundant mannan-based reserve polysaccharide of the cell wall hemicellulose fraction) is mediated by the action of α-galactosidases that catalyze the hydrolysis of terminal nonreducing α-D-galactosyl residues in alkyl, aryl or glucosyl (mono or oligo) groups (Dey and Pridham 1969, 1972). α-Galactosidases were classified as α-D-galactoside galactohydrolases (EC 3.2.1.22), according to the general classification based on the catalytic activity of the enzymes (I.U.B. 1984). Owing to the enormous quantity and structural diversity of carbohydrates and derivatives, a classification system of glycosidases based on their protein sequence and structural similarities were suggested (Henrissat 1991; Henrissat and Bairoch 1993) to complement the I.U.B. system, that allowed to group them according to their common evolutionary origin. α-Galactosidases have been extracted and purified from microorganisms (Mital et al. 1973; Ulezlo and Zaprometova 1982), animals including humans (Beier et al. 1990) and plants (Chinen et al. 1981) especially from seed tissues containing large amounts of sugars from the raffinose-family.

α-Galactosidases were studied in several fungi such as *Aspergillus ficuum* (Zapater et al. 1990), (Manzanares et al. 1998; Ademark et al. 2001), *Monascus pilosus* (Wong et al. 1986), *Penicillium purpurogenum* (Shibuya et al. 1998), *Penicillium simplicissimum* (Luonteri et al. 1998), *Thermomyces lanuginosus* (Puchart et al. 2000, 2004; Rezessy-Szabó et al. 2002, 2003, 2007), *Trichoderma reesei* (Margolles-Clark et al. 1996) and were obtained in crystalline form from the mycelium of *Mortierella vinacea* (Susuki et al. 1970).

Taking into account the importance of fungal enzymes, the purpose of the present study was the purification of an α-galactosidase from *Lenzites elegans* (Spreng.) *ex* Pat. MB445947, the investigation of the effect of several effectors and the physicochemical and kinetic properties of the enzyme. Its resistance to extreme temperatures and pH was also analyzed.

Materials and methods

Microorganism

Lenzites elegans (Spreng.) *ex* Pat. MB445947 was kindly supplied by Dr. A. R. Sampietro (Instituto de Estudios Vegetales, Universidad Nacional de Tucumán, Argentina). The control of the fungal species was performed by the morphology of its colonies, fruiting bodies, and spores. Colonies and fruiting bodies were examined in a stereoscopic microscope (×40, CETI, Belgium) and spores were examined using a light microscope (×400, ×600, and ×1000, CETI, Belgium). The fungal identity was verified according to Barnet and Hunter (1999) by Dormsch (1980), and Nelson et al. (1983), and named according to Rossman et al. (1994) and Hawksworth et al. (1995). The specimen was deposited in the collection of the Institute "Miguel Lillo", Tucumán, Argentina, as *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 collection number LILC No. 5699.

Maintenance of the strain

Lenzites elegans (Spreng.) *ex* Pat. MB445947 LILC No. 5699 was maintained on slants of basal medium (1 % (w/v) peptone, 0.3 % (w/v) yeast extract and 2 % agar). After growth at 30 °C in a humidified thermostat for 10 days plates were finally stored at 4 °C prior to use.

Materials

Sephadex G-150 and DEAE Sepharose CL-6B were obtained from Amersham Biosciences (Uppsala, Sweden). Unless otherwise stated, all other chemicals were of analytical grade and purchased from Sigma-Aldrich (St. Louise, USA).

Media and culture conditions for enzyme production

The fungus was cultivated in 300 ml of basal medium (1 % (w/v) peptone, 0.3 % (w/v) yeast extract) added

with 2 % citric pectin prepared in distilled water, and distributed in three Erlenmeyers of 250 ml. The fungus was grown at 30 °C. The pH values were adjusted to 6 prior to sterilization (121 °C, 20 min). Maximum activity of α-galactosidase was reached after 12 days of incubation at 30 °C.

Enzymatic assay

α-Galactosidase activity was quantitatively measured by the *p*-nitrophenol released from *p*-nitrophenyl-α-D-galactopyranoside (*p*-NP-α-D-Gal) as substrate. The reaction mixture consisted of 40 µl of 0.2 M sodium acetate buffer (pH 4.5), 20 µl of 0.02 M *p*-NP-α-D-Gal and 40 µl of suitably diluted enzyme preparation to initiate the reaction, in a final volume of 100 µl. Incubations were performed at 50 °C for 10 min. Reactions were stopped by adding 1.6 ml of 1 M Na_2CO_3, and the released *p*-nitrophenol was determined spectrophotometrically at 420 nm (Pastore and Park 1979). *p*-Nitrophenol was used as standard. One unit of α-galactosidase activity was defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenol per min at 50 °C and pH 4.5.

Protein determinations

Proteins were determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. All chromatographic fractions were monitored for proteins by absorbance at 280 nm.

Carbohydrate determinations

Total structural carbohydrates were measured by phenol–sulfuric acid method (Dubois et al. 1956) using a solution containing equal amounts of glucose and mannose as standard. Sugar content of the enzyme was determined as follows: enzyme solution (300 µl containing 450 µg of purified enzyme was added with 150 µl of 100 % trifluoroacetic acid and incubated at 100 °C for 4 h in a sealed hydrolysis tube. The mixture was cooled to room temperature and dried in a vacuum evaporator. The residue was washed three times with distilled water and individual sugars identified by High Performance Anion Exchange Chromatography (HPAEC) using CarboPac^{TM} PD10 (5 mm × 250 mm) column connected to a pulse amperometric detector (PAD) (DIONEX^{TM} system). Elution was performed according to the manufacturer instructions.

Alpha-galactosidase purification

After 12 days of incubation, the culture was filtered through Whatman No. 4 filter paper. The filtrate was centrifuged 10 min at 27,000×*g* and the supernatant heated at 60 °C during 1 h. After another centrifugation, pellets were discarded and solid ammonium sulfate to 80 % saturation was added to the supernatant. The pellet was collected by centrifugation at 27,000×*g* for 10 min, resuspended in 4 ml of 10 mM sodium acetate buffer, pH 5.5, containing 1 mM 2-mercaptoethanol plus 0.05 M NaCl (Buffer A) and dialyzed against the same buffer for 2 h. Fractions of 4 ml of the dialyzate were applied to a Sephadex G-150 column (25 × 400 mm) equilibrated and eluted with buffer A, and 2.5 ml fractions were collected. Their protein content was estimated by measurement of absorbance at 280 nm. Fractions eluted between 69 and 89 ml were pooled and kept at −20 °C. Fractions of 3 ml were dialyzed against 10 mM sodium phosphate buffer, pH 7, containing 0.05 M NaCl and 1 mM 2-mercaptoethanol (Buffer B) and applied to a DEAE-Sepharose CL-6B column (22 × 180 mm) equilibrated with Buffer B. Elution was performed at a flow rate of 12 drops min^{-1} with a linear gradient of 0.05–1.2 M NaCl in the same buffer. α-Galactosidase containing fractions, eluted at 0.75 M NaCl. They were pooled, dialyzed during 2 h against buffer A and kept at −20 °C until use.

Estimation of relative molecular mass

Molecular mass estimation of the native α-galactosidase was carried out by the molecular size exclusion technique (Andrews 1964) using a Sephadex G-150 column (25 × 400 mm) equilibrated and eluted with Buffer A. The column was calibrated with cytochrome *c* (12,400), carbonic anhydrase (29,000), ovalbumin (45,000), bovine serum albumin (66,000) and alcohol dehydrogenase (150,000).

Electrophoretic analysis

Fractions obtained at the various purification steps were analyzed by electrophoresis using 7 % (w/v) polyacrylamide gel slabs and 25 mM TRIS glycine buffer, pH 8.3, on a Bio-Rad Mini-Protean II system according to the manufacturer's manual. Bromophenol Blue was employed as a front marker. Gels were

run for 4 h at 80 Volts and proteins were stained by a silver nitrate method (Blum et al. 1987). Enzyme activity was detected as a yellow band by the *p*-nitrophenol released after incubation of the gel in 0.01 M *p*-NP-α-D-Gal in 0.2 M sodium acetate buffer pH 4.5 at 37 °C (Gabriel 1971). Electrophoresis in denaturing conditions was carried out according to (Laemmli 1970). Protein bands were detected by argentic impregnation (Blum et al. 1987). The molecular mass markers used were: cytochrome *c* (12,400), lyzozyme (14,300), carbonic anhydrase (29,000), ovalbumin (45,000) and bovine serum albumin (66,000).

Isoelectric focusing

The isoelectric point was determined in the pH range of 3–9 on a Bio-Rad Mini-Protean II gel apparatus. Approximately 5.25 μg of protein was loaded per lane. The gel was focused at 100 V for 30 min and run at 100 V for 15 min, 200 V for 15 min and 450 V for 90 min with methyl red as the front marker. The pH gradient was determined in another lane of the same slab. The gel without sample was run and cut into 0.5 cm slices. These were put in small tubes containing 0.5 ml of distilled water and left in a cold room overnight. The pH was measured with a microelectrode. The sample lane of the slab was soaked in 12 % (w/v) trichloroacetic acid to wash out the carrier ampholites and stained for the presence of proteins with Coomassie Brillant Blue R-250.

Substrate specificity

The substrate specificity of the purified α-galactosidase was determined using various p-nitrophenyl-glycosides. Each assay mixture contained 40 μl of 20 mM *p*-nitrophenyl-glycoside solution, 20 μl of 0.2 M sodium acetate buffer, pH 4.5, 40 μl of a suitably diluted enzyme preparation. The reactions were performed at 50 °C for 10 min and terminated by adding 1.6 ml of 1 M Na_2CO_3 (Pastore and Park 1979). The substrate specificity of the enzyme for alkyl glycosides and oligosaccharides was determined in a reaction mixture containing 40 μl of 150 mM alkyl-glycoside or oligosaccharide solution, 20 μl of 0.2 M sodium acetate buffer, pH 4.5 and 40 μl of a suitably diluted enzyme preparation. Reactions were performed in the same conditions as before. The substrate specificity of the enzyme for polysaccharides was determined in an incubation mixture constituted by 40 μl of 20 mg/ml of pectin, agarose or polygalacturonic acid or 40 μl of 5 mg/ml of locus bean galactomannan, glycogen, starch, xylan, dextran or arabic gum, 20 μl of 0.2 M sodium acetate buffer, pH 4.5 and 40 μl of a suitably diluted enzyme preparation. All reactions were performed at 50 °C for 10 min.

Hydrolysis of the methyl derivatives, oligosaccharides and polysaccharides were followed measuring reducing sugar release (Somogyi 1945; Nelson 1944), except with reducing disaccharides, in which case glucose oxidase method (Jorgensen and Andersen 1973) was used.

Kinetic studies

Km, Vmax and the effect of galactose on the enzyme activity were determined using different glycosides as substrates. With *p*-NP-α-D-Gal as substrate the reaction mixtures contained 20 μl of enzyme, 40 μl of 0.2 M sodium acetate buffer, pH 4.5, 20 μl of 1–20 mM *p*-NP-α-D-Gal and 0-20 μl of 0.2 M galactose in a final volume of 100 μl. The *p*-nitrophenol liberated was measured at 420 nm. When the substrate was α-D-melibiose the reaction mixture contained 40 μl of enzyme extract, 40 μl of 0.2 M sodium acetate buffer, pH 4.5, 10 μl of 0.2–0.6 M α-D-melibiose and 10 μl of 0–0.2 M galactose. The glucose liberated was followed according to (Jorgensen and Andersen 1973).

For raffinose and stachyose as substrates the reaction mixtures consisted in 40 μl of enzyme, 40 μl of 0.2 M sodium acetate buffer, pH 4.5, 5 μl of 0.025–0.4 M raffinose or 5 μl of 0.025–0.2 M stachyose and 15 μl of 0–0.2 M galactose in a final volume of 100 μl. The activity was measured by determining the reducing sugars liberated from the substrate (Somogyi 1945; Nelson 1944). All reactions were run at 50 °C for 30 min. Km and Vmax were determined from the Lineweaver–Burk method using the trial version of the Enzyme Kinetics module of Sigmaplot (System Software Inc. Version 1.2.0.0).

Effects of saccharides

The effect of saccharides on the activity of the α-galactosidase of *Lenzites elegans* LILC No. 5699 was assayed in a reaction mixture consisting of: 20 μl

of enzyme, 20 µl of 0.2 M sodium acetate buffer, pH 4.5, 20 µl of 20 mM *p*-NP-α-D-Gal and 10; 20 or 30 µl of 0.2 M saccharide in a final volume of 100 µl. The incubation was performed at 50 °C for 30 min. The sugars assayed were 0.2 M glucose, fructose, xylose, sucrose, cellobiose, lactose, melibiose, and galactose.

Effect of pH on enzymatic activity and stability

The effect of pH on α-galactosidase activity was determined by assaying the enzyme with *p*-NP-α-D-Gal as substrate for 15 min at 50 °C with the following buffer systems: 0.2 M glycine-HCl for pH 2.2–3.25; 0.2 M sodium acetate-acetic acid for pH 3.5–5.7; 0.2 M sodium phosphate for pH 6.3–7.6; 0.2 M barbital-HCl for pH 8.0–8.3. Enzyme stability at different pH values was determined by measuring the residual activity after preincubating the enzyme for 2 h at different pHs in the range 2–9 at 50 °C. Then, the pH was adjusted to the optimal pH of the enzyme action (pH 4.5) and the residual activity was measured as described above.

Optimal temperature of the reaction

Determinations of the optimal temperature for the reaction were performed by evaluating the enzymatic activity at different temperatures in the 35–95 °C range, at intervals of 5 °C.

Thermal stability

Enzyme aliquots were preincubated at the optimal pH (0.2 M sodium acetate buffer, pH 4.5) at different temperatures in the 0–90 °C range for 1 and 2 h. Following preincubation, the remaining activity was determined at 50 °C as described above.

Effect of chemicals

The sensitivity of the enzyme to chemical products was examined in the following reaction mixture: 20 µl of 0.2 M sodium acetate buffer (pH 4.5), 20 µl of 0.02 M *p*-NP-α-D-Gal, 20 µl of each solution of the chemical product and 40 µl of suitably diluted enzyme preparation to initiate the reaction, in a final volume of 100 µl. The selected substances were 10 mM $HgCl_2$ or EDTA; 100 mM $AgNO_3$, $MnCl_2$, $MgSO_4$, $CoCl_2$, $Ca(NO_3)_2$, urea, $CuSO_4$, Ba Cl_2, TRIS or *p*-chloromercuribenzoate that were dissolved to their respective concentration in 0.2 M sodium acetate buffer, pH 4.5.

Amino-acid sequence analysis

The α-galactosidase was subjected to SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, USA; Immobilon, pore size 0.45 nm). The membrane was stained with Coomassie Brilliant Blue R-250 and the blue band corresponding to the enzyme was cut from the membrane. Automated Edman degradation was performed using the Applied Biosystem Model 476A protein sequencer equipped with an on-line analysis system. The obtained amino acid sequence was aligned and compared using Basic Local Aligment Research Tool (BLAST; http://www.ncbi.nlm.nih.gov/BLAST/) and CLUSTALW Programmes (www.clustal.org).

Molecular properties

The enzyme was deglycosylated by removal of N-linked carbohydrates with peptide N-glycosidase H. The reaction mixture consisted of 400 µl of α-galactosidase (0.121 EU), 500 mU of peptide N-glycosidase H in 20 mM sodium phosphate buffer pH 6.8 with 20 mM EDTA, 1 mM sodium azide and 1 mM MPSF (methyl-phenyl-sulphonyl-fluoride) in 800 µl final volume. After incubation for 20 h at 37 °C the products were separated by Biogel P6 column (2 × 60 cm) using 10 mM sodium acetate buffer pH 4.8 with 0.2 M NaCl and 1 mM 2-mercaptoethanol. Two ml fractions were collected and absorbance at 280 nm and neutral sugars were measured.

Fractions containing sugars were dried in a vacuum evaporator. The residue was resuspended in 200 µl of distilled water and 100 µl of 100 % trifluoroacetic acid was added. The mixture was sealed in a hydrolysis tube and incubated at 100 °C for 4 h. After cooling to room temperature the mixture was transferred to a microcentrifuge tube (Eppendorf) and dried in a vaccum evaporator.

The identity of the sugars was determined by paper chromatography using Wathman No. 1 paper as fixed phase and butanol-pyridine-water (6:4:3, v/v) as mobile phase. The standards used were mannose, glucose, fructose and galactose.

Results

The screening for glycosidase activities secreted to the liquid culture medium was conducted using sucrose, stachyose, xylan, citric pectin or locus bean gum (galactomannan polysaccharide) as carbon sources in surface cultures. Among the glycosidases, α-galactosidase was produced in the highest amount in media supplemented with stachyose, sucrose or citric pectin as carbon source but not in that supplemented with galactomannan polysaccharide suggesting that short-chain oligosaccharides are the preferred substrates of the enzyme.

Purification of the enzyme

Native PAGE analysis of the crude extract showed several protein bands (data not shown) with only one fraction with α-galactosidase activity (Fig. 1). The enzyme was purified to apparent homogeneity from the culture medium through the procedures described in Table 1. A 76-fold purification was attained. The purified preparation produced a single protein band after polyacrylamide gel electrophoresis in denaturing conditions (Fig. 2).

α-Galactosidase was focused as a single peak (pI in the range 4.0–4.2) suggesting acidic characteristics for the enzyme and the absence of iso-enzyme contaminations in our highly purified preparation.

Molecular properties of the α-galactosidase

The enzyme is a glycoprotein. Carbohydrates (glucose and galactose) constitute the 26 % of its total molecular mass. The apparent molecular mass of the enzyme was 158 kDa as determined by gel filtration. When its mobility in SDS–polyacrylamide gel electrophoresis was compared with that of proteins of known molecular weights, the apparent molecular mass of the enzyme sub-unit appeared to be 61 kDa. It must be pointed out that given the glycoprotein nature of α-galactosidase, it is possible that this value do not reflect the true molecular mass. It is well known that glycoproteins behave anomalously in SDS–polyacrylamide gel electrophoresis.

Effect of pH on enzyme activity and stability

The optimal pH of the purified enzyme was about 4.5 (Fig. 3). Preincubations of the enzyme at various pHs showed that it is stable from pH 3 to 7.5 when the preincubation takes place at 60 °C for 2 h (Fig. 3).

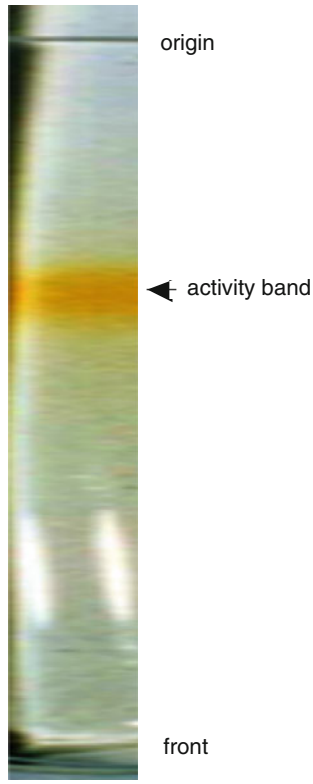

Fig. 1 Non-denaturing polyacrylamide gel electrophoresis (PAGE) of the crude extract of *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 α-D-galactosidase. The gel was stained for α-D-galactosidase activity. A *yellow band* of *p*-nitrophenol revealed after gel incubation in 0.01 M *p*-NP-α-D-Gal in 0.2 M sodium acetate buffer pH 4.5 at 37 °C (Gabriel 1971). (Color figure online)

Effect of temperature on enzyme activity and stability

The enzyme showed a high level of activity at temperatures from 60 to 80 °C (Fig. 4a). Moreover, it retained practically its full activity after 1 h of treatment at 75 °C and 2 h at 60 °C (Fig. 4b). The conventional Arrhenius plot for the α-galactosidase with *p*-nitrophenyl-α-D-galactoside as substrate was a straight line graph. The enzymatic activity was determined between 35 and 60 °C in the conditions described in enzyme assays. The activation energy of the hydrolytic reaction was calculated to be 17,100 cal/mol in the assayed temperature range.

Table 1 Purification of an α-galactosidase from *Lenzites elegans* (Spreng.) *ex* Pat. MB445947

Procedure	Enzyme units (EU)	Protein (mg)	Specific activity (units mg^{-1})	Purification	Yield (%)
Filtrate	90	257.0	0.35	1.00	100
Filtrate heated at 60 °C	86	242.0	0.36	1.02	96
80 % satn. $(NH_4)_2SO_4$	69	49.0	1.41	4.02	77
Sephadex G-150	33	1.4	23.57	65.70	37
DEAE-Sepharose CL-6B	25	0.9	27.77	75.70	28

Each value is the average of six determinations

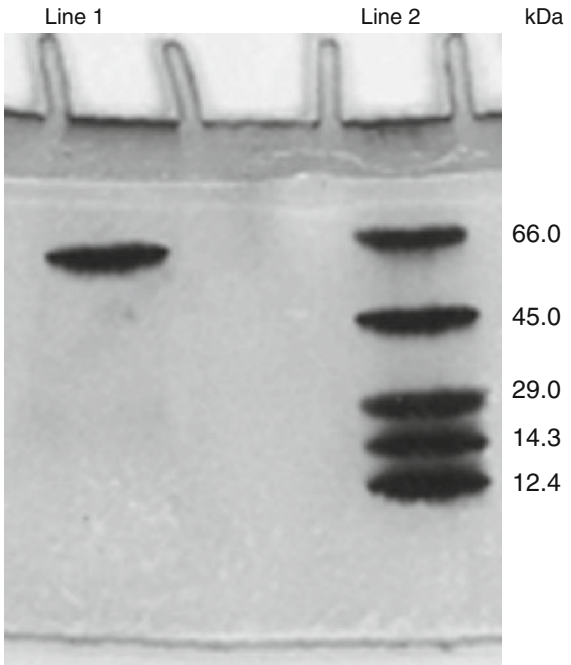

Fig. 2 SDS-PAGE of purified α-D-galactosidase of *Lenzites elegans* (Spreng.) *ex* Pat. MB445947. Protein bands were detected by argentic impregnation (Blum et al. 1987). *Line 1* purified α-D-galactosidase. *Line 2* standards of known molecular weight cytochrome *c* (12,400), lyzozyme (14,300), carbonic anhydrase (29,000), ovalbumin (45,000) and bovine serum albumin (66,000)

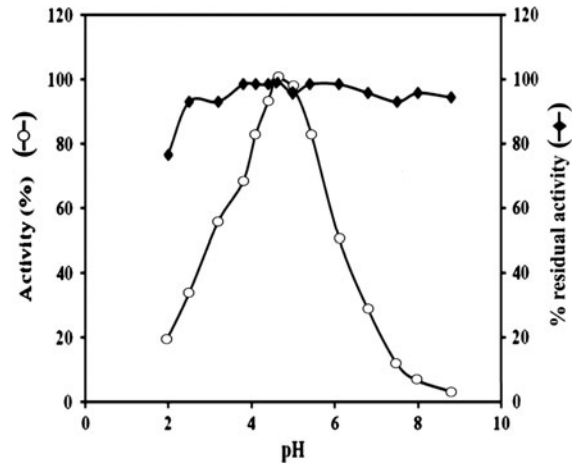

Fig. 3 Effect of pH on the activity of α-D-galactosidase from *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 (*open circle*). Enzyme stability of the enzyme at different pHs (*filled diamond*). The enzyme was preincubated at different pHs for 2 h at 50 °C. Then, the enzyme activity was measured at the optimal pH 4.5 at 50 °C

Substrate specificity

Table 2 shows the substrates hydrolyzed by the α-galactosidase. Other substrates assayed (not shown in the Table) were not hydrolyzed. The dependence of the reaction rate on the substrate concentration was assayed at pH 4.5 and 50 °C. The Michaelis–Menten constants (Km) and maximum velocities (Vm) for the reaction with *p*-nitrophenyl α-D-galactopyranoside, raffinose, stachyose and α-D-melibiose as substrates, were calculated from Lineweaver–Burk plots. These plots produced standard straight lines.

Effects of sugars on the enzyme activity

Glucose and galactose were inhibitors of this α-galactosidase. Galactose, a reaction product, produced a classical non-competitive inhibition. According to replots of slope against galactose concentration, the Ki calculated was 2 mM when the substrate was *p*-nitrophenyl α-D-galactopyranoside and 12 mM when the substrate was α-D-melibiose. Otherwise, glucose was a simple competitive inhibitor (Ki = 10 mM) with linear replots. Other effects of sugars are shown in Table 3. The strongest inhibitor appears to be α-D-melibiose (an α-D-galactoside), but this may be an

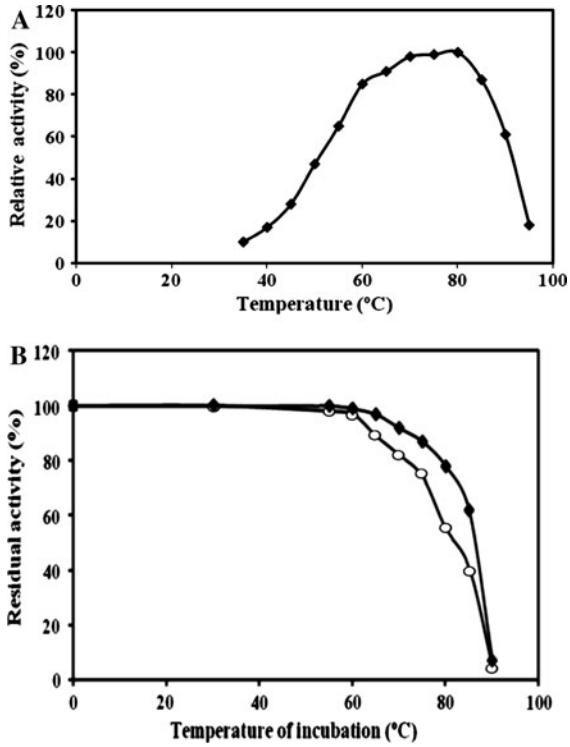

Fig. 4 **a** Influence of temperature on the activity of the α-D-galactosidase from *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 (*filled diamond*). **b** Stability of the α-D-galactosidase from *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 to the temperature after 2 h of pre-incubation at 60 °C (*filled diamond*), and after 1 h of preincubation at 75 °C (*open circle*)

Table 2 Kinetic properties of the α-galactosidase of *Lenzites elegans* (Spreng.) *ex* Pat. MB445947

Substrate	Km (mM)	Vmax (μmol min^{-1} mg^{-1})
p-Nitrophenyl α-D-galactopyranoside	5.0 ± 0.4	72.1 ± 0.7
Raffinose	2.5 ± 0.2	8.8 ± 0.5
Stachyose	2.0 ± 0.2	10.5 ± 0.2
α-D-Melibiose	60.0 ± 0.5	15.2 ± 0.2

Each value is the average of six determinations ± SD

apparent inhibition because this sugar is a substrate of the enzyme.

Effect of chemicals

Many metallic ions were inhibitors of the α-galactosidase from *Lenzites elegans* (Spreng.) *ex* Pat. MB445947, among them 1 mM $HgCl_2$ (90 %), and 10 mM *p*-chloromercuribenzoate (100 %) were strong inhibitors; also inhibitors were 10 mM $AgNO_3$ (65 %), 10 mM $CoCl_2$ (65 %), 10 mM urea (65 %), 10 mM $CuSO_4$ (60 %). Slight inhibitors were 10 mM EDTA (20 %), $MnCl_2$ (20 %), $MgSO_4$ (2 %), $Ca(NO_3)_2$ (20 %) and $BaCl_2$ (15 %). Moreover, 10 mM TRIS was a strong inhibitor of this enzyme.

Table 3 Effect of some saccharides on the activity of the α-galactosidase of *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 using *p*-nitrophenyl α-D-galactoside as substrate

Concentration (mM)	Sugar	Residual activity (%)
	None	100 ± 3
40	Glucose	82 ± 2
60	Glucose	78 ± 2
40	Fructose	108 ± 3
40	Xylose	109 ± 2
40	Sucrose	98 ± 4
40	Cellobiose	112 ± 2
40	Lactose	102 ± 5
20	Melibiose	67 ± 2
40	Melibiose	39 ± 2
20	Galactose	75 ± 3
40	Galactose	45 ± 2

The reaction mixture consisted of 20 μl of enzyme, 20 μl of 0.2 M sodium acetate buffer, pH 4.5, 20 μl of 20 mM *p*-NP-α-D-Gal and 10; 20 or 30 μl of 0.2 M saccharide in a final volume of 100 μl. The incubation was performed at 50 °C for 30 min. Each value is the average of six determinations ± SD

N-terminal amino acid sequence analysis

The sequence of the N-terminus of the α-galactosidase from *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 was SPDTIVLDGTNFALN. This sequence data was aligned and compared using Basic Local Aligment Research Tool (BLAST) and CLUSTALW Programmes. The obtained α-galactosidase showed a high sequence similarity to α-galactosidase A precursor from *Aspergillus niger* (accession number Q9UUZ4), *Aspergillus nidulans* FGSC A4 α-galactosidase precursor (accession number EAA58775) and *Thermomyces lanuginosus* CBS 395.62/b α-galactosidase (Fig. 5). These results suggest that the purified protein belongs to the Glycohydrolase Family 36 (GH 36).

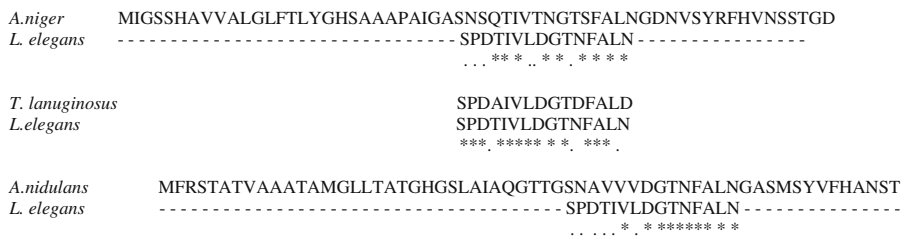

Fig. 5 Multiple sequence alignement of the α-D-galactosidase from *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 with α-D-galactosidases from *Apergillus niger*, *Thermomyces lanuginosus* and *Aspergillus nidulans*

Discussion

The ability of the microorganisms to produce glycosidases is well known. The enzymatic hydrolysis of the glycosidic bond continues to gain importance, reflecting the important functions that complex glycans play in health and disease as well as the rekindled interest in enzyme biomass conversion. Then, the studies on glycosidase production were intensified in recent years with the recognition of the importance of glycosides in many biological functions. Several glycosidases were detected in supernatants of culture media of *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 and other wood decaying fungi (Leal et al. 1994). As a contribution to the knowledge of the composition and function of these fungal enzymes two invertases were studied in our laboratory (Rojo et al. 1994; Quiroga et al. 1995). Although α-galactosidases from different sources have been studied; in this paper the purification, mechanism of action on different substrates and molecular characterization of the α-galactosidase isolated from *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 is described for the first time.

The enzyme purification was attained by gel filtration and ion-exchange chromatography and the purity of the preparation demonstrated in both non-denaturing and SDS-PAGE. The enzyme preparation produced a single protein band in native PAGE. This band had the same migration as the activity band and confirmed the presence of only one enzyme in the culture medium. The SDS-PAGE of the purified α-galactosidase showed the existence of two identical subunits without biological activity, at difference of α-galactosidases of other sources where there are a family of genes codifying galactosidases (de Vries et al. 1999).

The optimum pH for *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 α-galactosidase is acidic and stable in an ample temperature range as was reported for other α-galactosidases (Kotwal et al. 1998; King et al. 2002; Ozsoy and Berkkan 2003; Simerská et al. 2007). The isoelectric point is also acidic as others.

The enzyme is specific for short-chain α-galactosides. The synthetic substrate *p*-nitrophenyl-α-D-galactoside, and the natural sugars raffinose, α-D-melibiose and stachyose are substrates of the enzyme but galactomannan, agarose and *β*-galactosides are not. Galactose is a non-competitive inhibitor of the enzyme as reported for α-galactosidases of *Saccharum officinarum* (Chinen et al. 1981) and *Pycnoporus cinnabarinus* (Ohtakara and Masaru 1987), at difference of most of the α-galactosidases that show a competitive inhibition by galactose, for instance: *Trichoderma reesei* (Golubev et al. 2004), *Cucumis sativus* (Smart and Pharr 1980), *Saccharomyces carlsbergensis* (Lazo et al. 1978), *Vicia faba* (Dey and Pridham 1969), *Calvatia cyathiformis* (Kotwal et al. 1998), and others. Furthermore, galactose produces a mixed type inhibition for the *Mortierella vinacea* α-galactosidase (Susuki et al. 1970).

The activation energy of the enzyme is constant in the range of the assayed temperatures suggesting a simple structure for the enzyme (two identical subunits and a michaelian kinetics).

A useful characteristic of the enzyme is the ability to resist high temperatures, thus the α-galactosidase of *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 may be used at 60 °C during, at least, 2 h without activity loss. The heat resistance of the enzyme is comparable to these of *Pycnoporus cinnabarinus* (Ohtakara and Masaru 1987) and *Vicia sativa* (French 1953). The optimum action temperature reached 80 °C, while the highest level of activity occurred at 50 °C for *Calvatia cyathiformis* (Kotwal et al. 1998) and *Aspegillus nidulans* (Ríos et al. 1993), 60 °C for *Trichoderma reesei* (Zeilinger et al. 1993) and 75 °C for

Pycnoporus cinnabarinus (Ohtakara and Masaru 1987). It is worth noting that the activity of this enzyme is inhibited by Hg^{2+} and *p*-mercuribenzoate suggesting the presence of –SH groups in the active site.

Microorganisms, mainly fungi, have the ability to produce multiple α-galactosidases which show activity on oligo and polysaccharides. In the case of the α-galactosidase of *Lenzites elegans* (Spreng.) *ex* Pat. MB445947, in our work conditions, we found only one enzyme active on oligosaccharides. The comparison of its N-terminal amino acid sequence with the amino acid sequence of other α-galactosidases allowed us to suggest that the novel enzyme can be classified in family 36 glycosyl hydrolases.

Given the high optimum temperature, and heat and pH stability of *Lenzites elegans* (Spreng.) *ex* Pat. MB445947 α-galactosidase, this fungus may become a source of α-galactosidase for their use in the treatment of legume-based food for reducing gastric distress caused by oligosaccharides of the raffinose-family or other anti-nutritive oligosaccharides by converting them into sucrose and thus enhancing their nutritive value.

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