Vol.53, n. 3: pp. 719-729, May-June 2010
ISSN 1516-8913 Printed in Brazil

BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY
A N I N T E R N A T I O N A L J O U R N A L

Hydrolysis of Galacto-oligosaccharides in Soy Molasses by α-galactosidases and Invertase from *Aspergillus terreus*

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ABSTRACT

Two α-galactosidase (P1 and P2) and one invertase present in the culture of Aspergillus terreus *grown on wheat straw for 168 h at 28°C were partially purified by gel filtration and hydrophobic interaction chromatographies. Optimum pH and temperatures for P1, P2 and invertase preparations were 4.5-5.0, 5.5 and 4.0 and 60, 55 and 65°C, respectively. The $K_{M\ app}$ for ρ-nitrophenyl-α-D-galactopyranoside were 1.32 mM and 0.72 mM for P1 and P2, respectively, while the $K_{M\ app}$ value for invertase, using sacarose as a substrate was 15.66 mM. Enzyme preparations P1 and P2 maintained their activities after pre-incubation for 3 h at 50°C and invertase maintained about 90% after 6 h at 55 °C. P1 and P2 presented different inhibition sensitivities by Ag^+, D-galactose, and SDS. All enzyme preparations hydrolyzed galacto-ologosaccharides present in soymolasses.*

Key words: *Aspergillus terreus*, galactooligosaccharides, α-galactosidase, invertase, soy molasses

INTRODUCTION

α-Galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22) catalyzes hydrolysis of terminal non-reducing α-D-galactose residues in oligosaccharides such as melibiose, raffinose and stachyose; polysaccharides, glycolipids and glycoproteins (Varbanets et al., 2001). This enzyme is widely distributed in microorganisms, plants, and animals (Dey et al., 1993).

α-Galactosidases have a number of biotechnological applications. In the sugar beet industry these enzymes are used to remove raffinose from beet molasses and increase sucrose yields (Shibuya et al., 1995); they are also used to improve the gelling properties of galactomannans (Bulpin et al., 1990) and to degrade galacto-oligosaccharides (GO) in food materials such as soy derivatives (Guimarães et al., 2001, Viana et al., 2005; Viana et al., 2007). The GO, especially raffinose and stachyose, are considered the major factors responsible for flatulence and intestinal disturbances in humans and monogastric animals after ingestion of soybeans and other legumes. Enzymatic hydrolysis of GO by α-galactosidases may be an alternative to improve the nutritional quality of soy products for animal and human consumption (de Rezende et al., 2005).

α-Galactosidase has been purified from several microbial sources including *Aspergillus ficcum* (Zapater et al., 1990), *Trichoderma reesei* (Zeilinger et al., 1993) and *A. oryzae* (Prasshanth

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and Mulimani, 2005), with multiple forms of α-galctosidase being reported (Luonteri et al., 1998; Manzanares et al., 1998; Ademark et al., 2001), Although, α-galactosidases are the main enzymes required for GO hydrolysis, and invertases can also be important to increase the hydrolysis yield, since GO are substrates for both the enzymes.

The *Aspergillus* genus has been characterized as a good producer of several hydrolytic enzymes (de Rezende and Felix 1999; Noronha et al., 2002; Lemos et al., 2002; Souza-Mota et al., 2005). *A. terreus*, when grown on wheat straw, secreted multiple forms of α-galactosidase. One α-galactosidase form was partially purified and characterized, showing high hydrolysis GO potential in soy products (Falkoski et al., 2006). In the present study, the partial purification and characterization of new forms of α-galactosidases and invertase from *A. terreus*, aiming to elucidate their use in biotechnological applications was reported.

MATERIALS AND METHODS

Organism growth and enzyme production

Aspergillus terreus CCT 4083 and *Monascus rubber* were obtained from the André Toselo Tropical Research Foundation, Campinas–SP, Brazil, and *Aspegillus versicolor* was obtained from the Bromatology Laboratory's culture collection, DBB, Federal University of Viçosa, Viçosa, MG, Brazil.

The stock cultures were maintained on potato dextrose agar media at 4°C. In order to select the carbon source, spores (10^7/mL) were transferred to 25 mL of liquid medium containing (in g/L) 7.0 KH_2PO_4, 2.0 K_2HPO_4, 0.1 $MgSO_4.7H_2O$, 1.0 $(NH_4)_2SO_4$, 0.6 yeast extract, and 1% (w/v) of D-galactose (Sigma Chemical Co., St. Louis, MO) or wheat straw. After incubation at 28°C, 150 rpm for 24-240 h, the culture supernatants were collected by filter paper filtration.

The enzyme was produced in 5 L of the wheat straw medium. After 168 h of growth at 28 °C, the culture was filtered and the supernatants containing α-galactosidase activity were concentrated, approximately 10-fold, by lyophilization (de Rezende et al., 2005).

Enzyme assay

α-Galactosidase was assayed using ρ-nitrophenyl--α-D-galactopyranoside, ρNPGal, (Sigma) or other synthetic substrates, according to Falkoski et al., (2006). The reaction was carried out for 20 min at 40 °C, and ended by the addition of 1 mL 0.5 M Na_2CO_3. The amount of ρ-nitrophenol (ρNP) released was determined by spectrometry measurement at 410 nm. Invertase was assayed using sucrose, according de Rezende and Felix (1999). The reaction was carried out for 30 min at 40 °C using a reaction mixture containing 650 µL of 0.1 M sodium acetate buffer (pH 5.0) 100 µL of enzyme solution and 250 µL of sucrose. The amount of reducing sugar produced was determined according to Miller (1956).The activities against raffinose, stachyose, guar and locust bean gum were assayed for 30 min at 40 °C using a reaction mixture containing 650 µL of 0.1 M sodium acetate buffer (pH 5.0), 100 µL of enzyme solution and 250 µL of substrate solutions. The amount of reducing sugar produced was determined as above. The hydrolysis of melibiose, maltose, and lactose activities were evaluated by the glucose-oxidase method (Stemberg et al., 1970).

Protein Concentration

The protein concentration in the enzymatic samples was determined according to Bradford, (1976), with bovine serum albumin as the standard.

α-Galactosidase purification

The *A. terreus* culture supernatant was concentrated by lyophilization and chromatographed in a Sephacryl S-200 (Amersham Biosciences, Uppsala, Sweden) column (87.5 x 2.5 cm), equilibrated and eluted with 25 mM sodium acetate buffer, pH 5.0. Proteins were eluted at a flow rate of 20 mL/h and 4 mL fractions were collected. The active S2 protein fraction was pooled and concentrated using an Amicon ultrafiltration cell model 8400 (Bedford, MO) with a 10 kDa molecular cutoff membrane. The concentrated fraction was loaded in a Phenyl-Sepharose (Amersham Biosciences) column (20 x 2.0 cm), equilibrated with 50 mM sodium acetate buffer (pH 5.5), containing 1 M $(NH_4)_2SO_4$, and eluted with a negative linear

gradient consisting of 40 mL of the equilibration buffer and 40 mL of the equilibration buffer without ammonium sulfate. The proteins were eluted at a flow rate of 40 mL/h and active protein fractions were then pooled and concentrated by ultrafiltration. All purification procedures were performed at 4°C.

Effect of pH and temperature

The effect of pH and temperature on α-galactosidase activity was studied using the standard assay, including different McIlvaine buffer (McIlvaine, 1921) solutions (pH 3.0-7.0) at 40°C, and different incubation temperatures (35-70°C), pH 5.0. Thermal stability was evaluated by pre-incubation of 100 µL enzyme solutions and 650 µL 0.1 M sodium acetate buffer (pH 5.0) at 45°C for 0-3 h. After pre-incubation, 250 µL of 2 mM ρNPGal was added and the enzyme activity was determined.

Determination of Kinetic Parameters

The $K_{M\ app}$ and $V_{max\ app}$ values for ρNPGal and sucrose were calculated from the Michaelis Menten plot. Kinetic experiments were performed at 40°C, pH 5.0, with variable substrates concentrations.

Effect of ions, simple sugars and reducing agents

Enzyme samples were pre-incubated with each of the effectors (10 mM) in 0.1 M sodium acetate buffer (pH 5.0) for 15 min at 40°C. After pre-incubation, the effect of the compounds on the enzyme activity was determined using the standard assay.

Substrate Specificity

The reaction mixtures contained 680 µL of 0.1 M sodium acetate buffer (pH 5.0), 70 µL of enzyme solution (6.1 µg to P1 and 4.6 µg to P2), and either 250 µL of synthetic substrates (2 mM), or lactose, maltose and sucrose (10 mM), or raffinose, stachyose or melibiose (40 mM), or guar and locust bean gum solutions (1% w/v). The activities were measured under standard assay conditions at 40 °C. The data presented for all enzyme activities determined are mean values ± SD of the three measurements.

Enzymatic treatment of soy-molasses

Samples of soy molasses were mixed with distilled water (1:10 w/v). The mixture (10 g) was incubated for differing time periods several times at 40 °C, with an enzymatic preparation containing 8 U of α-galactosidases (P1 + P2) and 10 U of invertase. The control assay was carried out replacing the enzymatic preparation with water. Sugar hydrolysis efficiency was estimated by reducing sugar production (Miller, 1956).

Thin Layer Chromatography

A 0.25 mL sample from the soy molasses treated with enzymes for 4 h (reaction described above) was mixed with 0.3 mL of 80 % ethanol solution, stirred on a vortex for 10 s and centrifuged at 16,100 x g for 10 min. The supernatant (30 µL) was submitted to thin layer chromatography (TLC). TLC was performed on precoated silica gel plates (Sigma) at room temperature in a saturated chamber containing the solvent system *n*-propanol:acetic acid:water (1:1:0.1, v/v/v). The TLC plates were sprayed with absolute ethanol containing 1% α-naphthol (w/v) and 10% *ortho*-phosphoric acid (v/v) and placed in an oven at 140ºC for 5 min.

RESULTS AND DISCUSSION

Extracellular α-galactosidases were produced by *A. terreus* and *A. versicolor* in a medium containing wheat straw as carbon source (Table 1). α-Galactosidase activities were first detected in the *A. terreus* and *A. versicolor* cultures after 48h and 96h, respectively. Maximum extracellular α-galactosidase activity in *A. terreus* culture containing wheat straw was detected after 168 h of growth. According to these results, wheat straw was a good inducer of α-galactosidase in the *Humicola* sp (Kotwal et al., 1998). Although D-galactose requirements for α-galactosidase induction has been described in *A. nidulans*, *Bacillus stearothermophillus*, and *A. fumigatus* (Ríos et al., 1993; Gote et al., 2004 and de Rezende et al., 2005), in the fungi studied, D-galactose did not induce α-galactosidase activity. In *M. rubber*, D-galactose and wheat straw were readily converted into mycelial mass, but did not induce production of extracellular α-galactosidase.

The elution profile of lyophilized *A. terreus* supernatant culture grown on wheat straw for 168 h at 28 C, evaluated by Sephacryl S-200 chromatography showed three peaks of α-galactosidase activity, which were designated S1

(fractions 38-50) S2 (fractions 54-68) and S3 (fractions 80-96) (Fig. 1A).

The S2 enzymatic preparation showed the highest α-galactosidase activity and a final specific activity of 3.28 mM min^{-1} mg^{-1} (Table 2). S1 and S2 preparations presented α-galactosidase and invertase activity as shown in Figure 1A. Further chromatography of the S2 preparation in a Phenyl Sepharose column resulted in two main peaks P1 (fractions 48-60) and P2 (fraction 68-100), showing α-galactosidase activity (Fig. 1B). After concentration, the enzyme P1 and P2 presented α-galactosidase specific activity of 8.77 and 10.21 mM min^{-1} mg^{-1}, respectively (Table 2).

Even after gel filtration and hydrophobic interaction chromatographies, the P1 and P2 preparations presented invertase activity. Co-purification of α-galactosidases and invertase suggested that these enzymes presented similar molecular properties of size and hydrofobicity. Similarly, extracellular α-galactosidase from the culture filtrate of *A. flavipes* grown in melibiose was co-purified with invertase, after hydroxylapatite and DEAE-cellulose chromatographies (Ozsoy and Berkkan, 2003). The presence of invertase activity in the α-galactosidase preparations could contribute to the complete hydrolysis of the galactooligosaccharides (GO), because they are substrates for both enzymes.

Table 1 - α-Galactosidase specific activity in the culture medium of *Aspergillus terreus*, A*spergillus versicolor* and *Monascus ruber*, cultivated on D-galactose or wheat straw as carbon source, at 28 ºC.

Growth time (h)	*A. terreus*		*A. versicolor*		*M. rubber*	
	Gal	w. straw	Gal	w. straw	Gal	w. straw
	α-Galactosidase ($mM.min^{-1}.mg^{-1}$)					
24	0	0	0	0.003	0	0
48	0	3.15 ± 0.14	0	0.094	0	0
72	0.02	3.55 ± 0.21	0	0.56 ± 0.07	0	0.01
96	1.49±0.06	3.29 ± 0.18	0	1.03 ± 0.06	0	0.01
120	1.45±0.05	3.88 ± 0.16	0.22±0.012	1.62 ± 0.11	0	0.03
144	0.93±0.03	5.54 ± 0.22	0.33±0.016	2.81 ± 0.09	0	0.05
168	0.71±0.02	6.81± 0.17	0.45±0.018	3.41 ± 0.15	0	0.08
192	1.24±0.02	4.03 ± 0.14	0.18±0.006	1.36 ± 0.12	0	0.10
216	1.12±0.04	4.82 ± 0.18	0.09±0.014	1.76 ± 0.08	0	0.20
240	1.43±0.08	4.929 ± 0.21	0.13±0.005	1.69 ± 0.07	0.03	0.18

Table 2 - Summary of the purification steps for α-galactosidases from *Aspergillus terreus*.

Purification step	Total protein (mg)	Total activity ($mM\ min^{-1}$)	Specific activity ($mM\ min^{-1}mg^{-1}$)	Purification (fold)	Recovery (%)
Crude extract	16.10	53.60	3.33	1	100
Sephacryl S-200					
S1	0.69	9.75	14.10	4.20	18.20
S2	8.39	27.55	3.28	0.98	51.40
S3	2.25	5.63	2.50	0.75	10.50
Phenyl-Sepharose					
P1	0.99	8.69	8.77	2.62	16.23
P2	1.13	11.54	10.21	3.07	21.53

The optimal pH of P1 was in the range 4.5-5.0 while the P2 sample showed higher activity at pH 5.5. This optimum pH value of P1 was close to those for the α-galactosidases from several *Aspergillus* genera (de Rezende et al. 2005, Prasshanth and Mulimani, 2004). On the other hand, another α-galactosidase purified from *A. terreus* culture showed maximal activity at pH 4.0

(Falkoski et al., 2006). Generally, fungal and yeast α-galactosidases have optimal pH values in the acidic range from 3 to 5 (Ulezlo, 1982). The study of the effect of pH on P1 and P2 α-galactosidases showed that at pH 6.0, these enzymes retained about 60 and 80% of their activities, respectively. Since the natural pH of the soymilk is 6.2-6.4, these α-galactosidases could be used for GOS hydrolysis in soymilk.

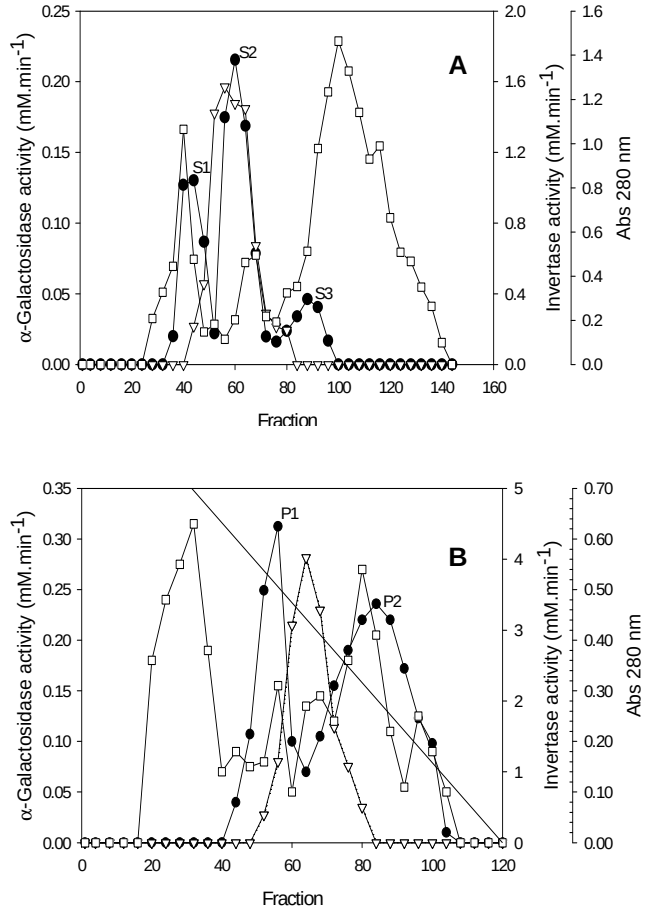

Figure 1 - Evolution profile of α-galactosidase and invertase activities from *Aspergillus terreus* in a (A) Sephacryl S-200 column and (B) Phenyl-Sepharose column. α-Galactosidase activity (●); invertase activity (∇), protein (□); and gradient of NaCl (─).

These enzyme preparations showed maximal invertase activity at pH 4.0 and at pH 6.0 only 40% of invertase activity was maintained. The P1 enzyme preparation exhibited maximum α-galactosidase activity at 60 °C while the optimum temperature of the P2 preparation was 55 °C. Maximum invertase activity was detected at 65°C. Thermostability is an important parameter for industrial applications of enzymes and the P1 α-galactosidase proved to be more thermostable than P2. The P1 enzyme preparation maintained about 100 % of its original activity after 3 h at 55 °C. When the α-galactosidase P1 was incubated at 60 °C, about 60 % of its original activity was maintained after 2.5 h (Figure 2A). The P2 α-galactosidase maintained about 90 and 30 % of its activity after 3 h of incubation at 50 and 55 °C, respectively. At 60 °C, its activity was completely lost after 90 min of incubation (Figure 2B). The half-life of the P1 α-galactosidase at 60 °C was 152.6 min, while the P2 preparation at 55 and 60°C showed half-life values of 80 and 32 min, respectively. These $t_{1/2}$ values were higher than those reported for soybean seeds α-galactosidases (Guimarães et al., 2001, Viana et al., 2005), but lower than those reported for S1 α-galactosidase

from *A. terreus* (Falkoski et al., 2006) and other microbial α-galactosidases (Duffaud et al., 1997; Viana et al, 2006). Invertase maintained about 90 % of its activity after 6 h at 55 °C, but its activity was lost after 2.5 h at 65 °C (Figure 2C).

The $K_{M\ app}$ values for the P1 and P2 α-galactosidase preparations, using ρNPGal, were 1.32 and 0.72 mM, respectively. These K_M values were comparable to those determined for hydrolysis of the same substrate by α-galactosidases purified from *A. fumigatus* (de Rezende et al., 2005) and *Penicillium* sp. 23 (Varbanets et al., 2001). The $K_{M\ app}$ value for invertase in these enzyme preparations, using sacarose as substrate, was 15.66 mM.

The enzymatic preparations P1 and P2 showed different sensitivities to simple sugars and mono and bivalent ions (Table 3).

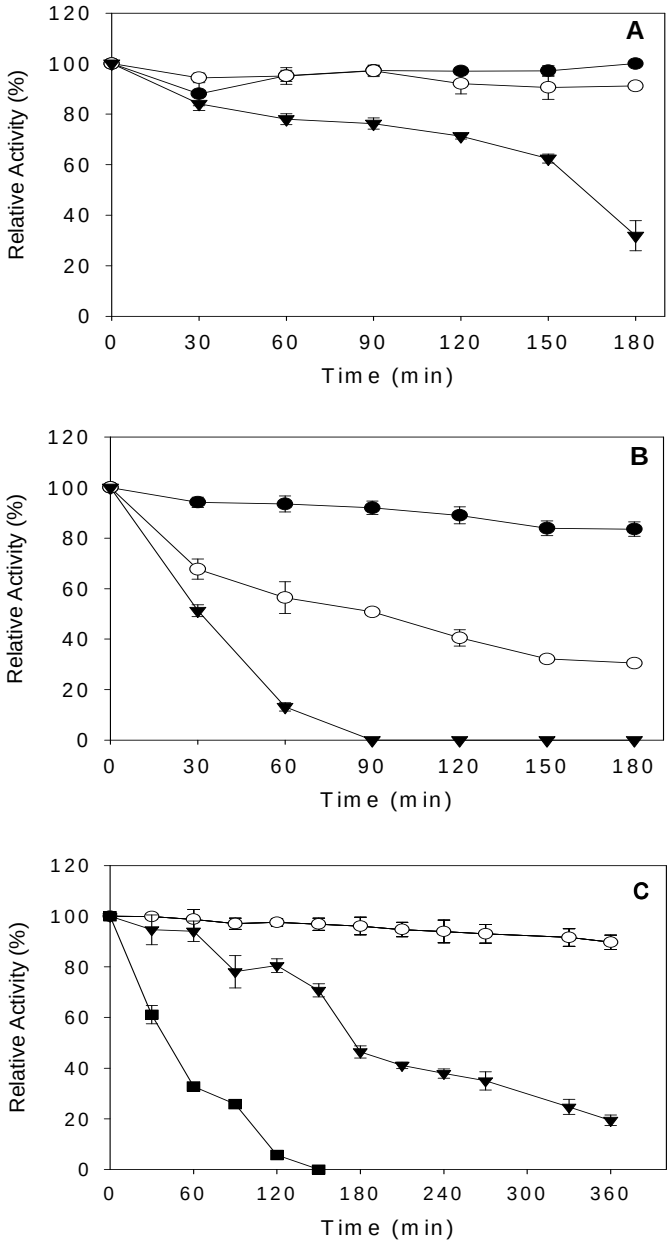

Figure 2 - Thermal stability of the α-galactosidases P1 (A), P2 (B) and invertase (C) from *Aspergillus terreus.* The samples P1 and P2 were pre-incubated for various time periods at 50 (●), 55 (○) and 60 (▼) °C and the residual α-galactosidase activity was determined by standard assay. The residual invertase activity was determined in the samples after pre-incubation at 55 (○), 60 (▼) and 65 °C (■)

The α-galactosidase in P2 was highly inhibited by SDS and Ag^+, while the P1 enzyme preparation was only partially inhibited by Ag^+ and showed very low inhibition by SDS. Reduction in the α-galactosidase activity by Ag^+ was reported for α-galactosidase purified from *B. stearothermophilus* NCIM 5146 (Gote et al., 2006). Participation of carboxyl and/or histidine imidazolium groups in the catalytic action was considered for the inhibitory effect (Dey and Pridham, 1972). The ionic detergent SDS is an extremely effective denaturing agent for proteins; in its presence most proteins lose their functionality either completely or partially with the disruption of tertiary and quaternary structures (Bischoff et al., 1998). It could be suggested that P1 and P2 α-galactosidase preparations did not have any metal cofactor requirements and that the sulfhydryl groups did not take part in catalysis, since there was no enzyme inhibition when applying EDTA or iodoacetamide treatments, respectively. The P1 enzyme was partially inhibited by Mn^{2+} and Cu^{2+}, but Mn^{2+} did not has any effect on the P2 α-galactosidase activity. D-galactose, the product of the reaction catalyzed by α-galactosidase on natural substrates, has been demonstrated to be a competitive inhibitor of several α-galactosidases (Guimaraes et al, 2001; Viana et al., 2006). The P1 α-galactosidase preparation was slightly inhibited by this sugar, but the P2 enzyme presented little inhibition from D-galactose. Invertase was completely inhibited by Cu^+ and Ag^+, and showed 50% inhibited by SDS. EDTA, β-mercaptoethanol and KCl promoted weak inhibitory effects on invertase activity (Table 3).

Table 3 - Effect of simple sugars, ions and reducing agents on the activity of the invertase and α-galactosidases of P1 and P2 from *Aspergillus terreus.*

Effector	**Relative activity (%) ± SD**		
	Invertase	**α-galactosidase P1**	**α-galactosidase P2**
---	100.0 ± 1.6	100.0 ± 1.2	100.0 ± 2.7
$CaCl_2$	93.7 ± 2.16	95.8 ± 1.0	105.5 ± 1.2
$MnCl_2$	92.7 ± 5.6	70.7 ± 0.8	103.9 ± 3.2
SDS	48.1 ± 2.5	94.7 ± 1.1	9.6 ± 0.9
NaCl	97.6 ± 2.2	102.5 ± 3.5	108.3 ± 2.7
$CuSO_4$	0	72.7 ± 2.8	84.5 ± 1.6
KCl	72.9 ± 2.9	98.8 ± 1.4	102.0 ± 1.7
Iodoacetamide	---	103.0 ± 0.9	101.1 ± 0.5
$AgNO_3$	0	52.6 ± 1.8	3.6 ± 2.7
β-mercaptoethanol	63.8 ± 3.5	107.2 ± 3.5	99.6 ± 3.7
EDTA	66.8 ± 6.4	102.3 ± 2.7	107.1 ± 2.2
D-Galactose	---	34.0 ± 0.7	67.9 ± 2.6

The final concentrations of all the effectors were 2 mM.

Under the experimental conditions, enzyme preparations P1 and P2 were more effective for raffinose and stachyose hydrolysis, followed by the synthetic substrates *ρ*NPαGal and *o*NPαGal (Table 4). Similar results were observed in previous study using *G. lucidum* α-galactosidase, which hydrolyzed raffinose, stachyose, *ρ*NPαGal and *o*NPαGal (Sripuan et al., 2003).

The more complete hydrolysis of raffinose and stachyose compared to *ρ*NPαGal, which is a specific substrate for α-galactosidases, could be explained by the presence of invertase in the enzyme preparations. These results suggested that the combined actions of invertase and α-galactosidase on the GOS showed to be more effective than only α-galactosidase action. Lactose and other synthetic substrates containing β-linkages or containing xylose, arabinose, and mannose residues were not hydrolyzed by these enzyme preparations. The expressive hydrolysis of sucrose by both P1 and P2 confirmed the presence of invertase in the enzyme preparations (Table 4). The preparations also exhibited the ability to hydrolyze polymers such as guar and locust bean gum. Accordingly, Gote et al., (2006) showed that α-galactosidase from *B. stearothermophilus* preferentially hydrolyzed stachyose and raffinose when compared to locust bean gum and guar gum polymers.

The combined effect from both α-galactosidases (P1 e P2) and invertases on GO present in soy-molasses was tested (Fig. 3).

Table 4 - Hydrolysis of several substrates with P1 and P2 enzyme preparations from *Aspergillus terreus.*

Substrate	Concentration[a]	Activity (mM min^{-1}) ± SD.	
		α -galactosidase P1	α -galactosidase P2
*ρ*NP α Gal[b]	0.5	0.24 ± 0.01	0.43 ±0.01
*o*NP α Gal[b]	0.5	0.11 ± 0.01	0.13 ± 0.01
*o*NP β Gal[b]	0.5	0	0
*ρ*NP α Glc[b]	0.5	0	0
*m*NP α Gal[b]	0.5	0	0
*ρ*NP β Gal[b]	0.5	0	0
*ρ*NP α Man[b]	0.5	0	0
*ρ*NP α Ara[b]	0.5	0	0
*ρ*NP β Xyl[b]	0.5	0	0
Sucrose	2.5	0.67 ± 0.01	0.79 ± 0.01
Raffinose	10	0.62 ± 0.02	0.65 ± 0.01
Stachyose	10	0.31 ± 0.01	0.30 ±0.01
Melibiose	10	0.01 ± 0.00	0.02 ± 0.01
Maltose	2.5	0.01 ± 0.00	0.02 ± 0.00
Lactose	2.5	0	0
Locust bean gum	0.5	0.10 ± 0.01	0.12 ± 0.01
Guar gum	0.5	0.10 ± 0.01	0.11 ± 0.01

[a] Final concentrations in mM, except for locust bean gum and guar gum (%). [b] *ρ*NPαGal, *para*-nitrophenyl-α-D-galactopyranoside, *o*NPαGal, *ortho*-nitrophenyl-α-D-galactopyranoside, *o*NPβGal, *ortho*-nitrophenyl-β-D-galactopyranoside, *ρ*NPαGlc, *para*-nitrophenyl-α-D-glucopiranoside, *m*NPαGal, *meta*-nitrophenyl-α-D-galactopyranoside, *ρ*NPβGal, *para*-nitrophenyl-β-D-galactopyranoside, *ρ*NPαMan, *para*-nitrophenyl-α-D-mannopyranoside, *ρ*NPαAra, *para*-nitrophenyl-α-D-arabinopyranoside *ρ*NPβXyl, *para*-nitrophenyl-β-D-xylopyranoside.

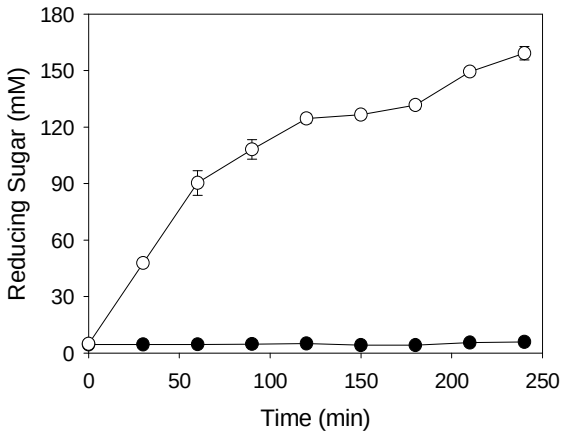

Figure 3 - Reducing sugar production during hydrolysis of soy molasses by *Aspergillus terreus* α-galactosidase (○). Soybean molasses mixed with water (1:10 w/v) (10 g) was incubated with an enzyme preparation containing 8 U of α-galactosidases and 10 U of invertase, at 40 °C, for 0, 30, 60, 90, 120, 150, 180, 210 and 240 min. For the control assay (●), the enzyme preparation was replaced with distilled water.

The amount of the reducing sugar in the control reactions did not change, in which the enzyme preparation was replaced by water. The maximum hydrolytic activity was observed during the first 60 min of treatment, since the reducing sugar content increased from 0 to about 90 mM. After 4 h of treatment, the reducing sugar content was approximately 150 mM. GOS hydrolysis in

soybean molasses was confirmed by Thin Liquid Chromatography (Fig. 4).

TLC analysis of a soy molasses sample treated with the enzymatic preparation for 4 h at 40 °C indicated the complete hydrolysis of stachyose and raffinose. As the enzyme preparations from *A. terreus* showed invertase activity, invertase and α-galactosidase probably acted synergistically, especially when using GOS, which were fully hydrolyzed. Complete GOS hydrolysis can be achieved by either α-galactosidase, invertase, or both. α-Galactosidase hydrolyzes the α-1.6 linkage of raffinose and produces galactose and sucrose, invertase hydrolyzes the β-1.2 linkage and produces melibiose and fructose (Guimaraes et al., 2001). On the other hand, the presence of sucrose after the enzymatic treatment (Fig. 4, line 3) may be due to the fact that sucrose is released after GOS hydrolysis by α-galactosidase. In agreement with other studies (Ozsoy and Berkkan, 2003; Falkoski et al., 2006), results indicated that the simultaneous use of both enzymes for GOS hydrolysis could be a more effective alternative in industrial applications.

The α-galactosidases and invertases described in this work could be interest because of their potential to convert GOS present in soybean products into digestible sugars. In general, the enzymes suggested for this purpose are of microbial origin and present the disadvantage of having no GRAS (generally recognized as safe) status. However, the fungal enzymes are relatively easy to produce and are suitable for most technological applications, mainly due to their extracellular localization, optimal acidic pH, and broad stability profiles. Therefore, the genes encoding these enzymes could be cloned and overexpressed in suitable organisms to produce the enzymes at low cost.

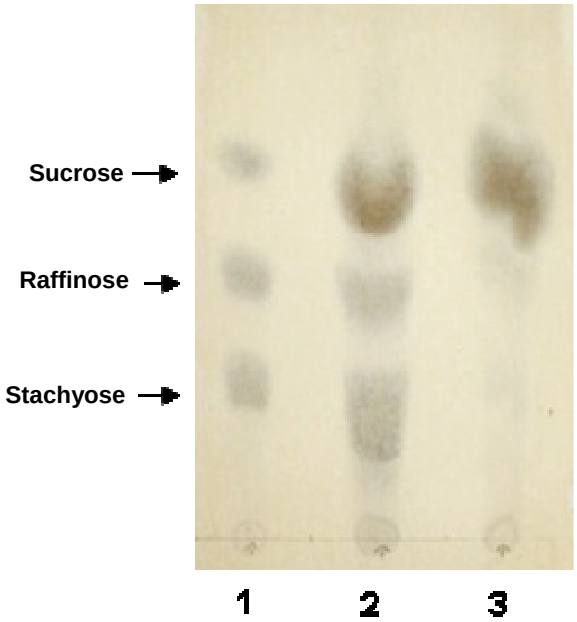

Figure 4 - TLC analysis of sugars present in soy molasses treated with an enzymatic preparation of *Aspergillus terreus.* Soybean molasses mixed with water (1:10 w/v) (10g) was incubated with the enzymatic preparation containing 8 U of α-galactosidase and 10 U of invertase, at 40 °C, for 4 h. Lane 1: standard mixture of sugars; Lane 2: untreated soy-molasses; Lane 3: soy-molasses treated with enzymatic mixture.

ACKNOWLEDGEMENTS

This study was supported by grants from the Fundação de Amparo a Pesquisa do Estado de Minas Gerais – FAPEMIG and Coordenação de Aperfeiçoamento de Pessoal de Nivel Superior – CAPES, Brazil.

RESUMO

Duas α-galactosidases (P1 e P2) e uma invertase produzidas no sobrenadante da cultura do fungo *Aspergillus terreus* quando crescido por 168 h a 28°C com farelo de trigo como fonte de carbono foram parcialmente purificadas por cromatografias de gel filtração e interação hidrofóbica. O pH e temperatura ótimos para as preparações P1, P2 e invertase foram entre 4,5-5,0, 5,5 e 4,0 e 60, 55 e 65°C, respectivamente. O $K_{M\ app}$ para ρ-nitrofenil-α-D-galactopiranosideo foi 1.32 mM e 0.72 mM para P1 e P2, respectivamente. O valor de $K_{M\ app}$ para invertase usando sacarose como substrato foi de 15,66 mM. As preparações enzimáticas P1 e P2 mantiveram suas atividades após 3 h de pré-incubação a 50 °C e a invertase manteve cerca de 90% após 6 h a 55 °C. P1 e P2 foram diferentemente sensíveis à inibição por Ag^{+}, D-galactose e SDS. As preparações enzimáticas hidrolisaram os galactooligossacarídeos presentes em melaço de soja.

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Received: November 05, 2007;
Revised: May 13, 2008;
Accepted: May 19, 2009.