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Processing of Soybean Products by Semipurified Plant and Microbial α-Galactosidases

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Galactooligosaccharides (GO) are responsible for intestinal disturbances following ingestion of legume-derived products. Enzymatic reduction of GO level in these products is highly desirable to improve their acceptance. For this purpose, plant and microbial semipurified α-galactosidases were used for GO hydrolysis in soybean flour and soy molasses. α-Galactosidases from soybean germinating seeds, *Aspergillus terreus*, and *Penicillium griseoroseum* presented maximal activities at pH 4.0−5.0 and 45−65 °C. The $K_{M,app}$ values determined for raffinose by the soybean, *A. terreus*, and *P. griseoroseum* α-galactosidases were 3.44, 19.39, and 20.67 mM, respectively. The enzymes were completely inhibited by Ag^{+} and Hg^{2+}, whereas only soybean enzyme was inhibited by galactose. *A. terreus* α-galactosidase was more thermostable than the enzymes from the other two sources. This enzyme maintained about 100% of its original activity after 3 h at 60 °C. The microbial α-galactosidases were more efficient for reducing GO in soybean flour and soy molasses than soybean enzyme.

KEYWORDS: Soybean; α-galactosidase; galactooligosaccharides; *Aspergillus*; *Penicillium*

INTRODUCTION

Human consumption of soy products is increasing, not only because of their high nutritional value but also in view of their health-promoting effects, such as reduction in cardiovascular disease, osteoporosis, and cancer risks (*1*). The consumption is limited partially due to the presence of α-galactooligosaccharides (GO) such as melibiose, raffinose, stachyose, and verbascose in soybean. Because humans and monogastric animals lack the enzyme α-galactosidase (EC 3.2.1.22, α-D-galactoside galactohydrolase), necessary to hydrolyze the α-linkages in these sugars, they pass on intact into the large intestine, where anaerobic microorganisms ferment them and cause gastrointestinal disturbances (*2*). Many attempts have been proposed to reduce soy GO contents through dehulling, soaking, cooking, γ irradiation, aqueous or alcoholic extraction, germination, and fermentation (*3*, *4*). However, the enzymatic processing of soy-derived products seems to be the most rational and effective alternative. The nutritional value of soy foods could be upgraded by procedures using microbial or plant α-galactosidases to hydrolyze the α-galactosides prior to consumption (*5*, *6*). The use of plant α-galactosidases for GO reduction in soy products, particularly from soybean sources, is advantageous because this enzyme is uniquely suited for the high-protein and strongly buffered environment of soybean products (*7*). On the other hand, fungal α-galactosidases are generally secreted in the culture facilitating the purification process. In addition, they present stability over a wide range of pH values and temperatures (*8*).

In the present investigation we describe the purification and characterization of α-galactosidases from soybean seeds and from the fungi *Aspergillus terreus* and *Penicillium griseoroseum* and the hydrolysis of soybean flour and soy molasses by these enzymes. Our long-term goal is to select efficient α-galactosidase sources for technological use.

MATERIALS AND METHODS

Enzyme Sources. Soybean seeds (*Glycine max* L. Merr. cv. Monarca) were provided by the soybean breeding program of BIOAGRO, Federal University of Viçosa, MG, Brazil. *A. terreus* was obtained from the André Toselo Tropical Research Foundation, Campinas, SP, Brazil. *P. griseoroseum* was obtained from the Fungal Collection of the Microbiology Department of the Federal University of Viçosa, MG, Brazil.

α-Galactosidase Production. Soybean seeds were germinated on water-soaked filter paper for 60 h at 27 °C and then kept at −20 °C. The pregerminated seeds (800 g of fresh weight) were ground in a blender and resuspended in 1.7 L of solution containing 0.1 M citric acid and 0.05 M sodium phosphate, pH 5.0, and kept under agitation for 1 h at 4 °C. The suspension was filtered through cheesecloth, and the filtrate was centrifuged at 15300*g* for 40 min at 4 °C. The supernatant was used as a crude enzyme preparation.

The stock cultures of *A. terreus* and *P. griseoroseum* were maintained on potato dextrose agar medium (PDA). For enzyme production, spores (10^7/mL) were transferred to 1.0 L of liquid medium containing (in g/L) KH_2PO_4, 7.0; K_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; yeast extract, 0.6; and wheat straw or locust bean gum, 10, for *A. terreus* and *P. griseoroseum*, respectively. The wheat straw was obtained from a local market, and locust bean gum was purchased from Sigma

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Chemical Co. (St. Louis, MO). Cultures were incubated under constant agitation at 120 rpm for 168 h at 28 °C (*A. terreus*) or for 132 h at 28 °C (*P. griseoroseum*). Culture filtrates were collected by filtration through filter paper, concentrated by lyophilization, and kept at −20 °C until use.

α-Galactosidase Purification. The crude soybean extract was kept at −20 °C for 24 h, thawed, and centrifuged at 15300*g* for 35 min at 4 °C. The pH of the supernatant was lowered to 4.0 with citric acid. The solution was stirred for 30 min at 4 °C and centrifuged as described above. The pH of the supernatant was adjusted to 5.0 with a NaOH saturated solution and then submitted to fractionation with 20−50% $(NH_4)_2SO_4$ (*7*). Protein precipitated at 50% saturation, which contained the α-galactosidase activity, was resuspended in 25 mM sodium acetate buffer, pH 5.0, and loaded onto a Sephadex G-100 (Amersham Biosciences, Uppsala, Sweden) column (95 × 2.5 cm) equilibrated with 25 mM sodium acetate buffer, pH 5.0. The proteins were eluted with the same buffer, and the fractions containing α-galactosidase activity were pooled and chromatographed on a CM-Sepharose Fast Flow (Amersham Biosciences) column (14 × 2.9 cm) equilibrated with 50 mM sodium acetate buffer, pH 5.0. The proteins were eluted with a linear gradient consisting of 200 mL of 50 mM sodium acetate buffer, pH 5.0, and 200 mL of the same buffer containing 0.8 M NaCl. Active protein fractions were pooled and concentrated by the use of an Amicon ultrafiltration cell model 8400 (Bedford, MA) with a 10 kDa molecular cutoff PM 10 Amicon membrane.

The *A. terreus* culture supernatant was concentrated by lyophilization and chromatographed on a Sephacryl S-200 (Amersham Biosciences) column (100 × 2.5 cm), equilibrated and eluted with 25 mM sodium acetate buffer, pH 5.0. The active protein fraction was concentrated by ultrafiltration using a PM 10 Amicon membrane filter. The concentrated fraction was loaded on a phenyl-Sepharose (Amersham Biosciences) column (15 × 2.9 cm) equilibrated with 25 mM sodium acetate buffer, pH 5.0, containing 1 M $(NH_4)_2SO_4$, and eluted by a negative linear gradient consisting of 40 mL of the equilibration buffer and 40 mL of the equilibration buffer without ammonium sulfate. Active protein fractions were pooled and concentrated by ultrafiltration using a PM 10 Amicon membrane filter.

The *P. griseoroseum* culture supernatant was submitted to fractionation with 40−100% $(NH_4)_2SO_4$. Protein precipitated at 100% saturation containing α-galactosidase activity was resuspended in 25 mM sodium acetate buffer, pH 5.0, and loaded onto a Sephacryl S-200 column (100 × 2.5 cm) equilibrated with 25 mM sodium acetate buffer and eluted with the same buffer. Active protein fractions were pooled and concentrated by ultrafiltration using a PM 10 Amicon membrane filter. All chromatography procedures were performed at 4 °C.

α-Galactosidase Assay. α-Galactosidase was assayed as described previously (*5*). The reaction mixture contained 650 μL of 0.1 M sodium acetate buffer, pH 5.0, 100 μL of enzyme solution, and 250 μL of 2 mM *p*-nitrophenyl-α-D-galactopyranoside (ρNPGal) or other synthetic substrates. The reaction was run for 20 min at 40 °C and ended by the addition of 1 mL of 0.5 M sodium carbonate. The amount of *p*-nitrophenol (pNP) released was determined at 410 nm. This procedure was defined as the standard assay.

The activities against melibiose, maltose, and lactose were evaluated according to the glucose−oxidase method (*9*). When sucrose, raffinose, and stachyose were used as substrate, the production of reducing sugars was determined using the 3,5-dinitrosalicylate reagent (*10*).

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) at 40 °C (*11*) under otherwise standard enzyme assay conditions. The optimum temperature was determined within the temperature range of 35−70 °C, at pH 5.0. Thermal stability was evaluated by preincubating 100 μL of enzyme solutions with 650 μL of 0.1 M sodium acetate buffer, pH 5.0, for different times and temperatures. Soybean and *P. griseoroseum* enzymes were preincubated at 40, 45, and 50 °C for 0−10 and 0−5 h, respectively. *A. terreus* enzyme was preincubated at 55, 60, and 65 °C for 0−36 h. After preincubation, 250 μL of 2 mM pNPGal was added, and the enzyme activity was determined.

Determination of Kinetic Parameters. Kinetic experiments were performed at 40 °C and pH 5.0. The Michaelis−Menten constant ($K_{M,app}$) and $V_{max,app}$ for pNPGal, raffinose, and melibiose hydrolysis were calculated by using the Michaelis−Menten plot. The substrate concentrations ranged from 0.01 to 2.00 mM in the case of pNPGal, from 0.1 to 40.0 mM for raffinose, and from 0.05 to 60.0 mM for melibiose.

Substrate Specificity. Enzymatic assays were performed with various synthetic and natural substrates. The reaction mixtures contained 650 μL of 0.1 M sodium acetate buffer, pH 5.0, 100 μL of enzyme solutions, and 250 μL of synthetic substrates (2 mM), or maltose, melibiose, lactose, and sucrose (16 mM), or raffinose and stachyose (28 mM). The activities were measured under standard assay conditions at 40 °C.

Effect of Ions, Simple Sugars, and Reducing Agents. The enzyme samples were preincubated with each of the compounds (10 mM) in 0.1 M sodium acetate buffer, pH 5.0, for 20 min at 40 °C. After preincubation, the effect of ions, simple sugars, and reducing agents on the enzyme activity was determined according to the standard assay.

Treatment of Soy Products with α-Galactosidase. Commercial defatted flour (Bunge Alimentos, RS, Brazil) (2 g) was mixed with water 1:10 (w/v) and was added with 8 units of α-galactosidases purified from soybean seeds, *A. terreus*, or *P. griseoroseum*. The mixtures were incubated for 0, 4, 6, and 8 h under agitation (100 rpm) at 40 °C. Each reaction mixture was lyophilized, and the soluble sugars were extracted from 30 mg of dried powder with 80% aqueous ethanol (v/v) (*5*). The solvent was evaporated at 45 °C, and the sugars were resuspended in 80% ethanol (1.2 mL) and analyzed by HPLC as described below. Soybean molasses was mixed with water (1:5 w/v), and a 10 g sample was incubated with 32 units of each α-galactosidase at 40 °C for several periods of time. The GO hydrolysis efficiency was estimated by the production of reducing sugars (*10*). One enzyme unit was defined as the amount of enzyme that released 1 μmol of product per minute under the assay conditions.

Determination of GO Content. Sugars 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. The sugars eluted were monitored by a refractive index detector model 6A from Shimadzu. They were automatically identified and quantified by comparison with retention times and known concentrations of the sugar standards sucrose, raffinose, and stachyose, which were purchased from Sigma Chemical Co. (*6*).

Protein Determination. The protein concentration in the enzymatic extract was determined according to the Coomassie Blue binding method with bovine serum albumin (BSA) as standard (*12*).

RESULTS AND DISCUSSION

The α-galactosidases from soybean germinating seeds and those secreted by the fungi *A. terreus* and *P. griseoroseum* were characterized in this work. α-Galactosidase from soybean germinating seeds have been proposed to reduce GOs in soybean-derived products (*5*). Although *A. terreus* and *P. griseoroseum* are not food grade fungi, *A. terreus* is a source of pharmaceutical products (*13*), and *P. griseoroseum* is a potential source of pectinolytic enzymes for the food industry (*14*). Enzyme production was done when α-galactosidase activity was maximum. In soybean germinating seeds this happened 60 h after imbibition (*5*, *6*). For *A. terreus* and *P. griseoroseum* maximum activity was reached after 168 and 132 h of culturing, respectively. These time points were previously determined in our laboratory (data not shown).

Fractionation of crude extract from germinating soybean seeds on gel filtration and ion exchange columns resulted in a 19.7-fold purification with a 36.6% recovery of the enzyme activity (**Table 1**). The α-galactosidases secreted by *A. terreus* were partially purified using gel filtration and hydrophobic chromatography procedures, with a purification factor of 20.2 and

Table 1. Summary of the Steps for α-Galactosidase Purification from Germinating Soybean Seeds Var. Monarca

purification step	total protein (mg)	total activity (mM min^{-1})	specific activity (mM min^{-1} mg^{-1})	purification (fold)	recovery (%)
crude extract[a]	13300.80	366.0	0.03	1.00	100
cryoprecipitation[b]	9036.71	312.6	0.04	1.25	85.4
acid precipitation[c]	5018.22	295.3	0.06	2.14	80.7
$(NH4)_2SO_4$ (20–50%)	990.00	170.3	0.17	6.25	46.5
Sephadex G-100	516.94	155.6	0.30	10.94	42.5
CM-Sepharose	246.84	134.1	0.54	19.74	36.6

[a] Crude extract was prepared from 800 g of pregerminated soybean seeds with 1.7 L of buffer solution. [b] Cryoprecipitation: crude soybean extract was kept at −20 °C for 24 h and then thawed, and the precipitate was removed by centrifugation at 15300*g* for 35 min at 4 °C. [c] Acid precipitation: the pH of the enzyme solution was lowered to 4.0 with citric acid, and the solution was stirred for 30 min at 4 °C and centrifuged as described above.

Table 2. Summary of the Steps for α-Galactosidase Purification 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[a]	16.1	53.6	3.33	1.00	100
Sephacryl S-200	0.69	9.75	14.1	4.20	18.2
phenyl Sepharose	0.11	7.40	67.3	20.20	13.8

[a] The purification process started from 1 L of culture medium.

Table 3. Summary of the Steps for α-Galactosidase Purification from *Penicillium griseoroseum*

purification step	total protein (mg)	total activity (mM min^{-1})	specific activity (mM min^{-1} mg^{-1})	purification (fold)	recovery (%)
crude extract[a]	70.0	170	2.02	1.00	100
$(NH_4)_2SO_4$ (40–100%)	27.0	140	5.67	2.81	82.2
Sephacryl S-200	1.94	108	55.0	27.2	63.1

[a] The purification process started from 1 L of culture medium.

recovery of 13.8% (**Table 2**). *P. griseoroseum* α-galactosidase was purified 27-fold with 63% recovery of the enzyme activity (**Table 3**).

Maximum α-galactosidase activities were detected at pH 5.0 (soybean), 4.0 (*A. terreus*), and 5.0 (*P. griseoroseum*) (**Figure 1A**). Similar optimum pH values were verified for α-galactosidases of other fungi, such as *Thermomyces lanuginosus* (*15*), *Aspergillus niger* (*16*), and *Aspergillus oryzae* (*17*) and for other soybean varieties (*5*, *6*). Optima pH values for crude extracellular α-galactosidase from *A. niger*, *A. oryzae*, *A. awamori*, and *Rhizopus oligosporus* grown in different carbon sources were also in the acidic range (*18*).

The temperature effect on the α-galactosidase activities is shown in **Figure 1B**. For α-galactosidases of germinating soybean seeds, *A. terreus*, and *P. griseoroseum*, highest enzyme activities were detected at 50, 65, and 45 °C, respectively. Optimal temperature values in the range of 50−60 °C were observed for crude extracellular α-galactosidases produced by fungi from four different species (*18*).

The *A. terreus* α-galactosidase was more thermostable than the enzymes from the other two sources (**Figure 2**). No reduction in *A. terreus* α-galactosidase activity was observed after a 24 h period at 55 °C. This enzyme maintained about 100 and 40% of its original activity after 3 and 36 h at 60 °C, respectively (**Figure 2B**). The enzyme showed significant stability for a wide range of temperatures, which is desirable for industrial applications. Four forms of purified *A. niger* ATCC 46890 α-galactosidases were identified, which retained about 95% of the original activity after incubation at 40 °C for 21 h (*16*).

α-Galactosidase from germinating soybean seeds retained 90 and 70% of its original activity after incubation for 4 and 10 h at 40 °C, respectively (**Figure 2A**). These values decreased to 60 and 33%, respectively, when incubation was performed at 45 °C. The half-life ($t_{1/2}$) of this enzyme at 45 °C was 6 h and 36 min. At 50 °C, 91% of its original activity was lost after 30 min of incubation. These $t_{1/2}$ values were higher than those reported for soybean α-galactosidases extracted from other varieties (*5*, *6*).

No loss in enzyme activity was observed after incubation of *P. griseoroseum* α-galactosidase for 5 h at 40 °C. The enzyme maintained about 65% of its original activity after 2 h at 45 °C, but about 90% of the activity was lost during an incubation period of 30 min at 50 °C (**Figure 2C**). The $t_{1/2}$ of *P. griseoroseum* α-galactosidase at 45 °C was 3 h and 27 min. This $t_{1/2}$ value was lower than that reported for α-galactosidase derived from *Bacillus stearothermophilus* NCIM 5146 (*19*).

The $K_{M,app}$ values for pNPGal, melibiose, and raffinose are displayed in **Table 4**. The K_M values determined in this study, using pNPGal as substrate, were similar to those obtained for α-galactosidases of germinating *Cyamopsis tetragonolobus* seeds (*20*), *Trichoderma reesei* RUT C-30 (*21*), and *Penicillium* species 23 (*22*). The K_M values determined with melibiose were close to that of α-gal I of *A. niger* (*16*), but lower than the K_M value observed for α-galactosidase of germinating soybean seeds of the vareity Doko (*5*). The K_M values determined for α-galactosidases of *A. terreus* and *P. griseoroseum* with raffinose, 19.39 and 20.67 mM, respectively, are close to that obtained for α-galactosidase of *B. stearothermophilus* NUB3621 (*23*). However, a lower K_M value, 3.44 mM, was determined, with raffinose, for soybean α-galactosidase (**Table 4**). Lower K_M values indicate that the enzyme has more affinity for the substrate and that the ES complex formation is not a limiting step for the reaction. In practical terms K_M is an important

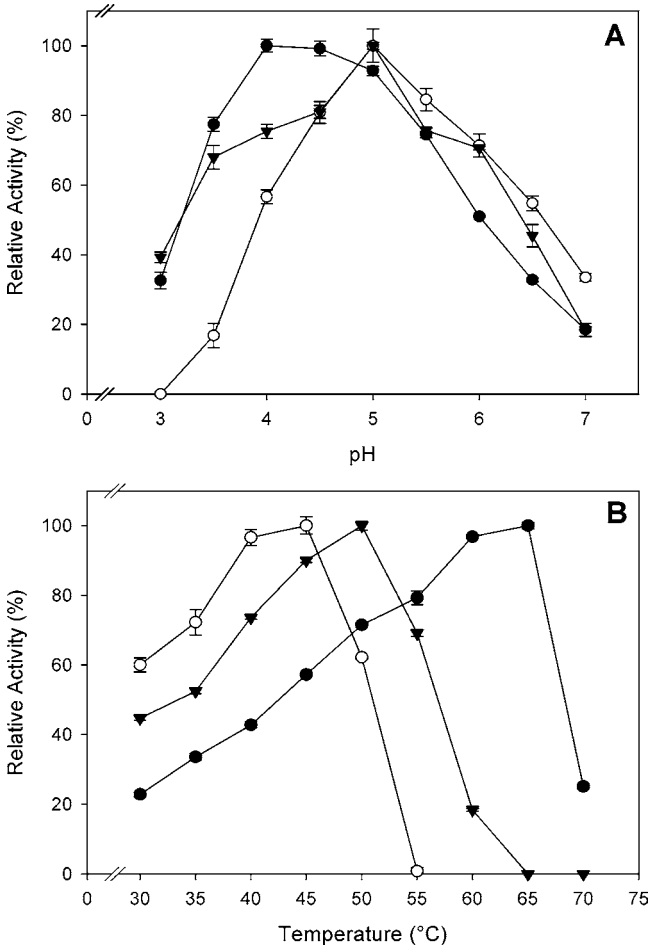

Figure 1. Effects of pH (**A**) and temperature (**B**) on the activity of soybean (▼), *Aspergillus terreus* (●), and *Penicillium griseoroseum* (○) α-galactosidase.

parameter; however, it is not the only characteristic determining enzyme efficiency.

Under the experimental conditions used, the soybean α-galactosidase was more effective in the hydrolysis of pNPαGal, followed by the GO raffinose, stachyose, and melibiose (**Table 5**). On the other hand, the substrate stachyose was more efficiently hydrolyzed than raffinose by enzyme preparations from *A. terreus* and *P. griseoroseum*. The more effective hydrolysis of the synthetic substrate *ρ*NPαGal compared to the natural oligosaccharide hydrolysis was also observed for grape flesh α-galactosidase (*24*). The three preparations containing α-galactosidase activity hydrolyzed raffinose more efficiently than melibiose. Lactose and synthetic substrates containing *β*-linkages or containing xylose, mannose, and glucose residues were poorly hydrolyzed by the enzyme preparations.

The microbial enzyme preparations contained other enzymes such as invertase, as they were able to hydrolyze sucrose (**Table 5**). The presence of invertase activity in the α-galactosidase preparations can contribute to the complete hydrolysis of the oligosaccharides, because these GOs are substrates for both enzymes.

The α-galactosidases differed in sensitivity to simple sugars and mono- and bivalent ions (**Table 6**). The enzymes were little or not at all inhibited by EDTA, Mg(II), iodoacetamide, Na(I), K(I), Ca(II), 2-mercaptoethanol, maltose, lactose, sucrose, D-glucose, and D-mannose. These results suggest that α-galactosidase is not a metalloenzyme and that sulfhydryl groups do not take part in the catalysis. This agrees with results reported

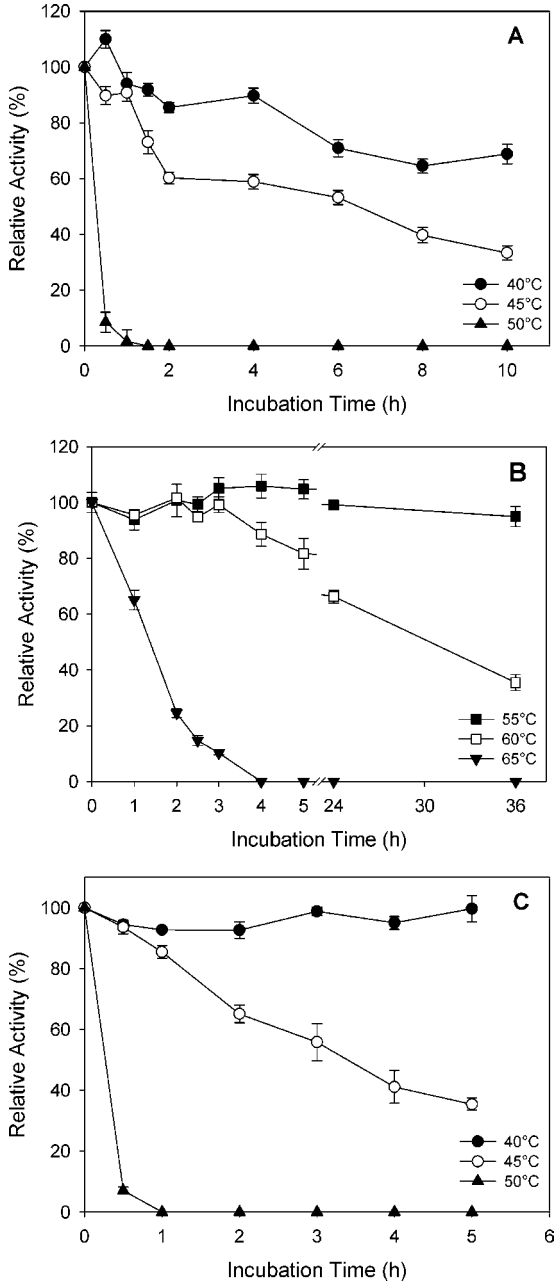

Figure 2. Thermal stability of the α-galactosidases from (**A**) soybean, (**B**) *Aspergillus terreus*, and (**C**) *Penicillium griseoroseum*. The enzyme samples were preincubated for several periods at different temperatures and assayed as described for the standard assay. The activity at 0 min of preincubation was considered to be 100%.

Table 4. $K_{M,app}$ Values Determined by the Michaelis–Menten Plot for α-Galactosidases from Soybean Seeds, *Aspergillus terreus*, and *Penicillium griseoroseum*

	$K_{M,app}$ (mM)		
substrate	soybean	*A. terreus*	*P. griseoroseum*
pNPGal	0.47	1.89	1.32
melibiose	1.47	0.45	2.06
raffinose	3.44	19.39	20.67

for α-galactosidase isolated from *Penicillium* sp. 23 (*22*), grape flesh α-galactosidase (*24*), and soybean α-galactosidase (*5*, *6*). The enzymes from soybean, *A. terreus*, and *P. griseoroseum* were completely inhibited by Ag(I) and Hg(II), whereas *A. terreus* and *P. griseoroseum* enzymes were completely inhibited

Table 5. Hydrolysis of Several Synthetic and Natural Substrates by Semipurified α-Galactosidases from Soybean Seeds, *Aspergillus terreus*, and *Penicillium griseoroseum*

substrate	concentration (mM)	hydrolytic activity[a] (%) ± SD soybean	*A. terreus*	*P. griseoroseum*
pNPαGal	0.5	100 ± 0.01	100 ± 0.03	100 ± 0.05
*o*NP*β*Gal	0.5	4.53 ± 0.01	0	0
pNPαGlc	0.5	0	0	3.30 ± 0.07
pNPαXyl	0.5	0	9.64 ± 0.02	0
pNPαMan	0.5	0	0	0
pNP*β*Gal	0.5	0	0	0
raffinose	7.0	30.60 ± 0.01	78.57 ± 0.06	60.65 ± 0.03
maltose	4.0	18.12 ± 0.05	6.07 ± 0.05	10.60 ± 0.03
stachyose	7.0	11.74 ± 0.02	85.71 ± 0.01	78.65 ± 0.02
melibiose	4.0	7.68 ± 0.05	15.36 ± 0.07	16.15 ± 0.01
lactose	4.0	2.30 ± 0.04	0	0
sucrose	4.0	0	35.71 ± 0.03	16.30 ± 0.05

[a] Relative activities were calculated in relation to the *ρ*NPαGal activity, which was considered to be 100%.

Table 6. Effect of Sugars, Salts, Sodium Dodecyl Sulfate, Iodoacetamide, EDTA, and 2-Mercaptoethanol on α-Galctosidases from Soybean, *Aspergillus terreus*, and *Penicillium griseoroseum*

effector[a]	α-galactosidase activity[b] (%) ± SD soybean	*A. terreus*	*P. griseoroseum*
	100 ± 0.05	100 ± 1.21	100 ± 2.91
lactose	89.51 ± 0.02	102.5 ± 1.09	88.00 ± 1.55
maltose	91.33 ± 0.02	92.80 ± 0.7	94.00 ± 3.78
melibiose	74.14 ± 0.01	39.70 ± 1.03	58.00 ± 3.56
raffinose	81.73 ± 0.05	80.60 ± 1.34	93.00 ± 0.80
D-mannose	90.05 ± 0.02	90.50 ± 0.67	94.00 ± 1.25
D-galactose	53.53 ± 0.01	107.2 ± 1.45	100.0 ± 2.34
stachyose	87.69 ± 0.04	95.73 ± 0.85	88.00 ± 3.81
sucrose	98.72 ± 0.07	116.5 ± 2.90	103.0 ± 2.80
D-glucose	94.40 ± 0.01	105.1 ± 2.57	96.00 ± 2.05
EDTA	97.21 ± 0.02	105.0 ± 1.02	101.0 ± 2.08
$MgCl_2$	94.15 ± 0.04	105.6 ± 1.25	98.00 ± 1.47
SDS	49.36 ± 0.01	46.90 ± 2.30	0
$CaCl_2$	96.25 ± 0.02	109.9 ± 2.79	94.00 ± 2.52
$CuSO_4$	59.16 ± 0.01	0	11.00 ± 1.88
KCl	96.48 ± 0.02	105.7 ± 1.80	102.0 ± 1.21
NaCl	94.20 ± 0.03	124.8 ± 0.82	96.0 ± 4.31
2-mercaptoethanol	98.66 ± 0.03	107.8 ± 2.35	95.0 ± 1.68
iodoacetamide	95.88 ± 0.03	104.3 ± 1.27	98.87 ± 0.56
$HgCl_2$	0	0	0
$AgNO_3$	0	0	0

[a] The final concentration of all effectors was 1 mM. [b] Relative activities were calculated in relation to the *ρ*NPGal activity, which was considered to be 100%.

by Cu(II) and SDS, respectively. Reduction in α-galactosidase activity by Cu(II) and Ag(I) was reported for α-galactosidases from *Torulaspora delbrueckii* IFO 1255 (*25*), *B. stearothermophilus* NCIM 5146 (*19*), and *D. hansenii* UFV-1 (*26*). Participation of carboxyl and/or histidine imidazolium groups in the catalytic action is presumed on the basis of the inhibitory effect (*27*). The ionic detergent SDS is an extremely effective denaturing agent for proteins; in its presence most proteins lose their functions either completely or partially with the disruption of tertiary and quaternary structures (*28*, *29*). The enzymes were partially inhibited by melibiose, raffinose, and stachyose. Soybean α-galactosidase was inhibited by D-galactose, an end product from GO hydrolysis. Galactose is a competitive inhibitor of purified soybean α-galactosidase (*5*). The microbial enzymes were not affected by this sugar (**Table 6**). This lack of galactose inhibition is likely to be an advantage for industrial applications of the *A. terreus* and *P. griseoroseum* α-galactosidases.

Table 7. Hydrolysis of Oligosaccharides Present in Fatfree Soybean Flour at Different Incubation Times by α-Galactosidase from Soybean Var. Monarca, *Aspergillus terreus*, and *Penicillium griseoroseum*

time (h)	GO enzymatic hydrolysis by α-galactosidase[a] (%) soybean raffinose	soybean stachyose	*A. terreus* raffinose	*A. terreus* stachyose	*P. griseoroseum* raffinose	*P. griseoroseum* stachyose
0	0	0	0	0	0	0
4	71.4 ± 3.9	34.7 ± 1.4	100	100	72.7 ± 2.6	100
6	78.1 ± 0.4	51.8 ± 8.12	100	100	73.7 ± 2.7	100
8	100	53.1 ± 2.67	100	100	75.5 ± 3.2	100

[a] The results were calculated on the basis of the chromatograms obtained from the GO analyses by HPLC. The assays were triplicate and represent the mean obtained values.

The data presented for α-galactosidase activity, GO, and reducing sugar determinations are means of triplicate assays in which the standard deviation values were smaller than 10%.

Despite the different procedures proposed for GO reduction in leguminous products, enzyme processing has proved to be more effective in terms of time and cost. It has been proposed that the use of crude or semipurified α-galactosidase is more cost-effective than other types of procedures (*30*, *31*). Hydrolysis time will depend on the amount of enzyme used during processing. The ability of soybean, *A. terreus*, and *P. griseoroseum* α-galactosidases to hydrolyze oligosaccharides in fatfree soybean flour was demonstrated (**Table 7**). Soybean flour (2 g) incubated with the soybean, *A. terreus*, and *P. griseoroseum* enzymes (8 units) for a period of 4 h promoted reductions of 71, 100, and 73% in the raffinose content, respectively, whereas stachyose was reduced by 35, 100, and 100%, respectively. No oligosaccharide hydrolysis was detected in control samples in which the enzyme extracts had been replaced by water. Using a much higher α-galactosidase amount, GOs present in pinto bean flour (4 g) were completely eliminated after 2 h of incubation with 40 mL of crude extracellular fungal enzyme (60 units mL^{-1}) (*18*). The extracellular α-galactosidase of *Debaryomyces hansenii* UFV-1 completely hydrolyzed soy milk GO in a 4 h treatment (*26*). Our results, especially with the microbial enzymes, indicate that the genes encoding α-galactosidases in these microorganisms can be potentially superexpressed in heterologous systems for optimum industrial use.

Soy molasses was also subjected to treatment with the α-galactosidases, and GO hydrolysis was evaluated by the production of reducing sugars (**Figure 3**). No oligosaccharide hydrolysis was detected in the control, in which the enzyme extracts had been replaced by water. The preparations containing microbial enzymes were more effective in hydrolyzing GO in soy molasses, as was also the case when soybean flour was used as the GO source.

As the enzyme preparations from *A. terreus* and *P. griseoroseum* showed invertase activity, it is possible that invertase and α-galactosidase acted synergistically, especially against stachyose, which was fully hydrolyzed in all treatment periods (**Table 7**). Complete GO hydrolysis can be achieved by either α-galactosidase, invertase, or both. Whereas 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 (*5*). Breaking down sucrose into glucose and fructose will also increase sweetness and increase the rate of Maillard browning reactions to darken the products. Although more research should be done, our results indicate that the

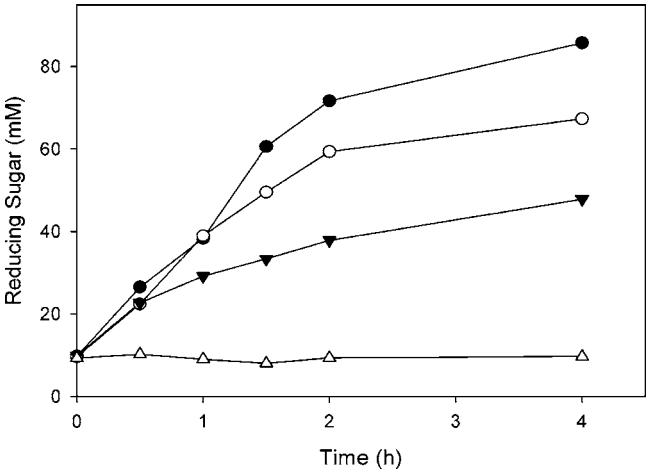

Figure 3. Production of reducing sugars during GO hydrolysis of soy molasses by soybean (▼), *Aspergillus terreus* (●), and *Penicillium griseoroseum* (○) α-galactosidases. Soybean molasses mixed with water (1:5 w/v) (10 g) was incubated with 32 units of each α-galactosidase at 40 °C for 0, 30, 60, 90, 120, and 240 min. For the control assay (△), the enzyme was replaced with distilled water.

simultaneous use of both enzymes for GO hydrolysis could be a more effective alternative in industrial applications.

The α-galactosidases described in this work are of interest because of their potential to convert oligosaccharides present in soybean products to digestible sugars. In general, the enzymes suggested for this purpose are of microbial origin and therefore present the disadvantage of having no GRAS (generally regard as safe) status. However, the fungal enzymes are relatively easy to produce and the most suitable for technological applications, mainly due to their extracellular localization, acidic pH optima, and broad stability profiles. On the other hand, soybean α-galactosidase is uniquely suited to use in the high-protein, strongly buffered environment of soymeal slurry. This enzyme should present no restriction regarding safety for use in food processing. However, the amount of α-galactosidase in soybean seeds is small, and the purification process is difficult. In both cases, we suggest the genes encoding these enzymes should be cloned and overexpressed in suitable organisms to produce the enzymes at low cost.

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