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# Characterization and biotechnological application of an acid α-galactosidase from *Tachigali multijuga* Benth. seeds

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ABSTRACT

*Tachigali multijuga* Benth. seeds were found to contain protein (364 mg $g^{-1}$ dwt), lipids (24 mg $g^{-1}$ dwt), ash (35 mg $g^{-1}$ dwt), and carbohydrates (577 mg $g^{-1}$ dwt). Sucrose, raffinose, and stachyose concentrations were 8.3, 3.0, and 11.6 mg $g^{-1}$ dwt, respectively. α-Galactosidase activity increased during seed germination and reached a maximum level at 108 h after seed imbibition. The α-galactosidase purified from germinating seeds had an $M_r$ of 38,000 and maximal activity at pH 5.0–5.5 and 50 °C. The enzyme was stable at 35 °C and 40 °C, but lost 79% of its activity after 30 min at 50 °C. The activation energy ($E_a$) values for *p*-nitrophenyl-α-D-galactopyranoside (pNPGal) and raffinose were 13.86 and 4.75 kcal $mol^{-1}$, respectively. The $K_m$ values for pNPGal, melibiose, raffinose, and stachyose were 0.45, 5.37, 39.62 and 48.80 mM, respectively. The enzyme was sensitive to inhibition by $HgCl_2$, SDS, $AgNO_3$, $CuSO_4$, and melibiose. D-Galactose was a competitive inhibitor ($K_i$ = 2.74 mM). In addition to its ability to hydrolyze raffinose and stachyose, the enzyme also hydrolyzed galactomannan.



## 1. Introduction

Plant α-galactosidases [α-D-galactoside galactohydrolase; E.C. 3.2.1.22] have been found in seeds, fruits, leaves and tubers. The α-galactosidases are classified as either acid or alkaline, according to the optimal pH for their activities (Keller and Pharr, 1996). Most of the α-galactosidases isolated from plant seeds and leaves are acidic enzymes and belong to the 27 glycosyl hydrolase cluster family. However, in plants which transport raffinose and stachyose in their phloem (Bachmann et al., 1994), α-galactosidases neutral to alkaline pH optima were found (Lee et al., 2004; Blöchl et al., 2008). Comparisons with other α-galactosidases showed that the alkaline enzymes were more similar to raffinose- and stachyose-synthases than to other α-galactosidases, suggesting that they belong to family 36 of the glycosyl hydrolases. It was suggested that alkaline and acidic plant α-galactosidases have different spatial and temporal expression patterns and different functions (Blöchl et al., 2008).

In germinating legume seeds, α-galactosidase plays a role in the mobilization of raffinose family oligosaccharides (RFOs), mainly raffinose and stachyose, which are galactosyl derivatives of sucrose containing one and two galactose moieties, respectively (Guimarães et al., 2001). Additionally, α-galactosidases also hydrolyze galactosyl cyclitols during legume germination (Horbowicz et al., 1998). RFO catabolism occurs primarily via α-galactosidase, which is an exo-glycosidase that hydrolyzes a variety of simple α-D-galactosides as well as more complex molecules, such as oligosaccharides and polysaccaharides. Seeds have a high demand for energy during early germination and RFOs rapidly decrease after seed imbibition (Guimarães et al., 2001). A proposed model for RFO breakdown in pea seeds suggested that acidic and alkaline α-galactosidases were sequentially active in the coordinated hydrolysis of RFOs (Blöchl et al., 2008). On the other hand, in corn and barley seeds (both endospermic seeds), the alkaline enzymes were either already present in the dry seed (Zhao et al., 2006) or expressed at early stages of germination (Carmi et al. 2003).

RFO breakdown is often completed before polysaccharides are mobilized, which indicates that they may play a special role in providing energy for early germination (Blöchl et al., 2007). α-Galactosidase acts together with endo-β-mannanase and β mannosidases to degrade plant seed galactomannans (Reid and Meier, 1973).

Different functions were proposed for coffee bean α-galactosidases, which might be associated with hardening of coffee fruit endosperm, through the removal of galactosyl residues from mannan chains in the galactomannan, leading to the settling of these polysaccharides in the cell walls (Bewley, 1997; Redgwell et al., 2003; Marraccini et al., 2005). α-Galactosidase could also be linked

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to the degradation of RFOs and galactosyl cyclitols during coffee bean germination (Marraccini et al., 2005).

Furthermore, α-galactosidase has attracted attention in the field of biotechnology, due to its capacity to hydrolyze raffinose in beet sugar syrups to facilitate crystallization and consequently improve the sucrose yield (Ganter et al., 1988). It has been proposed that α-galactosidase may be used to break down RFO in soybean milk and other legume-derived foods (Falkoski et al., 2006). The presence of RFO in soybean derivatives promotes intestinal disturbance and reduces nutrient absorption in human and monogastric animals after consumption (Karr-Lilienthal et al., 2006). This enzyme has been shown to modify the rheological properties of galactomannans by changing their long-chain structure and reducing their viscosity (Bulpin et al., 1990). Some plant α-galactosidases can remove the terminal galactose units (α-1,3-linked) from the blood group B cell surface carbohydrate, thus generating type O red blood cells (Zhu and Goldstein, 1994).

The seeds of the Brazilian Atlantic Forest species *Tachigali multijuga* Benth., a Fabaceae of the *Caesalpinioideae* subfamily, have been shown to have higher α-galactosidase activity during germination after breaking dormancy (Borges et al., 2004). This species is used as an ornamental tree. Its wood shows moderate density and is highly resistant and durable. However, few biochemical studies have examined this tropical species.

The objective of this study was to purify and biochemically/kinetically characterize an acid α-galactosidase from germinating *T. multijuga* seeds and to investigate possible biotechnological applications.

## 2. Results and discussion

### 2.1. Seed composition and α-galactosidase activity

Mature seeds of *T. multijuga* contained 364 mg $g^{-1}$ dwt protein, 24 mg $g^{-1}$ dwt lipids, 35 mg $g^{-1}$ dwt ash, and 577 mg $g^{-1}$ dwt total carbohydrates. Seed carbohydrates, such as starch or galactomannans and soluble sugars, make up most of the carbon reserves in many seeds (Bewley and Black, 1994). Polymeric carbohydrates are usually more abundant by weight than soluble sugars. Thus, soluble sugars have received relatively little attention as storage carbohydrates. Soluble sugars such as sucrose, raffinose and stachyose were present in *T. multijuga* seeds at concentrations of 8.3, 3.0 and 11.6 mg $g^{-1}$ dwt, respectively. They usually comprise only a minor fraction of seed carbohydrates (Ziegler, 1995). In *T. multijuga* seeds, the raffinose family oligosaccharides raffinose and stachyose (RFOs) and sucrose comprised 0.52%, 2.00%, and 1.44%, respectively, of the total carbohydrates. It is well known that RFOs are rapidly depleted after seed imbibition and that their breakdown is often completed before polymeric carbohydrates are mobilized (Vidal-Valverde et al., 2002; El-Adawy et al., 2003), indicating that they may play a special role during early germination. The co-occurrence of RFOs and α-galactosidases in dry seeds, germination time of 0 h (Fig. 1), could indicate a distinct cellular compartmentation or regulatory mechanisms, which prevent RFO degradation during maturation drying. α-Galactosidase activity increased with the germination of *T. multijuga* seeds and reached a maximum value at 108 h after seed imbibition. On the other hand, the RFO content in germinating *T. multijuga* seeds decreased during germination (Fig. 1).

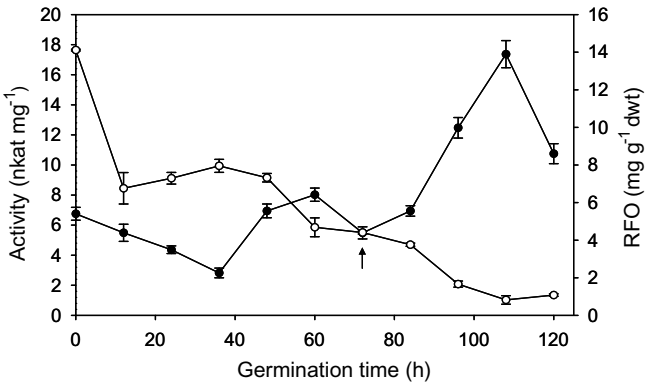


**Fig. 1.** (●) Specific activity of α-galactosidase against pNPGal and (○) raffinose and stachyose concentrations from *Tachigali multijuga* Benth. seeds during germination. The arrow indicates the time of seeds complete germination.

The profile of α-galactosidase activity after imbibing the *T. multijuga* seeds showed three activity peaks (0, 60, and 108 h after imbibition), that might be related to at least three different forms of α-galactosidases that were expressed during and following germination (Fig. 1). In this study, we purified only an acidic α-galactosidase that showed the maximal activity at 108 h after imbibition. These enzymes have been reported to be responsible for RFO breakdown and are required for cell wall galactomannan degradation. Inhibition of α-galactosidase in peas blocked RFO degradation, which led to a several-day delay in the completion of germination (Blöchl et al., 2007).

### 2.2. α-Galactosidase purification

The results of α-galactosidase purification from germinating *T. multijuga* seeds are summarized in Table 1. The specific activity of the enzyme increased from 5.77 nkat $mg^{-1}$ in crude extract to 1393.60 nkat $mg^{-1}$ after gel filtration chromatography. The resulting purification factor was 241.52, with an original activity recovery level of 6.16%. The profile of gel filtration chromatography showed one peak of protein with α-galactosidase activity (Fig. 2).

The molecular mass of the α-galactosidase was estimated 38,000 by SDS–PAGE (Fig. 3), and 34,000 by gel filtration in a Sephacryl S-200 column, indicating the monomeric form of the enzyme. Although a single protein band was observed in SDS–PAGE (Fig. 3, lane 4) after α-galactosidase purification, it is possible that enzyme isoforms with nearly equivalent molecular masses could be present. In a previous report, a similar molecular weight was determined for α-galactosidases from coffee bean seeds (Marraccini et al., 2005). However, a lower molecular weight was reported for α -galactosidase from *Sesbania virgata* (Cav.) Pers. (Tonini

**Table 1**
Summary of the steps in the purification of α-galactosidase from germinating *Tachigali multijuga* Benth. seeds

| Purification step | Total protein (mg) | Total activity (nkat) | Specific activity (nkat $mg^{-1}$) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 392.20 | 2263.62 | 5.77 | 1 | 100 |
| Acid precipitation | 278.78 | 2050.41 | 7.35 | 1.27 | 90.58 |
| Dialyzed sample | 256.00 | 2032.07 | 7.94 | 1.38 | 89.77 |
| DEAE-Sephacel | 20.49 | 1458.29 | 71.17 | 12.33 | 64.42 |
| Sephadex G-150 | 0.10 | 139.36 | 1393.60 | 241.52 | 6.16 |

One nkat of α-galactosidase activity was defined as the amount of enzyme that released 1 nmol of pNP per second under standard assay conditions.

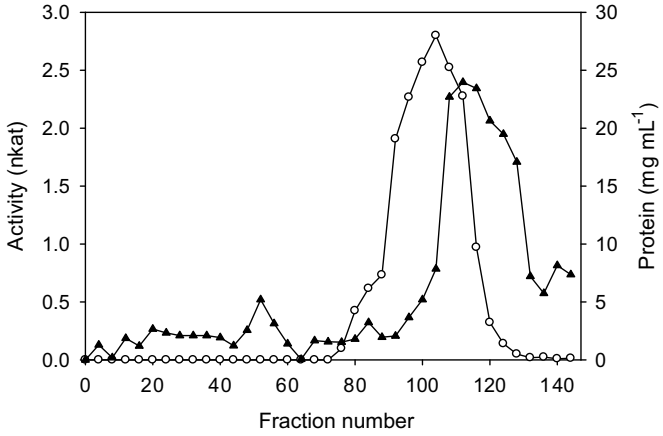


**Fig. 2.** Elution profile of α-galactosidase from germinating *Tachigali multijuga* Benth. seeds on a Sephadex G-150 column. (O) α-Galactosidase activity; (▲) Protein.

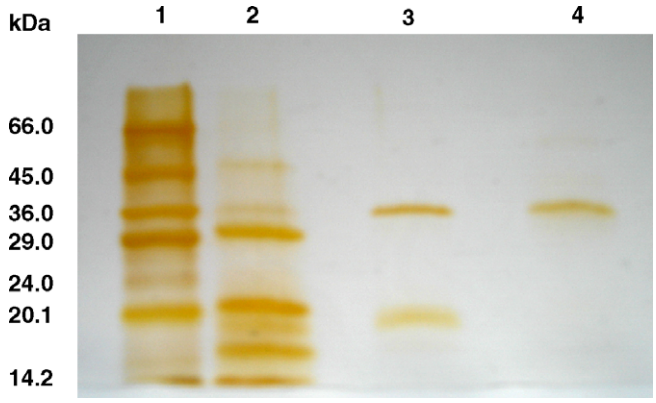


**Fig. 3.** SDS–PAGE (12.5% w/v) of different steps in the purification of α-galactosidase from germinating *Tachigali multijuga* Benth. seeds. Lane 1, molecular mass standards; lane 2, crude extract (25 μg protein); lane 3, DEAE-Sephacel fraction (20 μg protein); and lane 4, Sephadex G-150 fraction (20 μg protein). Protein gel was stained with silver.

et al., 2006). On the other hand, several α-galactosidases from microorganisms showed a higher molecular weight (de Rezende et al., 2005; Viana et al., 2006). Depending on the source, α-galactosidases can show different substrate specificities and activities (Kim et al., 2003).

### 2.3. Effects of pH and temperature on α-galactosidase activity

The purified α-galactosidase eluted from gel filtration chromatography was used for all enzymatic characterization assays. *T. multijuga* α-galactosidase was shown to be an acidic protein, with maximal activity at pH 5.0–5.5. At pH 7.0, the enzyme activity was completely lost (Fig. 4). Similar results were obtained for α-galactosidase from germinating *Platymiscium pubescens* Micheli seeds (Oliveira et al., 2005) and for the acid form of *Cucumis melo* L. cv C-8 α-galactosidase (Gao and Schaffer, 1999). The enzyme retained more than 70% of its maximal activity after pre-incubation at pH 4.0–6.0 (Fig. 4). This result suggested that, in this pH range, there was no significant change in the native structure of the enzyme. Pre-incubation of the enzyme for 30 min at pH 7.0 promoted an irreversible loss of activity. On the other hand, the activity regenerated after pre-incubation for only 15 min (Fig. 4).

This enzyme showed maximal activity at 50 °C. Heat-stability studies showed that the α-galactosidase from germinating *T. multijuga* seeds retained about 70% of its original activity after incubation for 12- or 200-h at 40 °C and 35 °C, respectively (Fig. 5). The half-life values of α-galactosidase at 45 °C and 50 °C were 117.8 min and 15.9 min, respectively (Fig. 5). Thermostability is an important characteristic of an enzyme for industrial purposes. Generally, plant α-galactosidases are less thermostable than those from microbial sources (Falkoski et al., 2006).

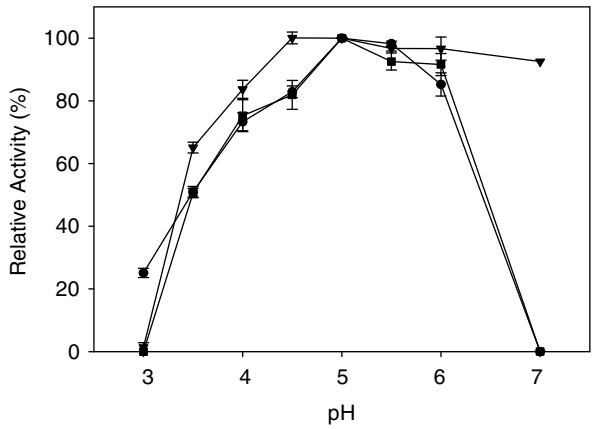


**Fig. 4.** Effects of pH on the activity (●) and stability for 15- (▼) and 30-min (■) of α-galactosidase from germinating *Tachigali multijuga* Benth., seeds. Relative activities were calculated in relation to activities determined in pH 5.0, that were considered as 100%.

### 2.4. Effects of metal ions and inhibitors on α-galactosidase activity

The sensitivity of purified α-galactosidase to various metal ions, sugars, and inhibiting reagents was tested (Table 2). The enzyme was completely inhibited by $Hg^{2+}$, $Cu^{2+}$, $Ag^{+}$, and partially inhibited by D-galactose and melibiose. A reduction in α-galactosidase activity by $Hg^{2+}$ and $Ag^{+}$ has been reported for several α-galactosidases (Itoh et al., 1986). The total reduction of *P. pubescens* α-galactosidase activity was also seen with $Cu^{2+}$ (Oliveira et al., 2005). This inhibition usually suggests a reaction with thiol groups and/or a carboxyl, amino and imidazolium group of histidine at the active site (Dey and Pridam, 1972). The ionic detergent SDS was a powerful enzyme inhibitor. SDS is an extremely effective denaturing agent for proteins; in its presence, most proteins lose their

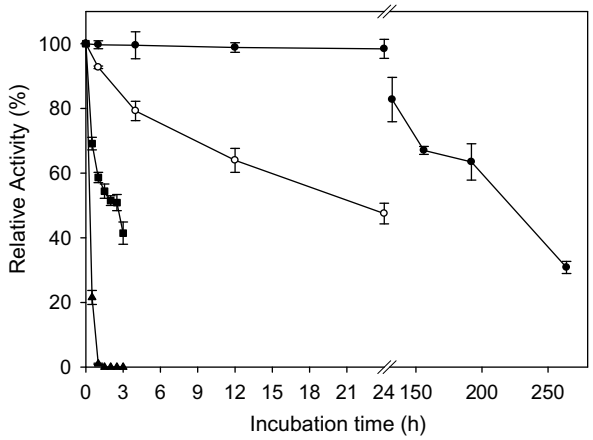


**Fig. 5.** Effect of temperature on the stability of α -galactosidase from germinating *Tachigali multijuga* Benth. seeds. The enzyme samples were pre-incubated at several temperatures [35 (●), 40 (O); 45 (■) and 50 °C (▲)], and assayed at 40 °C. Relative activities were calculated in relation to activities determined in the reaction without pre-incubation, that were considered as 100%.

**Table 2**
Effects of simple sugars, ions, and reducing agents on the activity of α-galactosidase from germinating *Tachigali multijuga* Benth. seeds

| Effector[a] | Relative α-galactosidase activity[b] (%) ±SD |
|---|---|
| – | 100.0 ± 0.31 |
| $HgCl_2$ | 0 |
| NaCl | 101.20 ± 0.02 |
| SDS | 0 |
| β-mercaptoethanol | 99.48 ± 0.02 |
| $CaCl_2$ | 93.94 ± 0.03 |
| KCl | 94.00 ± 0.03 |
| $CuSO_4$ | 1.43 ± 0.08 |
| $Na_2SO_4$ | 100.77 ± 0.62 |
| Iodoacetamide | 95.69 ± 0.02 |
| $AgNO_3$ | 0 |
| $NaNO_3$ | 100.59 ± 1.87 |
| EDTA | 113.83 ± 0.01 |
| Sucrose | 97.34 ± 0.01 |
| D-Glucose | 101.98 ± 0.01 |
| D-Galactose | 69.89 ± 0.01 |
| Melibiose | 75.56 ± 0.1 |

[a] The final SDS concentration was 1 mM. The final concentrations of all the other effectors were 2 mM.
[b] Relative activities were calculated in relation to activity determined in the reaction without effector, that was considered as 100%.

**Table 3**
Hydrolysis of several substrates by α-galactosidase from germinating *Tachigali multijuga* Benth. seeds

| Substrate | Concentration (mM)[a] | Relative activity[c] (%) ±SD |
|---|---|---|
| pNPGal | 0.5 | 100 ± 0.08 |
| Sucrose | 10 | 0 |
| Raffinose | 40 | 72.52 ± 0.16 |
| Melibiose | 6.0 | 13.04 ± 0.09 |
| Stachyose | 50 | 43.47 ± 0.05 |
| Locust bean gum | 0.5 | 2.80 ± 0.26 |
| Guar gum | 0.5 | 2.54 ± 0.29 |
| oNPβGal[b] | 0.5 | 0 |
| pNPβGal[b] | 0.5 | 0 |
| oNPβGlc[b] | 0.5 | 0 |
| pNPαGlc[b] | 0.5 | 0 |
| pNPαAra[b] | 0.5 | 0 |
| pNPβXyl[b] | 0.5 | 0 |
| pNPαMan[b] | 0.5 | 0 |

[a] Final concentrations in mM except the locust bean gum and guar gum substrates (% w/v).
[b] oNPβGal, *ortho*-nitrophenyl-β-D-galactopyranoside; pNPβGal, *para*-nitrophenyl-β-D-galactopyranoside; oNPβGlc, *ortho*-nitrophenyl-β-D-glucopyranoside; pNPαGlc, *para*-nitrophenyl-α-D-glucopyranoside; pNPαAra, *para*-nitrophenyl-α-D-arabinopyranoside; pNPβXyl, *para*-nitrophenyl-β-D-xylopyranoside; pNPαMan, *para*-nitrophenyl-α-D-mannopyranoside.
[c] Relative activities were calculated in relation to pNPGal activity, that was considered as 100%.

functions either completely or partially with the disruption of tertiary and quaternary structures (Bischoff et al., 1998). It has been suggested that α-galactosidase is not a metalloenzyme and that sulfhydryl groups do not take part in catalysis, since there is no enzyme inhibition under treatment with EDTA or iodoacetamide, respectively. This agrees with the results reported for α-galactosidase from germinating soybean seeds (Viana et al., 2005). Galactose, one of the products of the catalytic action of α-galactosidases on α-D-galactosides, was found to be a competitive inhibitor, with a $K_i$ value of 2.74 mM as determined by a Dixon plot. Galactose competitively inhibited the α-galactosidase from *C. melo* ($K_i$ of 13 mM) (Gao and Schaffer, 1999).

### 2.5. Substrate specificity and kinetic studies

The substrate specificities of α -galactosidase from germinating *T. multijuga* seeds were investigated by using oligosaccharides, polysaccharides, and synthetic substrates (Table 3). Under the experimental conditions, pNPGal was hydrolyzed most efficiently by the enzyme, followed by raffinose, melibiose, and stachyose. While the enzyme hydrolyzed pNPGal, it did not hydrolyze other synthetic substrates containing β-linkages or xylose, arabinose, mannose, and glucose residues (Table 3). This enzyme showed specificity for anomeric carbon and also seemed to be regiospecific for the galactoside configuration.

In addition, polymers such as locust bean gum and guar gum were hydrolyzed by α-galactosidase, which showed that it may play a physiological role in galactomannan degradation in *T. multijuga* seeds. The ability to hydrolyze locust bean gum and guar gum also showed that it may be useful industrially for improving the gelling properties of polysaccharides. Locust bean gum is more functional, but also more expensive than guar gum. The removal of a quantitative proportion of galactose moieties from guar gum, by α-galactosidase, improves its gelling properties and makes it comparable to those of locust bean gum (Bulpin et al., 1990).

α-Galactosidases are classified into two groups based on their substrate specificity. One group is specific for low-$M_r$ α-galactosides such as pNPGal, melibiose, and oligosaccharides. The other group acts on galactomannans and also hydrolyzes low-$M_r$ substrates to various extents (Dey et al., 1993). The results obtained in this study indicated that *T. multijuga* α-galactosidase can be grouped in the second category of α-galactosidases, like the enzyme from sunflower seeds, which effectively hydrolyzed melibiose, raffinose, stachyose, and galactomannan (Kim et al., 2003).

**Table 4**
$K_m$, $V_{max}$ and $V_{max}/K_m$ values determined by a Michaelis–Menten plot for α-galactosidase from germinating *Tachigali multijuga* Benth. seeds

| Substrates | $K_m$ (mM) | $V_{max}$[a] | $V_{max}/K_m$ ($s^{-1}$) |
|---|---|---|---|
| pNPGal | 0.45 ± 0.04 | 7.83 | $17.40 \times 10^{-9}$ |
| Melibiose | 5.37 ± 0.09 | 1.00 | $0.18 \times 10^{-9}$ |
| Raffinose | 39.62 ± 0.13 | 23.00 | $0.58 \times 10^{-9}$ |
| Stachyose | 48.80 ± 0.53 | 3.00 | $0.06 \times 10^{-9}$ |

[a] $V_{max}$ is expressed in nmol pNP. $s^{-1}$ for pNPGal; nmol glucose. $s^{-1}$ for melibiose and nmol reducing sugar. $s^{-1}$ for raffinose and stachyose.

The $K_m$ and $V_{max}$ values were calculated from a Michaelis–Menten plot for ρ NPGal, melibiose, raffinose, and stachyose (Table 4). The $K_m$ values were comparable to those determined for several α-galactosidases (Gao and Schaffer, 1999; Guimarães et al., 2001; Viana et al., 2005). The catalytic efficiency expressed by the $V_{max}/K_m$ ratio showed that the substrate pNPGal was used most efficiently by the enzyme, followed by raffinose, melibiose, and stachyose (Table 4). For the natural substrates tested, the lowest $K_m$ value was determined for melibiose, which indicated that *T. multijuga* α-galactosidase showed the highest affinity for melibiose and that complex ES formation was not the limiting step for the reaction.

The activation energy ($E_a$), estimated from an Arrenhius plot, was 13.86 ± 0.05 kcal $mol^{-1}$ for the substrate pNPGal and 4.75 ± 0.16 kcal $mol^{-1}$ for raffinose. In a previous study, Gote et al. (2006) showed $E_a$ values of 0.14 kcal $mol^{-1}$ and 14.30 kcal $mol^{-1}$ for the *Bacillus stearothermophilus* α-galactosidase reaction against raffinose and pNPGal, respectively. The state transition is more quickly reached when the $E_a$ value is smaller, and the velocity of the reaction toward product formation is faster. Although the $E_a$ value determined for the *T. multijuga* α-galactosidase reaction against pNPGal was higher than that against raffinose, the $V_{max}/K_m$ value for pNPGal was higher than that for raffinose. These results strongly suggest that, after ES complex formation, the hydrolysis of raffinose and product formation occurred faster.

### 2.6. Enzymatic hydrolysis of oligosaccharides present in soymilk

The ability of α-galactosidase from germinating *T. multijuga* seeds to hydrolyze the RFO present in fat-free soybean flour was demonstrated. The purified enzyme (133.4 nkat) reduced the raffinose and stachyose content in soybean flour (2 g) by 69 ± 0.30% and 48.5 ± 1.12%, respectively, after incubation for 6 h at 40 °C. No oligosaccharide hydrolysis was detected in control samples in which the enzymatic extract had been replaced by water. No invertase activity was determined in the extract, indicating that RFO hydrolysis in soybean flour was catalyzed exclusively by α-galactosidase. Reduction of 72.3% stachyose and 89.2% raffinose was observed in fat-free soybean flour (1.2 g) after treatment with α-galactosidase (83.4 nkat) from germinating soybean seeds for 6 h at 40 °C (Viana et al., 2005). Compared with *T. multijuga* α-galactosidase, the enzyme from soybean seeds was more effective for RFO hydrolysis in soybean flour. Soybean α-galactosidase has the advantage of being uniquely suited for the high protein and strongly buffered environment of soybean flour. On the other hand, the greater RFO hydrolysis by *T. multijuga* α-galactosidase could be achieved by using more enzyme.

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. 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 are the most suitable for technological applications, mainly due to their extracellular localization, acidic pH optima, and broad stability profiles (Falkoski et al., 2006). With the aim of developing probiotic microorganisms with high α-galactosidase activity and for manufacture of soy-derived products, an α-galactosidase gene (*melA*) from *Lactobacillus plantarum* was characterized and expressed in *Escherichia coli*. α-Galactosidase production reached amounts about 15 times higher than those found with the native producer *L. plantarum* (Silvestroni et al., 2002).

### 2.7. Enzymatic hydrolysis of locust bean gum and guar gum with α-galactosidase

Galactomannans from locust bean gum and guar gum were also treated with α-galactosidases. Galactomannan hydrolysis was evaluated by the reducing sugar production. No polysaccharide hydrolysis was detected in the control, in which the enzyme extracts had been replaced by water. The galactomannans were hydrolyzed by the enzyme to the extent of 61 ± 0.2%, after 24 h, in the assay conditions. The hydrolysis profile showed the progressive production of reducing sugar, which indicated that the enzyme was stable during the time period of the assay. When *Bacteroides ovatus* was grown on guar gum, it produced α-galactosidase I which is different from α-galactosidase II which it produced when grown on galactose, melibiose, raffinose, or stachyose. Neither enzyme was able to remove galactose residues from intact guar gum, but both were capable of removing galactose residues from guar gum which had been degraded into large fragments by mannanase (Gherardini et al., 1985).

Enzymatically modified guar also has tremendous potential in food applications. Guar is cheaper and more readily available, which makes it a suitable replacement for locust bean. Guar and locust bean both have an irregular distribution of α-1,6-D-galactosyl side groups along the β-1,4-D-mannanopyranose backbone. The primary difference is that guar gum contains 38–40% (w/w) galactose, whereas locust bean gum contains 22–24% (w/w) galactose. The α-galactosidase cleaves off the galactose branches from the backbone. Thus, it is of commercial interest to modify the structure of guar to convert it into a material with the desired functional properties of locust bean. The molecular interaction between guar and other food ingredients can be further controlled by debranching the guar and changing the mannose/galactose ratio to optimize food composition without harming functionality (Mahammad et al., 2007).

## 3. Concluding remarks

In the present study, we determined the biochemical composition of *T. multijuga* seeds, a tropical legume tree from the Brazilian Atlantic Forest. We characterized an acid α-galactosidase purified of seed and we also investigated the potential applications of *T. multijuga* α-galactosidase in industrial process.

Purified acidic α-galactosidase hydrolyzed raffinose, stachyose and galactomanans in vitro, suggesting its possible function in RFO and galactomanan degradation during germination. The study of the enzyme properties showed that *T. multijuga* α-galactosidase could be of potential interest to convert RFO in soybean flour, which are antinutritional factors for human and monogastric animals. In addition, this enzyme could be used for to modify the structure of guar gum, to convert it to a material having the desired functional properties of locust bean gum. However, α-galactosidases, especially of microbial origin, have more suitable properties in terms of stability for these applications.

## 4. Experimental

### 4.1. Materials

Sephadex G-150 Fast Flow and DEAE-Sephacel Fast Flow were obtained from Amersham Biosciences, Uppsala, Sweden. D-Galactose, melibiose, raffinose, stachyose, locust bean gum, guar gum, *p*-nitrophenol (pNP), and *p*-nitrophenyl-α-D-galactopyranoside (pNPGal) were purchased from Sigma Chemical Co. (St. Louis, MO). Sucrose and D-glucose were purchased from Merck S.A. Indústrias Químicas (São Paulo, Brazil). All other chemicals used in this study were of analytic purity. The seeds of *T. multijuga* Benth. were supplied by the Forest Engineering Department, Federal University of Viçosa, MG, Brazil.

### 4.2. Enzyme extraction

*T. multijuga* seeds were mixed with concentrated $H_2SO_4$ and shaken for 30 min to break dormancy. The seeds were then washed in tap $H_2O$ and dried with a paper towel. Dried seeds were then placed on a water-soaked filter paper and incubated in a germinating chamber for 108 h at 27 °C. After this period, the seeds were frozen at −20 °C. The germinated seeds were then powdered in a blender with 0.1 M sodium acetate buffer pH 5.0 (3 g fresh weight seeds per 10 ml buffer) and centrifuged (15,000*g*) for 30 min at 4 °C. Finally, the supernatant was used as crude enzymatic extract.

### 4.3. Protein determination and enzyme assays

The protein concentration in the enzymatic extracts was determined by the Coomassie Blue binding method (Bradford, 1976) with bovine serum albumin as a standard. α-Galactosidase was assayed in a reaction system containing 650–750 μl 0.1 M sodium acetate buffer, pH 5.0, 0–100 μl enzyme preparation and 250 μl 2 mM pNPGal or other synthetic substrates. The reaction was run for 15 min at 40 °C and ended with the addition of 1 ml 0.5 M $Na_2CO_3$. The amount of pNP released was determined at 410 nm. This procedure was defined as the standard assay. All results of the α-galactosidase activity analyses are presented as means ± SD for three measurements.

The activity against melibiose was evaluated by the glucose-oxidase method (Bergmeyer and Bernt, 1974). When sucrose, raffinose, stachyose, locust bean gum, and guar gum were used as the substrates, the reducing sugar production was determined using the 3,5-dinitrosalicylate reagent (Miller, 1956). Incubations were carried out for at least four different time periods and the initial rates of hydrolysis were calculated. Control assays without enzyme or without substrate were included.

### 4.4. α-Galactosidase purification

The pH of the crude enzymatic extract was lowered to 4.0 with citric acid. The solution was then stirred for 30 min at 4 °C and centrifuged (15,300 *g*) for 40 min at 4 °C. The supernatant was dialyzed overnight against 50 mM $NaOA_2$ buffer, pH 5.5, and applied to a DEAE-Sephacel Fast Flow (Amersham Biosciences, Uppsala, Sweden) column (17 × 3.0 cm) equilibrated with the same buffer. The proteins were eluted at 40 ml $h^{-1}$flow rate with a linear gradient of NaCl (0–1.0 M) in 50 mM $NaOA_2$ buffer, pH 5.5. The fractions containing α-galactosidase activity were pooled and concentrated by an Amicon ultrafiltration cell model 8400 (Bedford, MA) with a 10 kDa molecular mass cutoff PM 10 Amicon membrane, and then loaded onto a Sephadex G-150 Fast Flow (Amersham Biosciences, Uppsala, Sweden) column (90 × 2.6 cm) equilibrated with 25 mM $NaOA_2$ buffer, pH 5.5. The proteins were eluted at 20 ml $h^{-1}$ flow rate. All purification procedures were performed at 4 °C. The active fraction was pooled and analyzed for purity by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

### 4.5. Electrophoresis and molecular mass determination

Enzyme preparations were analyzed by SDS–PAGE, 12.5% w/v, as described by Laemmli (1970) and proteins were silver-stained according to Blum et al. (1987). The molecular mass standards (Amersham Biosciences, Uppsala, Sweden) were as follows: 66.0 kDa BSA, 45.0 kDa ovalbumin, 36.0 kDa glyceraldehyde-3-phosphate dehydrogenase, 29.0 kDa carbonic anhydrase, 24.0 kDa trypsinogen, 20.1 kDa trypsin inhibitor, and 14.2 kDa α-lactalbumin. The enzyme molecular mass was estimated by SDS–PAGE using a 12.5% w/v polyacrylamide gel.

### 4.6. Enzyme characterization

The enzyme samples were pre-incubated with each of the effectors (2 mM final concentration) in 0.1 M $NaOA_2$ buffer, pH 5.0, for 15 min at 40 °C. After pre-incubation, the effects of ions, simple sugars, and reducing agents on enzyme activity were determined by the standard assay. The effect of pH on enzymatic activities was determined within the pH range of 3.0–7.0 using McIlvaine buffer (citric acid/sodium phosphate) at 40 °C (McIlvaine, 1921).

Enzymatic assays were performed with various synthetic, natural, and polymeric substrates. The reaction mixtures contained 650 μl 0.1 M $NaOA_2$ buffer, pH 5.0, 100 μl enzyme solution, and 250 μl substrate solutions. The final substrate concentrations were: synthetic substrates (0.5 mM), sucrose (10 mM), melibiose (6 mM), raffinose (40 mM), stachyose (50 mM), locust bean gum (0.5% w/v), and guar gum solutions (0.5% w/v). The activities were measured under standard assay conditions at 40 °C.

Kinetic experiments were performed at 40 °C and pH 5.0. The Michaelis–Menten constant ($K_m$) and $V_{max}$ for pNPGal, raffinose, melibiose, and stachyose hydrolysis were calculated using a Michaelis–Menten plot. The substrate concentrations ranged from 0.2 to 2 mM for pNPGal, 5 to 120 mM for raffinose, 0.8–50 mM for melibiose, and 15–200 mM for stachyose. The inhibition constant ($K_i$) for galactose was calculated using a Dixon plot. The pNPGal concentrations ranged from 0.1 to 1.0 mM. The concentrations of inhibitor galactose were 1.0, 2.0, and 3.0 mM. The activation energy ($E_a$) for the reaction catalyzed by α-galactosidase against pNPGal and raffinose (30–50 °C) was calculated from the slope of the Arrhenius plot: log of velocity on the ordinate versus $1/T$ in $K$ on the abscissa. The Arrhenius equation used was: Slope = $-E_a/2.3R$, where $R$ is the universal gas constant (8.314 J $mol^{-1}$). All the assays were performed in triplicate.

### 4.7. Treatment of soybean flour with α-galactosidase

Commercial defatted flour (Bunge Alimentos, RS, Brazil) (2 g) was mixed with distilled $H_2O$ 1:10 (w/v) and α-galactosidase purified from germinating *T. multijuga* seeds (134 nkat) was then added. The mixture was 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 EtOH–$H_2O$ (4:1, v/v), as described by Guimarães et al. (2001). The solvent was then evaporated at 45 °C and the sugars were resuspended in EtOH–$H_2O$ (4:1, v/v, 1.2 ml) and analyzed using HPLC as described below.

One nkat was defined as the amount of enzyme that released 1 nmol product per second under standard assay conditions.

### 4.8. Determination of GO content

Sugars were analyzed by HPLC on a Shimadzu series 10A chromatograph (Kyoto, Japan), using a Supelcosil LC-NH2 25 cm × 4.6 mm analytical column (Supelco, Bellefonte, PA), eluted with an $CH_3CN$–$H_2O$ isocratic mixture (4:1, v/v) at 35 °C, at a flow rate of 1 ml $min^{-1}$. The sugars eluted were monitored by a refractive index detector (model 6A, Shimadzu). They were automatically identified and quantified by comparing the retention times and known concentrations of the sugar standards sucrose, raffinose, and stachyose (Viana et al., 2005).

### 4.9. Treatment of guar gum and locust bean gum with α-galactosidase

Enzyme solution (0.5 ml, 51.66 nkat) was mixed with 0.5 ml of 1% w/v galactomannan from guar or locust bean gum. The reaction mixture was then incubated at 40 °C for 0, 6, 12, and 24 h. After these incubation times, the reactions were stopped by boiling in water for 5 min, and the reducing sugar content was determined (Miller, 1956).

### 4.10. Seed composition determination

Total proteins (Kjeldahl method), lipid, carbohydrate, and ash contents were determined in seeds as described by the AOAC (1975). The extraction of soluble sugars from 100 mg of dried seed powder and the determination of sucrose, raffinose, and stachyose contents were carried out as described above.

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