Endo-β-mannanase from the endosperm of seeds of *Sesbania virgata* (Cav.) Pers. (Leguminosae): purification, characterisation and its dual role in germination and early seedling growth

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Abbreviations: ABA, abscisic acid; BSA, bovine serum albumin; CAP, micropylar area around the radicle emergence point or endosperm cap; DAB, diaminobenzidine; DEAE, diethylaminoethyl; IgG, immunoglobulin G; LAT, lateral coat plus endosperm; LBG, locust bean gum; η_{sp}, specific viscosity; PBS, phosphate buffer saline; pI, isoelectric point; RAD, radicle tip; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; Visc. U., viscometric units.

Galactomannans are storage cell wall polysaccharides present in seeds of some legumes. Their degradation is carried out by three hydrolases (α-galactosidase (EC 3.2.1.22), endo-β-mannanase (EC 3.2.1.78) and ß-mannosidase (EC 3.2.1.25)). In the present study we purified and characterised an endo-β-mannanase from seeds of *Sesbania virgata* and addressed its role in germination and seedling development. The polypeptide purified by Ion Exchange Chromatography and Affinity Chromatography on Sepharose-Concanavalin A, showed a pH optimum between 3.5 and 5 at 45°C and high stability at pH 7.8. The low stability at pH 5 appears to be associated with isoelectric precipitation, in view of the pI of the enzyme being 4.5. The purified enzyme is a glycoprotein with a molecular mass of 26 KDa by SDS-PAGE and 36 KDa by gel chromatography. The purified polypeptide attacked galactomannan from different sources, being more effective on polymers with a lower degree of galactosylation (from carob gum), in comparison with medium or highly galactosylated galactomannans (from guar, *S. virgata* and fenugreek), respectively. A peak of endo-β-mannanase activity was detected during radicle protrusion in the endosperm tissue surrounding the radicle and later on in the lateral endosperm. This second peak was associated with the period of reserve mobilisation. Using an antibody raised against coffee endo-β-mannanase, the enzyme could be detected in immunodot-blots performed with extracts of *S. virgata* endosperms. The results are consistent with the hypothesis that the peak of endo-mannanase during germination facilitates radicle protrusion through the surrounding endosperm by weakening it in the region close to the radicle tip.
Key words: *Sesbania virgata*, endo-β-mannanase, galactomannan, germination, Leguminosae.

Endo-β-mananase do endosperma de sementes de *Sesbania virgata* (Cav.) Pers. (Leguminosae): purificação, caracterização e seu duplo papel na germinação e crescimento inicial da plântula: Galactomananos são polissacarídeos de reserva de parede celular presentes em sementes de leguminosas. Sua degradação é efetuada por três hidrolases (α-galactosidase (EC 3.2.1.22), endo-β-mananase (EC 3.2.1.78) e β manosidase (EC 3.2.1.25)). No presente estudo, nós purificamos e caracterizamos uma endo-β-mananase de sementes de *Sesbania virgata* e focamos no seu papel na germinação e no desenvolvimento da plântula. A enzima foi purificada por cromatografia de troca iônica e cromatografia de afinidade em sepharose-concanavalina A, mostrando um pH ótimo entre 3,5 e 5 a 45 °C e alta estabilidade em pH 7,8. A baixa estabilidade em pH 5 parece estar associada à precipitação isoelétrica, pois o pI da enzima é 4,5. O polipeptídeo purificado é uma glicoproteína com massa molecular de 26 KDa em SDS-PAGE e 36 KDa em cromatografia em gel. O polipeptídeo purificado atacou galactomamano de diferentes fontes, sendo efetivo

sobre polímeros com grau de galactosilação mais baixo (goma caroba), em comparação com galactomamanos com médio e alto graus de galactosilação (guar, *S. virgata* e feno grego), respectivamente. Um pico de atividade de endo-β-mananase foi detectado durante a protrusão da radícula no tecido endospérmico ao redor da radícula e mais tarde nas porções laterais do endosperma. O segundo pico foi inversamente associado à mobilização de reservas. Usando um anticorpo feito contra endo-β-mananase de café, a enzima foi detectada por "immunodot-blots" feitos com extratos de endosperma de *S. virgata*. Os resultados são consistentes com a hipótese de que o pico de endo-mananase durante a germinação facilita a protrusão da radícula através do enfraquecimento do endosperma que circunda a área próxima à ponta da radícula.
Palavras-chave: *Sesbania virgata*, endo-β-mananase, galactomanano, germinação, Leguminosae.

INTRODUCTION

The seeds of lettuce, tomato and many legumes are known to accumulate galactomannan or galactoglucomannan in their endospermic cell walls (Buckeridge et al., 2000a; Buckeridge et al., 2000b; Hamer and Bewley, 1979). These polymers are broken down after germination, producing free galactose and mannose (and glucose in the case of galactoglucomannan) which serve as a source of carbon and energy for the growing seedling simultaneous with the biosynthesis of sucrose (Reid, 1971; Buckeridge and Dietrich, 1990).

Usually, galactomannan is deposited in the cell walls of the endosperm during seed development (Meier and Reid, 1977) and is later mobilised following germination by a well-documented mechanism involving production of hydrolases and secretion into living or non-living galactomannan-containing storage tissue (Reid and Davies, 1977; Reid and Meier, 1972; Buckeridge and Dietrich, 1996). Its mobilisation in endosperms of *Sesbania virgata* was followed during and after germination, and the enzymes α-galactosidase (EC 3.2.1.22), endo-β-mannanase (EC 3.2.1.78) and exo-mannanase (EC 3.2.1.25) were detected (Buckeridge and Dietrich, 1996). *S. virgata* (referred to in previous publications by our group under the synonym *Sesbania marginata* Benth.) is a legume shrub that occurs mainly in the gallery forests in tropical regions and is associated with early stages of ecological succession.

Although galactomannan has been considered to be a storage polysaccharide (Reid, 1985; Buckeridge and Dietrich, 2000a) it has also been demonstrated that this polymer can perform other functions, such as modulation of the mechanical strength of the endosperm of tomato seeds during radicle protrusion (Groot and Karssen, 1987), and to control water imbibition in seeds of *Trigonella foenum-graecum* (fenugreek) (Halmer and Bewley, 1979).

Groot and Karsen (1987) demonstrated that gibberellin regulates seed germination through the induction of endosperm weakening in tomato, and Spyropoulos and Reid (1985) suggested that the endosperm of legumes might be under control of auxin and gibberellin. When isolated and incubated in relatively small volumes of water, the endosperms of lettuce (Dulson, et al., 1988) and fenugreek (Spyropoulos and Reid, 1988) were not capable of completing galacto(gluco)mannan degradation. However, they did so when incubated in larger volumes of water. On this basis, these authors proposed the existence of a water-soluble inhibitor(s) which is somehow inactivated (or degraded) during germination and consequently liberates cell wall mobilisation.

Galactomannan mobilisation is inhibited by abscisic acid (ABA) in seeds of lettuce (Halmer and Bewley, 1979), tomato (Groot and Karssen, 1992), fenugreek (Kontos and Spyropoulos, 1995) and *Sesbania virgata* (Potomati and Buckeridge, 2002). In these studies, exogenously applied ABA has been demonstrated to interfere with the activity of galactomannan-hydrolysing enzymes. Dulson et al. (1988) detected endogenous ABA in seeds of lettuce and proposed that it plays a role in the regulation of endo-β-mannanase production in isolated lettuce endosperms.

In tomato seeds, the control of radicle protrusion by a thin endosperm layer has been investigated (Toorop et al., 1996) and the results revealed that endo-β-mannanase activity (one of the principal enzymes responsible for polymer degradation) appears first in the endosperm cap, near the radicle and later on after radicle protrusion in the lateral endosperm (Mo and Bewley, 2002). In this plant, two slightly different genes (*Leman 1* and *Leman 2*) have been cloned and sequenced, the first being spatially and temporally related to the radicle protrusion and the second to cell wall storage mobilisation in the lateral endosperm (Nonogaky and Bradford, 2000).

Although Leguminosae is the largest family with galactomannan storing seeds in nature (Buckeridge et al., 2000b), in our opinion no evidence has been produced that

corroborates or refutes the biological role of galactomannan in radicle protrusion of legumes. In the present work, we isolated and characterised an endospermic endo-β-mannanase from seeds of *Sesbania virgata* and performed time course experiments of activity and Western immunodot-blotting enzyme detection during imbibition, radicle protrusion and storage mobilisation. Our results suggest that a spatiotemporal heterogeneity appears to exist which is similar to the one observed in tomato and lettuce, i.e. one related to radicle protrusion and the other to storage mobilisation.

MATERIAL AND METHODS

Plant material: Seeds of *Sesbania virgata* (Cav.) Pers. were obtained from plants cultivated under natural conditions in the gardens of the Institute of Botany at São Paulo, Brazil. Seeds of *Trigonella foenum-graecum* were a kind gift from Professor J. S. Grant Reid from the University of Stirling, Scotland. The endo-β-mannanase antibody was prepared in rabbits against endo-β-mannanase purified from coffee seeds. The antibodies were kindly provided by Professor Jarbas Giorgini of the Department of Biology (Campus Ribeirão Preto) of the University of São Paulo, Brazil. Guar and carob gums were purchased from Sigma Chem. Co.

Germination: Seeds (30 per each period of imbibition time) were imbibed on Whatman filter paper in **9** cm-diameter Petri dishes (10 seeds per Petri dish) soaked in 5 mL of distilled water and incubated at 25°C under a 12 h photoperiod.

Imbibition and germination studies: Seeds (15 per each period of imbibition time) were imbibed on Whatman filter paper in 5 cm-diameter Petri dishes (5 seeds per plate) soaked in 2.5 mL of distilled water and kept at 25°C under continuous light. The seeds were collected following a time course (imbibition time) from 5 to 40 h at intervals of 5 h. To obtain the fresh mass, the imbibed seeds were dissected with scalpel into three parts [Rad, Cap and Lat (see dissection studies)] and weighed separately.

Dissection studies: Imbibed seeds were dissected with a scalpel separating the micropylar area around the radicle emergence point (*Cap*) (endosperm cap) from the remainder of the seed (*Lat)* (lateral coat plus endosperm) as previously described for tomato (Nonogaki et al., 1992). The radicle tip with approximately 5 mm (*Rad*) was separated from other embryonic tissues.

Galactomannan extraction: Galactomannan was extracted from powdered endosperms isolated from *S. virgata* seeds and from powdered endosperm plus seed coat isolates of fenugreek (*Trigonella foenum-graecum* L.) according to the procedure described by Buckeridge and Dietrich (1996). The powders were extracted with hot water (80°C, 0.1 $g.mL^{-1}$) for 6 h. After filtration through cheesecloth, the extract was centrifuged (10,000 g_n, 30 min, 5°C) followed by precipitation of the supernatant with 3 volumes of ethanol. The precipitate was left to form overnight at 5°C, collected by centrifugation, dried and weighed. This polysaccharide extract contained typically more than 95 % of galactomannan. Carob and guar gums were purchased from Sigma Chem. Co., St Louis, USA.

Extraction and detection of endo-β-mannanase: Activity of endo-β-mannanase was determined in homogenates of endosperm, endosperm plus seed coat, embryo and radicle tip. The homogenates were prepared in 50 mM sodium acetate pH 5 or 20 mM Tris-HCl pH 7.8 buffers. The enzyme was extracted in these two different buffers following a time course (imbibition time) from 10 to 168 h. This time course covered the periods of water imbibition, germination and early seedling growth until cotyledons were fully expanded.

To calculate the enzyme activity, the homogenates (50 μL) were mixed with 500 μL of a 0.5 % solution of *S. virgata*, carob, guar or fenugreek seed galactomannan (the substrates) and 50 μL of sodium acetate buffer (1 M, pH 5) at time 0, and 0.2 mL of the mixture was used for viscometric measurements. The number of units of endo-β-mannanase activity (viscometric units) in the homogenate was arbitrarily calculated from the reciprocal of the time required for the specific viscosity (η_{sp}) to fall to half of its initial value at time 0 in a section of a 0.1 mL glass pipette: [$\eta_{sp} = (t - t_0)/t_0$], where t_0 is the viscometer flow time, in seconds, for the solvent and t the flow time for the solution (adapted from Reid and Davies, 1977). For these calculations, the flow time through the pipette was measured at regular intervals. All our attempts to use activity measurements using Congo Red (Downie et al., 1994) failed.

A rapid method (running assay) to determine the presence of endo-β-mannanase was used in the chromatographic analysis. In this case, the presence of the enzyme in the fractions collected was estimated by a single measurement of the viscosity of the assay solution described above after 30 min of incubation. The differences in flow time before and after the incubation were used to follow enzyme activity during gel chromatography.

Extraction and detection of α*-galactosidase*: To determine α-galactosidase activity the samples were homogenised in 10 mL of 50 mM sodium acetate pH 5 or 20 mM Tris-HCl pH 7.8 buffers at 5ºC and centrifuged (10,000 g_n, 30 min, 5ºC). Aliquots of the supernatant were assayed for α-galactosidase activity using a 50 mM solution of ρ-nitrophenyl-α-D-galactopyranoside (Sigma Chem. Co., St Louis, USA) as substrate. The reaction was stopped by addition of 0.1 N Na_2CO_3 and the absorbance determined at 405 nm (Potomati and Buckeridge, 2002; Reid and Meier, 1973).

*Purification of endo-*β*-mannanase*: The crude homogenate prepared as described above was loaded onto a diethylaminoethyl (DEAE)-cellulose column (250 mL) which had been previously equilibrated with 200 mM Tris-HCl (pH 7.8). The column was washed with the same buffer for 1 h with 20 mM Tris-HCl (pH 7.8). Fractions of 1.5 mL were collected before and after the application of a NaCl gradient (0-0.5 M) in Tris-HCl buffer (20 mM pH 7.8). Aliquots of fractions were assayed for endo-β-mannanase and α-galactosidase activities as described above (running assay). Protein content for each fraction was monitored using the Bio Rad protein assay with BSA as standard (Bradford, 1976) or directly by measuring absorbance at 280 nm. The active fractions were pooled and after dialysis against 20 mM Tris-HCl pH 7.8, the pooled sample was loaded onto a Sepharose-Concanavalin A column (Sigma Chem. Co – 37 mL). The Concanavalin A resin was prepared in 20 mM Tris-HCl pH 7.8 containing 1 M NaCl, 1 mM MgCl, 1 mM $CaCl_2$ and 1 mM MnCl. The fractions with alpha-galactosidase and endo-β-mannanase activities were pooled after elution with 250 mM alpha-methyl-glucopyranoside.

*Endo-*β*-mannanase molecular mass*: To determine the molecular mass of native endo-β-mannanase, the concentrated enzyme preparation (active fractions from DEAE-cellulose) was chromatographed on a Bio Gel P-60 gel filtration column (245 mL of volume, 2 cm diameter, 97 cm height). The elution buffer was 20 mM Tris-HCl pH 7.8 and the column was calibrated with the protein standards BSA (67 kDa), ovalbumin (45 kDa), ß-lactoglobulin (39,5 kDa), myoglobin (17,8 kDa) and cytochrome C (12,4 kDa). The visualisation of the purified endo-β-mannanase enzyme and determination of its molecular mass in a denatured form were performed after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) in a vertical tray (9 x 6 cm glass plate) with 10 % (w/v) polyacrylamide-gel at pH 8.5. The gel was run at room temperature at 20 mA for 1.5 h and subsequently stained with 0.1 % Coomassie Brilliant Blue R250 in a mixture of water/methanol/acetic acid (5/5/2 – v/v/v) and the same gel (after destaining) stained with silver stain (based on Dion and Pometi, 1983).

*Endo-*β*-mannanase characterisation*: The optimum of pH of endo-β-mannanase activity was determined using the viscometric method described above. The crude extract of endosperms, obtained 110 h after imbibition, was incubated with 0.1 % galactomannan from *S. virgata* in McIlvaine phosphate-citrate buffer from pH 2.6 to 7.8. To investigate pH stability, the enzyme was incubated with substrate at pH 5 or pH 7.8 for 24 h at 30ºC and then assayed at pH 5. To detect the optimum temperature, enzyme extracts were incubated with substrate (0.1 % *S. virgata* galactomannan) for 15 min at 0, 10, 15, 28, 40, 45, 50 and 55ºC followed by enzyme assays. The isoelectric focusing of endo-β-mannanase was performed using the Bio Rad Rotofor System. The stability of endo-β-mannanase was determined by the extraction and incubation in two buffers: 20 mM sodium acetate pH 5 and 20 mM Tris-HCl pH 7.8.

Determination of the degrees of hydrolysis: The degree of hydrolysis of galactomannans by endo-β-mannanase was calculated by the viscometric method described above and expressed as a percentage of hydrolysis. Galactomannans from four species (*T. foenum-graecum, S. virgata, Cyamopsis tetragonolobus* and *Ceratonia siliqua*) were used as substrates. They were chosen because of their different D-galactose contents. The mannose/galactose ratios of these polysaccharides are 1:1 (Reid and Meier, 1970), 2:1 (Buckeridge and Dietrich, 1996), 2.6:1 (McCleary, 1983) and 3.8:1 (Dea and Morrinson. 1975) respectively. The galactomannans (500 μl, 0.5%) were incubated at 45 ºC in 1 M sodium acetate buffer (pH 4.5) for 0 to 2 h with three different fractions: purified endo-β-mannanase of *S. virgata*, partially purified endo-β-mannanase plus alpha-galactosidase (pool of active fractions from DEAE-cellulose) and the pool of active fractions from DEAE-cellulose with the addition of 62.5 mM of galactose, a concentration known to inhibit α-galactosidase (data not shown).

Western immunodot-blotting: Seeds incubated in water were harvested daily (30 seeds/plate) and dissected in order to separate seed coat, endosperm, cotyledons and embryo. These tissues were crushed in 50 mM Tris-HCl pH 7.8, filtered and

centrifuged (13,000 g_n, 10 min). Aliquots of the supernatants were assayed for protein (Bradford, 1976). For each extract a volume corresponding to a mass of 100 μg of proteins was separated, freeze-dried and subsequently resuspended in 50 mM Tris-HCl pH 7.4. A volume corresponding to a mass of 2 μg of protein was applied on the surface of a nitrocellulose membrane. It was incubated for 1 h in 100 mM potassium ferricyanide to inhibit seed peroxidase activity. The membrane was blocked for 3 h in a solution containing 0.1 % gelatin, 1 % BSA in phosphate buffer saline (PBS) pH 7.0 and Tween 20, followed by overnight incubation in a solution containing coffee anti-endo-β-mannanase antibody diluted 1:200 in PBS. The membrane was washed 5 times with 50 mM Tris-HCl pH 7.4 and subsequently incubated for 1 h in a solution containing goat anti-rabbit IgG conjugated to peroxidase diluted 1:200 in PBS. The membrane was washed again and the reaction was visualised by the addition of diaminobenzidine (DAB) solution (50 mg in 100 mL 50 mM Tris-HCl pH 7.4) followed by 0.03 % hydrogen peroxide. The extracts containing endo-β-mannanase appear darkest in the post-reaction of the Western immunodot-blot.

RESULTS

Purification of endo-β-mannanase: The purification procedure involved anion-exchange chromatography on DEAE-cellulose (figure 1A) and affinity-purification on a concanavalin A column (figure 1B). The supernatant was applied onto a DEAE-cellulose anion-exchange column and the activity was concentrated. The endo-β-mannanase and alpha-galactosidase activities eluted from DEAE-cellulose column in two peaks, the first one appearing in the wash volume and the second after the application of a NaCl gradient (0-0.5 M). The activity present in the wash volume contained only a small proportion of endo-β-mannanase, and it was not investigated further. On the other hand, the major peak of retained endo-β-mannanase coinciding with the largest peak of alpha-galactosidase activity was further investigated (figure 1A). The fractions containing activity were pooled and applied onto a Concanavalin A column and after elution with 250 mM α-methyl-glucopyranoside, the α-galactosidase activity was concentrated into a sharp peak (three fractions) whereas the endo-β-mannanase activity was broader (figure 1B). Due to this difference, it was possible to find fractions with endo-β-mannanase activity with virtually no α-galactosidase activity. The analysis of these pooled fractions by SDS-PAGE revealed a single band with molecular mass of 26 kDa (figure 2B).

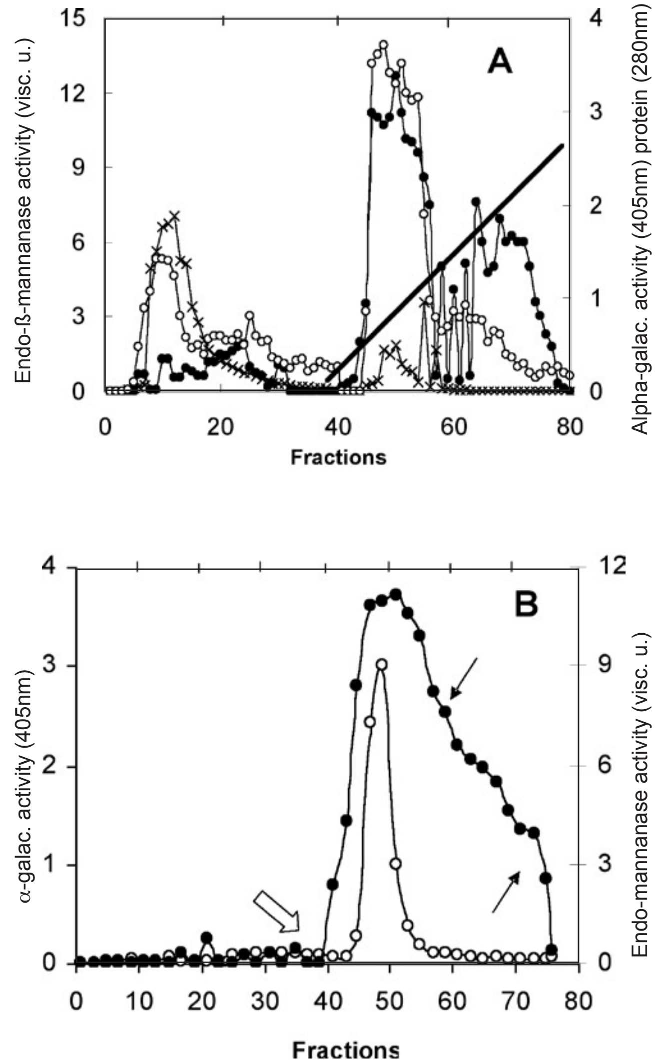

Figure 1. Endo-ß-mannanase (closed circles), α-galactosidase (open circles) activities and protein content for each fraction determined by measuring absorbance at 280 nm (x) during the first step of endo-ß-mannanase purification on DEAE-cellulose. The line represents a gradient of NaCl from 0 to 0.5 M (A). Second step of enzyme purification: step-wise elution with alpha-methyl-glucopyranoside (white arrow) on an affinity column of Sepharose Concanavalin A. The closed circles represent endo-ß-mannanase activity and the open circles α-galactosidase activity. The black arrows indicate the pooled fractions of endo-ß-mannanase free of alpha-galactosidase (B).

Characterisation of endo-β-mannanase

Estimation of molecular mass by gel-filtration: Concentrated (pool of active fractions from DEAE-cellulose) endo-β-mannanase activity was subjected to gel filtration on a Bio Gel P-60 column. As shown in figure 2A, the two enzymes were

well separated since the apparent molecular masses of alpha-galactosidase and the endo-β-mannanase were estimated to be 45 and 36 kDa, respectively. The molecular mass of α-galactosidase confirmed results from our laboratory with the purified enzyme (data to be published elsewhere).

Optimal pH: Figure 3A shows the optimal pH for activity of endo-β-mannanase, which corresponded to a range between pHs 3.5 and 4.5. The activity was low at pH 2.5 and increased strongly at pH 3 becoming fairly constant in the range pH 3.5 to 4.5. Thereafter, the enzyme activity decreased reaching less than half of its maximal activity at pH 5.5.

Optimal temperature: The enzyme was incubated at different temperatures at the pH optimum in the presence of substrate. According to figure 3B, the endo-β-mannanase activity increased linearly with temperature up to 40°C, then exponentially from 40 to 50°C followed by a decrease at higher temperatures. It was also observed that almost all activity was rapidly lost during incubation at 55°C for incubations longer than 15 min (data not shown).

Isoelectric focusing: When the non-denatured extract from pooled fractions of DEAE-cellulose was subjected to isoelectric focusing, only one peak was observed along the pH gradient. The isoelectric point (pI) value for endo-β-mannanase was 4.5 (figure 3C).

Enzyme stability : On storage for 96 h at 30°C, the enzyme showed no loss in activity at pH 7.8, but it lost more than 50 % of its activity at pH 5 (figure 4A) after incubation for 24 h. The differential responses to storage at different pHs were used to optimise the extraction conditions. In figure 4B, a time course of extraction of endo-β-mannanase using 20 mM sodium acetate pH 5 or 20 mM Tris-HCl pH 7.8 buffers showed that the latter was twice as efficient as the former regarding extraction.

Determination of the degree of hydrolysis: Endo-β-mannanase viscometric activity was determined with purified endo-β-mannanase from *S. virgata* endosperm homogenates, using as substrates the galactomannans from *S. virgata,* fenugreek, guar and carob (figure 5). We took advantage of the fact that the preparations of pooled fractions from DEAE-cellulose were contaminated with alpha-galactosidase, to test the specificity of endo-β-mannanase for galactomannans with different mannose/galactose ratios. We also took into

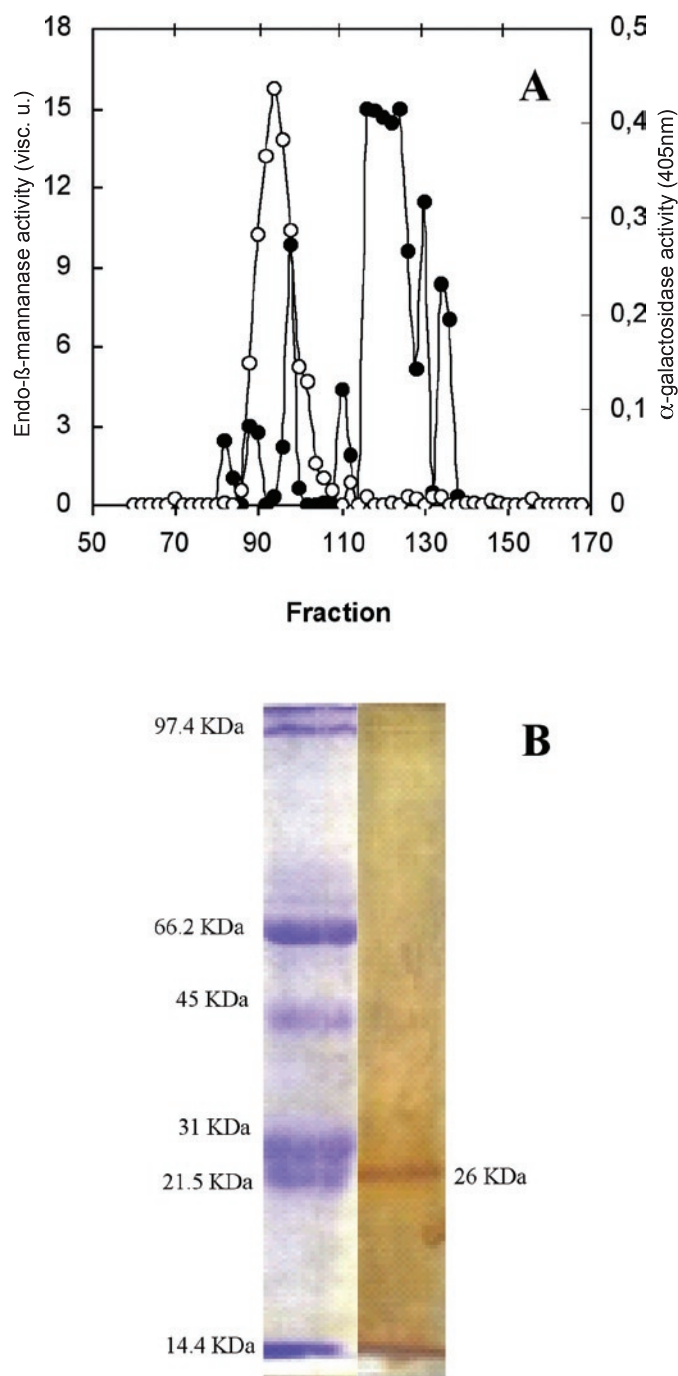

Figure 2. Determination of molecular mass of endo-ß-mannanase by gel chromatography (Bio Gel P-60) (A). Open circles represent the α-galactosidase activity (45 kDa) and the closed circles correspond to the endo-ß-mannase activity (39 kDa) without the α-galactosidase. Estimated denatured molecular mass of endo-ß-mannanase (26 kDa) by SDS-PAGE (B). The single band corresponds to the purified enzyme.

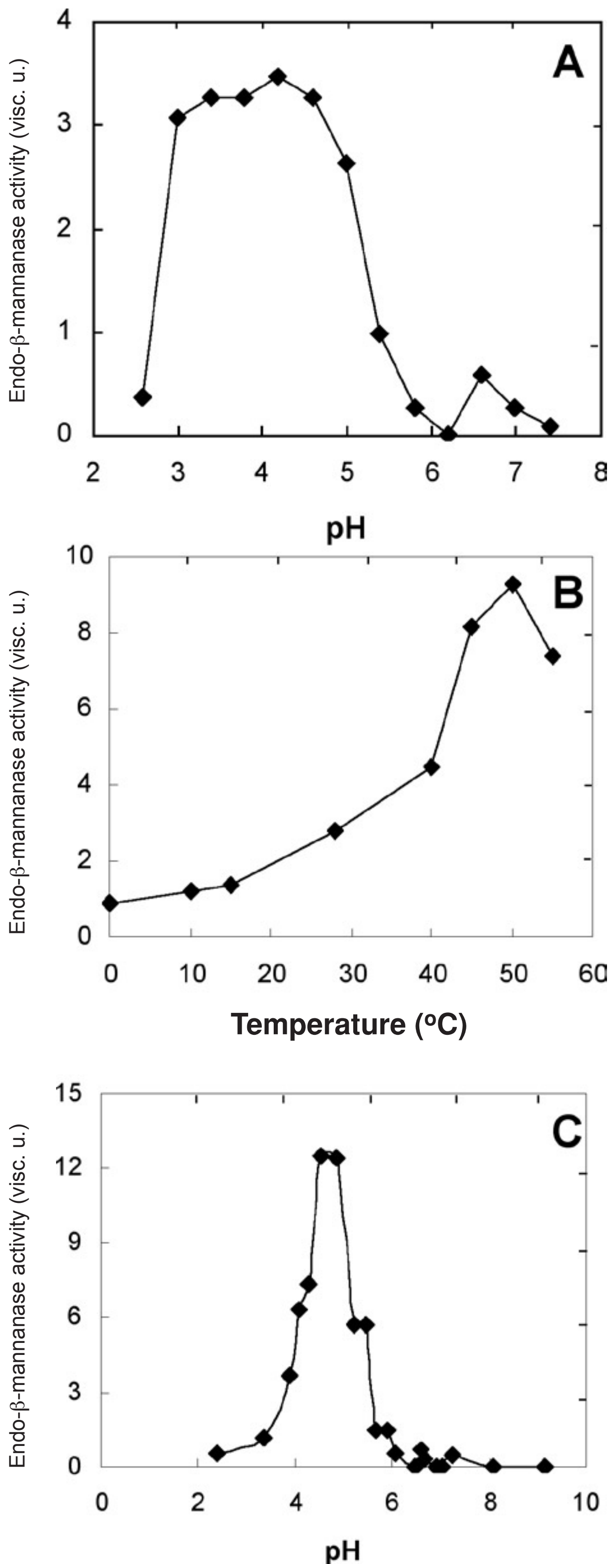

Figure 3. Endo-ß-mannanase characterisation. pH optimum of endo-β-mannanase activity (A). The activity was measured at different pHs using citrate-phosphate buffer. Optimum temperature or thermal stability of endo-β-mannanase (B). Isoelectric point of endo-ß-mannanase (C). The enzyme precipitated at pH 4.5.

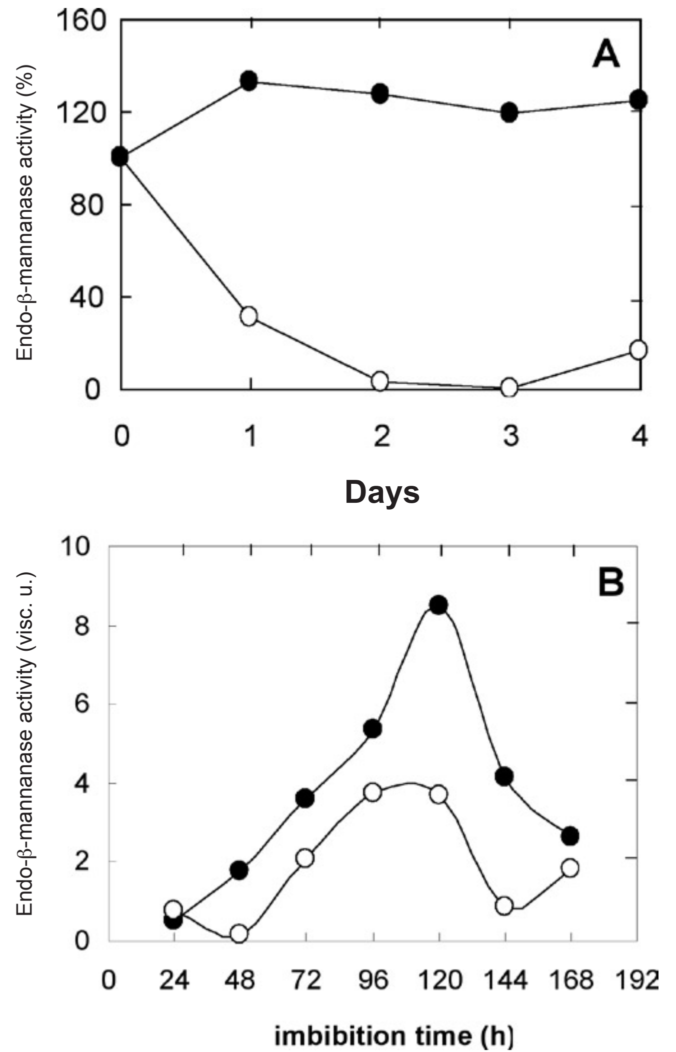

Figure 4. Stability of endo-β-mannanase at pH 5 (open circles) and pH 7.8 (closed circles) (A). The enzyme was stored in both pHs for up to 5 days at 30ºC, but the activity was measured at pH 5. Endo-β-mannanase extraction (B) in pH 5 buffer (20 mM sodium acetate) (open circles) and pH 7.8 buffer (20 mM Tris-HCl) (closed circles).

consideration the fact that the alpha-galactosidase from *S. virgata* is known to be inhibited by its own product (data not shown).

The purified endo-β-mannanase was capable of hydrolysing all galactomannans, with the exception of the one from *Trigonella foenum-graecum*, which is fully substituted. When the DEAE-cellulose pooled fraction (contaminated with α-galactosidase) was used as the source of enzyme, this galactomannan was then attacked by the endo-β-manannase. On the other hand, endo-β-mannanase was less active on *Trigonella foenum-graecum* galactomannan when in presence of 65.2 mM galactose, which prevents the attack

by α-galactosidase. These results clearly indicate that the presence of galactose branching prevents the action of the endo-β-mannanase from seeds of *S. virgata* towards the main galactomannan chain. Furthermore, our results also indicate that a proportion of at least 2:1 is necessary to permit access of the enzyme to the main chain of the galactomannan molecule (figure 5).

Time course of endo-β-mannanase activity: Fresh mass of the endosperm, enzyme activity and its presence (immuno-detected by a coffee anti-endo-β-mannanase antibody) were followed during germination and early seedling growth (figures 6A and 6B). Fresh mass of the endosperm was used as a means of detecting the period of bulk galactomannan degradation. A large decrease in fresh mass was observed after 96 h, ca. 30 h after radicle protrusion had taken place (figure 6A). The fresh mass of the endosperm decreased linearly between 96 and 144 h (5 days) to about 10 % of its initial value. This observation can be associated with galactomannan mobilisation (for details see Buckeridge and Dietrich, 1996) which is thought to be used as a storage compound.

Endo-β-mannanase activity increased transiently at 24 h, i.e. 6 h before detection of radicle protrusion (30 h) and then increased linearly to a much higher level from 48 to 120 h (this was our criterion to choose the ideal moment for enzyme extraction and purification). The detection of endo-β-mannanase by Western immunodot-blotting showed that in the endosperm the presence of enzyme was consistent with its increase in activity (figure 6B).

Detection of endo-β-mannanase activity in different regions of the S. virgata seed: The detection of a peak of endo-β-mannanase activity during the germination period before radicle protrusion, raised the possibility that the enzyme might be associated with the latter process. Therefore, the presence and pattern of changes in endo-β-mannanase activities were followed along with water imbibition in

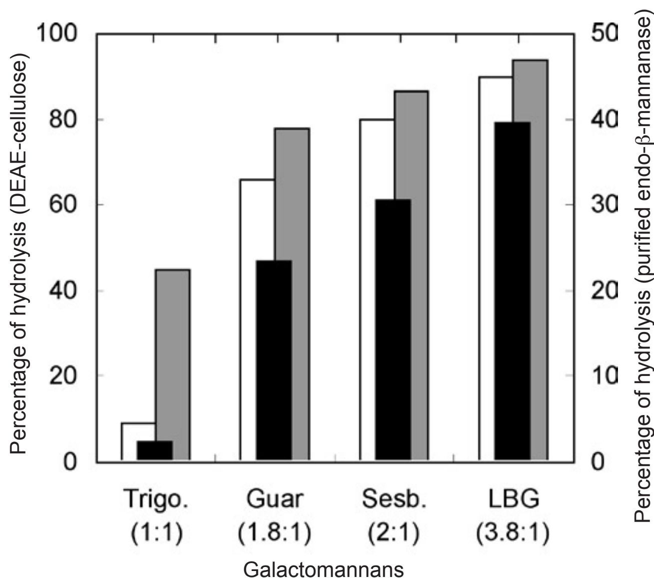

Figure 5. Degree of hydrolysis of galactomannans from different sources catalyzed by purified endo-β-mannanase (black bar), a mixture of α-galactosidase and endo-β-mannanase without addition of galactose (white bar) and a mixture of alpha-galactosidase and endo-β-mannanase with addition of 62.5 mM of galactose (gray bar). Aliquots of these three enzyme sources (60 μL) were incubated at 45°C in 1 M sodium acetate buffer (pH 5) with galactomannans from fenugreek, guar, *S. virgata*, carob (LBG), respectively (mannose:galactose ratios are expressed in the figure).

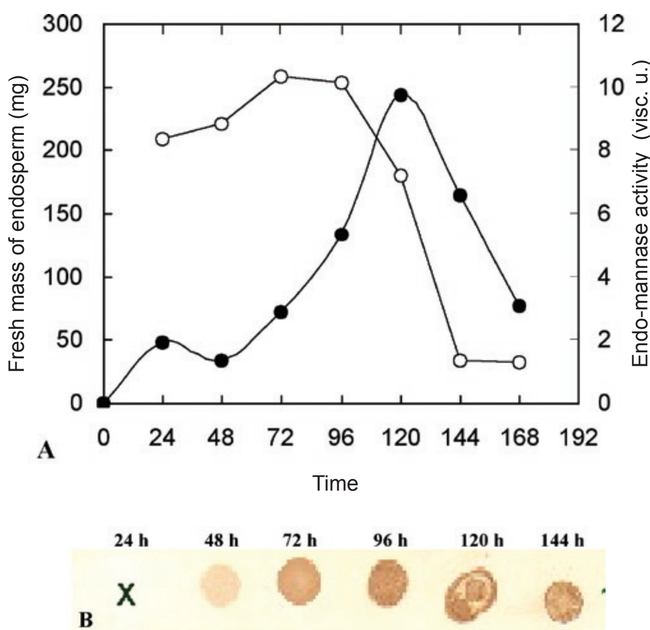

Figure 6. Storage mobilisation and hydrolase activity in endosperms of seeds of *Sesbania virgata* (A). Time course of galactomannan degradation (open circles represent fresh mass of the endosperm with galactomannan corresponding to 80 % of its dry mass) and endo-β-mannanase activity in the endosperm (closed circles). Western immunodot-blot (B) using endo-β-mannanase antibody prepared in rabbits against endo-β-mannanase purified from coffee seeds for detection of the presence of enzyme in the endosperm along the time course.

different parts of the seed in order to further investigate this possibility.

Figure 7 shows changes in fresh mass (figure 7A), compared with endo-β-mannanase activities, in different parts of the seed. Fresh mass of the radicle started to increase rapidly after 5 h (radicle length follows fresh mass – not shown), reached a plateau after 15 h and started to increase linearly after 25 h. Water imbibition was measured separately in the endosperm plus seed coat at the radicle region (*Rad*) and in the lateral endosperm (*Lat*). Whereas *Lat* fresh mass increased slowly in a linear fashion, the *Rad* showed a peak between 15 and 30 h and then decreased. These data can be compared to the changes in activity of endo-β-mannanase in the same seed parts (figure 7B). Enzyme activity was high in the radicle tissue (*Rad*) during early stages of imbibition. It decreased almost linearly up to 30 h, when radicle protrusion took place, and then decreased more rapidly up to 40 h, when it was no longer detected. The activities present in the lateral endosperm (*Lat* - the bulk storage tissue in *S. virgata*) and the *Cap* region (the endosperm layer surrounding the radicle) were followed separately (figure 7B). We observed that whereas in the *Lat* region the activity increased steadily, in the *Cap* region there was a clear peak of activity at 24 h which was followed by a decrease to the initial levels of activity.

DISCUSSION

Properties of the endo-β-mannanase from endosperm of Sesbania virgata: The principal endo-β-mananase from the endosperm of *Sesbania virgata* was purified and characterised. The enzyme has maximal activity at 50°C and is a polypeptide of ca. 26 kDa. The purified enzyme was shown to be active towards galactomannans, producing a decrease in viscosity characteristic of endo-β-mannanases from other legumes (Reid and Davies, 1977; McCleary, 1979). The *S. virgata* endo-β-mannanase was shown to be sensitive to the presence of galactose branching in the main chain of galactomannan, since it was not active on galactomannan from *Trigonella foenum-graecum*, which has a main chain fully substituted with galactose. This indicated that, in certain situations, α-galactosidase action is a necessary condition to grant access for endo-β-mannanase to the main chain of the galactomannan polymer. Thus, the action of α-galactosidase is probably fundamental for the completion of galactomannan degradation *in vivo* and the importance of this enzyme as a modulator of disassembly of galactomannan is increasingly important as galactose branching increases. The fact that α-galactosidase is strongly inhibited by galactose permits speculation that the debranching enzyme is possibly an important metabolic control point in storage mobilisation in seeds whose main storage carbohydrate is galactomannan. When galactomannan is attacked at the same time by the two enzymes (α-galactosidase and endo-β-mannanase) the galactose produced and accumulated in the cell wall will decrease the activity of the former by competitive inhibition (results to be published elsewhere). This still leaves the undegraded polymer with galactose branches, that are likely to interfere with the action of endo-β-mannanase. This type of control of the polysaccharide disassembling kinetics by the branches has been described for xyloglucan (Tiné et al., 2003) and implies that part of the

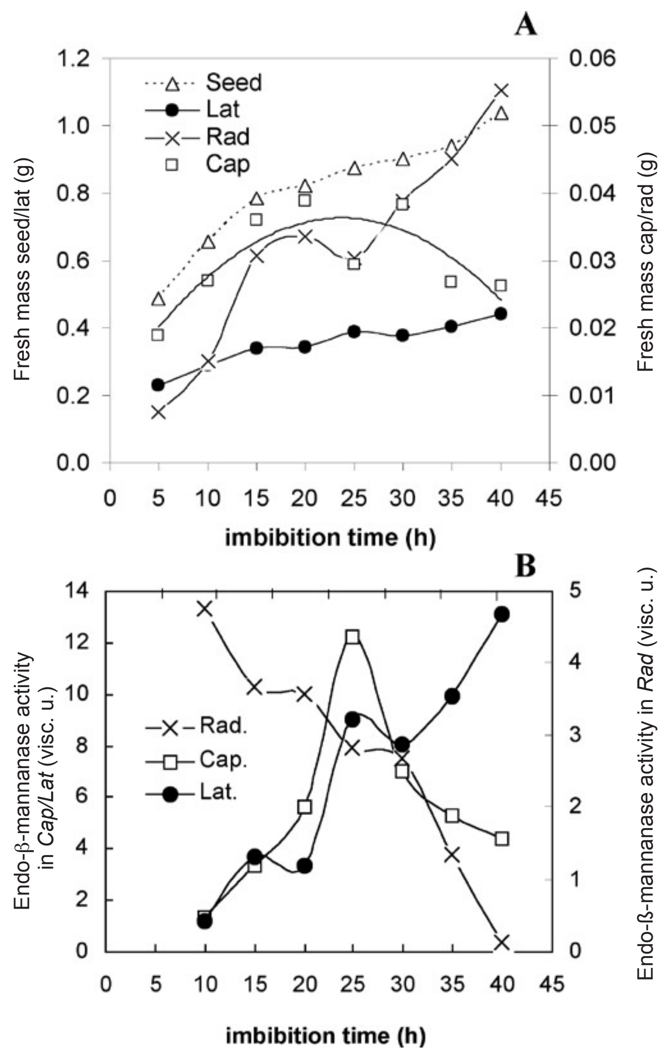

Figure 7. Endo-β-mannanase activity in different parts of the seed. The extracts were prepared in 20 mM Tris-HCl pH 7.8 from three distinct parts of the seeds: radicle tip (*Rad*), seed coat and lateral endosperm (*Lat*) and seed coat and endosperm cap (*Cap*) (endosperm at the radicle protrusion region).

control of the disassembling process is programmed in the substrate itself during biosynthesis.

Once degradation proceeds, the disassembling rate could be controlled by the flow of carbon through the endosperm cells. The internalisation and metabolism of galactose to sucrose by endosperm cells and the further transport and use of the latter by the growing embryo (McCleary, 1975; Buckeridge and Dietrich, 1996) would increase the activity of α-galactosidase, creating sites for the attack of the endo-β-mannanase upon the polymer. These events would probably increase the disassembling rate. From a physiological point of view, galactomannan mobilisation would be controlled by the intracellular metabolism, which is in close relation to the sink strength of the growing embryo.

Another point of control of galactomannan degradation in the endosperm of seeds of *S. virgata* might be related to some ionic properties of endo-β-mannanase. We have observed that its action on galactomannan from the same species is maximal at a range of pH between 3 and 4.5. We also found that whereas the activity was stable for up to 5 days in pH 7.8, it was rapidly lost at pH 4.5. These observations, together with the fact that the pI of endo-β-mannanase was 4.5, lead to the suggestion that the optimum pH of activity was also a condition that led the enzyme to isoelectric precipitation. A search in the literature for similar results (table 1) showed that endo-β-mannanases from different sources have similar properties (i.e. pH optimum=pI). It is possible that the fact that pI and pH optimum of endo-manannases are the same is physiologically relevant, but further investigation is necessary to confirm this hypothesis.

The dual functional character of endo-β-mannanase activity in seeds of Sesbania virgata: The endo-β-mannanase activity was detected in different tissue homogenates from seeds at different stages of germination and seedling growth (post germination). The enzyme was present in different tissues of *S. virgata* seeds: radicle tip (*Rad*), endosperm cap (*Cap*) and lateral endosperm (*Lat*). Endosperm and embryo tissues (radicle) were examined separately because storage galactomannan is present only in the former, and because the two organs perform quite different metabolic functions especially during germination (Reid, 1971; Buckeridge et al., 2000a). According to the activity measurements shown in figure 7B, this enzyme was present in the *Rad* and *Cap* prior to the first signs of emergence of the radicle. The highest activity in the *Rad* was observed 10 h after imbibition, it then decreased and was virtually undetectable at 40 h. In this region, after 36 h (6 h after radicle protrusion) no activity could be detected. This enzyme, as for the endo-β-mannanase of tomato seeds (Toorop et al., 1996; Mo and Bewley, 2002), was present in the mycropylar area (endosperm cap) during germination (figure 7B).

These observations suggest that in spite of the endosperm *Cap* being punctured by the radicle to complete germination, in seeds of *Sesbania virgata*, as in tomato (Nonogaki et al., 2000), enzymatic hydrolysis is necessary in the puncture zone to complete radicle emergence. Note that the fresh mass of the *Rad* increases up to15 h and levels off up to 25 h. It only starts to increase again when endo-β-mannanase activity in the *Cap* region reaches its peak of activity. Then, the fresh mass of the developing radicle increases linearly (figure 7A). This correlative evidence strongly suggests that endo-β-mannanase activity is related to puncture by the radicle, since this is indeed observed after 24 h. Apparently, *Cap* function as a mechanical constraint to radicle protrusion resumes after this period, since its fresh mass starts to decrease thereafter (figure 7A).

In contrast, lower mannanase activity was observed in *Lat* during germination (figure 7) and there was a detectable tendency for its activity to increase after radicle protrusion up to 120 h, when endo-β-mannanase reached its maximum catalytic activity (figure 6A). Even considering that the lateral endosperm fresh mass was approximately twice as high as the endosperm *Cap*, the total amount of enzyme activity in the *Lat* area was greater than in the micropylar endosperm. Endo-β-mannanase activity did not increase significantly in the lateral endosperm until after the completion of germination. This is consistent with previous findings for

Table 1. Comparison of endo-ß-mannanases from different species (data taken from McCleary, 1979).

Source of mannanase	Molecular Mass (KDa)	pI	Optimal pH	Optimal temperature (°C)
Medicago sativa	41	4.5	4.5	50
Basidiomycetes sp.	53	5.0/5.5	5.0	60
Aspergillus niger	45	4.0	3.0	70
Bacillus subtilis	37	5.1	5.0/6.0	50

seeds of tomato (Nonogaki et al., 2000; Toorop et al., 1996; Mo and Bewley, 2002). This observation confirms the hypothesis that galactomannan degradation in the endosperm *Lat* area during seedling growth is directly linked to reserve mobilisation for embryo development (Buckeridge and Dietrich, 1996).

Although it is not yet known whether *Cap* and *Lat* activities are the same polypeptide or not, and therefore encoded by the same or different genes, respectively, as has been observed for tomato seeds, our results suggest that endo-β-mannanase activity present in the legume seed of *Sesbania virgata* plays a dual physiological function, one related to radicle emergence and the other to storage (galactomannan) mobilisation after germination, depending on its location in the endosperm. This is the first observation of its kind for a plant belonging to the family Leguminosae, which is comprised of about 18,000 species and is the main one that accumulates relatively large amounts of galactomannan in its seeds. The finding of such a dual physiological role for the enzyme in a tropical wild legume species such as *S. virgata* highlights the importance of the specialisation of galactomannan during evolution as a multifunctional compound (Buckeridge et al., 2000a,b) playing a role during germination (in the release of radicle protrusion) and subsequently in the establishment of the seedling (as a carbon source). There appears to be no doubt that both specialisations maximise the ecophysiological performance of the species under natural conditions in the rain forest.

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