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Endo-β-mannanase and β-mannosidase activities in rice grains during and following germination, and the influence of gibberellin and abscisic acid

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Abstract

Grains of *indica* rice (*Oryza sativa* cv. Peiza 67) exhibit an increase in endo-β-mannanase activity, mostly after the completion of germination. According to tissue blots, the initial increase occurs in association with the embryo, and possibly the scutellum, although the largest sustained increase in activity is in the peripheral regions of the endosperm. The aleurone layer, being the only living region of the endosperm, is presumably the site of synthesis and secretion of the enzyme into the non-living, starch-laden region. β-Mannosidase activity is low throughout germination and subsequent seedling growth, particularly in the endosperm regions. Its activity profile does not mimic that of endo-β-mannanase. In the intact grain, gibberellin (GA) causes a relatively small increase in endo-β-mannanase activity, while abscisic acid (ABA) causes a large decrease; this inhibition is overcome to a considerable extent when GA is supplied along with ABA. β-Mannosidase activity is little affected by either GA or ABA. Embryoless half-grains imbibed in water exhibit only a small increase in endo-β-mannanase activity with time of imbibition, showing the necessity for a stimulus from the embryo for this to occur. Incubating half-grains in the presence of GA results in a large increase in enzyme activity; ABA reduces the amount of activity compared to the water controls. GA is capable of reversing the inhibitory effect of ABA with respect to endo-β-mannanase activity. As in the intact grains, β-mannosidase activity in the half-grains is unaffected by either GA or ABA. It is concluded that the major site for the production of endo-β-mannanase activity is the aleurone layer, and this event is influenced by the presence of the embryo; in the absence of the latter, the increase in enzyme activity is stimulated by GA. β-Mannosidase activity is low throughout germination and post-germination, it is not influenced by GA and ABA, and thus its activity is not regulated in a coordinated manner with that of endo-β-mannanase.

Keywords: abscisic acid, aleurone layer, β-mannosidase, endo-β-mannanase, gibberellin, *Oryza sativa*, rice

Introduction

The endosperm cell walls of many seeds contain mannan polymers, often in the form of galactomannans, although in some very hard seeds (e.g. ivory nut, *Phytelephas macrocarpa*) there is little galactose substitution in the mannose backbone (Bewley and Reid, 1985; Reid, 1985). These mannose polymers are a form of storage carbohydrate that is mobilized following germination. They may play additional roles: as mucilages to store water within the seed during germination and early seedling growth (e.g. in fenugreek), thus reducing susceptibility to drought, or to restrict germination until an appropriate signal is received to weaken the endosperm cell walls in the region around the radicle, to permit its emergence (Bewley, 1997).

The first enzyme involved in the mobilization of mannan polymers is endo-β-mannanase, which randomly cleaves the mannose backbone to release mannobiose and mannotriose; in turn, these are hydrolysed to mannose by β-mannosidase. Endo-β-mannanase is present in the seeds of many species, most frequently following the completion of

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germination (Dirk *et al.*, 1995). The majority of reports have been on the presence of this enzyme in seeds of dicots, and its presence in gymnosperm seeds during and following germination is known (Downie *et al.*, 1997). There are few reports of its presence in seeds of monocots, and the only detailed observations have been made in date palm seeds (DeMason *et al.*, 1985). A survey of cereal grains showed that the enzyme is present in imbibed oats, wheat, rice and barley, but not in maize (Dirk *et al.*, 1995). In isolated aleurone layers of the cv. Himalaya of barley, endo-β-mannanase activity is induced by gibberellic acid (GA) (M. Banik and J.D. Bewley, unpublished). A large number of hydrolases increase in cereal grains following germination, generally associated with the mobilization of the major storage reserves within the starchy endosperm (Bewley and Black, 1994); these include hydrolases to mobilize the starch and protein reserves (amylases and proteases) and to mobilize the cell walls of the aleurone layer and starchy endosperm (pentosanases and glucosidases). The synthesis and secretion of many of these enzymes is induced by GA, and is suppressed by abscisic acid (ABA). In germinated barley and rice grains, α-amylase is initially synthesized and released from the scutellum, and then later from the aleurone layer, under the regulation of GA (Gibbons, 1979; Okamoto and Akazawa, 1979).

Since endo-β-mannanase has been studied quite extensively in seeds of a variety of dicot families, but not in cereal grains, we undertook to determine when and where this enzyme is active in rice during and following its germination, and if hormones have any effect on enzyme activity. Galactomannans are known components of the endosperm cell walls of rice grains (Shibuya and Iwasaki, 1978), and hence the production of mannan-degrading enzymes was anticipated. Because activity of β-mannosidase was expected to be present concurrently with that of endo-β-mannanase, its activity was also followed.

Materials and methods

Rice grain germination conditions

Rice (*Oryza sativa* L. cv. Peiza 67, *indica* subspecies) grains were provided by the South China Agricultural University, Guangdong, China. Triplicate lots of 100 dehulled grains (lemma and palea removed by hand) were imbibed on 6 ml water or phytohormone solutions (GA and/or ABA, Sigma Chemical Co., St. Louis, Missouri, USA) on Whatman No. 1 filter paper in 9-cm-diameter Petri dishes at 25°C in the dark. The numbers of germinated grains were recorded every 4 h.

Endo-β-mannanase (E.C. 3.2.1.78) and β-mannosidase (E.C. 3.2.1.25) extraction and activity

Duplicate lots of five dehulled grains were imbibed on 2 ml water or phytohormone solution (100 μM GA, 100 μM ABA and 50 μM GA + 50 μM ABA) on Whatman No. 1 filter paper in 5-cm-diameter Petri dishes at 25°C in the dark. At the required intervals the imbibed grains were dissected into embryo, (starchy) endosperm and aleurone layer, and the separate parts transferred into a small mortar for grinding in 250 μl Hepes–NaOH buffer (0.1 M Hepes, 0.5 M NaCl, pH 8), and then centrifuged in a 1.5 ml Eppendorf tube at 4°C for 5 min at 13,000 rpm. The supernatant was used for enzyme assays. For experiments using half-grains, these were obtained by cutting dry dehulled grains in the mid-region of the starchy endosperm, and duplicates of five embryoless half-grains were used for treatment with phytohormone solutions to determine their influence on enzyme activity.

To assay endo-β-mannanase activity, duplicate 2 μl aliquots of the supernatant were loaded into 2-mm-diameter wells in an agarose gel into which locust bean gum (Sigma) was incorporated as substrate, and assayed using a gel-diffusion assay (Bourgault and Bewley, 2002). Modification or degradation of the substrate galactomannan, as shown by staining with Congo Red dye (Sigma), specific for endo-β-mannanase activity, was calculated against a serial dilution of *Aspergillus niger* endo-β-mannanase (Megazyme, Bray, Eire) as standard.

For assays of β-mannosidase (β-mannoside mannohydrolase) activity, duplicate 60 μl aliquots of extract were incubated with 90 μl 2 mM 4-nitrophenyl β-D-mannopyranoside (Sigma) in McIlvaine buffer (0.1 M citric acid, 0.2 M $Na_2HPO_4.7H_2O$, 1:1.1 ratio, pH 5) for 2 h at 37°C (Ouellette and Bewley, 1986). The reaction was terminated and colour developed by the addition of 75 μl 0.2 M aqueous sodium carbonate. The yellow colour produced was measured at 405 nm in a microplate reader (Molecular Devices Corp., Sunnyvale, California, USA). Assay controls involved adding enzyme extract after the sodium carbonate. The extinction coefficient for 4-nitrophenyl β-D-mannopyranoside was taken as 18,400 (Reid and Meier, 1973) to calculate the amount released in pmol min^{-1} $seed^{-1}$ or seed $part^{-1}$.

Tissue prints

Dehulled rice grains imbibed for different times were cut into halves longitudinally by using a freezing microtome (Leica CM 1850, Wetzlar, Germany) and laid with the cut surface down on the top of the activity gel described above. After incubation for

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5 min at room temperature, the seed halves were removed from the gel and the gel was stained with Congo Red as above. The clearing zones on the gel indicated where the enzyme activity was present.

Results

Germination of rice

Dehulled rice grains of the cv. Peiza 67 showed the first signs of radicle emergence at about 36 h from the start of imbibition, and germination of the population was almost completed (92%) by 72 h (Fig. 1).

Endo-β-mannanase and β-mannosidase activity and location in the grain

Endo-β-mannanase activity was measured during and following germination. The enzyme was determined to be soluble in low-salt buffer, i.e. the addition of 0.5 M NaCl to the extraction buffer did not increase the amount of enzyme obtained (not shown); this is as reported for other seeds, e.g. tomato, lettuce and fenugreek (Dirk *et al.*, 1995).

Determination of the location of endo-β-mannanase activity was conducted using dehulled grains. Enzyme activity in the whole grain was first detected very faintly after 24 h from the start of imbibition, and was prominent after 48 h (Fig. 2A), following completion of germination by approximately 60% of the grains in the population (Fig. 1), and continued to increase up to 120 h (data not shown). Endo-β-mannanase activity was present in the embryo, endosperm and aleurone layer (Fig. 2A). On a per seed (grain) part basis, most activity was present in the starchy endosperm, the largest tissue. The fresh weight of the aleurone layer was about 20% of that of the starchy endosperm, so the endo-β-mannanase content per unit weight was greatest in the former. A decline in activity occurred between 72 h and 96 h, perhaps related to the degradation of the aleurone layer, and continued growth of the embryo, with the growing seedling exhibiting less enzyme activity.

Since hydrolysis of mannan polymers to the monomer requires both endo-β-mannanase and β-mannosidase activities, the latter was also measured. It was routinely extracted using a high-salt buffer (containing 0.5 M NaCl), although this led to less than a 10% increase in activity compared to extraction with a low-salt buffer. Thus, β-mannosidase is a soluble enzyme in the rice grain, as in fenugreek (Reid, 1985), but unlike the enzyme in tomato and

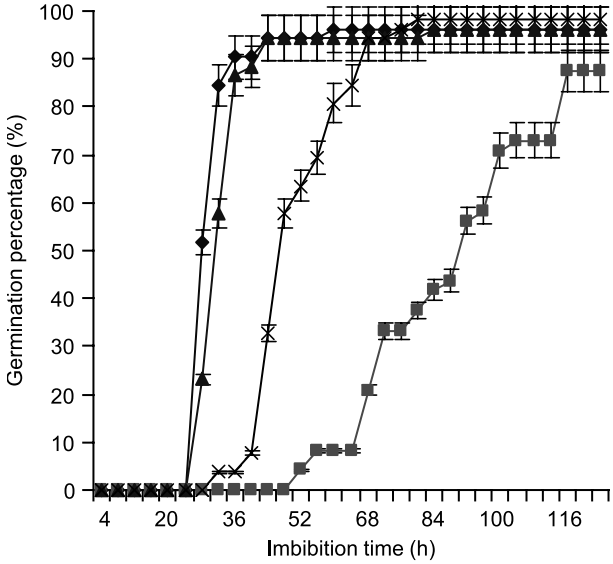

Figure 1. The germination time course of dehulled rice grains imbibed at 25°C on water (×) or 100 μM gibberellic acid (GA, ◆), 100 μM abscisic acid (ABA, ■) or 50 μM GA + 50 μM ABA (▲). Error bars show the variation in germination among triplicate samples.

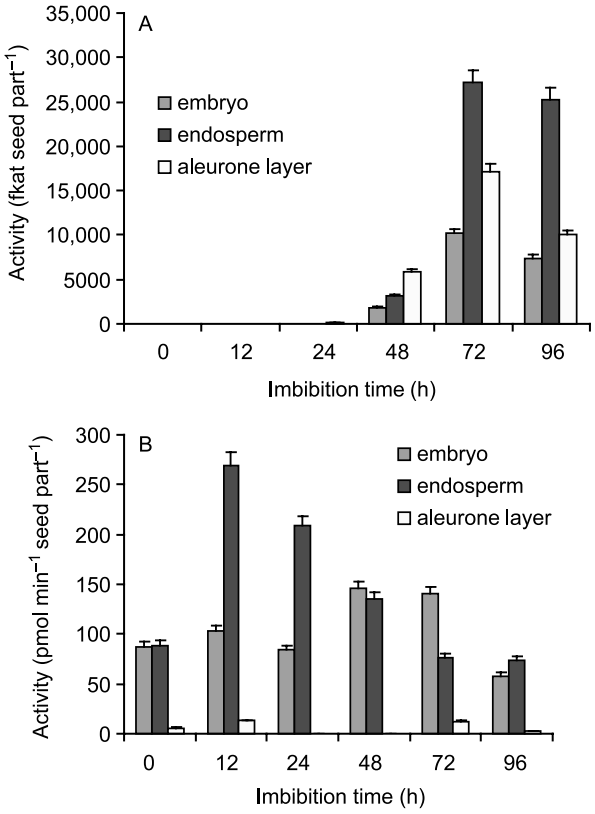

Figure 2. (A) Endo-β-mannanase and (B) β-mannosidase activities in dehulled rice grains imbibed for up to 96 h on water at 25°C before dissection into embryo, endosperm (starchy endosperm) and aleurone layer, and enzyme assay of the dissected parts. Error bars show the variation in activity between duplicate samples.

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lettuce (Ouellette and Bewley, 1986; Mo and Bewley, 2002). Its activity was present in the embryo and starchy endosperm throughout germination and early seedling growth (Fig. 2B), and was both unrelated to the time of completion of germination and to endo-β-mannanase activity.

Tissue prints were used also to determine the location and timing of increased endo-β-mannanase activity following germination. Dehulled grains were germinated for up to 72 h, cut in half longitudinally (Fig. 3A), and placed on a gel containing galactomannan substrate. Some endo-β-mannanase activity was detected very faintly in the embryo region in 36-h-imbibed grains, but more clearly after 48 h when the radicles of the grains were starting to elongate (Fig. 3C, D). At this latter time, some enzyme activity was also apparent in the outer layer of the starchy endosperm (most likely the aleurone layer), and within another 12 h it was present copiously in the area of the embryo/scutellum, and more obviously in the outer layer of the starchy endosperm (Fig. 3E). By 72 h, a clear ring of activity surrounded the starchy endosperm (Fig. 3F), most likely due to endo-β-mannanase secretion from the aleurone layer, which exhibited a large increase in enzyme activity at this time (Fig. 2A).

Endo-β-mannanase and β-mannosidase activities in response to plant growth regulators

Since cereal grains classically respond to GA and ABA with respect to production of hydrolases (Bewley and Black, 1994), rice grains were subjected to these growth regulators. Dehulled grains imbibed on 100 μM GA exhibited a faster time course of germination than those on water (Fig. 1). ABA at 100 μM did not prevent germination, but caused a

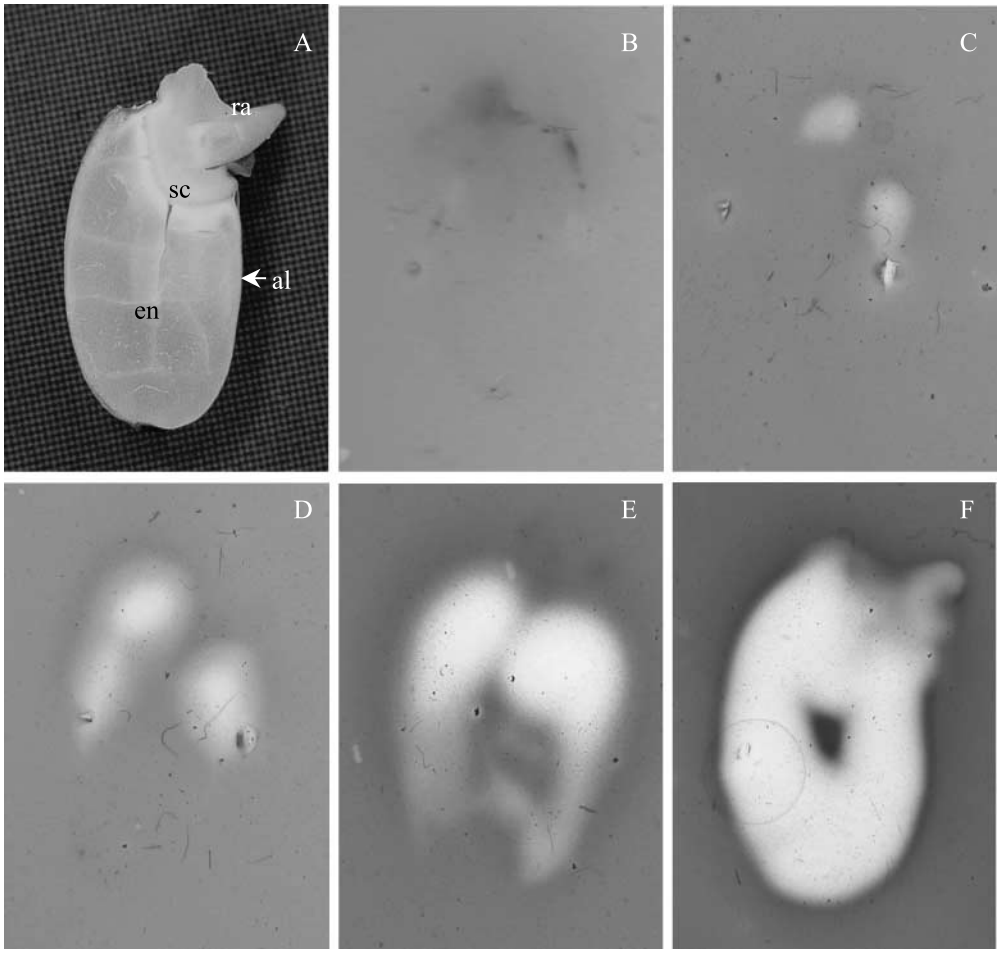

Figure 3. Structure (A) of the rice grain and tissue prints (B–F) of half-seeds obtained by cutting the seeds along the longitudinal axis into two mirror images and placement on agarose gels containing the galactomannan substrate for 5 min at room temperature. (B–F) Grains were imbibed for 12 h, 36 h, 48 h, 60 h and 72 h, respectively. The clearing zones (white) on the gel indicated where the enzyme activity was present. ra, radicle; sc, scutellum; al, aleurone layer; en, endosperm.

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delay in its completion by about 50 h, and the final germination percentage achieved was lower (85%) than for water-imbibed grains. The addition of GA along with ABA (both at 50 μM), increased the final percentage and rate of germination compared to that of dehulled grains on ABA.

Imbibition of dehulled grains in GA for 72 h resulted in a greater increase in endo-β-mannanase activity (Fig. 4A) in the embryo, aleurone layer and starchy endosperm compared to grains imbibed in water, but there was a greater decline in the starchy endosperm by 96 h (see Fig. 2A). β-Mannosidase activity also was increased by the presence of GA in all regions of the grain at the longer times from the start of imbibition (Fig. 4B). However, the activity recorded for β-mannosidase in all assays was close to the lower limit of detection by the assay, and hence the differences were regarded as being of limited consequence.

ABA markedly reduced endo-β-mannanase enzyme activity until at least 192 h from the start of imbibition (Fig. 5A), after the grains had germinated

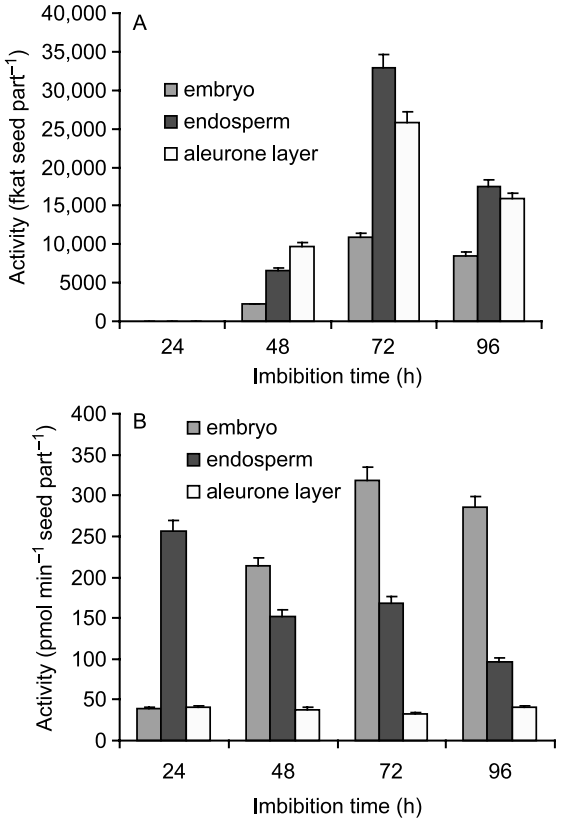

Figure 4. (A) Endo-β-mannanase and (B) β-mannosidase activities in dehulled rice grains imbibed at 25°C for up to 96 h on 100 μM gibberellic acid (GA) before dissection into embryo, endosperm (starchy endosperm) and aleurone layer, and enzyme assays of the dissected parts. Error bars show the variation in activity between duplicate samples.

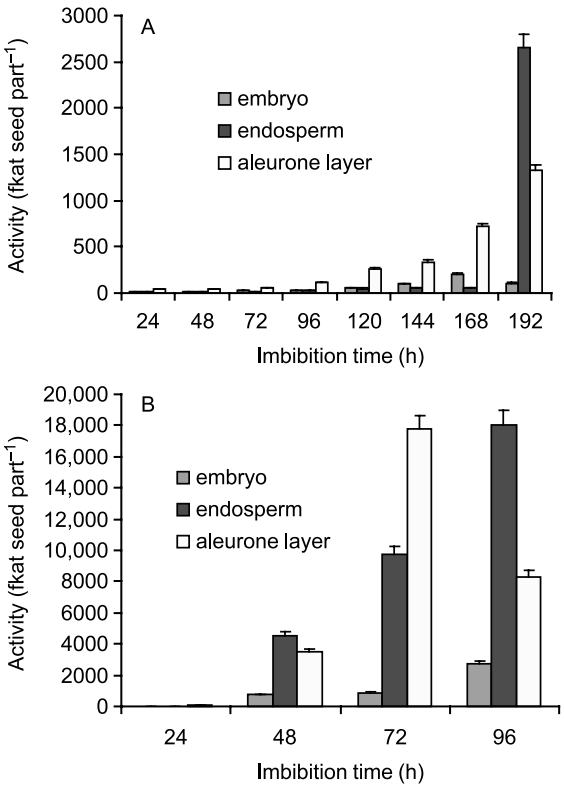

Figure 5. Endo-β-mannanase activity in dehulled rice grains imbibed at 25°C for (A) up to 192 h on 100 μM abscisic acid (ABA), or (B) up to 96 h on 50 μM gibberellic acid (GA) + 50 μM ABA, before dissection into embryo, endosperm (starchy endosperm) and aleurone layer, and enzyme assays of the dissected parts. Error bars show the variation in activity between duplicate samples.

(Fig. 1). β-Mannosidase activity in the embryo and starchy endosperm was largely unaffected by ABA from 24–96 h from the start of imbibition (not shown), compared to the water controls. When added to the rice grains along with ABA, GA had an ameliorating effect on endo-β-mannanase activity, which increased following the completion of germination (Fig. 5B), although even by 96 h it was not elevated compared to activity extracted from the water-imbibed control grains (Fig. 2A). β-Mannosidase activity changed little in the presence of both phytohormones (not shown).

To determine that the increase in enzyme activity due to GA was not mediated via the embryo, embryoless rice grains (half-grains) were imbibed in GA. A low amount of endo-β-mannanase activity was present in the starchy endosperm and aleurone layer of the half-grains imbibed in water, although an increase occurred between 48 h and 96 h, particularly in the aleurone layer (Fig. 6A). This increase was small, however, compared to that occurring in both regions in the presence of 100 μM GA (Fig. 7A). For comparison of activity on a tissue weight basis, the

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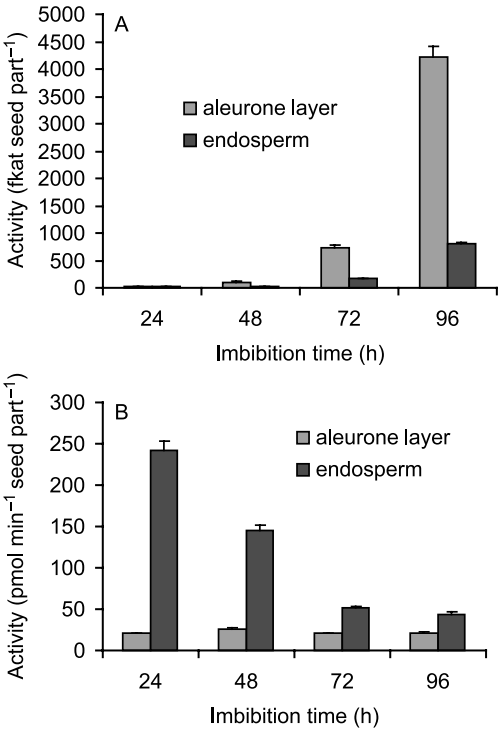

Figure 6. (A) Endo-β-mannanase and (B) β-mannosidase activities in embryoless rice half-grains imbibed at 25°C for up to 96 h on water before dissection into endosperm (starchy endosperm) and aleurone layer, and enzyme assays of both parts. Error bars show the variation in activity between duplicate samples.

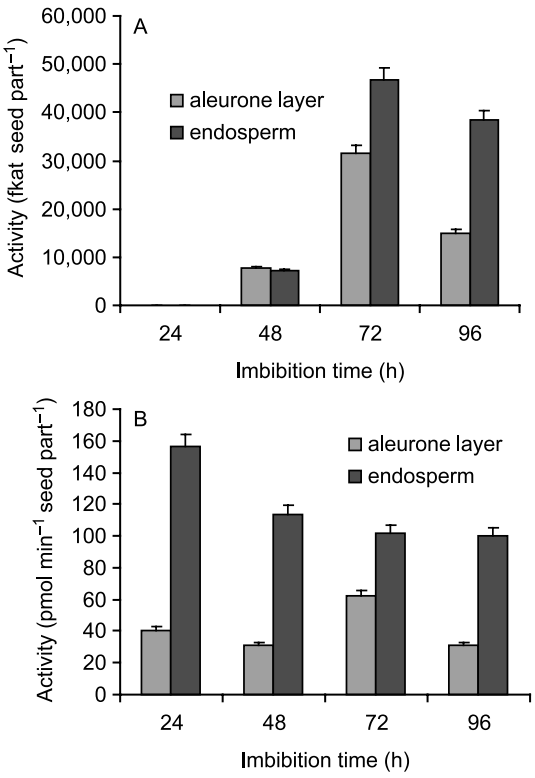

Figure 7. (A) Endo-β-mannanase and (B) β-mannosidase activities in embryoless rice half-grains imbibed at 25°C for up to 96 h on 100 μM gibberellic acid (GA) before dissection into endosperm (starchy endosperm) and aleurone layer, and enzyme assay of both parts. Error bars show the variation in activity between duplicate samples.

average fresh weight per grain of the aleurone layer was 2–2.5 mg, and of the starchy endosperm 9–10 mg, from 48 to 96 h. β-Mannosidase activity in the half-grain was similar in both GA and water (Figs 6B, 7B).

Incubation of the embryoless half-grains in ABA reduced endo-β-mannanase activity over the first 72 h (Fig. 8A), compared to those placed in water, and continued to suppress this activity up to 96 h in comparison to the water controls (Fig. 6A). When incubated on GA and ABA, there was strong promotion of enzyme activity (Fig. 8B), although not to the extent that was present in GA alone (Fig. 7A). This was presumably due to some inhibition of enzyme activity by the ABA that remained; nevertheless GA strongly overcame the negative effects of ABA. β-Mannosidase activity was not substantially affected by ABA, or by ABA and GA together, compared to the water controls (not shown).

Discussion

Rice grains of the *indica* cultivar Peiza 67 exhibited a large increase in endo-β-mannanase activity following the completion of germination. This is similar to the situation in a number of other species, particularly the endospermic legumes, in which the increase occurs after radicle emergence (Reid and Meier, 1973; McCleary and Matheson, 1975; Dirk *et al.*, 1999). The presence of this enzyme in cereal grains of barley, oats, wheat and rice has been noted previously (Dirk *et al.*, 1995), and several isoforms were identified in the embryo and endosperm of each species. Rice of the cv. Bengal was reported to exhibit endo-β-mannanase activity in the embryo and endosperm after only 4 and 8 h from the beginning of imbibition (Dirk *et al.*, 1995), although in the study reported here the earliest activity was detected using tissue blots at 24 h. The reason for this variation in timing of activity is unclear; it could be a cultivar difference.

An increase in activity of endo-β-mannanase following germination occurred in the dehulled grain (caryopsis), i.e. with surrounding fruit coat structures removed. This pattern of enzyme activity is similar to the more frequently reported

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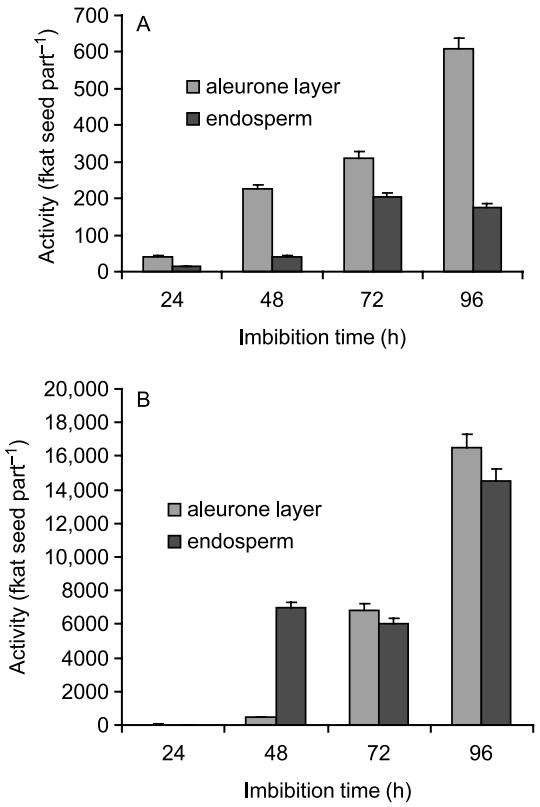

Figure 8. Endo-β-mannanase activity in embryoless rice half-grains imbibed at 25°C for (A) up to 96 h on 100 μM abscisic acid (ABA) or (B) on 50 μM gibberellic acid (GA) + 50 μM ABA before dissection into endosperm (starchy endosperm) and aleurone layer, and enzyme assays of both parts. Error bars show the variation in activity between duplicate samples.

and long-known changes in the starch-hydrolysing enzyme α-amylase (Murata *et al.*, 1968; Okamoto and Akazawa, 1979), which increases in the endosperm following germination, as do various other non-starch hydrolases (Palmiano and Juliano, 1972). Analysis of the cell walls of the rice endosperm reveals that the hemicellulose fraction is largely composed of xylose and arabinose (presumably as arabinoxylans), with a complex mixture of other polysaccharides, in which mannose and galactose predominate, possibly as galactomannans (Shibuya and Iwasaki, 1978). In total, mannose accounts for about 6% of the total cell wall sugars. The role of endo-β-mannanase in the breakdown of endosperm cell walls during the mobilization of the major carbohydrate and protein reserves could be to degrade the cell walls in the starchy endosperm, along with other hemicellulases, thus facilitating the passage of α-amylase and proteases. Such a role for cell-wall degrading hydrolases has been suggested in barley and wheat (Taiz and Jones, 1970; Fincher and Stone, 1974).

A slight increase in endo-β-mannanase activity occurred during germination, but it was far greater following germination, with the largest initial activity being in the aleurone layer (48 h; Fig. 2A), as would be expected since this is generally regarded as the major site of hydrolase production in the grains of cereals (Bewley and Black, 1994). The great subsequent accumulation of endo-β-mannanase in the starchy endosperm was presumably because of the diffusion of the enzyme from the aleurone layer, since the endosperm is a non-living tissue at grain maturity. The least enzyme activity was detected in the embryo. Tissue prints of the rice grain showed that the activity of endo-β-mannanase was first detectable at about the time of germination, towards the proximal end of the grain, in the area of the embryo. It is possible that the initial increase in enzyme occurred in the scutellum, since this is the initial region of appearance of α-amylase, as shown by immunolocalization; then over several days the enzyme was secreted into the starchy endosperm (Okamoto and Akazawa, 1979). Enzyme activity spread from the embryo to the starchy endosperm adjacent to the embryo, and along its periphery, the region of the aleurone layer. Eventually, by 72 h, a ring of endo-β-mannanase activity was observed surrounding the starchy endosperm, indicative of its synthesis and release from the aleurone layer.

β-Mannosidase is an enzyme that acts in concert with endo-β-mannanase to degrade polymeric mannans. Its activity was largely confined to the starchy endosperm and embryo, but it remained very low throughout germination and post-germinative growth. A general decline in activity occurred during and following germination, and was dissimilar to that of endo-β-mannanase. Lack of coordination of the activities of these two enzymes is also evident in other seeds, e.g. lettuce and tomato (Ouellette and Bewley, 1986; Mo and Bewley, 2003), although in the endosperm (aleurone layer) of fenugreek they increase simultaneously (Reid, 1985).

As with most hydrolases present in the endosperm of cereal grains, endo-β-mannanase activity was affected by GA and ABA, the former stimulating and the latter suppressing activity. GA did not cause a large increase in endo-β-mannanase in the dehulled grain, presumably because there was sufficient endogenous phytohormone present to promote full activity. However, ABA was strongly suppressive in these grains, and this occurred in the aleurone layer, starchy endosperm (the presence of endo-β-mannanase in the latter being dependent upon secretion from the former), and in the embryo. Eventually a small increase occurred in enzyme

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activity, but only to about 10% of the maximum activity present in water-imbibed grains; the increase was probably because of the eventual slow germination of the grains in ABA, which presumably led to an increased synthesis and/or release of endogenous GA, thus inducing an increase in enzyme activity. At the single concentration of GA applied to ABA-treated grains, a small increase occurred in the total number of grains completing germination, but the germination rate was more rapid; this was reflected in an increase in endo-β-mannanase activity in the aleurone layer, but to a lesser extent in the embryo. This could be related to a differential sensitivity of these sources of enzyme to these phytohormones.

By the use of embryoless half-grains, it was possible to show that the source of the stimulation for the increase in endo-β-mannanse in the aleurone layer (and secreted enzyme into the starchy endosperm) is the embryo. Presumably, this is because the embryo is the source of GA, since it was readily replaced by imbibing de-embryonated half-grains in this phytohormone. An antagonism between GA and ABA on enzyme activity was noted for the half-grains.

In conclusion, this study shows that in the rice grain, endo-β-mannanase increases in activity, predominantly following germination, a phenomenon not previously demonstrated in cereals. Initial increases occur in the embryo region, with the largest activity residing within the endosperm during seedling establishment, following secretion from the aleurone layer. Changes in this enzyme activity are affected by both GA and ABA, in a positive and negative manner, respectively. β-Mannosidase activity is low, and is not influenced substantially by phytohormones, nor coordinated with the activity of endo-β-mannanase.

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