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Analysis of acid invertase and comparison with acid phosphatase in the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf and Kernan

C. J. Straker[1], W. H. Schnippenkoetter[1], M.-C. Lemoine[2]

[1] Plant Molecular Biology Laboratory, Department of Botany, University of the Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, South Africa
[2] Laboratoire de Phytoparasitologie, Station d'Amélioration des Plantes, INRA BV 1540, F-21034 Dijon Cedex, France

Abstract. Fractions of acid invertase and acid phosphatase of the ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf & Kernan were compared by column chromatography and polyacrylamide gel electrophoresis. Acid invertase levels were measured during the exponential phase after 14 days growth in pure culture. Most acid invertase was wall associated (50%) with 41% forming an extracellular fraction and 9% a soluble, cytoplasmic fraction. The wall-bound fraction was partially solubilized by 1 M NaCl, bulked with the extracellular fraction and separated by gel filtration into two acid invertase activity peaks. These peaks corresponded closely to two acid phosphatase activity peaks measured in the same eluates. Anion exchange chromatography under a continuous salt gradient separated the invertase and phosphatase isoforms from each other. Non-denaturing polyacrylamide gel electrophoresis demonstrated that the more active isoforms of each enzyme have different electrophoretic properties and are high mannose-type glycoproteins with a high affinity for the lectin, concanavalin A. The results are discussed in terms of the functional aspects of the two enzymes and their cytochemical localization.

Key words: Ericaceae – Mycorrhizal fungus – Acid invertase – Acid phosphatase

Introduction

The ericoid mycorrhizal fungus *Hymenoscyphus ericae* (Read) Korf and Kernan produces high levels of acid phosphatase (Pearson and Read 1975; Straker and Mitchell 1986; Lemoine et al. 1992). The enzyme has been localized by both cytochemistry and immunogold labelling in the isolated fungus and in the fungus associated with a host plant, and shown to be a predominantly wall-bound and hyphal-surface glycoprotein (Gianinazzi-Pearson et al. 1986; Lemoine et al. 1992). Lemoine et al. (1992) isolated two isoforms of the enzyme with molecular weights of 78000 Da and approximately 180000 Da. Apart from its function in the catalytic release of P_i from organic and/or inorganic complexes, it has been proposed that the enzyme, as a glycoprotein, may be involved in attachment and/or recognition processes involving host and fungus (Straker et al. 1989; Lemoine et al. 1992).

Invertases are presumably required by the mycobiont for the hydrolytic breakdown of host sucrose to glucose and fructose, which is then converted to the storage sugars mannitol and trehalose (Stribley and Read 1974). If invertases are active in the interfacial apoplast (Smith and Smith 1990), it is probable that they are predominantly wall-bound and on the surface, and in pure culture would be secreted into the external medium. Acid invertases (β-D-fructofuranoside fructohydrolase, E.C. 3.2.1.26) have been investigated in the mycelia of *Neurospora crassa* (Chang and Trevithick 1972a, b), *Claviceps purpurea* (Dickerson 1972), *Ustilago maydis* (Billett et al. 1977; Callow et al. 1980), *Puccinia graminis* (Williams et al. 1984), *Botrytis cinerea* (Geissmann et al. 1991) and *Aspergillus nidulans* (Vainstein and Peberdy 1991), but little is known of the activity of invertases in ericoid mycorrhizal fungi. In plant-fungal pathogen interactions, variability in the carbohydrate components of invertase glycoprotein between different strains of the pathogen has been related to the compatibility of the host-pathogen interaction (Ziegler and Albersheim 1977; Albersheim et al. 1981), and it is possible that the enzyme may function similarly in determining specificity in ericoid mycorrhizas, as has been suggested for acid phosphatases.

This paper presents the first investigation into the acid invertase activity of *H. ericae* in culture and partial characterization of the enzyme. During this study, it was observed that acid invertase and acid phosphatase have similar molecular weights and comparative studies were pursued to establish whether the enzymes also have similar charge characteristics.

Correspondence to: C. J. Straker

Materials and methods

Culture procedures

An isolate of *H. ericae* (LPA2) was grown in 100-ml Pyrex bottles in 20 ml of a liquid culture medium (Mitchell and Read 1981) in which glucose was replaced by an equivalent carbon concentration of sucrose (10.26 g l^{-1}). The fungus was grown for 14 days on 50 ppm P with KH_2PO_4 as the P source. At 14 days fungal growth was in the exponential phase, which is the optimal period for the production of acid phosphatase (Straker and Mitchell 1986) and acid invertase by the fungus under these conditions.

Enzyme extraction

The extraction procedure was modified from that of Greenland and Lewis (1981). Fifty bulked mycelia were filtered under suction through Whatman No. 4 filter paper. The filtrate, which was dialysed against distilled water and then concentrated by polyethylene glycol (PEG 200000) and lyophilization, constituted an extracellular fraction. The residue was homogenized in 200 mM citrate-phosphate buffer (pH 8.0), centrifuged twice at 30000 × g for 20 min at 5° C with a further addition of buffer, and the supernatants bulked, dialysed against distilled water and lyophilized to form a cytoplasmic fraction. The remaining residue was suspended in 0.2% Triton X-100 in 50 mM citrate-phosphate buffer (pH 5.5) at 4° C for 3 h and washed through muslin. The membrane-bound fraction was found to contain negligible enzyme activity and discarded. The sediment was resuspended in 1 M NaCl overnight (1 g per 5 ml) with gentle stirring and centrifuged at 30000 × g for 20 min at 5° C. The supernatant was dialysed against distilled water and lyophilized to form a solubilized wall-bound fraction.

Assay for acid invertase

The glucose oxidase/peroxidase method for the determination of blood glucose (Peridochrom test-kit: Boehringer-Mannheim) was used to measure the hydrolytic ability of invertase. Enzyme extract (30 µl) was added to 320 µl 90 mM sucrose in 100 mM citrate-phosphate buffer (pH 5.5 for extracellular and wall-bound fractions and pH 4.0 for the cytoplasmic fraction) and the mixture incubated for 1 h at 30° C. The action of invertase was stopped by the addition of 2 ml Peridochrom reagent and after 5 min the glucose reaction was stopped with 1 ml 60 mM HCl. Optical density values were measured at 510 nm and activity values adjusted by control readings for non-enzymatic hydrolysis of sucrose and for glucose contamination of the enzyme extracts.

Assay for acid phosphatase

Acid phosphatase activity was determined by the method of Straker and Mitchell (1986).

Assay for protein

Protein content of fractions was determined by the method of Bradford (1976).

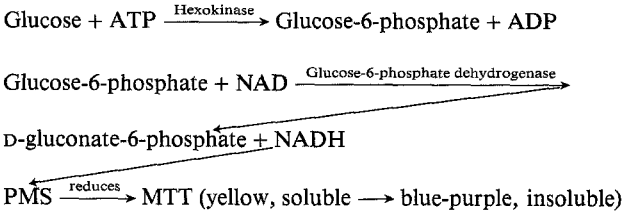
Gel filtration and ion exchange chromatography

Extracellular and solubilized wall-bound fractions dissolved in 10 ml 100 mM acetate buffer (pH 5.0) were loaded onto a Sephacryl S-400 column (2.6 cm × 60 cm) and eluted with the same buffer at 4° C at a flow rate of 40 ml h^{-1}. Fractions (5 ml) were collected by a Gilson 203 fraction collector. Active invertase and phosphatase fractions were bulked, dialysed against 25 mM imidazole buffer (pH 6.5), concentrated by lyophilization, loaded onto a Q Sepharose fast flow column (3 cm × 15 cm) and eluted in the same buffer at 4° C with 5 ml fractions being collected at maximum flow rate. Bound anions were displaced with a 0.0–0.4 M continuous NaCl gradient.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) and visualization of acid phosphatase activity were performed according to Straker et al. (1989). Protein bands were visualized by silver staining (Blum et al. 1987) and zones of acid invertase activity localized by the following method.

After electrophoresis, gels were rinsed in 50 mM citrate-phosphate buffer (pH 5.0) and incubated, covered, overnight at room temperature with 180 mM sucrose in 100 mM citrate-phosphate buffer (pH 5.0) as invertase substrate. The gels were not incubated directly in the sucrose solution but fed by paper wicks to prevent excessive diffusion of the reaction products from the gel. After washing carefully with 100 mM tris-HCl buffer (pH 8.0), a 0.6% agar overlay prepared in tris-HCl buffer (pH 8.0) with ethylenediamine tetraacetic acid (EDTA) disodium salt (3.65 mM), $MgCl_2$ (25 mM), adenosine-5′-triphosphate (ATP; 5 mM), β-nicotinamide-adenine dinucleotide (NAD; 0.75 mM), 3-[4,5-Dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT, Sigma; 1.5 mM), *n*-methylphenazonium methyl sulphate (PMS, Fluka; 0.82 mM), hexokinase (Boehringer Mannheim; 20 units) and glucose-6-phosphate dehydrogenase (Sigma, Type XXIII; 40 units) was poured gently over the gel and purple bands allowed to develop in the dark for up to 30 min. The purple bands represented sites of glucose localization revealed by the following reaction:



Electrotransfer to nitrocellulose and visualization of glycoproteins

Electrophoretic transfer was carried out with a Hoefer TE series Transphor electrophoresis unit at 60 V for 4 h with standard buffers (Straker et al. 1989). Glycoprotein visualization (affinoblotting) was performed according to Straker et al. (1989) and with a Glycan Differentiation Kit (Boehringer Mannheim).

Results

When mycelia of *H. ericae* were harvested after 14 days of growth in liquid culture, 91.5% of acid invertase activity was wall-associated or extracellular, with almost half of that occurring in the external medium (Table 1). Only 8.6% of the enzyme formed a soluble, cytoplasmic fraction which also showed the lowest specific activity (Table 1). Only 16.5% of the wall-bound enzyme was solubilized by incubating homogenized mycelia in 1 M NaCl overnight (Table 1). The localization of enzyme activity in the mycelium is likely to be growth phase-

Table 1. Acid invertase activity of partially-purified fractions of mycelia of *Hymenoscyphus ericae*

Fraction	Total activity (μg glucose g^{-1} fresh weight h^{-1})		Specific activity (μg glucose μg^{-1} protein h^{-1})
Extracellular			
Crude	1957±247	(41)[a]	8.8±2.1
Dialysate	1847±264		7.0±2.0
Lyophilisate	764±452		3.5±0.9
Wall-bound (solubilized)			
Crude	393±109	(8.4)	11.3±3.3
Dialysate	343± 68		8.5±1.8
Lyophilisate	131± 37		3.8±0.7
Cytoplasmic			
Crude	246± 69		1.3±0.4
Dialysate	407± 99	(8.6)	3.1±1.0
Lyophilisate	129[b]		1.2±0.3
Wall bound (unsolubilized)	1993[b]	(42)	
Total	4750	(100)	

[a] Figures in parentheses represent percentage contribution to total activity

[b] Values are the means from two separate extractions. All other values are the means from three or four separate extractions ± standard errors

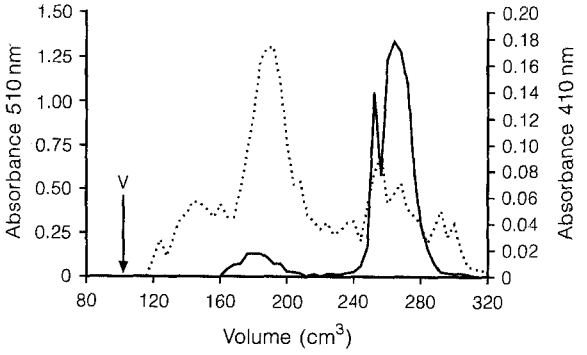


Fig. 1. Acid invertase (—) and acid phosphatase (· · · · ·) activity profiles from a solubilized wall-bound/extracellular fraction of *Hymenscyphus ericae* eluted through a Sephacryl S-400 gel filtration column. Incubation of eluate fractions occurred for 1 h at 30° C in pH 5.5 citrate buffer (acid invertase) and pH 4.5 acetate buffer (acid phosphatase). *V*, Void volume

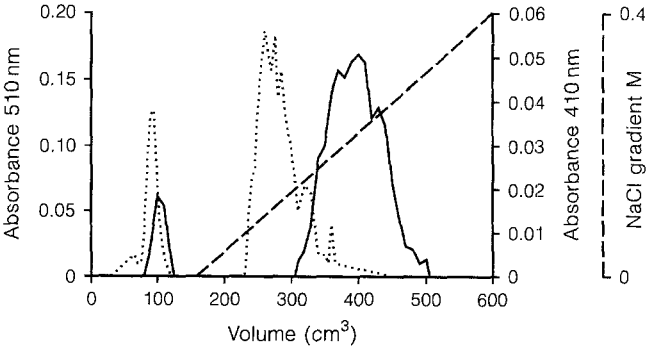


Fig. 2. Acid invertase (—) and acid phosphatase (· · · · ·) activity profiles from a solubilized wall-bound/extracellular fraction of *H. ericae* eluted through a Q-Sepharose anion exchange column, pH 6.5. Incubation of eluate fractions occurred for 1 h at 30° C in pH 5.5 citrate buffer (acid invertase) and pH 4.5 acetate buffer (acid phosphatase)

dependent as at 18 days there was a shift in activity by 30 percentage points from the wall-bound to the extracellular fraction (data not shown). This shift in activity suggested that the wall-bound and extracellular fractions contained the same enzyme, with more of it being secreted into the culture medium as the mycelia aged and started lysing upon entering the stationary stage of growth. Therefore, subsequent characterization by column chromatography and PAGE was performed on combined samples of solubilized wall-bound and extracellular fractions.

Table 1 shows that there were losses in activity during dialysis and lyophilization of 39%, 33% and 52.4% for the extracellular, solubilized wall-bound and cytoplasmic fractions, respectively.

When a solubilized wall-bound/extracellular extract was passed through a Sephacryl S-400 gel filtration column, two acid invertase activity peaks were eluted, a minor one of higher molecular weight and a major one of lower molecular weight (Fig. 1). The same eluted fractions when tested for acid phosphatase activity showed a major peak of higher molecular weight (HMW) and a minor one of lower molecular weight (LMW) (Fig. 1). The coincidence of the two peaks of the two enzymes suggested a strong similarity in their respective molecular weights. To ascertain whether such a similarity in charge properties also existed, bulked and concentrated active fractions from the gel filtration column were eluted through a Sepharose anion exchange column (pH 6.5). Minor activity peaks of both acid phosphatase and invertase were eluted together in the cation fraction (Fig. 2), but major activity peaks were eluted separately under a continuous salt gradient (Fig. 2).

Non-denaturing PAGE gels loaded with a partially-purified solubilized wall-bound/extracellular extract and assayed for sites of acid phosphatase and invertase activity produced a band of phosphatase activity at R_F 0.02 and a more diffuse area of invertase activity at R_F 0.15 (Figs. 3a, b). The activity bands represent the major forms of each enzyme eluted from the chromatography columns. This was confirmed when the major invertase peak eluted from the anion exchange column was concentrated, loaded onto the gel and stained for protein (Fig. 3d). There was a strong protein band at R_F 0.15, which corresponds to the invertase activity band in the same position in Fig. 3b, an unknown band at R_F 0.29 and a light band at R_F 0.02, which corresponds to the phosphatase activity band in Figure 3a; this indicates some contamination of the invertase sample with HMW acid phosphatase protein. Figure 3c shows migration sites of a partially-purified solubilized wall-bound/extracellular extract stained for protein. There is clear staining at R_F 0.02 and R_F 0.15, the sites of phosphatase

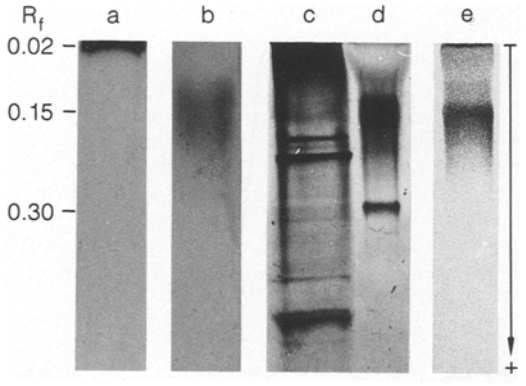


Fig. 3. PAGE separations of fractions of *H. ericae*: *lane a*, solubilized wall-bound/extracellular fraction stained for acid phosphatase activity; *lane b*, solubilized wall-bound/extracellular fraction stained for acid invertase activity; *lane c*, solubilized wall-bound/extracellular fraction stained for protein; *lane d*, purified high-activity acid invertase stained for protein; *lane e*, affinoblot of purified high-activity acid invertase after reaction with Concanavalin A

and invertase activity, respectively, seen in Figs. 3a, b, as well as other unknown protein bands. In Figure 3e, the affinoblot of the major acid invertase sample collected off the anion exchange column, positive staining with Concanavalin A (Con A) occurred slightly at R_F 0.02 and at R_F 0.15, indicating sites of glycoprotein transfer from the gel. These bands correspond to the phosphatase and invertase activity sites (seen in Figs. 3a, b), respectively. Affinoblots were also performed using a glycan differentiation kit and only *Galanthus nivalus* agglutinin (GNA) bound positively at these sites, which confirms that both acid phosphatase and invertase contain N-glycoside-linked carbohydrate chains (data not shown).

Discussion

This study shows that during the late exponential growth of *H. ericae* appreciable acid invertase activity was wall-associated (50%) or extracellular (41%) with only low activity associated with a soluble, cytoplasmic fraction (8.6%) (Table 1). The localization of enzyme activity in the mycelium is growth phase-dependent with greater activity being found in the external medium on aging of the culture. Many fungi produce wall-bound invertases which increase in activity as the mycelium ages. Chang and Trevithick (1972b) demonstrated an appreciable increase (8–23%) in wall activity of the invertase of *N. crassa* during exponential growth, while in *B. cinerea* there was a low secretion of invertase into the culture medium (0.5%) but a relatively high wall-bound fraction (45%) which increased in older cultures at the expense of the soluble enzyme (Geissmann et al. 1991). Other fungi which produce high levels of wall invertase activity include *P. graminis* (Williams et al. 1984) and *A. nidulans* (Vainstein and Pedberdy 1991), while in maize smut infections increased invertase activity was attributable to a soluble rather than a wall-bound enzyme of fungal origin (Callow et al. 1980).

In biotrophic infections one could expect wall-bound or extracellular invertase to be important in initializing cleavage of sucrose when this occurs before sugar uptake by the fungus, as with a *Botrytis* infection (Geissmann et al. 1991). The precise localization of host sucrose hydrolysis in mycorrhizal ericaeceous roots is not known, but Smith and Smith (1990) have postulated the interfacial apoplast between root cell and fungal plasma membrane as the site of host sucrose cleavage. This proposition presupposes that sucrose is hydrolysed before uptake by the endophyte and this is supported by the high levels of wall-associated or extracellular acid invertases observed in *H. ericae* in pure culture; these would facilitate sucrose hydrolysis external to the fungal plasma membrane rather than intracellularly, where very low levels of invertase activity were found. It is also noteworthy that cytochemically the presence of glucose-6-phosphate has been observed along the walls of intracellular hyphae in the interfacial zone in ericoid mycorrhizal roots (Gianinazzi-Pearson et al. 1984). Glucose-6-phosphate is an important intermediate molecule in both glycolysis and the pentose shunt. Its presence on the walls of intracellular hyphae could indicate that products of host sucrose hydrolysis (catalysed by fungal invertases in the interfacial matrix or periplasma space of the fungus) are being used immediately in pathways leading to the production of ATP.

The solubilization of wall-bound invertase of *H. ericae* by 1 M NaCl was low (16.5%). Chang and Trevithick (1972a) were unable to release more than 6% of wall-bound invertase by a similar method, whereas Callow et al. (1980) released over 50% of wall-associated enzyme with 1 M KCl leaving only 10% unsolubilized. It is possible that differences in incubation time or mass/volume ratios account of these variations, but the use of lytic digestion (Vainstein and Peberdy 1991) and hydrolytic enzymes such as chitinase, cellulose or glucanase (Chang and Trevithick 1972a) should be investigated to improve solubilization.

The high-activity invertase of *H. ericae* is a glycoprotein with a strong binding affinity for Con A, a lectin with a high affinity for mannose, and for GNA, which binds to N-glycoside-linked carbohydrate chains. The invertase isolated from *B. cinerea* shows similar glycoprotein characteristics (Geissmann et al. 1991) and strains of *Phytophthora megasperma* var. *sojae* all contained invertases with high levels of mannose and glucosamine residues in different proportions (Ziegler and Albersheim 1977).

Column chromatography of wall bound/extracellular extracts of *H. ericae* revealed two forms of acid invertase and phosphatase of similar molecular weights but different charge properties. However, when fractions were characterized by PAGE activity gels only the high activity isoforms of each enzyme were visualized and these probably correspond to the HMW acid phosphatase and LMW acid invertase forms. It is likely that the PAGE enzyme assay techniques were not sufficiently sensitive for visualization of the less active forms. Le-

moine et al. (1992) have, on the contrary, reported LMW acid phosphatase to be more active than the HMW form and were able to visualize a LMW phosphatase activity band at R_F 0.29. It appears from their study that LMW acid phosphatase is more sensitive to external P than HMW phosphatase. The LMW phosphatase is inducible at low P levels and repressible at high P levels with an optimum P concentration for activity/synthesis of 20 ppm. This is less than that used in the present study for growth of *H. ericae* (50 ppm P) and could explain the lower activity value of the LMW enzyme observed here. Lemoine et al. (1992) found that LMW acid phosphatase on hyphae within living roots which could not be detected by cytochemical localization was still observable by immunogold labelling. This observation suggested an inhibition of phosphatase activity (induced by higher pH and phosphate concentrations) rather than lack of enzyme synthesis. Lemoine et al. (1992) have, in fact, suggested that the dominant isozyme associated with external acid phosphatase activity at the hyphal surface where P levels will be low is LMW, with the HMW enzyme probably associated with the cytosol fraction characterized by higher P concentrations. Because differences in the inducible nature of the acid phosphatases are also suggested by the present study, caution is needed when deciding which isoform is dominant in cellular, wall-bound or extracellular fractions or the functional nature of each form in mycorrhizal roots.

The results presented here demonstrate the production of active wall-bound, extracellular and cytoplasmic invertases by *H. ericae*. Studies on the full characterization of the two isoforms of the enzyme are continuing. Insight into their functions will necessitate establishing to what degree they are constitutive and their precise sites of activity in mycorrhizal roots, especially at the host-fungus interface. Ziegler and Albersheim (1977) have shown how differences in the structures of the carbohydrate portions of the invertases between different races of *P. megasperma* var. *sojae* are related to their degree of pathogenicity and this is presently being investigated with regard to differences between high- and low-infective strains of ericoid mycorrhizal fungi (Gianinazzi-Pearson and Bonfante-Fasolo 1986).

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