Extra- and Intra-cellular α -L-Arabinofuranosidase of *Sclerotinia fructigena*

By F. LABORDA,* A. H. FIELDING AND R. J. W. BYRDE

Long Ashton Research Station, University of Bristol, Bristol

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SUMMARY

The production of both intracellular and extracellular α -L-arabinofuranosidase (AF) by *Sclerotinia fructigena* was demonstrated. They had different kinetic parameters, pH stability and optimal pH values. Two peaks of activity were found on gel filtration: maximum activity was associated with the peak of lower molecular weight in the culture filtrate, but with the higher molecular weight peak in the mycelial homogenate.

Isoelectric-focusing studies on culture filtrates revealed two peaks of AF activity with pI 3.0 and 6.5 respectively, maximum activity being associated with the latter. In the mycelial homogenate, most of the activity was associated with a third peak of activity with pI 4.5.

Physical and kinetic data suggest that the two AF activities present externally in the culture filtrates are identical with the two internal ones, with corresponding pI.

INTRODUCTION

The important role that host-cell-wall degrading enzymes produced by plant pathogenic fungi play in pathogenicity has been widely described (see reviews by Bateman & Millar, 1966; Albersheim, Jones & English, 1969). Among these lytic enzymes, production of α -L-arabinofuranosidase (AF) by several plant-pathogenic fungi has been reported (Fuchs, Jobson & Wonts, 1965; Akinrefon, 1968; Kaji & Yoshihara, 1969; Keegstra, English & Albersheim, 1972). Production of AF by *Sclerotinia fructigena* was also demonstrated by Byrde & Fielding (1965). Studies with isolates of this fungus obtained after mutagen treatment indicated an important role for AF during mycelial advance in apple tissue (Howell, 1972).

Although production of host-cell-wall degrading enzymes by plant-pathogenic fungi often seems to be dependent upon environmental conditions (Albersheim *et al.* 1969), in general biosynthesis and secretion of enzymes by fungi are not fully understood. There are indications that both in yeast and moulds secretion of enzymes may be associated with molecular changes in the active protein before they are secreted (Liras & Gascon, 1971; Trevithick & Metznerberg, 1964).

All studies to date on fungal AF enzymes have been concerned with extracellular activity. Because Calonge, Fielding & Byrde (1969) suggested the possibility that AF activity may be associated with multivesicular bodies within the cytoplasm of *Sclerotinia fructigena*, the relationship of intracellular and extracellular AF activity was studied.

A brief account of some of the present work has previously been published (Laborda, Hislop, Fielding & Byrde, 1972).

* Present address: Department of Plant Pathology, University of Wisconsin, Madison, Wisconsin 53706, U.S.A.

METHODS

Fungal culture. The culture of *Sclerotinia fructigena* Aderh. & Ruhl. was grown either on V-8 agar or in ammonium tartrate-sodium polypectate liquid medium as previously described (Byrde & Fielding, 1968).

Production of enzyme. Five-hundred-ml conical flasks containing 150 ml ammonium tartrate-sodium polypectate liquid medium were inoculated with a suspension of spores, from two 8–10 days old cultures on V-8 agar slopes, and incubated at 25 °C in a reciprocal shaker. After 4 days, cultures were harvested by filtration. Culture filtrates were dialysed against distilled water for 24 to 48 h at 4 °C. After washing several times with distilled water, mycelium was broken in a mortar at 0 °C by using washed sand as an abrasive. Breakage sequence was followed by phase-contrast microscopy. Unbroken mycelium, cell walls and sand were separated by centrifugation at 2000 g for 20 min. Supernatants were centrifuged for 30 min at 30000 g in a refrigerated MSE High-speed 18. When needed, both culture filtrate and mycelial homogenate were concentrated in a Leybold-Heraeus freeze drier, Model SB4.

Estimation of α -L-arabinofuranosidase (α -L-arabinofuranoside arabinohydrolase) activity. For routine assay, 25 mg of substrate (*p*-nitrophenyl- α -L-arabinofuranoside; Fielding & Hough, 1965) were dissolved in 100 ml of 0.1 M-sodium acetate buffer, pH 4.7; 2.5 ml of this solution were incubated with 1 ml of enzyme preparation at 30 °C. The reaction was stopped by addition of 1 ml of saturated solution of sodium carbonate; *p*-nitrophenol liberated was measured at 403 nm in a CE 272 Linear Readout Ultraviolet Spectrophotometer.

To study the pH activity and pH stability of the different enzymes, the borate-citrate-phosphate buffer system of Teorell & Stenhagen (1938) was used.

Gel filtration. The different AF activities were separated by using either Sephadex G-100 bead form or Biogel P-300 (50 to 150 mesh) columns (4 × 80 cm) equilibrated with 0.05 M-tris-hydrochloric acid buffer, pH 7.5, containing 0.1 M-potassium chloride and 0.02 % sodium azide. One ml of enzyme preparation containing sucrose was layered on the top of the column, and 3 ml fractions were collected. The Sephadex G-100 column was eluted by gravity, whereas the Biogel P-300 one was eluted with an LKB peristaltic pump. Flow rate through both columns was 12 ml/h. The same columns were used for molecular weight estimations of the different AF molecular forms by the method of Andrews (1964).

Isoelectric focusing. The method of Vesterberg & Svensson (1966) was followed using an LKB 110 ml column and carrier ampholytes at a final concentration of 1 % (w/v) to establish the pH gradient. The enzyme preparation was applied in the light solution of the sucrose gradient. A dense lower-anode solution was prepared using 0.6 % phosphoric acid and an upper-cathode solution of 2 % ethylene diamine. The voltage applied was usually 200 V giving a current of 10 mA, increasing to about 600 V with a current < 2.0 mA. Each run was for 64 h at 10 °C. The column was drained off by gravity flow through capillary tubing and collected in 4.5 ml fractions.

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine-serum albumin as standard.

Table 1. *AF distribution in a 4-day-old culture of Sclerotinia fructigena*

	Total activity ($\mu\text{mol/min}$)	Protein (mg)	Specific activity
Culture filtrate	140	216	0.64
Mycelial homogenate	19	170	0.13

Fig. 1. pH/stability relationships for intracellular (○) and extracellular (●) α -L-arabinofuranosidase of *Sclerotinia fructigena*.

RESULTS

Localization of AF activity

In a 4-day-old culture filtrate, AF activity was detected both in the culture filtrate ('E') and in the mycelial homogenate ('I'), the total activity detected in the culture filtrate being 7 to 8 times higher than in the mycelial homogenate (Table 1). During the incubation period the pH of the culture medium rose from 5.2 to 6.8.

Properties of intracellular and extracellular AF

The pH stability of both 'E' and 'I' preparations was measured by incubating 1 vol. of buffer+9 vol. of enzyme over the pH range 2 to 10 at 30 °C. After 30 min incubation, residual activity was measured at pH 4.7 under the standard conditions. 'E' was stable over the pH range 4 to 7, but 'I' was stable only at pH 6 to 7. Generally, 'E' was more stable under acid conditions, while 'I' was more stable at higher pH values (Fig. 1).

pH activity. One vol. of enzyme+2 vol. of buffer+0.5 vol. of substrate (1 mg/ml water) were incubated at 30 °C for 15 min. Activity was expressed as per cent of the maximum value. Results are plotted in Fig. 2. Maximum activity for 'E' was at pH 4.5, and that for 'I' was at pH 5.5 to 6.0 (Fig. 2). The Figure shows a similar pattern to that for pH stability, viz. 'E' was more active at acid pH, and 'I' was more active at high pH values.

The effect of substrate concentration was studied over a range from 0.25 to 2.0 mM at both pH 4.5 and 5.5 in 0.1 M-acetate buffer. The Lineweaver-Burk plots obtained gave K_m values for 'I' of 0.28 mM at pH 4.5 and 0.23 mM at pH 5.5, and for 'E' of 0.66 mM at pH 4.5 and 1.1 mM at pH 5.5.

Gel filtration. Either a culture filtrate or a mycelial homogenate was applied to a column of Sephadex G-100 and eluted as described under Methods. The patterns obtained for both elutions are shown in Fig. 3; the results are expressed as per cent of maximum activity

Fig. 2. pH/activity relationships for intracellular (○) and extracellular (●) α -L-arabinofuranosidase of *Sclerotinia fructigena*.

Fig. 3. Elution pattern of intracellular (---) and extracellular (—) α -L-arabinofuranosidase of *Sclerotinia fructigena* on Sephadex G-100. (Elution volumes are indicated for: 1, dextran blue; 2, γ -globulin; 3, serum-albumin fraction V; 4, trypsin inhibitor; 5, cytochrome c.)

obtained. Two peaks of AF activity were found in both preparations, but while in the culture filtrate maximum activity was associated with the peak which had a V_e/V_o value of 2.1, in the mycelial homogenate maximum activity was associated with the peak which was excluded with the V_o . The elution pattern of several proteins with known molecular weight is also shown.

Isoelectric-focusing studies. Isoelectric focusing over a wide pH range (pH 3 to 10) was used to separate the different AF activities from both culture filtrate and mycelial homogenate. The culture filtrate showed two peaks of AF activity with isoelectric points 3.0 and 6.5, maximum activity being associated with the peak with pI 6.5. The mycelial homogenate also showed peaks of activity with pI 3.0 and 6.5, but maximum activity was associated with a third peak which had pI 4.5 (Fig. 4).

Properties of the different AF enzymatic activities

Corresponding peak-activity fractions from several isoelectric-focusing experiments, either from mycelial homogenate or from culture filtrate, were pooled and dialysed against 0.1 M-phosphate buffer, pH 7.0, at 4 °C for 18 to 24 h. The different samples were studied on the basis of their pH activity, pH stability, thermal inactivation, K_m and apparent molecular weight.

Fig. 4. Isoelectric focusing of intracellular (---) and extracellular (—) α -L-arabinofuranosidase of *Sclerotinia fructigena*. (....), pH gradient.

Fig. 5. pH/stability relationships for α -L-arabinofuranosidase isoenzymes of *Sclerotinia fructigena*. (a) Isoenzymes with pI 3.0. (b) Isoenzymes with pI 6.5. (c) Isoenzyme with pI 4.5. (Open symbols, internal isoenzymes. Closed symbols, external; internal also where coincident.)

pH stability was studied as described previously. The two isoenzymes (using the term in a functional sense) with pI 3.0 and those with pI 6.5 were shown to be stable at pH 3 to 8 but not at alkaline pH values, while the internal isoenzyme with pI 4.5 was stable at pH values near neutrality but not at acid ones (Fig. 5).

pH activity was studied as previously described. Results plotted in Fig. 6 show a similar behaviour for those internal and external isoenzymes with the same isoelectric point, but while those with pI 3.0 showed maximum activity at pH 3 to 4, and those with pI 6.5 at pH 5.0, the isoenzyme with pI 4.5 had maximum activity at pH 5.7 to 6.0, being almost completely inactive at pH values below 4 (Fig. 6).

Thermal inactivation was studied by incubating 1 ml of enzyme at different temperatures

Fig. 6. pH/activity relationships for α -L-arabinofuranosidase isoenzymes of *Sclerotinia fructigena*. For details, see Fig. 5.

Fig. 7. Thermal inactivation (10 min exposure) of α -L-arabinofuranosidase isoenzymes of *Sclerotinia fructigena*. For details, see Fig. 5.

Fig. 8. Effect of pH on the K_m of α -L-arabinofuranosidase isoenzymes of *Sclerotinia fructigena*. For details, see Fig. 5.

for 10 min, then cooling rapidly to about 0 °C by immersion of the tube in acetone at -20 °C. Residual activity was measured at pH 4.7 under the standard conditions. The results show a similar behaviour for isoenzymes with the same pI (Fig. 7). Isoenzymes with pI 3.0 were completely inactivated at 60 °C, but isoenzymes with pI 6.5 were practically inactivated at 45 °C. The internal isoenzyme with pI 4.5 showed an intermediate behaviour.

Effect of substrate concentration on isoenzyme activity was determined over a range of substrate concentrations from 0.05 to 4 mM at different pH values; 0.1 M-acetate buffer was used over a range from pH 3.5 to 6.0. The Lineweaver-Burk plots were linear, within experimental error, for the different isoenzymes, with the exception of those with pI 6.5 that were linear only for substrate concentrations from 0.5 to 4 mM. The different K_m values obtained are represented in Fig. 8. Isoenzymes with the same isoelectric point behaved very similarly. Isoenzymes with pI 3.0 had K_m values between 0.12 mM at pH 3.5 and 0.9 mM at pH 6, those with pI 6.5 varied from 1.0 mM at pH 4.5 to 2.5 mM at pH 6, and the internal isoenzyme with pI 4.5 showed a variation from 0.19 mM at pH 6.0 to 2.5 mM at pH 3.5.

Molecular weight determinations. Assuming that the molecular shape of AF isoenzymes is similar to those of the marker proteins, the approximate molecular weights were: for both isoenzymes with pI 6.5, 40 000; for the internal isoenzyme with pI 4.5, 350 000; and for the external one with pI 3.0, 220 000. Samples of internal isoenzymes with pI 3.0 from several isoelectric-focusing runs always gave a very wide peak in Biogel P-300, with two apparent maxima corresponding to molecular weights of approximately 220 000 and 125 000.

DISCUSSION

In a previous paper, Fielding & Byrde (1969) showed that α -L-arabinofuranosidase can exist in culture filtrates of *Sclerotinia fructigena* in multiple forms and suggested a possible interconversion of these forms. In other fungi, such as *Neurospora crassa*, multiple forms of invertase that can be interconverted are related to biosynthesis and secretion of that enzyme (Metzenberg, 1964).

AF activity was found to be present both in the culture filtrate and in the mycelial homogenate of *Sclerotinia fructigena* when the fungus was grown in an ammonium tartrate-sodium polypectate liquid medium. Both activities had different physical and kinetic characteristics. The presence of at least two isoenzymes in the culture filtrate and three in the mycelial homogenate in different proportions in each preparation can account for these differences. Comparative studies suggest that the extracellular isoenzymes are identical to the internal ones with the same isoelectric point.

Isoenzymes with pI 3.0 and 6.5 were shown to have maximum activities and to be more stable at low pH values: this seems to be a general characteristic of extracellular α -L-arabinofuranosidase already studied from different plant-pathogenic fungi (Kaji & Yoshihara, 1969). By contrast, the internal isoenzyme with pI 4.5 was more stable and had maximum activity near or above pH 7.0.

While the molecular weight of the main AF isoenzyme in the culture filtrate was about 40 000, that of the other was 220 000. Secretion of isoenzymes with different physical and kinetic characteristics is not rare amongst plant-pathogenic fungi. These differences, such as size and diffusibility of enzyme molecules, may have an important role in sequential degradation of host-cell-wall polysaccharide (Goodman, Kiraly & Zaitlin, 1967; Olutiola & Ayres, 1973).

Trevithick & Metzenberg (1966) in studies of secretion of invertase in *Neurospora crassa* suggested that the high molecular weight isoenzyme underwent molecular sieving by fungus cell walls, so explaining why the 'heavy' isoenzyme was the main component within the mycelium and the 'light' isoenzyme the main one in the culture filtrate. Molecular weight determination of the different AF isoenzymes in *Sclerotinia fructigena* showed that the main isoenzyme in the mycelial homogenate, which was never detected in culture filtrates, also had a high molecular weight, while the main isoenzyme in the culture filtrate had a molecular weight approximately one-tenth of that of the internal one.

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