Studies on the α -L-Arabinofuranosidase Complex from *Sclerotinia fructigena* in Relation to Brown Rot of Apple

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SUMMARY

α -L-Arabinofuranosidase (AF) was detected in apple fruitlets experimentally infected by *Sclerotinia fructigena*. In extracts of such fruitlets, three AF isoenzymes were separated by preparative isoelectric focusing. When the fungus was grown in shake culture with different carbon sources, AF was detected in each culture filtrate and mycelial homogenate. Although fungus growth and total AF varied with the carbon source, the AF isoenzyme pattern was similar in each instance to that obtained when grown on sodium polypectate.

Each of the partially purified AF isoenzymes behaved differently in substrate specificity and inhibitor studies; however, each showed a specificity for α -L-arabinofuranosides. The two extracellular AF isoenzymes released monomeric arabinose when incubated with araban or apple cell walls. External AF III (pI 6.5) was more active on a substrate of apple cell wall material than external AF I (pI 3.0). The latter form of the enzyme was less susceptible to inhibition by either oxidized or unoxidized apple juice. Two isolates of *Sclerotinia fructigena* with low growth rate *in vivo* secreted no AF III *in vitro*.

INTRODUCTION

Sclerotinia fructigena is a plant pathogen capable of producing cell wall degrading enzymes, both *in vitro* and *in vivo*, which may well account for the rotting of infected apple tissues (Byrde, Fielding, Archer & Davies, 1973).

Among these enzymes, α -L-arabinofuranosidase (AF) was first described by Byrde & Fielding (1965) and was then believed to be involved in tissue maceration in a model system, but this gross effect was later shown to be due to another enzyme (Byrde & Fielding, 1968). Subsequent studies demonstrated the presence of AF in infected apple tissues (Calonge, Fielding, Byrde & Akinrefon, 1969). The growth rates *in vivo* of 160 *Sclerotinia fructigena* isolates, obtained after mutagen treatment, showed a higher positive correlation ($P < 0.001$) with AF secretion *in vitro* than with that of two other wall degrading enzymes (Howell, 1972).

Production of AF by several other plant-pathogenic fungi has also been reported (Fuchs, Jobson & Wonts, 1965; Akinrefon, 1968; Kaji & Yoshihara, 1969; Keegstra, English & Albersheim, 1972).

Isoelectric focusing studies showed that, when *Sclerotinia fructigena* was grown in a sodium polypectate-ammonium tartrate liquid medium, two peaks of AF activity with pI values of 3.0 and 6.5 respectively could be separated from the culture filtrate, greater activity being associated with the latter. Although AF isoenzymes with pI 3.0 and 6.5 were also found in mycelial homogenates, maximum activity was associated with a third isoenzyme with pI 4.5. Physical and kinetic data showed that the two AF activities present in the culture

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filtrate were identical to the internal ones with corresponding pI, but the internal AF isoenzyme with pI 4.5 had characteristics different from those of the other isoenzymes and a higher molecular weight (Laborda, Fielding & Byrde, 1973).

This paper describes further comparative studies with AF isoenzymes, designated AF I (pI 3.0), AF II (pI 4.5) and AF III (pI 6.5); different roles are suggested for the two extra-cellular isoenzymes (I and III) during rotting of apple by *Sclerotinia fructigena*.

METHODS

Materials. These were obtained as follows: L-arabinose, D-galactose, D-glucose, D-mannose, D-xylose, L-arabitol, acrylamide, *N,N'*-methylenebisacrylamide, riboflavin and 1,2-diaminoethane from BDH Chemicals Ltd, Poole, Dorset; araban, *p*-nitrophenyl α -D-galactopyranoside, L-arabono- γ -lactone, D-arabono- γ -lactone and D-galactono- γ -lactone from Koch-Light Laboratories Ltd, Colnbrook, Buckinghamshire; arabinogalactan and L-galactono- γ -lactone from Calbiochem Ltd, London; Carrier Ampholytes from LKB Instruments Ltd, South Croydon, Surrey; sodium polypectate from Sunkist Growers Inc., Ontario, California, U.S.A.; D-galacturonic acid and Fast Garnet GBC salt from Sigma Ltd, London; Sephadex G-100 from Pharmacia (G.B.) Ltd, London; Biogel P-300 from Bio-Rad Laboratories, Richmond, California; *p*-nitrophenyl α -L-arabinopyranoside from Dr M. L. Sinnott, University of Bristol.

Organisms. *Sclerotinia fructigena* Aderh. & Ruhl. ATCC26106 was grown either on V-8 agar or in sodium polypectate-ammonium tartrate liquid medium as previously described (Laborda *et al.* 1973). In some studies, several *S. fructigena* isolates obtained after mutagen treatment (Howell, 1972), were used and were cultured as above.

Production of enzyme. The general procedure was as previously described (Laborda *et al.* 1973).

For larger-scale enzyme production, 500 ml conical flasks, each containing 100 ml of ammonium tartrate-sodium polypectate liquid medium, were inoculated with a suspension of spores from two slopes of V-8 agar 8 to 10 days old and incubated at 25 °C in a reciprocal shaker (100 strokes/min). After 2 days, cultures were transferred to a 3 l conical flask containing 1 l of ammonium tartrate-sodium polypectate liquid medium, incubated at 25 °C in a reciprocal shaker, and treated as before.

For studies on the effect of different carbon sources on enzyme production, 150 ml conical flasks, containing 50 ml ammonium tartrate-sodium polypectate liquid medium or the same medium in which sodium polypectate was substituted by different carbon sources (each at 1.0 %, w/v), were inoculated with a suspension of spores from one V-8 agar slope culture 10 days old. Mycelium was collected by centrifugation at 1000 g in a refrigerated MSE 'High Speed 18' centrifuge for 15 min, and after being washed was broken in a manual Ten Broeck glass homogenizer (Jencons, Hemel Hempsted, Hertfordshire).

Estimation of α -L-arabinofuranosidase (α -L-arabinofuranoside arabinohydrolase) activity. Unless otherwise stated, activity was estimated by hydrolysis of *p*-nitrophenyl α -L-arabinofuranoside as previously described (Laborda *et al.* 1973).

Enzyme-fractionation procedures. Gel filtration with either Sephadex G-100 or Biogel P-300 and isoelectric focusing with a 110 ml column (LKB Instruments Ltd) were conducted as previously described (Laborda *et al.* 1973).

For isoelectric focusing on polyacrylamide gels, the general method of Vesterberg & Svensson (1966) was followed; a Shandon apparatus (Shandon Southern Instruments Ltd, Camberley, Surrey) was used for polyacrylamide electrophoresis, the apparatus being

supplemented with a spacer ring in order to run 110 mm gels. Each gel tube was filled with a mixture of a solution containing: 30 % (w/v) recrystallized acrylamide and 1 % (w/v) recrystallized *N,N'*-methylenebisacrylamide, 0.5 ml; 40 % (w/v) carrier ampholytes, 0.06 ml; riboflavin (0.14 mg/ml), 0.16 ml; enzyme solution, containing 30 to 300 µg of protein; and distilled water to 2.4 ml. After polymerization, up to eight gel tubes were run simultaneously. The upper reservoir (anode) was filled with 0.2 % (v/v) sulphuric acid, and the lower reservoir (cathode) with 0.4 % (v/v) 1,2-diaminoethane. The voltage applied was usually increased to 200 V with a current of < 2.0 mA/tube. Routine runs were done overnight in a cold room (4 °C). For briefer runs, voltage was gradually increased to 400 V while keeping the current < 2.0 mA/tube. Gels were removed from the tubes by the immersed-irrigation method, and before being stained for activity were soaked in 1 M-sodium acetate buffer, pH 6.0, for 2 h and then for 1 h in distilled water, both with several changes of liquid. AF activity in the gels was visualized by immersing them in 0.1 M-acetate buffer, pH 5.0, containing 2-naphthyl α -L-arabinofuranoside (0.6 mg/ml) (Fielding & Hough, 1971) and Fast Garnet GBC salt (1 mg/ml).

Preparation of apple fibre and apple cell walls. Apple fruits to be used for the isolation of cell walls were stored at -20 °C until required. The deep-frozen tissue was excised with a scalpel, finely chopped, and dropped into acetone at -20 °C. A mixture of 1 vol. fruit and 3 vol. acetone was blended together in an Atomix homogenizer for 3 min at full speed. The slurry was filtered free of acetone and the process repeated with a further 3 vol. of solvent. This was continued until the filtrates appeared free of pigment.

The crude preparation (pressed free of as much acetone as possible), designated apple fibre, was homogenized in 10 vol. of cold 0.1 M-potassium phosphate buffer (pH 7.0) for 5 min. Solid matter was filtered off through a coarse glass sinter and re-suspended in a further quantity of buffer. This was repeated a further four times with buffer and five times with distilled water, all at 4 °C, by which time filtrates were clear. Remaining solid matter (largely cell wall) was then blended for 5 min with 10 vol. chloroform-methanol (1:1, v/v), before filtration through Whatman no. 41 paper. The cell walls were washed with chloroform-methanol and finally with redistilled acetone. They were dried at 60 °C, allowed to cool in a desiccator and ground to a fine grey-white powder before storing at room temperature.

Apple-fruit extraction. Climacteric apple fruits, cultivar Lord Lambourne, were chopped and then macerated in an Atomix homogenizer (1 g chopped apple: 1 ml distilled water) for 10 min. The liquid was strained through muslin and stirred at room temperature to ensure complete oxidation. The oxidized dark-brown suspension was centrifuged at 15000 g in a MSE High Speed 18 centrifuge for 30 min and the sediment discarded.

The unoxidized juice was obtained by a similar procedure, but by using a 0.1 % solution of sodium dithionite instead of distilled water during maceration.

Extraction of apples rotted by Sclerotinia fructigena. Apple fruitlets, cultivar Lord Lambourne, were swabbed with 70 % (v/v) ethanol and artificially inoculated with a wild-type isolate of *Sclerotinia fructigena*. The resulting rots developed rapidly and, after 6 days, infected tissue was excised and homogenized with half its weight of extraction medium (0.2 M-NaCl; 0.1 M-K, Na-phosphate buffer, pH 7.6). The slurry obtained was filtered under reduced pressure through Whatman no. 4 paper and the filtrate dialysed, first against the extracting buffer and then against distilled water (both at 4 °C), and finally concentrated by freeze-drying.

Chromatography of sugars. Both descending paper and ascending thin-layer chromatography were used. For descending paper (Whatman no. 1) chromatograms, ethyl acetate:pyridine:acetic acid:water (5:5:1:3, by vol.) was used as solvent (Gee & McCready, 1957).

Fig. 1. Isoelectric focusing (preparative column) of extract of rotted apple. ·····, α -L-Arabinofuranosidase (AF) detected; ---, pH gradient. For comparison, AF patterns of (—) intracellular and (— · —) extracellular AF of *Sclerotinia fructigena* are included. (From Laborda, Fielding & Byrde, 1973.)

For thin-layer chromatograms, Whatman cellulose powder CC 41 was used as support and butan-1-ol:acetic acid:water (3:1:1, by vol.) as solvent (Shah & Loewus, 1967). All chromatograms were run in solvent-equilibrated tanks at 22 °C. Reducing sugars on chromatograms were visualized by spraying the paper or plates with *p*-anisidine hydrochloride (Hough, Jones & Wadman, 1950) or silver nitrate in acetone followed by alcoholic sodium hydroxide (Trevelyan, Procter & Harrison, 1950). Migration distances were assessed relative to the distance travelled by glucose.

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

Reducing sugars were determined as arabinose with a low-alkaline Cu reagent (Somogyi, 1952) and the arsenomolybdate chromogen of Nelson (1944).

RESULTS

AF pattern in vivo

An extract of rotted apple fruitlets subjected to wide range (pH 3 to 10) isoelectric focusing in a preparative column gave a pattern (Fig. 1) broadly similar to that for the mycelial homogenate obtained when *Sclerotinia fructigena* was grown in an ammonium tartrate-sodium polypectate liquid medium (Laborda *et al.* 1973). The main activity was associated with the isoenzyme AF II, but the proportion of activity associated with each isoenzyme was different from that for the mycelial homogenate.

Effect of different carbon sources on AF production and isoenzyme pattern in vitro

Sclerotinia fructigena was grown on different liquid media, and culture filtrate (E) and mycelial homogenate (I) were obtained. Total AF activity was measured in E and I. Total protein was measured in I. Table 1 shows the results of a typical assay.

Fungus growth (expressed as total internal protein) and AF in E and I (expressed as total amount or per unit mycelial protein) were dependent upon the carbon source. Araban, sodium polypectate and apple fibre were among the best carbon sources for fungus growth and AF production. With galactose and arabinogalactan the fungus grew poorly, but the relative AF in both E and I was high (Table 1).

Table 1. *Effect of different carbon sources on AF production by Sclerotinia fructigena*

Carbon source (each at 1 %, w/v)	Growth, as total internal protein (mg)	Extracellular AF		Intracellular AF	
		Total activity (μ mol/min)	AF/mg protein (μ mol/min)	Total activity (μ mol/min)	AF/mg protein (μ mol/min)
Polymers:					
Apple fibre*	0.57	3.00	5.26	0.16	0.26
Araban	0.90	5.40	6.00	1.35	1.50
Arabinogalactan	0.07	0.34	4.86	0.09	1.29
Sodium polypectate	0.77	5.00	6.49	0.35	0.45
Monomers:					
L-Arabinose	0.35	0.50	1.43	0.10	0.29
D-Galactose	0.07	0.30	4.29	0.15	2.14
D-Glucose	0.47	0.24	0.51	0.10	0.21
D-Mannose	0.70	0.30	0.43	0.12	0.17
D-Xylose	0.68	0.32	0.47	0.07	0.10
None	No growth				

* Obtained as described under Methods.

The AF isoenzyme pattern in E and I for each carbon source was studied by using wide range (pH 3 to 10) polyacrylamide gel isoelectric focusing. The pattern for each carbon source tested was similar to that with sodium polypectate.

With this substrate, two main sets of bands with AF activity were found for E (Fig. 2A). Comparative studies showed that the upper set corresponded with a similar pattern for the partially purified isoenzyme AF I and the lower set with the partially purified external isoenzyme AF III (see below). In the mycelial homogenate too, sets of active bands corresponding to the external AF I and AF III isoenzymes were detected, but a single active band corresponding to that of partially purified internal isoenzyme AF II was also found (Fig. 2B). Although good reproducibility in different runs was achieved, a drift towards the cathode, similar to that described by Fawcett (1968), was sometimes observed for the sets of active bands corresponding to the isoenzymes AF II and AF III.

Partial purification of extracellular isoenzymes

To the culture filtrate, 10 % (v/v) of a solution of 10 % (w/v) tannic acid was added and precipitation was allowed to occur overnight at 4 °C. The precipitate was collected by centrifugation at 30000 g for 30 min and washed several times with cold acetone, then extracted several times with 0.1 M-sodium acetate buffer, pH 5.0, and the sediment discarded. During this step a fivefold purification was achieved (Table 2).

After dialysis for 48 h at 4 °C against distilled water, the sample was subjected to wide range (pH 3 to 10) preparative isoelectric focusing. Two peaks of activity were separated. Appropriate fractions corresponding to nos. 4 to 9 and 16 to 24 (pH values 2.0 to 3.8 and 5.5 to 7.6 respectively; Fig. 1) were pooled and, after dialysis at 4 °C for 24 h against distilled water, protein and AF activity were measured. Although the amount of protein in each sample was reduced, purification was not improved (Table 2).

Both samples were concentrated to 2 ml by freeze-drying and further purified by gel filtration. Concentrated sample A was applied to a Biogel P-300 column, while concentrated sample B was applied to a Sephadex G-100 column. From both columns 5 ml fractions were

Fig. 2. Isoelectric focusing on polyacrylamide gels of AF from *Sclerotinia fructigena* grown in ammonium tartrate-sodium polypectate liquid medium. Gels were run and stained for AF activity as described under Methods. A, culture filtrate; B, mycelial homogenate.

collected and AF activity measured in each fraction. Active fractions were pooled and total AF and protein measured. Both samples were shown to have a low protein content, but because of loss of activity during the preliminary steps no great overall increase in specific activity was achieved (Table 2).

Table 2. *Partial purification of external AF isoenzymes of Sclerotinia fructigena*

Stage	AF units (μ mol/min)	Protein (mg)	Specific activity
Culture filtrate	659	956	0.69
Tannic acid precipitate	318	102	3.12
Isoelectric focusing			
Sample A (AF I)	30	14	2.14
Sample B (AF III)	120	39	3.08
Gel filtration			
Sample A (AF I)	25	6.4	3.91
Sample B (AF III)	67	8.7	7.70

Table 3. *Partial purification of the main internal AF isoenzyme of Sclerotinia fructigena*

Stage	AF units (μ mol/min)	Protein (mg)	Specific activity
Mycelial homogenate	68	388	0.18
Ammonium sulphate fractionation (30 to 80 % saturation)	66	132	0.50
Isoelectric focusing (AF II)	24	64	0.38
Gel filtration	19	7.7	2.47

Partial purification of the main internal AF isoenzyme

A mycelial homogenate was obtained by following the general procedure described under Methods, but by using 0.05 M-tris-HCl buffer, pH 7.0, instead of distilled water. This mycelial homogenate was brought to 30 % saturation with ammonium sulphate and allowed to stand at 4 °C for 4 h; after centrifugation at 30 000 g for 20 min the precipitate was discarded and the supernatant was then brought to 80 % saturation and stood at 4 °C overnight. The sediment, redissolved in the extracting buffer, was shown to have the main bulk of AF activity (Table 3).

Before further purification by wide-range preparative isoelectric focusing (pH 3 to 10), the sample was dialysed at 4 °C against the extracting buffer, of decreasing molarity, and finally against distilled water. Appropriate fractions corresponding to nos. 11 to 15 (pH 4.2 to 5.2; Fig. 1) were pooled, and after dialysis, first against extracting buffer and then against distilled water, total activity and protein were measured. Although inactivation may well have taken place, the low recovery in this step (Table 3) is also attributable to separation of AF II from other internal AF isoenzymes.

The enzyme was concentrated to 2 ml by freeze-drying and further purified by filtration through Biogel P-300. Fractions (5 ml) were collected and assayed for activity. Active fractions were pooled and assayed for total activity and protein. During this step a large amount of protein was removed (Table 3).

Attempts to purify the enzyme further were abandoned because of great loss of activity, possibly associated with the instability of this isoenzyme at pH values of 5.0 and below (Laborda *et al.* 1973).

Table 4. *Relative activity of partially purified AF isoenzymes of Sclerotinia fructigena on different substrates**

	Activity (%)		
	AF I	AF II	AF III
<i>p</i> -Nitrophenyl α -L-arabinofuranoside† (Fielding & Hough, 1965)	100	100	100
<i>p</i> -Nitrophenyl α -L-arabinopyranoside†	0	0	0
<i>p</i> -Nitrophenyl β -L-arabinopyranoside†	0	0	0
2-Naphthyl α -L-arabinofuranoside‡	102	410	94
<i>p</i> -Nitrophenyl α -D-galactopyranoside†	0	0	0
Araban§	50	32	37
Arabinogalactan§	0	0	0

* Enzyme (0.5 ml) was incubated with 0.5 ml of substrate in 0.2 M-sodium acetate buffer, pH 5.0 (1 mg/ml) at 30 °C.

† Measure of *p*-nitrophenol liberated.

‡ Data of Hislop, Barnaby, Shellis & Laborda (1974).

§ Measure of arabinose liberated.

Table 5. *Inhibition of partially purified AF isoenzymes of Sclerotinia fructigena**

Substance	Concentration (M)	Inhibition (%)†		
		AF I	AF II	AF III
L-Arabeto- γ -lactone	10 ⁻²	93	60	97
	10 ⁻³	63	12	80
L-Galactono- γ -lactone	10 ⁻²	0	16	0
	10 ⁻³	0	0	0
D-Galactono- γ -lactone	10 ⁻²	48	7	33
	10 ⁻³	7	0	5
DL-Galactaro- γ -lactone (Lewis, Smith & Stephen, 1963)	10 ⁻²	24	0	29
	10 ⁻³	0	0	6

None of the following substances gave any inhibition up to 10⁻² M: Mg²⁺, Ca²⁺, D-xylose, L-arabinose, D-glucose, D-mannose, D-galactose, galacturonic acid, L-arabitol, D-arabeto- γ -lactone.

* Reaction mixture (3.5 ml) contained enzyme, 0.01 u.; *p*-nitrophenyl α -L-arabinofuranoside, 0.5 $\times$ 10⁻³ M; inhibitor, 10⁻² or 10⁻³ M in 0.1 M-sodium acetate buffer, pH 5.0. Residual activity was measured in the standard conditions.

† Expressed relative to control (as in *, but without inhibitor).

Activity of partially purified isoenzymes on different substrates

The relative activity of partially purified isoenzymes towards different compounds was studied by incubating 0.5 ml enzyme (0.01 u.) with 0.5 ml of a solution of the respective substrate (1 mg/ml) in 0.2 M-sodium acetate buffer, pH 5.0 at 30 °C. In each test, release of either *p*-nitrophenol or arabinose was determined.

Results, expressed as a percentage of the activity on *p*-nitrophenyl α -L-arabinofuranoside, are summarized in Table 4. All three isoenzymes had specificity towards compounds with α -L-arabinofuranoside groups, but the rate of hydrolysis of these compounds was different for each isoenzyme. The external AF I had the highest activity towards araban.

Table 6. *Inhibitory effect of apple juice on extracellular AF isoenzymes of Sclerotinia fructigena**

	Inhibition (%)†	
	AF I	AF III
Unoxidized juice‡	10	35
Oxidized juice‡	28	55

* Enzyme (0.5 ml) was incubated with 0.5 ml juice and 2.5 ml substrate in 0.1 M-sodium acetate buffer, pH 4.7. Substrate concentration and determination of residual activity were as in the standard procedure.

† Expressed relative to control (as in *, but distilled water instead of apple juice).

‡ Obtained as described under Methods.

Inhibition studies on the partially purified isoenzymes

Approximately 0.01 u. of each isoenzyme in turn was incubated with *p*-nitrophenyl α -L-arabinofuranoside (final concentration 0.5×10^{-3} M in 0.1 M-acetate buffer, pH 5.0) in the presence of several substances at final concentrations of 10^{-3} or 10^{-2} M. The final volume of each incubation mixture was 3.5 ml. The reaction was stopped and release of *p*-nitrophenol measured as in the standard procedure. Results are summarized in Table 5.

None of the likely precursors of apple cell-wall polymers had any inhibitory effect on the different AF isoenzymes. D-Arabinono- γ -lactone was also ineffective, but its L-isomer was the most inhibitory compound tested against all three isoenzymes. This, and other lactones tested, inhibited the three isoenzymes to differing extents.

Effect of partially purified extracellular AF isoenzymes on apple cell walls

Both external isoenzymes were incubated at 25 °C with isolated apple cell walls. Incubates contained: apple cell walls, 25 mg; 0.01 M-sodium acetate buffer (pH 4.7), 2.5 ml; enzyme, active or boiled for 5 min, 1 ml (approx. 0.6 u.). Samples were withdrawn after 1, 2, 4 and 6 h and examined by both descending paper and ascending thin-layer chromatography.

For the isoenzyme AF III, gradually increasing amounts of a spot with R_G corresponding to monomeric arabinose were detected with the active, but not with the inactivated, enzyme incubates. For the isoenzyme AF I, trace amounts of arabinose were liberated after 6 h incubation, together with a second spot with R_G corresponding to galacturonic acid: this was probably released from the apple cell walls by some contaminating polygalacturonase in this isoenzyme preparation.

Parallel chromatographic studies from araban incubated with each external AF isoenzyme always showed a single spot with R_G corresponding to that of monomeric arabinose.

Effect of apple juice on extracellular AF isoenzymes

Enzyme (0.5 ml) was incubated with *p*-nitrophenyl α -L-arabinofuranoside, in the standard conditions, in the presence of 0.5 ml of either oxidized or unoxidized apple juice. Both external AF isoenzymes were partially inactivated, whether the juice was oxidized or not, but isoenzyme AF III was inactivated to a greater extent than AF I. On both isoenzymes apple juice had a more inhibitory effect when oxidized (Table 6).

A 0.1 % (w/v) solution of sodium dithionite had no inhibitory effect on either external AF isoenzyme.

Table 7. Relationship between growth rate *in vivo* and AF secretion *in vitro* of *Sclerotinia fructigena* isolates

Isolate	Relative growth rate <i>in vivo</i> * (%)	Relative AF <i>in vitro</i> * (%)	External AF isoenzymes	
			AF I	AF III
Wild type	100	100	+	+
601	50	52	+	+
695	100	21	+	+
768	26	0†	+	—
799	25	39	+	—

+, Isoenzyme detected; —, not detected.

* Derived from Howell (1972).

† Slight AF production was found during the present work.

*AF isoenzyme pattern in culture filtrates from different
Sclerotinia fructigena isolates*

Several *Sclerotinia fructigena* isolates with a relatively low AF level in the culture filtrate and with various growth rates *in vivo* were grown in an ammonium tartrate-sodium polypectate liquid medium, and culture filtrate was collected as described for the wild type. After 50-fold concentration by freeze-drying the AF isoenzyme pattern was studied by gel filtration through Sephadex G-100.

For those isolates with a relatively high growth rate *in vivo* (isolates no. 601 and 695) a similar AF pattern to that for the wild type was found, while for those with a low growth rate *in vivo* (isolates no. 768 and 799) a single peak of activity corresponding to isoenzyme AF I was found (Table 7).

DISCUSSION

Sclerotinia fructigena produced AF when grown in liquid medium containing apple fibre as carbon source. Activity of this enzyme was also found in artificially-inoculated apple fruitlets. These results, together with the positive correlation found by Howell (1972) between fungus growth *in vivo* and production of AF *in vitro*, suggest an important role for this enzyme during rotting of apple by this fungus.

All three AF isoenzymes could be demonstrated when extracts of rotted apple were studied by isoelectric focusing. The presence of AF II as the main isoenzyme in these studies indicates a large contribution of intracellular activity to the total AF extracted. It was not possible to determine the contribution of extracellular activity to the total amount of both AF I and AF III detected.

When *Sclerotinia fructigena* was grown on different carbon sources, AF activity was found in each culture filtrate and mycelial homogenate. Polymers (araban, sodium polypectate and apple fibre) were found to be better inducers of this enzyme than any of the monosaccharides tested; a similar result was obtained for AF production by another plant pathogenic fungus (Kaji & Yoshihara, 1969).

Isoelectric focusing studies with these culture filtrates and mycelial homogenates showed that the AF isoenzyme pattern was similar in each instance to that previously described (Laborda *et al.* 1973). However, by the use of wide range (pH 3 to 10) isoelectric focusing in polyacrylamide gels instead of a preparative column, several AF bands within both AF I and AF III could be demonstrated. Their presence may explain the occurrence of diffuse staining patterns after normal gel electrophoresis. Further evidence for multiplicity of

molecular forms within both AF I and AF III was obtained from narrow-range isoelectric focusing in a preparative column (unpublished results). A similar multiplicity was found by Marshall & Cohen (1972) for bovine-liver ornithine transcarbamylase. They suggested that all the related active proteins were originally identical and that the observed multiplicity arose as a consequence of deamination of the original molecule which led to small differences in pI but did not affect the binding of the substrate or the kinetics of the enzymes.

The biochemical basis for multiple forms within AF I and AF III, and their physiological significance, have not yet been elucidated; all proteins with a similar pI and molecular weight and showing AF activity are therefore considered as a single AF isoenzyme in the present Discussion (see Tables 2 and 3).

Among plant pathogenic fungi, production of multiple forms of an enzyme, each able to break a specific glycosidic bond, has been widely described. In some instances a different mode of action (e.g. exo- or endo-activity) was associated with each of the different enzymes (Bateman, 1972). In others, each enzyme seemed to be specialized to attack the substrate at different stages of degradation (Olutiola & Ayres, 1973).

In *Sclerotinia fructigena* AF, each isoenzyme showed specificity for α -L-arabinofuranoside substrates: they were unable to break Larix arabinogalactan, where arabinose is linked by β -bonds (Aspinall, Fairweather & Wood, 1968), *p*-nitrophenyl α -L-arabinopyranoside, its β -anomer, or *p*-nitrophenyl α -D-galactopyranoside. The almost complete inhibition of AF isoenzymes by L-arabono- γ -lactone and an inhibitory effect of the structurally-related D-galactono- γ -lactone support this α -L-arabinofuranoside substrate specificity (Levy & Snaith, 1972).

As only monomeric arabinose was found to be released when araban was incubated with the external AF isoenzymes, an exo- α -L-arabinofuranosidase activity was considered to be associated with each of these isoenzymes. Since pectic araban is composed of both α -1,3- and α -1,5-linked arabinofuranose units (Rees & Richardson, 1966), a more defined substrate would be desirable in order to assess whether each one of the external AF isoenzymes has any specificity for a given α -linked arabinofuranoside.

Both external isoenzymes seem to have the same substrate specificity, but a comparison of their characteristics show that: (i) AF III was more active than AF I in releasing arabinose from apple cell walls, even though the latter was more active when incubated with araban and had some polygalacturonase associated with it that could help in cell-wall degradation (Jones, Anderson & Albersheim, 1972); (ii) AF III was more susceptible than AF I to inhibition by either oxidized or unoxidized apple juice; and (iii) AF III had a lower molecular weight, lower stability at low pH and a higher pH optimum than AF I (Laborda *et al.* 1973).

These results suggest a preferential effect for the external AF III in the margin of the apple rotted tissue, in association with other lytic enzymes in the degradation of the intact apple cell walls, thus helping the advance of the fungal hyphae. The lack of AF III secretion by those isolates with low growth rate *in vivo* is in accord with this suggestion. The external AF I may act preferentially in the rotted tissue once partial digestion of the apple cell walls allows a better diffusion of high molecular weight enzymes towards the substrate (Goodman, Király & Zaitlin, 1967). At the same time, the release of the acidic intracellular contents of the apple cells would lead to an environment where the activity of AF I would be favoured over that of AF III.

The internal AF II, also present *in vivo*, showed different physical and kinetic characteristics and behaved slightly differently during inhibitor and substrate specificity studies. However, no obvious role has emerged for it other than its possible relationship with the external isoenzymes during their biosynthesis and secretion (Laborda *et al.* 1973).

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