

**$\alpha$ -L-Arabinofuranosidase from *Sclerotinia sclerotiorum*: Purification, Characterization, and Effects on Plant Cell Walls and Tissue**

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**ABSTRACT**

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*Sclerotinia sclerotiorum* produced  $\alpha$ -L-arabinofuranosidase when grown at 25 C on a medium of mineral salts containing 0.4% L-arabinose plus 0.2% Difco yeast extract. Enzyme in filtrates from 14-day-old cultures was concentrated and dialyzed by Amicon ultrafiltration. The specific activity of the  $\alpha$ -L-arabinofuranosidase was increased 26 $\times$  by a four step procedure: (i) preparative electrofocusing in granulated gel using ampholytes with a pH of 7-9; (ii) column ion-exchange chromatography (CM-Sephadex [C-50])

in 20 mM sodium acetate at pH 5.0; and (iii, iv) two cycles of gel filtration on Ultrogel (AcA 54) in 72 mM phosphate buffer at pH 7.0 containing 100 mM NaCl. Purified  $\alpha$ -L-arabinofuranosidase had a pH optimum of 4.0-4.5, a molecular weight of about 63,000 and a pI of about 7.5. This enzyme released arabinose from arabinan and from isolated cell walls of bean and rice. Highly active preparations of this  $\alpha$ -L-arabinofuranosidase failed to macerate potato tuber or cucumber endocarp tissue.

Decomposition of plant cell walls during tissue invasion and pathogenesis is a characteristic feature of numerous plant diseases incited by facultative, necrotrophic pathogens. The role of pectic enzymes in tissue maceration, cell wall disruption, and cell death is now generally appreciated (7,23). The involvement of cellulases, galactanases, xylanases, and arabinase in pathogenesis is less well understood. Many pathogens produce these enzymes during pathogenesis, however, and in many instances, their substrates in invaded hosts are degraded (2,6-8,18).

The significance of enzymes that digest structural polysaccharides in the plant cell wall remains to be elucidated with respect to pathogen ingress. How important are they to this process? Do these enzymes play an essential role in pathogen ramification of host tissues? Before these questions can be adequately approached experimentally, we need to understand better the precise chemical structure of the plant cell wall, as well as the involvement of polysaccharidases in cell wall disruption. The availability of purified enzymes that attack each of the major glycosidic linkages in cell walls is essential for the studies needed to answer the above questions.

Several reports have concerned the purification of arabinosidase from various sources (10-13,17). However, the purity of these preparations in regard to trace amounts of other cell wall-degrading enzymes has not been sufficiently demonstrated.

We previously reported source organisms and procedures for purifying endopectate lyase (5), endo- $\beta$ -1,4 galactanase (9), and endo- $\beta$ -1,4 xylanase (4). Here we report the purification and characterization of an  $\alpha$ -L-arabinofuranosidase produced by *Sclerotinia sclerotiorum* and some of its effects on isolated cell walls and on excised potato tuber and cucumber endocarp tissues.

**MATERIALS AND METHODS**

**Substrates and chemicals.** The *p*-nitrophenyl- $\alpha$ -L-arabinofuranoside was a gift from A. H. Fielding, Long Ashton Research Station, Bristol, U. K. The arabinan, galactan, xylan, polygalacturonic acid, carboxymethylcellulose, and mannan used as potential enzyme substrates were the same as those used by Baker et al (4). L-arabono-1, 4-lactone was purchased from Koch-Light Lab., Ltd., Colnbrook, U. K. D-galactono-1, 4-lactone, D-

glucono-1, 5-lactone and D-mannono-1, 4-lactone were purchased from Pfanstiehl Lab., Inc., Waukegan, IL 60085. All other chemicals used were reagent grade.

Cell walls from kidney bean (*Phaseolus vulgaris* L.) and rice (*Oryza sativa* L.) cell suspension cultures in log phase growth were prepared by the procedure of Talmadge et al (25).

**Enzyme assays.** The  $\alpha$ -L-arabinofuranosidase activity was routinely assayed by measuring release of *p*-nitrophenol (PNP) from PNP- $\alpha$ -L-arabinofuranoside in 50 mM sodium acetate buffer at pH 4.5 (9). One unit of  $\alpha$ -L-arabinofuranosidase was defined as the amount of enzyme that released 1  $\mu$ mol of product per minute under the above conditions. Galactanase and polygalacturonase activities were determined by measuring the release of reducing groups from their respective substrates as previously described (9). In all enzyme assays lasting longer than 2 hr, 0.02% sodium azide was routinely included to inhibit microbial activity. Protein was determined by the procedure of Lowry et al (21), using bovine serum albumin as the standard.

**Production and purification of  $\alpha$ -L-arabinofuranosidase.** *Sclerotinia sclerotiorum* (Lib.) deBary (isolate 214) was stored in distilled water at 4 C (9). This fungus was grown on a mineral salts medium in the manner described by Bauer et al (9), except that the medium was supplemented with 0.4% L-arabinose instead of D-galactose. After a 14-day incubation at 25 C, cultures were filtered through several layers of cheesecloth, followed by vacuum filtration through two layers of Whatman GF/A glass fiber paper.

Approximately 2.5 L of crude culture filtrate was chilled to 4 C, concentrated to 92 ml, and dialyzed against distilled water by pressure ultrafiltration in an Amicon pressure cell (Amicon Corp., Lexington, MA 02173) at 3 atmospheres (3.16 kg/cm<sup>2</sup>) using a UM-10 membrane. All enzyme purification steps were performed at 4 C. The concentrated, dialyzed culture filtrate was subjected to preparative electrofocusing in a granulated gel bed of Ultrodex using an LKB Multiphor apparatus (Model 2117, LKB Instruments, Inc., Rockville, MD 20852). The protocol suggested by the manufacturer (LKB application Note 198) was followed using pH 7-9 Ampholine carriers (2%). The power source was initially set for constant power at 8 W with a maximum of 1500 V and 20 mA. After 15 hr the gel bed (10.7  $\times$  23 cm) was divided into 30 equal zones and the gel from each zone placed into a 1.5  $\times$  5 cm column. Each column was eluted with 3 ml of distilled water and the pH of the eluate measured. Each column then was rinsed with three 1-ml amounts of 100 mM sodium acetate buffer at pH 4.5.

The water and buffer washes from each column were combined and assayed for  $\alpha$ -L-arabinofuranosidase, polygalacturonase, and galactanase activities.

Fractions containing  $\alpha$ -L-arabinofuranosidase activity were pooled, dialyzed against 20 mM sodium acetate buffer at pH 5.0, and applied to a 1.5  $\times$  20 cm CM-Sephadex (C-50) ion-exchange column equilibrated with the same buffer. The column was eluted at a flow rate of 12 ml/hr with a 200-ml linear salt gradient in buffer (0–100 mM NaCl). Five-milliliter fractions were collected and assayed for  $\alpha$ -L-arabinofuranosidase, galactanase, and polygalacturonase. The peak fractions of  $\alpha$ -L-arabinofuranosidase were pooled and concentrated by ultrafiltration. The enzyme concentrate was applied to an Ultrogel (AcA 54) gel filtration column (2.5  $\times$  70 cm), equilibrated with 72 mM phosphate buffer at pH 7.0 containing 100 mM NaCl. The column flow rate was adjusted to 15 ml/hr and 2-ml fractions were collected and then assayed for  $\alpha$ -L-arabinofuranosidase, galactanase, and polygalacturonase. Reaction mixtures for the latter two enzymes were allowed to incubate 24 hr before assaying. The fractions that degraded galactan were subjected to paper chromatography to distinguish release of galactose and galactose oligomers from release of arabinose. Fractions containing only  $\alpha$ -L-arabinofuranosidase activity were pooled. In some cases, the gel filtration step had to be repeated, using the pooled  $\alpha$ -L-arabinofuranosidase fractions, in order to free this enzyme from polygalacturonase and/or galactanase.

The molecular weight of the purified  $\alpha$ -L-arabinofuranosidase

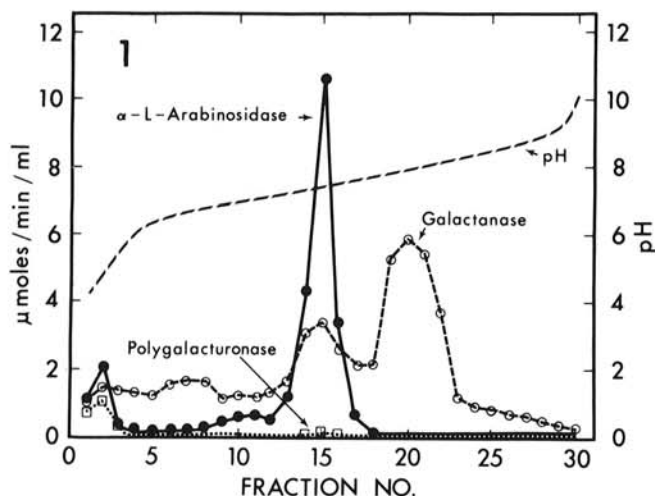


Fig. 1. Electrofocusing of culture filtrate from *Sclerotinia sclerotiorum* grown for 14 days at 25 C on a minimal salts medium supplemented with 0.4% arabinose. The filtrate was dialyzed, concentrated, and subjected to electrofocusing with pH 7–9 ampholytes. Fractions were assayed for  $\alpha$ -L-arabinosidase, galactanase, and polygalacturonase.

TABLE 1. Purification of  $\alpha$ -L-arabinofuranosidase from culture filtrates of *Sclerotinia sclerotiorum* grown for 14 days on a minimal medium supplemented with 0.4% arabinose

Purification Step	Volume (ml)	Total protein (mg)	Specific activity ( $\mu$ mol/min/mg of protein)	Recovery (%)
Culture filtrate	2,655	328	1.1	...
Culture filtrate concentrate	92	319	1.1	94
Electrofocusing	24.4	...	...	29
CM-Sephadex	28.0	6.72	9.0	16
1st Ultrogel (AcA 54)	26.0	3.02	13.4	11
2nd Ultrogel (AcA 54)	34.9	0.66	28.5	5

was estimated by sucrose density-gradient ultracentrifugation (22) and SDS discontinuous gel electrophoresis (4). The reference compounds (and their molecular weights) used in the sucrose density-gradient studies were: cytochrome C (12,300), horseradish peroxidase (40,000), and human  $\gamma$ -globulin (153,100). Lysozyme (14,300), soybean trypsin inhibitor (21,000), carbonic anhydrase (30,000), ovalbumin (45,000), bovine serum albumin (68,000), and phosphorylase B (94,000) were used as references in the SDS gel electrophoretic studies. Low-pH gel electrophoresis (4) was used to study homogeneity of the purified enzyme.

## RESULTS

**Purification and characterization of  $\alpha$ -L-arabinofuranosidase from *S. sclerotiorum*.** The  $\alpha$ -L-arabinofuranosidase activity in crude culture filtrates of *S. sclerotiorum*, grown at 25 C on a mineral salts medium supplemented with 0.4% arabinose, increased over a 19-day culture period. This enzyme was purified from 14-day-old culture filtrate (2,655 ml) that contained 0.136 units of  $\alpha$ -L-arabinofuranosidase activity per milliliter (Table 1).

Narrow pH range electrofocusing (pH 7.0–9.0) of crude dialyzed enzyme concentrate showed that the  $\alpha$ -L-arabinofuranosidase had a pI of 7.5 and could be resolved from a minor peak of polygalacturonase (pI 4.7) and galactanase (pI 8.0) (Fig. 1). The apparent galactanase activity associated with the  $\alpha$ -L-arabinofuranosidase peak occurred primarily because of the release of arabinose from the galactan substrate (Table 2). In this particular assay, therefore, only the presence of galactose in the reaction mixture was taken as evidence for galactanase activity.

In the next purification step, the peak  $\alpha$ -L-arabinofuranosidase fractions were applied to a CM-Sephadex column and eluted at about 30 mM NaCl (Fig. 2). This procedure further resolved the  $\alpha$ -L-arabinofuranosidase from polygalacturonase that eluted at 45 mM NaCl. Paper chromatography of reaction products revealed that arabinose was released from lupin galactan by fractions containing  $\alpha$ -L-arabinofuranosidase, and galactose and oligomers of galactose were released by fractions following the  $\alpha$ -L-arabinofuranosidase peak (Fig. 2).

The remaining traces of polygalacturonase and galactanase associated with the  $\alpha$ -L-arabinofuranosidase peak eluted from the

TABLE 2. Tests for hydrolysis of selected compounds by the purified  $\alpha$ -L-arabinofuranosidase from *Sclerotinia sclerotiorum*

Compound tested	Reducing groups released in reaction mixtures <sup>a</sup> at pH:	
	4.5	8.0
Polysaccharide	$\mu$ mol/24 hr	
Arabinan (araban)	28.1 <sup>b</sup>	0.08
Carboxymethylcellulose	0.00	0.00
Galactan	1.01 <sup>c</sup>	0.00
Mannan	0.00	0.00
Polygalacturonic acid	0.00	0.00
Xylan	0.00	0.00
Glycoside	$\mu$ mol/2 hr	
PNP <sup>d</sup> - $\alpha$ -arabinoside	3.60 <sup>e</sup>	...
PNP- $\alpha$ -galactoside	0.00	...
PNP- $\beta$ -galactoside	0.00	...
PNP- $\alpha$ -glucoside	0.00	...
PNP- $\beta$ -glucoside	0.00	...

<sup>a</sup> Reaction mixtures contained 0.1% polysaccharide in 500  $\mu$ l of 50 mM sodium acetate (pH 4.5) with 0.07 units of purified enzyme or 0.05% glycoside in 1 ml of 50 mM sodium acetate (pH 4.5) with 0.03 units of enzyme. Reaction mixtures were incubated at 30 C and then assayed for release of reducing groups or PNP.

<sup>b</sup> Calculated activity based on assays of reaction mixtures incubated for 1 hr.

<sup>c</sup> Reaction products were monomeric arabinose by paper chromatography.

<sup>d</sup> PNP = *p*-nitrophenol.

<sup>e</sup> Calculated activity based on assays of reaction mixtures incubated for 10 min.

CM-Sephadex (C-50) column were removed by two cycles of Ultrogel (AcA 54) gel filtration in 72 mM phosphate buffer at pH 7.0 containing 100 mM NaCl (Fig. 3). In this system polygalacturonase preceded, and galactanase followed, the elution of  $\alpha$ -L-arabinofuranosidase. This purification procedure resulted in a 26-fold increase in the specific activity of the  $\alpha$ -L-arabinofuranosidase and a 5% recovery (Table 1). The purified enzyme was stored at  $-20^{\circ}\text{C}$  in 50 mM sodium acetate buffer at pH 5.0 containing 100 mM NaCl and 0.02% sodium azide for up to 1 yr without significant loss of activity.

Low-pH, discontinuous gel electrophoresis of 2–34  $\mu\text{g}$  of the purified enzyme revealed a single diffuse protein band when the gels were stained with Coomassie Brilliant Blue R. This band coincided with the location of  $\alpha$ -L-arabinofuranosidase activity eluted from nonstained gels. SDS electrophoresis of 9.5  $\mu\text{g}$  of the purified enzyme revealed a single protein with a molecular weight of 62,000; the molecular weight estimated by sucrose density-gradient ultracentrifugation was 64,000. When 19 or 38  $\mu\text{g}$  of enzyme was subjected to SDS gel electrophoresis, the stained gel revealed a faint protein band that migrated slightly ahead of the major band.

The pH optimum for the hydrolysis of PNP- $\alpha$ -L-arabinofuranoside by the purified enzyme was between pH 4.0 and 4.5 when determined in 50 mM sodium citrate–50 mM potassium

phosphate buffer (pH 3–7) or in 50 mM sodium acetate buffer (pH 3.5–5) (Fig. 4). When 0.2 units of enzyme per milliliter were incubated with 0.1% arabinan in 50 mM sodium acetate buffer at pH 4.5, the only product detected by paper chromatography (4) was arabinose. Chromatograms were prepared after 0, 5, 8.5, 9, and 16% hydrolysis of the arabinan substrate as determined by the release of reducing groups. When similar reaction mixtures contained PNP glycosides or various polysaccharides, no reducing groups or hydrolytic products were detected except for the reaction mixtures containing lupin galactan (Table 2). The product released from this substrate was arabinose. Hydrolysis of PNP- $\alpha$ -L-arabinofuranoside by the purified enzyme was inhibited 70% and 24%, respectively, by 1.48 mM L-arabonolactone and 1.48 mM D-galactonolactone. Neither D-gluconolactone nor D-mannonolactone inhibited this enzyme.

**Effects of  $\alpha$ -L-arabinofuranosidase on isolated plant cell walls and tissues.** The ability of the purified  $\alpha$ -L-arabinofuranosidase to solubilize constituents of isolated bean and rice cell walls was examined (Table 3). The carbohydrates in the supernatants from buffer (control) and enzyme treatments were hydrolyzed by

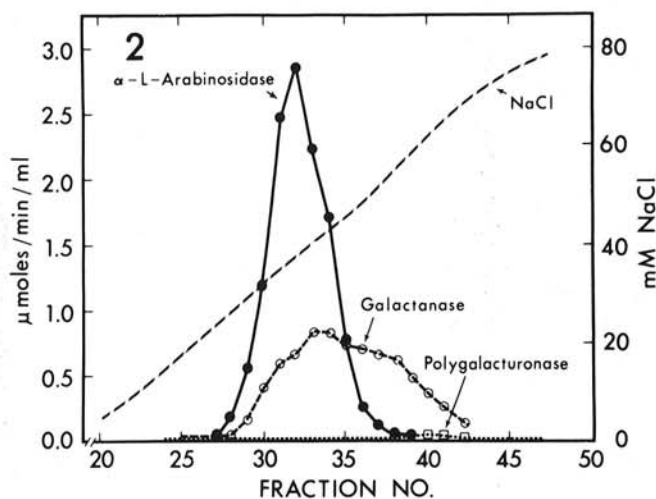


Fig. 2. Fractionation of the enzyme activities in the peak fractions of  $\alpha$ -L-arabinosidase from the electrofocusing column on CM-Sephadex (C-50) equilibrated with 20 mM sodium acetate buffer at pH 5.0.

TABLE 3. Solubilization of neutral sugars in isolated cell walls from suspension cell cultures of bean and rice by trifluoroacetic acid (TFA) and  $\alpha$ -L-arabinofuranosidase ( $\alpha$ -L-ara)

Sugars	Neutral sugars solubilized (% by weight) <sup>a</sup> from:			
	Bean Walls		Rice Walls	
	TFA <sup>b</sup>	$\alpha$ -L-ara <sup>c</sup>	TFA	$\alpha$ -L-ara
Rhamnose	0.86	0.00	1.08	0.00
Fucose	0.48	0.00	0.46	0.00
Arabinose	11.51	2.38	18.40	2.53
Xylose	2.71	0.00	13.74	0.00
Mannose	0.42	0.01	0.25	0.00
Galactose	3.98	0.38	6.03	0.13
Glucose	3.65	0.00	12.70	0.11

<sup>a</sup> Reaction mixtures contained 10 mg of cell walls and 0.28 units of  $\alpha$ -L-arabinofuranosidase in a 2.0-ml volume of 50 mM sodium acetate buffer at pH 4.5 and 0.02% sodium azide. Reaction mixtures were incubated for 16 hr at 25 C. Controls contained all ingredients except enzyme.

<sup>b</sup> Sugars released by TFA hydrolysis of buffer-treated cell walls as determined by the procedure of Albersheim et al (3).

<sup>c</sup> Sugars released from cell walls by  $\alpha$ -L-arabinofuranosidase ( $\alpha$ -L-ara) into the supernatant of reaction mixtures.

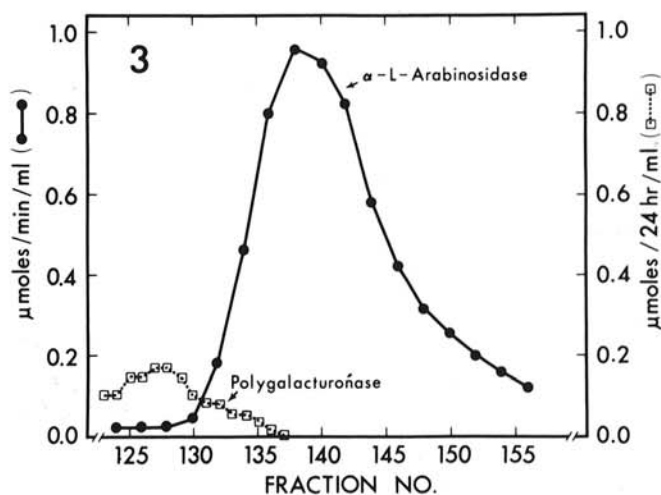


Fig. 3. Fractionation of the enzyme activities in the  $\alpha$ -L-arabinosidase peak from the CM-Sephadex (C-50) column by gel filtration in Ultrogel (AcA 54).

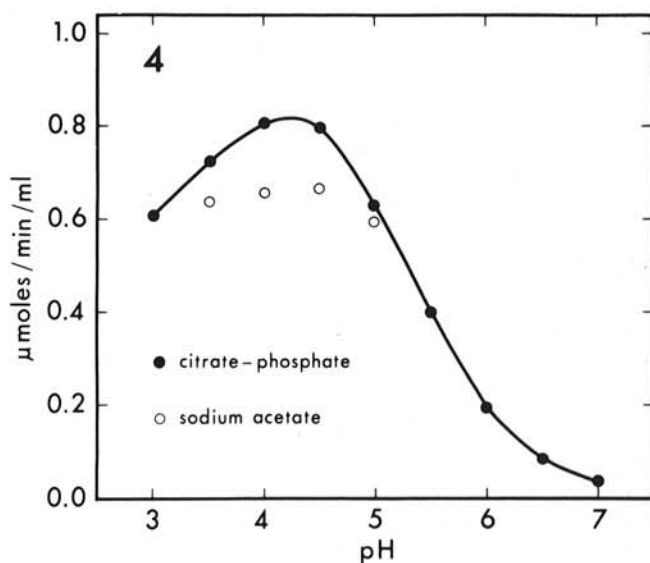


Fig. 4. Hydrolysis of *p*-nitrophenol- $\alpha$ -L-arabinofuranoside by purified  $\alpha$ -L-arabinosidase from *Sclerotinia sclerotiorum* in relation to pH at 30 C in 50 mM buffers.

trifluoroacetic acid (TFA) and analyzed by gas chromatography for neutral sugars using the procedures of Albersheim et al (3).

Only traces of arabinose, mannose, galactose, and glucose were detected in the supernatant of control reaction mixtures containing either bean or rice cell walls. The supernatant of bean walls treated with  $\alpha$ -L-arabinofuranosidase contained traces of mannose and glucose plus 2.38 and 0.38% of the cell wall weight as arabinose and galactose, respectively. The number and quantities of sugars released by the enzyme from rice cell walls and bean cell walls were similar (Table 3). The enzyme released 20.7% and 13.8%, respectively, of the arabinose in bean and rice cell walls as determined by TFA hydrolysis of buffer-treated cell walls.

The potential for  $\alpha$ -L-arabinofuranosidase to macerate plant tissue was examined in reaction mixtures containing 0.26 units of  $\alpha$ -L-arabinofuranosidase per milliliter, six tissue discs ( $9 \times 0.5$  mm) of potato tuber tissue or cucumber endocarp tissue, and 0.02% sodium azide in a 3-ml volume of 50 mM sodium acetate buffer at pH 5.0. Controls consisted of reaction mixtures without enzyme and reaction mixtures containing 0.3 units of endopectate lyase (5) instead of  $\alpha$ -L-arabinofuranosidase. Reaction mixtures were incubated for up to 24 hr at 25 C and examined periodically for tissue maceration (22). The purified  $\alpha$ -L-arabinofuranosidase did not macerate either plant tissue during these experiments. The endopectate lyase completely macerated the potato and cucumber tissue discs within 1 hr. Assay of reaction mixtures for  $\alpha$ -L-arabinofuranosidase activity after 24-hr incubation showed that 90% of the initial enzyme activity was present.

## DISCUSSION

*S. sclerotiorum* produced  $\alpha$ -L-arabinofuranosidase and galactanase plus smaller amounts of polygalacturonase when cultured on a medium containing 0.4% L-arabinose as the carbon source. Cultures of *Verticillium albo-atrum* and *Fusarium oxysporum* behave in a similar manner when grown on low concentrations of L-arabinose (14).

A large number of plant pathogenic fungi produce  $\alpha$ -L-arabinofuranosidases (10,12,16,18,24). The cell walls in host tissues invaded by some of these pathogens lose cell wall arabinose during pathogenesis (8,18). In addition, the virulence of *S. fructigena* isolates correlates with their ability to produce  $\alpha$ -L-arabinofuranosidase (19). The role and significance of this enzyme in plant cell wall decomposition or pathogenesis remains to be determined, however. There are conflicting reports about the ability of  $\alpha$ -L-arabinofuranosidase to cause plant tissue maceration (10-13,17). These reports may conflict owing to the presence of pectic enzymes in some of the  $\alpha$ -L-arabinofuranosidase preparations used in tissue maceration studies. The  $\alpha$ -L-arabinofuranosidases from *S. rolfsii* (12) and *Penicillium digitatum* (13) both failed to macerate plant tissues when freed of polygalacturonase. The  $\alpha$ -L-arabinofuranosidase of *S. sclerotiorum* purified in this study also failed to macerate either potato or cucumber tissue.

The properties of the purified  $\alpha$ -L-arabinofuranosidase from *S. sclerotiorum* were quite similar to those of  $\alpha$ -L-arabinofuranosidases produced by a number of other plant pathogenic fungi (15,16,24), except for its pI, which is about pH 7.5; those reported for other fungal  $\alpha$ -L-arabinofuranosidases range from pH 3.0 to 6.5 (15).

The  $\alpha$ -L-arabinofuranosidase of *S. sclerotiorum* was inhibited in a competitive manner by L-arabono-1, 4-lactone and D-galactono-1, 4-lactone but not by D-glucono-1, 5-lactone or D-mannono-1, 4-lactone. The  $\alpha$ -L-arabinofuranosidases from *Phytophthora palmivora* and *S. fructigena* responded similarly to these compounds (1,20).

Only L-arabinose was released from arabinan or lupin galactan by the purified  $\alpha$ -L-arabinofuranosidase produced by *S. sclerotiorum*. This enzyme released only 16% of the arabinose in beet arabinan, which suggests that this enzyme is either specific for certain bonds in the substrate or that the presence of branches in the substrate and/or the occurrence of anomalous sugars within the arabinan chain block further action of this exoenzyme. An  $\alpha$ -L-arabinofuranosidase from *Aspergillus niger* has been reported to

release about 90% of the arabinose in beet arabinan (26). The quantity and pattern of release of arabinose from arabinan by the  $\alpha$ -L-arabinofuranosidase of *S. sclerotiorum* resembled that reported for a similar enzyme produced by *Sclerotium rolfsii* (12).

*S. sclerotiorum*  $\alpha$ -L-arabinofuranosidase released significant amounts of arabinose from isolated bean or rice cell walls in the absence of other polysaccharide-degrading enzymes (Table 3). Since this enzyme can be purified and is highly stable, it should be a useful tool for examining plant cell wall structure and the role of  $\alpha$ -L-arabinofuranosidase in cell wall decomposition by plant pathogens.

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