Production of Cell Wall-Degrading Enzymes by the Phytopathogenic Fungus *Sclerotinia sclerotiorum*

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The range of polysaccharide-degrading enzymes and glycosidases formed by the phytopathogenic fungus *Sclerotinia sclerotiorum* was monitored following growth on 16 carbohydrate substrates. Endo- and exoenzymes capable of degrading cellulosic, hemicellulosic, and pectinolytic polysaccharides were secreted. Pectinolytic activities were produced constitutively on all of the substrates tested. Cellulolytic enzymes were not induced in simple sugar (i.e., glucose or xylose) media. Polysaccharide growth substrates and cellulase inducers increased all of the enzyme activities tested. Gel filtration analysis revealed the appearance of new molecular forms of pectinase, β-xylosidase, and cellobiosidase during induction on pectin and carboxymethyl cellulose media.

Plant pathogens produce a range of enzymes capable of degrading plant cell wall components. Fungi frequently secrete several molecular forms of hydrolases which attack the same substrate but differ in isoelectric point and molecular weight. This multiplicity confers flexibility, increasing the efficiency of the hydrolytic complex.

Among the economically important groups of plant pathogens, *Sclerotinia sclerotiorum* is a ubiquitous phytopathogenic fungus which attacks a wide range of plants. Extracellular proteins secreted by the fungus are able to macerate tissues and degrade cell wall components. They must thus contain all of the enzymes corresponding to the types of glycosidic linkages present in the cell wall polysaccharides. *S. sclerotiorum* is known to produce pectinolytic and cellulolytic enzymes (1, 2, 7, 9, 10, 16, 19). The level of these enzyme activities correlates with the development of disease symptoms (16, 17). Aside from pectic and cellulolytic enzymes, the diversity of polysaccharidases produced by *S. sclerotiorum* and the mechanisms controlling expression of cell wall-degrading enzymes are poorly understood.

The objective of this study was to examine in detail the range of polysaccharide depolymerases and glucoside hydrolases secreted by *S. sclerotiorum*. Cultures were grown on a range of monosaccharides, disaccharides, and polysaccharides so that the effects of the available carbon sources on enzyme production and activity could be evaluated. Gel filtration of extracellular proteins was used to characterize the isozymes which are produced depending on the nature of the carbon substrate.

MATERIALS AND METHODS

Chemicals. The polysaccharides used as the carbon source and as enzyme substrates were cellulose microcrystalline (Avicel) from Merck (Darmstadt, Federal Republic of Germany), hydroxymethyl cellulose (HEC), citrus pectin (esterification, 63 to 66%), and apple pectin (esterification, 70 to 75%) from Fluka (Buchs, Switzerland), laminarin (larch wood) and arabinogalactan from Sigma (St. Louis, Mo.), and xylan (oat spelts), Na$^+$ polygalacturonic acid (M_r, 25,000 and 50,000), carboxymethyl cellulose (CMC; degree of poly-

merization, 500 to 520), and galactan from Serva (Heidelberg, Federal Republic of Germany). All other reagents, glycosides used as carbon sources, and nitrophenyl and methylumbelliferyl used as substrates were purchased from Sigma.

Culture conditions. *S. sclerotiorum* (strain ssm 1; Rhône Poulenc Agrochimie) was grown on a liquid minimal medium supplemented with a 0.5% (wt/vol) carbon source. The minimal medium contained NH$_4$NO$_3$ (2 g/liter), KH$_2$PO$_4$ (1 g/liter), MgSO$_4$ (0.1 g/liter), yeast extract (0.5 g/liter), NaOH (1 g/liter), and DL-malic acid (3 g/liter). Cultures were maintained on potato dextrose agar. For enzyme production, 200-ml cultures inoculated with 20 plugs (4-mm diameter) removed from the growing edge of 4-day-old colonies were grown for 6 days at 24°C under constant agitation.

Cultures were harvested by filtration through Whatman no. 1 filter paper. Filtrates were dialyzed against distilled water overnight at 4°C and then freeze-dried. Mycelia were freeze-dried to determine dry weights and estimate fungal growth.

Enzyme methods. Freeze-dried culture media were solubilized in 4 ml of 0.1 M Na$^+$ acetate buffer, pH 6, and then precipitated to 25% saturation of ammonium sulfate and centrifuged at 10,000 × *g* for 10 min. The supernatant was brought to 85% saturation of (NH$_4$)$_2$SO$_4$ and then centrifuged. Pellets dissolved in Na$^+$ acetate buffer and dialyzed against distilled water and then against the Na$^+$ acetate buffer were used as enzyme sources. Protein content was determined by the method described by Bradford (3) with bovine serum albumin as the standard.

Glycoside hydrolase activities were determined by measuring the rate of *p*-nitrophenol released from the appropriate *p*-nitrophenyl derivatives (see Tables 1 to 4). The standard reaction mixture (1 ml) contained 20 μl of enzyme solution and 2 mg of substrate dissolved in 0.1 M Na$^+$ acetate buffer, pH 6. After 15 min of incubation at 50°C, reactions were stopped by the addition of 2 ml of 0.1 M Na$_2$CO$_3$, and the *p*-nitrophenol liberated was determined spectrophotometrically at 399 nm (11).

Polysaccharidase activities were determined by measuring the amount of reducing sugar released from various substrates (see Tables 1 to 4). A 20-μl volume of enzyme solution was incubated at 50°C for 30 min in 1 ml of substrate

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solution polysaccharide (2 mg · ml^{-1}) dissolved in 0.1 M Na$^+$ acetate buffer, pH 6. Reactions were stopped by the addition of 3 ml of dinitrosalicylic reagent (21). Tubes were placed in a boiling-water bath for 8 min. The A_{528} was read with appropriate single sugars as standards (11).

Enzyme and substrate controls were included in all assays. All enzyme reactions were linear over the period of assay. Enzyme activities are expressed as nanomoles of *p*-nitrophenol or the equivalent micrograms of reducing sugar.

Chromatography methods. Gel filtrations of concentrated culture media were done on a column (1.8 by 90 cm) of Ultrogel AcA 34 (IBF, Paris, France) matrix equilibrated in 0.01 M Na$^+$ acetate buffer, pH 6. Elution was performed at a flow rate of 12 ml/h with equilibrating buffer containing 0.1 M NaCl. Fractions (2.3 ml) were collected and assayed for enzyme activities.

Electrophoresis methods. Analytical isoelectric focusing (IEF) gel electrophoresis was performed by using Servalyt Precotes (Serva) containing 5% ampholine, pH 3.0 to 6.0. Gels were prefocused up to 500 V before application of the samples, and then proteins were focused at a constant power (4 W) for 2 h up to a final power of 1,700 V. Gels were cut in parallel slices for protein staining with Coomassie brilliant blue R 250 and detection of enzyme activities (12). Gels were incubated in 5 ml of substrate solution containing 2 mg of methylumbelliferyl glycoside dissolved in 0.1 M Na$^+$ acetate buffer, pH 6. After 5 to 10 min of incubation at room temperature, activities were revealed by fluorescence, under UV light exposure at 365 nm, of the methylumbelliferone released from the substrate. Photographs were taken by using a Polaroid camera and a yellow filter.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of extracellular proteins was performed on slabs by using a 7.5 to 15% gradient separating gel and a 4.5% stacking gel. Running conditions were the same as those described by Laemmli (15). Samples were boiled for 5 min in denaturing buffer before being loaded. Electrophoresis was carried out at 30 mA per gel. Proteins were located by staining with Coomassie brilliant blue R 250 or silver staining (Stratagene Kit; Stratagene, La Jolla, Calif.). High- and low-molecular-weight standards were from Sigma and Bio-Rad (Richmond, Calif.).

RESULTS

Effect of the carbohydrate growth substrate on enzyme activity and protein secretion. Enzymes capable of degrading a wide range of glucosides and polysaccharides were detected in cell-free culture supernatants. Growth of *S. sclerotiorum* on various polysaccharides used as the sole carbon source demonstrated that the fungus secretes enzymes that convert cellulosic, pectinolytic, and hemicellulolytic substrates to assimilable simple sugars. The biomasses produced in these cultures were much lower than those obtained on glucose or xylose media (Tables 1 and 2).

Glucose- and xylose-grown cultures exhibited nearly all of the enzyme activities tested except for xylanase and cellobiohydrolase, which were not detected. The levels of the other enzymatic activities were low except those for pectinase, polygalacturonase, and β-1,3-glucanase, indicating that glucose and xylose did not completely prevent synthesis of all of the enzyme activities and that some enzymes are formed constitutively.

The abilities of various substrates to modify enzyme production were studied by growing *S. sclerotiorum* on

TABLE 1. Glycosidase activities of extracellular preparations from *S. sclerotiorum* grown on a range of carbon sources and expressed amounts of *p*-nitrophenol released per milliliter of culture supernatant

Carbohydrate growth substrate	Derivative released (nmol/ml of culture supernatant)							Biomass produced (dry wt)
	β-D-Glucopyranoside (β-glucosidase)[a]	β-D-Galactopyranoside (β-galactosidase)	β-D-Fucopyranoside (β-fucosidase)	β-D-Xylopyranoside (β-xylosidase)	β-D-Lactopyranoside (cellobiohydrolase)	α-L-Arabinopyranoside (α-arabinosidase)	β-D-Cellobiopyranoside (β-cellobiosidase)	
Glucose	26.9	36.5	21.1	33.3	ND[b]	31.7	14.7	15.2
β-Methylglucoside	177.0	85.9	37.8	45.8	4.6	57.3	16.1	3.4
Xylose	35.4	52.0	26.9	39.6	ND	42.6	17.2	7.8
Sorbose	31.5	221.0	34.0	ND	ND	51.0	4.3	0.5
Sophorose	90.4	128.4	36.7	ND	ND	51.5	14.8	0.7
Cellobiose	23.9	27.6	ND	ND	3.2	53.1	6.4	7.1
Avicel	70.0	5.9	70.0	ND	7.5	62.5	17.5	NM[c]
CMC	52.5	133.5	57.6	49.9	21.1	94.7	14.1	1.0
HEC	280.2	89.6	152.3	54.4	12.1	70.7	68.6	0.5
Citrus pectin	165.9	285.6	379.7	163.2	46.3	180.6	138.2	1.9
Apple pectin	111.0	1,697.3	317.1	59.9	13.6	122.4	90.3	1.8
Na$^+$ polygalacturonic acid	204.8	1,251.2	553.0	147.2	59.5	236.8	268.8	2.8
Galactan	63.8	2,048.0	205.6	103.5	2.7	101.0	63.8	4.5
Arabinogalactan	72.6	1,132.1	227.0	111.5	ND	124.8	53.0	5.2
Laminarin	10.3	1,373.5	9.1	25.4	ND	57.4	6.1	6.3
Xylan	74.1	351.1	217.6	228.5	83.4	219.1	19.6	3.1

[a] Enzyme type is shown in parentheses.
[b] ND, not detected.
[c] NM, not measured.

TABLE 2. Polysaccharidase activities of extracellular preparations from *S. sclerotiorum* grown on a range of carbon sources and expressed as amounts of reducing sugar released per milliliter of culture

Carbohydrate growth substrate	Sugar released (μg/ml of culture)						Biomass produced (dry wt)
	Citrus pectin (pectinase)[a]	Apple pectin (pectinase)	Na$^+$ polygalacturonic acid (polygalacturonase)	Xylan (xylanase)	CMC (β-1,4-glucanase)	Laminarin (β-1,3-glucanase)	
Glucose	482.8	146.8	344.5	ND[b]	ND	412.2	15.2
β-Methylglucoside	1,293.5	313.2	551.7	ND	20.0	1,212.6	3.4
Xylose	588.8	339.2	652.8	ND	32.0	329.6	7.8
Sorbose	195.0	30.0	115.5	ND	ND	ND	0.5
Sophorose	876.3	165.8	262.1	ND	ND	405.0	0.7
Cellobiose	1,078.1	670.3	965.6	4.7	33.7	815.6	7.1
Avicel	441.2	251.5	204.7	ND	ND	573.6	NM[c]
CMC	864.0	1,016.5	1,136.2	271.1	90.4	333.2	1.0
HEC	873.6	324.5	297.2	137.3	126.7	936.0	0.5
Citrus pectin	1,920.0	1,289.3	2,803.2	520.3	120.0	1,329.6	1.9
Apple pectin	1,843.2	1,930.4	2,169.6	115.2	67.2	950.4	1.8
Na$^+$ polygalacturonic acid	1,886.1	1,665.2	2,688.0	406.6	129.9	1,253.6	2.8
Galactan	1,288.8	806.4	656.2	18.7	37.5	843.7	4.5
Arabinogalactan	581.2	548.4	550.4	168.9	102.4	748.8	5.2
Laminarin	1,307.0	1,520.6	1,350.1	ND	ND	720.0	6.3
Xylan	608.0	690.7	554.7	196.0	135.0	916.0	3.1

[a] Enzyme type is shown in parentheses.
[b] ND, not detected.
[c] NM, not measured.

polysaccharides and sugars which are known to regulate enzyme production in other fungal species. In the presence of pectinolytic substrates (pectins, Na$^+$ polygalacturonic acid, galactan, and arabinogalactan), large amounts of all of the enzymes tested were produced. In comparison with glucose medium, polygalacturonic acid medium showed the highest production of exoenzymes (56 times the β-galactosidase, 26 times the β-fucosidase, and 18 times the cellobiosidase). Maximal pectinolytic activities were also measured in these media. Pectinase activities against citrus pectin, apple pectin, and polygalacturonase were, respectively, 4, 18, and 8 times higher in these media than those activities in glucose medium. Xylanase and β-1,4-glucanase activities which were not detected in glucose medium were recovered from these media. Different levels of exoenzyme activities, i.e., of β-1,3-glucanase and pectinolytic enzymes, were present in media containing the cellulosic substrates crystalline cellulose (Avicel) and their soluble derivatives (HEC and CMC), but xylanase and endo-β-1,4-glucanase activities were present only in HEC and CMC media.

The increased secretion of extracellular proteins in the presence of pectinolytic and cellulolytic substrates was confirmed by the revelation of several heavily stained bands on SDS-polyacrylamide gels (Fig. 1). Among the hemicellulolytic polysaccharides used, laminarin was a very poor substrate for enzyme production; activities were very low and only few a proteins were detected by using SDS-PAGE. In contrast, in medium containing xylan, the other hemicellulolytic substrate, all of the enzyme activities were present and SDS-PAGE revealed numerous polypeptides.

The induction of cellulolytic enzymes by crystalline cellulose or soluble derivative molecules which cannot enter the cell is held to be mediated by low-molecular-weight cellulose degradation products or their transglycosylation products (20). Several potent inducers in other organisms, i.e., sophorose (26), L-sorbose (13), cellobiose (18), and β-methylglucoside (27), were used as the sole carbon source. Among these known cellulolytic enzyme inducers, only β-methylglucoside and cellobiose were efficient for the production of β-1,4-glucanase activities degrading CMC and β-D-lactopy-

ranoside and, surprisingly, of pectinases, polygalacturonases, and β-1,3-glucanases. In contrast, sophorose and sorbose were poor substrates because notable increases occurred in only a pectinase, β-glucosidase, and β-galactosidase in sophorose-grown cultures and in β-galactosidase in sorbose-grown cultures as compared with the increases in glucose-grown cultures. This lack of enzyme production by sophorose was confirmed by SDS-PAGE of extracellular proteins since only a few polypeptides were detected on gels.

When comparisons of enzyme production were made at the level of the specific activity (i.e., enzymatic activity per microgram of protein), maximal activities were generally obtained in cultures grown on the appropriate structure-related polysaccharides (Tables 3 and 4). The highest spe-
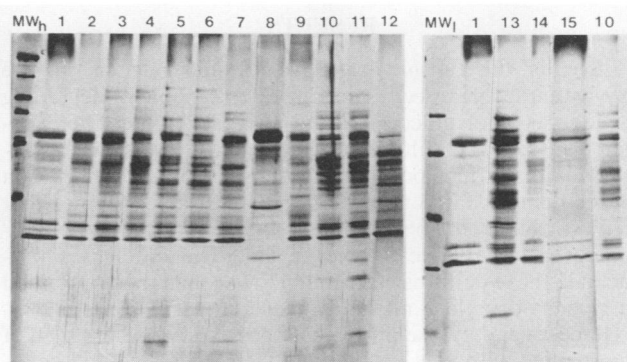

FIG. 1. SDS-PAGE of extracellular proteins secreted by *S. sclerotiorum* grown on a range of carbon sources. Equal volumes of culture medium were loaded. Lanes 1 to 15 correspond to the different media used: 1, glucose; 2, citrus pectin; 3, apple pectin; 4, Na$^+$ polygalacturonic acid; 5, galactan; 6, arabinogalactan; 7, sophorose; 8, laminarin; 9, methylglucoside; 10, Avicel; 11, HEC; 12, CMC; 13, xylan; 14, cellobiose; 15, sorbose. Molecular sizes of markers: for MW$_h$, 200, 116, 97, 66, and 45 kDa; for MW$_l$, 97, 66, 45, 31, and 21 kDa.

cific activities of β-glucosidase, β-1,4-glucanase, β-galactosidase, β-1,3-glucanase, and β-xylosidase were obtained, respectively, in HEC-, arabinogalactan-, laminarin-, and xylan-grown cultures. However, increases of enzyme activity unrelated to the carbon source used were also frequently observed. α-Arabinosidase, cellobiosidase, cellobiohydrolase, and a pectinase had the highest specific activities in sorbose-, polygalacturonic acid-, and xylan-grown cultures.

This survey showed that enzyme activities and protein secretion increased following growth on polysaccharides, but the possibility that multiple forms of each enzymatic activity were regulated differently had to be considered.

Effect of polysaccharide on the synthesis of multiple forms of hydrolases. Fungi produce isoenzymes in culture, and differences have been found between the isozymes produced by phytopathogenic fungi in cultures and those produced in infected tissues (19). The complexity of the exoenzyme system of *S. sclerotiorum* was shown by analytical IEF of secreted proteins and revelation of their enzymatic activity by using fluorogenic substrates (Fig. 2). β-Glucosidase, β-xylosidase, β-cellobiosidase, β-galactosidase, β-fucosidase, and α-arabinosidase activities were revealed at different pHs on the IEF gels. Several activities were detected at the same pH, possibly indicating that the enzymes are aspecific and hydrolyze a wide range of substrates or that different enzymes have similar pIs. However, specific bands characterized each enzyme activity.

A study was undertaken to investigate the pattern of enzymes produced during different culture conditions. Extracellular fluid from CMC- and citrus pectin-grown cultures were fractionated by gel filtration to compare the effects of these inducers. A 2-ml volume of concentrated culture medium was chromatographed on Ultrogel AcA 34 which allows the separation of proteins ranging in size from 20 to 350 kDa.

The β-galactosidase and β-glucosidase activities of each medium exhibited similar profiles. They eluted, respectively, as a single peak (molecular size, 110 kDa) and two peaks (molecular sizes, 120 and 70 kDa). The levels of these peaks correspond to the levels of total activity secreted in the presence of the polysaccharides (data not shown).

Differences were observed in the distribution of other enzymatic activities. Pectinase activity measured against apple pectin corresponded to a single peak which was recovered in different fractions (i.e., corresponding to different apparent molecular weights) according to the nature of the medium. Pectinase activity from pectin medium, measured against citrus pectin, was recovered in two peaks, while only one peak of intermediate molecular weight was found in CMC medium (Fig. 3). That these pectinolytic activities were collected in different fractions indicates that *S. sclerotiorum* is able to produce several isozymes differing in molecular weight and substrate specificity. Xylanase and β-1,3-glucanase activities were eluted as single peaks. However, enzymes from pectin-grown cultures had apparent molecular weights that were higher than those of the enzymes from CMC-grown cultures (Fig. 3). This may indicate that different enzymes were synthesized depending on the carbon source of the medium.

β-Xylosidase and cellobiosidase activities from CMC cultures were separated in two peaks, while only one peak of each enzyme was present in pectin cultures (Fig. 3). Thus, isozymes were specifically induced in the presence of the cellulosic substrate.

Cellobiohydrolase activity from each of the culture media was resolved in two peaks (Fig. 3). However, the activity of

TABLE 3. Glycosidase activities of extracellular preparations from *S. sclerotiorum* grown on a range of carbon sources and expressed as amounts of *p*-nitrophenyl released

Carbohydrate growth substrate	Derivative released (nmol)							Amt of protein (mg)
	β-D-Glucopyranoside (β-glucosidase)[a]	β-D-Galactopyranoside (β-galactosidase)	β-D-Fucopyranoside (β-fucosidase)	β-D-Xylopyranoside (β-xylosidase)	β-D-Lactopyranoside (cellobiohydrolase)	α-L-Arabinopyranoside (α-arabinosidase)	β-D-Cellobiopyranoside (β-cellobiosidase)	
Glucose	4.2	5.7	3.3	5.2	ND[b]	5.0	2.3	6.40
β-Methylglucoside	29.2	14.2	6.3	7.6	0.8	9.5	2.7	6.06
Xylose	4.5	6.7	3.4	5.0	ND	5.5	2.2	8.96
Sorbose	31.5	221.0	34.0	ND	ND	51.0	4.3	1.00
Sophorose	26.4	37.5	10.8	ND	ND	15.1	4.3	3.42
Cellobiose	3.8	4.4	1.0	ND	0.5	8.5	1.0	6.25
Avicel	18.3	35.0	18.3	ND	2.0	16.4	4.6	3.82
CMC	8.7	14.9	9.6	8.3	3.5	15.7	2.3	6.02
HEC	67.3	68.7	36.6	13.1	2.9	17.0	16.5	4.16
Citrus pectin	16.7	171.1	38.3	16.5	4.7	18.2	13.9	9.92
Apple pectin	8.7	97.8	24.8	4.7	1.1	9.6	7.1	12.80
Na+ polygalacturonic acid	16.0	160.0	43.2	11.5	4.7	18.5	21.0	12.80
Galactan	13.6	241.5	43.9	ND	0.6	21.6	13.6	4.70
Arabinogalactan	15.5	292.7	48.4	ND	ND	26.6	11.3	4.70
Laminarin	3.6	16.0	3.2	8.9	ND	20.2	2.1	2.84
Xylan	12.0	57.0	35.3	37.1	13.6	35.6	3.2	6.16

[a] Enzyme type is shown in parentheses.
[b] ND, not detected.

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TABLE 4. Polysaccharidase activities of extracellular preparations from *S. sclerotiorum* grown on a range of carbon sources and expressed as amounts of reducing sugar released

Carbohydrate growth substrate	Sugar released (μg)						Amt of protein (mg)
	Citrus pectin (pectinase)[a]	Apple pectin (pectinase)	Na⁺ polygalacturonic acid (polygalacturonase)	Xylan (xylanase)	CMC (β-1,4-glucanase)	Laminarin (β-1,3-glucanase)	
Glucose	75.4	23.0	53.9	ND[b]	ND	64.4	6.40
β-Methylglucoside	213.5	51.7	91.1	ND	3.4	200.1	6.06
Xylose	65.7	37.9	72.9	ND	3.6	36.8	8.96
Sorbose	195.0	30.0	115.5	ND	ND	ND	1.00
Sophorose	266.3	48.5	76.7	ND	ND	118.4	3.42
Cellobiose	172.5	107.2	154.5	0.8	5.4	130.5	6.25
Avicel	115.5	65.8	53.6	ND	ND	150.0	3.82
CMC	143.5	168.9	188.7	45.0	15.0	55.4	6.02
HEC	210.0	78.0	71.5	33.0	30.5	225.0	4.16
Citrus pectin	193.5	130.0	282.6	52.4	12.1	134.0	9.92
Apple pectin	144.0	150.8	169.5	9.0	5.3	74.3	12.80
Na⁺ polygalacturonic acid	147.4	130.1	210.0	31.8	10.2	98.0	12.80
Galactan	274.2	171.6	139.6	4.0	8.0	179.5	4.70
Arabinogalactan	123.7	116.7	117.1	36.0	21.8	159.3	4.70
Laminarin	460.0	535.4	475.4	ND	ND	253.5	2.84
Xylan	98.7	112.1	90.1	318.0	21.9	151.3	6.16

[a] Enzyme type is shown in parentheses.
[b] ND, not detected.

the first peak, compared with that of the second, was dependent on the carbon source of the culture and was stimulated in the presence of CMC.

DISCUSSION

Previous studies on *S. sclerotiorum* have demonstrated its potential to degrade the principal plant structure polysaccharides (1, 2, 9, 10, 16, 17). The data presented here confirmed that this pathogenic fungus has a very wide range of polysaccharidase and glycosidase activities. This fungus possesses the glycoside hydrolase activities that complement the polysaccharidase enzymes which are also formed, conferring an enzymatic potential to release monosaccharides from each plant cell wall polymer. Glycosidases may also remove
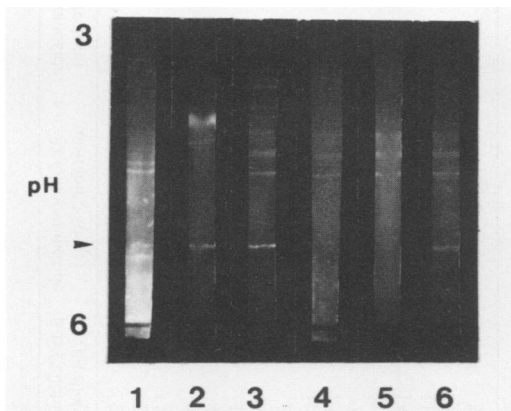

FIG. 2. Glycosidase activities of extracellular proteins secreted by *S. sclerotiorum* grown in citrus pectin medium. Proteins (10 μg) were separated by analytical IEF-PAGE in a pH range of 3.0 to 6.0. β-Galactosidase (lane 1), β-glucosidase (lane 2), β-cellobiosidase (lane 3), β-fucosidase (lane 4), α-arabinosidase (lane 5), and β-xylosidase (lane 6) activities were revealed by hydrolysis of the related methylumbelliferyl glycoside viewed under UV illumination (365 nm).

side groups of heteropolysaccharides, facilitating the action of endoenzymes.

Isozymes of various hydrolytic activities secreted by *S. sclerotiorum* were revealed by electrophoretic and chromatographic analyses. Several proteins migrating at different pHs or differing in their molecular weights exhibited the same enzyme activity. Multiplicity of enzyme forms is widespread in fungi, and it has been suggested that host specificity of pathogens might be associated with specific isoenzymes (25). However, the origin of this multiplicity is still controversial (6). Postsecretional modifications are implicated in the existence of different molecular forms of enzymes in *Trichoderma* and *Fusarium* spp. Proteolysis in late culture stages contribute to the multiplicity of cellulases found in *Trichoderma reesei* culture fluids (8). The release of sugar chains from the glycosylated α-fucosidase by an endo-β-N-acetylglucosaminidase leads to a deglycosylated enzyme found in the culture broth of *Fusarium oxysporum* (28).

Our results showed that, in *S. sclerotiorum*, aspecificity of the enzymes could be implicated in the apparent exoenzyme multiplicity revealed by analytical IEF. Different activities were exhibited at the same pH, indicating the nonspecific action of an enzyme on several substrates. For example, parts of β-xylosidase, β-cellobiosidase, β-fucosidase, and α-arabinosidase activities were associated with identical proteins which could represent multifunctional glycosidases. In other fungi, it has been demonstrated that a single protein is responsible for β-glucosidase, β-fucosidase, and β-galactosidase activities (12, 23). Aspecificity of glycoside hydrolases has also been observed in other fungi; β-xylosidases from *T. reesei* (24) and from *Neurospora crassa* (5) exhibit α-arabinosidase and β-glucosidase activities, respectively. The substrate cross-specificity of the enzymes could increase adaptability of the phytopathogen, but evidence for the separate identities and roles of the different enzymatic forms necessitates the study of purified enzymes.

The mechanism controlling expression of cell wall-degrading enzymes in fungi is not well understood. In culture, the production of hydrolytic enzymes by many fungi requires
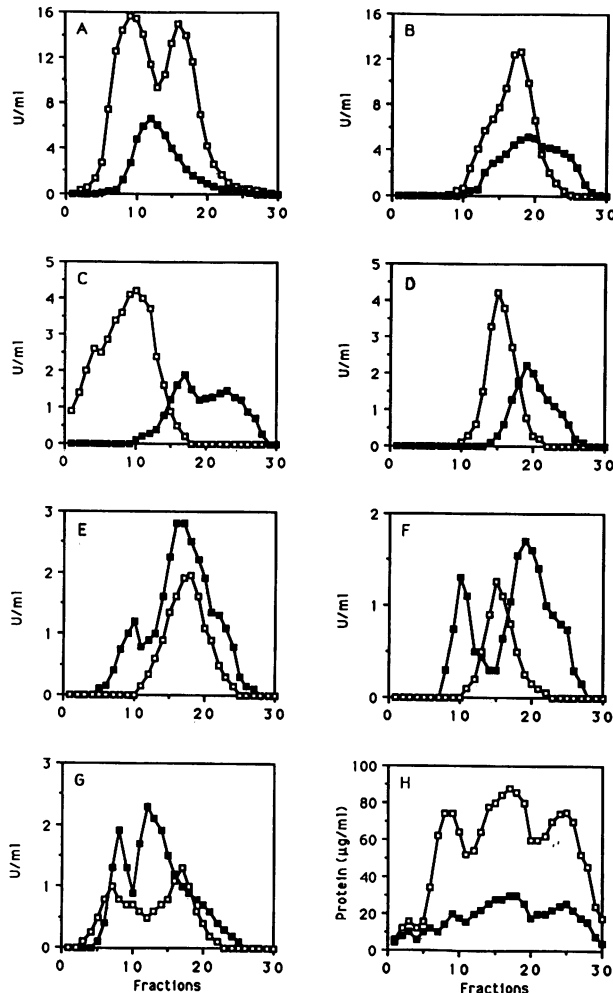
FIG. 3. Gel filtration on an Ultrogel AcA 34 matrix of the extracellular enzymes secreted by *S. sclerotiorum* grown in citrus pectin (□) and CMC (■) media. Supernatant (2 ml) was loaded onto a column (1.8 by 90 cm) and eluted with Na⁺ acetate buffer, pH 6. Fractions (2.3 ml) were collected and assayed for the following enzyme activities: A, pectinase activity against citrus pectin; B, pectinase activity against apple pectin; C, β-1,3-glucanase; D, xylanase; E, β-xylosidase; F, cellobiosidase; G, cellobiohydrolase; H, proteins.

acterized by chromatographic analysis, were recovered following growth on media containing CMC and pectin. Supplementary peaks of pectinase in pectin medium and of β-xylosidase and cellobiosidase in CMC medium were detected. This may indicate that different forms of these enzymes are regulated by different controls. Similar observations have been made for the production of polygalacturonase isoenzymes by *S. sclerotiorum* in culture and in infected tissues. One peak of activity was found in pectin or polygalacturonic medium, while two peaks were obtained from infected tissues (19). In *T. reesei*, endoglucanase activity is separated in five forms. Two of the forms are controlled by induction, while two others are regulated by carbon catabolite repression (20). Different controls of isoenzyme production seem to exist in different fungi, but the biological significance of this is unknown and must await the purification of the isozymes and the determination of their properties.

The apparent molecular weights of several enzymes such as β-1,3-glucanase and xylanase, estimated by gel filtration, were different in CMC and pectin media. Differences have also been observed for the polygalacturonase produced by *S. sclerotiorum* in pectin and polygalacturonic media (19). In *T. reesei*, the most active endoglucanase (M_r, 43,000) is modified following release in the culture medium, yielding another enzymatic form (M_r, 56,000 to 62,000) (22). These modifications are not understood, but the realization of multienzymatic complexes as they exist in *Neocallimastix frontalis* (30) could explain the important increase in molecular weight observed depending upon the culture medium used. Heterogeneous glycosylation has also been mentioned as an explanation of the existence of these multiple forms (6, 29).

In the present work we have shown that *S. sclerotiorum* produces polysaccharide depolymerases and glucosidases necessary to degrade the important structural cell wall polysaccharides, i.e., cellulose, pectin, and hemicellulose. The secretion of this wide range of enzymes provides this pathogenic fungus with the ability to attack hosts which differ in their polysaccharide cell wall compositions and could explain the lack of host specificity of this fungus. Because of the variety of enzymes produced, this fungus may also offer commercial potential. Optimization studies will serve to increase the understanding of factors that control the production, activity, and consequently the role of the enzymes.

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substrate and is repressed by preferred carbon sources such as glucose (4, 14).

The induction and repression of the hydrolytic enzymes were studied by growing *S. sclerotiorum* on a variety of carbon sources. The enzyme activities, except those of pectinases and β-1,3-glucanase, were low or absent in glucose-grown cultures. The activities increased considerably when various polysaccharides were used as the carbon source. Pectinolytic enzymes seem to be produced constitutively, while β-1,4-glucanases are induced by polysaccharides. Cellulolytic or pectinolytic substrates were able to induce cellulolytic and pectinolytic enzymes, indicating an aspecificity of the induction or a common regulatory system. This was also illustrated by the high pectinolytic activities of cultures obtained on cellobiose or methylglucoside, inducers of cellulolytic enzymes.

Different molecular forms of the enzyme activities, char-

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