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Purification and Characterization of Extracellular Pectinolytic Enzymes Produced by *Sclerotinia sclerotiorum*

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An exopolygalacturonase (exoPG) and an exopolymethylgalacturonase (exoPMG) produced by *Sclerotinia sclerotiorum* have been purified by ammonium sulfate precipitation, gel filtration, and ion exchange chromatography. The exoPG and the exoPMG were purified 66- and 50-fold, respectively, by using a series of separation procedures that included ammonium sulfate precipitation and gel chromatography. Molecular masses of the native proteins were 68 kDa for exoPG and 140 kDa for exoPMG. The pH optima of the enzymes were about pH 5, and their optimum temperature was 45°C. Activities of both enzymes were inhibited by Hg^{2+}, Zn^{2+}, Cu^{2+}, and *p*-chloromercuribenzoate. ExoPMG activity, in contrast to exoPG activity, was stimulated by Mn^{2+} and Co^{2+}. ExoPMG hydrolyzed only citrus pectin, while exoPG degraded sodium polygalacturonate and, to a lesser extent, citrus pectin. The exo mode of action of the enzymes was revealed by thin-layer chromatography of substrate hydrolysates. Antibodies raised against each purified protein exhibited no cross-reaction, thus confirming the biochemical specificities of the enzymes.

Decomposition of plant cell walls during tissue invasion and pathogenesis is a characteristic feature of numerous plant diseases caused by phytopathogenic fungi (9, 18). These fungi are able to produce a large array of cell wall-degrading enzymes, both in culture and in diseased plant tissues. They may constitute ideal organisms to characterize and to produce enzymes which could be involved in industrial bioconversions. Among the phytopathogenic fungi, *Sclerotinia sclerotiorum* is a ubiquitous fungus that is able to infect a wide range of plants and that secretes a complete set of enzymes to macerate healthy plant tissues (16, 22, 31). Although endo-β-1,4-galactanase, α-L-arabinofuranosidase, and endopolygalacturonases (endoPGs) from this fungus have been investigated previously (2, 6, 13, 18, 24), extracellular pectinolytic enzymes have not yet been characterized. Studies of microorganisms able to grow on a cell wall polymer raise the question of how these microbes can recognize the substrate in their environment even though large molecules cannot penetrate the cell wall and membrane. It has been suggested that they secrete a low basal level of hydrolytic enzymes which may release low-molecular-weight products that serve as the inducers of the synthesis of more enzymes. Cellobiose has been identified as the most potent inducer of cellulases (14, 23). Galacturonic acid has been claimed to be the inducer of pectic enzyme activity of *Fusarium* species and *Botrytis* species (1, 10, 21). However, the mechanisms by which an extracellular polymer can induce its own catabolism are still poorly understood. Since exoenzymes are candidates for the enzymes able to release simple sugars from polymers, we have focused our attention on the properties of extracellular pectinolytic enzymes secreted by *S. sclerotiorum*.

Here we report the purification and characterization of an exopolygalacturonase (exoPG) and of an exopolymethylgalacturonase (exoPMG) produced in vitro by the phytopathogenic fungus *S. sclerotiorum*.

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MATERIALS AND METHODS

Organism and culture conditions. *S. sclerotiorum* (Lib.) de Bary was provided by R. Pépin (Rhône-Poulenc Agrochimie, Lyon, France). The isolate was maintained on potato dextrose agar. Enzymes were produced in Erlenmeyer flasks containing 500 ml of liquid minimal medium supplemented with 0.5% citrus pectin as the carbon source, as previously described (21). The cultures were inoculated with mycelial disks cut from the margins of 4-day-old colonies and incubated for 6 days at 24°C under gentle agitation. The mycelium was harvested by filtration through bolting cloth, and the culture filtrates were pooled and dialyzed against distilled water for 24 h at 4°C. This preparation was freeze-dried, and the powder was used for enzyme purification.

Enzyme purification. All procedures were done at 4°C.

(i) Ammonium sulfate precipitation. The powder from freeze-dried filtrate was solubilized in 0.1 M sodium acetate buffer (pH 5). The preparation was brought to 20% saturation with ammonium sulfate and centrifuged at 8,000 × *g* for 30 min. The supernatant was brought to 80% saturation with ammonium sulfate. The resulting precipitate was collected by centrifugation (8,000 × *g* for 30 min), dissolved in the smallest possible volume of sodium acetate buffer, and dialyzed first against distilled water and then against sodium acetate buffer.

(ii) Ultrogel AcA 34 (Industrie Biologique Française [IBF]) chromatography. The dialyzed material (2 ml) was applied to an Ultrogel AcA 34 column (2 by 95 cm) equilibrated with 0.01 M sodium acetate buffer (pH 6). Elution was performed with sodium acetate buffer at a flow rate of 20 ml/h, and 2.5-ml fractions were collected. The fractions containing the enzyme activities were pooled and concentrated by centrifugation by using CF 25 Centriflo ultrafiltration membrane cones (Amicon Corp.).

(iii) CM Bio-Gel A (Bio-Rad Laboratories) chromatography. Concentrated exoPMG fractions were applied to a CM Bio-Gel A column (1.5 by 50 cm) equilibrated with 0.01 M sodium acetate buffer (pH 5) containing 0.05 M NaCl. The column was washed with the equilibrating buffer, and the

retained proteins were eluted with a linear NaCl gradient (0 to 0.3 M) in the same buffer at a flow rate of 15 ml/h. Active fractions (1.25 ml) were pooled and concentrated as described above.

(iv) DEAE Bio-Gel A (Bio-Rad Laboratories) chromatography. Concentrated fractions of exoPG were applied to a DEAE Bio-Gel A column (2.5 by 30 cm) equilibrated with 0.01 M sodium acetate buffer (pH 7). The column was washed with the same buffer and then eluted with a linear gradient of NaCl (0 to 0.3 M). Fractions (2.5 ml) were collected at a flow rate of 30 ml/h.

(v) Ultrogel AcA 44 (IBF) chromatography. The concentrated active fractions were chromatographed on an Ultrogel AcA 44 column (2 by 60 cm) equilibrated with 0.01 M sodium acetate buffer (pH 6). Elution was carried out with the equilibrating buffer containing 0.05 M NaCl at a flow rate of 10 ml/h. The active fractions (1.25 ml) with high specific activities were pooled and used as the final preparation of the enzymes. The native M_r of the purified enzyme was estimated by gel filtration through a column equilibrated with blue dextran and standard M_r marker proteins (Pharmacia).

Analytical electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done with slab gels (10 by 15 cm) composed of 4% (wt/vol) stacking gels and 10% (wt/vol) resolving gels by the method of Laemmli (20). Molecular weight markers (Sigma or Bio-Rad) were used to determine the subunit M_r of the enzyme.

Analytical isoelectric focusing-PAGE was performed with Servalyt Precotes (Serva) containing 5% ampholytes (pH range, 3.0 to 10.0; Serva). The gels were prefocused at up to 500 V before samples were applied. The samples were focused at a constant power for 2 h at a final voltage of up to 1,700 V. A part of the slab gel was stained with Coomassie brilliant blue R-250 for protein determination, and the other part was used to detect the enzyme activities.

Protein and enzyme assays. Pectinolytic activities were determined, as previously described (31), by measuring the production of reducing sugars by the dinitrosalicylic acid procedure (27). The reaction mixtures contained 0.95 ml of 2-mg/ml polygalacturonic acid Na (Serva) or citrus pectin (Fluka) in 0.01 M sodium acetate buffer (pH 5) and 50 μmol of enzyme. Incubations were performed at 45°C for 10 min. Activity is expressed as micromoles of reducing sugar equivalents released. Protein concentrations were determined by the method of Bradford (7), with bovine serum albumin used as the standard.

Enzyme characterization. Estimates of optimal temperature and pH as well as of thermal and pH stability were made by using a temperature range of 20 to 80°C and a pH range of 2.0 to 8.0. The K_m values were determined from Lineweaver-Burk plots by using substrate concentrations of 0.05 to 4 mg ml^{-1}. The effects of various metallic ions and reagents on enzyme activities were determined at a concentration of 2 mM. Enzyme activity assayed in the absence of an additive was considered to be 100%. The ability of the purified enzymes to hydrolyze various substrates was assayed by using different *p*-nitrophenyl (pNP)-glycosides and polysaccharides at concentrations of 5 mM and 2 mg ml^{-1}, respectively, and by using the conditions described above.

Analysis of reaction products. The purified enzymes were incubated with 2% polygalacturonic acid Na (Serva) and citrus pectin or apple pectin (Fluka) for different periods. After heat inactivation, samples (20 μl) were analyzed by thin-layer chromatography (TLC). The plates were developed for 4 h with ethyl acetate-acetic acid-H_2O (40:20:40), dried, and sprayed with 2.5% (wt/vol) aniline phosphate in acetone.

Amino acid sequencing. The purified enzyme samples were run on an SDS-polyacrylamide gel, as described above. The polypeptides from the polyacrylamide gel were electrophoretically transferred to an Immobilon PVDF transfer membrane (26). The membrane was stained with Coomassie brilliant blue R-250, and, after destaining, the polypeptide bands were cut from the membrane and used for amino acid sequencing by automated micro-Edman degradation with an Applied Biosystems model 470-A vapor-phase sequencer (Centre de Microanalyse du Centre National de la Recherche Scientifique, Solaize, France).

Immunological methods. Antisera to enzymes were prepared by immunizing rabbits with intradermal injections of 50 μg of purified enzymes dissolved in 1 ml of Freund's complete adjuvant. The rabbits were injected three times at 20-day intervals. For immunoblotting (Western blotting), 2 μg of extracellular proteins and purified enzymes was separated on SDS-polyacrylamide gels and then electroblotted to nitrocellulose, as described by Towbin et al. (35). Remaining binding sites of nitrocellulose were blocked for 2 h with 5% nonfat dry milk in Tris-buffered saline (TBS). After three washes in TBS, protein blots were incubated for 2 h in TBS with appropriate antisera (1:1,000 or 1:2,000 dilution) and washed three times in TBS. Horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:1,000 dilution) was used as a second antibody, and color was developed in TBS with 0.5 mg of 1-chloro-1-naphthol per ml and 0.01% H_2O_2.

RESULTS

Enzyme purification. Purification of an exoPG and an exoPMG was achieved in a series of three separation steps: (i) gel filtration, (ii) cation or anion exchange chromatography, and (iii) gel filtration. Gel filtration of the crude exocellular preparation gave two peaks of pectinase activity. The second peak was associated with polygalacturonase activity. In the second step, exoPMG was not retained on anion exchange column. A main peak of polygalacturonase activity was retained on a cation exchange column and collected at 0.11 M NaCl during elution with an NaCl gradient. A lower peak of polygalacturonase and pectinase was not retained on this type of column but was not further studied. In the third step, gel filtration of the pooled active fractions yielded a single peak of each enzyme activity that coincided with the maximal protein concentration. The purification data are summarized in Table 1. The specific activities of purified exoPG and exoPMG represented purities 66- and 50-fold greater than that of the crude extract, respectively.

Determination of molecular weight. The molecular mass of each enzyme was estimated to be 70 kDa for the exoPG and 140 kDa for the exoPMG by the Ultrogel AcA 34 gel filtration column. After SDS-PAGE and silver staining of the purified materials, a single protein band in each preparation was observed. Molecular masses assessed by SDS-PAGE were 68,000 Da for the exoPG and 74,000 Da for the exoPMG (Fig. 1). This indicates that the exoPG is likely to be a single polypeptide protein, while the native exoPMG is composed of two identical subunits. The M_rs of these exoenzymes are higher than those of the endoPGs which are secreted by this fungus and which are in the range of 28,000 to 43,000 (18, 24).

In analytical gel isoelectric focusing, exopectinase and exoPG had pI values of pH 4.1 and 4.8, respectively.

TABLE 1. Purification of exoPMG and exoPG from *S. sclerotiorum*

Purification step	Total activity[a]	Sp act[b]	Yield (%)	Purification (fold)
exoPMG				
Culture filtrate	3,568	5.1	100	1
Ammonium sulfate precipitation	3,371	9.5	95	1.9
Ultrogel AcA 34	3,058	80.5	85	15.8
CGMC Bio-Gel A	2,602	153.0	73	30.0
Ultrogel AcA 44	1,684	259.1	47	50.8
exoPG				
Culture filtrate	8,472	12.1	100	1
Ammonium sulfate precipitation	7,744	22.1	91	1.8
Ultrogel AcA 34	6,783	357.0	81	29.5
CGMC Bio-Gel A	5,213	473.9	61	39.0
Ultrogel AcA 44	3,212	803.2	38	66.4

[a] Expressed as micromoles of reducing sugar released during a 10-min incubation.
[b] Expressed as micromoles of reducing sugar formed per minute per milligram of protein.

EndoPGs secreted by this fungus also have acidic pI values in the range of pH 4.8 to 5.1 (13, 24, 32).

Enzyme characteristics. The optimum temperature for activity (measured after a 10-min incubation) of these two purified enzymes was 45°C. The exoPG was mostly inactive at 65°C, while at that temperature the exoPMG exhibited 40% of its maximal activity. The exoPMG showed a pH optimum at pH 5.0, and 50% of the maximal activity was found at pH 7.5. ExoPG had a narrow pH activity curve; the optimal pH was 4.5. Fifty percent of the maximal activity was found at about pH 5.5, and the enzyme was inactive at pH 7.

The thermostabilities were investigated by measuring the residual activities of the enzymes after 4 h of incubation at temperatures ranging from 20 to 80°C. The exoPG was stable below 30°C. Treatment at 45°C resulted in a loss of 50%, and complete inactivation was obtained at 60°C. The exoPMG was more heat stable, being fully active at 45°C and exhibiting 50% activity after treatment at 60°C. Complete inactivation was observed when the enzyme was treated at 80°C.

The affinities of the purified enzymes for the two substrates, citrus pectin and polygalacturonic acid Na, were examined with a Lineweaver-Burk plot. The K_ms of exoPMG against citrus pectin and of exoPG against Na polygalacturonate were 0.8 and 0.83 mg of substrate per ml, respectively.

The effects of various reagents on both enzymatic activities were investigated (Table 2). Significant inactivation of both enzymes was observed with Hg^{2+}, *p*-chloromercuribenzoate (pCMB), Cu^{2+}, and Zn^{2+}. Inhibition of activity by thiol-group-blocking agents such as pCMB and $HgCl_2$ suggests the possible involvement of thiol groups in the active site of the enzyme. ExoPG was also inhibited by Ca^{2+}, Mg^{2+}, and SDS, none of which modified exoPMG activity. On the other hand, this activity was stimulated by Co^{2+} and Mn^{2+} (Table 2).

Substrate specificity. The actions of the purified enzymes were tested with a large number of substrates; they had no detectable glycosidase activity against the pNP-linked sugars pNP-β-galactoside, pNP-β-fucoside, pNP-β-glucoside, pNP-β-xyloside, pBP-β-cellobioside, pNP-β-lactoside, and

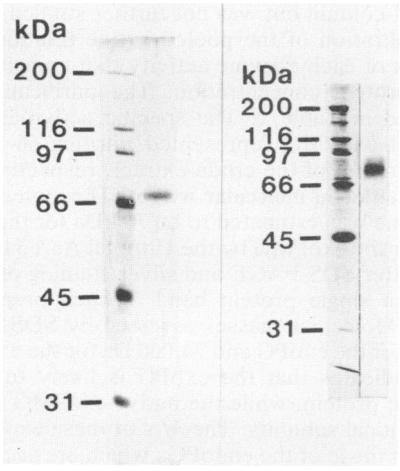

FIG. 1. SDS-PAGE of purified extracellular pectinolytic enzymes from *S. sclerotiorum*. Left panel, exoPG (2 μg); right panel, exoPMG (2 μg). Molecular mass markers are in the left lane of each panel.

TABLE 2. Effects of cations and reagents on extracellular pectinolytic activities from *S. sclerotiorum*

Cation or reagent	Activity (%)[a]	
	exoPMG	exoPG
—[b]	100	100
KCl	101	86
LiCl	101	92
NaCl	105	99
$HgCl_2$	3	1
$MgCl_2$	102	79
$CaCl_2$	112	54
$ZnCl_2$	69	48
$MnCl_2$	242	125
$CuCl_2$	57	54
$CoCl_2$	156	98
$FeCl_3$	103	86
EDTA	96	87
SDS	94	15
pCMB	8	52

[a] Activities of the purified enzymes were assayed in the presence of various cations and reagents used at 2 mM. Activity assayed in the absence of added substances (control) was considered to be 100%.
[b] —, Control.

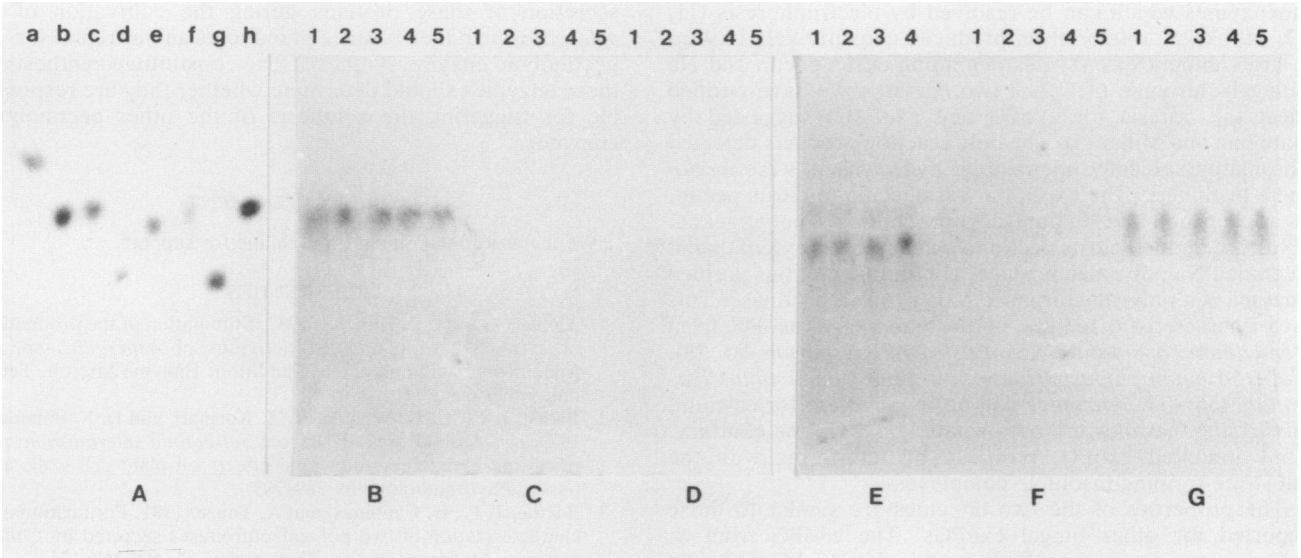

FIG. 2. Patterns of action of purified extracellular pectinolytic enzymes on pectic polymers. TLC of products released when purified exoPMG (B, C, and D) and exoPG (E, F, and G) were incubated for 10, 20, 30, 60, and 120 min (lanes 1 to 5) with citrus pectin (B and G), apple pectin (C and F), or polygalacturonate (D and E) is shown. (A) TLC of sugar standards. Lanes: a, rhamnose; b, glucose; c, arabinose; d, digalactose; e, galactose; f, galacturonic acid; g, methyldigalactose; h, methylgalactose.

pNP-α-arabinoside. Among the polymers tested, only citrus pectin was hydrolyzed by the exoPMG. ExoPG had maximal activity on Na polygalacturonate and showed 40% activity on citrus pectin. Neither enzyme attacked the highly esterified pectin from apple or was active against carboxymethylcellulose, xylan, or laminarin.

The exo mode of action of these enzymes was confirmed when reaction products resulting from the incubation of purified enzymes with polygalacturonic acid Na, citrus pectin, or apple pectin were analyzed by TLC (Fig. 2). A single reaction product was released from the beginning of the incubation of exoPMG with citrus pectin. ExoPG released two low-molecular-weight products when incubated with polygalacturonate or citrus pectin. These products accumulated during the course of incubation, but no high-molecular-weight products were detected.

N-terminal sequences. The N-terminal amino acid sequences of the two enzymes blotted from the SDS-polyacrylamide gel were different, being Ser-Val-Asp-Ser-Phe-Ile-Ala-X-Glu-Pro-Ile-Ala for the exoPG and Ala-Leu-Leu-Gly-Thr-Ser-Phe-Gly-Val-Pro-Gly for the exoPMG. This latter sequence showed some homologies with the N-terminal sequence, Ala-Thr-Thr-Gly-Thr-Phe-Ser-Gly-Ser-Ser-Gly-Ala, determined for an endoPG secreted by *S. sclerotiorum* (18), but comparison with other sequences in the National Biomedical Research Foundation protein sequence data base revealed no significant homologies with previously reported proteins.

Immunological specificity. Specific antibodies against purified exoPMG and exoPG were raised in rabbits. They did not show cross-reaction when tested against the purified enzymes (Fig. 3). Detection of 1 ng of purified proteins was obtained with 1:1,000 dilutions of both antisera. To determine the immunological specificities of the antisera, the abilities of the antibodies to cross-react with proteins from the crude culture filtrate were examined by SDS-PAGE and immunoblotting of 2 μg of extracellular proteins. The anti-exoPMG detected only one band of about 74 kDa, corresponding to the molecular mass of the purified enzyme subunits. The anti-exoPG detected one band of 68 kDa, corresponding to the molecular mass of the enzyme, along with faint traces of a band at about 60 kDa. This latter polypeptide showing weak reactivity may correspond to a protein with pectinolytic activity or to an unrelated extracellular protein with which it has epitopes in common.

DISCUSSION

Exocellular pectic enzymes from *S. sclerotiorum* consist of a complex mixture of pectinase and polygalacturonase

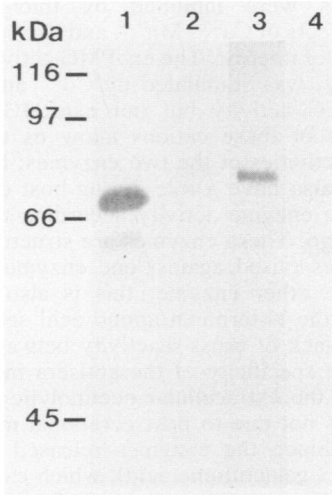

FIG. 3. SDS-PAGE Western blot of extracellular proteins and purified extracellular pectinolytic enzymes from *S. sclerotiorum*. Two micrograms of extracellular proteins (lane 1) and exoPMG (lane 2) was probed with the antiserum (1:1,000) against purified exoPG. Two micrograms of extracellular proteins (lane 3) and exoPG (lane 4) was probed with the antiserum (1:2,000) against purified exoPMG.

isoenzymes which can be resolved by electrophoresis (11, 13, 24, 32). *S. sclerotiorum* produced constitutively in vitro a large amount of enzymes degrading citrus pectin and Na polygalacturonate (31). The two enzymes we have purified meet the criteria for exoPG and exoPMG suggested by Bateman and Millar (5). The only reaction products detected chromatographically in substrate hydrolysates were monomers or dimers. The exoPMG was specific to citrus pectin, as apple pectin and Na polygalacturonate were not attacked. ExoPG degraded citrus pectin to a much lower extent than it degraded Na polygalacturonate, confirming that this purified enzyme is a polygalacturonase rather than a pectinase. This is a characteristic feature of the endo- and exoPG from *Penicillium capsulatum* (15), *Fusarium oxysporum* (25, 28), *Colletotrichum gloeosporioides* (30), and *Cochliobolus carbonum* (36). The enzymes had no requirement for calcium, confirming that they are not pectate lyase. On the contrary, Ca^{2+} inhibited exoPG, probably by interfering with the substrate-forming insoluble complexes.

The properties of the two enzymes are similar to those reported for other fungal exoPGs. The exoPG from *S. sclerotiorum* is a single-subunit protein with an M_r of 68,000, close to those reported for *F. oxysporum* (25, 28), *Colletotrichum lindemuthianum* (3), and *C. gloeosporioides* (30). They are larger than most endoPGs, which exhibit M_r values ranging from 32,000 to 56,000 (15, 18, 19, 28, 36).

EndoPGs have been exhaustively studied because of their random attack of the substrate and their possible involvement in tissue maceration (for reviews, see references 4, 5, 8, 9, and 17). Exoenzymes have received less attention; recently, however, exoPMG together with other extracellular pectinolytic enzymes of phytopathogenic fungi has been described (12, 19, 25, 28, 29). Contradictory evidence exists concerning the role of exoPG in pathogenicity, but in some cases the presence of this type of enzyme alone or in the presence of pectin methylesterases is enough to macerate tissues (28, 34). The exoenzymes described here exhibit a very high level of activity on pectin and polygalacturonate. Various metallic ions and reagents modify the activities of these two exoenzymes. Sulfhydryl groups are probably involved in the catalytic active centers of the enzymes, since their activities were inhibited by thiol-group-blocking agents. The effects of Ca^{2+}, Mn^{2+}, and Co^{2+} on the enzyme activities were of interest. The exoPMG activity, but not the exoPG activity, was stimulated by Co^{2+} and Mn^{2+}. Ca^{2+} inhibited exoPG activity but not exoPMG activity. The inverse effects of these cations allow us to discriminate between the activities of the two enzymes; however, these cations could also have a role during host colonization by modulating the enzyme activity according to the environmental condition. These enzymes are structurally different, since antibodies raised against one enzyme do not cross-react with the other enzyme; this is also illustrated by differences in the N-terminal amino acid sequences of the proteins. The lack of cross-reactivity between the two enzymes and the specificity of the antisera indicate that the multiplicity of the extracellular pectinolytic enzymes in *S. sclerotiorum* is not due to postsecretional modifications of the proteins. Since the enzymes released dimers and/or monomers (i.e., galactutonic acid), which can act as inducers of pectinolytic enzyme synthesis in *S. sclerotiorum* (13a) and in other fungal systems (1, 33), the exoPG and exoPMG we have characterized may be involved in the induction of the other pectinolytic enzymes secreted by *S. sclerotiorum*. The availability of specific polyclonal antibodies against these enzymes will permit us to follow the time course secretion of these proteins during the cultivation of *S. sclerotiorum* in the presence of inducers and/or repressors of pectinolytic enzyme synthesis. The constitutive synthesis of these enzymes should determine whether they are responsible for triggering the synthesis of the other pectinolytic enzymes.

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