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Purification and Characterization of an Exo-polygalacturonase from *Botrytis cinerea*

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***Botrytis cinerea* T91-1 has been shown to produce at least four different polygalacturonases into a liquid medium containing citrus pectin, a carbon source. One of the enzymes, which had an apparent molecular weight of 66 kDa estimated by denatured polyacrylamide gel electrophoresis, was purified to electrophoretic homogeneity by a series of procedures including acetone precipitation, ion exchange, heparin affinity, and reverse phase column chromatographies. The molecular weight of native enzyme was determined to be 64 kDa by gel permeation chromatography indicating the enzyme to be a single polypeptide chain. By viscometric analysis, the enzyme was revealed as exo-polygalacturonase. The enzyme activity was inhibited by divalent cations such as Ca^{2+}, Mg^{2+}, and Cu^{2+}. Km and Vmax for polygalacturonic acid hydrolysis were 0.33 mg/ml and 28.6 nM/min, respectively. The optimum temperature for enzymatic activity was 50°C. And the enzyme showed optimal pH values between 4.0 and 5.0. The enzyme was stable upto 12 hours in the range of pH 3 to 8 and at temperature below 30°C.**

Key words: *Botrytis cinerea*, Enzyme production, exo-polygalacturonase

Botrytis cinerea, a plant pathogen, is a necrotrophic fungus that causes grey mold in many crop plants, the most important post-harvest disease of soft fruits and vegetables (3, 19). This pathogen produces a number of cell wall-degrading enzymes such as pectin methylesterase (EC 3.1.1.11), pectate lyase (EC 4.2.2.2), and polygalacturonases (PGs) (EC. 3.2.1.15) (23, 26).

The PG which hydrolyzes polygalacturonide components of the plant cell wall is the first enzyme secreted by various plant pathogens including *B. cinerea* when cultured on isolated cell walls (8, 11, 12 22). Degradation of plant cell wall by PGs facilitates the attack of other cell wall-degrading enzymes on their substrates. Therefore, the role of PGs has been proposed to be critical in the initial infection stage as well as extensive degradation of plant cell wall leading to the marceration of host tissue. There are two classes of PGs according to their mode of action on the polygalacturonic acid chain; namely, endo-PG which cleaves the substrate at random, and exo-PG which hydrolyze the substrate at the reducing ends (11). In most studies on the plant-pathogen interaction, an endo-PG produced by the pathogen is widely viewed as the predominant form of PGs involved in pathogenesis (10, 25, 27). Although endo-PGs are generally considered to be more significant in the tissue marceration and pathogenesis than exo-PG in plants, there are many controversies in the role of PGs by *B. cinerea* in infected tissues (7, 15). Recently, Tobias *et al* (24) showed that an exo-PG was produced in an apple tissue decayed by *B. cinerea*, which indicates the possibly of a crucial role of exo-PGs in pathogenesis. However, only a few data are available on the properties and the working mechanism of exo-PG from *B. cinerea*. In this study, exo-PG was purified from *B. cinerea* T91-1, isolated from a lesion of a tomato leaf. Then its chemical and physical properties as well as kinetic parameters were investigated.

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Materials and Methods

Fungal isolates and culture condition

Botrytis cinerea T91-1 obtained from a lesion of a tomato leaf was maintained on potato dextrose agar (PDA, Difco Co.) at 20°C. To produce PGs in liquid culture, 10 mycelial plugs (0.5 cm, in diameter) of *B. cinerea* grown on PDA were inoculated in 1 L of medium containing 0.5% citrus pectin (Sigma Co.), 1% $NaNO_3$, 0.5% KH_2PO_4, 0.25% $MgSO_4 \cdot 7H_2O$, 0.1%

$CaCl_2$, and 0.05% yeast extract. The culture was incubated on a rotary shaker (180 rpm) at 20°C for 12 days (22).

Polygalacturonase assay and protein determination

PG activity was determined by measuring release of reducing end-groups from polygalacturonic acids using DNS solution containing 0.75% 3,5-dinitrosalicylic acid 1.4% NaOH, 0.22% sodium potassium tartarate, 0.59% $Na_2S_2O_5$ and 0.54% phenol. Reaction mixture (1 ml) containing 200 mM potassium acetate buffer (pH 4.5), 2 mM EDTA, 0.5% (w/v) sodium polygalacturonic acid and enzyme source was incubated at 45°C for 20 min. The reaction was terminated by adding 3 ml of DNS solution and the color of end product was developed by boiling for 10 min. The absorbance was measured at 530 nm. One enzyme unit was defined as the amount of enzyme which released 1.0 μmol of reducing end groups from the polygalacturonic acid in 1 min.

Protein concentration was measured according to Bradford (2) using bovine serum albumin as a standard.

Preparation of culture filtrate and acetone precipitation

The mycelium was removed by fitering through two layers of cheesecloth. The filtrate was then centrifuged at 10,000 g at 4°C for 30 min. To the supernatant cold acetone was added to a final concentration of 30% (v/v) at 4°C, and centrifuged at 10,000 g for 30 min to remove the precipitates. To the obtained acetone fluid more cold acetone was added to a final concentration of 60% (v/v), and centrifuged again at 10,000 g for 30 min. The precipitates was collected and suspended in 10 mM Hepes buffer (pH 7.2) containing 1.5 M ammonium sulfate.

Purification of Exo-polygalacturonase

All procedures were carried out at 4°C unless otherwise specified. The crude protein fraction obtained above was applied directly to a Phenyl Sepharose CL-4B column (2.6 × 20 cm) equilibrated with 10 mM Hepes buffer (pH 7.2) containing 1.5 M ammonium sulfate. The column was washed with the same buffer, and then the proteins were eluted with a reverse linear gradient of ammonium sulfate (1.5-0 M) in 10 mM Tris-HCl buffer (pH 7.4). In this step, four active fractions were detected, designated PG-I, -II, -III, and -IV. Each fractions were collected separately. One of the active fractions, PG-IV, was concentrated by Centriprep (Amicon) and washed with 10 mM Hepes buffer (pH 7.2). The PG fraction obtained from Phenyl Sepharose CL-4B column was injected into a HPLC Phenyl-5PW column equilibrated with 10 mM Hepes buffer containing 1. 5 M ammonium sulfate (pH 7.2). The column was washed with the same buffer to remove unbound protein. The enzyme was then eluted with a reverse linear gradient of ammonium sulfate (1.5-0 M). Active fractions were concentrated by Centricon (Amicon) and dialyzed against 10 mM Hepes buffer (pH 7.2). Active enzyme fraction was injected into HPLC DEAE-5PW column equilibrated with 10 mM Hepes buffer (pH 7.2). The column was washed with same buffer; and the enzyme was eluted with linear gradient of KCl (0-0. 15 M). Active fraction was collected, and concentrated by Centricon (Amicon) and washed with 10 mM Hepes buffer (pH 7.2). Finally, the enzyme fraction was further purified by HPLC Heparin-5PW column chromatography. The enzyme fraction was injected into column and eluted with linear gradient of KCl (0-0.15 M). Purified enzyme was concentrated by Centricon and stored at -20°C until used.

Determination of molecular weight of exopolygalacturonase

The molecular size of native exo-PG was determined by gel filtration with FPLC Superose 6 HR 10/30 (Pharmacia-LKB) gel permeation column. The column was equilibrated with 10 mM Hepes buffer (pH 7.2) containing 0.5 M KCl and calibrated with blue dextran 2,000 standard proteins such as alcohol dehydrogenase (150,00), bovine serum albumin (66,000), trypsin inhibitor I-S (20, 000) and trypsin inhibitor I-P (6,500). The molecular weight of denatured enzyme was analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as described elsewhere (14).

Modes of action

The mode of action of the enzyme fractions on the substrate was determined by measuring the decrease in the relative viscosity of a 1.2% (w/v) solution of polygalacturonic acid in 0.1 M acetate buffer (pH 5.2) using Cannon-Fensk viscometer to which 8 units of enzyme were added. The viscometer was immersed in a water bath at 30°C.

Results

Purification of exo-polygalacturonase

Table 1. Purification of extracellular polygalacturonase produced by *Botrytis cinerea* T91-1

Steps	Total protein (mg)	Total activity	Sp. activity (U/mg)	Recovery (%)	Folds
Culture filtrate	256	4725	18.5	100	1
Acetone precipitation (30~60%)	33	3215	97.4	68	5.3
Phenyl Sepharose CL-4B	3.8	1672	440	35	23.8
Phenyl-5PW	0.27	1287	4767	27	258.3
DEAE-5PW	0.14	1043	7450	22	403.6
Heparin-5PW	0.12	918	7650	19	414.5

A crude enzyme extract was obtained from filtering a culture of *B. cinerea* grown in liquid medium. The specific activity of this crude filtrate was about 18.5 U/mg. Bulk of the polygalacturonases in the culture filtrate was recovered in the protein precipitated by the addition of cold acetone (30~60%). This step resulted in nearly a 5.3-fold purification with 68% recovery of the total activity (Table 1). On a Phenyl-Sephadex CL-4B column, four fractions containing polygalacturonase activities were resolved, designated as PG-I, -II, -III, and -IV in the order of elution from the column (Fig. 1). Of the four polygalacturonases fractions, PG-IV had the highest activity. PG-IV was further purified and 35% recovery of the original enzyme was recovered from this step with 23.8-fold purification. When the enzyme solution obtained from the above step was applied to Phenyl-HPLC column, most of the polygalacturonase were adsorbed. The proteins were eluted as five major protein peaks by decreasing the concentration of ammonium sulfate (1.5-0 M). This step resulted in a 258.3-fold purification with 27% recovery of total activity. When the enzyme solution obtained from above step was applied on a DEAE-HPLC column, most of the enzyme activity was absorbed. The proteins were eluted as two major protein peaks by increasing the concentration of KCl (0-0.2 M). This step resulted in a 403.6-fold purification with 22% recovery of the enzyme. Finally, the enzyme fraction was applied on a heparin-HPLC column. The proteins were eluted as one major protein peak with one minor impurity peak with increasing concentration of KCl, 0 to 0.2 M. This step resulted 414.5-fold purification with 19% recovery of total activity. Thus, the combination of the five steps resulted in 414.5-fold purification of polygalacturonase with overall recovery of 19% as summarized in Table 1.

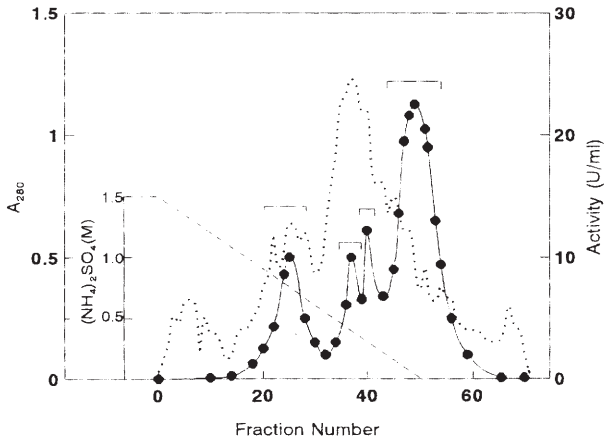

Fig. 1. Phenyl Sepharose CL-4B Chromatography of polygalacturonases from *Botrytis cinerea* T91-1. Polygalacturonase activity (-●-) was traced by measuring the amount of reducing sugars, and proteins (·····) were monitored by determining the absorbance at 280 nm.

Criteria of the enzyme purity

The purity of the polygalacturonase prepared from the final step of heparin-HPLC was examined by polyacrylamide SDS-gel electrophoresis (Fig. 2). Activity staining was performed to determine the presence of enzyme activity by overlaying the gel

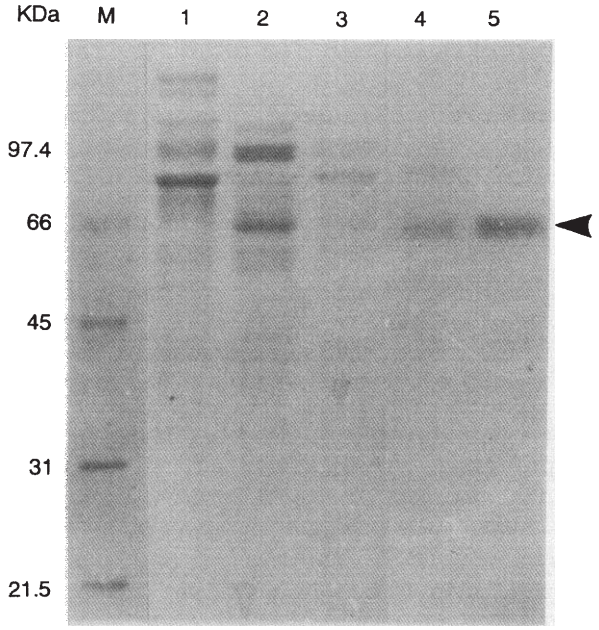

Fig. 2. SDS polyacrylamide gel electrophoresis of the protein fractions containing polygalacturonase activity. Lane 1; acetone precipitation, 2-5; active fractions from Phenyl Sepharose CL-4B, HPLC Phenyl, HPLC DEAE, HPLC Heparin column chromatography, respectively. M; molecular weight markers (phosphorylase b, bovine serum albumin, ovalbumin, carbonic anhydrase and soyben trypsin inhibitor). Arrow head indicates the position of exo-polygalactruonase.

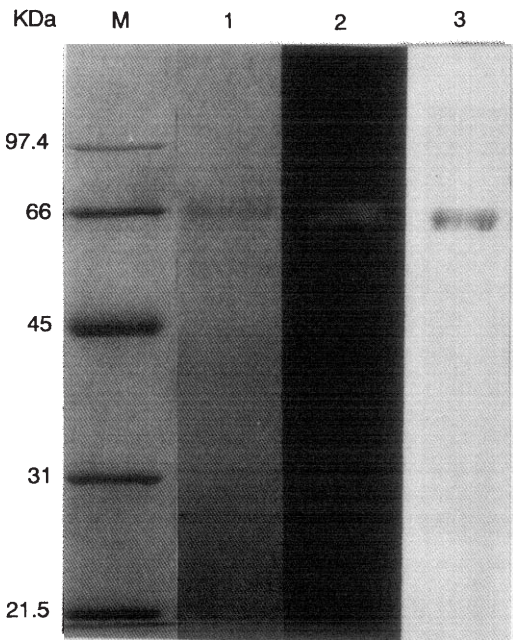

Fig. 3. Activity staining and PAS staining of purified exo-polygalacturonase produced by *B. cinerea* T91-1. Lane 1; Coomassie blue stained protein, 2; activity stained overlay, 3; PAS stained protein, M; molecular weight markers.

containing polygalacturonic acid as substrate as described previously (13). As shown in Fig. 3 (lane 2), the degradation of polygalacturonic acid on the position of the protein band was consistent as shown Coomassie staining (Fig. 3, lane 1). In addition, the purified protein was also stained with periodic acid and Shiff's reagent (PAS) (Fig. 3, lane 3). Molecular weight of the enzyme obtained by SDS-polyacrylamide gel electrophoresis was 66,000 Da (Fig. 2 and Fig. 3). Meanwhile, molecular size of the purified enzyme estimated by gel permeation chromatography was 64,000 Da (Data not shown).

Properties of polygalacturonase

The mode of cleavage of polygalacturonic acid by the purified enzyme was studied by comparison of the rate of reduction in viscosity with the corresponding increase in appearance of reducing end-groups in solution. The enzyme yielded a high percentage increase in reducing sugar end-groups for a relatively low drop in viscosity over a 3 hr period, confirming that this protein functioned as an exo-enzyme (Data not shown).

The effect of pH on polygalacturonase was studied using four buffer systems over a pH range of 3.0 to 11.0. As the pH increased from 3.0 to 5.0, the enzyme activity increased gradually with optimum pH at 5.0 in potassium phosphate buffer. At the same pH the activity in acetate buffer was better than that in phosphate buffer (Fig. 4A). The effect of temperature on the enzyme activity was investigated over a range of 4°C to 70°C. The optimum temperature for the enzyme was 50°C as shown in Fig. 4B. The enzyme was stable at the range of pH 3 to 10. The loss of activity was observed after 12 hr treatment above pH 9 (Fig. 5A). The thermostability was investigated by measuring the residual activity of the enzyme after 12 hr incubation at temperatures ranging from 4 to 80°C.

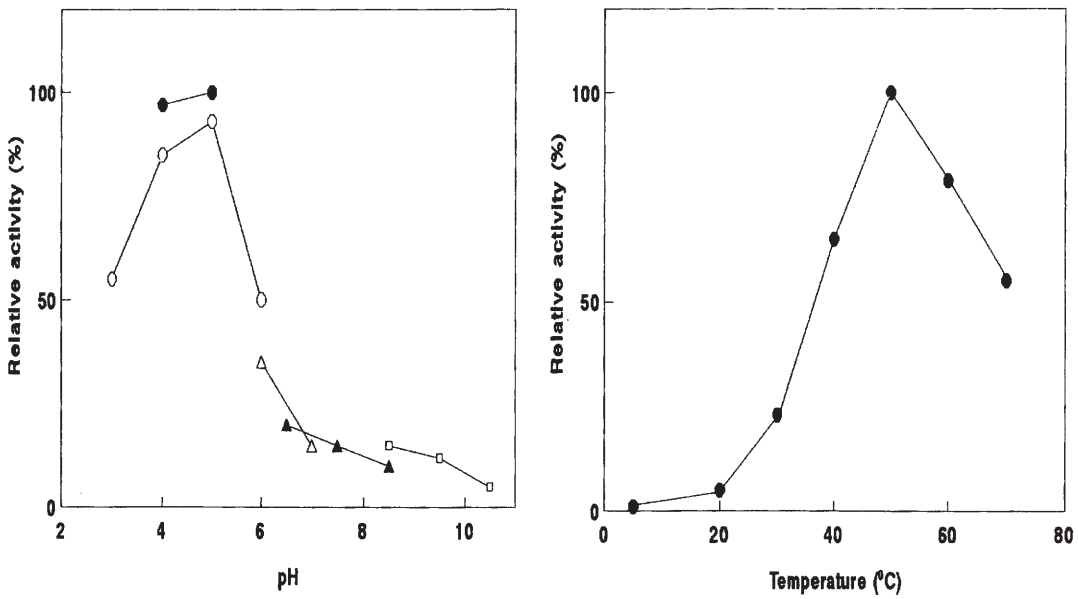

Fig. 4. Effects of pH and temperature on the enzymatic activity of exo-polygalacturonase purified from *B. cinerea* T91-1. polygalacturonase was assayed using 0.1 M citrate (○), acetate (●), phosphate (△), Tris-HCl (▲), and Glycine-NaOH (□) buffers.

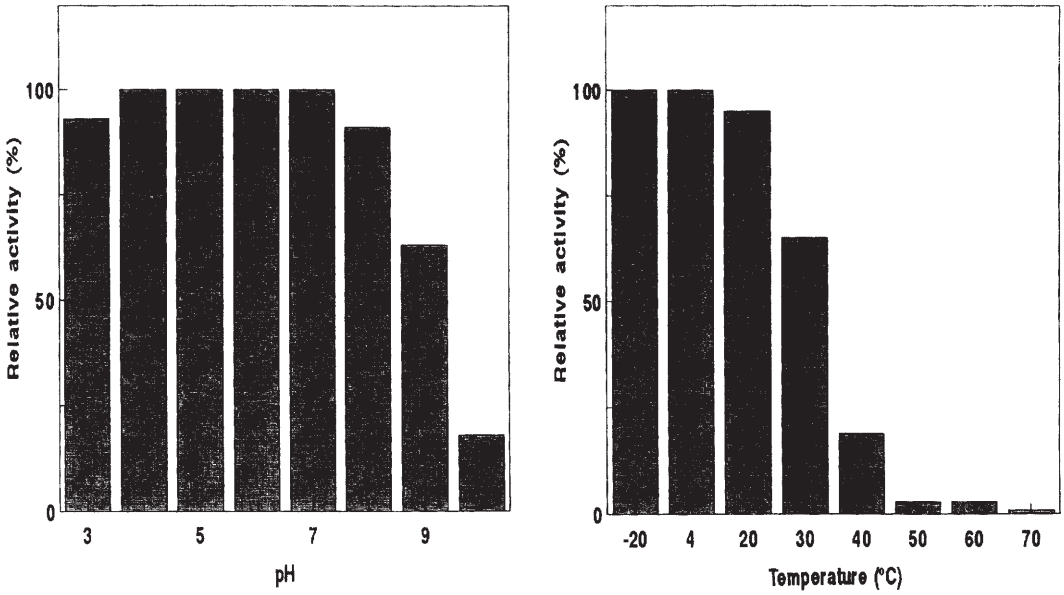

Fig. 5. Effects of pH and temperature on the stability of exo-polygalacturonase.

The enzyme was stable, being most active at 20°C and exhibiting 70% activity after treatment at 30°C. Complete inactivation of the enzyme was observed at 80°C (Fig. 5B).

The effects of cations and reagents on polygalacturonase were examined. As shown in Table 2, the enzyme activity decreased in the presence of divalent cations such as Ca^{2+}, Mg^{2+}, and Cu^{2+}. There was no inhibitory effect by EDTA, PMSF and DTT. However, the enzyme was severely inhibited by SDS.

By increasing the concentration of polygalacturonic acid, the rate of polygalacturonase increased and typical Michaelis-Menten type substrate saturation pattern was obtained as shown in Fig. 6. The reciprocal plots were linear. From such plot, Km and Vmax values for polygalacturonic acid were 0.33 mg/ml and 28.6 nM/min, respectively.

Table 2. Effects of cations and reagents on polygalcturonase activity from *B. cinerea* T9-1

Cations and reagents	Concentration (mM)	Relative activity (%)
Control	0	100
$CaCl_2$	2	71
$MnCl_2$	2	44
$CoCl_2$	2	50
NH_4Cl	2	88
$FeCl_3$	2	73
$MgCl_2$	2	69
$CuSO_4$	2	58
$ZnSO_4$	2	74
SDS	2	3
EDTA	5	96
PMSF	5	96
DTT	5	96

Discussion

The production of polygalacturonase has been investigated in a wide range of plant pathogens (4). Because of its peculiar infection mechanism, *B. cinerea* has been subjected to particularly intense studies (15, 16, 17). Since many reports on the production of polygalacturonases by *B. cinerea* and their possible role in pathogenesis, numerous investigators have contributed to the increasing

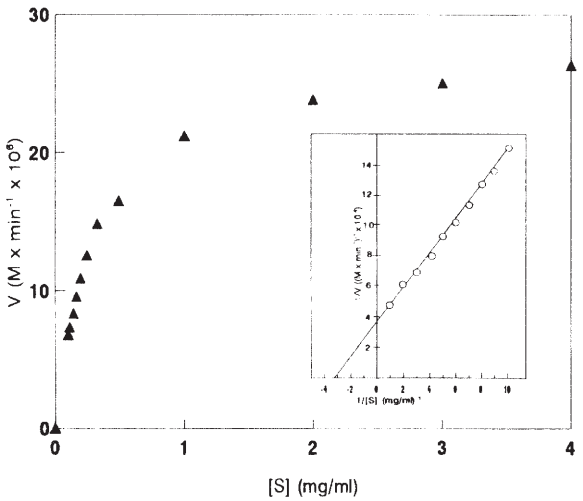

Fig. 6. K_m and V_{max} values of exo-polygalacturonase purified from *B. cinerea* T91-1. (Inset : Lineweaver-Burk plot of the data)

knowledge of polygalacturonases from this pathogen (10, 11, 25, 27). However, in spite of these considerable research efforts, it is still unclear how many polygalacturonases are produced and which of them are important during plant infection. Numerous investigators have shown that the number and patterns of PG isozymes produced by *B. cinerea* varied markedly. Difference in the PG isozymes produced by *B. cinerea* have been attributed to the differences in the isolates used, growing conditions, the age of cultures, and the inherent pathogenic variability of the pathogen (16). It also remains unknown whether different isozymes correspond to different proteins or are due to variations in the carbohydrate moieties of the same enzyme.

Most previous reports on polygalacturonase from *B. cinerea* showed endo-polygalacturonase being produced in culture filtrate and in infected tissue (15, 16, 17). Even though Johnston and Williamson (11) reported the isolation of two exo-PGs with two endo-PGs from *B. cinerea*, the properties of the enzyme was not studied in detail. Infection process of *B. cinerea* differs from that of most other fungi because the success of infection by germinating conidia requires the presence of certain sugars in the inoculum (13). Galacturonic acid has been claimed to be the inducer of polygalacturonase of Botrytis species. However, the mechanisms by which an extracellular polymer can induce its own catabolism are still poorly understood. Since exo-PG is a candidate for enzyme able to release simple sugars from polymers, we had focused on the properties of exo-PG secreted by *B. cinerea*.

The properties and the kinetic parameters are similar to those reported for other fungal exo-PGs. The exo-PG from *B. cinerea* T91-1 is a single-subunit protein with a Mr of 66,000, close to those reported from *Penicillin capsulatum* (9), *Fusarium oxysporum* (18), *Colletotrichum lindemuchicurum* (1), and *C. gloeosporioides* (21). They are larger than most endo-PGs, which exhibit Mr values ranging from 32,000 to 56,000. The purified enzyme was inhibited by Ca^{2+}, Mg^{2+}, Cu^{2+}, and sodium dodecylsulfate like exo-PGs examined in other fungi. The optimum temperature of the exo-enzyme from *B. cinerea* is close to the optima of 40°C determined for two constitutive PGs from *C. lindemuchicurum* (1). There was no inhibitory effect of dithiothreotol and phenylmethylsulfonyl fluoride, indicating that sulfhydryl group or serine residue is not involved in the active center of the enzyme. Interestingly, the exo-PG purified in this study is glycosylated like PGs investigated in several phytopathogen (11, 21); however, its physiological significance has not been elucidated; it has been suggested that the ability to form complexes with host proteins of *B. cinerea* is likely to depend on the glycosylation nature of the PGs.

To elucidate the role of exo-PG in the infection process of *B. cinerea*, it is necessary to obtain an antibody against this enzyme. The availability of specific polyclonal antibody against the enzyme will permit us to follow the time course secretion of the proteins during the cultivation of *B. cinerea* in the presence of inducers and/or repressors of the enzyme synthesis. The constitutive synthesis of the enzyme should determine whether it is responsible for triggering the synthesis of other pectinolytic enzymes including endo-PG.

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