# Characterization of an Exo-β-1,3-Glucanase Produced by *Pichia anomala* Strain K, Antagonist of *Botrytis cinerea* on Apples

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## ABSTRACT

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The exo-β-1,3-glucanase (EC 3.2.1.58) activity of *Pichia anomala* strain K, an antagonistic yeast of *Botrytis cinerea* on postharvest apples, was studied in a synthetic medium supplemented with laminarin, a cell wall preparation (CWP) of *B. cinerea*, or glucose. The highest enzyme activity was detected in culture media containing a CWP of *B. cinerea* as the sole carbon source, whereas the lowest activity was observed in culture media supplemented with glucose. Exoglc1, an exo-β-1,3-glucanase, was purified to homogeneity from culture filtrates of strain K containing a CWP. The molecular mass of exoglc1 was estimated to be under 15 kDa. Optimum activity of exoglc1 was recorded at 50°C and pH 5.5. The exoglc1 $K_m$ value was estimated at 22.4 mg/ml. Exoglc1 showed in vitro a stronger inhibitory effect on germ tube growth of *B. cinerea* than on conidia germination and caused morphological changes such as leakage of cytoplasm and cell swelling. Exo-β-1,3-glucanase activity was detected on apples treated with strain K and was similar to exoglc1 on the basis of activity on native gel. Moreover, the addition of a CWP to a suspension of *P. anomala* stimulated both in situ exo-β-1,3-glucanase activity and protective activity against the pathogen, strengthening the hypothesis that exo-β-1,3-glucanase activity is one of the mechanisms of action involved in the suppression of *B. cinerea* by *P. anomala* strain K.

Since the early 1970s, postharvest diseases of apple have caused losses of 15 to 25% despite the use of modern storage facilities (5,6). Among the different postharvest pathogens of apple, *Botrytis cinerea* Pers.:Fr. (causes gray mold) is one of the most devastating worldwide, and it is controlled primarily with chemical fungicides (6,13,31). There is a critical need to develop alternative methods, because of the possibility of the loss of effective and widely used fungicides and the development of fungicide-resistant strains of *B. cinerea* (17,31). Biological control of postharvest diseases appears to be an attractive and realistic approach, because of the defined and stable environmental conditions in storage rooms and the high value of apples. Numerous microbial strains that are antagonistic to *B. cinerea* have been reported in the literature (38). Furthermore, biocontrol products such as Biosave (*Pseudomonas syringae*, Esc-11) and Aspire (*Candida oleophila*, I-182) already have been commercialized by Ecogen Inc. (Langhorne, PA) and Ecoscience Corp. (Worcester, MA), respectively, and are used to protect apples against *B. cinerea* after harvest.

We previously isolated the yeast *Pichia anomala* (Hansen) Kurtzman strain K from the surface of an apple and demonstrated that it had high and reliable biocontrol activity against *B. cinerea* on wounded 'Golden Delicious' apples at 5 and 25°C (20). The mass production of strain K in a fermentor did not affect its antagonistic activity against gray mold on apples (21).

Understanding the modes of action of biocontrol agents is a prerequisite to developing rational procedures to select more effective antagonistic microbial strains; developing appropriate production and formulation methods that enhance biocontrol activity; and fulfilling some requirements of the toxicological and registration packages needed for commercial development. Elucidation of the mechanisms of action often is hampered by the complex interactions between host-pathogen-antagonist. Moreover, the mechanisms studied in vitro do not necessarily reflect in situ activity.

A variety of mechanisms have been reported to contribute to the biocontrol of postharvest pathogens by introduced microorganisms. For example, *Burkholderia cepacia* and *Bacillus subtilis* produced pyrrolnitrin and iturin, respectively, which appeared to be involved in the suppression of *B. cinerea* and *Penicillium expansum* by *Burkholderia cepacia*, and *Penicillium digitatum* by *Bacillus subtilis* (19,39). Competition for nutrients frequently has been cited as a mechanism of biocontrol by antagonistic yeasts such as *Pichia*, *Candida*, and *Cryptococcus* (8,12,30,39). Droby and Chalutz (11) observed the production of ethylene in tissues of different plants treated by *P. guilliermondii*, suggesting a possible induction of resistance in plant tissues.

The production of hydrolytic enzymes that degrade cell walls of phytopathogens has been reported as a mechanism of suppression of soilborne pathogens by some biocontrol agents such as *Trichoderma* spp. (3,4,24,33), but lytic enzymes have rarely been studied in the biological control of postharvest diseases (39).

To determine the possible role of β-1,3-glucanases in the antagonism of *B. cinerea* by *P. anomala* strain K, we studied the conditions that affect production of β-1,3-glucanase(s) by *P. anomala* strain K in vitro, purified and characterized the enzyme(s), and detected production of β-1,3-glucanase(s) in vivo.

## MATERIALS AND METHODS

**Organisms and culture conditions.** *P. anomala* strain K was stored at 5°C on potato dextrose agar (PDA) (E. Merck AG, Darmstadt, Germany). For each experiment, strain K was grown on PDA for 24 h at 25°C. After three successive subcultures under the same conditions, yeast colonies were flooded with sterile distilled water and scraped from plates. The concentration of yeast suspensions was adjusted to $10^7$ CFU/ml (unless otherwise specified) on the bases of optical density measurements in an UltrospecII spectrophotometer (LKB Biochron Ltd., Uppsala, Sweden) at 595 nm.

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*B. cinerea* was isolated from a rotting strawberry and stored on PDA at 5°C in the dark. The pathogen was cultivated on PDA at 25°C for 12 to 14 days with 16 h of light per day. To obtain spore suspensions, cultures were flooded with sterile distilled water containing 0.05% Tween 20. Spores were scraped from fungal colonies with a rubber spatula, and suspensions were filtered through sterile nylon gauze. The spore suspension was diluted to the required concentration as determined with a haemacytometer.

**CWP.** Hyphal cell walls were prepared from 5-day-old cultures of *B. cinerea* grown at 25°C in 50 ml of potato dextrose broth (PDB) (Difco Laboratories, Detroit) and shaken on a R020 shaker (Gerhardt, Bonn, Germany) at 60 rpm. The mycelium was centrifuged for 10 min at 16,260 × *g* (Sorvall RC-5B; Dupont Instruments, Wilmington, DE), the supernatant was discarded, and the pellet was homogenized with an Ultra Turrax $T_{25}$ (Janke & Kunkel GmbH & Co., Staufen, Germany) for 2 min at 24,000 rpm. The material was subjected to five successive cycles of centrifugation, resuspension, and homogenization. The final pellet of fungal material was frozen at –20°C for 24 h. The thawed pellets were sonicated twice with a probe-type sonicator (Branson Sonifier 250; Branson Ultrasonic Corp., Danbury, CT) for 10 min. The CWP was washed six times with distilled water and centrifuged at 16,260 × *g* for 10 min before being lyophilized and stored at –20°C.

**Effect of time course and carbon source on β-1,3-glucanase production.** Strain K was cultured in yeast nitrogen broth (YNB) (Difco Laboratories) (6.7 g/liter) amended with glucose (Sigma Chemical Co., St. Louis), laminarin (from *Laminaria digitata*, Sigma L-9634; Sigma Chemical Co.), or a CWP as the sole carbon source (2 mg/ml) and adjusted to pH 6.3. Flasks (100 ml) containing 50 ml of culture media were incubated with shaking (120 rpm) at 25°C for various time periods. Culture filtrate from each flask was collected by filtration through Whatman No. 1 filter paper (Schleicher & Schuell, Inc., Dassel, Germany). Three separated flasks were used for each carbon source and incubation time. The experiment was arranged in a completely randomized design and conducted three times.

**Enzyme assays and protein measurement.** The total activity, endo- (EC 3.2.1.39) and exo-β-1,3-glucanase (EC 3.2.1.58), was assayed by the method of Nelson (26) and Somogyi (34). The reaction mixture was made by adding 250 µl of 0.05 M potassium acetate buffer (pH 5.0) containing 2.5 mg of laminarin per ml to 250 µl of strain K culture filtrate or enzyme solution. The exo-β-1,3-glucanase (EC 3.2.1.58) activity was determined by following the release of free glucose from laminarin using a commercial glucose oxidase kit (Sigma 510-A; Sigma Chemical Co.). The difference between the results of total and exo-β-1,3-glucanase activities represented the endo-β-1,3-glucanase (EC 3.2.1.39) activity. One unit (U) of β-1,3-glucanase was defined as the amount of enzyme releasing 1 µg of glucose equivalent per min per ml of enzyme solution. Specific activity was expressed in units per milligram of protein (specific units [SU]). Protein content was measured by the Bradford method (7) using bovine serum albumin (Sigma A-9647; Sigma Chemical Co.) as the standard.

**Polyacrylamide gel electrophoresis (PAGE).** Native PAGE and sodium dodecyl sulfate (SDS)-PAGE were performed according to the method of Laemmli (22) with 4% (wt/vol) polyacrylamide in the stacking gel and 15% (wt/vol) acrylamide in the separating gel. The gels for SDS-PAGE were stained with silver nitrate using the commercial kit 17-1150-01 from Pharmacia Biotechnology Inc. (Uppsala, Sweden). β-1,3-glucanase activity was detected in situ by overlaying a native gel with 0.2% (wt/vol) MUG (4-methylumbelliferyl-β-D-glucoside, Sigma M-3633; Sigma Chemical Co.) in 0.05 M acetate buffer (pH 5.0) and incubating for 45 min at 30°C before UV illumination (25).

**Purification of exo-β-1,3-glucanase.** *P. anomala* strain K was grown in YNB supplemented with a CWP for 3 days as de-

TABLE 1. Scheme of the purification of exo-β-1,3-glucanase secreted by *Pichia anomala* strain K

| Purification step[x] | Total protein (mg) | Total activity[y] (U) | Specific activity[z] (SU) | Purification (-fold) | Recovery (%) |
|---|---|---|---|---|---|
| Culture filtrate | 141.42 | 647.80 | 4.6 | 1 | |
| $(NH_4)_2SO_4$ fractionation (40 to 80%) | 33.57 | 560.62 | 16.7 | 3.6 | 86.2 |
| Anion-exchange (DEAE A25) | 10.01 | 424.42 | 42.4 | 9.2 | 65.2 |
| Gel filtration (Sephadex G75) | 1.05 | 315.80 | 300.8 | 65.4 | 48.5 |
| Cation-exchange (MonoQ) first peak | 0.15 | 106.65 | 711.0 | 154.6 | 16.4 |
| Electro-elution first peak | 0.045 | 90.66 | 2,014.8 | 438.0 | 13.9 |
| Cation-exchange (MonoQ) second peak | 0.820 | 41.49 | 50.6 | 11.0 | 6.4 |

[x] Measurements were carried out after dialysis.
[y] One unit (U) of β-1,3-glucanase is defined as the amount of enzyme causing the release of 1 µg of glucose equivalent per min under the conditions described in Material and Methods.
[z] Specific activity was expressed in units (U) per milligram of protein (specific units [SU]).

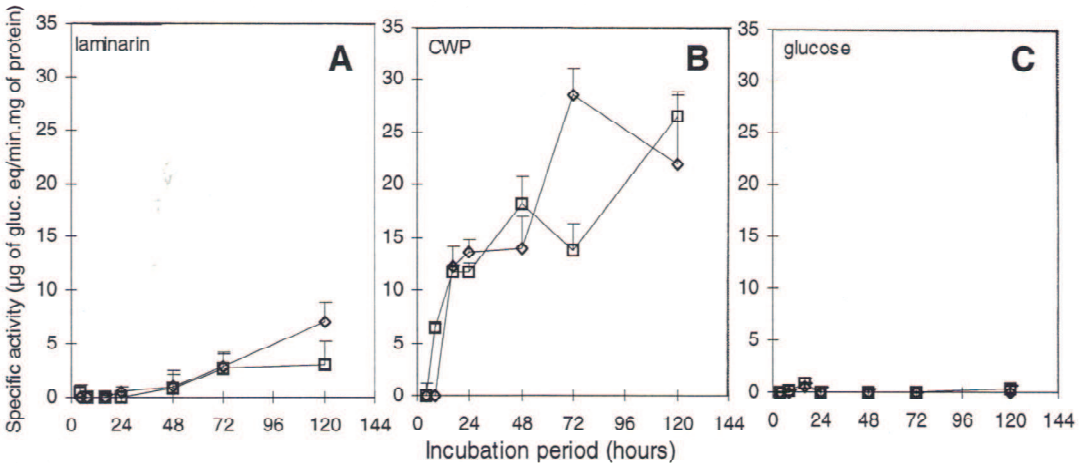


**Fig. 1.** Time courses of endo-β-1,3-glucanase (◇) and exo-β-1,3-glucanase (□) production by strain K in yeast nitrogen broth medium containing **A,** laminarin; **B,** *Botrytis cinerea* cell wall fragments (CWP); or **C,** glucose (gluc.) as the sole carbon source (2 mg/ml). One specific unit of β-1,3-glucanase (endo- or exo-) is defined as the amount of enzyme causing the release of 1 µg of glucose equivalent per milligram of protein per minute. Assays were performed in triplicate, and the experiment was repeated three times. Values presented are averages of the three experiments. The error bars represent standard deviations of the means of the three experiments.

scribed above. The culture (4,725 ml) was filtrated through Whatman No. 1 filter paper, and proteins in the supernatant fluid were precipitated with $(NH_4)_2SO_4$ (40 to 80% saturation) at 4°C. The precipitate was recovered by centrifugation (16,260 × *g* for 10 min), dissolved in 10 mM of Tris-HCl (pH 8.0), filtrated through a Millipore membrane (0.2 μm), and dialyzed three times against 5 liters of the same buffer at 4°C. The protein solution was loaded onto a column of DEAE A25 (Pharmacia Biotechnology Inc.) that had been equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The column was eluted with a nonlinear gradient of 0 to 1 M NaCl at a flow rate of 1 ml/min. The β-1,3-glucanase–positive fractions that eluted at 0.18 to 0.35 M NaCl were pooled, dialyzed against 0.05 M acetate ammonium buffer (pH 6.0), and freeze-dried. Proteins were dissolved in 3 ml of the same acetate ammonium buffer (pH 6.0) and loaded onto a gel filtration column (Sephadex G75; Pharmacia Biotechnology Inc.). The gel was eluted with the same buffer at a flow rate of 0.3 ml/min. The β-1,3-glucanase–positive fractions were pooled and freeze-dried.

The proteins were dissolved in 2 ml of 0.02 M L-histidine buffer (pH 5.7) and loaded onto a cation-exchange column (MonoQ; Bio-Rad Laboratories, Hercules, CA). The column was eluted with a nonlinear gradient of 0 to 0.5 M NaCl at a flow rate of 0.5 ml/min. Two fractions with enzymatic activity were obtained.

The first fraction was dialyzed against acetate ammonium buffer (pH 6.0) and freeze-dried. The proteins were dissolved in native Laemmli buffer (22), and aliquots of 20 μl were loaded onto PAGE gels. After electrophoresis, the two external lanes were incubated in 0.2% (wt/vol) MUG for in situ detection of bands revealing β-1,3-glucanase activity. Gel slices from the others lanes were cut at the same height as the bands showing activity. Proteins were electro-eluted from gel slices using the Bio-Rad apparatus (model 422) and Bio-Rad method 165-2977 (Bio-Rad Laboratories) (10 mA per electro-elution tube during 6 h, followed by 1 min of inversed polarity). Numerous similar purifications of exo-β-1,3-glucanase were conducted.

**Calculating kinetic constants and pH and temperature optima.** Kinetic values were calculated by using various laminarin

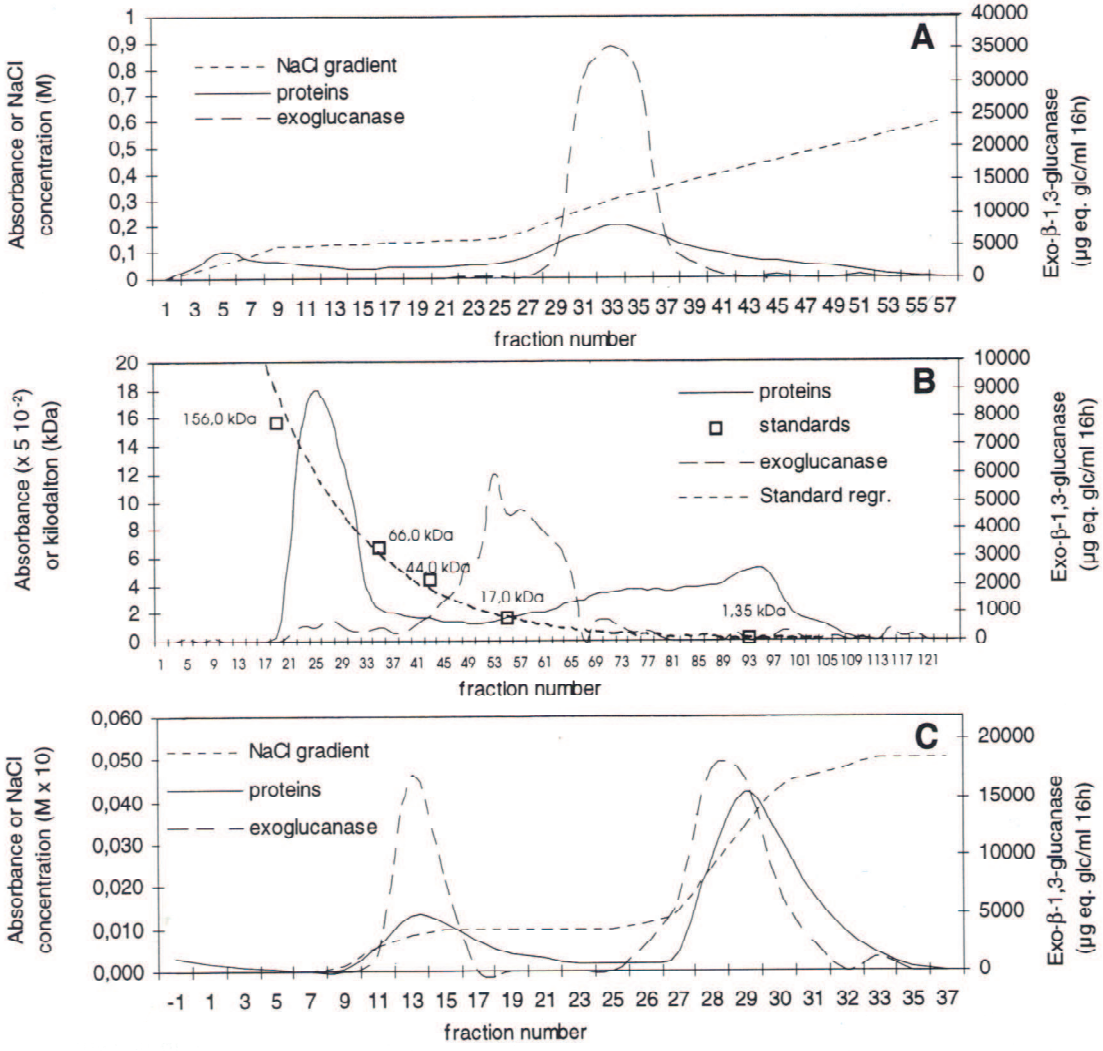


**Fig. 2.** Fractionation of the exo-β-1,3-glucanase enzymes from *Pichia anomala* strain K. Proteins in the culture filtrate of strain K were precipitated with $(NH_4)_2SO_4$ (40 to 80% saturation). **A,** After dialysis in 10 mM of Tris-HCl (pH 8.0), the protein solution was loaded onto a column of DEAE A25 that was equilibrated with the same buffer and eluted with a nonlinear gradient of 0 to 1.0 M NaCl at a flow rate of 1 ml/min. The exo-β-1,3-glucanase–positive fractions that eluted at 0.18 to 0.35 M NaCl were pooled, dialyzed against 0.05 M acetate ammonium buffer (pH 6.0), and freeze-dried. **B,** Proteins were dissolved in 3 ml of the same acetate ammonium buffer and loaded onto a gel filtration column of Sephadex G75 and eluted at a flow rate of 0.3 ml/min. The exo-β-1,3-glucanase–positive fractions were pooled and freeze-dried. **C,** The proteins were dissolved in 2 ml of 0.02 M L-histidine buffer (pH 5.7) and loaded onto a cation-exchange column, MonoQ. The column was eluted with a nonlinear gradient of 0 to 0.5 M NaCl at a flow rate of 0.5 ml/min. Proteins = proteins detected at 280 nm. Exoglucanase = exo-β-1,3-glucanase activity; one unit of exo-β-1,3-glucanase activity is defined as the amount of enzyme causing the release of 1 μg of glucose equivalent per ml per 16 h of incubation with laminarin at 37°C. Standards = Sephadex G75 was calibrated with molecular mass standards (Bio-Rad 261-52: γ-globulin [158 kDa], ovalbumin [44 kDa], myoglobin [17 kDa], and B12 vitamin [1.35 kDa]). Standard regression = the calibration curve was subjected to exponential regression ($y = 66.23\ e^{-0.1306\ x}$).

concentrations of 1 to 20 mg/ml of potassium acetate buffer (0.05 M, pH 5.5). A Lineweaver-Burk plot was used to calculate $K_m$ and $V_{max}$ values. The effects of pH on exo-β-1,3-glucanase activity were measured using 0.05 M citrate buffer and 0.05 M phosphate buffer at a pH ranging between 3 to 5 and 5.5 to 9, respectively. The effects of temperature on enzyme activity were assayed in phosphate buffer (0.05 M, pH 5.5) at temperatures of 20, 30, 34, 37, 45, 50, 60, 70, and 80°C. In all cases, purified enzyme was used at a concentration of 0.16 µg/ml of final solution. Assays at each pH, each temperature, or each laminarin concentration were performed in triplicate and averaged, and the experiment was repeated. Values presented are the means and standard deviations of two pooled experiments.

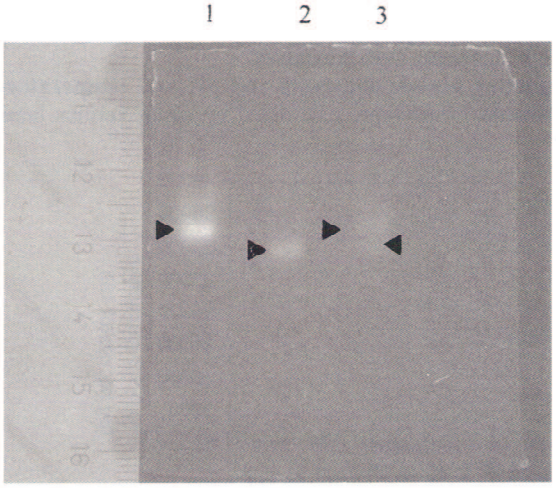


**Fig. 3.** Detection of β-1,3-glucanase activity on native gel after different purification steps. Lanes 1 and 2, Pooled and concentrated fractions after elution on MonoQ (Fig. 2C), respectively, from the first peak (2 µg) eluting between 0.06 and 0.1 M NaCl and the second peak (8 µg) eluting between 0.11 and 0.46 M NaCl with exo-β-1,3-glucanase activity. Lane 3, Pooled and concentrated fractions after elution on gel filtration (Fig. 2B). β-1,3-glucanase activity was detected by overlaying the native gel with 0.2% (wt/vol) MUG in acetate buffer (pH 5.0) and incubating for 45 min at 30°C before UV illumination.

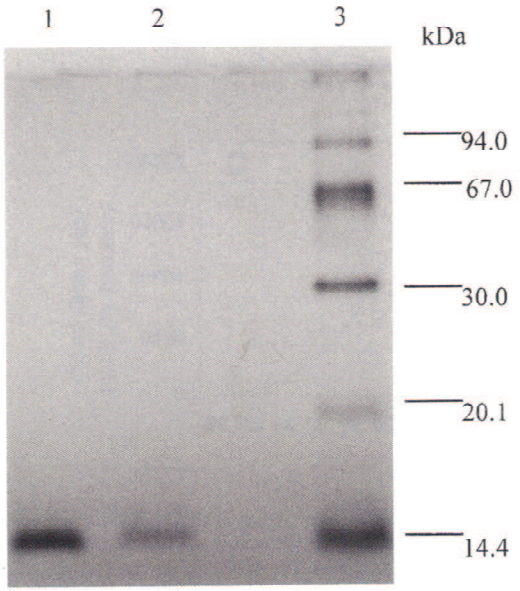


**Fig. 4.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the purified exo-β-1,3-glucanase (exoglc1). After electrophoresis, the gel was stained as described in the commercial kit from Pharmacia 17-1150-01. Lanes 1 and 2, Purified exoglc1 loaded at a concentration of 2 and 1 µg per well, respectively. Lane 3, Molecular mass markers (2 µg): phosphorylase b (94.0 kDa), bovine serum albumin (67.0 kDa), carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), and lactalbumin (14.4 kDa).

**Assay of antifungal activity.** All assays were performed under sterile conditions as described by Lorito et al. (23). Equal volumes of a spore suspension ($3 \times 10^5$ spores per ml), 3× PDB, and enzyme test solution were mixed in sterile Eppendorf tubes and incubated at 25°C for various periods (2 h to 24 h) before microscopic examination. The enzyme solution was replaced with sterile distilled water or boiled enzyme solution in the controls. The number of germinating conidia was determined with the first 100 spores observed on a microscope slide. In addition, the length of 20 germ tubes was measured and averaged. The values obtained for the control (no enzyme) were taken as 0% inhibition, and all other values were divided by these values and multiplied by 100 to obtain percent inhibition. Data from two experiments with four replicates per treatment were pooled. For each enzyme concentration, the mean percent inhibition and the standard deviation were calculated. To determine the effective dose (ED) values, dosage response curves were calculated by probit analysis and subjected to regression analysis by using a polynomial regression of the second order ($r^2$ ranged between 0.97 and 0.99).

**Assay of in vivo detection of β-1,3-glucanase activity.** Apple fruits (cv. Golden Delicious) were bought in a commercial market and stored for 1 week in a cold room at 3°C before being used for

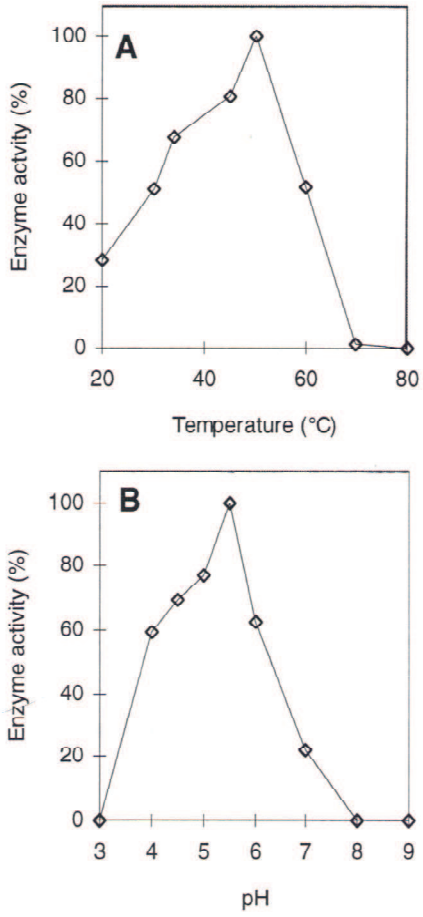


**Fig. 5.** Effects of temperature and pH on purified exo-β-1,3-glucanase from *Pichia anomala*. **A,** Exo-β-1,3-glucanase activity was measured at various incubation temperatures. The enzyme (0.16 µg/ml of final solution) was dissolved in 250 µl of phosphate buffer (0.05 M, pH 5.5) containing laminarin (10 mg/ml). **B,** Exo-β-1,3-glucanase activity of the enzyme was measured at various incubation pH under the following assay conditions: the enzyme (0.16 µg/ml of final solution) was dissolved in 250 µl of 0.05 M citrate buffer (pH 3.0 to 5.0) or 0.05 M phosphate buffer (pH 5.5 to 9.0) containing laminarin (10 mg/ml). Assays at each temperature or pH were performed in triplicate and averaged. Values presented are pooled means of two experiments and are expressed in relative activity (percent of the greatest activity detected).

bioassays. Fruits were surface-disinfected with sodium hypochlorite (10% commercial product for 2 min), rinsed with sterile water, and wounded with a cork-borer (nine wounds of 6 mm diameter and 3 mm deep for each apple). The wounded sites were treated with 50 µl of a suspension of the antagonistic strain K ($10^5$ CFU/ml) or with 50 µl of distilled water for the control, followed by the inoculation of either 50 µl of the CWP (2 mg/ml) or 50 µl of distilled water for the control after 24 h. Wounded sites were rinsed with 50 µl of distilled sterile water 24 h after treatments. Each treatment was replicated three times. Ten apples per replicate were used, and the washing water of wounds were pooled before detection of exo-β-1,3-glucanase activity with the colorimetric method (26,34) or PAGE (25). Treatments were arranged in a completely randomized design. The experiment was conducted three times. Data were subjected to analysis of variance, and means were separated by Fisher's least significant difference (LSD) test at $P = 0.01$.

To evaluate subsequent protection against *B. cinerea*, surface-sterilized wounded apples (two wounds of 6 mm diameter and 3 mm deep at the equator of each fruit) were treated with 50 µl of strain K suspension ($10^5$ CFU/ml). The inoculation of 50 µl of a suspension of *B. cinerea* ($10^6$ spores per ml) or the application of 50 µl of the CWP (2 mg/ml) was carried out 24 h after the application of the yeast. Inoculated fruits were maintained in plastic boxes for 5 days at 25°C before measuring the diameters of the lesions resulting from tissue decay. Five apples were used per treatment. Treatments were arranged in a completely randomized design. The experiment was conducted twice, and data were subjected to analysis of variance. Means were separated by Fisher's LSD at $P = 0.01$.

## RESULTS

**Effect of incubation time course and carbon source on β-1,3-glucanase production.** When strain K was grown in YNB media supplemented with glucose as the sole carbon source, exo- and endo-β-1,3-glucanase activities were not detected or were lower than 0.8 SU regardless of the incubation period (Fig. 1C). When strain K was grown in YNB media supplemented with laminarin, the exo- and endo-β-1,3-glucanase activity level increased with time and reached a maximum of 7.1 and 3.0 SU, respectively, after 5 days (Fig. 1A). Higher specific activities for both enzymes were obtained in media with a *B. cinerea* CWP (Fig. 1B) as compared with media with laminarin or glucose (Fig. 1A and C). After 8 h of incubation in media with the CWP, the activity of endo-β-1,3-glucanase was greater than 6 SU, whereas exo-β-1,3-glucanase activity was not detected before 16 h of incubation (12.6 SU). After the 16-, 24-, 48-, and 120-h incubation periods, each enzyme type accounted for about 50% of the total activity. The specific activity of endo-β-1,3-glucanase reached a maximum of 28.6 SU after 72 h of incubation of strain K; exo-β-1,3-glucanase specific activity (26.5 SU) reached a maximum after 5 days of incubation of strain K (Fig. 1B).

**Purification of exo-β-1,3-glucanase produced in vitro from *P. anomala* strain K.** The purification scheme is summarized in Table 1. The partially purified enzyme precipitated by $(NH_4)_2SO_4$ at 40 to 80% saturation contained 86% of the total activity and consisted exclusively of exolytic activity. In contrast, the endolytic activity was very low and was detected only in the 0 to 40% saturation fractions. Only the fractions showing exo-β-1,3-glucanase activity were pooled and further purified by anion-exchange (DEAE A25) and gel filtration (Sephadex G75) (Pharmacia Biotechnology Inc.) (Fig. 2A and B). The elution profile of this third purification step revealed two peaks of exo-β-1,3-glucanase activity. The molecular mass corresponding to the maximal activity values of both peaks was determined to be about 19 and 15 kDa, respectively. The next purification method (MonoQ) allowed the total separation of both peaks (Fig. 2C). That last step, as well as activity detection after electrophoresis on native gel (Fig. 3), revealed two different exo-β-1,3-glucanases, the first one (exoglc1) showing a 14-fold higher activity using laminarin as the substrate than the second one (exoglc2). The enzyme (exoglc1) exhibiting the highest exo-β-1,3-glucanase specific activity was purified to homogeneity by electro-elution from native electrophoresis gel with a recovery of 16.4% and a specific activity of 2,014.8 SU.

**Characterization of exoglc1.** The molecular mass of the purified exoglc1 was about 15 kDa as determined by SDS-PAGE (Fig. 4). The effect of temperature was studied by incubating the enzyme at temperatures between 20 and 80°C (Fig. 5A). The enzyme was active from pH 4.0 to 7.0 with an optimum at pH 5.5 (Fig. 5B). At pH 5.5, the enzyme was most active at 50°C when laminarin was used as the substrate. A Lineweaver-Burk plot was used to determine the $K_m$. The $K_m$ and $V_{max}$ values with laminarin as the substrate were estimated to be 22.41 mg/ml and 48.54 µg of glucose equivalent per ml per h after regression analysis ($1/v = 0.461 \times 1/S + 0.021$; in which $v$ = µg of glucose equivalent per ml

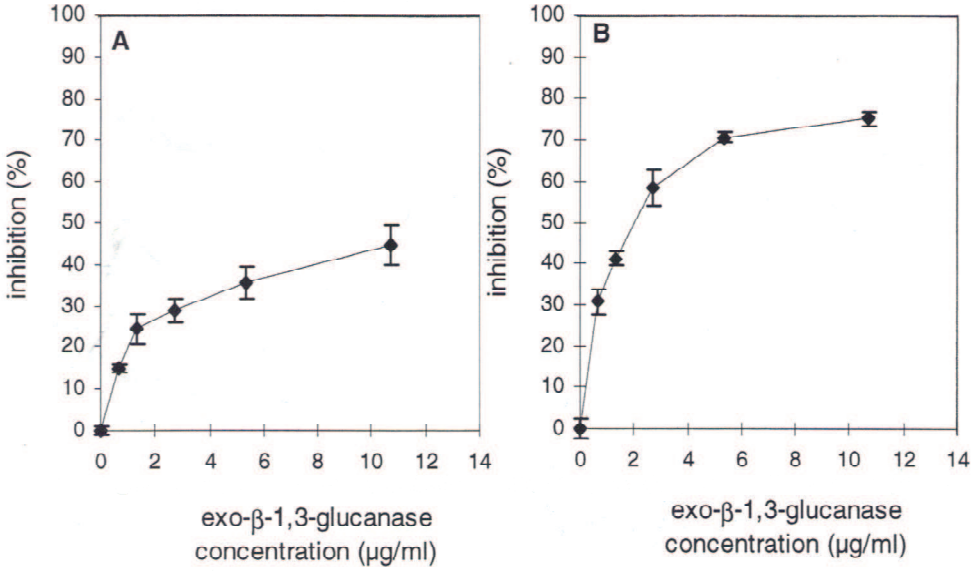


**Fig. 6.** Effect of purified exo-β-1,3-glucanase from *Pichia anomala* on **A,** spore germination and **B,** germ tube elongation of *Botrytis cinerea*. Percent inhibition was calculated from the first 100 spores observed on a microscope slide. The length of 20 germ tubes was measured and averaged. Error bars represent standard deviations. The data from two experiments with four replicates per treatment were pooled.

per h and $S$ = mg of laminarin per ml of enzyme solution), respectively.

**In vitro antifungal activity of purified exoglc1 from *P. anomala* strain K.** The biological activity of the purified enzyme was tested for activity against the spore germination and hyphal growth of *B. cinerea*. The percent conidial germination and length of germ tubes were similar in the control (no enzyme) and in the suspension containing denaturated boiled enzyme (10.72 µg/ml).

The lowest exoglc1 concentration tested (0.7 µg/ml) weakly inhibited conidial germination (14.9%) (Fig. 6). Inhibition increased slowly with increasing concentration of exoglc1; 10.7 µg of exoglc1 per ml resulted in a 44.8% inhibition of conidia germination. In contrast, the same range of exoglc1 concentrations strongly inhibited germ tube elongation. Purified exoglc1 at a concentration of 10.7 µg/ml resulted in 75.2% inhibition of germ tube growth. The $ED_{50}$ for the inhibition of germ tube length was 3.5 µg of exoglc1

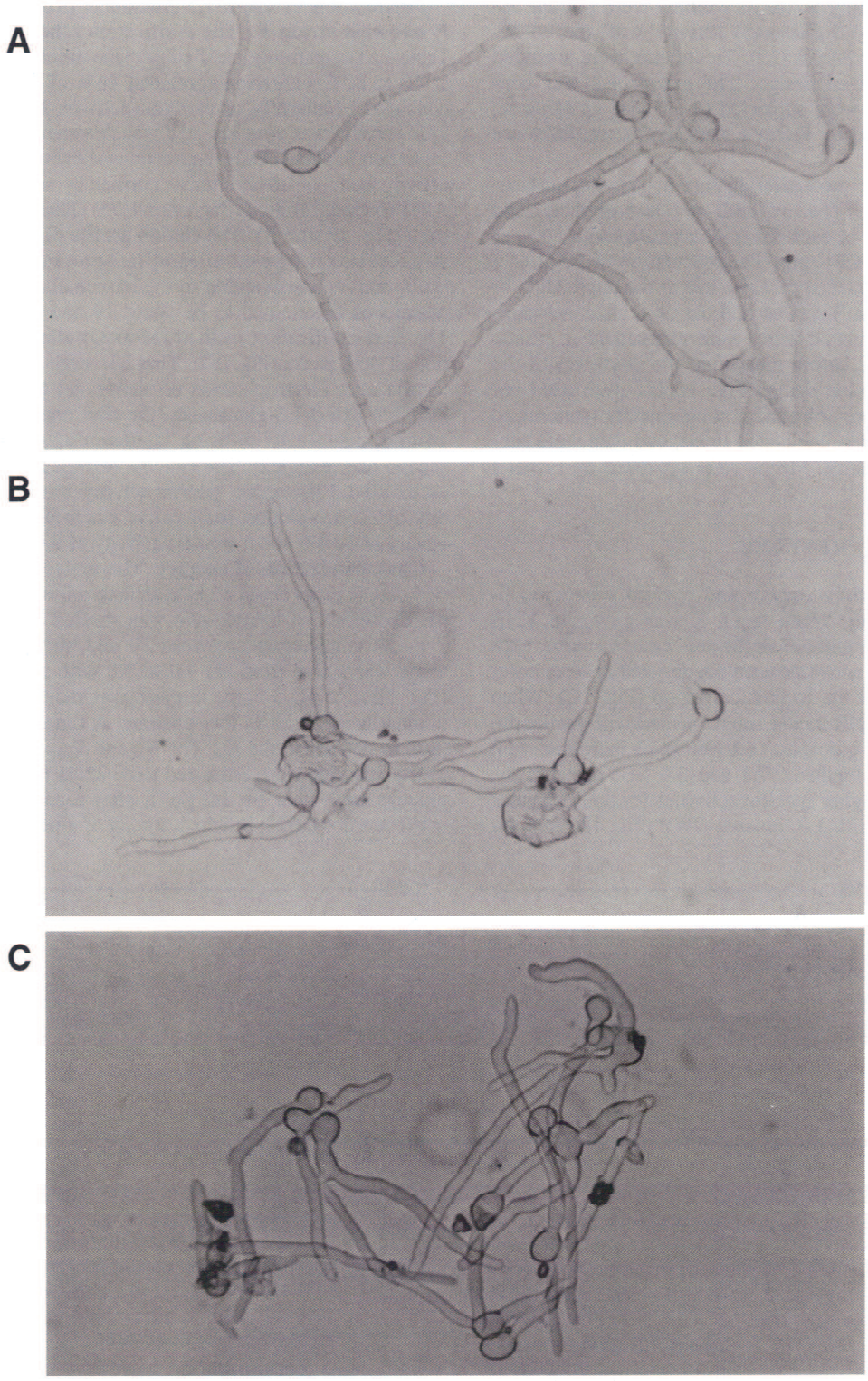


**Fig. 7.** Reduction of *Botrytis cinerea* germ tube elongation and induction of morphological changes by *Pichia anomala* exo-β-1,3-glucanase (exoglc1). Equal volumes of *B. cinerea* spore suspension ($3 \times 10^5$ spores per ml); 3× potato dextrose broth; and **A,** sterile water (control), or enzyme test solution at **B,** 3 × 10.72 µg/ml; or **C,** 3 × 2.68 µg/ml were mixed in sterile Eppendorf tubes and incubated at 25°C for 16 h. The enzyme induced **B,** leakage of cytoplasm; or **C,** cell swelling. —— = 20 µm.

per ml. Moreover, the enzyme caused morphological changes in *B. cinerea* such as leakage of cytoplasm and cell swelling (Fig. 7).

**In vivo detection of exo-β-1,3-glucanase activity from *P. anomala* strain K.** To detect the presence of exo-β-1,3-glucanase activity in apples treated with strain K, wounded sites were rinsed with water and washings were pooled and assayed for enzyme activity.

Using colorimetric methods, exo-β-1,3-glucanase was detected only at a low level in rinse waters obtained from nontreated wounded sites. In contrast, exo-β-1,3-glucanase activity in rinse water was 10.8 μg of glucose equivalent per wound when strain K was applied to wounded apples. However, the greatest activity (32.7 μg of glucose equivalent per wound) was detected from apples treated both with antagonistic strain K and the CWP (Table 2).

In situ detection of β-1,3-glucanase activity on native gel revealed at least one band from the wash water of wounded sites inoculated with *P. anomala* and the CWP. This band migrated to the same position as the band of purified exoglc1 (Fig. 8).

**Protection of apples against *B. cinerea* by strain K in the presence of the CWP.** A significant reduction in the size of the lesion caused by *B. cinerea* occurred when strain K at $10^5$ CFU/ml was applied on wounded sites as compared with the control (inoculated only with spores of the pathogen) (Table 2). The greatest reduction in lesion size occurred when apples were treated with strain K and the CWP (2 g/ml); the CWP applied alone provided no protection.

## DISCUSSION

The possible role of β-1,3-glucanases in the antagonism of *P. anomala* strain K against *B. cinerea* was investigated on apples. Water used to rinse apple wounds treated with strain K and with *B. cinerea* CWP were assayed for glucanase activity. Exo-β-1,3-glucanase activity was detected on strain K-treated apples. This activity appears to be related to exoglc1, an exo-β-1,3-glucanase purified from culture filtrates of strain K, on the basis of its electrophoretic mobility in native gel. Moreover, the addition of the CWP to a suspension of *P. anomala* stimulated both in situ exo-β-1,3-glucanase activity and suppression of the pathogen. To our knowledge, this is the first demonstration of in vivo glucanase production by a biocontrol yeast.

TABLE 2. In vivo detection of exo-β-1,3-glucanase activity from apples treated with *Pichia anomala* strain K and suppression of *Botrytis cinerea*

| Treatment[x] | exo-β-1,3-glucanase activity (μg eq. gluc./wound)[y] | Decay lesion (mm) after *B. cinerea* inoculation[z] |
|---|---|---|
| Control | 3.4 ± 0.47 a | 35.5 ± 8.43 a |
| CWP | 2.1 ± 1.58 a | 40.3 ± 4.65 a |
| Strain K | 10.8 ± 0.52 b | 15.6 ± 4.31 b |
| Strain K + CWP | 32.7 ± 5.51 c | 6.9 ± 3.65 c |

[x] Wounded sites of apples were treated with 50 μl of distilled water (control) or 50 μl of a suspension ($10^5$ CFU/ml) of strain K. After 24 h, 50 μl of the cell wall preparation (CWP) was applied at the wound sites treated with water (CWP) or strain K (strain K + CWP). Treatments were arranged in a completely randomized design.

[y] Twenty-four hours after the treatments were applied, exo-β-1,3-glucanase activities were measured. Means of enzymatic activity (± standard deviation) were expressed as micrograms of glucose equivalent per wound. Treatments were arranged in a completely randomized design (three replicates per treatment). The experiment was conducted three times and the data were pooled. After analysis of variance, means were separated by Fisher's least significant difference test. Means with a common letter do not differ significantly at $P = 0.01$.

[z] Twenty-four hours after the treatments were applied, wounded sites were inoculated with a *B. cinerea* suspension ($10^6$ spores per ml). The diameter of the lesions was measured 5 days after inoculation of *B. cinerea*. Treatments were arranged in a completely randomized design. Data from two experiments were pooled. After analysis of variance, means were separated by the Fisher's least significant difference test. Means with a common letter do not differ significantly at $P = 0.01$.

Production of both endo- (EC 3.2.1.39) and exo-β-1,3-glucanases (EC 3.2.1.58) by *P. anomala* also was stimulated by the presence of *B. cinerea*, given that YNB containing the CWP yielded substantially greater enzyme activity than YNB with glucose. Our findings are similar to those obtained with *Saccharomyces cerevisiae* and *T. harzianum*, which also produced both endo- and exo-β-1,3-glucanases (10,18,24). Furthermore, endo- and exo-β-1,3-glucanase activity from *T. harzianum*, *P. guilliermondii*, and *Serratia marescens* also was higher in media supplemented with fungal cell walls than in media containing laminarin (10,15,28,39).

Two exo-β-1,3-glucanases were identified from culture filtrates of *P. anomala* strain K following the MonoQ purification step and on the basis of enzymatic activity detected on native gel. The first enzyme, exoglc1, showed a 14-fold higher specific activity than the second, exoglc2, and was further purify to homogeneity with an electro-elution step. That exoglc1 precipitated at a high level of $(NH_4)_2SO_4$ saturation (for the first purification step) was an indication of its hydrophilic properties. These properties were confirmed by the lack of retention on hydrophobic interaction chromatography (t-Butyl) (M. H. Jijakli, *unpublished data*). Similarly, Aono et al. (2) and Chui and Tzean (9) observed precipitation of β-1,3-glucanase at a high level of $(NH_4)_2SO_4$ saturation. The results from the ion-exchange steps (second and fourth steps) suggested that the pI of exoglc1 ranges between pH 5.7 to 8.0 (Fig. 2).

The β-1,3-glucanases from yeasts can be placed in three classes based on their molecular mass. The first class is produced by *Oerskovia scanthineolytica* (32), *Candida utilis* (27,37), *Hanseluna anomala* (1), and *Fabospora fragilis* (1) and includes low molecular mass proteins (from 12 to 30 kDa) with optimal activities ranging from pH 5 to 5.5 and high $K_m$ values ranging from

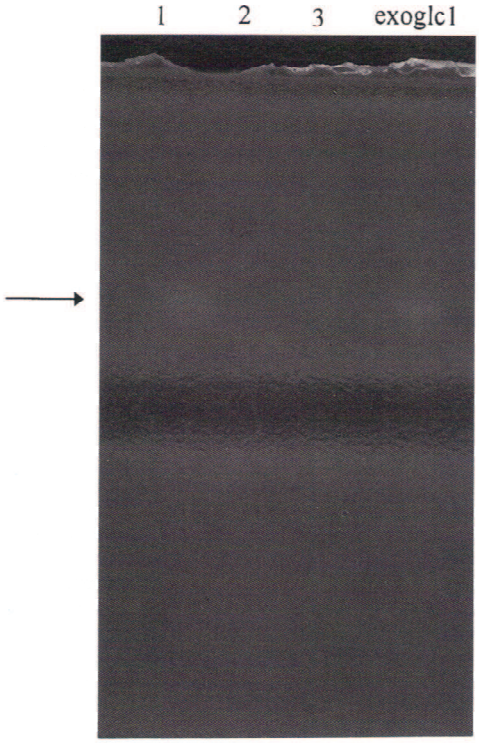


**Fig. 8.** Comparison of β-1,3-glucanase activity on native gel exoglc1 and in the wash water of wounded sites. Wounds of apples were treated with 50 μl of distilled water (3) or 50 μl of strain K suspension ($10^5$ CFU/ml) (2). Fifty microliters of cell wall preparation (2 mg/ml) was applied 24 h after treatment with the antagonist (1). Ten apples were used per treatment, and the wash water of wounds was pooled. The detection of exo-β-1,3-glucanase activity was conducted by overlaying a native gel with 0.2% (wt/vol) MUG in 0.05 M acetate buffer (pH 5.0) and incubating for 45 min at 30°C before UV illumination (25).

1.24 to 14 mg of laminarin per ml. The second and third classes of β-1,3-glucanase contain, respectively, medium molecular mass (from 41 to 75 kDa) and high molecular mass (from 76 to 220 kDa) proteins with lower $K_m$ (from 0.15 to 0.69 mg/ml) and with an optimal enzymatic activity either at an acidic pH (5.5) or at a higher pH (7.5) (16,18,29,32). Exoglc1 belongs to this first category with its molecular mass of 15 kDa (determined after gel filtration chromatography and SDS-PAGE), optimum pH of 5.5, and high $K_m$ of 22.4 mg/ml.

Saeki et al. (32) reported that β-1,3-glucanases of high molecular mass (>50 kDa) from *Oerskovia scanthineolytica* were not able to degrade yeast cell walls without the addition of protease, whereas β-1,3-glucanases of lower molecular mass (between 12 and 20 kDa) applied alone degraded yeast cell walls. Application of the purified exoglc1 to germinating spores and germ tubes of *B. cinerea* in vitro caused hyphal swelling and leakage of cytoplasm, indicating that exoglc1, which has low molecular mass and similar biochemical properties as β-1,3-glucanases produced by *O. scanthineolytica*, had the ability to degrade *B. cinerea* cell walls. Hydrolytic enzymes from other antagonists such as *Stachybotrys elegans* and *Schizophyllum commune* (9,35,36) caused similar effects on the hyphal growth of *Rhizoctonia solani* and *Fusarium moniliforme*. The $ED_{20}$ for inhibition of spore germination and the $ED_{50}$ for inhibition of germ tube growth by exoglc1 was 3.5 μg/ml, indicating that the enzyme is more inhibitory to hyphal elongation. These results contrast with these obtained with an exo-β-1,3-glucanase from *T. harzianum*, which showed similar inhibition of spores germination and of germ tubes elongation of *B. cinerea* (24). Furthermore, the exo-β-1,3-glucanase from *T. harzianum* is less active, with an $ED_{50}$ for inhibition of germ tubes growth of 94.5 μg/ml.

The involvement of cell wall-degrading enzymes produced by antagonists in the control of *B. cinerea* has been doubted (14). In our case, however, the possible production of exoglc1 on apples and its effect on *B. cinerea* in vitro strengthen the case that exo-β-1,3-glucanase contributes to the biocontrol of *B. cinerea* by *P. anomala* strain K.

Antibodies specific for exoglc1 could be used to provide further evidence that the in vivo activity of exo-β-1,3-glucanase is due to exoglc1. Furthermore, cloning the exoglc1 gene is ongoing in order to provide a molecular tool necessary to evaluate exo-β-1,3-glucanase synthesis in situ. Cloning the exoglc1 gene is also a prerequisite to using the genetic approach of gene disruption to determine the role of exo-β-1,3-glucanase in the biocontrol activity of *P. anomala*.

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