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Purification and Characterization of a Novel Extracellular Esterase from Pathogenic *Streptomyces scabies* That Is Inducible by Zinc

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Native polyacrylamide gels of extracellular proteins produced by several *Streptomyces* isolates grown with suberin were assayed in situ for esterase activity. Two pathogenic isolates of *Streptomyces scabies* from different geographical regions were found to produce a similar esterase activity that was not produced by nonpathogenic strains. After treatment with EDTA, suberin no longer induced esterase production. Expression was restored when EDTA-treated suberin was supplemented with zinc. The optimal concentration of zinc required for esterase production was 2 μM. This esterase was purified from one of the pathogenic isolates and characterized. The enzyme was 38,000 daltons when determined by gel filtration on Sephadex G-100 and 36,000 daltons when determined by denaturing polyacrylamide gel electrophoresis. The esterase showed maximal activity in sodium phosphate buffer above pH 8.0, was stable to temperatures of up to 60°C, and had an apparent K_m of 125 μM *p*-nitrophenyl butyrate.

Certain species of *Streptomyces*, collectively referred to as *Streptomyces scabies*, are pathogenic on a variety of underground vegetables, including potatoes (6). These organisms infect the tuber and cause the formation of scab lesions. Little is known about the mechanism of pathogenicity, but the ability of the organism to break down or modify molecules on the surface of the tuber may be important for its invasion of the host plant.

Cutin and suberin are insoluble waxy polyesters that cover the aerial or subterranean parts of plants, respectively, and may provide a protective barrier to pathogen invasion (10). A 26,000-dalton esterase that is capable of degrading cutin has been isolated from a number of fungi and from one strain of *S. scabies* (1, 7, 14). Specific inhibition of this cutinase activity prevented the infection of plants by the fungal pathogens, suggesting the importance of this esterase in pathogenicity (12, 15). The necessity of the 26,000-dalton *S. scabies* enzyme for pathogenicity has not been tested.

To determine whether other esterases that may be important to pathogenicity are produced by *S. scabies*, an in situ esterase assay was used to analyze native polyacrylamide gels of extracellular proteins produced by pathogenic and nonpathogenic *Streptomyces* isolates. A novel extracellular esterase produced by two different pathogenic strains that were isolated from different geographical regions was detected. An esterase with similar mobility on native polyacrylamide gels was not detected in the two nonpathogenic strains that were tested.

This esterase was purified from one of the pathogenic strains to greater than 95% purity. Several properties of this extracellular enzyme such as molecular weight, heat stability, effect of pH, and kinetics of the enzyme reaction were determined. The inducibility of esterase production by zinc was also studied.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. scabies* 10246 was obtained from the American Type Culture Collection, Rockville, Md. *S. lividans* 1326 and *S. coelicolor* M124 (2) originated in the laboratory of David Hopwood, John Innes Institute, Norwich, England. The remaining strains of *Streptomyces* were obtained from potato tubers of various cultivars, as described previously (16). To obtain spore preparations, the *Streptomyces* isolates were grown on either R2 regeneration medium (17) or oatmeal agar medium (21) at 30°C. The spores and mycelia were harvested by adding 10 ml of tryptone soy broth (TSB; Difco Laboratories, Detroit, Mich.) and by scraping the surface of the plate with an inoculation loop. The spores and mycelia were pelleted and suspended in 1 ml of TSB. Glycerol, at a final concentration of 20%, was added to the spores for long-term storage at −20°C.

Spores were germinated by diluting a concentrated spore stock into 50 ml of TSB to a final concentration of about 2×10^8 spores per ml and by incubating at 30°C with shaking. The culture was harvested while in late log or early stationary phase (optical density at 650 nm of about 2.0), washed once in minimal medium, and then suspended in 10 ml of minimal medium (8) without added KOH or $FeSO_4$. This preparation was used for culture inoculation.

Suberin was added to cultures at a final concentration of 2 mg/ml, and glucose was added at a final concentration of 56 mM. Unless otherwise indicated, zinc was added to cultures at a final concentration of 2 μM. In some experiments, suberin was treated with either 50 mM Tris buffer (pH 8; buffer-suberin) or 50 mM Tris (pH 8)–100 mM EDTA (EDTA-suberin) in 10-ml plastic tubes with gentle agitation for about 12 h and then washed five times with 10 ml of deionized distilled H_2O before it was added to the minimal medium.

Esterase assay. Esterase activity, with *p*-nitrophenyl butyrate (PNB) used as the substrate, was measured by a spectrophotometric assay essentially as described by Purdy and Kolattukudy (18). A sample of cells was centrifuged in a microfuge (Eppendorf). The resulting supernatant (20 to 100 μl) was added to 3 ml of 80 mM sodium phosphate buffer (pH 8.0). The reaction was started by the addition of substrate to a final concentration of 0.4 mM. The substrate was prepared by adding 18 μl of a commercially prepared 1.2-g/ml solution of PNB to 2.5 ml of acetonitrile. PNB hydrolysis was

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followed by determination of the absorption at 420 nm. An extinction coefficient for *p*-nitrophenyl of 15,000 at 420 nm was used (3).

Cutin and suberin extraction. Cutin was extracted from Golden Delicious apples as described by Walton and Kolattukudy (23). By using the method described by Kolattukudy and Agrawal (11), suberin was extracted from potato tubers of the cultivar Red Pontiac. We typically obtained 10 g of cutin from 23 kg of apples and 10 g of suberin from 10 kg of potato tubers. The final preparations of cutin and suberin were ground to a 60-mesh powder in a mill (Wiley).

Native polyacrylamide gels. Native polyacrylamide gels were run by the method described by Davis (5). A volume of culture filtrate containing 20 μg of total protein was lyophilized and suspended in 20 μl of water plus 20 μl of dye containing 20% glycerol, 0.125 M Tris (pH 6.8), and 0.01% bromophenol blue. A 4.4% (pH 6.8) stacking gel and 12.8% (pH 8.8) running gel were used. Electrophoresis was carried out at 4°C at 100 V of constant voltage. Unstained gels were assayed in situ for esterase activity by using α-naphthyl acetate (α-NA) or α-naphthyl butyrate (α-NB) as the substrate by the method described by Rosenburg et al. (20).

Denaturing gels. Sodium dodecyl sulfate (SDS)-polyacrylamide gels (4.4% stacking gel [pH 6.8], 12.8% running gel [pH 8.8]) were run by the method described by Laemmli (13). Protein (20 μg) was boiled for 5 min in the presence of 10% glycerol, 2.3% SDS, 0.35 M 2-mercaptoethanol, 0.005% bromophenol blue, and 0.062 M Tris (pH 6.8). Gels were run at 100 V of constant voltage at 4°C. The gels were stained with Coomassie blue, and the relative migration of the protein bands was calculated by dividing the distance of protein migration by the distance of dye migration from the top of the running gel.

Electroelution of protein. The outer two lanes of a preparative native polyacrylamide gel were removed, stained with α-NB, and realigned with the unstained portion of the gel to localize the esterase activity. The region of the gel corresponding to the esterase activity was removed from the gel. The gel slice was placed into dialysis tubing along with 1 ml of 80 mM sodium phosphate buffer (pH 8). The sealed dialysis tubing containing the gel slice was placed in a horizontal gel apparatus that was filled with 80 mM sodium phosphate buffer (pH 8). The protein was electroeluted from the gel slice into the buffer inside the dialysis bag at 125 V for 3 h. The protein sample was removed from the dialysis bag and dialyzed against 50 mM sodium phosphate buffer (pH 8).

Protein determination. The protein concentration was determined by the method described by Bradford (4).

Metal ion analysis. Metal ion analysis was carried out by the Research Analytical Laboratory of the University of Minnesota by using inductively coupled plasma-atomic emission spectrometry (ICP-AES).

Materials. *Aspergillus niger* cellulase, fungal pectinase, α-NB, α-NA, and PNB were obtained from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Screening isolates for esterase activity. Four different strains of *Streptomyces* were tested for the production of extracellular esterase activity when grown on either suberin or glucose as a carbon source. Suberin was chosen as a carbon source because of the possibility that enzyme production by *S. scabies* may be specifically induced by exposure to this natural insoluble polyester compound.

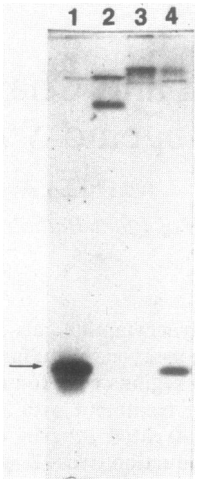

FIG. 1. Native polyacrylamide gel stained with α-NA. Each lane was loaded with 20 μg of protein from the culture filtrate. Lane 1, *S. scabies* FL1; lane 2, *S. lividans* 1326; lane 3, *S. scabies* ME2; lane 4, *S. scabies* PNT1. The arrow indicates the position of the esterase activity that is common to the two pathogenic isolates (FL1 and PNT1).

The number and similarity of esterases produced by these *Streptomyces* strains was determined by an in situ esterase assay of extracellular proteins that were separated on nondenaturing polyacrylamide gels (Fig. 1). Two pathogenic isolates (*S. scabies* FL1 and PNT1 [16]) and two nonpathogenic isolates (*S. scabies* ME2 and *S. lividans* 1326) were grown with suberin as the sole carbon source. The FL1 and PNT1 isolates (Fig. 1, arrow, lanes 1 and 4, respectively) secreted an esterase into the culture medium that had the same relative migration on the native polyacrylamide gel. This band of activity was not observed in the culture filtrates of the nonpathogenic strains (Fig. 1, lanes 2 and 3). Cultures grown in glucose showed very little esterase activity (data not shown), indicating that suberin may specifically induce production of this esterase in the pathogens. The FL1 isolate also produced this esterase activity when it was grown with cutin as a carbon source (data not shown).

The protein band of esterase activity was electroeluted and found to have PNB esterase activity. A comparison of the PNB esterase activity that was present in lysed cells with the activity that was present in the culture medium revealed that greater than 95% of the esterase activity was extracellular (data not shown).

Induction of esterase activity by zinc. The esterase was not detected when FL1 was grown in a glucose minimal medium, but supplementation of the glucose medium with a trace element solution (21) resulted in the production of the esterase (W.-S. Hu and J. Gonzalez, personal communication). Different divalent cations were added individually and in combination to cultures grown with glucose as the carbon source to determine which trace element was necessary for the expression of the esterase. Copper, manganese, calcium, borate, and magnesium had no effect on esterase production (data not shown). However, when zinc was added to the glucose medium, the esterase was expressed at levels comparable to that observed in the growth medium with suberin (Table 1). The optimal concentration of zinc that was required for esterase production in glucose minimal medium was found to be 2 μM (data not shown).

Suberin was treated with 100 mM EDTA in 50 mM Tris

(pH 8). The EDTA-treated suberin no longer induced esterase production. The addition of 2 μM zinc to the EDTA-treated suberin restored esterase production to a level higher than that found in cultures grown with untreated suberin, buffer-treated suberin, or glucose-zinc. Maximal esterase activity was obtained by adding zinc to a suberin-containing culture (Table 1). These results suggest that suberin may contain a second component which enhances the induction of esterase by zinc. This second component did not seem to induce esterase activity alone, as evidenced by the low amount of esterase produced with EDTA-treated suberin. In addition, this second component did not seem to be completely removed by EDTA treatment of the suberin because high levels of esterase activity were restored by the addition of only zinc.

Ion analysis of suberin and the purified esterase. The ion content of buffer-treated and EDTA-treated suberin was analyzed by ICP-AES. Buffer-treated suberin contained 65 ppm (μg/g) zinc, whereas EDTA-treated suberin contained 9 ppm zinc (data not shown). It is interesting that 65 ppm zinc in suberin corresponded to 2 μM zinc in the growth medium, which is the concentration of zinc that is optimal for esterase induction. The only other metals whose concentration was significantly reduced in suberin by EDTA treatment were calcium, magnesium, and manganese. These elements, however, were shown not to influence the levels of esterase production in the glucose minimal medium either alone or in conjunction with zinc (data not shown). These results indicate that the zinc associated with the suberin is the main component responsible for induction of the esterase. A second component in suberin which enhances the induction of esterase by zinc does not appear to be completely removed by EDTA treatment of the suberin (Table 1).

Purification of the esterase from the FL1 isolate. The major esterase activity produced by the FL1 isolate was purified. A culture of FL1 grown in glucose minimal medium with 2 μM zinc was harvested by filtering through Whatman no. 1 filter paper. The filtrate was concentrated 20 times by ultrafiltration with a filter (PM10; Amicon Corp., Lexington, Mass.) and dialyzed against 50 mM Tris hydrochloride (pH 7.5)–0.1 M NaCl. The concentrated filtrate was loaded onto a DEAE Sephadex column and washed with several bed volumes of the same buffer. Proteins were eluted with a 0.1 to 1.5 M NaCl gradient. Fractions from the DEAE Sephadex column that contained esterase activity eluted at 0.6 M NaCl and were pooled. A total of 10 μg of protein from the pooled esterase fractions was electrophoresed on an SDS-polyacrylamide gel. By Coomassie blue staining, the esterase was found to be greater than 95% pure (Fig. 2). The

TABLE 1. Effect of divalent cations on the expression of the esterase in FL1

Addition[a]	Sp act (nmol/min per mg mycelium[b])
Glucose	19
Glucose-Zn^{2+}	190
Suberin (untreated)	220
Buffer-suberin–glucose	180
Buffer-suberin–glucose–Zn^{2+}	390
EDTA-suberin–glucose	14
EDTA-suberin–glucose–Zn^{2+}	320

[a] Minimal medium, with the indicated additions, was inoculated with pregerminated spores and incubated for 24 h.
[b] Total dry weight of mycelium in the culture was determined. The results are the average of two experiments.

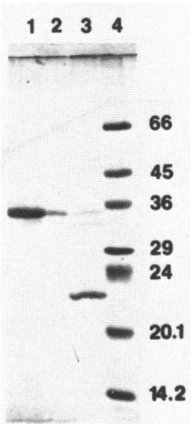

FIG. 2. SDS-polyacrylamide gel analysis of the purified esterase. Lane 1, 10 μg of purified esterase; lane 2, 2 μg of purified esterase; lane 3, 3 μg of culture filtrate protein; lane 4, molecular weight markers (10^3).

results of the purification procedure are summarized in Table 2. The esterase was purified 22-fold, with an overall yield of 53%.

The PNB esterase was also purified from FL1 cultures that were grown with either suberin or cutin as the sole carbon sources. Each esterase preparation contained a single protein species on denaturing polyacrylamide gels and had the same relative migration. In addition, the three esterases comigrated on a native polyacrylamide gel. Sephadex G-100 chromatography of the esterase produced with cutin indicated that it had a native molecular weight of 38,000 (data not shown). These results suggest that the same esterase activity is produced under the three different culture conditions.

ICP-AES analysis of the purified esterase did not detect zinc associated with the enzyme. In addition, dialysis of the esterase against Tris buffer (pH 8) containing 100 mM EDTA did not affect esterase activity (data not shown). These results suggest that the esterase does not require zinc for activity. The zinc requirement for esterase production may therefore be important for a process other than facilitating the reaction of the enzyme with the substrate.

Molecular weight determination of the esterase. The denatured molecular weight of the esterase was measured on SDS-polyacrylamide gels by using molecular weight standards and was found to be approximately 36,000 (Fig. 2). Native molecular weight was determined by gel filtration through a calibrated Sephadex G-100 column. From the standard curve, the native molecular weight of the esterase was estimated as 38,000 (data not shown), indicating that the PNB esterase is a monomeric protein.

TABLE 2. Esterase purification from FL1[a]

Fraction from the purification steps	Activity (nmol/min)	Protein (mg)	Sp act (nmol/min per mg)[b]	Recovery (%)
Culture filtrate	292,000	38	7,700	(100)
Concentrated culture filtrate	177,000	3.5	51,000	61
DEAE Sephadex	154,000	0.9	171,000	53

[a] The FL1 isolate was grown in glucose minimal medium with 2 μM zinc.
[b] Esterase activity was measured by using PNB as the substrate. Protein was measured by the method described by Bradford (4).

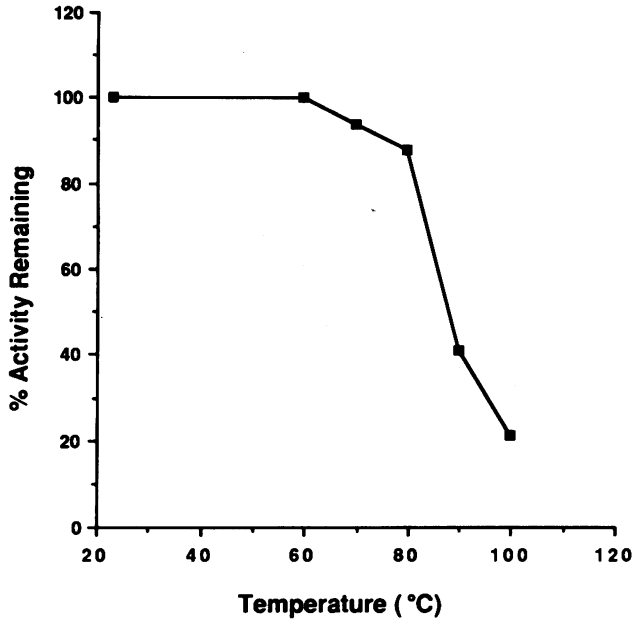

FIG. 3. Heat sensitivity of the esterase. A total of 0.4 μg of the purified esterase in 50 μl of 50 mM Tris (pH 7.5)–0.1 M NaCl was treated for 10 min at different temperatures and placed on ice, and the PNB esterase activity was measured.

Heat sensitivity of the esterase. The stability of the esterase at temperatures ranging from 23 to 100°C was determined (Fig. 3). This esterase is resistant to temperatures of up to 60°C and loses only about 10% of its activity when exposed to temperatures of up to 80°C. While the stability of the esterase sharply decreased at temperatures above 80°C, 20% of the activity survived incubations at 100°C.

pH optimum of esterase activity. Esterase activity was measured in sodium citrate buffer (pH 3.5 to 6.0), sodium phosphate buffer (pH 6.0 to 8.6), and Tris hydrochloride buffer (pH 6.5 to 9.0) (Table 3). The enzyme activity increased with increasing pH in sodium citrate and sodium phosphate buffers. When the enzyme reaction was carried out in Tris hydrochloride buffer, the activity appeared to decrease above pH 7.6. Maximum esterase activity was obtained in sodium phosphate buffer at pH 8.6.

Kinetics of esterase activity. The velocity versus PNB substrate concentration relationship for the esterase is shown in Fig. 4. The data do not show typical Michaelis-Menten kinetics, but rather the curve is sigmoidal in nature. The apparent K_m was determined from this graph to be 125 μM PNB, and the V_{max} was estimated to be 130,000 nmol/min per mg.

TABLE 3. Effect of pH on esterase activity[a]

Buffer	pH	Activity (nmol/min per mg [10^3])[b]
Sodium citrate	3.7	14
	4.1	33
	4.6	34
	5.0	61
	5.6	65
	6.0	73
	6.6	75
Tris	7.2	95
	7.6	102
	8.0	93
	8.5	88
	9.0	86
Sodium phosphate	6.0	88
	6.6	95
	7.0	115
	7.6	105
	8.0	120
	8.6	137

[a] The esterase was purified from FL1, as outlined in Table 2.

[b] The reaction mixture consisted of 3 ml of 50 mM buffer, 400 μM substrate, and 0.4 μg of esterase. At approximately 1-min intervals, 200 μl was removed and added to 800 μl of 0.08 M sodium phosphate buffer (pH 8). The optical density at 420 nm was immediately measured. Values are the average of two determinations.

DISCUSSION

Two pathogenic isolates of *S. scabies* were compared with two nonpathogenic isolates of *Streptomyces* to detect esterases that were common to the pathogenic isolates. The in situ gel assay provides an efficient method for the detection of esterases. If several esterases are present in a single sample, comparisons can be made on the basis of mobility and on the basis of the intensity of the activity bands. A similar extracellular esterase was observed on native polyacrylamide gels that was produced by the two pathogenic strains of *S. scabies* that were isolated from different cultivars and from different geographical regions. The production of this esterase by two different pathogens suggests that this protein may be important to pathogenicity. An esterase activity with a similar gel mobility was not observed in the nonpathogenic isolates.

A PNB esterase has been isolated and characterized from an *S. scabies* strain by Lin and Kolattukudy (14). Their enzyme was 26,000 in molecular weight, was inactivated by

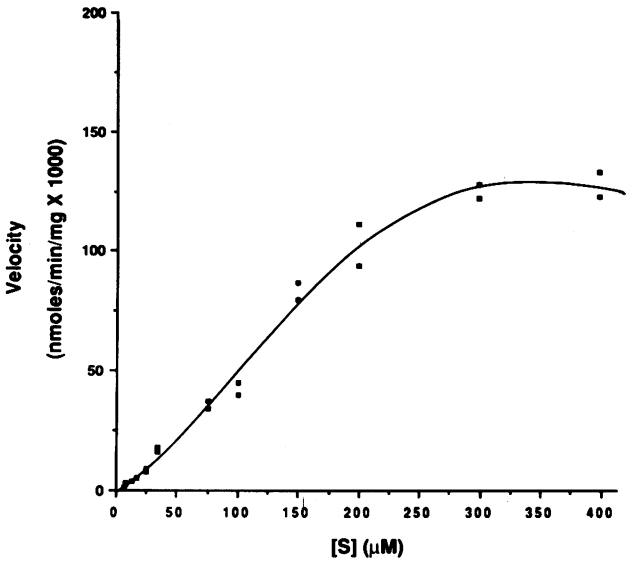

FIG. 4. Kinetics of esterase activity. Dilutions of the PNB substrate were made in acetonitrile. A total of 30 μl of each dilution was added to 3 ml of 0.08 M sodium phosphate buffer (pH 8) containing 0.3 μg of purified esterase. The reaction was followed at an optical density of 420 nm. [S], Final concentration of substrate in the reaction.

temperatures above 50°C, and showed Michaelis-Menten saturation kinetics with PNB as the substrate. These differences in molecular weight, heat stability, and effect of substrate concentration on reaction velocity suggest that the FL1 enzyme is unique to the previously reported PNB esterase. It would be of interest to determine whether these two proteins share any similarity in amino acid sequence or cross-reactivity with antibody.

The release of long-chain fatty acids from either cutin or suberin by the purified FL1 esterase was not detected. These results suggest the possibility that if cutin and suberin are substrates for this enzyme, other types of yet to be identified compounds are the products of esterase attack on these polyesters. The sigmoidal relationship between velocity and PNB substrate concentration suggests that there is cooperativity between substrate-binding sites on the esterase. A similar type of kinetics has been observed for trypsin, which also has esterase activity (22). A more detailed kinetic analysis of the PNB esterase is needed to study this apparent cooperativity of substrate binding.

PNB esterase activity from *S. scabies* is barely detectable in glucose minimal medium, and the levels of enzyme activity increase at least 10-fold with the addition of zinc to the growth medium, indicating that zinc may be important in the regulation of this enzyme. The induction of enzyme activities by micronutrients is found in other systems. For example, the expression of ligninases by *Phanerochaete chrysosporium* has been reported to increase by twofold with the addition of copper or manganese to the culture medium (9). The basis for this stimulation of ligninase activity has not been determined.

Esterase production was also induced by the growth of the organism on medium containing suberin. Our results indicate that the zinc associated with suberin is the main component responsible for induction of the esterase. The zinc is apparently not easily released by washing the suberin with buffer; buffer-washed suberin retained the ability to induce esterase production. This suggests that the bacterium is capable of actively extracting zinc from suberin during growth. A second component associated with the suberin does not induce esterase activity by itself but appears to enhance the induction of esterase by zinc. One possibility is that this second factor may facilitate the uptake of zinc by the bacterium.

The mechanism by which zinc induces esterase activity in *S. scabies* remains to be determined. Zinc was not found as an integral part of the purified esterase as determined by ICP-AES analysis, and dialysis of the purified esterase against EDTA did not affect enzyme activity. This trace element may be important in other aspects of esterase production such as gene transcription (19) or protein secretion.

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