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Production by *Streptomyces viridosporus* T7A of an Enzyme Which Cleaves Aromatic Acids from Lignocellulose†

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The lignocellulose-degrading actinomycete *Streptomyces viridosporus* T7A produced an extracellular esterase when grown in a mineral salts-yeast extract medium. Extracellular esterase activity was first detected during the late stationary phase and typically followed the appearance of intracellular activity. When the organism was grown in lignocellulose-supplemented medium, esterase activity was not increased, but lignocellulose-esterified *p*-coumaric acid and vanillic acid were released into the medium. Polyacrylamide gels showed that several extracellular esterases differing in substrate specificity were produced. Ultrafiltration was used to concentrate the esterase prior to purification. Activity was recovered mostly in the molecular weight fraction between 10,000 and 100,000. Concentrated esterase was further purified by DEAE-Sepharose anion-exchange chromatography to a specific activity 11.82 times greater than that in the original supernatant. There were seven detectable esterase active proteins in the partially purified enzyme solution. Three were similar esterases that may be isoenzymes. The partially purified esterase had a pH optimum for activity of 9.0, a temperature optimum of 45 to 50°C, and a K_m and V_{max} of 0.030 mM and 0.097 μmol/min per ml, respectively, when *p*-nitrophenyl butyrate was the substrate. The enzyme was unstable above 40°C but retained activity when stored at 4 or −20°C. It lost some activity (20%) when lyophilized. Substrate specificity assays showed that it hydrolyzed ester linkages of *p*-nitrophenyl butyrate, α-naphthyl acetate, α-naphthyl butyrate, and lignocellulose. Vanillic and *p*-coumaric acids were identified as products released from lignocellulose. The enzyme is thought to be a component of the lignocellulose-degrading enzyme system of *S. viridosporus*.

Lignin is a complex biopolymer composed of phenylpropane subunits linked by a variety of stable carbon-carbon and carbon-oxygen linkages (10, 18). Lignin is resistant to microbial degradation, although there are known lignin-degrading fungi, actinomycetes, and bacteria (14, 15). Within the actinomycetes, certain mesophilic *Streptomyces* species have been shown to degrade lignin (7, 27, 33), as have several thermophilic *Thermomonospora* species (22, 23). *Streptomyces* species degrade the polysaccharide components of lignocellulose as their primary carbon and energy source as they solubilize lignin (2, 7, 11). Known ligninolytic *Streptomyces* and *Thermomonospora* species degrade lignin oxidatively, solubilizing the lignin as they totally degrade the carbohydrate components of lignocellulose (12, 23). The lignin typically accumulates in the aqueous phase of growth media as a water-soluble, modified lignin polymer (11).

When lignin is degraded by *Streptomyces* species, a substantial loss of its structural integrity is observed both in the insoluble lignin (present in partially degraded lignocellulosic residues) and in the solubilized lignin (9). Chemical changes observed include a reduction in the aromaticity of the polymer and an increase in the number of carboxyl, carbonyl, and phenolic hydroxyl groups in the modified lignin. There is also a significant amount of aromatic ring demethylation and cleavage of aromatic acid-lignin ester linkages (5, 9). Some of the single-ring aromatic intermediates released from lignin by *Streptomyces* species are probably derived from the phenolic acids naturally esterified to the lignin polymer (8). These acids may be hydrolytically cleaved from the polymer by extracellular lignin esterases. The solubilized lignin retains some esterified aromatic acids, but these linkages are slowly cleaved as the *Streptomyces* species continue to attack the modified lignin (5).

Deobald and Crawford (16) have shown that *Streptomyces viridosporus* produces an extracellular coumarate ester esterase that may be involved in hydrolyzing esterified acids from lignin. They did not, however, show whether the enzyme acted on lignin. Other *Streptomyces* species have been found to produce extracellular esterases. Lin and Kolattukudy (19) reported on an esterase of *S. scabies* which acts as a cutinase. MacKenzie et al. (21) have reported the presence of an esterase that releases ferulic acid from wheat bran in *S. olivochromogenes*. McQueen et al. (24, 25) have recently reported the presence of extracellular esterases in several *Streptomyces* species. There have also been some reports of esterases being produced in fungal cellulolytic systems which act with xylanases in hydrolyzing acetylxylan (3, 4).

Here we report on the partial purification and characterization of a novel extracellular esterase of *S. viridosporus* T7A. The esterase activity appears to result from activities of several isoenzymes. It utilizes several ester-containing compounds, including lignocellulose, as substrates. The enzyme releases esterified aromatic acids from lignocellulose.

MATERIALS AND METHODS

Chemicals. Aromatic acids, esterase substrates, and other chemical reagents were obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.; Sigma Chemical Co., St. Louis, Mo.; or J. T. Baker Chemical Co., Phillipsburg, N.J. Yeast extract was from Difco Laboratories, Detroit, Mich. Dialysis tubing (molecular weight [MW] cutoff <10,000) was from Spectrum Medical Industries, Inc., Los Angeles, Calif. The hollow-fiber columns were from Amicon Corp., Danvers,

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Mass., and the DEAE-Sepharose was from Pharmacia, Uppsala, Sweden. Ethylcoumarate was synthesized from *p*-coumaric acid by standard procedures (16). The acetylated xylan was prepared from larch wood by the method of Biely et al. (4).

Culture. *S. viridosporus* T7A (ATCC 39115) was isolated by Sinden (D. L. Sinden, M.S. thesis, University of Idaho, Moscow, 1979). It conforms to the taxonomic description of *S. viridosporus* by Shirling and Gottlieb (31). Stock cultures were maintained on yeast extract-malt extract-dextrose agar (29).

Esterase assay. Extracellular esterase activity was measured spectrophotometrically at 420 nm at 25°C. The assay, which was modified from that of McQueen and Schottel (25), used 80 mM sodium phosphate buffer (pH 7.0). During the reaction, the change in A_{420} was measured at 30-s intervals for 5.5 min after an initial 15-s lag period, on a double-beam spectrophotometer (Spectronic 2000; Bausch & Lomb, Inc., Rochester, N.Y.). One unit of activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol from the substrate per min. The substrate used, unless otherwise specified, was *p*-nitrophenyl butyrate (PNB) dissolved in acetonitrile.

Inducibility of the esterase. Cultures were inoculated with a 0.1% (vol/vol) spore inoculum of *S. viridosporus* in 100 ml of the 0.6% (wt/vol) yeast extract-mineral salts medium in 250-ml flasks. The medium was supplemented with possible esterase inducers including *p*-coumaric acid (0.01%, wt/vol) ferulic acid (0.01%, wt/vol), vanillic acid (0.01%, wt/vol), ground and extracted corn stover lignocellulose (0.1%, wt/vol) (26), milled-wood lignin (MWL; 0.01%, wt/vol) (14), or ethylcoumaric acid (0.01%, wt/vol). A culture grown in unamended medium served as the uninduced control. Flasks were incubated with constant shaking (100 rpm) at 37°C for 5 days. Then cultures were harvested, the cells were collected by filtration, and cell extracts were prepared by using a French pressure cell, as previously described (13). Soluble cell extracts and culture supernatant solutions were immediately assayed for esterase activity as described above. The pH of each culture supernatant solution was also recorded. Each activity value was calculated as the mean of four replicates ± standard deviation. The extracellular enzyme control was uninoculated medium.

Esterase production over time. A spore suspension from one stock slant of *S. viridosporus* T7A was inoculated into a 7.5-liter fermentor (model 43-100; The VirTis Co., Inc., Gardiner, N.Y.) containing 5 liters of 0.6% (wt/vol) yeast extract-mineral salts solution. After 24 to 48 h of aerated incubation at 37°C, when a dense cell suspension had appeared, the culture was transferred to a second 7.5-liter fermentor containing 25 g of sterile, ground, and extracted corn stover lignocellulose. With this fermentor a draught tube (1) was used for aeration to provide aeration with minimal agitation. Inoculation of the second fermentor was recorded at time zero. Samples (100 ml) were removed aseptically from the fermentor every 3 days for 30 days. Each sample was filtered (through glass wool) to remove cells and lignocellulose, and 50 ml of the filtrate was retained for assay of its aromatic acid content. The remaining 50 ml was concentrated to 5.0 ml by use of polyethylene glycol (MW 15,000 to 20,000) incubated at 4°C. Concentrated filtrates, adjusted to an exact 5.0-ml volume, were then assayed for esterase activity. Each sample was analyzed for protein content by the procedure of Lowry et al. (20).

In a second experiment a spore suspension was inoculated into 3 liters of yeast extract-mineral salts solution in the 7.5-liter fermentor. After 24 h the log-phase cells were transferred to an 80-liter fermentor (Lab-Line Bioengineering, Ltd., Melrose Park, Ill.) containing sterile medium of the following composition: 10 g of $MgSO_4$, 10 g of NaCl, 2.5 g of $CaCl_2$, 0.6% (wt/vol) yeast extract, 25 g of ground and extracted corn stover lignocellulose, and 50 ml of the trace elements solution of Pridham and Gottlieb (29) in 50 liters of distilled H_2O. The temperature was maintained at 37°C, and the pH was controlled at 7.1. The agitation rate was 400 rpm. At time zero, a 2-liter sample of medium was withdrawn. At all following sampling times, 1 liter was withdrawn. Three 100-ml portions of each sample (500 ml for time zero) were filtered through preweighed filter paper (no. 54; Whatman, Inc., Clifton, N.J.), and the filters were then air dried and weighed to determine the weight of cell mass present. A 250-ml portion of each sample was filtered through glass wool and concentrated 10-fold by the polyethylene glycol method. These samples were assayed for esterase activity. Cell extracts prepared from cells from 12 of the samples were assayed for esterase (three replicates). Protein determinations (20) were performed on both the cell extracts and the concentrated filtrates. This experiment was repeated once with a medium lacking lignocellulose. Controls included a 28-h filtrate sample and 20-h cell extract sample, each of which was boiled for 15 min before the assay.

Aromatic acid extraction and identification. The single-ring aromatic acids present in culture supernatants after growth of *S. viridosporus* in media containing lignocellulose were identified as follows. The extraction procedure was essentially as described by Crawford (8). Prior to analysis by high-pressure liquid chromatography, acetonitrile was added to each extractive-containing beaker (10 mg of extractives per ml of acetonitrile). This sample was diluted 1:2 with water, filtered through a membrane filter (pore size, 0.45 μm), and then chromatographed (in duplicate). Standards of suspected products were prepared in acetonitrile and diluted 1:2 in water to a final concentration of 0.01% (wt/vol). They included *p*-coumaric acid, gentisic acid, syringic acid, syringaldehyde, vanillic acid, and protocatechuic acid. Two samples, containing 1.0 ml of the day 11 filtrate and 75 μl each of three standards, were also run. One sample contained *p*-coumaric acid, gentisic acid, and vanillic acid, and the other contained syringic acid, syringaldehyde, and protocatechuic acid. The high-pressure liquid chromatography analysis procedure was as described by Pometto and Crawford (28), except that pilot chromatograms were run only at 276 nm. By use of a diode array detector, specific aromatic compounds present in the samples were identified by their retention times, lambda maxima, and UV-visible spectra, as compared with those of authentic standards. The standards had the following retention times and lambda maxima: *p*-coumaric acid, 5.15 min and 308 nm; gentisic acid, 1.13 min and 250 nm; vanillic acid, 3.11 min and 260 nm; syringic acid, 3.57 min and 274 nm; syringaldehyde, 5.33 min and 308 nm; and protocatechuic acid, 0.98 min and 258 nm.

PAGE gel assays. Nondenaturing polyacrylamide gels were run at 25°C with a 7.5% (pH 8.8) resolving gel and a pH 6.8 stacking gel. A tracking dye solution containing sucrose and bromophenol blue was added to the samples. The gels were stained for esterase by a method modified from Rosenberg et al. (30) with α-naphthyl acetate (α-NA) or α-naphthyl butyrate (α-NB) as substrate. Coomassie brilliant blue was used to stain the gels for proteins.

Esterase purification. *S. viridosporus* was grown as described above in the lignocellulose-containing medium in the 80-liter fermentor for 30.5 h. The supernatant was then used

as the source of enzyme for purification. At each purification step, protein concentrations were determined by the Bradford method (6) and esterase activity was measured spectrophotometrically as described above. Nondenaturing PAGE gels were stained with Coomassie blue to determine the purity of the enzyme preparation. Nondenaturing gels were also activity stained for esterase bands with α-NA and α-NB as substrates. All purification steps were performed at 4°C. The steps are described below.

(i) Amicon hollow-fiber concentration. The supernatant solution was concentrated in a hollow fiber column by using two cartridges with differing MW cutoffs. The supernatant was first passed through a column with an MW cutoff of 100,000. The concentrated sample (872 ml) was collected and labeled >100,000. The column was then washed with mineral salts-buffer solution to ensure that protein was not adhering to the column. The supernatant that passed through the membrane was passed through a second column with an MW cutoff of 10,000. The concentrated sample (710 ml) was labeled >10,000. This column was also washed afterwards with mineral salts-buffer solution. The remaining supernatant was labeled <10,000.

(ii) DEAE-Sepharose ion-exchange chromatography. The >10,000 sample, which contained the majority of the esterase activity, was used for ion-exchange chromatography on DEAE-Sepharose. The resin was equilibrated with 60 mM Tris buffer (pH 8.5). After an enzyme sample had been loaded, the column was rinsed with 60 mM Tris (pH 8.5) until the A_{280} of the eluant was <0.100. A salt gradient (0 to 1.0 M NaCl in 60 mM Tris buffer [pH 8.5]) was then set up. Fractions (2.5 ml) were collected and assayed for esterase activity. The >100,000 sample was also passed through a DEAE-Sepharose column, but the buffer was 10 mM Tris (pH 8.0) and the Tris-buffered salt gradient was from 0 to 0.25 M NaCl.

Enzyme characterization. The pH optimum for activity of the partially purified esterase was assayed over a pH range of 4.0 to 9.0, in increments of 0.5, and also at pH 10.0. To a test tube, 2.67 ml of 30 mM buffer (10 mM sodium acetate, 10 mM sodium phosphate, 10 mM ammonium chloride) of known pH was added, along with 300 μl of the partially purified enzyme. The reaction was initiated by addition of 30 μl of PNB. The solution was mixed and incubated at 25°C for 3 min. Reaction rates were constant over this period. At 1, 2, and 3 min, 300 μl of sample was removed and transferred to 1.0 ml of 80 mM sodium phosphate buffer (pH 7.0), and the A_{420} of this solution was immediately measured. Owing to interference caused by the chemical hydrolysis of the substrate, neither acid nor base could be added to the sample. Therefore, the measurements were taken while the reaction was in progress. Controls at each pH substituted buffer for enzyme. The rate of activity at each pH was calculated in units per milliliter (triplicate samples).

Triplicate samples were assayed at temperatures between 20 and 60°C to determine the temperature optimum. Each sample contained 2.67 ml of 80 mM sodium phosphate buffer (pH 7.0) and 300 μl of partially purified enzyme equilibrated to the appropriate temperature. PNB substrate (30 μl) was added to initiate the reaction, and activity was then measured as described above.

The esterase stability was tested at 20 to 60°C. At each temperature, 2.67 ml of 80 mM sodium phosphate buffer (pH 7.0) and 300 μl of partially purified enzyme were incubated for 1 h. The samples were then equilibrated to 50°C (the temperature optimum for activity) and assayed for activity in triplicate.

The K_m and V_{max} of the partially purified enzyme were determined by measuring the reaction rate for 60 s at 1.0-s intervals, including an initial 15-s lag period, at different substrate (PNB) concentrations (0.04 to 0.8 mM). The Lineweaver-Burk equation was used to determine the K_m and V_{max} values.

Substrate specificity. Spectrophotometric assays for esterase activity were run with the following *p*-nitrophenyl substrates at a final concentration of 0.4 mM: *p*-nitrophenyl acetate (PNA), PNB, and *p*-nitrophenyl palmitate (PNP). PNA solutions contained 0.725 mg of PNA per 10.0 ml of acetonitrile. PNB solutions contained 18 μl of PNB per 2.5 ml of acetonitrile. PNP-buffer solutions were prepared by heating a mixture of 16 mg of PNP, 0.37 g of Triton X-100, and 1.0 ml of *t*-butanol until the PNP dissolved. This volume was brought up to 100 ml by the addition of 80 mM sodium phosphate buffer (pH 7.0). When PNP was used as substrate, the assay was run with 2.7 ml of the PNP-buffer solution in 300 μl of enzyme solution. Activity (release of *p*-nitrophenol) was monitored at 420 nm for all substrates.

Other potential esterase substrates were also tested, including ethylcoumarate, MWL, acid-precipitable polymeric lignin (APPL) previously produced from corn stover lignocellulose by *S. viridosporus* T7A (11), and ground and extracted corn stover lignocellulose. A 1-ml portion of partially purified esterase was incubated with 4.0 ml of substrate solution or suspension for 12 h with constant shaking (100 rpm) at 30°C. The substrate preparations were in 4.0 ml of 80 mM sodium phosphate buffer (pH 7.5) and included 0.5 g of ground and extracted corn stover lignocellulose, 30 mg of MWL, 100 mg of APPL, or 10 mg of ethylcoumarate. Controls substituted buffer for enzyme, and assays were run in triplicate. After incubation, the pH of each reaction mixture was lowered to pH 2 with concentrated H_2SO_4. Each acidified mixture was extracted with ether (two 5.0-ml extractions) and ethyl acetate (two 5.0-ml extractions). The extractive-containing solvents were dewatered over anhydrous sodium sulfate and transferred to screw-cap tubes, and the solvents were allowed to evaporate. The residues were then analyzed for aromatic acid products by gas chromatography (28). Potential products of esterase action included *p*-coumaric acid, ferulic acid, vanillic acid, and/or *p*-hydroxybenzoic acid. Standard solutions of each were prepared in absolute methanol at various concentrations. Each was then analyzed in the same manner as unknowns, and a standard curve was prepared for each.

Acetylated xylan from larch wood was tested as a substrate by a modified method of Biely et al. (4). A 2% (wt/vol) suspension of acetylated xylan in 0.4 M phosphate buffer was added to an equal volume of the partially purified esterase and incubated for 12 h at 32°C with shaking at 260 rpm. Any acetic acid released was then measured spectrophotometrically as described by Holz et al. (17) with an acetate kinase-hydroxylamine assay system. The samples were run in quadruplicate, and the controls were run in duplicate. In control A, buffer was substituted for the partially purified esterase. In control B, heat-denatured esterase was used. In control C, pH 10.5 buffer was substituted for esterase. This control was incubated with shaking at 260 rpm at 37°C for 1 h and then assayed for acetic acid. Control D was a 0.1-ml supernatant sample from control A spiked with 0.1 ml of acetic acid standard. Control E contained 0.1 ml of acetic acid in 0.1 ml of control B supernatant solution. These last two controls were used only in the acetate kinase assay. The esterase activity in the

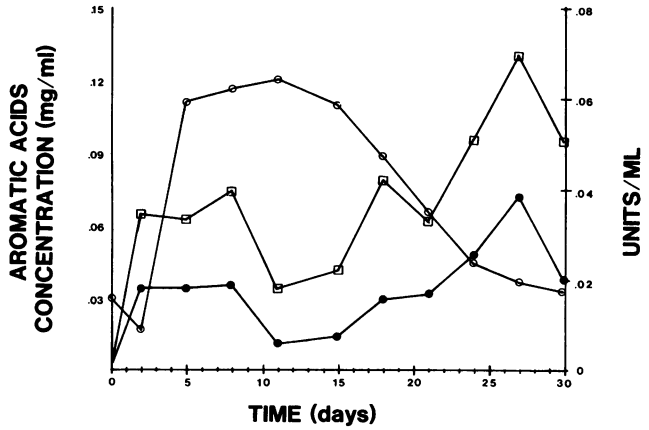

FIG. 1. Extracellular esterase activity and aromatic acid production over time in lignocellulose-containing medium. Symbols: ○, units of extracellular activity per ml □, *p*-coumaric acid concentration (milligrams per milliliter); ●, vanillic acid concentration (milligrams per milliliter).

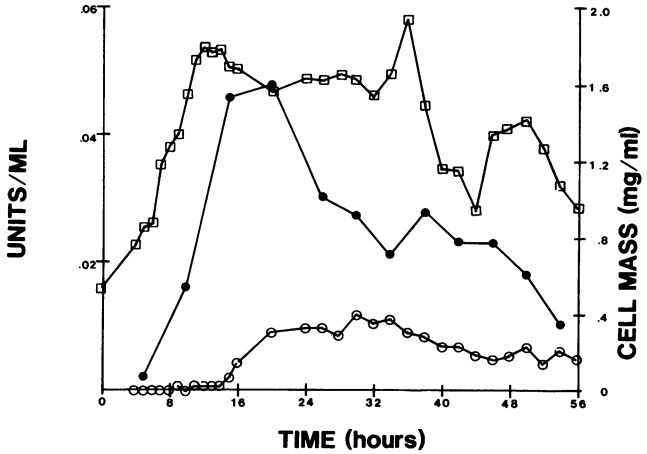

FIG. 2. Cell mass versus esterase production in the 80-liter fermentor over time in medium containing lignocellulose. Symbols: ○, extracellular esterase activity; ●, intracellular esterase activity; □, cell mass.

samples and in controls A and B was measured before and after the assay.

RESULTS

Esterase assay. When PNB was used as the substrate in the enzyme assay at 25°C, the nonenzyme controls showed no detectable esterase activity over the 5.5-min assay period. If the reaction mixture was incubated for several additional minutes, the controls showed a low level of activity. Therefore, a nonenzyme control (buffer substituted for enzyme) was routinely incorporated into the standard assay to correct activity values for any nonenzymatic release of *p*-nitrophenol. Because the rate of nonenzymatic release increased further at 37°C, the standard assay was performed at 25°C.

Inducibility of the esterase. PNB esterase activity was detected after 5 days in both cell extracts and 10-fold-concentrated extracellular filtrates of cultures grown with or without potential inducers. Analysis of variance ($P < 0.05$) testing in a completely randomized design revealed no statistically significant differences between the esterase activities in either the cell extracts or the culture filtrates for any of the compounds tested as potential inducers.

Esterase production over time. In the 7.5-liter fermentor culture, where cells were grown in medium supplemented with lignocellulose, there was a sudden appearance of extracellular PNB esterase activity after 2 days of incubation (Fig. 1). Activity remained high through day 15, after which it gradually decreased through day 30. In unconcentrated supernatant samples, the extracellular protein concentration fluctuated between 0.73 and 1.69 mg/ml, with no distinct pattern.

Intracellular esterase activity correlated with cell growth and peaked as the cells entered the stationary phase (Fig. 2). This was followed by a gradual decline in activity. Extracellular esterase activity was not detected until the cells had entered the stationary phase, but it then remained fairly constant until the cells entered the death phase. In a second run, but with medium lacking lignocellulose, similar results were observed. Extracellular esterase activity appeared at 20 h, peaked at 28 h, and then gradually decreased until 42 h, after which it remained steady through the remainder of the experiment. Intracellular esterase activity was detected at 5 h, peaked after 30 h, and then gradually decreased through 42 h. A second increase of activity followed, peaking at 54 h. Boiled controls showed no activity. Cell mass increased gradually until hour 13, when it peaked at 1.3 mg/ml. This was followed by a slow decrease through hour 56, when the cell mass had declined to 0.4 mg/ml.

Aromatic acid extraction and identification. Both *p*-coumaric acid and vanillic acid were detected by high-pressure liquid chromatography in culture supernatant samples taken as early as day 2. There was an erratic but steady increase in their concentrations through day 30 of the incubation (Fig. 1). *p*-Coumaric acid was the dominant compound present in solvent extracts. Vanillic acid was also present. Vanillic acid and *p*-coumaric acid, which were present on day 27, were absent on day 0 (Fig. 3). The UV-visible spectra of both *p*-coumaric and vanillic acids in the culture supernatant extracts, as recorded by the diode array detector, were identical to those of the corresponding standards (Fig. 3). Other unidentified compounds were present in small amounts. Both *p*-coumaric acid and vanillic acid showed the same pattern of accumulation in that both peaked in accumulated concentration on day 27 (Fig. 1), at vanillic acid and *p*-coumaric acid concentrations of 0.073 ± 0.002 and 0.131 ± 0.002 mg/ml, respectively.

PAGE gel assays. PAGE gel esterase staining assays showed that there were substrate specificity differences among the several esterase bands. When α-NA was the substrate for a 100-fold-concentrated crude enzyme sample, seven esterase bands were present (Fig. 4). However, only five of these bands reacted with α-NB (Fig. 5). The bands hydrolyzing α-NB were also reactive with PNB. The PNB-reactive esterase was chosen for purification since it possessed the broadest substrate specificity.

Esterase purification. The starting supernatant had 0.001 U of esterase activity per ml. Its protein concentration was 0.02 mg/ml. Specific PNB-esterase activity was 0.062 U/mg of protein, and total activity was 78.94 U. Ultrafiltration concentrated the activity in the >10,000 fraction (Table 1). The buffer washings contained insignificant activity, an indication that the enzyme was not adhering to the hollow-fiber membranes. Ultrafiltration resulted in a 1.9-fold purification of the enzyme. When native PAGE gels were activity stained for esterase with α-NA and α-NB as substrates, they

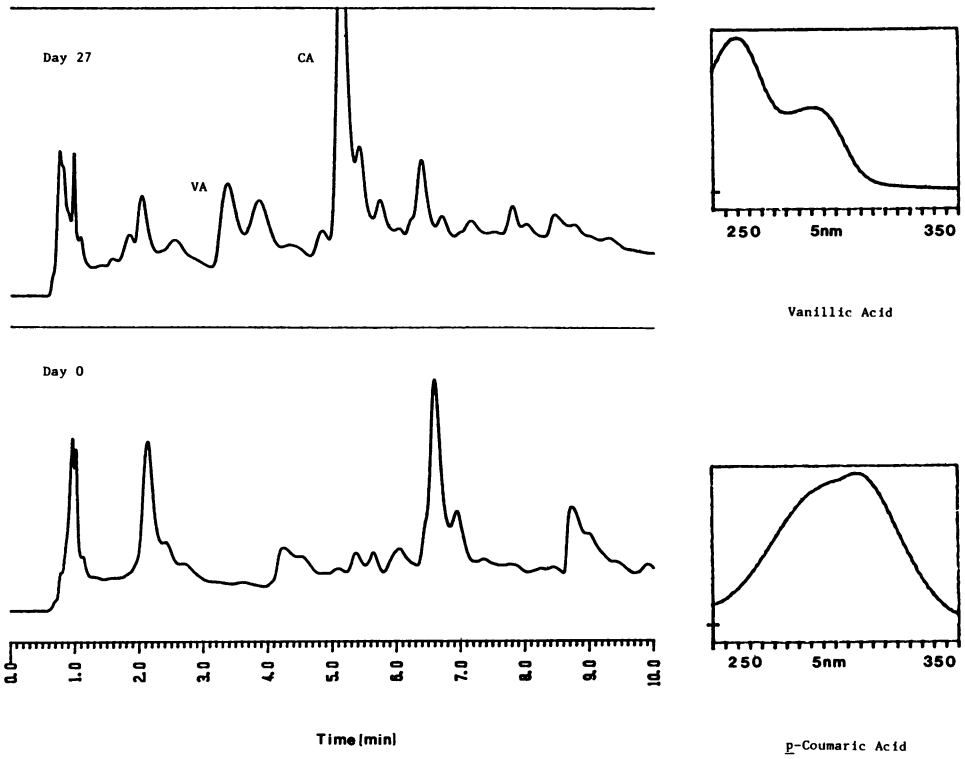

FIG. 3. UV spectra of selected aromatic acids.

showed that all of the esterase bands were present in both the >100,000 and >10,000 fractions. Numerous protein bands were observed in the >10,000 fraction when the gels were stained with Coomassie blue. Although the esterases were present in low concentrations, they appeared to be among the major extracellular protein bands detected by Coomassie blue staining.

Active fractions were eluted from the DEAE-Sepharose column when the NaCl concentration reached 0.19 M. When pooled, these fractions had a protein concentration of 0.28 mg/ml and contained 85.75% of the original esterase activity (Table 1). On native PAGE gels activity stained for α-NA esterase, seven active protein bands were detected in the partially purified enzyme preparation. Three of the bands also hydrolyzed α-NB. Coomassie blue protein stains of the native gels revealed only four protein bands (Fig. 6).

Enzyme characterization. The pH curve for activity of the partially purified esterase showed little activity in the low pH range, but activity was high at high pHs. The pH optimum was 9.0 with PNB as the substrate. The activity-versus-temperature curve for the partially purified enzyme showed a broad temperature optimum at 45 to 50°C. An examination of the temperature stability of the enzyme showed that after 1 h of incubation at 20, 30 or 40°C, the esterase still retained more than 85% of its original activity. However, after incubation for 1 h at 50°C or higher, all activity was lost. As

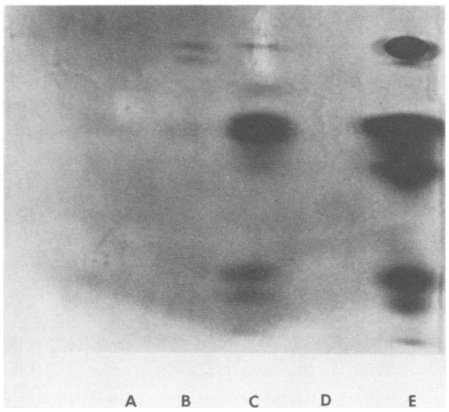

FIG. 4. Nondenaturing polyacrylamide gel stained for esterase activity with α-NA as the substrate. Lanes: A, original filtrate; B, >100,000 fraction; C, >10,000 fraction; D, <10,000 fraction; E, eluant from the DEAE-Sepharose ion-exchange column.

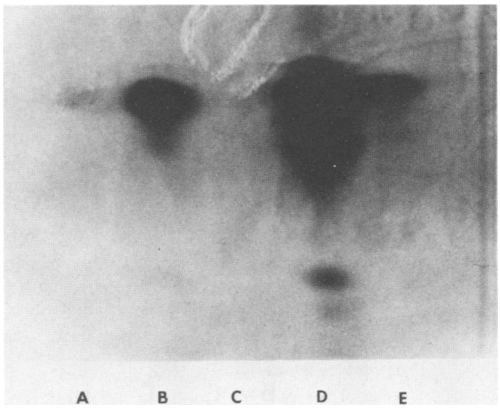

FIG. 5. Nondenaturing polyacrylamide gel stained for esterase activity with α-NB as the substrate. Lanes: A, original filtrate; B, >100,000 fraction; C, <10,000 fraction; D, >10,000 fraction; E, eluant from the DEAE-Sepharose ion-exchange column.

TABLE 1. Purification results for *S. viridosporus* extracellular esterase purified by ultrafiltration and ion-exchange chromatography

Procedure	Vol (ml)	Total activity (U)	Sp act (U/mg of protein)	% Recovery	Purification (fold)
Original filtrate	53,000	78.94	0.06	100	1
Hollow-fiber filtrates					
>100,000	872	16.14	0.08	20.45	1.30
>100,000 BW[a]	709	0.45	0.04	0.57	0.68
>10,000	710	53.93	0.12	68.32	1.91
>10,000 BW[a]	1,089	3.94	0.11	4.99	1.71
<10,000	49,620	0.00	0.00	0.00	0.00
DEAE-Sepharose	229.5	46.24	0.73	58.58 (85.75)[b]	11.82

[a] BW, Buffer wash.
[b] Percent recovery of the esterase actually loaded on to the ion-exchange column.

determined by Lineweaver-Burk analysis, the K_m for the partially purified esterase was 0.030 ± 0.004 mM with PNB as the substrate. The V_{max} was 0.097 ± 0.003 μmol/min per ml when 300 μl of partially purified esterase was added to a total volume of 3.0 ml of reaction mixture.

Substrate specificity. Substrate specificities of the partially purified esterase are shown in Table 2. The enzyme was tested separately for activity on PNA, PNB, and PNP. It cleaved only the ester linkage in PNB. This indicates that the multiple PNB-specific bands present in the partially purified esterase, as observed on PAGE gels, probably represent isoforms of the same enzyme. MWL, APPL, and ethylcoumarate were not substrates for the partially purified esterase under the conditions used. Lignocellulose, however, did serve as a substrate. Vanillic acid and *p*-coumaric acid were released from the lignocellulose on incubation with the esterase for 12 h (0.021 ± 0.001 and 0.032 ± 0.001 mg/ml, respectively), whereas the no-enzyme control showed the release of only background levels of the two acids (0.010 and 0.016 mg/ml, respectively). Acetylxylan did not serve as a substrate for the partially purified esterase (Table 3). The controls showed that the xylan was adequately acetylated, since acetic acid was released from the xylan at pH 10.5 in the absence of enzyme by base-mediated chemical hydrolysis. Acetic acid in the spiked no-enzyme sample was also readily detected. Thus, if acetic acid had been produced by action of the esterase, it would have been detected. Active PNB-esterase was still present in the assay solution after the acetylxylan assay; however, its activity had decreased by 68%.

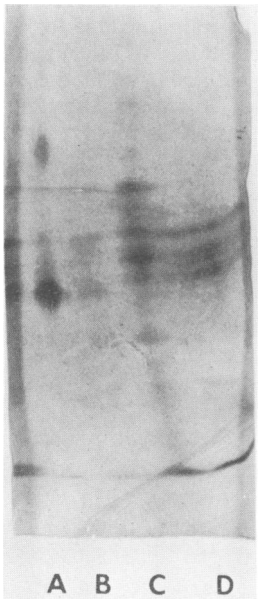

FIG. 6. Coomassie blue protein stain on a native polyacrylamide gel. Lanes: A, bovine serum albumin protein standard; B, original filtrate; C, >10,000 fraction from the hollow-fiber concentration step; D, eluant from the DEAE-Sepharose ion-exchange column.

TABLE 2. Substrate specificity of the partially purified esterase

Substrate	Reactivity	Units/ml
PNA	−	0.001 ± 0.000
PNB	+	0.101 ± 0.006
PNP	−	0.009 ± 0.001
α-NA	+	
α-NB	+	
Lignocellulose	+	
APPL	−	
MWL	−	
Ethyl coumarate	−	
Acetylxylan	−	

DISCUSSION

The partially purified esterase utilized lignocellulose as a substrate, releasing esterified *p*-coumaric acid and vanillic acid. This was true even though neither lignocellulose or any other compound tested acted as an inducer of the esterase. The esterase did not remove esterified aromatic acids from MWL or APPL. It is possible that APPL was a poor substrate because most of the readily available aromatic acid ester bonds had already been cleaved during initial production of APPL by *S. viridosporus* (5, 11). The reason for the lack of activity on MWL is less clear. MWL is a purified form of native lignin that does contain esterified aromatic acids, but MWLs are essentially carbohydrate free. The type of specific ester bonds present in the MWL may differ from those of intact lignocellulose. Aromatic acids, such as ferulic acid, are known to be esterified to hemicelluloses in some grasses (32). Therefore, it is possible that the esterase of *S. viridosporus* preferentially hydrolyzes aromatic acid ester bonds within hemicelluloses rather than lignin. Additional research is needed to determine the exact nature of the linkages that this esterase attacks in native lignocellulose. The release of aromatic acids from lignocellulose, however, indicates that the partially purified enzyme possesses aromatic acid-ester esterase activity.

TABLE 3. Esterase assay with acetylxylan as substrate

Sample[a]	A_{492}
Partially purified esterase	0.025 ± 0.007
Control A	0.033 ± 0.003
Control B	0.034 ± 0.002
Control C	0.304 ± 0.001
Control D	0.077 ± 0.000
Control E	0.082 ± 0.001

[a] For ingredients of controls A to E, see Materials and Methods.

Because the esterase was not released from the cell until the stationary phase of growth, it is probably not involved in the primary degradation of lignocellulose by *S. viridosporus*. The organism utilizes other readily available nutrients such as cellulose or hemicellulose for its primary growth (8a). Breakage of aromatic ester bonds in the lignocellulose might, however, still be beneficial, since it might expose more cellulose or hemicellulose to hydrolytic enzymes (K. Grohmann, H. E. Himmel, D. Mitchell, B. Dale, and H. Schroeder, Abstr. 10th Symp. Biotechnol. Fuels Chemicals, paper no. 4, 1988). If so, this would be advantageous to cells entering the stationary phase and running out of readily available cellulosic carbon. Alternatively, it is possible that the esterase actually has some totally unrelated intracellular function, since the intracellular esterase was present in higher concentrations than the extracellular esterase. Intracellular activity first appeared during the log phase and continued to increase until the cells entered the stationary phase. In contrast, the appearance of extracellular activity lagged and was not detected until the stationary growth phase. Therefore, since the cells enter the stationary phase and esterase is excreted extracellularly, the esterase may fortuitously attack aromatic acid esters in the lignocellulose. Even if this is so, the enzyme appears to be involved in the lignocellulose-degradative process.

It was interesting that both fractions from the hollow-fiber concentration step contained all of the esterase bands; however, different conditions were needed to elute the esterases from the ion-exchange column. For elution, the >10,000 fraction required a more alkaline buffer of higher ionic strength (60 mM Tris [pH 8.5]) than did the >100,000 fraction (10 m*M* Tris [pH 8.0]). This suggests there was something else present in the medium in the >100,000 fraction which was competing for the charged sites on the resin or that the enzymes in this fraction had different physical properties.

The three α-NB esterase proteins present in the partially purified enzyme stained on native PAGE gels in close proximity to one another. They are probably of similar MW and charge, because on a native gel proteins migrate according to these two properties. Ion-exchange chromatography also separates proteins according to their charge, and these three esterases eluted off the column at essentially the same time. Therefore, it is likely that the proteins are isozymes. Further purification and characterization will be required to confirm this possibility. Although the esterases had to be concentrated for detection in routine assays, Coomassie blue protein staining indicated the esterase to be a major protein component in culture fluids. Therefore, this enzyme may be similar to the first extracellular fungal ligninases discovered (34), which were initially found only after culture supernatants had been concentrated 100-fold. The cumulative data indicate that this esterase is a peripheral but participatory component of the lignocellulose-degrading enzyme system of *S. viridosporus*.

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