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# Biochemical and Molecular Characterization of the Extracellular Esterase from *Streptomyces diastatochromogenes*

CORNELIA TESCH,[1] KLAUS NIKOLEIT,[1] VOLKER GNAU,[2] FRIEDRICH GÖTZ,[1] AND CHRISTIANE BORMANN[1]*

*Mikrobielle Genetik[1] and Institut für Organische Chemie,[2] Universität Tübingen, D-72076 Tübingen, Germany*

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**An esterase of *Streptomyces diastatochromogenes* was purified to homogeneity from culture filtrate. The purified enzyme had a molecular mass of 30,862 ± 5.8 Da, as determined by electrospray mass spectrometry. The esterase-encoding gene was cloned on a 5.1-kb *Mbo*I fragment from *S. diastatochromogenes* genomic DNA into *Streptomyces lividans* TK23 by using plasmid vector pIJ702. Nucleotide sequence analysis predicted a 978-bp open reading frame, *estA*, encoding a protein of 326 amino acids, a potential ribosome binding site, and a putative 35- or 36-residue signal peptide for secretion in *S. lividans* or *S. diastatochromogenes*, respectively. The transcriptional initiation site was mapped 29 nucleotides upstream from the predicted translational start codon of *estA* in *S. diastatochromogenes*. The protein sequence deduced from the *estA* gene was similar to that of the esterase from the plant pathogen *Streptomyces scabies*. Both enzymes lacked the conserved motif GXSXG carrying the active-site serine of hydrolytic enzymes. A serine modified by [1,3-$^3$H]diisopropyl fluorophosphate was located at position 11 of the mature enzyme in the sequence GDSYT. This finding and results obtained by site-directed mutagenesis studies indicate that serine 11 may be the active-site nucleophile.**

Streptomycetes are gram-positive, saprophytic soil microorganisms that use a wide variety of extracellular hydrolytic enzymes, including chitinases, cellulases, xylanases, proteases, lipases, and nucleases (50), to degrade organic material in the soil. Polysaccharidases, proteases, and enzymes exhibiting unusual catalytic activities have been studied extensively (35). However, except for phospholipase D, only a few streptomycete lipolytic enzymes and their corresponding genes have been analyzed so far, although Sztajer et al. (46) reported high lipolytic activity in *Streptomyces* strains. An esterase secreted by the plant-pathogenic strains of *Streptomyces scabies* has been characterized at the protein (31) and DNA (40) levels. The enzyme is believed to be important in pathogenicity and penetration of *S. scabies* by hydrolyzing ester bonds in suberin, a waxy polyester covering the external portions of the plants. Recently, genes encoding extracellular lipases from *Streptomyces* sp. strain M11 (36) and *Streptomyces albus* G (17) have been cloned and sequenced.

Esterases and lipases are carboxylic ester hydrolases (EC 3.1.1). The carboxylesterases (EC 3.1.1.1) hydrolyze water-soluble or emulsified esters with relatively short fatty acid chains, whereas lipases (triacylglycerol acyl hydrolases; EC 3.1.1.3) preferentially act on emulsified substrates with long-chain fatty acids. The hydrolytic mechanism of most of the known esterases and lipases resembles that of serine proteases. These hydrolases all contain a similar catalytic triad, generally consisting of a nucleophilic serine residue that acts in conjunction with a histidine and an aspartic acid residue (8, 10, 18, 51). Microbial lipolytic enzymes have become biotechnologically important enzymes used for hydrolysis and synthesis of esters and transesterification reactions with a wide range of applications (7, 27). To investigate lipolytic enzymes from streptomycetes, we screened *Streptomyces* strains on agar plates for extracellular lipolytic activity toward Tween 20, olive oil, and egg yolk. For further studies, *Streptomyces diastatochromogenes* Tü20, which exhibited high hydrolytic activity toward Tween 20, was selected. Here, we report the purification and biochemical properties of an extracellular esterase from *S. diastatochromogenes* and the cloning and characterization of the encoding *estA* gene. Sequence analysis revealed that the deduced protein lacks the motif GXSXG that contains the active-site serine residue conserved in most esterases and lipases (11). We describe the consequences of changing selected amino acid residues by site-directed mutagenesis and present evidence by chemical modification of the esterase that serine 11 of the mature enzyme within the sequence GDSYT may be the active site nucleophile.

* Corresponding author. Mailing address: Mikrobielle Genetik, Universität Tübingen, Ob dem Himmelreich 7, D-72074 Tübingen, Germany. Phone: 49-07071-294640. Fax: 49-07071-87815.

## MATERIALS AND METHODS

**Bacterial strains, plasmid and cloning vectors, and cultivation conditions.** *S. diastatochromogenes* Tü20 was obtained from H. Zähner (University of Tübingen, Tübingen, Germany). *Streptomyces lividans* TK23 (24) was kindly provided by D. A. Hopwood (John Innes Institute, Norwich, England). *Escherichia coli* JM83 (52) was used as host for cloning procedures. Plasmids used in this study are listed in Table 1.

For esterase production, liquid medium consisting of 1% mannitol and 1% soy peptone (pH 7.2) was used. For purification of the esterase, 100-ml cultures were grown for 36 h in 500-ml Erlenmeyer flasks on a rotatory shaker at 100 rpm. The inoculum was 1% of a preculture grown under the same conditions. Esterolytic activity of *S. lividans* transformants was detected by screening for zones of hydrolysis around colonies growing on HA plates, which consisted of 0.4% glucose, 1% malt extract, 0.4% yeast extract, 1% Tween 20, and 1.8% agar (pH 7.2). For preparation of protoplasts and DNA isolation, *Streptomyces* strains were grown in CRM (10.3% sucrose, 2% tryptic soy broth, 1% yeast extract [pH 6.6] [37]) liquid medium. For protoplast regeneration, R3 agar plates were used (43). Thiostrepton was added to solid media at 30 μg/ml and to liquid media at 10 μg/ml for selective pressure when recombinant strains were grown. Thiostrepton was a gift from S. J. Lucania (E. R. Squibb & Sons, Princeton N.J.). *Streptomyces* strains were grown at 27°C. *E. coli* strains were grown at 37°C in LB liquid medium and on LB agar plates (41) supplemented with ampicillin (100 μg/ml).

**General protein techniques.** Proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using a 12% polyacrylamide resolving gel and a 4% polyacrylamide stacking gel (29). Molecular weight markers were obtained from Bio-Rad and Sigma. Proteins were visualized in the gels by staining with Coomassie brilliant blue. When activity staining was performed, the sample applied to the gel was not boiled, and after electrophoresis, SDS was removed by washing the gel for 20 min with 20% isopropanol and twice

TABLE 1. Plasmids used in this study[a]

| Plasmid | Relevant characteristics | Source and/or reference |
|---|---|---|
| pIJ702 | 5.686 kb, HC, Thio$^r$, Mel$^+$ | D. A. Hopwood (24) |
| pWHM3 | 7.2 kb, *E. coli-Streptomyces* shuttle vector, HC, Thio$^r$, Amp$^r$ | C. R. Hutchinson (47) |
| pUC19 | 2.686 kb, HC, Amp$^r$ | Yanish-Perron et al. (52) |
| pCT1 | 10.8 kb, pIJ702 with 5.1-kb *Mbo*I cloned DNA fragment from *S. diastatochromogenes* Tü20, confers strong Tween 20-hydrolyzing activity in *S. lividans* | This work |
| pUCT7 | 5.39 kb, pUC19 with a 2.7-kb *Pst*I fragment from pCT1 (carrying part of pIJ702 as well as 2.289 kb of *S. diastatochromogenes* DNA containing the *estA* gene), the direction of *estA* transcription is opposite that of p*lacI* | This work |
| pUCT11 | 5.39 kb, pUCT7 carrying the Ser-11→Cys mutation in *estA* | This work |
| pUCT56 | 5.39 kb, pUCT7 carrying the Ser-56→Cys mutation in *estA* | This work |
| pUCT113 | 4.83 kb, pUCT7 without *Eco*RV fragments, carrying the Thr-113→Ala mutation in *estA* | This work |
| pUCT130 | 4.83 kb, pUCT7 without *Eco*RV fragments, carrying the Ser-130→Cys mutation in *estA* | This work |
| pWCT11 | 9.84 kb, pWHM3 carrying the 2.64-kb *Sph*I fragment from pUCT11 with the mutated *estA* | This work |
| pWCT56 | 9.84 kb, pWHM3 carrying the 2.64-kb *Sph*I fragment from pUCT56 with the mutated *estA* | This work |
| pWCT113 | 9.28 kb, pWHM3 carrying the 2.08-kb *Sph*I fragment from pUCT113 with the mutated *estA* | This work |
| pWCT130 | 9.28 kb, pWHM3 carrying the 2.08-kb *Sph*I fragment from pUCT130 with the mutated *estA* | This work |

[a] Abbreviations: HC, high-copy-number plasmid; Thio$^r$, thiostrepton resistance; Amp$^r$, ampicillin resistance; Mel$^+$, production of melanin.

for 10 min with distilled water. The gel was then transferred onto a plate containing 1% Tween 20, 25 mM Tris-HCl (pH 8.0), and 1.3% agar and incubated for approximately 3 h at 37°C. Hydrolysis of Tween 20 was detected by bands of precipitated fatty acids. Activity staining for proteolytic activity was performed by incubating the gel for approximately 3 h at 37°C on a plate consisting of 0.25% Hammersten casein, 0.5% peptone, 0.3% meat extract, 0.5% NaCl, 0.015% $Ca(OH)_2$, 0.005% $CaCl_2$, and 1.3% agar. Proteolytic activity was detected by clearing bands of casein hydrolysis. Protein concentrations were measured according to Bradford (9), using bovine serum albumin as a standard. For N-terminal sequencing of proteins separated by SDS-PAGE, proteins were transferred onto an 0.2-μm-pore-size polyvinylidene difluoride membrane (Immobilon P; Millipore) and sequenced in a model 477A pulsed-liquid protein sequencer (Applied Biosystems) equipped with a model 120A on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems).

**Measurement of lipolytic activity.** Esterase activity was measured with *p*-nitrophenyl caprylate (PNC), *p*-nitrophenyl laurate, and *p*-nitrophenyl palmitate. The reaction was started by adding supernatant from liquid cultures or enzyme preparations to 400 μl of 25 mM Tris-HCl (pH 9.0)–0.1% Triton X-100 plus 5 mM substrate dispersed by sonication. The total volume of the reaction mixture was 500 μl. The amount of *p*-nitrophenol liberated during the reaction was monitored at 25°C by determination of the $A_{405}$. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of *p*-nitrophenol per min at 25°C. To determine the hydrolytic activities of the purified esterase with lipase and phospholipase A1 substrates, we used (*R*,*S*)-2,3-dibutyryl dithiopropanol 1-butyrate and 2-hexadecanoylthiopropane 1-phosphocholine, respectively, as described by Nikoleit et al. (32). Both substrates were kindly provided by H. M. Verheij (University of Utrecht, Utrecht, The Netherlands). Determination of the pH optimum of the purified esterase was performed in esterase reaction buffer containing 0.1% Triton X-100, 5 mM PNC, and 25 mM BisTris adjusted to pH 5.5 to 7.0 with HCl or 25 mM Tris adjusted to pH 7.0 to 9.0 with HCl. The effects of metal ions and of inhibitors were tested against the PNC-hydrolyzing activity of purified esterase. For the latter assays, the enzyme was preincubated with 1 mM inhibitor for 30 min at 24°C, with phenylmethylsulfonyl fluoride (PMSF) for 30 min at 50°C, and with 5 mM diisopropyl fluorophosphate (DFP) for 60 min at 24°C before the residual enzyme activity was measured.

**Purification of the esterase.** Mycelia from a 1-liter culture were removed by centrifugation, and protein in the supernatant was precipitated at 80% saturation with solid ammonium sulfate. After centrifugation (20 min at 12,000 × *g*), the precipitate was redissolved in buffer A (20 mM Tris-HCl [pH 8.0]) and dialyzed overnight against the same buffer. The dialyzed enzyme solution was applied to a DEAE-Sepharose fast-flow column (3.2 by 15 cm) equilibrated with buffer A. Esterase was eluted with a linear gradient of 0 to 1 M NaCl–buffer A, using a flow rate of 2.5 ml/h. The active fractions eluted at 0.5 M NaCl were pooled, dialyzed against buffer A, and lyophilized. Protein was resuspended in buffer A and chromatographed on a gel filtration column (Ultrogel AcA44; 2.3 by 45 cm) equilibrated with buffer A, using a flow rate of 9 ml/h. Esterase-containing fractions were pooled and further purified by chromatography on an anion-exchange column (MonoQ HR10/10; Pharmacia), using a linear gradient of 0 to 1 M NaCl–buffer A. Active fractions eluted at 0.4 M NaCl and were adjusted to 2 M $(NH_4)_2SO_4$ and chromatographed on a hydrophobic-interaction column (phenyl-Superose, HR5/5; Pharmacia), using a linear gradient of 2 to 0 M $(NH_4)_2SO_4$–buffer A. Esterase-containing fractions eluted at 0.05 M $(NH_4)_2SO_4$ and were lyophilized, redissolved in 1 ml of buffer A, and desalted by chromatography on a gel filtration column (Superdex 75, Hiload 16/60; Pharmacia). Purification was monitored by activity assays and SDS-PAGE. All protein purification steps were performed at 4°C.

For determination of the molecular mass by electrospray mass spectrometry, the esterase solution was adjusted to 0.5% trifluoroacetic acid and chromatographed on a reverse-phase column (μRPC C2/C18, SC2.1/10), using a linear gradient of 0 to 50% acetonitrile–0.5% trifluoroacetic acid in 3.8 ml with a flow rate of 200 μl/min. The esterase was collected by the peak detection method, dried with a vacuum concentrator, and dissolved in a concentration of 0.01 to 0.1 of mg protein per ml in double-distilled water. Samples (5 to 20 μl) were kindly analyzed by J. Metzger, Organische Chemie, Universität Tübingen, on an API III triple-quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray source (Sciex, Thornhill, Ontario, Canada) and operated in a positive ion mode.

**Chemical modification of esterase.** Esterase (about 1.6 nmol) in supernatant from *S. lividans*(pCT1) was modified by incubation with 10.5 nmol of [1,3-$^3$H]DFP (specific activity, 222 MBq/μmol) in 400 μl for 90 min at 24°C. After addition of 600 μl of distilled water, incubation was continued for 90 min. Protein in the incubation mixture was transferred onto a polyvinylidene difluoride membrane of a ProSpin column (Applied Biosystems) by centrifugation (five times) for 15 min at 5,600 × *g* and was subjected to sequence analysis using a model 477A protein sequencer (Applied Biosystems) equipped with a model 120A on-line phenylhydantoin amino acid analyzer (Applied Biosystems). The amount of esterase analyzed by Edman degradation was 90 pmol. The fraction of each cycle was collected, and ethyl acetate was added to a final volume of 100 μl. Radioactivity of samples (5 μl) was measured with a model LS1801 Beckman liquid scintillation spectrometer.

**General DNA techniques.** Recombinant DNA techniques for *Streptomyces* spp. were performed as described by Hopwood et al. (24). Total DNA from *Streptomyces* cells was isolated by the large-scale method reported by Hunter (25). To clone the esterase-encoding gene from *S. diastatochromogenes*, total DNA was partially digested with *Mbo*I, and 5- to 10-kb fragments were ligated with the multicopy plasmid pIJ702 that was cleaved at the unique *Bgl*II site within the *mel* gene. The ligation mixture was used to transform *S. lividans* TK23. Thiostrepton-resistant Mel$^-$ transformants were grown for 48 h on Tween 20-containing complex medium plates. Colonies hydrolyzing Tween 20 produced zones of precipitated fatty acids. The halos of Tween 20 hydrolysis from *S. lividans* TK23 colonies were visible only after several days of incubation. Standard procedures were used for *E. coli* (41). *E. coli* cells were transformed by electroporation (22) using a Gene Pulser apparatus (Bio-Rad, Munich, Germany). Plasmid DNA was isolated by using Nucleobond AX100 columns (Macherey-Nagel, Düren, Germany). DNA fragments were isolated from agarose gels by using GeneClean (Bio 101). Labeling of the 20-mer oligonucleotide (5′-GC[ACGT]GA[TC]TTG CA[AG]TGGGT[ACGT]GC-3′) used to localize the *estA* gene on pCT1, hybridization, and detection were done according to the specifications of the Dig 3′-End Labeling and Detection kit supplied by Boehringer (Mannheim, Germany). Hybridization and the stringent wash step were performed at 58°C. Nucleotide sequence was determined by the dideoxy-chain termination method (42) with a T7 sequencing kit, deaza-dGTP reaction mixtures (Pharmacia), and [$^{35}$S]dATP or with an AutoRead sequencing kit (Pharmacia) for automated sequencing with an A.L.F. sequencer (Pharmacia). A combination of subclones constructed in pUC19 (52) with M13/pUC universal and reverse primer and synthetic oligonucleotide primers was used. The DNA and deduced amino acid sequences were analyzed by using the sequence analysis software package of the Genetics Computer Group, University of Wisconsin, Madison, Wis. (20), and MacDNAsis Pro (Hitachi Software Engineering, San Bruno, Calif.). Similarity of the deduced amino acid sequence from *estA* with proteins in databases (PDP, release April 1995; SwissProt, release 31; PIR, release 45; GenPept, release 91) was determined with the FASTA (34) and BLASTP (1) programs.

TABLE 2. Purification of esterase from *S. diastatochromogenes*

| Step | Total protein (mg) | Total activity[a] (U) | Sp act (U $mg^{-1}$) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|
| Culture filtrate[b] | 120 | 191 | 1.6 | 100 | 1 |
| $(NH_4)_2SO_4$ precipitation (80%) and dialysis | 88 | 158 | 1.8 | 82.7 | 1.1 |
| DEAE-Sepharose | 21 | 90 | 4.3 | 47.5 | 2.7 |
| Ultrogel AcA44 | 4.9 | 44 | 9.0 | 23.3 | 5.6 |
| MonoQ | 0.75 | 12 | 16.0 | 6.2 | 10.0 |
| Phenyl-Superose | 0.45 | 10 | 22.2 | 5.2 | 13.9 |
| Superdex 75 | 0.28 | 6.5 | 23.2 | 3.4 | 14.5 |

[a] Esterase activity was measured by using PNC as the substrate.
[b] Supernatant from a 1-liter culture grown for 36 h in soy peptone-mannitol medium.

To map the 5′ end of *estA* mRNA, total RNA was isolated from *S. diastatochromogenes* grown for 36 h in soy peptone-mannitol medium. Mycelia of a 25-ml culture quickly chilled by adding ice (approximately one-half of the volume) were harvested by centrifugation (10 min for 4,000 × *g*), frozen with liquid nitrogen, and then homogenized by using a mortar. Total RNA was isolated as described by Chomczynski and Sacchi (16), using guanidinium thiocyanate-phenol-chloroform. A 30-base primer (PE2; 5′-TGCGGCCGAGCCGAGCAGGGCCAGAC GAGT-3′) was designed to hybridize to an RNA sequence in the predicted EstA-coding region (between nucleotides [nt] 377 and 406 in Fig. 4). Nucleic acids of the hybridization mixture (10 μl) consisting of 18 μg of total RNA and 0.6 pmol of 5′-$^{32}$P-end-labeled primer in 10 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES; pH 6.4)–0.4 M NaCl were denatured for 5 min at 85°C, and primer annealing was performed for 1 h at 42°C. After addition of 90 μl of extension buffer (50 mM Tris-HCl [pH 8.2], 6 mM $MgCl_2$, 1 mM each deoxynucleoside triphosphate [dNTP], 10 mM dithiothreitol, 25 μg of actinomycin per ml, 10 U of avian myeloblastosis virus reverse transcriptase [Pharmacia] [final concentrations]), incubation was carried out for 2 h at 42°C. After precipitation of nucleic acids with ethanol-acetone (1:1) and several washing steps with 70% ethanol, the pellet was redissolved in 4 μl of distilled water plus 4 μl of stop mix (T7 sequencing kit; Pharmacia). A sample (2 μl) was heated at 95°C for 3 min, chilled on ice, and electrophoresed on a denaturing sequence gel in parallel with the dideoxy sequencing reactions from pUCT7 and primer PE2.

**Site-specific mutagenesis.** DNA was amplified by PCR using mutagenic oligonucleotide primers to generate the desirable mutations in the *estA* gene. PCR mixtures contained 25 pmol of each primer, DNA template pUCT7 (80 ng), 2 U of Vent DNA polymerase (New England Biolabs), 333 μM each dNTP, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, and 0.1% Triton X-100. To introduce a serine 11-to-cysteine mutation, we performed two PCRs that yielded overlapping fragments, each containing the mutation. The primer sets were (i) O1s (5′-CATAGACGCACCGCGCACTCGCAC-3′; nt 251 to 274) and O501a (5′-GCGGTGTAG**C**[G]AGTCGCCCAGGGCAACC-3′; nt 510 to 484) and (ii) O501s (5′-GTGGGTTGCCCTGGGCGACT**G**[C]CTA CACCGC-3′; nt 481 to 510) and O3a (5′-TTCGCCGCCCAGCTCCTGG CACTT-3′; nt 856 to 832). The positions of the primers indicated in brackets correspond to those in Fig. 4, and the bases in boldface represent deviations from the sequence (shown in brackets) used to create the mutation. Products of both PCRs (200 ng each) were mixed, denatured, and annealed, and the overlapping ends were filled by Vent DNA polymerase in three cycles. Primers O1s and O3a were then added to amplify a 605-bp fragment carrying the Ser-11→Cys mutation by PCR. The obtained product digested with *Nae*I and *Apa*I (349 bp) was used to replace the corresponding fragment in pUCT7 (see Fig. 3), yielding pUCT11. To create the serine 56-to-cysteine mutation, the following two primer sets were used to generate overlapping fragments: (i) O1s and O635a (5′-GCCGCAGC**A**[T]GACATTGGTCAGGTCGAAGAG-3′; nt 644 to 613) and (ii) O635s (5′-ACCTGACCAATGTC**T**[A]GCTGCGGCG-3′; nt 621 to 650) and O3a. The fragments were annealed, and the overlapping ends were filled by Vent DNA polymerase followed by PCR using primers O1s and O3a. The obtained fragment was cloned into pUCT7 as described above. As the mutation Ser-56→Cys led to a loss of a *Pvu*II site, plasmid DNA from the resulting transformants was checked by restriction with *Pvu*II. The plasmid carrying the *estA* gene with the Ser-56→Cys mutation was designated pUCT56. To mutate threonine 113 to cysteine, we used primers O806s (5′-AGGCGGTGGGCCCC GGCACCGACGTCATCACCGTCGGCGTGGGCGGAAAC**G**[C]CCCTCGG CTTCG-3′; nt 756 to 818) and O2a (5′-AACTGCGGGATTCCCTGATATC AAGTGCCC-3′; nt 1375 to 1346), which have an *Apa*I and an *Eco*RV site (underlined), respectively. The PCR product was restricted with *Apa*I and *Eco*RV and ligated to pUCT7 digested with *Apa*I and *Eco*RV, yielding pUCT113. Plasmid DNA from proper clones lost two *Eco*RV fragments of 77 and 487 bp (see Fig. 3).

To construct the serine 130-to-alanine mutation, we used two primer sets that yielded overlapping fragments: (i) O875s (5′-CAAGTGCCAGGAGCTGGG CGGCGAA**T**[A]GCGGCGGAG-3′; nt 832 to 866) and O2a and (ii) O635s and O3a. The products of the PCRs were mixed, denatured, and annealed, and the overlapping ends were filled in by Vent DNA polymerase. PCR was then performed with primers O635s and O2a, which bound to the opposed end of the fragments. The resulting product was restricted with *Apa*I and *Eco*RV and cloned into pUCT7 digested with *Apa*I and *Eco*RV, yielding pUCT130. The complete DNA sequences of all of the mutated *estA* genes were determined. The mutated genes were isolated from pUCT11, pUCT56, pUCT113, and pUCT130 by restriction with *Sph*I, which cut within the multiple cloning site and in the *mel* fragment originating from pUCT7 (see Fig. 3) and by cloning the fragment into the unique *Sph*I site of pWHM3 (47). The resulting constructs (pWCT11, pWCT56, pWCT113, and pWCT130) were transformed into *S. lividans* TK23.

**Nucleotide sequence accession number.** The DNA sequence data described in this report have been deposited at the EMBL nucleotide sequence database under accession number X92765.

## RESULTS

**Purification and biochemical properties of the extracellular esterase from *S. diastatochromogenes*.** An esterase was isolated from the supernatant of an early-stationary-phase culture of *S. diastatochromogenes* grown in soy peptone-mannitol medium. Fractions were tested during purification for hydrolytic activity toward PNC and after separation by SDS-PAGE for hydrolytic activity toward Tween 20 and casein.

The results of the purification procedure are summarized in Table 2 and Fig. 1. The PNC-hydrolyzing fractions obtained from the hydrophobic chromatography step on phenyl-Superose still contained protease activity. Homogeneous enzyme free of impurities was obtained after gel filtration on Superdex 75. The yield of protein was 0.28 mg from 1 liter of culture, and the recovery was 3.4%. SDS-PAGE of purified esterase stained by Coomassie brilliant blue showed a single protein band with an apparent molecular mass of 33,000 Da. The molecular mass calculated by electrospray mass spectrometry was 30,862 ± 5.8 Da. When the esterase was applied to the SDS-polyacrylamide gel without heat denaturation, it migrated with an apparent molecular mass of 20 kDa (Fig. 2), indicating a compact and

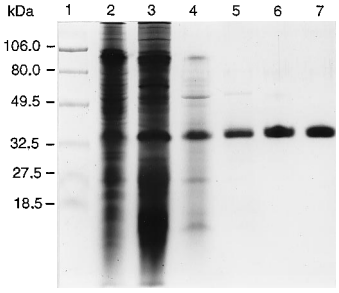


FIG. 1. Purification of the extracellular esterase from *S. diastatochromogenes*. Shown is a Coomassie blue-stained SDS-polyacrylamide gel of culture filtrate concentrated by ammonium sulfate precipitation (lane 2) and of pooled fractions eluted from DEAE-Sepharose (lane 3), Ultrogel AcA44 gel (lane 4), MonoQ (lane 5), phenyl-Superose (lane 6), and Superdex 75 (lane 7). Lane 1, protein standards; molecular masses are shown on the left.

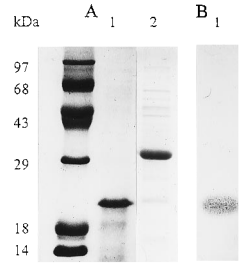


FIG. 2. SDS-PAGE of supernatant from *S. lividans* TK23(pCT1). Protein (7 μg) was applied without (A, lane 1; B, lane 1) and after heat denaturation (lane 2) and stained with Coomassie brilliant blue R250 (A) and for Tween 20-hydrolyzing activity (B). After removal of SDS, the gel was incubated on Tween 20-containing agarose for 3 h at 37°C. The band of precipitated fatty acids indicates hydrolysis of Tween 20. The molecular masses of the protein standards are shown on the left.

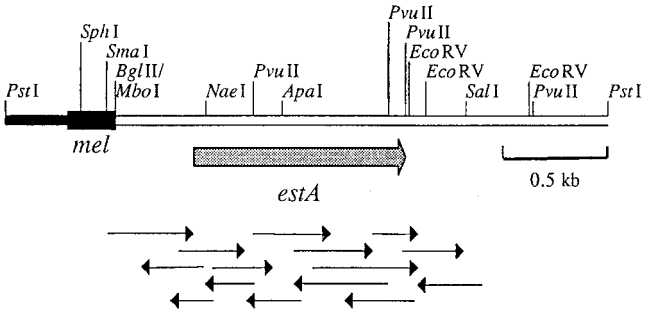


FIG. 3. Restriction map of the 2.7-kb *Pst*I insert of pUCT7 and the sequence strategy. The orientation of the *estA* gene is indicated by a wide arrow. The black segment of the insert is from pIJ702 containing the 5′ region of the *mel* gene. The positions and lengths of nucleotide sequences determined for the 1,622-bp (*Mbo*I)-*Sal*I fragment are indicated by arrows below the map.

tight conformation that was retained in the presence of SDS. A sample of esterase after SDS-PAGE was subjected to N-terminal sequence analysis, and the first 17 amino acids were determined to be ADLQWVALGDSYTAGVI.

The substrate specificity of the purified enzyme was studied with various *p*-nitrophenyl esters of fatty acid. The enzyme showed optimum activity with the water-soluble PNC (C-8), whereas activity decreased to 10 and 2%, respectively, with water-insoluble *p*-nitrophenyl laurate (C-12) and *p*-nitrophenyl palmitate (C-16). Substrates for lipases and phospholipase A1 (2,3-dibutyryl dithiopropanol 1-butyrate and 2-hexadecanoylthiopropane 1-phosphocholine, respectively [32]), were not hydrolyzed by the purified enzyme. These results and the observed high hydrolytic activity toward Tween 20 suggested that the purified enzyme from *S. diastatochromogenes* is an esterase. The esterase showed optimum activity at temperatures between 40 to 50°C, decreased to 60% of its value at 60°C, and had no activity at 70°C. The enzyme had optimum activity at pH 8.0 to 9.0 (data not shown). Higher pH values were not tested because of spontaneous hydrolysis of PNC. The esterase was resistant to temperatures up to 50°C and lost about 30% of its activity when incubated for 30 min at 60°C. The stability of the enzyme sharply decreased at temperatures above 60°C. PNC-hydrolyzing activity of the esterase was not significantly affected by the following divalent cations: 1 mM $Cu^{2+}$, $Zn^{2+}$, $Mn^{2+}$, $Mg^{2+}$, and $Ca^{2+}$. Esterolytic activity was reduced to 2 and 10% by PMSF and DFP, respectively, indicating that serine groups are necessary for catalysis. However, enzyme activity was not significantly inhibited by thiol group inhibitors, such as iodoacetamide and ethylmaleimide, or by disodium EDTA.

**Cloning of the *S. diastatochromogenes* esterase gene in *S. lividans*.** From approximately 1,700 thiostrepton-resistant $Mel^-$ *S. lividans* TK23 transformants, one clone was observed to have a large zone of Tween 20 hydrolysis when grown on Tween 20-containing complex medium plates. Plasmid DNA from the transformant carried an insert of 5.1 kb and was designated pCT1. When pCT1 was used to retransform *S. lividans* TK23, all of the subsequent transformants produced zones of Tween 20 hydrolysis.

SDS-PAGE analysis of culture supernatant from TK23 (pCT1) revealed a prominent protein band that exhibited Tween 20-hydrolyzing activity (Fig. 2). Protein sequence analysis revealed that except for an additional alanine residue, the N-terminal sequence of the recombinant esterase was identical with that of the esterase purified from supernatant of *S. diastatochromogenes*. The esterase secreted by TK23(pCT1) accounted for more than 90% of total extracellular proteins. On the basis of Coomassie blue staining, the amount of esterase produced by a culture grown for 120 h in soy peptone-mannitol medium was calculated to be about 120 mg/liter; comparison of activity (units per milliliter) indicated that this was 13-fold higher than the amount produced by *S. diastatochromogenes* grown under similar conditions.

**Analysis of the *estA* gene and the deduced protein.** A synthetic 20-mer oligonucleotide was designed on the basis of the N-terminal amino acid sequence determined for the extracellular *S. diastatochromogenes* esterase and used as a hybridization probe to localize the esterase-encoding gene on pCT1. As the probe bound to a 2.7-kb *Pst*I fragment (Fig. 3) and a 2.0-kb *Pvu*II-*Bam*HI fragment, the second of which cut a single site within the vector DNA, its binding site was mapped on an 0.64-kb (*Mbo*I)-*Pvu*II fragment (Fig. 3). For DNA sequencing, a 2.7-kb *Pst*I fragment of pCT1 containing 2.3 kb of insert and 0.5 kb of vector DNA was cloned into *Pst*I-digested pUC19, yielding pUCT7. Its restriction map and the sequencing strategy used are shown in Fig. 3. Analysis of the nucleotide sequence of a 1,627-bp (*Mbo*I)-*Sal*I fragment (Fig. 4) by using the CODONPREFERENCE program revealed the existence of an open reading frame (*estA*) of 978 nt. The *estA* gene starts at an ATG start codon at nt 362, which is preceded by a potential ribosome binding site (GGAGG; nt 353 to 357) with a good complementarity to the 3′ end of the 16S rRNA from *S. lividans* (5) and terminates at a TGA stop codon at nt 1340. *estA* could encode a protein of 326 amino acids and a deduced molecular mass of 34,250 Da. Amino acid residues 36 to 53 and 37 to 53 of the deduced gene product were identical to the N-terminal amino acid sequence determined for the mature esterase secreted by *S. lividans* TK23(pCT1) and *S. diastatochromogenes*, respectively, indicating putative 35- and 36-amino-acid signal peptides, respectively. Cleavage of the signal peptide would yield mature proteins of 291 and 290 amino acids, respectively. The molecular mass deduced for the mature esterase of *S. diastatochromogenes* was 30,865 Da and corresponded with that determined for the purified enzyme by electrospray mass spectrometry (30,862 ± 5.8 Da).

The G+C contents in the first, second, and third positions of the *estA* codons are 68.9, 49, and 88.8 mol%, respectively, values which are in agreement with the biased codon usage of streptomycetes (6). The G+C content in codon position 3 is lower than the >90 mol% found in other previously described streptomycete genes. The overall G+C content of *estA* was 68.9 mol%. Downstream of the *estA* coding region, there is an inverted repeat sequence beginning at nt 1392 (−11 kcal [1 cal

```
   1 GATCGCTTACGGCCTTCGGCGCTGGCGTTCGGCCAGGACAGCACAGGCGCGATGTGCGCA
  61 GTTCGCGACAACTTCTCTAAGGCCGTCCAGGACGGAGGCCAGTGGCTGAGAACAAGACAG
 121 TCACGGAGAGGATGCGTGTCCGACCAGGTCGCGCAGTCGATCAAGAACCTTCTCCGCGCT
 181 GCGCAAGTACCGGACCAGGGCGGTCAGGTGTGTGGGGCGAGCGATCGACACGCCGAGCCT

 241 CAATCACCCACATAGACGCACCGCGCACTCGCACCCGTGACTGTCCGTATTGGATCTAGT
     -35          -10        *          SD
 301 GCATTAAGTGACGAGTCTTTTATGGTCAAAGCGACGCCAACCCCGTATCTCTGGAGGATT

 361 CATGGCACGCTTCCGTACTCGTCTGGCCCTGCTCGGCTCGGCCGCAGCTCTCGCCGCCGG
       M  A  R  F  R  T  R  L  A  L  L  G  S  A  A  A  L  A  A  G

 421 CGCAGTCGTCCCCGCCCAGCTCGCCGGGGCTCAACCCGCTGCCGCGGCTGCTGACCTCCA
       A  V  V  P  A  Q  L  A  G  A  Q  P  A  A  A  A  A  D  L  Q
                                      s.l.▲  ▲s.d.
 481 GTGGGTTGCCCTGGGCGACTCCTACACCGCCGGGGTCATCCGGGCCGCGGGCGACGCCAT
       W  V  A  L  G  D  S  Y  T  A  G  V  I  R  A  A  G  D  A  I

 541 CGACTACCCGCGCGACGGCTGCGAGCCGGACCGACCGGTCCTACCCTCAGGTCATCGAACG
       D  Y  P  R  D  G  C  E  R  T  D  R  S  Y  P  Q  V  I  E  R

 601 CGACCTCTACGGTCTCTTCGACCTGACCAATGTCAGCTGCGGCGCCGCCACCATCGAGAA
       D  L  Y  G  L  F  D  L  T  N  V  S  C  G  A  A  T  I  E  N

 661 CGTCTCCGACACGCCCCAGTACCCGATCGGCCGTCACATGCCGCCCTTCTCCGAGGACCC
       V  S  D  T  P  Q  Y  P  I  G  R  H  M  P  P  F  S  E  D  P

 721 GGACCACCCCTTCCCTCCGGTGCCGCCCCAGTCCGAGGCGGTGGGCCCCGGCACCGACGT
       D  H  P  F  P  P  V  P  P  Q  S  E  A  V  G  P  G  T  D  V

 781 CATCACCGTCGGCGTGGGCGGAAACACCCTCGGCTTCGCCGACATCCTCACCAAGTGCCA
       I  T  V  G  V  G  G  N  T  L  G  F  A  D  I  L  T  K  C  Q

 841 GGAGCTGGGCGGCGAAAGCGGCGGAGAAGGAACCCCGTGCAAGGGCGCCCTGGGCTCCGG
       E  L  G  G  E  S  G  G  E  G  T  P  C  K  G  A  L  G  S  G

 901 CATCAAGGCCCGGCTGGACAAGGTGAGCCAGGGCTACGACCGGATGCTCACCGTTCTCCA
       I  K  A  R  L  D  K  V  S  Q  G  Y  D  R  M  L  T  V  L  H

 961 CGAGCGCGGTCCGAAGGCGAAGATCCTGACCGTCGGCTACCCCACGGTCATCCCCGAGAA
       E  R  G  P  K  A  K  I  L  T  V  G  Y  P  T  V  I  P  E  N

1021 CACCGCCGACTGCGGCTACGGCGACCTGACGAAGTTCGGCACGATCACCCAGGGCGACCT
       T  A  D  C  G  Y  G  D  L  T  K  F  G  T  I  T  Q  G  D  L

1081 GGCCTGGCTGCGCCAGGACGTTCTGGAACCCCTCAACAAGACCATCGAGGACTCGGCGGC
       A  W  L  R  Q  D  V  L  E  P  L  N  K  T  I  E  D  S  A  A

1141 CACACAGGAAGCCGCCGACTTCGTCAACCTCTACGCCCCCTCCCAGGGCCACAGCGTCTG
       T  Q  E  A  A  D  F  V  N  L  Y  A  P  S  Q  G  H  S  V  C

1201 CGACGCCGACAAGTGGGTGGAAGGCATCCTCACCGTCTCCCCTAACCAGGACCCCCAGCT
       D  A  D  K  W  V  E  G  I  L  T  V  S  P  N  Q  D  P  Q  L

1261 GTCCTTCGTACATCCCAACGCCTCCGGCCACCGCAACGCCGCCGACCACGTCGAAGAAGC
       S  F  V  H  P  N  A  S  G  H  R  N  A  A  D  H  V  E  E  A

1321 GATGCTGAACGCCATCAGCTGAGCCGGGCACTTGATATCAGGGAATCCCGCAGTTCTGGG
       M  L  N  A  I  S  -
          ------>  <------
1381 AGTGAGGCGGGCGGTTTCGAGGGTGAAACCGTGGTGGACGAGGTGGCTGGGATATCCACC
1441 GGCCGTGGTGAAAGCGCGCGTCACATGAGGGTCCGAGATGGGTCGCCATCGTGTGCTGGC
1501 GCTTATTCAGTGAGGAAGAGCAGTGCAGTCTGTGACGGGCCAGGACGTACTCAATTTCTA
1561 CCATGCACGTAACGACTGGCTCGTCCTCACTGAGAGAGGGGAGCTGGAATGCATGAAGAG
1621 TGTCGAC
```

FIG. 4. Nucleotide sequence of the *S. diastatochromogenes* esterase gene and the predicted amino acid sequence. A putative ribosome binding site (SD) is overlined. The translational termination codon is indicated by a dash. The putative −35 and −10 nucleotides upstream of the *estA* transcriptional start site (asterisk) are overlined. Amino acids determined experimentally by direct N-terminal sequencing of extracellular esterase purified from *S. diastatochromogenes* (*s.d.*) and the recombinant esterase secreted by *S. lividans*(pCT1) (*S.l.*) are underlined, and the arrowheads indicate the proposed sites of cleavage between the signal sequence. →← indicates an inverted repeat sequence.

= 4.184 kJ]/mol) (Fig. 4). A structure forming a long and stable stem-loop structure of the mRNA (−30 to −80 kcal/mol) known to mediate transcription termination of *Streptomyces* genes (19, 30, 38) was not observed at the 3′ end of *estA*.

Primer extension analyses were performed to determine the transcriptional start site of the *estA* mRNA in *S. diastatochromogenes*. The primer extension reaction resulted in a single extended product, and alignment with its sequencing ladder (Fig. 5) indicated an apparent transcription start site 29 bp upstream of the ATG start codon of *estA* (Fig. 4). Potential −10 (TATGGT) and −35 (GTGCAT) sequences were identified. The −10 region corresponded to the consensus sequence of streptomycete E$\sigma^{70}$-like promoters, while the −35 region differed from the consensus sequence TTGAC(Pu) (45).

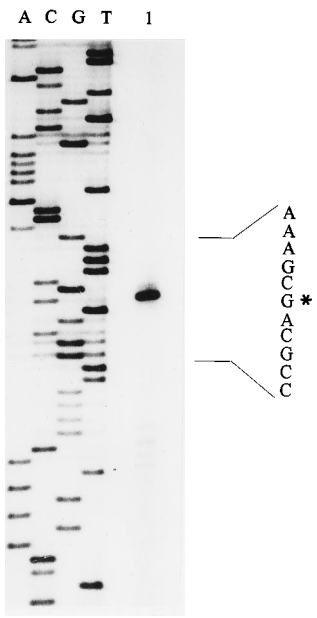


FIG. 5. Primer extension mapping of the transcription initiation site of the *estA* gene. A primer complementary to the 5′ end of the coding region of *estA* (between nt 406 and 377 in Fig. 4) was hybridized to total RNA from *S. diastatochromogenes*. Lane 1, primer extension product compared on a sequencing gel alongside the products of a dideoxy sequencing reaction (A, C, G, T) obtained by using the same primer and pUCT7 as the template. The complements of the bases are shown; the asterisk indicates the 5′ base of *estA* mRNA.

**EstA has similarity to the plant pathogen *S. scabies* esterase.** A search in databases by using the deduced amino acid sequence of *estA* (EstA) revealed no notable similarities with members of the family of esterases and lipases (18). However, there was striking similarity to the esterase from *S. scabies* (40). The proteins exhibited 31.4% identity in a region of 156 amino acids. A comparison of the deduced amino acid sequence of EstA with the *S. scabies* EstA sequence revealed conserved regions throughout the entire length (Fig. 6). In addition, as is the case for the *S. scabies* esterase, *S. diastatochromogenes* EstA lacked the GXSXG sequence conserved in most of the known esterases and lipases (11). Furthermore, the mature esterases from *S. diastatochromogenes* and *S. scabies*, with sim-

```
SDESTA   A---DLQWVALGDSYTAGVIRAAGDAIDYPRDGCERTDRSYPQVIERDL-   46
SSESTA   APADPVPTVFFGDSYTANFGIAPVTNQDSERGWCFQAKENYPAVATRSLA   50

SDESTA   ---YGLFDLTNVSCGAATIENVSDTPQYPIGRHMPPFSEDPDHPFPP--V   91
SSESTA   DKGITLDVQADVSCGGALIHH---------------FWEKQELPFGAGEL   85

SDESTA   PPQSEAVGPGTDVITVG-VGGNTLGFADILTKCQE-------LGGESGGE  133
SSESTA   PPQQDALKQDTQL-TVGSLGGNTLGFNRILKQCSDELRKPSLLPGDPVDG  134

SDESTA   GTP---CKGALGSG-----IKARLDKVSQGYDRMLTVLHERGPKAKILTV  175
SSESTA   DEPAAKCGEFFGTGDGKQWLDDQFERVGAELEELLDRIGYFAPDAKRVLV  184

SDESTA   GYPTVIPENTADC-----GYGDLTKFGTITQGDLAWLRQDVLEPLNKTIE  220
SSESTA   GYPRLVPEDTTKCLTAAPGQTQLP-FADIPQDALP-----VLDQIQKRLN  228

SDESTA   DS---AATQEAADFVNLYAPSQGHSVCD-ADKWVEGILTVSPNQ--DPQL  264
SSESTA   DAMKKAAADGGADFVDLYAGTGANTACDGADRGIGGLLEDSQLELLGTKI  278

SDESTA   S-FVHPNASGHRNAADHVE---EAMLNAIS                     290
SSESTA   PWYAHPNDKGRDIQAKQVADKIEEILN--R                     306
```

FIG. 6. Alignment of amino acid sequences of mature esterases from *S. diastatochromogenes* (SDESTA) and *S. scabies* (SSESTA [40]), using MULTIPLE EDIT (MacDNAsis Pro). Identical amino acid residues are indicated in boldface. Residues of SDESTA mutated by site-directed mutagenesis are indicated by asterisks.

ilar lengths of 290 and 306 amino acids, respectively, were resistant to temperatures of up to 60°C (31).

**Site-directed mutagenesis and chemical modification studies implicate Ser-11 as the active-site nucleophile.** Since the hydrolytic activity of the purified esterase was inhibited by PMSF and DFP, a serine residue was believed to be required for enzyme activity. To investigate the active-site nucleophile of the *S. diastatochromogenes* esterase, an alignment of the deduced *S. diastatochromogenes* EstA protein and *S. scabies* Est was performed to identify nucleophilic amino acid residues located within conserved regions (Fig. 6). Serines at positions 11 and 56 of the mature *S. diastatochromogenes* esterase were present within conserved motifs in the *S. scabies* enzyme. In addition, Thr-113 was located in a sequence conserved in both enzymes. To investigate the importance of Ser-11, Ser-56, and Thr-113 in enzyme activity, these residues were changed by site-directed mutagenesis. Furthermore, Ser-130, located within a sequence (GESGG) similar to the consensus motif of esterases and lipases, but without a homologous sequence in the *S. scabies* esterase, was mutated. Thr-113 was replaced with Ala, and Ser-11, Ser-56, and Ser-130 were replaced with Cys. After verification of the DNA sequence, the mutated genes were cloned into the *E. coli-Streptomyces* plasmid pWHM3 (47), yielding pWCT11, pWCT56, pWCT113, and pWCT130, which were used to transform *S. lividans*. Supernatants from the resulting transformants were examined for extracellular esterase protein by SDS-PAGE and Coomassie blue staining and for PNC-hydrolyzing activity and its sensitivity toward serine-active and thiol group inhibitors. *S. lividans*(pWCT113) secreted the esterase with the replacement Thr-113→Ala in an amount similar to that secreted by *S. lividans*(pCT1) (data not shown). The mutated enzyme still exhibited about 50% of the PNC-hydrolyzing activity of the wild-type enzyme, suggesting that Thr-113 does not participate in catalytic reaction. Each of the substitutions of Ser with Cys led to a drastic reduction of extracellular esterase proteins (data not shown). The supernatants from *S. lividans*(pWCT56) and *S. lividans*(pWCT130) had 1.7 and 1.3% of the PNC-hydrolyzing activity (units per milliliter) of *S. lividans*(pCT1), whereas no activity was detected in supernatant from *S. lividans*(pWCT11). Despite the replacements Ser-56→Cys and Ser-130→Cys, PNC-hydrolyzing activities of the mutant esterases were inhibited by PMSF but unaffected by cysteine-specific inhibitors such as iodoacetamide and ethylmaleimide (data not shown). These results indicated that neither Ser-56 nor Ser-130 is involved in catalytic activity.

Since no conclusion could be drawn as to the role of Ser-11, chemical modification of the esterase was performed with [1,3-$^3$H]DFP, an inhibitor known to modify active-site serine residues of serine hydrolases covalently, using supernatant from *S. lividans*(pCT1). By taking advantage of the close proximity of Ser-11 to the N terminus of the mature protein, the modified enzyme was centrifuged onto a polyvinylidene difluoride membrane and directly subjected to Edman degradation (Fig. 7). The high amount of radioactivity detected in the first cycle was due to [1,3-$^3$H]DFP washed from the membrane. A significant amount of radioactivity was released in cycles 10 to 12. Each fraction contained a mixture of three amino acid residues that coincided with the degradation of a nested set of esterase species with different N termini, consisting of 25% of the recombinant esterase secreted by *S. lividans*(pCT1), 50% of the mature protein lacking the N-terminal alanine residue, and 25% which lacked two alanine residues. Obviously, a proteolytic processing at the amino terminus of the esterase occurred during incubation with [1,3-$^3$H]DFP catalyzed by peptidases produced by *S. lividans* (2, 13). In each case, [1,3-$^3$H]DFP labeling detected in fractions 10 to 12 was specific to Ser-11, indicating that this residue in the sequence GDSYT might be the active serine.

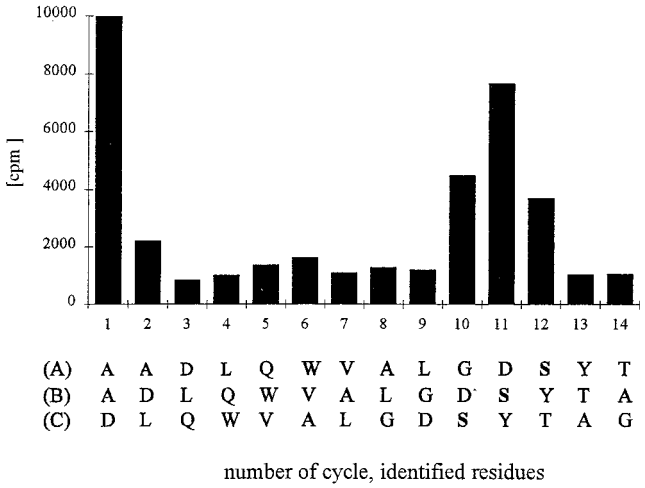


FIG. 7. Edman degradation of [1,3-$^3$H]DFP-labeled *S. diastatochromogenes* esterase. Amino acid residues identified in each cycle are indicated. The labeled esterase (90 pmol) from the supernatant consisted of three different species, A, B, and C, originated by sequential processing. The relative amounts of A, B, and C were 25, 50, and 25%, respectively.

## DISCUSSION

Using a 30-mer degenerated oligonucleotide probe designed on the basis of the N-terminal amino acid residues of the secreted esterase, we failed to isolate the corresponding gene from an *S. diastatochromogenes* gene bank constructed in pBR322. The hybridization conditions applied led to the isolation of an incorrect DNA fragment that contained two possible binding sites for the oligonucleotide probe within a 100-bp sequence. The esterase-encoding gene, *estA*, was cloned in *S. lividans* by using the multicopy plasmid pIJ702 and by screening the resulting transformants for Tween 20-hydrolyzing activity. The deduced amino acid sequence of the *estA* gene composed of 326 residues comprised a potential 36-residue signal peptide that was removed during secretion by *S. diastatochromogenes* and exhibited the features of other known signal peptides (48). The N terminus contained three arginine residues that are generally preferred as positively charged amino acids in the hydrophilic N-terminal region of *Streptomyces* leader peptide sequences (26) and was followed by a hydrophobic region. The proposed cleavage site was preceded by a proline residue at a distance of four residues and followed the −1, −3 rule for a signal peptidase of type I having alanines in both positions. The recombinant esterase secreted by *S. lividans* TK23(pCT1) had an additional alanine residue at its N terminus, indicating that the precise cleavage sites of the signal peptidase may differ in *S. lividans* and *S. diastatochromogenes*, as reported for the subtilisin inhibitor gene from *Streptomyces albogriseolus* S-3253 expressed in *S. lividans* 66 (33).

The protein sequence deduced from the *S. diastatochromogenes estA* gene had significant identity with the esterase from the plant pathogen *S. scabies*, which was discussed as a causative agent of pathogenicity and so far was unique among known proteins (40). In addition, the enzymes, both of which are exported, were similar in their biochemical properties, such as temperature and pH optima for catalytic activity and thermostability (31). However, the corresponding genes differed in the regulation of expression. *S. diastatochromogenes* secreted

the esterase during exponential growth, reaching a maximum amount in the stationary phase (data not shown), whereas the *S. scabies* esterase gene was expressed when the culture was in the late exponential or early stationary phase (40). Temporal differences in gene expression might be mediated at the level of transcription by RNA polymerases with different sigma factors, as the *S. scabies est* promoter resembles the *dagAp3* promoter of *Streptomyces coelicolor*, which is recognized by $E\sigma^{49}$-like RNA polymerase, whose activity predominates in stationary-phase cultures (14). In contrast, the *S. diastatochromogenes estA* promoter is similar to $E\sigma^{70}$-like promoters. The −10 region conforms with streptomycete promoters recognized by $E\sigma^{70}$-like RNA polymerase (45), and the −35 region is identical to that of the *rrnDp1* promoter of the rRNA gene cluster of *S. coelicolor*, classified as an $E\sigma^{70}$-like promoter (4). Furthermore, there was a difference in response of gene expression to the presence of zinc in the culture medium. In *S. scabies*, an increased transcription of the *est* gene occurs when zinc is present (40). A protein-binding sequence in the 5′ region of the *est* gene has been shown to be involved in positive regulation of transcription (3). In contrast, esterase production was not influenced by zinc in *S. diastatochromogenes* (data not shown), and in accordance, there was no sequence in the 5′ region of the *estA* gene homologous to the protein-binding sequence of the *S. scabies est* gene. Differences observed in the regulation of gene expression may be due to differences in physiological roles of the esterases, especially since *S. diastatochromogenes* Tü20 (28) is not considered to be a plant pathogen.

When the sequences of the *S. diastatochromogenes* EstA and the *S. scabies* Est proteins were aligned, the highly conserved sequence GXSXG of hydrolytic enzymes containing an active-site serine (11) was not found. A similar motif, GNTLG with threonine in position 113 of the mature EstA, was also conserved in the *S. scabies* esterase. The mutant enzyme with the Thr-113-to-alanine replacement retained approximately 50% of catalytic activity, indicating that Thr-113 does not function as an active-site nucleophile. This result was in agreement with the finding that mutants of pancreatic cholesterol esterase and human lipoprotein lipase in which the active-site serine residue is replaced by threonine are totally inactive (21, 23). All mutations of Ser-11, Ser-56, and Ser-130 to cysteine resulted in a drastic reduction of the amount of secreted esterases. Introduction of a new cysteine residue in addition to six cysteines present in the wild-type enzyme might have caused incorrect folding of the proteins. Recently, Wei et al. (49) reported the crystal structure of the *S. scabies* esterase, revealing that its three-dimensional structure is stabilized by three disulfide bridges formed between the six cysteines of the protein by linking two subsequent residues. Given the close similarity to the *S. diastatochromogenes* esterase, it seems likely that cysteine bridges link residues 31 to 57, 123 to 137, and 188 to 244 in the mature *S. diastatochromogenes* esterase. In the mutated esterases, disulfide bridges could have formed with the new cysteine residue, resulting in an altered conformational structure that could hamper secretion and induce proteolytic degradation (12). The results obtained by chemical modification of the esterase with the serine group inhibitor [1,3-$^3$H]DFP, which has been used to identify the active site serine of fungal cutinase (44), thioesterases (15, 39), and pancreatic cholesterol esterase (21), supported evidence that serine 11 within the sequence GDSYT is the catalytic active serine. This hypothesis has been confirmed by the crystal structure of the *S. scabies* esterase recently reported by Wei et al. (49). They show that the tertiary fold of the *S. scabies* esterase is different from that of the α/β hydrolases. The catalytic site of the esterase consists of a dyad of Ser-14 and His-283 and lacks a carboxylic acid as the third member of a typical triad. These residues are located within sequences that are also conserved in the *S. diastatochromogenes* esterase (Fig. 6). The similarity between the enzymes suggests that the esterase from *S. diastatochromogenes* is another member of hydrolases that has an unusual tertiary fold.

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