JOURNAL OF BACTERIOLOGY, Apr. 1992, p. 2087–2094
0021-9193/92/072087-08$02.00/0

Vol. 174, No. 7

# Identification of a Cocaine Esterase in a Strain of *Pseudomonas maltophilia*

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Received 2 October 1991/Accepted 13 January 1992

**A strain of *Pseudomonas maltophilia* (termed MB11L) which was capable of using cocaine as its sole carbon and energy source was isolated by selective enrichment. An inducible esterase catalyzing the hydrolysis of cocaine to ecgonine methyl ester and benzoic acid was identified and purified 22-fold. In the presence of the solubilizing agent cholate, cocaine esterase had a native $M_r$ of 110,000 and was shown by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be a monomer. In the absence of cholate, cocaine esterase had a native $M_r$ of 410,000 and probably existed as a tetramer. The pH optimum of the enzyme was 8.0, and the $K_m$ values for cocaine, ethyl benzoate, and ethyl 2-hydroxybenzoate were 0.36, 1.89, and 1.75 mM, respectively. Inhibition studies indicated that the enzyme was a serine esterase, possibly possessing a cation-binding site similar to those of mammalian acetylcholinesterase and the atropine esterase of *Pseudomonas putida* PMBL-1. The cocaine esterase of *P. maltophilia* MB11L showed no activity with atropine, despite the structural similarity of cocaine and atropine.**

Microbial enzyme activities against alkaloids have been investigated as models of mammalian metabolism and as potential sources of new therapeutic compounds (16, 17, 38). Of the tropane alkaloids, the microbial metabolism of atropine has received the most significant attention. *Corynebacterium belladonae* catabolizes atropine via esterolytic hydrolysis of the tropic acid moiety followed by dehydrogenation, ring opening, and deamination of the tropane ring (27, 28). A wide range of pseudomonads were also found to utilize atropine as their sole source of carbon and nitrogen (34). Further studies with nine of these strains identified two distinct classes of atropine esterase which possessed different physical and chemical properties (31). The atropine esterase from *Pseudomonas putida* PMBL-1 has been purified and extensively characterized (15, 40, 43–45). Probing of the active site of the enzyme indicated an active serine residue and a classical charge relay system (43, 44), although sequence analysis (15) and structure prediction (42) implied little homology to the serine protease families. Atropine esterase showed stereospecificity toward the (−)-isomer of atropine, (−)-hyoscyamine, and appeared to favor esters of tropic acid over those of acetic acid (34). Interestingly, the enzyme displayed no activity with the structurally related tropane alkaloid cocaine (34). In this paper, we describe the isolation of a strain of *Pseudomonas maltophilia* capable of using cocaine as its sole carbon and energy source and the partial purification and characterization of a cocaine esterase from this organism.

## MATERIALS AND METHODS

**Chemicals.** All chemicals were purchased from Aldrich Chemical Company Ltd. (Gillingham, United Kingdom), BDH Ltd. (Poole, United Kingdom), Sigma Chemical Company Ltd. (Poole, United Kingdom), or FSA Laboratory Supplies (Loughborough, United Kingdom) unless otherwise stated and were of analytical grade or better. Pharmaceutical-grade cocaine hydrochloride and seized cocaine (90% [wt/wt] pure by high-performance liquid chromatography [HPLC] analysis) were gifts from Peter Baker, Laboratory of the Government Chemist, London, United Kingdom. *cis-cis*-Muconate, (+)-muconolactone, and β-ketoadipate (potassium salt) were gifts from R. B. Cain, Department of Agricultural and Environmental Science, University of Newcastle, Newcastle upon Tyne, United Kingdom. All enzymes and proteins were from Sigma except where stated. Solutions and buffers were prepared by using water purified by a Milli-RO-60 system (Millipore Waters UK Ltd., Watford, United Kingdom). Ecgonine hydrochloride was prepared from cocaine by using the method of Findlay (11). The purity of the product was established by thin-layer chromatography (TLC), gas chromatography (GC), and HPLC analyses. Ecgonine methyl ester hydrochloride was prepared from ecgonine hydrochloride (0.3 g) by stirring with dry methanol (2.5 ml) and thionyl chloride (0.5 g) at 50°C for 48 h. Melting points and $^1H$ nuclear magnetic resonance spectra of ecgonine and ecgonine methyl ester were in agreement with those previously reported (11, 22).

**Analytical methods.** TLC was performed on silica (LK6, 250-μm-thick coating; Whatman UK Ltd.) or C-8 reverse-phase plates (Merck, RP8 F254; BDH Ltd.). Mobile phases, adapted from Misra et al. (24), were ethyl acetate-methanol-ammonia (13:7:1, vol/vol/vol; solvent A) and butan-1-ol-water-glacial acetic acid (35:10:3, vol/vol/vol; solvent B). All amines were detected with acidified iodoplatinate (25). HPLC separations were made on a Waters 600 HPLC system (Millipore Waters UK Ltd.) consisting of a 600E System Controller connected to a 484 Absorbance Detector set to 218 or 275 nm, 0 to 1 V fsd (full-scale deflection). Injections (50 μl) were performed with a WISP 712 Autoinjector and data processing was done with Maxima 820 software. The column (4.6 by 250 mm) was constructed of 5-μm-particle-size C18 Spherisorb obtained from Anachem Ltd. (Luton, United Kingdom). A guard column (4.6 by 40 mm) made of the same packing was used to protect the main column. The mobile phase was 3:1 (vol/vol) HPLC-grade methanol–sterile-filtered 80 mM sodium dihydrogen orthophosphate (23, 29) with a flow rate of 1.1 ml/min. The mobile

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phase was sparged with helium at 30 ml/min throughout the operation. GC was performed by using a Perkin Elmer 8410 Gas Chromatograph (Perkin Elmer Ltd., Beaconsfield, United Kingdom) fitted with a flame ionization detector and a DB-5 capillary column (15 m by 0.25 mm) (J&W Scientific; obtained from Jones Chromatography Ltd., Hengoed, United Kingdom). Injections (1 μl) were performed manually, and elution was with nitrogen gas held at a constant pressure of 6 lb/in$^2$. The following temperature profile was developed: injection port, 280°C; oven, 100°C (held 2 min); ramped to 130°C at 5°C/min; held 1 min at 130°C; ramped to 280°C at 30°C/min; held 2 min at 280°C; detector, 300°C. Cocaine, atropine, and aromatic acid concentrations (benzoic acid, 2-hydroxybenzoic acid, 3-hydroxybenzoic acid, 4-hydroxybenzoic acid, tropic acid) were determined by HPLC analyses of samples. Comparison of peak heights with calibration curves obtained with standard samples treated in a manner identical to that of the assay samples allowed estimation of ester and acid contents. $^1$H nuclear magnetic resonance was performed at 250 MHz by the Department of Chemistry, University of Cambridge, on a Bruker WM-250 Spectrometer.

**Organisms and culture conditions.** *P. maltophilia* MB11L was isolated by enrichment in 250-ml Erlenmeyer flasks containing 50 ml of defined medium A, which consisted of 0.5 g of $(NH_4)_2SO_4$ per liter, 0.5 g of $KH_2PO_4$ per liter, 2 g of $K_2HPO_4$ per liter, 0.1 g of $MgSO_4$ per liter, 1 ml of mineral salts per liter (2), and 10 mM cocaine at 30°C in a shaking incubator (250 rpm). Fermentations were performed under identical conditions in 2-liter Erlenmeyer flasks containing 750 ml of medium B, which consisted of 4.33 g of $Na_2HPO_4$ per liter, 2.65 g of $KH_2PO_4$ per liter, 2 g of $NH_4Cl$ per liter, 0.1 g of nitrilotriacetic acid per liter, 4 ml of mineral salts per liter (35), and 10 mM cocaine. Larger-scale fermentations for enzyme production were performed in a 10-liter Biocul system (LH Fermentation, United Kingdom) at 30°C and stirred at 300 rpm and were forcibly aerated at 18 liters/min. DL-Methionine was added to a final concentration of 40 mg/liter. Cells were harvested at 10,000 × *g* for 15 min at 4°C in a Sorvall RC-5C centrifuge (Du Pont Instruments Ltd., Stevenage, United Kingdom) fitted with a GS-3 rotor. The cell broth was concentrated to 2 to 3 liters in a Membrex Benchmark Rotary Concentrator (obtained through Anachem Ltd.) fitted with a 200-cm$^2$ membrane with a pore size of 0.45 μm. Seeds for 750-ml cultures were prepared by resuspending cells harvested from a 750-ml culture after 48 h in 15 ml of 50 mM morpholinepropanesulfonic acid (MOPS), pH 7.0, plus 7% (vol/vol) dimethyl sulfoxide and storing at −80°C as 1-ml aliquots. A number of alternative bacteria were screened for constitutive cocaine esterase activities. Growth was on 1% (wt/vol) nutrient agar (Oxoid Ltd.) prior to inoculation into a liquid medium (taken from the National Collection of Industrial and Marine Bacteria [NCIMB] 1986 catalogue) consisting of 2 g of yeast extract per liter, 5 g of Bacto Peptone per liter, 5 g of NaCl per liter, and 10 g of glucose per liter. The following bacteria were obtained from NCIMB, Aberdeen, United Kingdom: *Arthrobacter oxydans* 9337, *Corynebacterium* sp. strain 10406, *Pseudomonas aeruginosa* K ATCC 25102, *Pseudomonas fluorescens* 9815, *P. maltophilia* RH873-3, and *P. putida* ATCC 17464; *P. putida* PMBL-1 was a gift from A. C. M. van der Drift, TNO Medical Biological Laboratory, Rijswijk, The Netherlands. The same range of organisms was screened for growth in medium B containing 10 mM cocaine as the sole source of carbon and energy.

**Analysis of fermentation broths and whole-cell incubations.** Samples (1 ml) from 10-liter fermentations were removed, and cells were pelleted by centrifugation in an MSE Micro-Centaur centrifuge. Samples of supernatant were analyzed by HPLC. Whole-cell incubations were performed with washed *P. maltophilia* MB11L cells resuspended in 10 ml of growth medium containing 10 mM cocaine and incubated at 30°C. Samples were removed at timed intervals, and cells were pelleted prior to analysis of the supernatant by HPLC and GC. Similar whole-cell incubations were performed in a stirred $O_2$ electrode cell (Rank Brothers, Cambridge, United Kingdom) containing 3 ml of reaction mixture at 30°C.

**Preparation of crude extract.** Harvested cells were resuspended in 50 mM MOPS-NaOH buffer, pH 7.0 (0.5 g [wet weight] per ml of buffer). Disruption of the cells was by sonication in an MSE Soniprep (Fisons Instruments, FSA Ltd.) with 18 bursts (12 μm) of 15 s alternated with 30 s of cooling in melting ice. Cell debris and unbroken cells were removed by centrifugation at 48,000 × *g* for 20 min at 4°C in a Sorvall RC-5C using an SS-34 rotor to give a clarified cell extract.

**Incubations with crude extracts.** An aliquot (0.5 ml) was incubated with 10 ml of 50 mM MOPS buffer, pH 7.0, containing 5 mM cocaine at 30°C. Samples (1 ml) were removed at timed intervals, and protein was precipitated with 10 μl of concentrated hydrochloric acid. After centrifugation to remove the protein, samples of the supernatant were analyzed by HPLC and TLC. Reaction products were identified by comparing the chromatograms obtained with those of authentic standard compounds. Control samples containing boiled extract were treated and analyzed in an identical fashion.

**Enzyme assays.** In each case, 1 U of enzyme activity was defined as that amount of enzyme producing 1 μmol of product in 1 min at 30°C.

**(i) Cocaine esterase.** The reaction mixture contained 2 mM cocaine and enzyme in 1 ml of 50 mM MOPS buffer, pH 7.0. The assay mixture was shaken at 30°C, aliquots were removed at intervals, and the reaction was stopped by the addition of concentrated $H_3PO_4$. The protein precipitate was removed by centrifugation in a Minifuge before samples (50 μl) of the supernatant were analyzed by HPLC. All standard curves were linear in the range investigated (0 to 1 mM), and recoveries were reproducible and quantitative when $H_3PO_4$ was used to precipitate the protein. All incubations were performed in duplicate. Hydrolytic activity against amido esters was determined by similar incubations and analyses.

**(ii) Enzymes of aromatic metabolism.** Catechol 1,2-dioxygenase (EC 1.13.11.1) was assayed by using the method of Kojima et al. (19). Catechol 2,3-dioxygenase (EC 1.13.11.2) activity was determined by using the method of Nakazawa and Yokota (26). Protocatechuate 3,4-dioxygenase (EC 1.13.11.3) activity was determined by using the method of Cain et al. (7). *cis-cis*-Muconate cycloisomerase (EC 5.5.1.1) activity was detected by monitoring the rate of decrease of $A_{260}$ ($\Delta\varepsilon_{260}$ of muconolactone assumed to be 17,500 $M^{-1}$ $cm^{-1}$ according to Kojima et al. [19]) at 30°C upon adding extract (10 μl) to an assay mixture containing 2.6 ml of 50 mM Tris HCl buffer (pH 8.0), 0.3 ml of 1 mM *cis-cis*-muconate, and 0.1 ml of 60 mM $MnCl_2$. Combined muconolactone isomerase (EC 5.3.3.4) and β-ketoadipate enol-lactone hydrolase (EC 3.1.1.24) activity was assayed by the addition of cell extract (10 μl) to an assay mixture containing 2.7 ml of 100 mM Tris HCl buffer (pH 8.0) and 0.3 ml of 5 mM (+)-muconolactone. The decrease in $A_{230}$ at 30°C was monitored and used to calculate the net activity of the two enzymes ($\Delta\varepsilon_{230}$ of muconolactone assumed to be 1,650 $M^{-1}$

$cm^{-1}$ according to Cain et al. [7]). β-Ketoadipate coenzyme A (CoA)-transferase (EC 2.8.3.6) was assayed by using the method of Katagiri and Hayaishi (18). The increase in $A_{305}$ due to the formation of the magnesioenol derivative of ketoadipate CoA was monitored at 30°C upon the addition of extract (10 μl) to a reaction mixture containing 0.7 ml of 100 mM Tris HCl buffer (pH 8.0), 0.1 ml of 200 mM $MgCl_2$, 0.1 ml of 5 mM succinyl CoA, 0.1 ml of 200 mM β-ketoadipate, and potassium salt. As the extinction coefficient of the reaction product is unknown, an arbitrary $\Delta\varepsilon_{305}$ of 1,000 $M^{-1}$ $cm^{-1}$ was assumed. Protein concentration was determined by using the method of Bradford (6).

**Column chromatography.** All columns were obtained from Pharmacia/LKB Biotechnology Ltd. (Milton Keynes, United Kingdom). Elutions were monitored with an LKB 2238 SII Uvicord which was set to monitor $A_{280}$ and which was connected to a 2210 chart recorder. Fractions were collected by using an LKB 2211 Superrac fraction collector. Samples were loaded and eluted by using a Gilson Minipuls II pump (Anachem Ltd.).

**Purification of cocaine esterase.** All buffers contained 1 mM β-mercaptoethanol and 2% (vol/vol) glycerol as stabilizing agents. All steps were performed at 4°C. Solubilization of cocaine esterase activity was afforded by the addition of cholic acid to the crude extract at a final concentration of 0.5% (wt/vol) followed by the addition of 1 M NaOH to adjust the pH to 7.0 and that of NaCl to give a final conductivity of 13 mS/cm, equivalent to that of 50 mM MOPS–0.5% (wt/vol) cholate–0.1 M NaCl (pH 7.0) (buffer A). Treated extract, typically 150 ml containing 1 g of protein and 160 U of cocaine esterase activity, was then loaded onto a DEAE-Sephacel (Pharmacia/LKB Biotechnology) column (27 by 2.6 cm) preequilibrated with buffer A. The column was washed with buffer A until no further elution of protein monitored at 280 nm was detected. After adsorption, the esterase was eluted by a linear gradient containing 125 ml of buffer A and 125 ml of buffer B (50 mM sodium borate, 0.5% [wt/vol] cholate, 0.6 M NaCl [pH 9.0]) and then washed with 100 ml of buffer B. Fractions (10 ml) were collected at a flow rate of 15 ml/$cm^2$/h and assayed for cocaine esterase activity and protein. Active fractions (eluting at a salt concentration of approximately 0.4 M) were pooled and dialyzed against 2-liter quantities of buffer C (10 mM potassium phosphate buffer, 0.5% [wt/vol] cholate, 0.1 M NaCl [pH 6.8]) until the pH and conductivity of the protein solution were identical to those of buffer C. Dialyzed material was loaded onto a Bio-Gel HT (Bio-Rad Labs Ltd.) hydroxylapatite column (14 by 1.6 cm) preequilibrated in buffer C. After adsorption, the column was washed with buffer C until no further elution of protein was seen. Elution of the cocaine esterase was afforded with a gradient of 30 ml (each) of buffer C to buffer D (identical to buffer C except that the potassium phosphate concentration was 300 mM) followed by a wash of 30 ml of buffer D. Fractions (3 ml) were collected at a flow rate of 15 ml/$cm^2$/h and assayed for cocaine esterase activity and protein. Active fractions (eluting at a phosphate concentration of approximately 80 mM) were pooled and concentrated against polyethylene glycol 4000 to a final volume of approximately 4 ml. This concentrated material was loaded onto a column (60 by 1.6 cm) of Ultrogel AcA44 (Life Science Labs Ltd.) preequilibrated in buffer A. Fractions (2 ml) were collected at a flow rate of 4 ml/$cm^2$/h and assayed for cocaine esterase activity. Active fractions were pooled prior to dilution (1:10) with buffer E (50 mM MOPS [pH 7.0]). This material was dialyzed twice against 10 volumes of buffer E to remove salt and cholate. The dialyzed material was concentrated against polyethylene glycol 4000 to a volume of approximately 4 ml and loaded onto a Sephacryl S-300 (Pharmacia/LKB Biotechnology) column (75 by 1.6 cm) preequilibrated in buffer E. Subsequent elution of the enzyme was performed at 4 ml/$cm^2$/h, and the protein was collected as 2-ml fractions prior to assaying for cocaine esterase activity.

**Polyacrylamide gel electrophoresis (PAGE).** Electrophoretic analyses were performed by using a Bio-Rad Mini Protean II system (Bio-Rad Laboratories Ltd.) by the method of Laemmli (21). Vertical slab gels (84 by 60 by 0.75 mm) containing 7.5% (wt/vol) acrylamide were run at 200 V constant voltage. Native gels, also containing 7.5% (wt/vol) acrylamide, were run as above, except that they were run at 4°C to retain enzyme activity and sodium dodecyl sulfate (SDS) was omitted. Protein was detected by staining with Coomassie blue R-250 (0.1% [wt/vol] in 40% [vol/vol] methanol–10% [vol/vol] glacial acetic acid) for 45 min. Gels were destained in 40% (vol/vol) methanol–10% (vol/vol) glacial acetic acid. Esterase activity was detected by using the method of Sobek and Görisch (39); gels were soaked in 50 mM MOPS buffer, pH 7.0, for 30 min and then stained with 50 ml of 1 mg of fast blue (from Sigma Chemical Co. Ltd.) per ml in the buffer to which 0.5 ml of 10 mg of either α- or β-naphthyl acetate per ml in methanol was added.

**$M_r$ determinations.** The following enzymes were used as markers in gel filtration experiments: bovine liver catalase ($M_r$, 240,000), yeast alcohol dehydrogenase ($M_r$, 150,000), and yeast C300 hexokinase ($M_r$, 100,000). Assays for their activity were as described by Bergmeyer (4). Cytochrome *c* ($M_r$, 13,000) was detected by its $A_{505}$. Horse spleen apoferritin and pumpkin seed globulins ($M_r$s, 440,000 and 300,000, respectively) were detected by their $A_{280}$. The $M_r$ of the enzyme in crude extract was determined by using the method of Andrews (1); 1 ml of treated extract was mixed with 10 U of bovine liver catalase and loaded onto a Sephacryl S-300 column (1.6 by 75 cm) preequilibrated with 50 mM MOPS, pH 7.0. When the treatment of the extract involved the addition of a detergent, identical additions were made to the equilibration and elution buffers. A flow rate of 4 ml/$cm^2$/h was maintained in each case, and eluting protein was collected as 2-ml fractions. Comparison of the elution volumes of the proteins with those obtained for standard proteins (horse spleen apoferritin, pumpkin seed globulins, and bovine liver catalase) with the same column and conditions allowed an estimation of the $M_r$ of the cocaine esterase. The $M_r$ of purified cocaine esterase in the presence of salt and cholate was determined by the addition of salt and cholate to 0.1 M and 0.5% (wt/vol), respectively, to purified cocaine esterase (2 mg of protein, 7 U of activity). After standard proteins (bovine liver catalase, yeast alcohol dehydrogenase, bovine serum albumin, and cytochrome *c*) were added, the mixture (volume, 2 ml) was loaded onto a Sephacryl S-200 column (1.6 by 75 cm) preequilibrated in buffer A. Elution of protein with this buffer was at 4 ml/$cm^2$/h, and the eluting protein was collected as 1.3-ml fractions. The same method was used to determine the $M_r$ of purified cocaine esterase in the absence of salt and cholate, except that these agents were not added to the purified material or equilibration and elution buffers and the column contained Sephacryl S-300. SDS-PAGE of purified cocaine esterase (30 μg of protein in 10 μl diluted 1:3 with sample buffer) and standard proteins (10 μg (each) of Bio-Rad high-molecular-weight standards) as described previously allowed determination of the $M_r$ of the denatured cocaine esterase by the method of Shapiro et al. (36).

TABLE 1. Rates of oxidation of cocaine, ecgonine, and benzoate by washed cells[a]

| Growth substrate | Oxygen uptake (nmol/min/mg of cells)[b] upon addition of: | | |
|---|---|---|---|
| | Cocaine | Ecgonine | Benzoate |
| Cocaine | 40 | 60 | 80 |
| Ecgonine | <10 | 20 | <10 |
| Glucose | <10 | 0 | <10 |

[a] Concentrations of cocaine, ecgonine, and benzoate as both growth substrates and additives were 10 mM.
[b] Dry weight of cells was determined, and uptake was measured at 30°C.

## RESULTS

**Degradation of cocaine by whole cells of *P. maltophilia* MB11L.** *P. maltophilia* MB11L was isolated from industrial waste by selective enrichment in medium A containing 10 mM cocaine as its sole carbon source. Subculturing and serial dilution on agar plates containing the same growth medium resulted in the isolation of two distinct organisms. The organism showing the fastest growth was identified as NCIMB as *P. maltophilia* MB11L. This organism utilized both the benzoate and ecgonine moieties of cocaine as its sole carbon and energy sources in liquid and solid media. Analysis of the culture medium by HPLC when *P. maltophilia* was grown at the expense of cocaine demonstrated the disappearance of cocaine (HPLC retention time, 9.46 min) with the concomitant production of benzoic acid (HPLC retention time, 2.93 min), which was metabolized further. Samples (1 μl) from whole-cell studies of *P. maltophilia* MB11L analyzed by GC showed that cells grown on cocaine were able to metabolize cocaine (GC retention time, 9.23 min) to benzoate (GC retention time, 6.15 min) and ecgonine methyl ester (GC retention time, 10.15 min), while cells grown solely on ecgonine hydrolyzed cocaine at a slower rate. The ability of whole cells of *P. maltophilia* MB11L to metabolize cocaine, benzoate, and ecgonine was investigated polarographically. The results, listed in Table 1, indicate that cells grown on cocaine oxidized cocaine and benzoate more rapidly than those grown on ecgonine or glucose. Similarly, cocaine- and ecgonine-grown cells oxidized ecgonine at a greater rate than those grown on glucose.

**Studies with crude extracts.** Crude extracts from cells of *P. maltophilia* MB11L grown on cocaine were seen to degrade cocaine to give a compound whose retention time under HPLC analysis corresponded with that of authentic benzoic acid (Fig. 1). TLC analysis of the same incubation mixture resolved a compound with an $R_f$ of 0.93 in solvent A on silica plates which coincided with that of ecgonine methyl ester. No hydrolysis of cocaine was seen when the crude cell extract was replaced with an identical quantity of boiled extract. These results and those observed with the whole cells show that cocaine esterase hydrolyzes cocaine to benzoate and ecgonine methyl ester (Fig. 2). Cocaine esterase was induced by cocaine, since specific activities of the enzyme in crude extracts from cells of *P. maltophilia* MB11L grown on glucose or benzoate as their sole carbon sources were only 20% of that observed with extracts from cocaine-grown cells (0.02 U/mg), while cells grown on citrate possessed no cocaine esterase activity. DL-Methionine, although not essential for growth, increased the specific activity of cocaine esterase in crude extracts by approximately 20%.

**Metabolism of benzoate in *P. maltophilia* MB11L.** Growth on 10 mM cocaine or benzoate, but not glucose or ecgonine, elicited high activities (more than 0.15 U/mg of protein) of catechol 1,2-dioxygenase, muconate cycloisomerase, muconate isomerase-enol lactone hydrolase, and β-ketoadipate CoA transferase in cell extracts of *P. maltophilia* MB11L. Lower levels (approximately 0.02 U/mg of protein) of catechol 2,3-dioxygenase activity were detected in these extracts. No protocatechuate 3,4-dioxygenase activity was detected in any of the extracts, indicating that benzoate metabolism is solely via catechol and predominantly through the β-ketoadipate pathway in *P. maltophilia* MB11L.

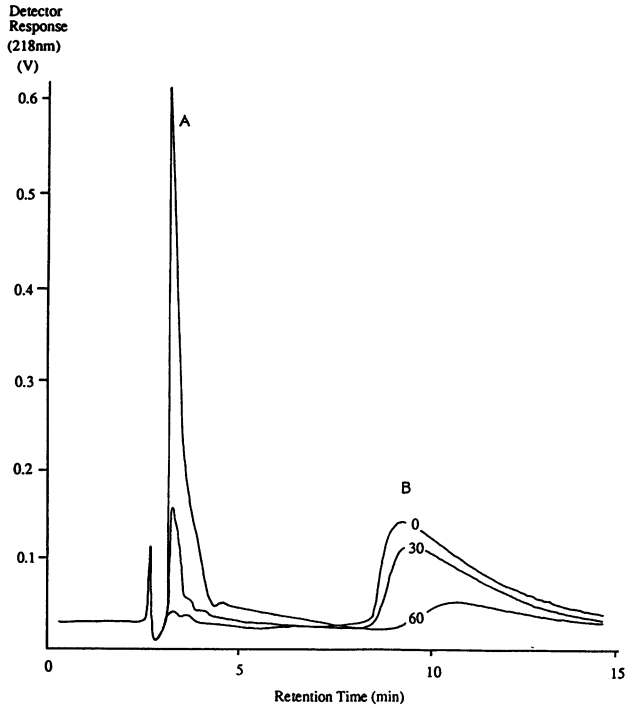
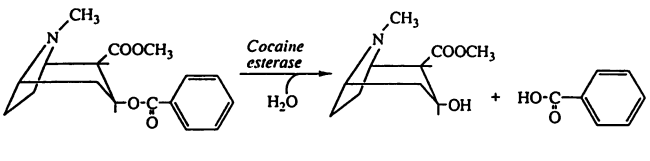



FIG. 1. Breakdown of cocaine by cell extracts monitored by HPLC. Samples removed at 0, 30, and 60 min from an incubation of cocaine with cell extract showed the breakdown of cocaine (B) with the concomitant production of benzoic acid (A).

**Partial purification of cocaine esterase.** It was observed that the cocaine esterase activity in crude extracts from *P. maltophilia* MB11L failed to bind to ion-exchange, hydrophobic interaction, and affinity chromatography media under a wide range of conditions. The $M_r$ of the enzyme in the crude extract determined by gel filtration chromatography on Sephacryl S-300 was observed to be greater than 400,000. Thus, it was suspected that the enzyme was aggregating or interacting with some cell component and thereby preventing binding to the chromatography media. A range of treat-




COCAINE — ECGONINE METHYL ESTER — BENZOIC ACID

FIG. 2. Cocaine esterase reaction.

TABLE 2. Typical purification protocol for cocaine esterase[a]

| Purification step | Total vol (ml) | Total activity (U) | Overall recovery (%) | Total protein (mg) | Sp act (U/mg) | Overall purification (fold) |
|---|---|---|---|---|---|---|
| Solubilization | 150 | 163 | 100 | 1017 | 0.16 | 1.0 |
| DEAE-Sephacel anion-exchange chromatography | 120 | 98 | 60 | 141 | 0.70 | 4.4 |
| Hydroxylapatite HT chromatography | 64 | 57 | 35 | 57 | 1.00 | 6.3 |
| AcA 44 Ultrogel gel filtration chromatography | 28 | 46 | 28 | 20 | 2.30 | 14 |
| S-300 Sephacryl gel filtration chromatography | 23 | 14 | 9 | 4 | 3.50 | 22 |

[a] Starting material was from the sonication of approximately 50 g (wet weight) of *P. maltophilia* MB11L cells grown on cocaine.

ments was performed on the crude extract, and the $M_r$ of the cocaine esterase was determined after each treatment in order to detect any solubilization of the enzyme which might enable it to interact with chromatographic media. Treatments involving solvent extractions, lipase and neuraminidase additions, denaturation with urea followed by renaturation, raising of pH, reduction of disulfide bridges, or the addition of saponin all failed to produce a change in the $M_r$ of cocaine esterase. However, the addition of Triton X-100 to 1% (vol/vol) reduced the $M_r$ to approximately 300,000, while the addition of 0.5% (wt/vol) cholate in the presence of 0.1 M NaCl resulted in an $M_r$ of 110,000. This latter solubilization technique was adopted as a first step in the purification of cocaine esterase, as enzyme treated in this manner bound to chromatography media under the conditions described in Materials and Methods. Subsequent separations by DEAE-Sephacel, hydroxylapatite, and gel filtration chromatographies gave a 22-fold purification of cocaine esterase, with an overall 9% recovery of activity. The purification scheme is summarized in Table 2.

**Analysis by native PAGE.** Purified cocaine esterase in the absence of cholate and salt was analyzed by PAGE under nondenaturing conditions. Staining with Coomassie blue showed a major band ($R_f$, 0.2) plus several minor bands. The major band failed to show acetylesterase activity when stained with α- or β-naphthyl esters, while a minor band ($R_f$, 0.91) showed β-esterase activity (by the definition of Goullet and Picard [14]). Sections of an unstained gel run under identical conditions were assayed for cocaine esterase activity by incubation with cocaine (2 mM in 1 ml of 50 mM MOPS buffer, pH 7.0, 30°C), and the reaction was monitored by HPLC. Only the section corresponding to the major band ($R_f$, 0.2) possessed activity. These results indicate that the cocaine esterase and acetylesterase activities are due to distinct proteins.

**Heat inactivation studies.** The thermal stabilities of cocaine esterase and acetyl esterase activities in the purified protein preparation (0.015 mg of protein) in the presence and absence of cholate and salt at 47°C were compared. The results, shown in Fig. 3, demonstrate that solubilization of cocaine esterase markedly decreases its stability at this temperature (half-life, 2.5 min), while little instability is seen at this temperature in the absence of salt and cholate. This suggests that some physical change is induced by the binding of cholate to the enzyme. The different heat inactivation profiles of cocaine esterase and acetylesterase activities provide further evidence that the two activities are due to distinct enzymes.

***$M_r$* of cocaine esterase.** Purified cocaine esterase subjected to SDS-PAGE yielded a single protein band with an $M_r$ of 129,000 by the method of Shapiro et al. (36). Gel filtration analysis of the purified enzyme by using the method of Andrews (1) gave $M_r$s for cocaine esterase of 110,000 in the presence of salt and cholate and 410,000 in their absence, implying that the enzyme exists as a tetramer in the absence of the solubilizing agents.

**pH optimum of cocaine esterase activity.** Purified cocaine esterase activity (5 μg of protein) was assayed in a range of buffers (50 mM Bis-Tris propane, 50 mM MOPS, and 50 mM bicine) covering a pH range of 6.0 to 10.0. The pH optimum of the enzyme was 8.0. It was observed during the course of this experiment that the enzyme activity in tertiary amine buffers (MOPS and bicine) at equivalent pH values was between 25 and 60% of that in Bis-Tris propane, a secondary amine.

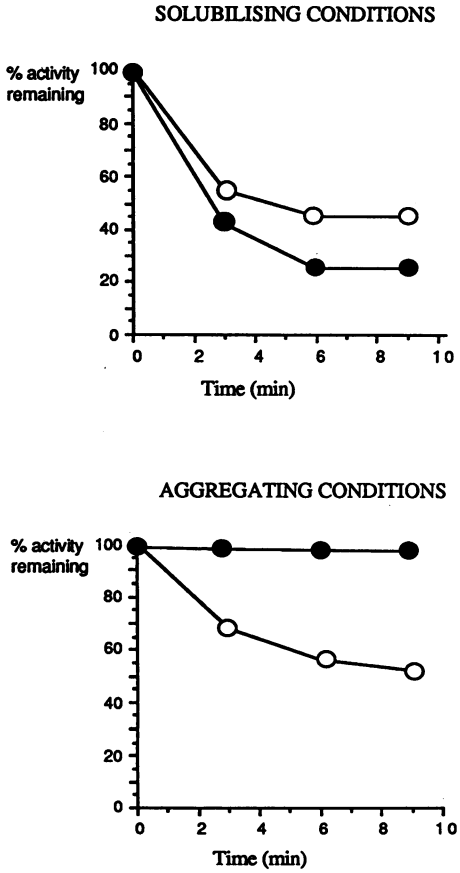



FIG. 3. Thermal denaturation profiles of cocaine esterase. Purified cocaine esterase (0.05 U) was held at 47°C in the presence and absence of 0.1 M NaCl and 0.5% wt/vol cholate (solubilizing and aggregating conditions, respectively). Samples were removed at timed intervals and assayed for cocaine esterase (●) and acetylesterase (○) activities.

TABLE 3. Kinetic constants of cocaine esterase[a]

| Substrate | Apparent $K_m$ (mM) | Apparent $V_{max}$ (U/mg) |
|---|---|---|
| Cocaine | 0.36 | 29.5 |
| Ethyl benzoate | 1.89 | 65.0 |
| Ethyl 2-hydroxybenzoate | 1.75 | |
| Ethyl 3-hydroxybenzoate | None | None |
| Ethyl 4-hydroxybenzoate | None | None |

[a] Esterase activities were determined in duplicate assays by using 1.25 μg (protein) of purified cocaine esterase as described in Materials and Methods. Average apparent $K_m$ and maximum rate of metabolism ($V_{max}$) values were determined from both double-reciprocal and Eadie-Hofstee plots with lines fitted to the data by regression analysis.

**Substrate specificity and kinetic studies.** The ability of various esters to serve as substrates was investigated by replacing cocaine with each compound in the reaction mixture. Apparent $K_m$ values for each of the esters were obtained by varying the substrate concentrations within the range of 0 to 2 mM (Table 3). The lack of activity with ethyl 3-hydroxybenzoate and ethyl 4-hydroxybenzoate indicates that the enzyme shows a strong specificity against benzoate esters substituted at the 3 and 4 positions of the aromatic ring, while it accepts unsubstituted benzoates and those substituted at the 2 position.

**Inhibition studies.** Purified cocaine esterase was not inhibited by 1 mM eserine or 1 mM *p*-hydroxymercuribenzoate; however, 1 mM phenylmethylsulfonyl fluoride caused total inhibition of activity. These results suggest the presence of a catalytically reactive serine in the esterolytic site of cocaine esterase (12). The enzyme is probably a type B carboxylesterase by the nomenclature used by Sobek and Görisch (39). Cocaine esterase showed product inhibition with neither benzoate nor ecgonine methyl ester.

**Activity of cocaine esterase against lipids, peptides, and amido esters.** Cocaine esterase showed no lipolytic or proteolytic activities when assayed according to the methods described by Veeraragavan (46) and Bergmeyer (4), indicating that it is a true esterase. This is in contrast to the esterase isolated from *Escherichia coli* by Pacaud (32), which showed proteolytic activity. Sulpiride, dibucaine, procainamide, bupivacaine, lidocaine, diethyltoluamide, and metoclopramide (amido esters of aromatic acids possessing a C-3 and/or a C-4 substituted aromatic ring) all failed to act as substrates for purified cocaine esterase. However, the enzyme showed equal activity with dimethylbenzamidine, an amido ester without a substitution on its ring, and cocaine. This implies that cocaine esterase has activity against amido esters of benzoic acid, with a specificity against C-3 and C-4 substituted benzamidines similar to that observed with substituted benzoates.

**Alternative organisms.** None of the organisms screened possessed a constitutive cocaine esterase or were capable of utilizing cocaine as their sole carbon and energy source.

## DISCUSSION

A strain of *P. maltophilia* (termed MB11L) capable of utilizing cocaine as its sole carbon and energy source was isolated by selective enrichment. Growth on cocaine conferred on crude extracts of *P. maltophilia* MB11L the ability to hydrolyze cocaine to benzoic acid and ecgonine methyl ester. Both reaction products supported growth of *P. maltophilia* MB11L; however, only cocaine induced high activities in the cocaine esterase. This is in distinct contrast to the constitutive phthalate esterase from a *Micrococcus* species described by Eaton and Ribbons (9) and the range of soil bacteria described by Engelhardt and Wallnöfer (10); in both cases, the phthalate esterases were induced by the free aromatic acid as well as by its esters. The atropine esterase in *P. putida* PMBL-1 was induced by the tropic acid metabolite phenylacetaldehyde (40). The benzoic acid produced by cocaine esterase is further metabolized in *P. maltophilia* MB11L via catechol and, thence, via the β-ketoadipate pathway. A low level of catechol 2,3-dioxygenase activity was detected, indicating either that *P. maltophilia* MB11L possesses an enzyme with both intra- and extradiol activities, as is observed for *Pseudomonas arvilla* C-1 (13), or that enzymes of both *ortho* and *meta* cleavage pathways are present, as has been described for benzoate metabolism in *P. putida* mt-2 (26).

In the crude extract, cocaine esterase appeared to aggregate as a tetramer with an $M_r$ of 410,000. When the enzyme was complexed in this manner, it proved exceptionally difficult to purify, as it failed to interact with a standard range of chromatography media. The addition of salt (final concentration, 0.1 M) and cholate (0.5% [wt/vol]) in combination with cocaine esterase solutions caused deaggregation of the enzyme, which resulted in a monomer with an $M_r$ of 110,000. Subsequent removal of these agents by dialysis caused a reaggregation of the enzyme. In the deaggregated form, the purified cocaine esterase showed a decrease in thermal stability at 47°C, implying that some destabilizing physical change was induced by the binding of cholate or the deaggregation of the enzyme. This is in contrast to the mammalian lipases, whose thermal stabilities have been reported to increase in the presence of bile acids; however, these enzymes do not undergo a bile acid-induced deaggregation (8, 41).

The cocaine esterase from *P. maltophilia* MB11L had a pH optimum of 8.0, similar to the pH optima of other mammalian and microbial carboxylesterases; Krisch (20) reported pH optima in the range of 7.9 to 9.0 for mammalian enzymes, while microbial esterases with similar pH optima have been reported (30, 37, 39).

The existence of a cation-binding site in close proximity to the esterolytic site has been proposed for acetylcholinesterase (5, 12, 33) and atropine esterase (3). The inhibitory effect of tertiary amine buffers on the cocaine-hydrolyzing activity of cocaine esterase implies that a similar site may be present in this enzyme. Such a site would aid in the binding of cocaine to the enzyme, resulting in a greater apparent affinity for cocaine than for simple aromatic esters, and would result in a slower release of reaction products after hydrolysis had occurred. Kinetic characterization of the purified enzyme demonstrated a lower apparent $K_m$ for cocaine than for ethyl benzoate and 2-hydroxybenzoate and a slower rate of hydrolysis of cocaine. The activity of the enzyme against ethyl 2-hydroxybenzoate but not ethyl 3- or 4-hydroxybenzoate indicates the existence of either a steric or an electrostatic effect due to the presence of a hydroxyl group on the benzoate ring at the 3 or 4 position.

Although atropine esterase and cocaine esterase are both serine hydrolases, atropine esterase from *P. putida* PMBL-1 and cocaine esterase from *P. maltophilia* MB11L showed no activity against cocaine and atropine, respectively, despite the apparent structural similarities between the two aromatic tropane esters. Neither enzyme showed activity with acetyl esters; this was ascribed to the existence of a hydrophobic cleft in the active site in the case of atropine esterase (34, 43, 44). Sequence data obtained by Hessing (15) allowed a

structural prediction of atropine esterase (42), indicating that it showed no homology to either the trypsin family or the subtilisin family. Without similar data for cocaine esterase, it is not yet possible to describe its family membership. Repeated and further purifications of cocaine esterase should allow sequence data to be determined, permitting a more detailed comparison of the enzymes.

## ACKNOWLEDGMENTS

We thank Peter Baker of the Food and Bioscience Division at the Laboratory of the Government Chemist for technical help and advice. We warmly acknowledge Alfons van der Drift of TNO Medical Biological Laboratory, Rijswijk, The Netherlands, for the resurrection and donation of *P. putida* PMBL-1.

A.J.B. acknowledges the award of an SERC studentship for this study.

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