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Identification of a Heroin Esterase in *Rhodococcus* sp. Strain H1

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A strain of a *Rhodococcus* sp. (termed H1) capable of utilizing heroin as its sole carbon and energy source was isolated by selective enrichment. An inducible heroin esterase was partially purified and shown to catalyze the hydrolysis of both of the acetylester groups of heroin. The enzyme displays optimum activity at pH 8.5 and appears to be a trimer of identical subunits with an M_r of 39,000 and a native M_r of 120,000.

Heroin (3,6-diacetylmorphine) possesses potent analgesic properties but is probably best known as one of the most powerfully addictive and commonly abused narcotic drugs. Bacterial enzymes mediating degradative pathways for the metabolism of heroin and other morphine alkaloids could be utilized as specific biorecognition systems in the development of a heroin biosensor. We have previously identified a highly specific $NADP^+$-dependent morphine dehydrogenase which has been purified from *Pseudomonas putida* M10 and characterized (3, 4). The structural gene for morphine dehydrogenase, *morA*, has been cloned and overexpressed in *Escherichia coli* (9). Our laboratory is particularly interested in microbial acetyl esterases because, for morphine dehydrogenase to function in an illicit-drug biosensor, heroin has firstly to be deacetylated to morphine (Fig. 1); commercially available esterases were unable to hydrolyze rapidly the 6-acetyl ester group of heroin. In this article we describe the isolation of a *Rhodococcus* species capable of growth on heroin as a sole carbon and energy source and the partial purification of an acetylmorphine carboxyesterase (heroin esterase).

Isolation of the organism and culture conditions. *Rhodococcus* sp. strain H1 was isolated from Cambridge garden soil by selective enrichment in 250-ml Erlenmeyer flasks containing 50 ml of defined medium (4) and 10 mM heroin (Macfarlan Smith Ltd., Edinburgh, United Kingdom). The enrichment cultures were incubated at 30°C in a shaking incubator (160 rpm). *Rhodococcus* sp. strain H1 was identified by the National Collection of Industrial and Marine Bacteria (Aberdeen, United Kingdom). All subsequent fermentations were carried out in a minimal medium containing (grams per liter) Na_2HPO_4 (4.33), KH_2PO_4 (2.65), NH_4Cl (2.0), and nitrilotriacetic acid (0.1), 50 mM acetate, and 2 mM heroin, which was added during the late exponential growth phase. Trace elements were as described by Rosenberger and Elsden (7) and were added at 4 ml/liter. Large-scale fermentations were carried out in a 75-liter Chemap CMF400 fermenter (Alfa-Laval Engineering, Brentford, United Kingdom), monitored by a Bio-Data Manager software. Culture broth was analyzed by the aseptic withdrawal of 1-ml samples at timed intervals. The samples were clarified by centrifugation, and the supernatant was subjected to high-performance liquid chromatography (HPLC) analysis on a Waters component system (Millipore Waters UK Ltd., Watford, United Kingdom) consisting of a 600E system controller and a model 712 WISP autoinjector connected to a 484 absorbance detector set to 235 nm (full-scale deflection, 0 to 1 V). Separation of samples (50 μl) was achieved on a 5-μm Techsphere octadecyl silane column (4.6 by 50 mm), guarded by a column (4.6 by 40 mm) containing the same material with an isocratic solvent system (6), delivered at a flow rate of 1 ml/min. Data processing was done with Maxima 820 software. Analysis of the culture medium by HPLC when *Rhodococcus* sp. strain H1 was grown at the expense of acetate and heroin demonstrated the disappearance of heroin (HPLC retention time, 3.40 min) with the concomitant production of 6-acetylmorphine (HPLC retention time, 1.70 min) after a lag phase of 30 to 40 min (Fig. 2). There was negligible morphine (HPLC retention time, 1.10 min) in the culture supernatant until 90 min; then, coincidently with the decrease in 6-acetylmorphine concentration, the stoichiometric production of morphine was monitored. The growth study implies that *Rhodococcus* sp. strain H1 hydrolyzed the 3-acetylester group from the heroin molecule to yield 6-acetylmorphine; this product was then excreted into the supernatant. When the heroin was nearly exhausted, the 6-acetylmorphine was reinternalized into the cell and the 6-acetylester group was hydrolyzed to give morphine. No esterase activity was detected extracellularly. The organism grew at the expense of 2 mol of acetate per mol of heroin.

Heroin esterase activity in crude extract. Harvested cells were resuspended in buffer A (50 mM morpholinepropanesulfonic acid [MOPS] [pH 7.0]) at a density of 0.5 g (wet weight) of cells per ml of buffer. Disruption of the cells was by sonication in a Lucas Dawe continuous-flow Soniprobe 7535A at full power and a flow rate of 80 ml · min^{-1}. The cell slurry was passed through the sonication chamber a total of eight times. Cell debris was removed by centrifugation at 27,500 × *g* for 45 min at 4°C in a Sorvall RC5C centrifuge using a GSA rotor to give the crude cell extract. Crude extracts from cells of *Rhodococcus* sp. strain H1 grown on heroin were seen to degrade heroin and 6-acetylmorphine to morphine (generous gifts from M. J. Davies, Macfarlan Smith Ltd.). The reaction mixtures contained 1 mM heroin (or 6-acetylmorphine), 50 mM bicine buffer (pH 8.5), and 30 μl of crude extract in a total volume of 1 ml and incubated at 30°C. The reactions were stopped at intervals by the addition of 2 μl of glacial acetic acid. The protein precipitate was removed by centrifugation in an MSE Microfuge before samples (50 μl) of the supernatant were analyzed by HPLC. No hydrolysis of heroin was seen when the crude cell extract was replaced with an identical quantity of extract that had been boiled for 3 min. Heroin esterase activity was routinely measured with 6-acetylmorphine as the substrate for the enzyme. Standard curves of 6-acetylmorphine and morphine were linear in the range investigated

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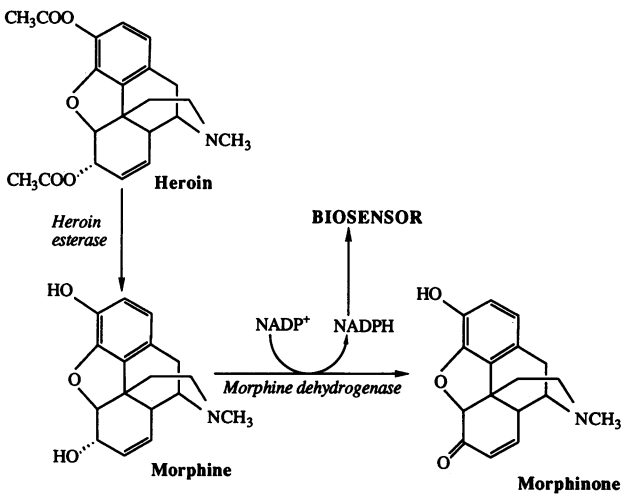

FIG. 1. Coupled enzyme assay for the detection of heroin.

(0.02 to 1.0 mM), and all analyses were done in triplicate. A unit of enzyme activity was the amount of enzyme producing 1 μmol of morphine in 1 min at 30°C. In crude extracts prepared from cells grown on heroin in shaken-flask cultures, the heroin esterase was present at activities of 0.31 U · mg of protein^{-1}, some 20-fold greater than that in acetate-grown cells, indicating that the heroin esterase activity was induced by heroin. Assays for heroin esterase activities performed on separate batches of extracts generally agreed to within 10%. Protein concentrations were measured in solution by the method of Bradford (2), using bovine serum albumin as the standard.

Purification of the heroin esterase. To verify that a single heroin esterase was responsible for the hydrolysis of the 6-acetylester group, the heroin esterase preparation was purified by anion-exchange and hydrophobic-interaction chromatography. Heroin esterase was purified 23-fold from 100 g (wet weight) of cells of *Rhodococcus* sp. strain H1 grown at the expense of acetate and heroin, with a 28% overall recovery. The buffer used throughout was buffer A, and all the steps were performed at 4°C. Enzyme activity was measured during the purification procedure by monitoring the increase at 270 nm caused by the liberation of phenol (assumed ε_{270}, 1,480 M^{-1} cm^{-1}) from phenylacetate. The reaction mixture contained 0.3 mM phenylacetate and 50 mM bicine buffer (pH 8.5) in a total volume of 1 ml. This activity assay was used only to detect the heroin esterase in column elutions. The pooled fractions were then assayed by measuring the rate of hydrolysis of 6-acetylmorphine, as described previously. Crude extract (210 ml, containing 609 mg of protein with a specific activity of 0.31 U/mg) was applied to a DEAE-Toyopearl (Supelchem, Saffron Walden, United Kingdom) column (5.0 by 24 cm) preequilibrated with buffer A. The column was washed at a linear flow rate of 10 ml/cm^2/h with 0.1 M NaCl in buffer A until no further elution of protein monitored at 280 nm was detected. After adsorption, the heroin esterase was eluted by a linear gradient between 0.1 and 0.5 M NaCl in buffer A in a total volume of 600 ml. Fractions (10 ml) were collected and assayed for activity and for protein. Two peaks of activity against phenylacetate were detected; the first passed straight through the column in the load wash, while the second activity was eluted at a salt concentration of 0.3 M NaCl. The first esterolytic activity was found to hydrolyze slowly the C-3 acetylester group of heroin but possessed no activity with 6-acetylmorphine. The heroin esterase activity resided wholly in the DEAE-Toyopearl adsorbed material. The active heroin esterase fractions were pooled, and the salt concentration was raised to 2 M NaCl. The active material was loaded onto an octyl-agarose 6XL (Affinity Chromatography Ltd., Ballasalla, United Kingdom) column (2.6 by 9.0 cm) preequilibrated in 2 M NaCl in buffer A. The column was washed at a linear flow rate of 20 ml/cm^2/h with 2 M NaCl in buffer A until no further elution of protein was detected at 280 nm (approximately 300 ml). After adsorption, the heroin esterase was eluted by a decreasing linear gradient between 2 and 0 M NaCl in buffer A in a total volume of 200 ml. The active fractions were pooled and dialyzed extensively at 4°C against 50 mM ammonium acetate buffer (pH 7.0); then, the solution was lyophilized. Separations by DEAE-Toyopearl and octyl-agarose chromatographies gave a partially purified heroin esterase, with a specific activity of 7.0 U/mg.

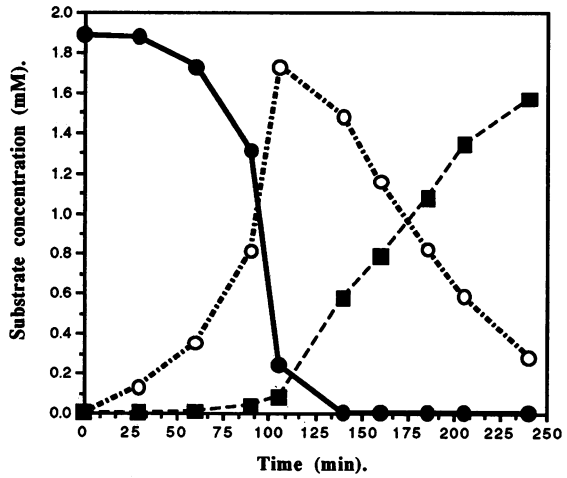

FIG. 2. Degradation of heroin by *Rhodococcus* sp. strain H1. Erlenmeyer flasks containing minimal media supplemented with 50 mM acetate were inoculated with cultures pregrown with 50 mm acetate as the sole carbon source. When the culture approached late exponential phase, 2 mM heroin was added. Heroin (●), 6-acetylmorphine (○), and morphine (■) concentrations were quantified by HPLC analysis.

Analysis by PAGE. Partially purified heroin esterase (200 μg) was analyzed by polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions. Electrophoretic analyses were carried out by the method of Laemmli (5). Vertical slab gels (17 cm by 14.7 cm by 2 mm) containing 10% (wt/vol) acrylamide were run at a 200-V constant voltage at 4°C to retain enzyme activity. 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 60 min. Gels were destained in 40% (vol/vol) methanol–10% (vol/vol) glacial acetic acid. Acetyl esterase activity was detected by using the method of Sobek and Görisch (8) with α-naphthylacetate as the substrate, and a single active band, which comigrated with a major band in a Coomassie-stained gel, was evident. Sections cut from an unstained gel, run under identical conditions, were assayed for heroin esterase activity by incubation with 6-acetylmorphine (1 mM in 1 ml of buffer A at 30°C), and the reaction was monitored by HPLC. Only the section corresponding to the major band possessed activity.

***M_r* of the heroin esterase.** Sodium dodecyl sulfate (SDS)-PAGE analysis of the heroin esterase, cut from a native polyacrylamide gel, gave a single band with an M_r of 39,000. M_r determinations of the purified enzyme were obtained by using

Bio-Rad SDS–PAGE low-molecular-mass standards. Gel filtration analysis on a Sephacryl S-200 column (1.6 by 77 cm) of the purified enzyme by the method of Andrews (1) gave a native M_r for the heroin esterase of 120,000, implying that the enzyme exists as a trimer of equal subunits.

pH optimum of heroin esterase activity. Partially purified heroin esterase activity (2 μg of protein) was assayed in buffers (50 mM 1,3-bis(tris[hydroxymethyl]-methylamine) propane, 50 mM MOPS, and 50 mM bicine) covering a pH range of 6.0 to 10.0 with 6-acetylmorphine as the substrate. The pH optimum of the enzyme was pH 8.5 in bicine buffer.

Determination of the N-terminal amino acid sequence. Partially purified heroin esterase (200 μg) was subjected to SDS-PAGE as described previously and then to electroblotting onto a Problott membrane in 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid]–10% (vol/vol) methanol (pH 11) using an LKB 2005 Transphor kit. The N-terminal sequence of the major band corresponding to heroin esterase was determined by automated Edman degradation with an Applied Biosystems 470A sequencer at the Cambridge Centre for Molecular Recognition, University of Cambridge, Cambridge, United Kingdom. The N-terminal sequence of the heroin esterase was determined to be Thr-Thr-Phe-Pro-Thr-Leu-Asp-Pro-Glu-Leu-Ala-Ala-Ala-Leu-Thr-Met-Leu-Pro-Lys-Val-Asp-Phe.

These results demonstrate the ability of *Rhodococcus* sp. strain H1 to catabolize heroin, yielding morphine. A heroin esterase which has considerable potential for application in a heroin biosensor has been identified and partially characterized. To this end, continuing studies in our laboratory are directed towards cloning and overexpressing the structural gene for the heroin esterase.

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