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The Development of Bacterial Carboxylesterase Biological Recognition Elements for Cocaine Detection

Suhad A. Mustafa[1]

Abstract

Enzyme recognition element-based biosensors are very attractive for biosensor application due to a variety of measurable reaction products arising from a catalytic process. In this study, biosensor recognition elements have been developed via engineer bacterial enzymes (*carboxylesterases* (*CEs*)) which will used for narcotic detection because of their role in narcotics metabolism. The modification (insertion of cys-tag) allows the enzyme to bind into a transducer surface of a biosensor which will translate the reaction product into the detection system. The results demonstrate the successful isolation, cloning, expression, and purification of recombinant (*pnbA1* and *pnbA2*), and engineered (*pnbA1*-cys and *pnbA2*-cys) bacterial carboxylesterases. Enzyme capability to hydrolyse cocaine into benzoylecgonine and methanol was quantified using HPLC. Both enzymes showed broad maximal activity between pH (8.0, 8.5, and 9.0), PnbA1 temperature stability ranging between (25 and 45 °C); however, PnbA2 stability range was (25–40 °C). Insertion of cys-tag at the N-terminal of the enzyme did not limit entrance to the active site which is located at the base of a cavity with dimensions 20 by 13 by 18 Å, and did not prevent substrate hydrolysis. Bacterial carboxylesterases pnbA1 and pnbA2 mimic hCE1 and not hCE2 in its reaction pathways hydrolysing cocaine into benzoylecgonine and methanol rather than ecgonine methyl ester and benzoic acid. These results are the first experimental evidence confirming the capability of bacterial carboxylesterase to hydrolyse cocaine into its main metabolites, therefore opening up the possibility to use these enzymes in numerous biotechnological applications in addition to a cocaine biosensor.

Keywords Bacterial carboxylesterases · Biological recognition elements · Enzymes · Cocaine · Biosensor

Introduction

Carboxylesterases (CES) are ubiquitous enzymes that have been identified from different sources ranging from mammals [1] to bacteria [2], and even in plants [3], they achieve ester bond hydrolysis with the aid of these enzymes [4]. These enzymes exhibit broad substrate specificities, they cleave carboxylic esters into the corresponding alcohol and carboxylic acid, and they are members of α/β hydrolase family that share a common α/β hydrolase fold, a regulatory domain, and the catalytic triad of Ser, His, and Asp/Glu [5–9]. They catalyse hydrolytic and transesterification reactions of narcotics (heroin and cocaine) [10], anticancer drugs such as irinotecan (CPT-11) [11], and detoxify numerous organophosphate and carbamate compounds used as insecticides [12–14] or chemical weapons (Sarin, Tabun, and Soman) [15], and catalyse several reactions in cholesterol and fatty acid metabolism [16–18]. Cocaine (COC), an alkaloid obtained from *Erythroxylum coca* plant, has become one of the most notably abused drugs, and remains one of the most common health problems [19]. Human CEs catalyse the rapid hydrolysis of ester linkages in COC to the inactive benzoylecgonine (BZE), the main metabolite in both blood and urine [20]. The role of CEs in narcotics metabolism and the illegal use of narcotics have encouraged considerable interest in the development of methods for their detection [21, 22]. There has been a great increase in the use of microbial carboxylesterases as biocatalysts in biomedical applications because of their tremendous abilities to achieve regio-,

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✉ Suhad A. Mustafa
chem.bangor@gmail.com

[1] Scientific Research Center, Salahaddin University-Erbil, Erbil, Kurdistan Region, Iraq

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stereo-, and enantio-specific reactions [23–25]. *p*-Nitrobenzyl esters act as protecting groups on intermediates in the production of clinically important oral β-lactam antibiotics [26]. A *Bacillus* carboxylesterase has been used for stereo-specific resolution of *R,S*-naproxen esters, which is an important anti-inflammatory drug [27], and a *p*-nitrobenzyl esterase was genetically engineered in order to synthesise cephalosporin-derived antibiotics [28]. The crystal structure of *Bacillus subtilis* Pnb CE has been solved and it is homologous to rCE and shows activity with the prodrug CPT-11. So far, studies concerning the role of *Bacillus* or other microbial carboxylesterases in narcotic metabolism are scarce. The key purpose of this study was to develop biological recognition elements for a sensor for cocaine detection by way of isolation of two different bacterial carboxylesterases (CEs) PnbA from Bacillus subtilis subsp. subtilis strain 168 designated in this study as PnbA1, and PnbA from *Bacillus licheniformis* ATCC 14580 designated as PnbA2, based on the role of human liver carboxylesterases hCE to hydrolyse cocaine into its main metabolites: ecgonine methyl ester and benzoic acid, or benzoylecgonine and methanol. Then confirm their activity via the catalysis of cocaine into its main metabolites, followed by engineering of the enzymes through integration of the cys-tag (6 cysteine amino acids) at the enzyme N-terminus to allow the enzyme to be immobilised on the biosensor electrode surface and subsequently study the effect of cys-tag insertion on hydrolysis reaction pathways achieved by the enzyme.

Materials and Methods

DNA Extraction and PCR Amplification of Target Gene

The genomic DNA was extracted using Wizard® genomic DNA purification kit [29]. PCR primers incorporating *Bam*H1, *Hind*III, and *Sac*I restriction sites (underlined) were designed for amplification of the target genes, and were supplied by MWG Biotech (London, UK). Forward primer: 5′-AAGGGATCCAACACAATGACTCATCAAATAG-3′ Reverse primer: 5′-CAGCACAAGCTTATCCTGTTTTCC CCA-3′ regarding *Bacillus subtilis* and Forward primer: 5′-AAGGGATCCAATAAAATGTATGATACAACTGTC GAA-3′ Reverse primer: 5′- GGCGAGCTCCACAGGTCA GGCCCG-3′ regarding *Bacillus licheniformis*. Carboxylesterase genes *pnbA* from *Bacillus subtilis*, and *pnbA* from *Bacillus licheniformis* were amplified by PCR master mix, *Taq* DNA Polymerase [30]. The PCR solution comprised 10 µl 5 × Phusion HF buffer, 1 µl template DNA, 1.5 µl forward primer, 1.5 µl reverse primer, 1 µl from 10 mM dNTP, 0.5 µl Phusion DNA polymerase, and 34.5 µl distilled water. The PCR program consisted of two steps: 15 cycles of 98 °C for 30 s (initial denaturation), 98 °C for 10 s (denaturation), 64–54 °C for 30 s (annealing), 72 °C for 30 s (extension) followed by 72 °C for 10 min (final extension) were used for the first step, while the second step included 20 cycles of 98 °C for 10 s, 54 °C for 30 s, 72 °C for 30 s followed by 72 °C for 10 min, finally the temperature was lowered to 4 °C overnight.

Cloning, Expression, and Purification of Target Gene

The amplified PCR products were cloned into pET28a vector and transformed into *E. coli* DH5α competent cells. Plasmid DNA was purified from transformants using a QIAprep Spin Miniprep TM kit. The ligation mixture was digested with the appropriate restriction enzymes and subjected to agarose gel electrophoresis to confirm the success of the ligation. A sample of the purified plasmid DNA was sequenced to confirm the identity of the amplification product. The pET28a construct was then transformed into competent cells of the *Rosetta* strain of *E. coli* and expression of the His-tagged recombinant protein was carried out using IPTG induction LB medium. A single colony was used to inoculate 500 ml of medium, which was incubated at 37 °C and 180 rpm until stationary phase was reached. The resulting cell pellet was obtained via centrifugation at 8000 rpm for 10 min. Expressed protein was purified using 10 ml, 10 mM imidazole solution, consisting of phosphate buffer (PB) (pH 7.4, 6.25 ml, 0.1 M) and imidazole (2 M, 0.25 ml), made up to 50 ml with distilled water. The resulting suspensions were then sonicated four times for 30 s, and centrifuged (35,000 rpm, 5 °C, for 45 min). The supernatant was filtered through a 0.4 µm filter to remove any residual particles prior to loading the sample onto the column.

PCR Protocol for the Incorporation of the Cys-Tag Sequences

Using purified His-tagged plasmids as the template DNA; the protocols for PCR were repeated using primers (Forward primers) designed to containing six adjacent codons for Cys_6 between the His_6-tag determined by pET28a(+) and the start codon of the enzymes. The same protocols were used for cloning the modified gene, transforming it into *E. coli* DH5α, and for the expression and purification.

Enzyme Assays

Carboxylesterases ability to hydrolyse cocaine was measured by quantifying the amount of benzoylecgonine produced from cocaine by Agilent 1100 series HPLC system. The mobile phase was Isocratic 50% Solvent A: NaH_2PO_4 buffer 80 mM pH5.5 made up in HPLC grade water, and 50% Solvent B: Acetonitrile. The column used was

Phenomenex polar Rp Synergi 4 µm 80 A 250 × 2.0 mm; detector was UV 320 nm; and temperature was 30 °C. The flow rate was 0.3 ml/min, run time per sample was 15 min, and the injection volume was 5 µl. The standard assay conditions were 100 mM MOPS at pH 7.0, with 2 mM cocaine in a total volume of 1 ml at room temperature. The reaction mixture contained 2 mM cocaine and 20 µl cell extract in 1 ml of 50 mM MOPS buffer, pH 7.0 at room temperature, aliquots were removed at intervals (0 min, 6 h, 12 h, and 20 h). All assay results were compared with those from control assays containing no extract.

pH and Temperature Dependence

The optimum temperature and pH effect on carboxylesterases activity were established at 400 nm for 1 min at a series of different pH values (50 mM sodium citrate for pH range 5–6, 50 mM phosphate buffer for pH range 7–9, 50 mM CAPS for pH range 10–11) and different temperature values (25–60 °C) in 50 mM, pH 8.0 phosphate buffer.

Results and Discussion

CEs proliferate in higher eukaryotes, but appear infrequently in bacteria and lower eukaryotes [31]. To date there is no experimental evidence concerning cocaine metabolism by bacterial carboxylesterases; however, the capability of hCE-1 [32] and hCE-2 [33] to hydrolyse cocaine, and the crystal structures of hCE1 in complex with the cocaine analogue homatropine have been determined [2, 34] (Fig. 1).

The PCR experiments resulted in the isolation of CEs genes (*PnbA1* and *PnbA2*) having a *Bam*H1 restriction site in the 5′ end regarding both enzymes, and a *Hin*dIII restriction site and *Sac*I in the 3′ end regarding pnbA1 and pnbA2, respectively. The second PCR experiment resulted in the isolation of modified CEs genes having six adjacent codons for cysteine (cys-tag). The plasmids containing the cloned genes were digested and the expected DNA sequences were obtained (Fig. 2). The results from SDS-PAGE electrophoresis of the purified protein (Fig. 3) show an estimated mass of 54 kD, which agrees with the molecular weight of the gene product of pnbA1 and pnbA2.

The optimum pH and temperature dependence were estimated for the carboxylesterase activity. Both enzymes were

Fig. 1 Amino acid alignment of hCE-1, hCE-2, PnbA1, and PnbA2 using clustalX2. Shading indicates amino acid conservation of 100% (green), 80% (orange), and 60% (grey)

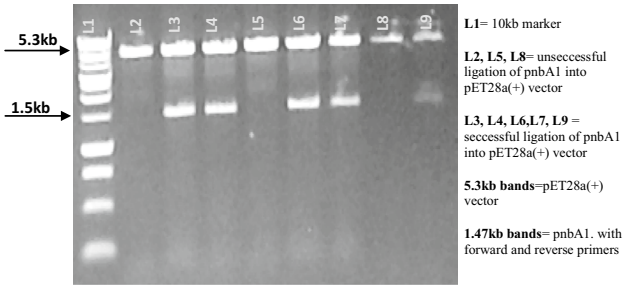

Fig. 2 Ligation of recombinant bacterial carboxylesterases (pnbA1) into the plasmid vector pET28a(+)

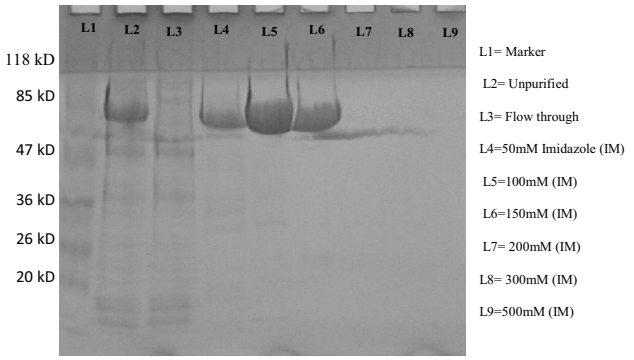

Fig. 3 SDS-PAGE of fractions containing the purified bacterial CEs expressed from pET28a(+)-PnbA1

active at a broad range of pH from 6.0 to 11.0, but more favourable in a basic pH, and a broad maximal activity for both enzymes between pH 8.0 and 9.0 was found. However, both enzymes were completely inactivated at acidic pH (5.0). The results indicate that PnbA1 remained stable at temperatures ranging from 25 to 45 °C. However, stability of the enzyme decreased sharply above 45 °C, while PnbA2 remained stable at temperatures ranging from 25 to 40 °C, and stability of the enzyme decreased gradually above 40 °C. The activity of PnbA1 sharply decreased when the temperature moved up 40 °C, while PnbA2 activity gradually decreased with temperature increased, and the enzyme retained a considerable part of its activity even at a temperature of 55 °C, indicating that PnbA1 has a lower denaturing temperature than that of PnbA2. This property (thermostability) of the enzyme can be exploited for different biotechnological purposes specifically gene therapy due to the role of the enzyme in cancer therapy, which requires active enzyme at 37 °C and some enzymes once isolated cannot remain active at the particular temperature. Carboxylesterase activity in cocaine was measured by quantifying the amount of benzoylecgonineand methanol produced from cocaine using HPLC. All assay results were compared with those from control assays containing no extract. The two compounds were detected by UV absorbance at wavelengths of 230 nm due to maximum absorption of both compounds at this wavelength, and the peak positions for cocaine and benzoylecgonine were confirmed by using the pure compounds. The identity of each compound was established by comparing the retention times and UV spectra obtained from samples with those obtained from standards. Cocaine and benzoylecgonine were well resolved and the retention times were approximately 4.4, and 2.0 min respectively. (Fig. 4) The stability of cocaine during 20 h at room temperature and 100 mM MOPS buffer pH 7.0 is noticeable and it is remains stable for 24 h at the same condition after that spontaneous degradation of cocaine will start even in a buffer with pH 7.0.

The efficiency of PnbA1 to hydrolyse cocaine was established using the reaction conditions (100 mM MOPS at pH 7.0, with 2 mM cocaine, and 20 µl cell extract in a total volume of 1 ml) and Fig. 5 shows the typical profile elution of cocaine hydrolysis and the concomitant formation of benzoylecgonine by bacterial carboxylesterases PnbA1 during 20 h, with approximate retention times of 4.6 and 2.0 min.

Figure 5 clarifies that a: at 0 = time there is only one peak with retention time roughly 4.4 min. This peak represents cocaine as demonstrated by the control sample. At 0 = min the reaction mixture contains 100 mM MOPS pH 7.0, with 2 mM cocaine, the reaction initiated by addition of fresh purified CEs enzyme, b: later after 6 h it is clear from the same figure that the cocaine peak at retention time 4.6 was driven down due to the cocaine break down, and the appearance of a new peak with retention time 2.0 min representing the production of benzoylecgonine, the main cocaine metabolite, as confirmed by the control sample. Hence the ability of the enzyme to break down cocaine and formation of its main metabolites; c: after 12 h a great drop in the cocaine peak and noticeable increase in the benzoylecgonine peak were found indicating the continuous enzymatic reaction, d: next after 20 h there was no cocaine peak and a greater peak (in comparison with the peak after 12 h) with retention time 2.0 min signifying break down of all the cocaine into benzoylecgonine, hence the continuous catalytic efficiency of the enzyme. The capability of engineered CEs (PnbA1-cys and PnbA2-cys) to hydrolyse cocaine was also established. Different reaction conditions have been used to enhance the catalytic efficiency of the enzymatic reaction, and all the assays for recombinant and engineered CEs were done at room temperature and 30 °C with and without shaking. Identical results were obtained related to shaking and standing (without shaking) reaction condition, while 30 °C was found to speed up the reaction progress.

Carboxylesterases are serine esterase involved in both drug metabolism and activation. The substrate specificity of CEs is significantly different. Some of them, namely human CE-1 (hCE-1) essentially hydrolyses a substrate with a small alcohol group and large acyl group, but

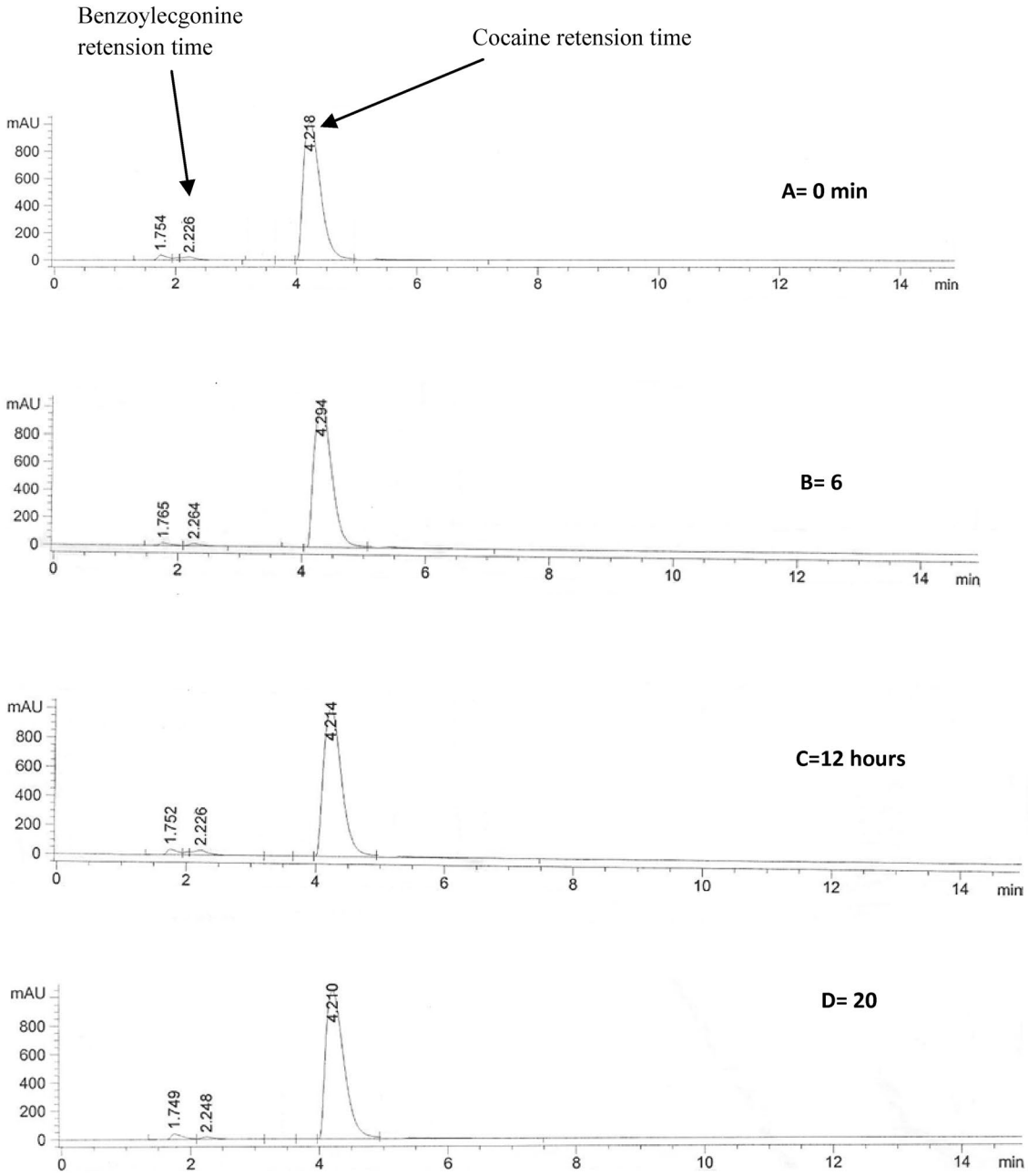

Fig. 4 Control assay of 2 mM cocaine in 100 mM MOPS buffer pH 7.0, aliquots were taken at intervals (*A* 0 min, *B* 6 h, *C* 12 h, and *D* 20 h). The peak positions for cocaine and benzoylecgonine were confirmed by using the pure compounds

sometimes act on structurally distinct compounds with either a large or small alcohol group due to its wide active pocket. In contrast, other CEs specifically hCE-2 identifies a substrate with a large alcohol group and small acyl group due to the occurrence of conformational interference in the active pocket. The different catalytic efficiencies of hCE-1 and hCE-2 are associated with the relative size of the substrate substituents versus that of the enzyme active sites. hCE-1 and hCE-2 catalyse the hydrolysis of cocaine; cocaine has two ester groups which can be hydrolysed. The hCE-1 enzyme hydrolyses the methyl ester of cocaine. This suggests that the hCE-1 acyl sites recognise large multi-ring substituents, and its alcohol site accommodates smaller methyl groups. In contrast, hCE-2 prefers the benzoyl ester of cocaine. This suggests that the hCE-2 acyl site recognises the relatively smaller benzoyl groups and its alcohol site recognises the bulky multi-ring groups, and the substrate specificity of cocaine esterase (CocE) resembles that of hCE-2.

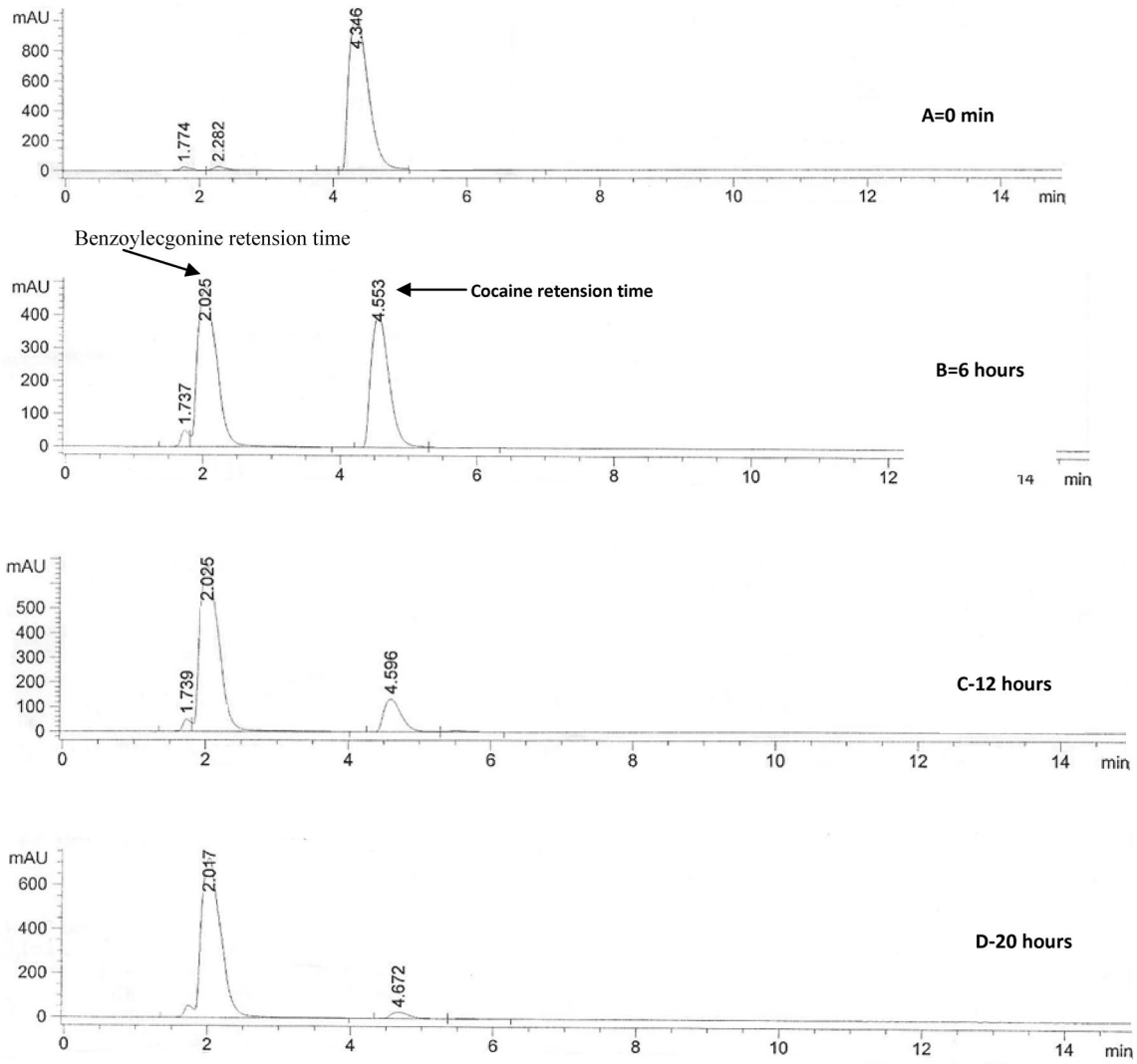

Fig. 5 Breakdown of cocaine with the concomitant production of benzoylecgonine by PnbA1. Samples removed at 0 min, 6, 12, and 20 h from an incubation of 2 mM cocaine with cell extract

Conclusion

These results revealed that bacterial carboxylesterases hydrolysed cocaine into benzoylecgonine rather than ecgonine methyl ester which results as a consequence of hydrolysis of methyl ester of cocaine and this explains that PnbA-1 and PnbA-2 resemble hCE-1 in cocaine metabolism. These results pave the way to use bacterial carboxylesterases as a model of hCEs, since proteins from bacterial origin are relatively easy to overexpress, purify, and engineer.

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