Biochemistry and Biophysics Reports 4 (2015) 44–51

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Application of termite hindgut metagenome derived carboxyl ester hydrolases in the modification of cephalosporin substrates

Nobalanda Mokoena [a], Kgama Mathiba [a], Tsepo Tsekoa [a], Paul Steenkamp [a,b], Konanani Rashamuse [a,*,1]

[a] CSIR Biosciences, Biomanufacturing Technology Competence Area, Brummeria, Pretoria 0001, South Africa
[b] Department of Biochemistry, University of Johannesburg, P.O. Box 524, Auckland Park 2006, South Africa

ARTICLE INFO

Article history:
Received 18 March 2015
Received in revised form
20 August 2015
Accepted 24 August 2015
Available online 28 August 2015

Keywords:
Functional metagenomics
Antibiotic synthesis
Esterases
Deacetylated cephalosporins

ABSTRACT

In the pharmaceutical industry, de-acetylated cephalosporins are highly valuable starting materials for producing semi-synthetic β-lactam antibiotics. In this study a fosmid metagenome library from termite hindgut symbionts was screened for carboxyl ester hydrolases capable of de-acetylating cephalosporins. Recombinant *Escherichia coli* clones with esterolytic phenotypes on tributyrin agar plates were selected and further tested for de-acetylating activity against Cephalothin and 7-aminocephalosporanic acid (7-ACA). Two clones displaying de-acetylating activity were sequenced and the corresponding two carboxyl ester hydrolase encoding genes (*axeA* and *axeB*) belonging to the carbohydrate esterase family 7 (CE7) were identified. The primary structure of both the axeA and axeB revealed the presence of G-X-S-X-G sequence motif and respective subunit molecular masses of 40 kDa. In addition to de-acetylating cephalosporin based molecules, the two enzymes were also shown to be true esterases based on their preferences for short chain length fatty acid esters.

© 2015 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

1. Introduction

The de-acetylated cephalosporins are highly valuable starting materials for producing semi-synthetic β-lactam antibiotics in pharmaceutical industry. Chemical processes for the production of de-acetylated cephalosporins involve general hydrolysis of the ester bond under alkaline conditions resulting in low yields and by-products that are toxic to the environment [1]. A white biotechnology process based on the use of ester hydrolysing enzymes has been suggested as an alternative approach to the chemical process for the production of de-acetylated cephalosporins [2]. The de-acetylation of cephalosporins using an enzymatic process offers several advantages including mild reaction conditions of pH, temperature, and high yields [3].

Carboxyl ester hydrolases (3.1.1.1) capable of de-acetylation of cephalosporins have been reported from two different esterase families, namely carbohydrate esterase family 7 (CE7) [4,5,6] and family VIII lipolytic enzymes [7]. However, carboxyl ester hydrolases belonging to these two families share no similarities in terms of the amino acid sequence identities and other biochemical properties. For example, the primary structure of family VIII esterases resemble that of class C β-lactamases, peptidases, and penicillin binding proteins [7,8], while that of CE7 is related to the carbohydrate esterases [6]. Moreover, the substrate specify profile of family VIII esterases reveal a broad specificity range against fatty acid esters (C2-C8) [9], in contrast to carbohydrate esterase family 7 enzymes that show a limited substrate preference to acetate (C2) and propionate (C3) fatty acid esters [10]. Furthermore, family VIII lipolytic esterases generally display monomeric subunits, while CE7 display multi-oligomeric quaternary structures [6].

The P3 section of termites' hindgut is a metabolic engine involved in the degradation of a wide variety of compounds with the aid of obligate symbionts [11]. As a result, hindguts of termite species provide an ideal source for bio-prospecting for novel enzyme genes. In this study, we have screened a previously constructed termite hindgut metagenomic library [12] for esterases/cephalosporin C-deacetylases that can be applied in a chemo-enzymatic synthesis of deacetylated β-lactam molecules. The two identified carboxyl ester hydrolase encoding genes (*axe*A and *axe*B) were recombinantly expressed in *Escherichia coli* and their deacetylating catalytic abilities against acetylated β-lactam

* Corresponding author.
E-mail address: KRashamuse@csir.co.za (K. Rashamuse).
[1] Drs. Mokoena and Rashamuse contributed equally to the work.

http://dx.doi.org/10.1016/j.bbrep.2015.08.016
2405-5808/© 2015 Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

substrates were demonstrated.

2. Material and methods

2.1. Metagenomic library screening

The construction and screening of a metagenomic library from termite hindgut symbionts have been described previously [12]. Briefly, following the extraction of a total High molecular weight community DNA from the P3 section of termite hindgut, a large insert DNA library was generated using the copy control fosmid library production kit (Epicentre Biotechnologies, WI, USA). The library was screened for clones displaying esterolytic activity by tributyrin hydrolysis on LB tributyrin agar plates [12]. Esterase genes (AxeA and AxeB) from the positive fosmids were identified by generating pUC19 subclone libraries from fosmids partially digested with *Sau*3A1. Functional screening of the pUC19 recombinant esterase clones in *E. coli* was performed on LB agar plates supplemented with tributyrin 1% (v/v) and Gum Arabic 0.1% (w/v), followed by incubation at 37 °C. Esterase positive clones were identified by the presence of zone of clearance around the colony margins.

The nucleotide sequences of the pUC19 derived esterase positive clones were determined using the Sanger-Sequencing method. Open reading frames were identified using the GeneMark gene prediction tool (http://exon.gatech.edu/GeneMark/) and the amino acid sequences determined using the DNA translation tool available on the CLC Combine Workbench software (CLCBIO, Denmark). A similarity search was performed using the Basic Local Alignment Search tool (BLASTP) [13]. Sequence alignments and editing were done using the Bio-Edit software [14].

2.2. Protein expression and purification

The primer pairs, (AxeAF: 5′-ATCCATATGCCGTATATTGATATGC-3′/,AxeAR: 5′-GATCTCGAGTGCCGGATAGCTAACTG-3′) and (AxeBF: 5′-ATCCATATGGCA-CAATTTG-ACCTG-3′/, AxeBR”: 5′-GATCTCGAG-GGTGCTTACGCAGCCATG-3′) were designed using available sequence information. The restriction sites (underlined) were incorporated into primers for directional cloning of the two esterase genes (AxeA and AxeB) into the pET20b and pET28a expression vector, respectively. Recombinant proteins were co-expressed with a C-terminus 6x His-tag. Plasmid DNA from pUC19-derived esterase positive clones was used as template for PCR amplification of *axeA and axeB* genes, using KAPA HiFi DNA Polymerase (KAPA Biosysyems). The resulting PCR products were ligated into pET20b and pET28a vectors restricted with the same enzymes, resulting pET20AxeA and pET28AxeB expression constructs which were used to transform *E. coli* BL21 (DE3) expression host.

Expression studies were performed using the EnBase technology [15,16] with EnPresso™ tablet cultivation set (BioSilta, Finland) according to the supplier's recommended instructions. Recombinant protein production was induced with ITPG (1 mM) followed by additional overnight incubation at 20 °C. Cultures were pelleted and cells lysed using B-PER (in phosphate buffer, 50 mM (pH 7.5)) bacterial protein extraction reagent (Pierce, USA) according to the manufacturer's instructions, to release the intracellular proteins. Supernatants were then recovered following centrifugation (22,000*g* for 30 min).

Purification of recombinant proteins was performed using immobilized metal affinity chromatography with Protino Ni-TED 2000 pre-packed columns (Macherey-Nachel, Germany). Protein supernatants were first buffer exchanged into an equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8) and AxeA and AxeB proteins were purified following manufacturer's instructions. The protein was eluted with the equilibration buffer containing additional 250 mM imidazole. Elution fractions were transferred into 20 mM Tris–HCl (pH 8) using VIVASPIN 10 kDa cut-off spin columns (Vivascience, U.K.). Protein concentrations were determined by Bradford method [17], using bovine serum albumin (BSA) as a standard. Purified products were analyzed on denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [18].

2.3. Determination of native molecular weights

Molecular weights of native AxeA and AxeB proteins were determined with Superdex™ 200 10/30 GL (GE, Healthcare) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl. The following molecules were used as reference standards: thyroglobulin (670,000 Da), γ-globulin (160,000 Da), oval bumin (44,000 Da), myoglobin (17,000 Da) and vitamin B12 (135 Da) (Bio-Rad, USA).

2.4. Standard esterase assay

Routine esterase activity assays were performed by a standard assay measuring the release of *p*-nitrophenol from *p*-nitrophenyl ester at 410 nm [19] using a Beckman DU850 UV/visible spectrophotometer, equipped with a temperature controller. Unless otherwise described, enzyme activity was measured at 30 °C with 1 mM *p*-nitrophenyl acetate as the substrate dissolved in 50 mM Tris–HCl, pH 8. The extinction coefficient of *p*-nitrophenol under these conditions was 13,800 M^{-1} cm^{-1}.

2.5. Biochemical characterization

2.5.1. Substrate profiling

Substrate specificity of the AxeA and AxeB enzymes was determined using 1 mM *p*-nitrophenyl esters of various chain lengths: *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenylcaprylate (C8) and *p*-nitrophenyllaurate (C12). Described enzyme activity was measured at 30 °C in 20 mM Tris–HCl, pH 7.5 with 1 mM *p*-nitrophenylesters (dissolved in isopropanol) as the substrate. The extinction coefficient of *p*-nitrophenol under these conditions was 13,800 M^{-1} cm^{-1}. Experimental initial velocity data *versus* substrate concentration, with coefficients of variation of $\leq 5\%$, were fitted to the Michaelis–Menten equation.

2.5.2. Temperature optima and stability profiles

The temperature optima of the two enzymes were determined between 30 and 80 °C using esterase standard assay. The thermostability profiles of the AxeA and AxeB were determined by incubating the enzyme at a range of temperatures (30–80 °C) in Tris–HCl (50 mM, pH 7.5) and the residual activity determined at 30-min time intervals using the standard assay.

2.5.3. Activity on cephalosporin based substrates

Deacetylase activity of AxeA and AxeB against β-lactam substrates was determined using high-performance liquid chromatography (HPLC) by measuring de-acetylated forms of Cephalothin and 7-aminocephalosporanic acid. A β-lactamase from *Bacillus cereus* (Sigma) was used as a positive control. The reaction mixture contained purified enzymes (AxeA and AxeB) or β-lactamase positive control incubated with 1 mM substrate solution in 50 mM Tris–HCl (pH 8) at 30 °C for 1 h. The reaction was stopped by adding 0.2 mL of stop solution (100 mM H_2SO_4 and 30 mM crotonate). HPLC analyses of antibiotics reaction samples catalysed by either AxeA, AxeB or β-lactamase were carried out using a Hewlett Packard 1100 HPLC (Agilent Technologies Incorporated, Lovedale, CO, USA). The instrument was equipped with a binary pump

Download English Version:

https://daneshyari.com/en/article/1941797

Download Persian Version:

https://daneshyari.com/article/1941797

Daneshyari.com