Functional characterization of a metagenome derived family VIII esterase with deacetylation activity on β-lactam antibiotics

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ABSTRACT

Family VIII esterases represent a poorly characterised esterase family, with high sequence identity to class C β-lactamases, peptidases and penicillin binding protein. In this study we report on the metagenomic screening and biochemical characterisation of a novel esterase (Est22) derived from an acidic Leachate environment. The enzyme is 423 amino acids in length and contained 22aa signal peptide. Analysis of the Est22 primary structure revealed the presence of N-terminus S-x-x-K sequence, which is highly conserved in class C β-lactamases, peptidases as well as carboxylesterases belonging to family VIII. Phylogenetic analysis using representative sequences from class C β-lactamases and family VIII esterases indicated that Est22 clustered mainly with family VIII esterases. Substrate specificity profiling using *p*-nitrophenyl esters (C2-16) indicated that Est22 preferred shorter chain *p*-nitrophenyl esters (C2-C5), a characteristic typical of true carboxylesterase. In addition of hydrolysing nitrocefin, Est22 also hydrolysed first generation cephalosporin derivatives. Detailed selectivity study using cephalosporin revealed that Est22 selectively hydrolyse the ester bond of a cephalosporin derivatives leaving the amide bond of the β-lactam ring intact. The selective nature of Est22 makes this enzyme potential candidate for use in the synthesis and modification cephalosporin based molecules.

Keywords: Metagenomics, familly VIII carboxylesterase, β-lactam substrate

Introduction

Microbial lipolytic enzymes comprise of two enzyme groups, namely lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) and carboxylesterases (carboxylester hydrolases, EC 3.1.1.1) [1]. Lipases catalyze the hydrolysis of triacylglycerols with subsequent release of diacylglycerols, monoacylglycerols, free fatty acids and glycerol [2]; while carboxylesterases catalyze the hydrolysis of ester bonds of carboxyl ester substrate molecules to form an alcohol and carboxylic acid [3]. The growing number of primary structures has provided a clearer picture of the evolutionary relationships between lipolytic enzymes of different origin. Currently, microbial lipolytic enzymes (including both esterases and lipases) are classified into eight families (Family I-VIII) [1]. This classification scheme is based on a comparison of amino acid sequences and some fundamental biological properties of these enzymes.

Family VIII esterases (F-VIII-Ests) represent an ill-defined family with high sequence identity to class C β-lactamases, peptidases, and penicillin binding protein [1]. A large proportion of F-VIII-Ests members that have been isolated to-date through culture enrichment methods are mainly from eubacteria domain from a number of genera including: *Arthrobacter* [4], *Brevibacterium* [5], *Burkholderia* [6, 7], *Streptomyce* [8], *Pseudomonas* [9] and *Marinobacter* [10]. Although there is only one report on F-VIII-Ests member from eukaryotic domain [11], the representative members of F-VIII-Ests from archael domain are yet to be reported. Recently, there has been an increase in a number F-VIII-Ests representatives from metagenomic libraries have been reported from diverse ecosystems including: marine sponge [12], cold adapted environments [13], Soil environment [14, 15], Compost environment [16], leachate environment [17] and drinking water biofilm environment [15].

Like all other reported carboxylesterases, all members belonging to F-VIII-Ests are serine hydrolases [2]. The primary structures of these enzymes contain a highly conserved S-x-x-K motifs (where x-denotes any amino acid) located at the N-terminus part of the primary structure. This S-x-x-K sequence is also highly conserved in a number of peptidases [18]

and β-lactamases [19] and has been shown to harbour the catalytic serine residue in these enzyme families. In addition to the S-x-x-K sequence motif, some members belonging to F-VIII-Ests also contain C-terminus located pentapeptide (G-x-S-x-G) sequence motif. The classical (G-x-S-x-G) signature motif harbours catalytic serine residues in a number of other esterase families (Family I, III, IV, V, VI, VII) and many other hydrolytic enzymes that exploit serine residue as a catalytic nucleophile. In F-VIII-Ests, a number of site directed mutagenesis studies have shown that the serine residue located within the S-x-x-K motif (as opposed to the one located within the G-x-S-x-G motif) is responsible for catalysis [10].

Despite high sequence identity to class C β-lactamases, most members of family VIII esterases reported to date lack activity against standard β-lactam substrates. The exception is with EstC [17], EstM-N1 and EstM-N2 [13] which exhibit low hydrolyzing activity towards nitrocefin (a chromogenic substrate used to determine β-lactamase activity). In addition of showing activity against nitrocefin, EstB [7] and EstU1 [14] also exhibits β-lactamase hydrolytic activity towards cephalosporins class of β-lactam substrates. In this study we report on metagenomic isolation and characterization of an esterase (Est22) which selectively hydrolyzes the ester bond and not the amide bond of the β-lactam substrates.

Materials and Methods

Library construction and esterase screening

A metagenomic library used for screening esterase activities was previously constructed from an aqueous acidic leachate (pH 4.2) collected from the Chloorkop landfill site East of Johannesburg South Africa (26˚03´17.50´´S) using the EpiFOS™ Fosmid Library Production Kit (Epicentre Biotechnologies, USA) [17]. Functional screening of the recombinant esterase

positive clones in *E. coli* EPI100-T1^{R} was performed on LB agar plates supplemented with isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM), chloramphenicol (12.5 µg ml^{-1}), 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.

DNA manipulation and sequencing

Recombinant DNA techniques were carried out as described by Sambrook and Russell [20]. DNA sequencing and oligonucleotide synthesis services were provided by Inqaba Biotech (South Africa). Sequence analysis and manipulation were performed using CLC Combine Workbench software (CLCBIO, Denmark) and Bioedit [21] with the aid of BLASTP search [22]. The signal peptide predictions were conducted using SignalP 3.0 server located at http://www.cbs.dtu.dk/services/SignalP/ [23].

Phylogenetic analysis

The evolutionary relationsip between Est22, family VIII esterases and class C β-lactamases was inferred using the Neighbor-Joining method conducted with MEGA5 [24, 25]. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method [26], and are in the units of the number of amino acid substitutions per site.

Sub-cloning and expression

Primers specific for the *est*22 esterase gene were designed using available pyrosequencing data. The *est*22 gene was amplified with and without signal peptide using F8F (ATC CAT ATG CCA CAC ATA AAT AAA CTT CTT G) / F8R (GAT CTC GAG ATC CAC AAT CGC CTG ATT CAC) and F8FF (ATC CAT ATG CAA ACG CCA CCC TCT GGC CTG) / F8R (GAT CTC GAG ATC

CAC AAT CGC CTG ATT CAC) primer pairs, respectively (Table 1). DNA extracted from the fosmid was used as template and both primer pairs separate introducing the *Nde*I and *Xho*I sites at the 5´- and -3´ end of the gene respectively. The amplified PCR products were digested with *Nde*I/*Xho*I, followed by ligation into pET28a linearized with the same enzymes. The recombinant *est*22 gene was expressed in-frame with the 6x-His tag sequence at the 3´-end of the gene. Expression vectors were first propagated in *E. coli* DH5α cells and the recombinant clones were selected on Kanamycin (50 µg/ml). Clones containing the correct inserts were then used to transform the expression host, *E. coli* BL21 (DE3).

Expression studies were performed using the EnBase technology, which uses an enzyme to release glucose from solubilised polysaccharide allowing a slow and consistent release of nutrients. The procedure was carried as follows using EnPresso^{TM} tablet cultivation set (BioSilta, Finland). A single colony was used to inoculate 5 mL media (containing 50 µg/mL Kanamycin) followed by incubation at 37°C for 6-8 hrs. The pre-inoculum culture (1 % V/V) was then used to inoculate EnBase tablet media containing enzyme mixtures (EnZ I'm (600 U/L)) to a final concentration of 0.3 U/L and the antibiotic (50 µg/mL Kanamycin). The culture was incubated at 16°C overnight and then booster tablet and additional enzyme mixture (0.6 U/L) were added to the culture. Est22 protein expression was induced with ITPG (1mM) followed by additional overnight incubation at 16 °C. Cultures were pelleted and cells lysed using B-PER (in phosphate buffer, 50mM (pH 7.5)) bacterial protein extraction reagent (Pierce, U.S.A.) following the manufacturer's instructions, to release the intracellular proteins. Supernatants were then ultra-centrifuged at 22 000 x g for 30 minutes using the JA-14 rotor (Beckman, Avanti^{R}J-26XPI).

Est22 Purification

The soluble Est22 esterase protein fraction was loaded onto the immobilised metal affinity column (IMAC) packed with Protino Ni-TED resin (Macherey-Nagel, Germany). A 25 ml Ni-TED column was equilibrated (50 mM NaH_2PO_4, 300 mM NaCl, pH 8) with 3X column

volumes at a flow rate of 5 ml/min. The Est22 protein was bound and unbound proteins were removed by washing the column with 3X bed volumes of the equilibration buffer. The protein was eluted with the equilibration buffer containing additional 250 mM imidazole. Eluted fractions were passed through VIVASPIN 10 kDa cut-off spin columns (Vivascience, U.K.) for imidazole removal and protein concentration. Protein concentration of the purified samples was determined by the Bradford [27] method, using bovine serum albumin (BSA) as a standard, while the purity of the samples was analysed on denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [28]. Molecular weight of Est22 was determined with Superdex ^{TM} 200 10/30 GL using 30% Acetonitrile with 0.1% TFA buffer. The gel filtration standard (Bio-Rad, USA) contained the mixture of thyroglobulin, γ-globulin, ovalbumin, myoglobin, and vitamin B12.

Functional assays

Esterase Assays: Unless otherwise stated all enzyme assays were performed in triplicate. Routine esterase activity assays were performed by a standard colorimetric method measuring the release of p-nitrophenol from p-nitrophenyl esters at 410 nm [6], using a Beckman DU850 UV/visible spectrophotometer with a temperature controller. Described enzyme activity was measured at 30ºC in 20 mM Tris-HCl, pH 7.5 with 1 mM p-nitrophenyl butyrate (dissolved in isopropanol) as the substrate. The extinction coefficient of p-nitrophenol under these conditions was 13800 $M^{-1} \cdot cm^{-1}$.

β-lactam hydrolysis

The β-lactam hydrolytic activity of Est22 was determined spectrophotometrically using 0.1 mM Nitrocefin [3-(2, 4 dinitrostyrl) - (6R, 7R-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E-isomer)] as a substrate (Oxoid kit manual, 6th edition 1990, Unipath Ltd., Basingstoke, UK). The enzyme was incubated with Nitrocefin (0.01-0.1 mM) solution in (0.1 M phosphate; 1mM EDTA, pH 7.0) at 30ºC and the rate change at 486 nm was recorded.

The molar extinction coefficient of Nitrocefin under these conditions was 20,500. The Activity of Est22 against non-chromogenic β-lactam substrates (7-Aminocephalosporanic acid, Cephalosporin C and Cephalotin) was also measured as described by Avison et al. [2000]. A β-lactamase from *Bacillus cereus* (Sigma) was used as a positive control. Purified Est22 and β-lactamase were incubated with antibiotic substrates at 1 mM in 50 mM Tris-HCl (pH 8.0) for 1 h at 30°C.

The reaction mixture were analysed by HPLC, using a Hewlett Packard 1100 HPLC (Agilent Technologies Incorporated, Lovedale, CO, USA) equipped with a binary pump autosampler, column thermostart, UV diode array detector and ChemStation Chromatography Management software (Revesion B.03.02, Agilent Technologies Incorporated). Reaction mixtures containing Cephalothin as substrate were separated on a Phenomenex Luna C_{18} (150 x 4.60 mm 5 µm inner dimension, Phenomenex, USA) and those containing 7-Aminocephalosporanic acid, Cephalosporin C on 250 × 4.60 mm, 5 µm inner dimension,) columns, coupled with the corresponding guard columns (C18, 4 × 3.0 mm, Phenomenex, USA). The samples were analysed with a pump in isocratic mode using $NH_4H_2PO_3$ buffer (125 mM, pH 4.5) and methanol (HPLC grade), 68:32 % v/v, as the mobile phase. The mobile phase was pumped at a flow of 1.0 ml/min and the sample injection volume was 10 µl, the column temperatures was 25°C. The β-lactam substrates hydrolysis was identified by comparing peak retention times of substrates only to that of reaction mixtures.

Biochemical characterization

To investigate substrate specificity of Est22, enzyme activity was determined using standard assay in the presence of 1 mM of the specified p-nitrophenyl esters of various chain lengths: p-nitrophenyl acetate (C2), p-nitrophenyl butyrate (C4), p-nitrophenyl caprylate (C8) and p-nitrophenyl laurate (C12). Experimental data of initial velocity versus substrate concentration were fitted to the Michaelis-Menten equation.

Optimum temperature of the Est22 was determined by measuring the rate of p-nitrophenyl butyrate hydrolysis over a temperature range (30 - 70°C). Substrate, 1 mM in 50 mM Tris-

HCl buffer (pH 7.5), was incubated at desired temperatures and followed by addition of enzyme. Where necessary the pH adjustments at set temperatures were performed to take into account the effect that changing temperature has on pH. Thermostability profile of Est22 was measured by incubating the enzyme at three temperature points 30-, 50- and 70°C, followed by measuring residual activities after every 30 min using the standard esterase assay. The influence of pH on the p-nitrophenyl butyrate hydrolysis was tested from pH 4.0 to 9.5 at 30°C.

Molecular modelling of Est22 and structure analysis

The amino acid sequence of Est22 was used to search non-redundant (at 95% redundance) protein structures with BLASTp in order to identify suitable templates for homology modeling. Chain B of PDB entry 1CI9, corresponding to the crystal structure of esterase ESTB from *Bukholderia gladioli* with a bound ligand was selected as a template. Alignment of Est22 with this protein resulted in 12% sequence identity and 33% sequence similarity. On the basis of this alignment, Est22 was modelled against 1CI9 using the modeller engine in Discovery Studio 3.1 (Accelrys). A total of 50 models were created with molecular dynamics optimization set to perform high levels of sampling in simulated annealing. Amino acid residues that fell outside aligned regions in the models were subjected to side-chain refinement using the DOPE method with optimization level again set to high. The best model selected on the basis of DOPE score and PDF total energy was validated using PROCHECK (Ramachandran Plot) [29] and the WHAT-IF server.

Accession number

The esterase (*est*22) gene nucleotide coding sequence has been deposited in the GenBank under the Accession number: KF052088.

Results

Library screening and sequencing

A fosmid library from leachate environment (library size, average insert size) was previously constructed using a copy-controlled pEpiFOS-5 vector and subsequently screened for esterase positive colonies resulting in 87 positive colonies [17]. To specifically target esterases belong to family VIII, 87 identified esterase colonies were further screened on a secondary liquid assay using nitrocefin as a substrate. A total of three nitrocefin positive (pFOS8, pFOS827 and pFOS8Y) clones were indentified. Based on RLFP pattern using *Bam*HI and *Hind*III restriction enzymes, pFOS827 and pFOS8Y were shown to harbour the same DNA insert. To exclude the EstC previously identified from the same library, primer pair targeting EstC was used and confirmed the presence of EstC fragment in fosmid pFOS827 and pFOS8Y. Fosmid (pFOS8) was therefore selected for further study. In order to locate the gene(s) encoding both esterolytic/β-lactam activities within the pFOS8 fosmid, a random shotgun sequencing of a complete insert DNA was performed. Sequence analysis of the insert DNA revealed 38.4kb insert size, with an average GC content of 54 %. Translational analysis of the nucleotide sequence of the 38.4 kb insert fragment revealed 25 complete open reading frames. Identified ORFS we manually examined for classical serine containing motifs (G-x-S-x-G, G, G-DS-X and S-X-X-K), resulting in five 5 orfs (orf3, orf12, orf16, orf22, orf 23). Following PCR sub-cloning and screening using primer pairs targeting the five orfs, ORF22 was shown to contain both esterolytic and β-lactam activity.

Primary structure analysis and phylogenetic classification

The *est*22 gene (ORF22 which is located at the -3 strand at position 31174-29906) which is 1269 pb has a GC content of 52%. Translational analysis of the ORF22 revealed a polypeptide of 423 amino acids encoding a putative protein of 47 kDa. The *est*22 also encoded a pre-protein containing a 22 amino acid signal peptide as predicted with SignalP 3.0 [23]. The maximum cleavage site was predicted to be 0.9 between Ala22 and Gln23, which could be cleaved to form a mature protein of 402 aa with predicted molecular weight and pI values of 44.7 kDa and 5.0 respectively.

Deduced Est22 protein sequence revealed the S-M-T-K sequence (amino acid positions 100-103), compatible with the conserved S-x-x-K motif in class C β-lactamases (Figure 1A) [30], penicillin binding proteins (PBPs) [31]. The Est22 protein was identified as β-lactamase by BALST analysis with the top five highest identity score from *Methylobacterium* sp. (46%, 187/407; ACA19666.1), *Opitutus terrae* (46%, 187/409; ACB74974.1), *Methylobacterium nodulans* (46%, 191/404; ACL60913.1), *Acetobacteraceae bacterium* (45%, 184/409; EHM01916.1) and *Candidatus Koribacter* (43%, 180/423; ABF39642.1). However, Arpigny and Jaeger [1999] esterase classification scheme indicated that ORF22 is related to family VIII esterases. Furthermore neighbour joining analysis of Est22 using MEGA indicated that this enzyme is a member of family VIII esterases, as it clustered with previously reported sequences belonging to this family (Figure 1B).

3D-structure and identification of active site residues in modelled Est22

A homology model of Est22 was created using EstB from *B. gladioli* as template. Superposition of the two structures revealed a RMSD of only 0.82 Å, with 277 C-α atoms from each protein superposed (supplementary data). The model structure had two major domains, an all α domain and a α/β domain with each domain composed of amino acid residues form both the C-terminal and N-terminal regions of the protein. Structure elements typically found in the active site region of b-lactamases and peptidases were identified in the model. These included a Ser-Xaa-Xaa-Lys motif (Ser^{100}-Met^{101}-Thr^{102}-Lys^{103} in Est22) that contains the nucleophillic catalytic serine. An additional motif identified was a Tyr^{212}-Ser^{213}-Ala^{214} loop similar to Tyr^{181}-Ser^{182}-Leu^{183} in EstB [Wagner et al., 2001]; and analogous with what is sometimes termed the SDN loop in Class A β-lactamases. In depth analysis of the Est22 structure also revealed that this enzyme lacks the (K-T/S-G) and (YAN) sequences which are well conserved at the C-terminus of class C β-lactamases [30].

Est22 expression and purification

In order to study the biochemical properties of the recombinant enzyme, the esterase (*est*22) gene was directionally cloned into pET28a to allow the expression of the gene under a strong T7 promoter, with and without the signal peptide. Recombinant Est22 was produced in a biologically active form in the soluble cytoplasmic fraction of *E. coli* cells using EnBase technology with EnPresso^{TM} tablet cultivation set [32, 33], which allows an enzymatic slow release of nutrients. Production of the extracellular Est22 with the signal peptide was not successful with this technology and with conventional LB culturing. The intracellular Est22 enzyme was purified in a single step IMAC procedure) since the expression construct was designed to allow recombinant protein to be fused with the C- terminal 6x histidine tag (Figure 2A). The molecular mass of the purified Est22 was estimated to be 45 kDa, consistent with the estimated molecular weight calculated from the translated nucleotide sequence of the mature protein. The monomeric 43 kDa molecular state of Est22 was within the 42-45 kDa range which has been reported for other family VIII esterases [13-17]. The protein was purified with 2-fold enrichment and a yield of 87% and specific activity of 60.0464 U/mg. A zymogram assay involving the staining of PAGE gel α-naphthol acetate substrate solution in the presence of fast blue B dye exhibited single dark-red protein band (Figure 2B), indicating the biological activity of the purified Est22. However, the size of Est22 band on the native gel was migrating at higher molecular weight than the calculated subunit. This necessitated investigation of the globular structure of Est22 using size exclusion chromatography. Based on the analytical size exclusion the molecular weight of Est22 was 153 kDa, suggesting a global trimetric structure of Est22 (supplementary data).

Biochemical characterization

Substrate specificity: When hydrolytic activity toward p-nitrophenyl esters was examined, Est22 enzyme showed the highest activity with p-nitrophenyl butyrate (Figure 3A). Activity

towards medium- and long-chain acyl substrates was poor substrates; Est22 showed no activity against p-nitrophenyl ester substrates with acyl chains C10 and longer (Figure 3A).

pH, temperature and thermostability profile: Kinetic parameters were determined with p-nitrophenyl acetate, and Est22 had the optimal temperature and pH at 30°C (data not shown) and 8 (Figure 3B), respectively. The enzyme was not active at higher temperatures (supplementary data). Thermostability studies revealed that the Est22 enzyme is thermo-labile with an estimated half-life of 30 minutes at temperatures 50°C and higher (supplementary data).

β-lactamase activity: Deduced Est22 protein sequence revealed the S-x-x-K motif which is conserved in class C β-lactamases. Therefore Est22 β-lactam hydrolytic activity was preliminarily determined using nitrocefin as the substrate. Est22 showed β-lactam activity against nitrocefin as evidenced by colour change from yellow to red (data not shown). Hydrolytic activity of Est22 against nitrocefin promoted further investigation against non chromogenic β-lactam cephalosporin derivatives (7-Aminocephalosporanic acid, Cephalosporin C and Cephalothin). Est22 showed activity towards these three cephalosporin derivatives. While the HPLC profile of the β-lactamase positive control indicated hydrolysis of the amide bond of the β-lactam ring, the Est22 profile indicated that the enzyme is hydrolysing a different bond of the three β-lactam cephalosporin derivatives as the profile was differed from that of β-lactamase. HPLC spectra showed that retention times obtained with the β-lactamase positive control and the Est22 reactions were different (Figure 4A, B, C). These results suggested that Est22 is hydrolyzing the ester bond only on the substrates and not the amide bond. To validate the results acetic acid was measured, as one of the by products from the deacyltelation reaction of cephalosporin substrates. Results of the acetic acid production were inconclusive hence the products from the cephalosporin derivatives hydrolysis were identified using UPLC-TOF MS analysis. Cephalosporin alcohols

were identified from all the reactions indicating that Est22 is selectively hydrolysing the ester bond and not the amide bond (supplementary data).

Discussions

Traditionally esterase gene candidates from the environment have been discovered using culture enrichment approaches [34]. The classical culture enrichment approaches, generally involve the cultivation of microorganisms and the subsequent screening of the pure strains for the desired catalytic activity [34]. This is typically achieved by simply including the substrate of interest in the agar plate, usually tributyrin (1% v/v) for screening of esterase activities and olive oil and rhodamine B for lipase activities [35].

A number of culture-independent approaches have therefore been developed to access the diversity of novel esterase gene sequences that fit a particular industrial performance profile [36]. These include strategies that involve a combination of functional and sequence-based screening approaches designed to rapidly clone and express genes from metagenome libraries and the PCR-based sequence-independent techniques that prospect for novel enzyme genes directly from community DNA. In this study we demonstrated the application of functional and sequenced-based metagenomic approaches combined in the isolation of novel esterase Est22 with a primary structure related to C β-lactamase [19], transpeptidases [18] and penicillin binding protein PBPs [37].

The Est22 protein was identified as a class β-lactamase with BLAST analysis. However phylogenetic analysis with class C β-lactamases and family VIII esterases showed that Est22 protein cluster together with family VIII esterases and not with class C lactamases. Like all other previously reported carboxylesterases belonging to family VIII, Est22 is a serine hydrolase with the serine residue located within the conserved S-x-x-K sequence (S^{100}-M^{101}-T^{102}-K^{103}) (Figure 1A). The motif is typically conserved in class C β-lactamases [30] and family VIII esterases [1]. From the EstB crystal structure Wagner et al [38] the proposed mechanism of ester hydrolysis involving Ser75 within the S-x-x-K as a catalytic

nucleophile. The nucleophilicity of Ser is enhanced by highly conserved Tyr (often located at position 180-200), which acts as a general base and is presumably stabilized as the phenolate group due to the proximity of the side chains of Lys (within the S-x-x-K sequence). Multiple sequence analysis revealed that Tyr212 in Est22 primary structure was highly conserved, suggesting that this residue plays a role analogous to that proposed for Tyr150 from the X-ray crystal structure of the *C. freundii* class C β-lactamase [30, 39, 40] and Tyr219 [17]. In addition to the S-x-x-K sequence motif, some members belonging to F-VIII-Ests also contain C-terminus located pentapeptide (G-x-S-x-G) sequence motif [4, 17] which harbours catalytic serine residues in a number of other esterase families (Family I, III, IV, V, VI, VII). The primary structure of Est22 lacked the C-terminus sequences corresponding to the (G-x-S-x-G sequence, further emphasizing the role of serine within S-x-x-K as a possible catalytic serine. Furthermore, the primary structures of some of F-VIII-Est members such as that of EstU1 [14], Est2K [16] and EstC [17] also encode 25-29 aa N-terminal leader (secretion signal) peptide. Analysis of Est22 primary structure also revealed the presence of a 23 aa N-terminal leader peptide consistent with the primary structure of EstC, Est2k and EstU1. This observation suggests that Est22 could be an extracellular enzyme that is secreted through the Xep-dependent pathway mechanism mediated by the N-terminal leader peptide.

Esterases are generally differentiated from lipase counterparts on the basis of substrate preferences, and they only hydrolyze water-soluble short acyl chain esters of less than 10 carbon atoms [Jaeger et al., 1999]. Substrate specificity profiling with p-nitrophenyl esters (pNP-esters) of different carbon chain lengths (C2-C16) revealed that Est22 is a “true” esterases with a strong preference for short acyl chains esters, C4. Due to high sequence identity of Est22 to class C β-lactamases; the enzyme was further investigated for its ability to hydrolyze β-lactam substrates. Although some members of family VIII esterases reported to date exhibit activity towards nitrocefin (a chromogenic substrate used to determine β-lactamase activity) namely; EstC [17], EstM-N1 and EstM-N2 [13] very few esterases have

been reported to date that catalyze the hydrolysis of β-lactam substrates. The exception is EstB [7] and EstU1 which exhibit low hydrolyzing activity towards [14] these substrates. With EstB it was demonstrated clearly that the enzyme selectively deacetylate cephalosporin base substrates leaving the amide bond of the β-lactam ring intact. However with EstU1 it was not clear from the HPLC data if the observed pattern against cephalosporin substrates was due to deacetylation or amide bond hydrolysis of the β-lactam ring. Our Est22 was only specific for the ester bond of the β-lactam substrates (7-Aminocephalosporanic acid, Cephalosporin C and Cephalothin), as an HPLC spectrum obtained with Est22 was different from that of β-lactamase control (Figure 4A, B and C). Furthermore only cephalosporin alcohols were identified with UPLC-TOF MS (supplementary data) and none of the β-lactamase products were identified. The UPLC-TOF MS analysis further proved that Est22 only show deactylating activity against these substrates. Jeon and co-workers [14] proposed that the active pocket of EstU1 is changed to accommodate β-lactam antibiotics allowing EstU1 to bind the antibiotics and cleave the β-lactam rings and this factor differentiate it from other carboxylesterases, including our Est22, EstB, EstC, EstM-N1, and EstM-N2. Est22 enzyme displayed similar properties to EstB from *Burkholderia gladioli* [7]; however did not show closest similarity at primary sequence level with this specific enzyme. Despite structural homology to β-lactamases; nature and arrangement of active-site residues of EstB and Est22 were unable to hydrolyze the amide bond of β-lactam antibiotics. Modeling studies suggested steric hindrance to account for the EstB enzyme's selectivity for ester hydrolysis versus β-lactam cleavage [38]. Hence in this study we have shown that Est22 selectively deacetylate the first generation β-lactam antibiotics and not the amide bond in these β-lactam substrates. Docking of β-lactam substrates on Est22 (which only deacetylate the sustrates) and Estu1 which can hydrolyze both bonds might provide answers on the plylogenetic link between family VIII carboxylesterases and β-lactamases. Furthermore due to its deactylating selectivity Est22 has a potential for use in the modification of β-lactam antibiotics.

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Figure legends

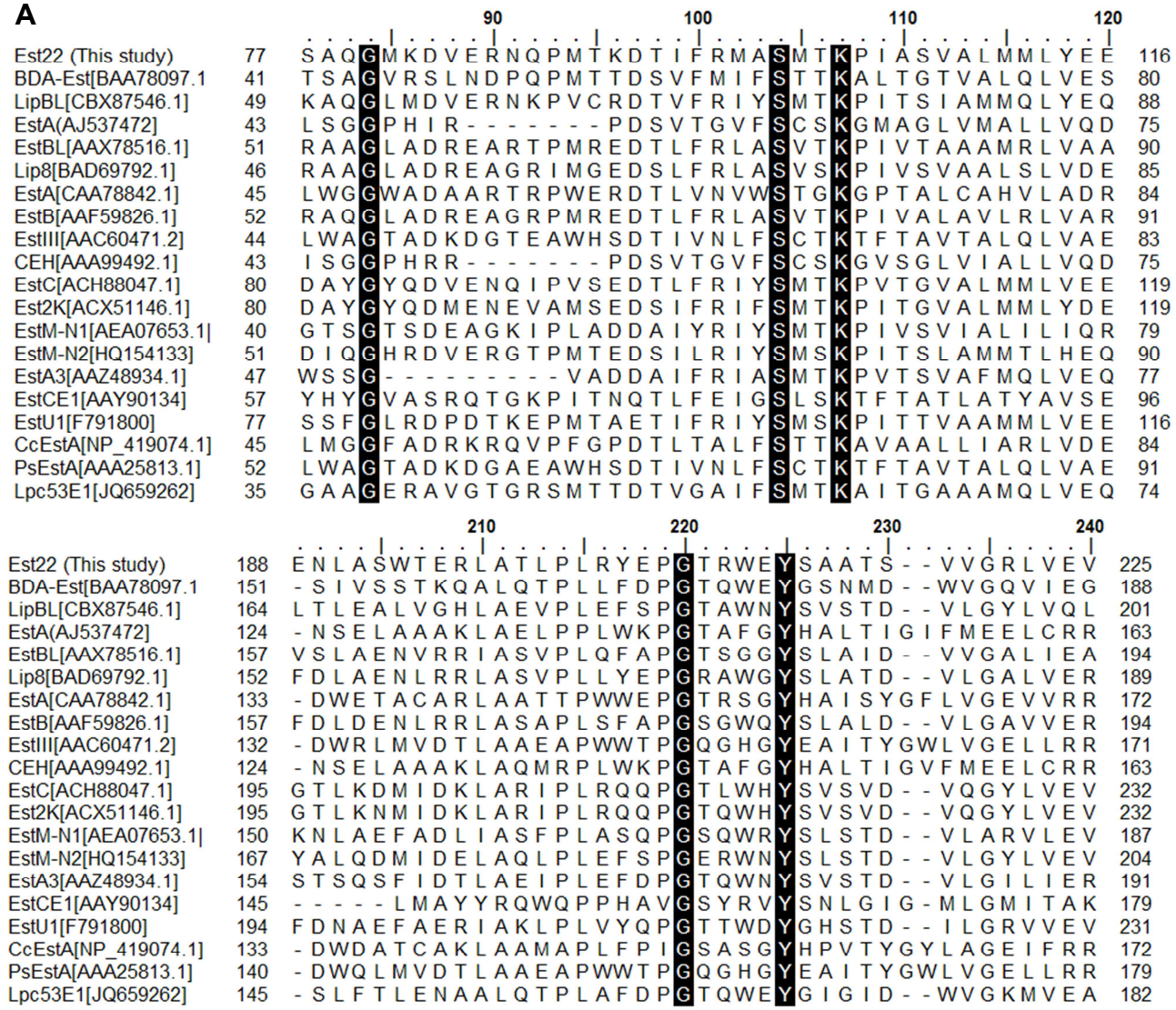
Figure 1: a) Multiple alignment of Est22 with related family VIII esterases and class β-lactamases showing the S-X-X-K motif and other conserved residues. Family VIII carboxylesterases are represented by BDA-Est, LipBL, EstA, EstBL, Lip8, EstB, EstIII, CEH, EstC, Est2K, EstM-N1 EstM-N2, EstA3, EstCE1, pLR1 and EstU1 (accession numbers; (BAA78097.1, CBX87546.1, AJ537472, AAX78516.1, BAD69792.1, AAF59826.1, AAC60471.2, AAA99492.1, ACH88047.1, ACX51146.1, AEA07653.1, HQ154133, AAZ48934.1, AAY90134, AEM45130.1 and F791800, respectively). Beta-lactamases are represented by CcEstA, PsEstA and Lpc53E1 (accession numbers; NP419074.1, AAA25813.1 and JQ659262, respectively). b) The evolutionary history of Est22 in relation to family VIII esterases and class C β-lactamases inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances and the percentage of replicate trees in the bootstrap test (500 replicates) are shown next to the branches. The evolutionary distances are in the units of the number of amino acid substitutions per site.

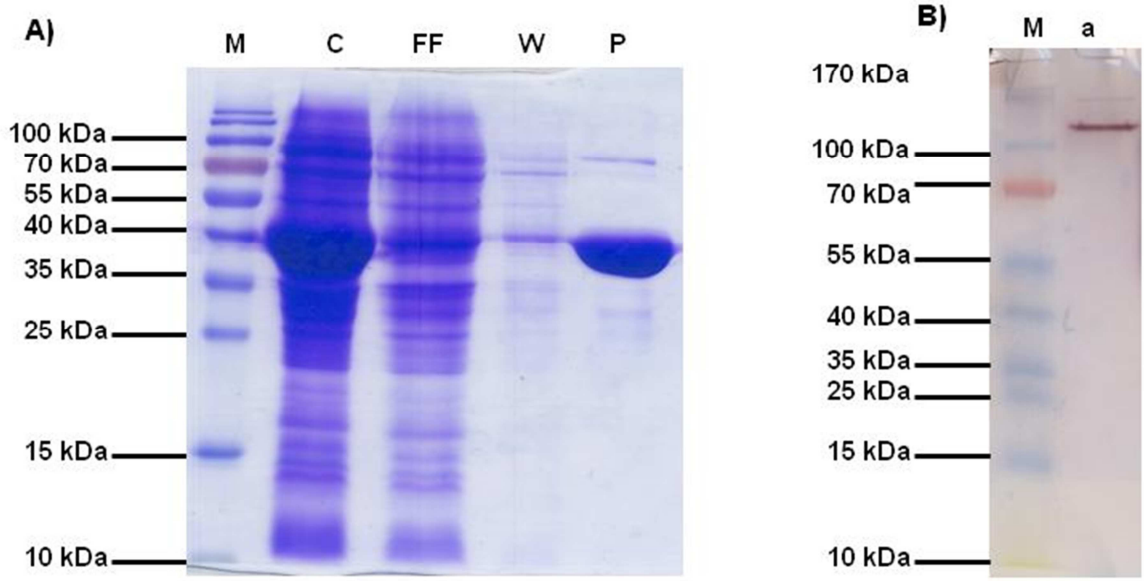
Figure 2: Denaturing SDS-PAGE and native PAGE for activity analysis, A) IMAC purification of Est22, crude sample is represented on lane 1, the flow through and the wash fractions on lanes 2 and 3, respectively and the purified Est22 is on lane 4. B) Purified Est22 zymogram, lane a) indicate Est22 stained with α-naphthol acetate/fast blue B-stained and lanes M on both gels represents protein ladders.

Figure 3: Biochemical characterization of recombinant Est22. A) Est22 substrate specificity B) Thermostability profiling using *p*-nitrophenyl butyrate as a substrate. C) Effect of pH on Est22 activity using *p*-nitrophenyl butyrate as a substrate.

Figure 4: HPLC analysis of the cephalosporin derivatives reactions, A) Cephalothin substrate with β-lactamase and Est22 separately. B) 7-amino-cephalosporanic acid with with β-lactamase and Est22 separately, and C) Cephalosporin C hydrolyzed with β-lactamase and Est22 separately. Substrates only were also analyzed with the reaction products to compare retention times (as indicated on all the peaks).

Figures




A)
M
C
FF
W
P
100 kDa
70 kDa
55 kDa
40 kDa
35 kDa
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B)
M
a
170 kDa
100 kDa
70 kDa
55 kDa
40 kDa
35 kDa
25 kDa
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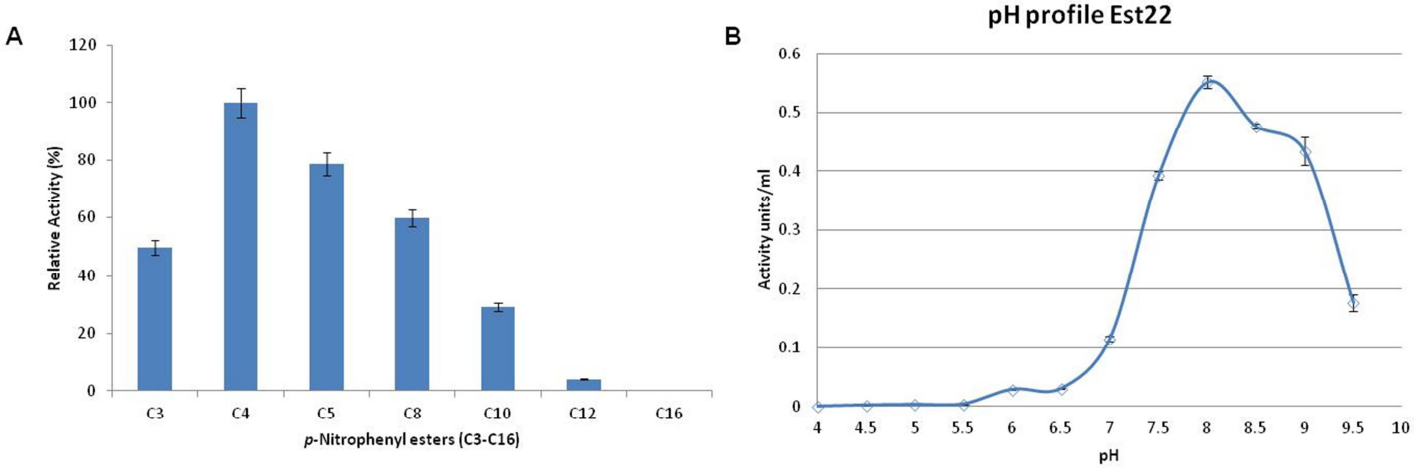
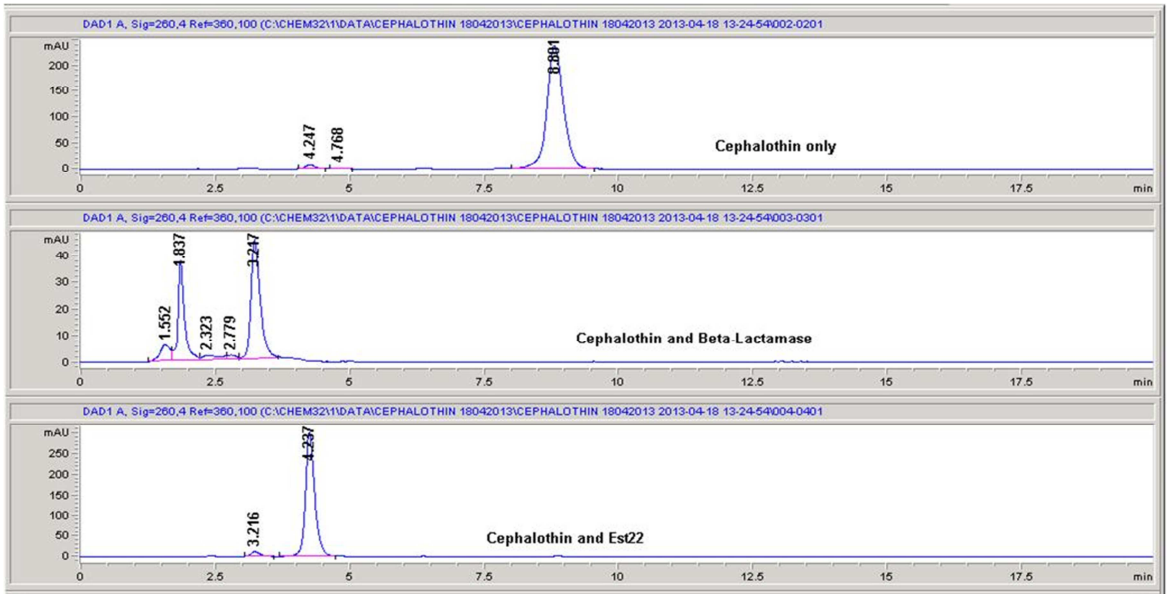
10 kDa


A
Relative Activity (%)
120
100
80
60
40
20
0
C3
C4
C5
C8
C10
C12
C16
p-Nitrophenyl esters (C3-C16)
B
pH profile Est22
Activity units/ml
0.6
0.5
0.4
0.3
0.2
0.1
0
4
4.5
5
5.5
6
6.5
7
7.5
8
8.5
9
9.5
10
pH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min
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Cephalothin and Beta-Lactamase
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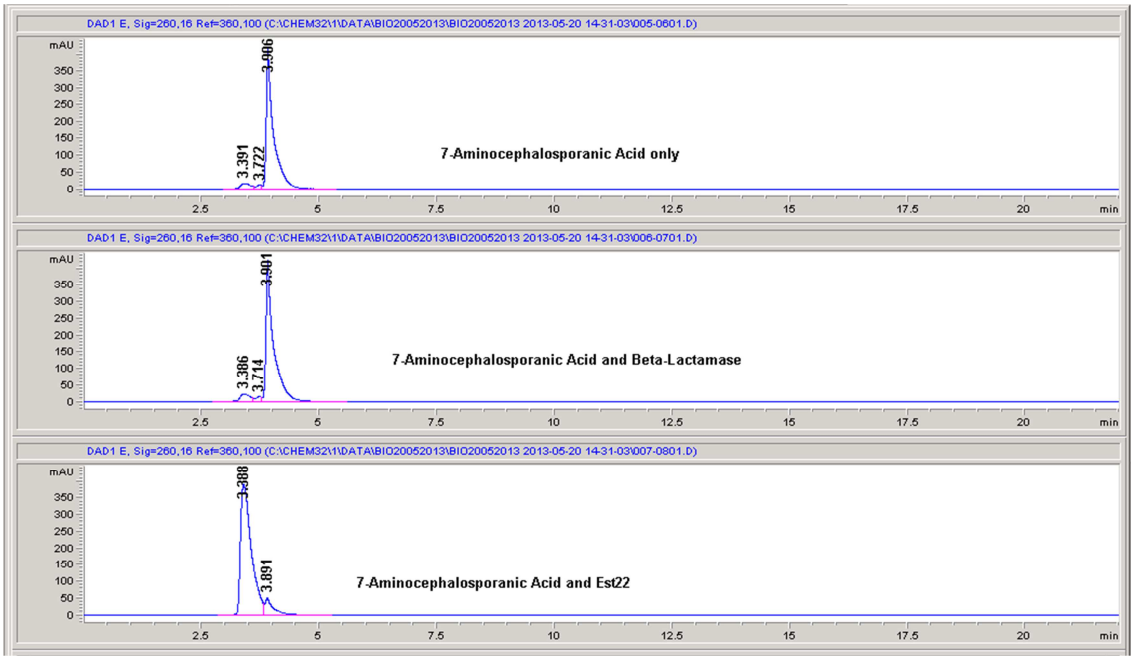
min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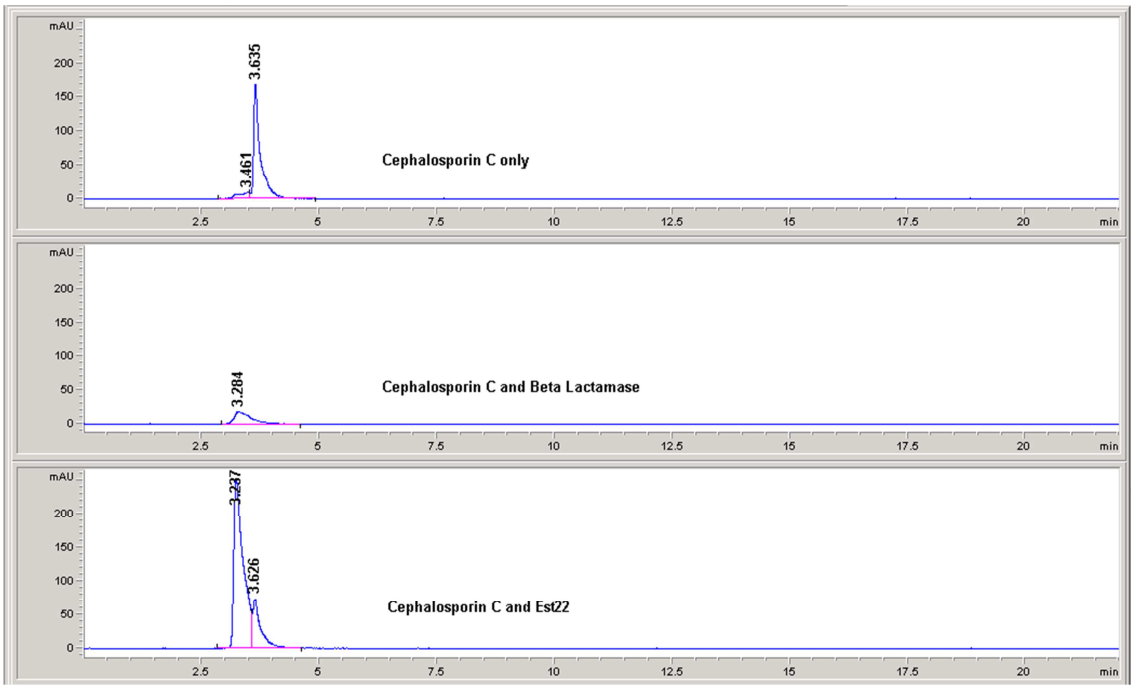
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15
17.5
20
min
mAU
200
150
100
50
0
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Cephalosporin C and Beta Lactamase
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10
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20
min