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A feruloyl esterase derived from a leachate metagenome library

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A feruloyl esterase encoding gene (designated *fae6*), derived from a leachate metagenomic library, was cloned and the nucleotide sequence of the insert DNA determined. Translational analysis revealed that *fae6* consists of a 515 amino acid polypeptide, encoding a 55 kDa pre-protein. The Fae6 primary structure contained the G-E-S-A-G sequence, which corresponds well with a typical catalytic serine sequence motif (G-x-S-x-G). The *fae6* gene was successfully over-expressed in *E. coli* and the recombinant protein was purified to 8.4 fold enrichment with 17% recovery. The K_M data showed Fae6 has a high affinity to methyl sinapate while thermostability data indicated that fae6 was thermolabile with a half life ($T_{1/2}$) < 30 min at 50°C. High affinity for Fae6 against methyl sinapate, methyl ferulate and ethyl ferulate suggest that the enzyme can be useful in hydrolyzing ferulated polysaccharides in a biorefinery process. [BMB reports 2012; 45(1): 14-19]

INTRODUCTION

Feruloyl esterases (EC 3.1.1.73) are a subclass of the carboxylic ester hydrolases (EC 3.1.1.-), which catalyse the hydrolysis of the ester linkages between hydroxycinnamic acids and carbohydrates in the process of plant cell biodegradation (1, 2). In addition to catalysing ester hydrolysis reactions, a number of feruloyl esterases (FAEs) have been reported that catalyse the transesterification reactions under non-aqueous conditions (3, 4). Like other carboxyl ester hydrolases, all reported FAEs are serine hydrolases, with α/β fold tertiary structures (5). FAEs are sub-classified into four types (type A-D), based on the amino acid sequence identities, substrate specificity profiles against methyl esters of hydroxycinnamic acids and the varying ability to release diferulic acids from esterified substrates (6).

The applications of FAEs in the industry cover a broad spectrum, including in the food industry as food additives (7), the pharmaceutical industry for the synthesis of bioactive phytochemicals (8), cosmetic industry for the production of flavour and fragment precursors (9), in animal feeds as feed additives (10, 11) and in pulp and paper industry as bleaching agents (12). There is a growing interest from both academic and industrial researchers to develop robust FAEs that can be part of enzyme "cocktails" for the conversion of plant biomass for biofuel production (1), as well as in the process of deriving value added chemicals from lignocellulosic substrates (13).

There is a widely held notion that the classical enzyme screening approaches involving the cultivation of microorganisms and the subsequent screening of the pure strains for the desired catalytic activity (14), fail to capture the majority of enzyme genes due to the limitations in microbial cultivation (15). In recent times, culture-independent metagenomic approaches have been developed that circumvent the problems of microbial culturing by directly accessing the total genetic pool in a given environment (16, 17). A number of studies on the metagenomic library creation coupled with functional screening have demonstrated the usefulness of the metagenomic approaches as a powerful tool in discovering novel bio-molecules with potential industrial applications (18, 19).

Domestic landfills provide a unique and varied environment in which a wide range of natural and synthetic compounds are found, potentially providing a basis for broad microbial diversity identification and evolutionary development (20). Hence, they are of interest as sources of novel biomolecules discovery. We have previously reported on the discovery of a novel esterase gene (estC) from leachate environment (20). In this study we further report on the screening and characterisation of a feruloyl esterase (Fae6) from the leachate environment which could potentially be used in degrading plant cell wall biomass for biofuel production.

RESULTS

Library screening

A total of three feruloyl esterase positive fosmid clones were identified in this study on ethyl ferulate agar plates (21). To identify feruloyl esterase open reading frame (ORF) in one of the active fosmid clones, a pUC19 subclone library was generated from a partially digested *Sau*3AI fosmid DNA. The subclone library was re-screened on ethyl ferulate agar and the insert DNA of the confirmed active clone was sequenced.

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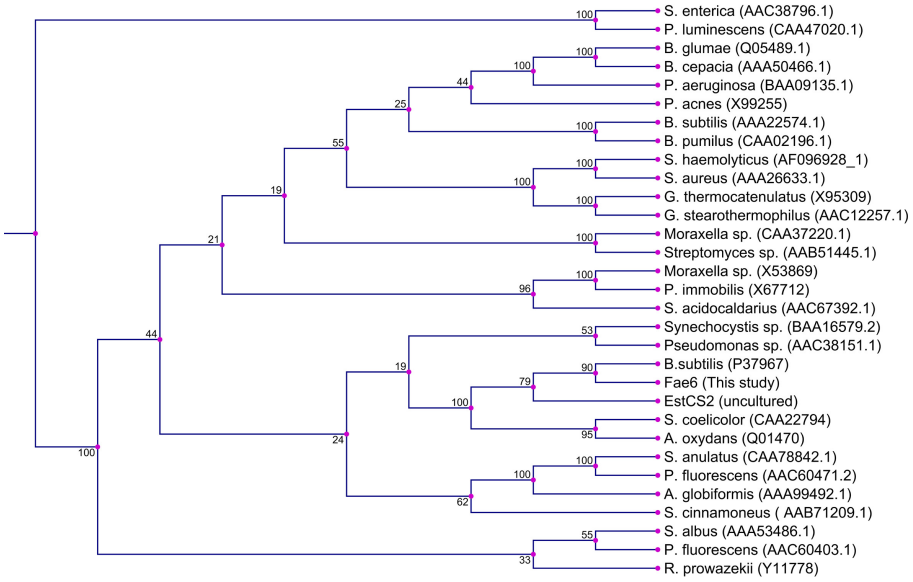

Fig. 1. Evolutionary distance phylogram showing the position of Fae6 from leachate metagenomic library in relation to other microbial enzymes from eight families described by Arpigny and Jaeger (26).

Nucleotide sequence analysis

The ORF of *Fae6* consisted of 1545 nucleotides in length and encoded a polypeptide of 515 amino acid residues with an estimated molecular mass of 55 kDa. The Fae6 primary structure encoded a pre-protein with a putative 22 amino acid leader peptide (with a maximum cleavage site probability of 0.9 between the Glu22 and Lys23, which would be cleaved to form a 52 kDa mature protein of 495 aa (predicted using SignalP3 Program; 22). The highest sequence identity scores for Fae6 in the Genbank database, from a global amino acid alignment (23), were with carboxylesterase type B of *Solibacter usitatus* (49%), *Opitutus terrae* (48%), *Acidobacterium capsulatum* (49%) and *Verrucomicrobiae bacterium* (43%).

Primary structure analysis

The deduced amino acid sequence of Fae6 contained a -G-E-S-A-G- sequence (position 217-221), which corresponds well with the pentapeptide -G-x-S-x-G- signature motif that is generally conserved in many esterolytic enzymes (5, 24). Multiple sequence alignment of Fae6 with a recently published metagemomic derived esterase (25) from family VII and the other related sequence from the BLASTp hits showed that the following residues were highly conserved: Ser253 within the G-E-S-A-G sequence motif, as well as the C-terminus located Glu307 and His507 (Fig. S1; Supplementary material). Based on the complete conservation Ser261, Glu290 and His525 residues, it can be deduced that corresponding residues (Ser219, Glu331 and His422) within the Fae6 amino acid sequence constitute the catalytic triad residues.

To gain further understanding regarding the evolutionary relationship of Fae6, a neighbouring-joining distance analyses was performed between Fae6 sequence and the amino acid sequences corresponding to the eight (I-VIII) lipolytic families described by Arpigny and Jaeger (26). The phylogenetic tree reveals that Fae6 primary structure clusters together with esterases belonging to family VII (Fig. 1).

Purification and biochemical characterisation

In order to study the biochemical properties of the recombinant enzyme, the *fae6* gene (lacking the N-terminal leader peptide coding sequence) was directionally ligated into pET20b(+) to allow the expression of the gene under a strong IPTG inducible T7 promoter and the fusion of the recombinant proteins with the C-terminal 6x histidine tag. Following an induction with IPTG for 6 h, the recombinant Fae6 was produced in a biologically active form in the soluble cytoplasmic fraction of *E. coli* cells.

An attempt to purify the enzyme in a one-step immobilised metal affinity chromatography (IMAC) purification procedure was unsuccessful. As a result a classical protein purification procedures (involving ammonium sulphate precipitation, followed by hydrophobic and size exclusion chromatography) were then used (detailed procedure in the supplementary material). About 1.8 mg of Fae6 (specific activity of 1,043 U mg^{-1} on pNP-C4) with an 8-fold enrichment and a yield of 17% were obtained (Table S1, supplementary material). An SDS-PAGE analysis of the purified sample resulted in a single protein band being observed, corresponding well to the predicted molecular weight of 53 kDa (Fig. S2; supplementary material). Based on the analytical Superdex^{TM} 200 size exclusion column (GE Halthcare), the globular size of the Fae6 was determined to be monomeric (Data not shown).

Biochemical characterisation

Activity of Fae6 was investigated over a temperature range of

5-50°C (Fig. 2A). The derived results indicate that Fae6 had a temperature optimum at 35°C. Thermostability data showed that Fae6 was thermo-labile with a half life ($T_{1/2}$ < 30 min) at 50°C (Fig. 2B). The pH profile of Fae6 revealed a pH optimum between 6.5-7.0 (Fig. S3, supplementary material).

The initial rates of hydrolysis of Fae6 were determined for different hydroxycinnamate esters (Table 1). Maximal hydrolytic rates were obtained with ethyl ferulate (100.0%), followed methyl ferulate (91.8%) and methyl sinapate (83.8%). Fae6 showed lower conversion rates (less than 50%) against methyl-*p*-coumarate (42.1%) and methyl caffeate (26.7%). No significant activity against chlorogenic acid was observed. The K_M values indicated that Fae6 has a high substrate affinity for MSA, followed MFA and EFA. Fae6 displayed high catalytic turnover (K_{cat}) against MSA. The catalytic efficiencies (as measured by K_{cat}/K_M) showed the following order of preference: MSA > MFA > EFA.

The hydrolytic activity of Fae6 against different fatty acid esters was investigated using a range of *p*-nitrophenyl (*p*-NP) esters of varying chain lengths (Table 1). The Fae6 hydrolytic patterns against *p*-NP esters showed a strong preference towards short to medium length acyl chains of C4 and C5, with *p*-NP-C5 being the most easily hydrolysed substrate (100.0%). The hydrolytic activity of Fae6 against long chain esters decreased

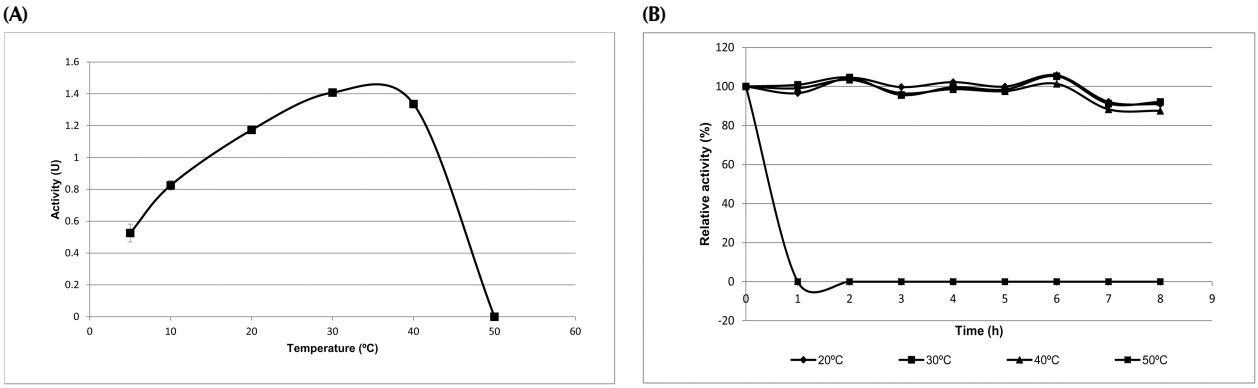

Fig. 2. (A) Temperature optimum profile of Fae6. (B) Temperature stability profile of Fae6. The temperature optimum of Fae6 was determined with reaction mixtures containing 1 mM methyl ferulate in 50 mM MOPS buffer (pH 6.5). Fae6 (10 μg) was added to initiate the reaction. After 1 h of incubation at temperatures between 5 and 50°C, the reaction was stopped by the addition of 200 μl of a 32% HCl solution. Ferulic acid release was analysed by HPLC. All experiments were performed in triplicates and the values represent the means ± standard error from the triplicate values measured.

Table 1. Substrate specificity and kinetic parameters of the purified Fae6 from leachate metagenomic library against hydroxycinnamate ethyl/methyl esters and *p*-nitrophenyl esters. All experiments were performed in triplicates and the values represent the means ± standard error from the triplicate values measured

Substrate	Relative activity (%)	K_M (mM)	K_{cat} (s^{-1})	K_{cat}/K_M (s^{-1} mM^{-1})
Hydroxycinnamate ester				
Ethyl ferulate (EFA)	100.0 (±0.5)	3.9 (±0.7)	42,483 (±5,290)	10,840 (±1,349)
Methyl ferulate (MFA)	91.8 (±0.8)	1.4 (±0.3)	20,758 (±1,887)	15,422 (±1,402)
Methyl sinapate (MSA)	83.8 (±0.7)	0.7 (±0.1)	12,159 (±1,120)	18,851 (±1,737)
Methyl *p*-coumarate (MpCA)	42.1 (±5.0)	ND	ND	ND
Methyl caffeate (MCA)	26.7 (±0.7)	ND	ND	ND
Chlorogenic acid (ChA)	0.7 (±26.7)	ND	ND	ND
p-nitrophenyl esters				
p-NP-acetate (C2)	12.0 (±3.9)	ND	ND	ND
p-NP-butyrate (C4)	84.8 (±7.9)	0.2 (±0.1)	46,710 (±276)	217,148 (±643)
p-NP-valarate (C5)	100.0 (±3.2)	0.1 (±0.01)	42,975 (±1,548)	592,580 (±11,155)
p-NP-caprylate (C8)	16.1 (±9.6)	ND	ND	ND
p-NP-laurate (C12)	7.5 (±3.7)	ND	ND	ND
p-NP-palmitate (C16)	0.0	ND	ND	ND

ND = not determined

drastically with only 7.5% conversion rate against *p*-NP-C12 and zero conversion against *p*-NP-C16. The kinetic parameters of Fae6 were determined for two (*p*-NP-C4, *p*-NP-C5) readily hydrolysed fatty acid esters. The K_M values suggested that Fae6 has high affinity for *p*-NP-C5, while the catalytic efficiency constant (K_{cat}/K_M) also showed that *p*-NP-C5 was also the most preferred substrate (Table 1).

DISCUSSION

In this study a metagenome library from an aqueous leachate sample was functionally screened for recombinant clones conferring to feruloyl esterase phenotypes. Following shotgun subcloning and sequencing a full length Fae6 encoding gene was indentified.

The Fae6 amino acid sequence revealed the presence of the G-E-S-A-G sequence corresponding to the catalytic G-x-S-x-G motif which has also been found in a number of other FAE primary structures. The solved feruloyl esterase crystal structures from *Aspergillus niger* (27) and *Clostridium thermocellum* (28) has shown that, the serine residue within the G-x-S-x-G motif harbours the catalytic serine, which attacks (through its hydroxyl oxygen) the carbonyl carbon atom of the ester bond; a reaction that yields a tetrahedral intermediate stabilized by a basic His residue and an acidic residue that could either be Asp or Glu (5). Based on the multiple sequence alignment the catalytic triad residues for the Fae6 were deduced to be: Ser219, Glu331 and His422.

The Fae6 primary structure analysis also showed the presence of a putative 22 amino acid N-terminal leader peptide characterized by a positively charged N-terminus, a hydrophobic core region and a polar C-terminal region (22). This observation suggests that Fae6 could be a periplasmic or extracellular enzyme, which is directly exported through the membrane with the aid of the leader sequence via the two step Xcp-dependent secretion pathways which are normally mediated by the N-terminal signal peptide (22).

Fae6 showed no lipase activity on the olive oil/Rhodamine B lipase specific assay of Kouker and Jaeger (29), suggesting that the enzyme was a "true" carboxylesterase. This observation was further confirmed by substrate profiling (using *p*-nitrophenyl esters of different chain lengths) which revealed that Fae6 prefers short to medium substrate chain lengths, a characteristic typical of true carboxylesterases (5). The application of Crepin's feruloyl esterases classification scheme (6) suggests that Fae6 belongs to either type A or type D. Both type A and D feruloyl esterases show high preferences to MFA and MSA and very little or no activity to M*p*CA and MCA (6). In addition of hydrolysing MFA and MSA substrates, type D also release ferulic acid dimers from the natural substrates such as FAX (2-*O*-[5-*O*-trans-feruloyl)-β-L-arabinofuranosyl]-D-xylopyranose) and FAXX (*O*-[5-*O*-(trans-feruloyl)-α-L-arabinofuranosyl]-(1->3)-O-β-D-xylopyranosyl-(1->4)-D-xylopyranose) (6). However, due to the lack of the availability of the FAX and FAXX substrates, we could not study the ability of Fae6 in releasing ferulic acid dimmers, which would have allowed Fae6 to be defined into a specific classification category.

The primary structure of Fae6 contains a G-G-G-F (112-115) sequence, which corresponds to the G-G-G-X (X-denoting hydrophobic residue) motif. Henke *et al*. (30) have previously shown that the presence of the G-G-G-X motif in lipolytic enzymes is linked with specificity for tertiary alcohols. All enzymes bearing this motif, including pig liver esterase, several acetyl choline esterases and an esterase from *Bacillus subtilis* were found to be active towards acetates of tertiary alcohols, while enzymes bearing the more common G-X motif did not catalyze these model substrates (30).

The presence of the G-G-G-F sequence prompted us to investigate the activity of Fae6 against tertiary alcohol ester using linalyl acetate as a substrate. Fea6 showed no activity against the linalyl acetate. In contrast to Fae6, a recently reported esterase estCS2 from compost metagenomic library which also belongs to family VII lipolytic enzyme converted linalyl acetate to linalool (19). Interestingly, the estCS2 primary structure contained the G-G-A-F sequence, in contrast to a common G-G-G-F sequence which has been shown to provide enantiorecognition of tertiary alcohols (5, 31).

Previous studies have shown that in addition of showing relatively high molecular weights relative to other microbial lipolytic families, enzymes belonging to family VII are very versatile enzymes with the ability to accept wide range of substrates (26). The classical examples include esterase from *Arthrobacter oxydans* which is active against phenylcarbamate herbicides (32), a *Bacillus subtilis* esterase which hydrolyses *p*-nitrobenzyl esters (33) and a compost metagenome derived esterase, which in addition to hydrolyzing polyurethane and tertiary alcohol esters, also shows enantioselectivity against (R/S)-ketoprofen ethyl ester (25). Thus esterases belonging to family VII constitute one of the most versatile lipolytic enzyme groups.

In concluding, this work illustrates the advantage of applied metagenomic approach coupled with a function-based screening approach in mining novel enzyme genes. The unique primary structure of Fae6 (moderate identity 49% to any known carboxylesterases in the database) demonstrates the usefulness of the technology in the discovery of novel enzyme genes that could otherwise have been missed by classical enrichment technology.

MATERIALS AND METHODS

Library construction and screening

A metagenomic library used for screening feruloyl esterase activities, was previously constructed from an aqueous acidic leachate sample collected from the Chloorkop landfill site, East of Johannesburg, South Africa (26°03′17.50″S) (20).

The purified eDNA was subjected to metagenome library construction using the EpiFOS™ Fosmid Library Production Kit (Epicentre Biotechnologies, USA). Primary screening of recom-

binant clones in *E. coli* EPI100-T1^R was performed on LB agar plates supplemented with isopropyl-β-D-thiogalactoside (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^{o}C. Esterase positive clones were identified by the presence of zone of clearance around the colony margins.

Designing of the expression vector

The *fae6* gene was PCR amplified from the plasmid (pUCFae6) template using the Fae6F (5′-GAGCTCATATGCAGCAGAATG GTGTTACCAC-3′) and Fae6R (5′-CCTCGAGGGTTTCTGCAA AACCGCTCAGAC-3′) primer pair, which introduces the *Nde*I and *Xho*I restriction site at the 5′- and 3′- end of the gene, respectively. The Fae6F/Fae6R primer pair targeted the truncated *fae6* gene without the leader peptide coding sequence and allowed the recombinant gene to be expressed in-frame with the 6x-His tag sequence at the 3′-end of the gene. The amplified PCR product was digested with *Nde*I/*Xho*I, followed by ligation into pET20b(+) linearized with the same restriction enzymes. The resultant p20Fae6 expression construct was placed under the control of the T7 promoter which is IPTG inducible.

Analytical methods

Qualitative agar assays: Primary screening for general esterase activities was performed using tributyrin agar assays (34). Secondary screening for feruloyl esterase activities was performed on ethyl ferulate agar assay as essentially described by Donaghy *et al*. (21).

Quantitative assays: All quantitative assays were performed in triplicate. Feruloyl esterase activity was determined by HPLC method as previously described by Andersen *et al*. (35). Unless otherwise stated, routine feruloyl esterase activities were performed in triplicate. One unit of enzyme activity was defined as the amount of enzyme that releases 1 μmol of ferulic acid per minute from methyl ferulate and the specific activity was given in units per milligram of protein. The reaction mixture (1 ml) was carried out in MOPS buffer (50 mM, pH 6.5) that contained 1 mM methyl ferulate (dissolved in acetonitrile/ K_2HPO_4 (90 : 10)). The reaction was started upon the addition of 10 μl of the appropriately diluted enzyme solution. Reactions were incubated at 40^{o}C for 5 min and were stopped by addition of 200 μl HCl (36 % (v/v)). The identical reaction mixture excluding the enzyme was included as a control to correct for autohydrolysis of the substrate.

General esterase assays were performed by measuring the release of *p*-nitrophenol as described by Petersen *et al*. (36). Protein concentrations were determined by the method of Bradford (37) using bovine serum albumin (BSA, Sigma Aldrich) as a standard.

Biochemical characterisation: Substrate preferences for the purified Fae6 against hydroxycinnamate ester derivatives were determined using the standard assay in the presence of 1 mM of the following esters: Ethyl ferulate (EFA), methyl ferulate (MFA), methyl *p*-coumarate (M*p*CA), methyl sinapate (MSA), and methyl caffeate (MCA). Kinetic parameters for the purified Fae6 were determined for EFA, MFA and MSA as starting substrates. Initial substrate concentrations were varied between 0.5 mM and 3.0 mM. In addition, activity against chlorogenic acid (ChA) was tested.

Selectivity of Fae6 against *p*-nitrophenyl esters were performed by a standard calorimetric method measuring the release of *p*-nitrophenol from the specified *p*-nitrophenyl esters of various chain lengths (dissolved in isopropanol): *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl valarate (C5), *p*-nitrophenyl caprylate (C8), *p*-nitrophenyl laurate (C12) and *p*-nitrophenyl palmitate (C16) as substrates. The extinction coefficient of *p*-nitrophenol under these conditions was 13,800 mM cm^{-1}. The experimental data of initial velocities *versus* substrate concentrations were used to determine the kinetic constants of the purified enzyme.

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