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A novel recombinant ethyl ferulate esterase from *Burkholderia multivorans*

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Key words: Ferulic acid esterase, Ethyl ferulate, Gene cloning; Esterase purification and characterization.

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Aims: Isolation and identification of bacterial isolates with specific ferulic acid esterase activity and cloning of a gene encoding activity

Methods and Results: A micro-organism with ethyl ferulate hydrolysing activity was isolated by culture enrichment techniques. Detailed molecular identification based on species-specific primers and two conserved genes (16S rRNA and *rec*A) led to the identification of the isolate as *B. multivorans* UWC10. A gene (designated *estEFH5*) encoding an ethyl ferulate hydrolysing enzyme was cloned and its nucleotide sequence determined. Translational analysis revealed that *estEFH5* encoded a polypeptide of 326 amino acids with an estimated molecular weight of 34.83 KDa. The EstEFH5 primary structure showed a typical serine hydrolase motif (G-H-S-L-G). The *estEFH5* gene was over-expressed in *E. coli* in insoluble form. Following urea denaturation and *in vitro* refolding, the enzyme was purified using one-step His Select ™ Nickel chromatographic column.

Conclusion: Purified EstEFH5 showed a preference for short chain ρ-nitrophenyl esters (C2 and C3) a typical feature for carboxylesterase. Furthermore, the recombinant enzyme also retained the activity against ethyl ferulate.

Significance of the study: A biocatalytic process for the production of ferulic acid from ethyl ferulate as a model substrate was demonstrated. This is the first report that describes the cloning and expression of a gene encoding ferulic acid esterase activity from the genus *Burkholderia*.

Introduction

Agro-processing industries, such as South Africa's Germiston African Products (Afprod) plant, process tons of corn per day, generating large amounts of plant cell wall waste materials. As for many other plant waste materials, such wastes have a high carbohydrate and phenolic content (Faulds *et al*., 1997). Such products are also potentially valuable resources for biotechnology because of the presence of high levels of hydroxycinnamic acids (ferulic, ρ-coumaric and ρ-cafferic acid) which are valuable compounds (Ishii *et al*., 1997). However, these phenolic acids are not readily accessible since they are typically covalently linked to polysaccharides (Saulnier *et al*., 1995; Kroon and Williamson, 1996). Furthermore, ferulic acid (FA) may also cross-link to form diferulic acid bridges, a process reported to be important in resistance to plant pathogens (Micard *et al*., 1997).

There is a growing interest from both the academic and industrial researchers to develop biotechnological processes to recover high value compounds from plant waste materials (Faulds *et al*., 1997). The recovery of FA in particular has been of a major interest, since FA is potentially a versatile substrate for biotransformation, and can be converted with added value to vanillic acid and vanillin (Falconnier *et al*., 1994, Kroon and Williamson, 1999, Andersen *et al*., 2002). FA itself has a wide range of industrial applications, based both on its antioxidant properties (Graft, 1992) and its use in the food industry (Andreoni *et al*., 1984).

Ferulic acid esterases (FAE) (EC 3.1.1.73) are a subclass of the carboxylic ester hydrolyses, which hydrolyze ester linkages between hydroxycinnamates and sugars (Williamson *et al.,* 1998). In addition of catalyzing transesterifcation reaction (Hatzakis *et al*., 2003), FAEs also play an important role in hydrolyzing ethyl ferulate esters to ferulic acid (Donaghy *et al*., 1998). These enzymes have been characterized from a number of microbial hosts including

the fungi *Aspergillus niger* (Christov and Prior, 1993, de Vries *et al*., 1997), *A. awamori* (McCrae *et al*, 1994,) *A. oryzae (*Tenkanen *et al*, 1991), *Neocallimastix MC-2 (*Borneman *et al*, 1992), *Penicillium pinophilum* (Castanares *et al*, 1992), *Butyrivibrio fibrisolvens* (Dalrymple *et al*, 1996) and the bacteria *Streptomyces olivochromogenes (*Faulds and Williamson, 1991), *Pseudomonas fluorescens* (Faulds *et al*., 1995), *Bacillus* and *Lactobacillus* sp.(Donaghy *et al*., 1998).

A number of studies have demonstrated ferulic acid esterase activities from different micro-organisms using model synthetic substrates such as ethyl and methyl ferulate (Vafiadi *et al*., 2006, Topakas *et al*., 2005, Anderson *et al*., 2002, Couteau *et al*., 2001). Here we report the isolation, screening and identification of a *Burkholderia multivorans* isolate possessing ferulic acid esterase activity using ethyl ferulate (ethyl-3-(4-hydroxy-3-methoxyphenyl)-2-propenoate) as a model substrate and the subsequent cloning and expression of a gene encoding the activity.

Materials and Methods

Unless stated otherwise reagents used in this study were supplied by Sigma Aldrich, Germany. Ethyl ferulate was kindly provided by CSIR (South Africa). Minimal medium 9 (M9) was prepared by the method of Russell and Sambrook, (2001). M9-EF medium was prepared by replacing glucose with ethyl ferulate as a carbon source.

Selective enrichment

Maize silage samples used for isolation of ethyl ferulate hydrolyzing microorganisms were collected from a maize processing farm in Stellenbosch (South Africa). Maize silage samples (1.0 g) were resuspended in 10 ml of sterile milli-Q water containing 2% (v/v) Tween 20.

Samples were vortexed for 5 min to dislodge microorganisms and allowed to stand for 1 h at room temperature before aliquots (100 µl) of serially ($10\text{-}10^{-5}$) diluted samples were aseptically spread onto M9-EF minimal medium plates containing various concentrations of ethyl ferulate (0.1-0.5%) as a sole carbon source. Filter sterilized cyclohexamide 0.1% (w/v) was added to the medium to inhibit fungal growth. The plates were incubated aerobically at a range of temperatures (25, 30, 37, 45 and 50 ^{o}C). Colonies were repeatedly streaked on the same minimal nutrient medium until pure cultures were obtained.

Assays

Agar assays

Ethyl ferulate agar assay were prepared essentially as described by Donaghy *et al*. (1998). Qualitative tributyrin (Ro *et al*., 2004) and Olive oil-Rhodamine B (Kouker and Jaeger, 1987) agar assays were used to screen for esterase and lipase activities, respectively.

Quantitative Assays

All assays were performed in triplicate. Ethyl ferulate hydrolyzing assay was performed using method of Andersen *et al*. (2002). Quantitative esterase and lipase assays were performed by measuring the release of ρ-nitrophenol as described by Petersen *et al*. (2001) and Gupta *et al*. (2002), respectively. Protein concentrations were determined by the method of Bradford, (1976) using bovine serum albumin (BSA, Sigma Aldrich) as a standard.

Thin layer chromatography (TLC) analysis

After biotransformation of EF by selected isolates, supernatants were acidified to pH<2 with 10 N HCl, extracted twice with diethyl ether, then dried over anhydrous Na_2SO_4 and concentrated by evaporation under N_2:CO_2 (80:20). Extracts were dissolved in a minimal volume of methanol and chromatographed on 5 x 10 cm silica gel F_{20} TLC plates (Merck), together with commercial ferulic acid as a positive control. The mobile phase was chloroform: methanol: formic acid (85:15:1 v/v/v). The spots were visualized at 335 nm and R_f values were measured.

High performance liquid chromatography (HPLC)

Supernatants after biotransformation were analyzed with a LaChrom HPLC system equipped with UV detection and an 80-position auto-injection sampler. Separation was achieved on a Wakosil II C18 reverse phase column with a gaurd column. The detector wavelength was set at 315nm. The mobile phase was water: methanol: acetic acid (80:20:2.5 v/v/v) at a flow rate of 1 ml min^{-1}.

Construction and screening of *B. multivorans* UWC10 genomic library

Chromosomal DNA isolated according to the method of Marmur, (1963), was partially digested with *Sau*3A1 and fractionated by electrophoresis on 0.7% agarose gels. The 2-8 kb fraction was recovered and ligated into pUC18, which was previously digested with *Bam*HI and desphosphorylated with shrimp alkaline phosphatase. The ligation products were used to transform chemically competent *E. coli* DH5α cells. Recombinants were screened on LB agar plates supplemented with 0.1 mM isopropyl-β-D-thiogalactoside (IPTG), 100 µg ml^{-1} ampicillin, 1% (v/v) tributyrin and 0.1% (w/v) Gum Arabic, incubated at 37 ^{o}C.

Construction of expression vector

To generate the His tagged *estEFH5* gene construct, the plasmid-encoded gene was amplified by PCR using oligonucleotides EstN and EstHis carrying *Nde*I and *Hind*III restriction sites, respectively (Table1). The PCR product was purified and cloned into the corresponding sites of pET22b (+), resulting in the $pETEFH5_{tag}$ expression plasmid, which was used to transform *E. coli* BL21 (DE3) cells.

Protein purification

Cultures expressing the recombinant protein were harvested and disrupted by sonication using a Sonoplus HD-070 (Bandelin, Germany) set at: 5x cycle burst, 50% max. amplitude for 2 min, at 30 sec interval. Cells debris was recovered by centrifugation (15 000 x g, 20 min, 4 ^{o}C) then washed twice with Tris-HCl buffer (50 mmol l^{-1}, pH 8.0) containing 1% Triton x100.

The insoluble inclusion bodies were resuspended in denaturing buffer (100 mmol l^{-1}, NaH_2PO_4, 100 mmol l^{-1} Tris, 8 mol l^{-1}urea, pH 8.0) at a density of 0.2 g ml^{-1} followed by incubation at 25 ^{o}C for 1 h with gently stirring. The clear supernatants (20 ml) obtained after centrifugation (20 000 x g; 20 min, 20 ^{o}C) were loaded onto a His Prep^{TM} FF 16/10 column (Amersham Biosciences). The unbound proteins were washed with 3 column volumes of wash buffer (100 mmol l^{-1} NaH_2PO_4, 100 mmol l^{-1}Tris, 8 mol l^{-1}, urea, pH 7.0) at a flow rate of 0.5 ml min^{-1}. Bound proteins were refolded over 5 column volumes at a flow rate of 0.5 ml min^{-1} with a linear gradient of a decreasing urea concentration (8-0 mol l^{-1}), generated with gradient buffer (500 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris, 20% (v/v) glycerol, pH 7.4). The refolded bound proteins were eluted with a linear gradient of increasing imidazole concentration (0-250 mmol l^{-1}) generated with elution buffer (500 mmol l^{-1} NaCl, 20 mmol l^{-1} Tris, 20% (v/v) glycerol, 250 mmol l^{-1} imidazole, pH 7.4) at a flow rate of 1 ml min^{-1}. The imidazole was removed by dialysis and the protein fraction was concentrated by membrane filtration (Centriprep YM-10, cutt-off, 10 kDa, Millipore) before analysis by SDS-PAGE according to the method of Laemmli, (1970).

DNA sequencing and sequence analysis

Sequence analyses, manipulation and annotation were performed on the Gene construction kit 2 (Textco BioSoftware, Inc.), and Bioedit (Hall, 1990) software programs. Multiple sequence alignments were performed with CLUSTALX (Thompson *et al*., 1997). Theoratical Mw and pI were prected using the EXPASY web site (http://www.expasy.ch) (Appel *et al*., 1994). Nucleotide and amino acid sequences were obtained from the following databases: GenBank, DDBJ, EMBL, SWISSPROT and PDB. Homology searches were performed using the basic local alignment search tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al*., 1997). Oligonucleotides used in this study were synthesized by Inqaba Biotech (South Africa).

Results

Isolation and screening strategies

Maize silage sample were used to isolate ethyl ferulate hydrolyzing bacteria. Samples spread on M9-EF plates were incubated aerobically at various temperatures (30-50 ^{o}C) for up to eight days. Colonies were observed only on media containing 0.1% (v/v) EF incubated at 30 ^{o}C. Re-plating of selected colonies was repeated until pure cultures were obtained, based on the light microscopic observations (data not shown). Rapid growth in the presence of the EF as a sole carbon source was taken as an indication of the presence of ethyl ferulate hydrolyzing (EFH) enzymes.

Thirteen pure bacterial isolates, selected for their ability to grow on EF as a sole carbon source, were further examined for their ability to produce EF-hydrolyzing enzyme(s). In order to confirm the presence of EFH enzyme(s) in these isolates, a qualitative agar-screening assay (Donaghy *et al.* 1998) was used. The appearance of a zone of clearance around wells containing culture supernatants was indicative of the presence of EF-hydrolyzing esterase(s).

One isolate (designated UWC 10) consistently showed high EFH activity as indicated by a large zone of clearance. This isolate also tested positive for non-specific esterase and lipase activities using qualitative tributyrin and olive oil plate assays, respectively. Cell fractionation studies indicated that EFH activity was intracellularly located (data not shown). Similar observations have been made for a number of other micro-organisms including *Bacillus*, *Lactobacillus* (Donaghy *et al*., 1998), *E. coli* and *Bifidobacterium* strains (Couteau *et al*., 2001)

Molecular Identification of isolate UWC10

In order to determine the identity of isolate UWC10, molecular identification based on the 16S rRNA gene sequence as a molecular marker was performed. Genomic DNA from strain UWC10 was used as a template for PCR amplification using the universal primer pair E9F and U1510R (Table 1). A BLAST search with the 1498 bp sequence revealed that the 16S rRNA gene of UWC10 had a very high degree of nucleotide sequence identity to the genus *Burkholderia* (Table 2). The 16S rRNA gene sequence of *B. multivorans* UWC10 was deposited in GenBank under the accession number DQ103700

Since 16S rRNA gene analysis revealed very high levels of nucleotide sequence identity (99%) to a number of *Burkholderia* species, further phylogenetic analysis was required to establish the identity of UWC10 to species level. Species–specific primers (G1, G2, SPR3, SPR4), based on a region of heterogeneity within the 16S-23S spacer sequence of members of the *Burkholderia cepacia* complex were used (Table 2).

Combinations of primer pairs (G1/G2, G1/SPR3, and G1/SPR4) were evaluated for their ability to amplify UWC10 genomic DNA. No amplification was achieved with the G1/G2 and G1/SPR4 primer pairs under the conditions used. Based on the PCR algorithm for identification of *Burkholderia* species (Whitby *et al*., 2000), the absence of a PCR product with the G1/G2 primer pair suggested that the UWC10 was neither *B. cepacia* nor *B. concepacia*, while the absence of a PCR product with G1/SPR4 suggested that UWC10 was not *B. stabilis*. The absence of the PCR amplicon with G1/SPR3 indicated that the organism was not *B. vietnamiensis*, while amplification with G1/SPR3 supported the conclusion that strain UWC10 was *B. multivorans*.

In order to establish conclusively that UWC10 belonged to the species *B. multivorans*, a primer pair BCR1/BCR2 (Table 1), specifically targeting the *recA* gene of *B. multivorans* (Mahenthiralingam *et al*., 2000), was evaluated. UWC10 genomic DNA was amplified with the BCR1/BCR2 primer pair and the predicted PCR product of 1043 bp was observed. The ability of the BCR1/BCR2 primer pair to produce a strong signal under these conditions strongly suggests that UWC10 is *B. multivorans*.

Biotransformation of ethyl ferulate

Ethyl ferulate deesterification was monitored during the growth of UWC10 in M9-EF minimal medium containing 0.1% (v/v) EF. The liquid medium was inoculated with washed 10% (v/v) of a stationary phase culture pre-grown in nutrient medium. Ethyl ferulate hydrolyzing activity was confirmed by the production of ferulic acid in the culture broth (Fig. 1). The decrease in FA concentration during extended periods of growth suggests that FA was being further metabolized. Under similar conditions without inoculation of the medium, EF was very stable.

Supernatant of the cultures grown on EF-containing media were analysed by Thin Layer Chromatography (Fig. 2). A single spot (R_f =0.85) was observed at time zero (Fig. 2, lane 2). After 12 h, three spots were observed: based on the R_f values, these spots were identified as EF (R_f =0.85), ferulic acid (R_f = 0.54) and ρ-coumaric acid (R_f = 0.41). After 24 h of growth the substrate was completely utilized as indicated by the absence of EF spot (Fig. 3, lanes 4 and 5). However, both ferulic acid and ρ-coumaric acid were still detectable. Although both ferulic acid and ρ-coumaric acid were still observed after 48 h of growth, an additional spot (R_f =0.76) was also observed. Based on the likely metabolic products of FA, this spot was

tentatively assigned as one of the vanillin derived compounds: i.e., vanillin, vanillic acid or vinyl guaiacol (Fig.2, Lane 6).

Chromatoplates were developed and sprayed with ferric chloride and ethanolic-vanillin reagents (Fried and Sherma, 1996). The R_f (0.76) product failed to react with ferric chloride but yielded a violet colour with ethanolic-vanillin. Such a reaction is characteristic of vinyl guaiacol. Furthermore, when vanillin was included as a standard, it showed an R_f value of 0.68 (data not shown).

Library screening

Approximately 10 000 ampicillin-resistant recombinants were screened on tributyrin, olive oil-Rhodamine B and FAE plate assays. Several halo-forming clones were identified, and one highly active clone produced large halo on ethyl ferulate agar plates after three days of incubation at 37 ^{o}C was selected. The clone was designated TEND5 and its recombinant plasmid (pTEND5) isolated. This clone formed halo in the absence of the IPTG, indicating that the gene encoding esterolytic activity was being expressed in *E. coli* under the control of the intrinsic promoter.

Nucleotide sequence analysis of the cloned pTEND5 DNA insert

Nucleotide sequence analysis of the 2.1 kb DNA insert in the clone TEND5 revealed a single open reading frame (ORF1), predicted to encode an esterolytic enzyme. ORF1 consisted of 978 bp, commencing with an ATG start codon and extending to a TGA stop codon. Possible nucleotide sequences corresponding to typical elements of *Burkholderia* or *E. coli* promoter sequences were not found in the region upstream the predicted ORF1. However, an -AGGC- sequence located 4 bp upstream the ATG start codon was presumed to be the putative

ribosome binding site (RBS), based on its close similarity to the consensus Shine-Dalgano (-AGGA-) sequence. Translational analysis of ORF1 revealed a polypeptide of 326 a.a. encoding a protein of 34.83 kDa with a predicted pI value of 10.02. ORF1 had a very high GC content (74%).

Analysis of deduced amino acid sequence of ORF1

A classical signature motif, the pentapeptide G-H-S-L-G motif (a.a. positions 133-137), corresponding to the G-x-S-x-G motif which harbours the catalytic serine for many esterases, lipases and other hydrolases (Jaeger *et al*., 1999) was observed. The serine (Ser135) within the G-H-S-L-G motif is likely to be the catalytic serine. Further amino acid sequence analysis of ORF1 revealed a PIVFVHG motif (a.a. positions 61-67) corresponding well with a putative oxyanion region (Arpingy and Jaeger, 1999) comprised of the HG dipeptide and a short hydrophobic upstream stretch. A putative oxyanion binding motif (HGDAL, a.a. positions 175-179), which corresponds well with the HXDXZ motif (Arpingy and Jaeger, 1999) (where X represents any amino acids and Z a hydrophobic residue) was also observed. In subfamilies I.1 and I.2 lipolytic enzyme, this segment is conserved as HLDEI or HILDEV, respectively and normally harbours the catalytic histidine (Arpingy and Jaeger, 1999).

Sequence comparison of ORF1 with other esterases

The deduced amino acid sequence (326 a.a.) of ORF1 was employed to search for homologous proteins from protein databases. The search report revealed that the ORF1 had high sequence identity (46-64%), to a number of lactone hydrolyzing esterases (Table 3). ORF1 was designated EstEFH5 and the corresponding gene was designated *estEFH5*. The EstEFH5 amino acid sequence was deposited in GenBank under the accession number AAV97951.

In order to further analyse the primary structural features of EstEFH5, multiple sequence alignments were performed with the five closely related esterase sequences (Fig. 3). Alignment of the EstEFH5 amino acid sequence with Est1, EstF1, EstMA EstPF5 and SS3 sequences clearly demonstrates the conservation of the classical G-x-S-x-G pentapeptide signature motif (a.a. 137-141). From the alignment it could be deduced that Ser139, Asp219, and His279 form the catalytic triad, which correspond to S135, His 275, and Asp215 as the putative catalytic triad in the EstEFH5 primary structure (Fig. 3).

The HG-dipeptide (a.a. positions 70-71) typical of the oxyanion region in esterases and lipases was also conserved. Among the eight residues of the oxyanion region, three were identical and four were similar within the consensus sequence. High sequence identity between EstEFH5 and other esterases were noted at a.a. positions 223-229 (GGGLLG) and 299-301 (GHM). The functions of these motifs have yet to be elucidated.

Application of the Arpingy and Jaeger, (1999) classification scheme suggested that EstEFH5 belongs to Family V. Esterases in this family share significant homology to non-lipolytic enzymes such as epoxide hydrolase, peptidases, dehalogenases and haloperoxidases, all of which possess the α/β hydrolase fold (Arpingy and Jaeger, 1999).

Protein expression and purification

Analysis of the reverse primer sequence (EstR1) corresponding to the 18-nucleotide coding sequence of the *estEFH5* gene at the 3´-end revealed the presence of direct repeat sequence and showed a very high GC content (83%). From these observations we concluded that PCR amplification of the *estEFH5* gene amplification would be affected by secondary structure formation and/or premature chain termination in the repeat regions. The reverse primer sequence (EstR2) with a lower GC content (66%) was designed in-frame against a non-coding sequence downstream of the TGA stop codon. As a result, the $EstEFH5_{tag}$ protein had a predicted molecular weight of 37 KDa, due to the additional 18 amino acid residue extension at the C-terminus, including the vector encoded polyhistidine sequence (MTVRCKLAAALEHHHHHH).

Several attempts (including the removing of a His-tag, the use of the weaker tac promoter of pMS470Δ70 (Balzer *et al*., 1992) and lowering the temperature during expression), were made to express EstEFH5 in a biologically active form. These attempts showed that EstEFH5 activity could be detected in the intracellular fractions although SDS-PAGE analysis showed no corresponding protein band. However, the corresponding protein band was observed in the pellet fractions, suggesting that the protein was accumulating in an insoluble form (data not shown).

In vitro refolding studies were used to recover EstEFH5 in a biological active form (Fig. 4A). The purified EstEFH5 had a specific activity of 13.4 U mg^{-1} against ρ-nitrophenyl acetate. Activity staining gave a single band on the zymogram corresponding exactly with the purified protein band and confirming that no other esterases were present in the purified sample (Fig. 4B).

Substrate specificity

The substrate specificity of the purified EstEFH5 against different fatty acyl substrates was determined using ρ-nitrophenyl esters of C2-C16 (Fig. 5). EstEFH5 selectivity was highest against shorter acyl chain lengths. EstEFH5 showed maximum activity (100%) against ρ-NP-C3 followed by ρ-NP-C2 at 74%. The hydrolytic activity of EstEFH5 decreased dramatically against medium chain length esters (C6-C10). EstEFH5 was not active against ρ-nitrophenyl esters with *n*-acyl chain lengths of greater than 10 (C14 and C16).

Using two readily hydrolysed ρ-nitrophenyl ester substrates (C2 and C3), kinetic constants for EstEH5 were calculated (Table 4) using the Michaelis-Menten non-linear regression hyperbola plot in GraphPad Prism version 4.0. The K_M and V_{max} values for ρ-NP-C2 were approximately 1.7-fold lower than that of ρ-NP-C3. Catalytic turnover (k_{cat}) for ρ-NP-C2 was 1.6-fold higher than that of ρ-NP-C3. Specificity constant (k_{cat}/K_M) values for the two substrates suggested that ρ-NP-C3 was a marginally preferred substrate.

EstEFH5 activity against ethyl ferulate was first investigated using the qualitative plate assay. EstEFH5 showed a zone of clearance (data not shown), indicating ethyl ferulate hydrolyzing activity. To further confirm this activity, the reaction products were analysed by HPLC. HPLC traces showed the presence of free ferulic acid (data not shown) confirming that the recombinant EstEFH5 was capable of hydrolysing ethyl ferulate.

Discussion

The primary goal of this study was to identify novel enzyme(s) capable of the conversion of ethyl ferulate to ferulic acid. Plate screening assays led to the isolation of *B. multivorans* UWC10 which consistently demonstrated high EFH activity.

Both growing and resting cells of *B. multivorans* released ferulic acid from ethyl ferulate, which was further metabolized to vinyl guaiacol. The mechanisms of FA bioconversion are well documented and include (i) non-oxidative decarboxylation, (ii) side-chain reduction, and (iii) coenzyme-A-independent deacetylation and coenzyme-A-dependent deacetylation (Priefert *et al*., 2000). Based on the mechanisms of ferulic acid metabolism by *B. cepacia* (Andreoni *et al*., 1984), *Ps. fluorescens* (Andreoni *et al*., 1995) and *B. coagulans* (Karmakar *et al*., 2000), a mechanistic model of ethyl ferulate hydrolysis by *B. multivorans* UWC10 was postulated. Our data suggest that *B. multivorans* UWC10 hydrolyses the ester bond between the ethyl and ferulate groups, releasing ferulic acid. The released ferulic acid is subsequently decarboxylated to vinyl guaiacol by endogenous decarboxylase activity (Andreoni *et al*., 1984; 1995; Karmakar *et al*., 2000).

Unique features of EstEFH5 and the related lactone hydrolysing esterases (Khalameyzer *et al*., 1999) include the presence of a 30 a.a. N-terminal extension and a very high pI value (10-11). Hydropathy plots (data not shown) indicate that the N-terminal region of EstFH was highly hydrophobic, suggesting that this sequence may anchor the enzyme in the cell membrane. It has been noted that lactone hydrolyzing enzymes possessing this sequence typically require detergents to maintain the protein in a soluble state during expression and/or purification (Khalameyzer *et al*., 1999).

The presence of an ORF upstream the EstEFH5 encoding a putative monooxygenase was also noted (data not shown). The location of monooxygenase genes adjacent to the lactone hydrolyzing esterase genes has been previously reported in *Ps. fluorescens* (Khalameyzer *et al*., 1999) and *Acinetobacter* sp. (Taschner *et al.,* 1988). A mechanistic model has been proposed, suggesting that esterases located adjacent to monooxygenases are involved in the hydrolysis of lactones formed by Baeyer-Villiger monoxygenases (Onakunle *et al*., 1997).

The primary structure of EstEFH5 contains a GGGL sequence, which corresponds to the G-G-G-X (X-denoting hydrophobic residue) motif. Henke *et al*., (2002) have shown that the presence of the GGGX motif is linked with specificity for tertiary alcohols. All enzymes bearing this motif, including pig liver esterase, several acetyl choline esterases and an esterase from *B. subtilis* were found active towards acetates of tertiary alcohols, while enzymes bearing the more common GX motif did not catalyze the model substrates (Henke *et al*., 2002). It is noted however that the GGGX motif in these esterases was located adjacent to the oxyanion region, whereas in EstEFH5, this motif was located towards the C-terminus of the protein. While the significance of the positioning of the GGGX motif is unclear, the ability of these enzymes to accept bulky substrates is consistent with the activity of EstEFH towards ethyl ferulate.

The presence of predicted membrane anchor domain (Leu5-Val30) within the EstEFH5 primary structure was presumed to adversely affect protein expression in *E. coli.* This was based on the observation that the esterase activity could be detected in the intracellular fractions although no corresponding protein band could be observed on SDS-PAGE. It was therefore hypothesized that either the expressed protein was largely unfolded and inactive (presumably present as inclusion bodies) or was correctly folding but anchored to the membrane in an active form.

In order to address these questions, an expression construct lacking the 30 N-terminal amino acids corresponding to the putative membrane domain was designed. The removal of this domain resulted in complete loss of esterase activity, stressing the importance of this domain in maintaining the catalytic function or folding of this enzyme. However, the protein was still found in the membrane fraction even after the removal of the putative membrane anchor domain. Attempts to use detergents to facilitate the recovery of the enzyme from the membrane were unsuccessful.

In vitro refolding experiments were attempted to recover the EstEFH5 from the insoluble fractions. *In vitro* refolding attempts based of previously published reports were unsuccessful (Siritapetawee *et al*., 2004). However, a series of *in vitro* refolding trials showed that glycerol was necessary to stabilise the refolding EstEFH5, and was therefore incorporated in all purification buffers.

Although the recombinant EstEFH5 esterase was shown to release FA from the model substrate (ethyl ferulate), further development of this enzyme as a possible biocatalyst for biotechnological production of ferulic acid would require additional engineering to enhance the solubility of the protein.

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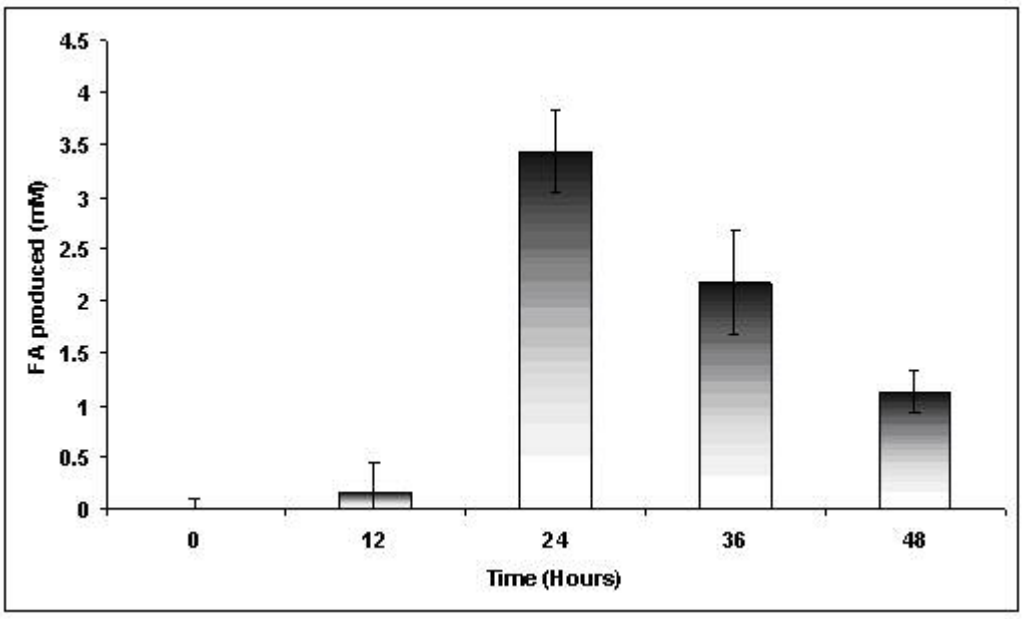


Figure 1: Ferulic acid produced in ethyl ferulate containing minimal medium. Data are mean values of triplicate assays. The initial EF concentration was 0.1% (w/v) (4.5 mM).

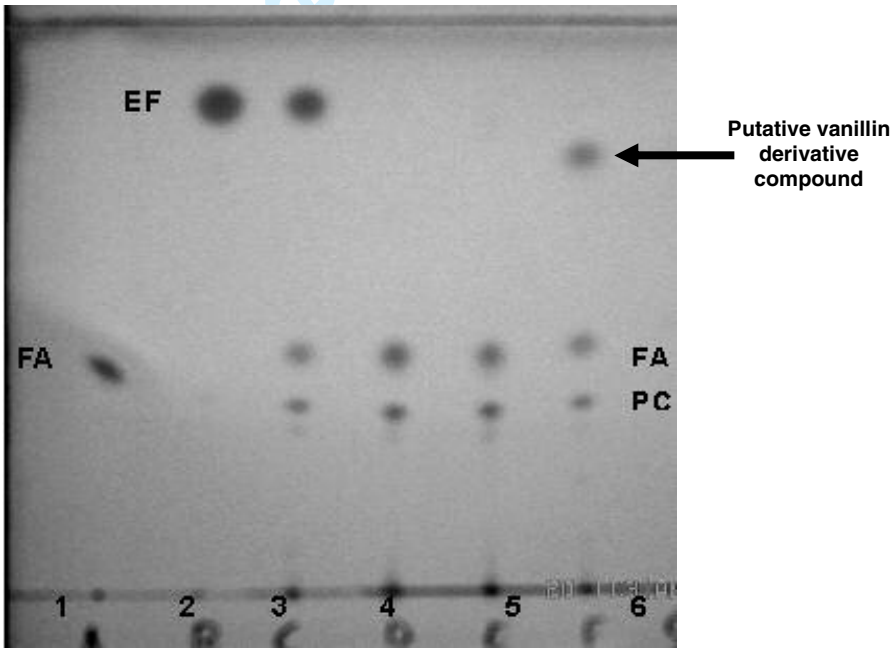


Figure 2: Thin layer chromatoplate showing ethyl ferulate (0.1% v/v) biotransformation products. Lane 1: ferulic acid (1µg μl^{-1}), Lane 2: 0 h, Lane 3:12 h, Lane 4: 24 h, Lane 5: 36 h, and Lane 6: 48 h. The chromatogram was developed in chloroform:methanol:formic acid (85:15:1:v/v/v) as a mobile phase and was visualized under UV light at 366 nm. FA = ferulic acid, EF=ethyl ferulate, PC=ρ-coumaric acid.

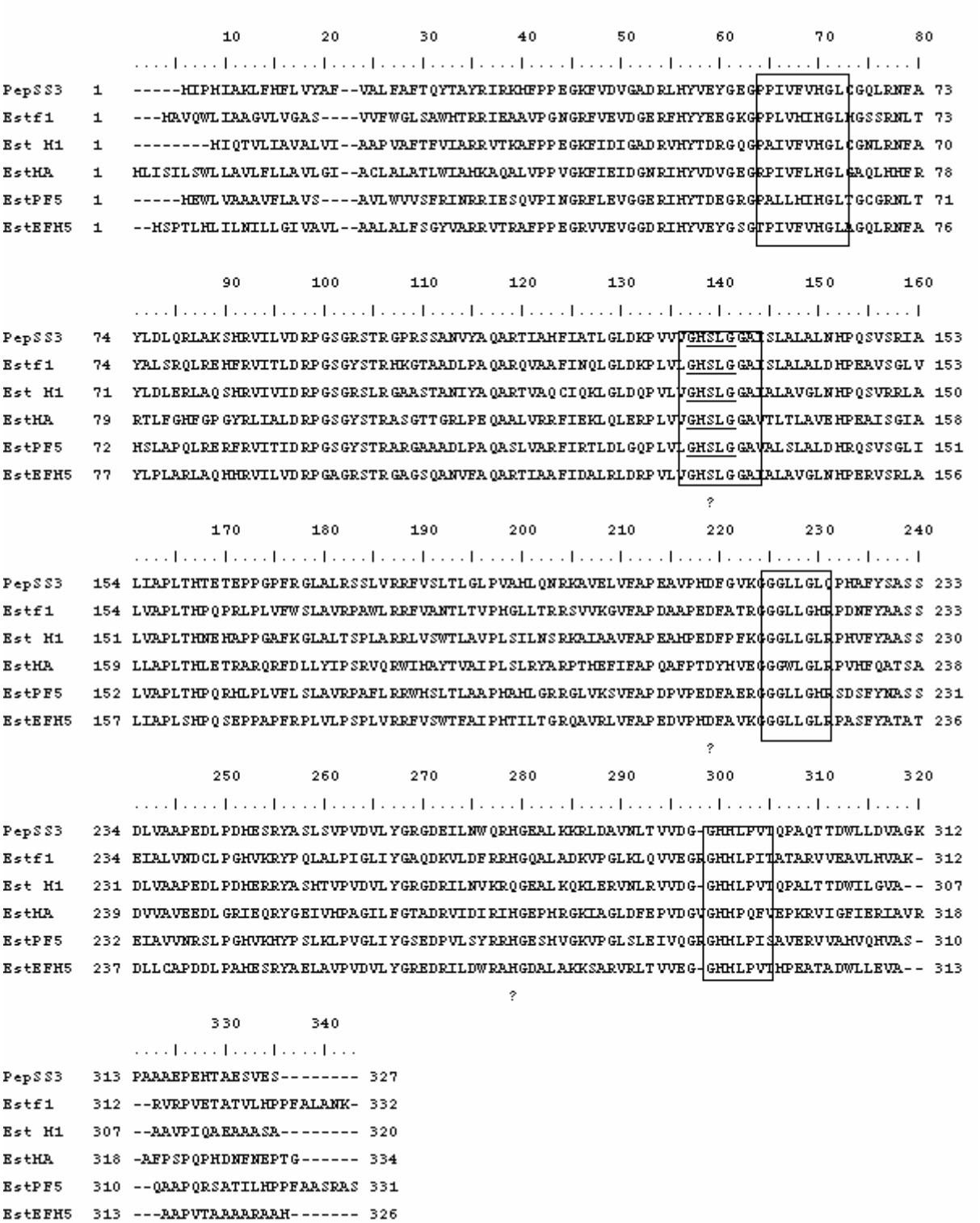
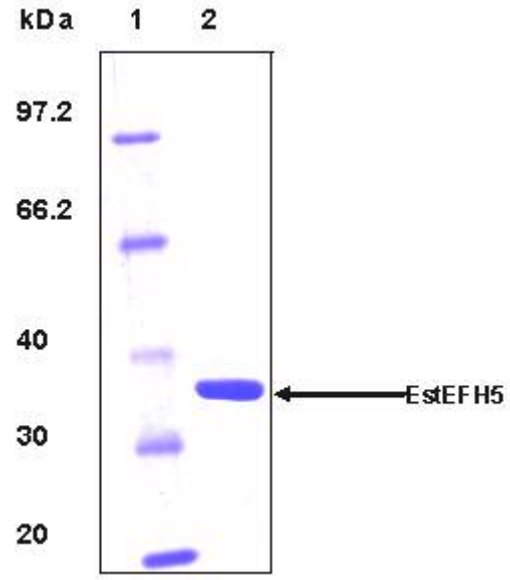


Figure 3: Multiple sequence alignment of *B. multivorans* esterase EstEFH5 (AAV97951) and other related proteins represented by SS3 from *B. pseudomallei* (YP11976), Estf1 *Ps. fluorescens* DMS01016 (AAC36352), Est1 from *R. mannitoliltyica* M1 (AAQ83881), EstMA from *Mesorhizobium loti* (BAB47791) and EstPF5 from *Pseudomonas* sp. (AAY92206). Catalytic triad residues are indicated by solid triangle. Motifs referred to in the text are boxed.

A



B

Figure 4: (A) 12% SDS-PAGE analysis of (1) Molecular weight marker (2) purified EstEFH5 and (B) zymogram assay showing activity stained EstEFH5.

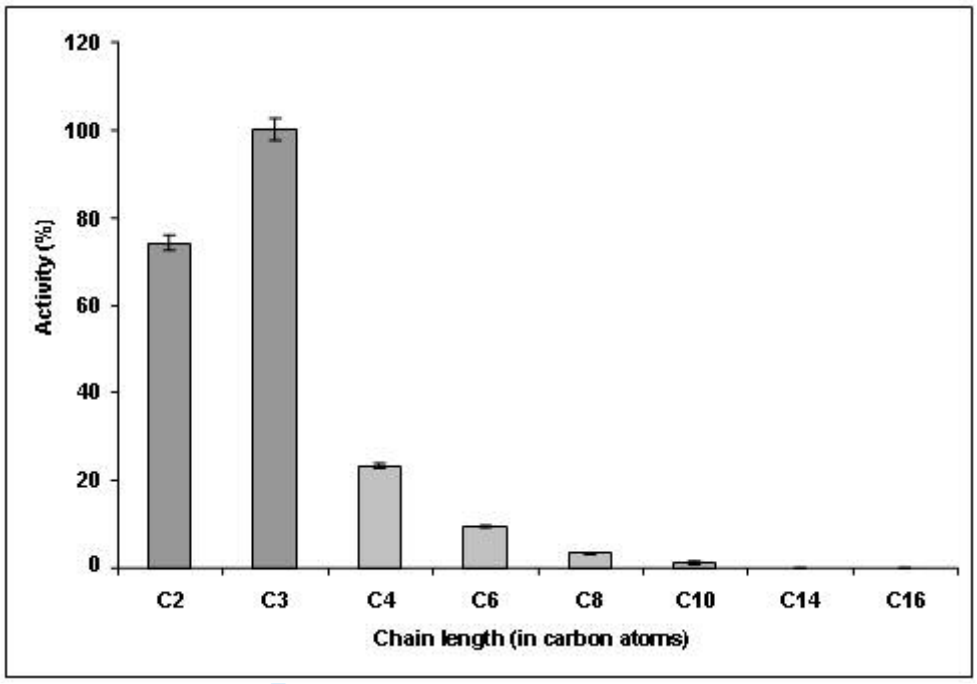


Figure 5: Hydrolysis of ρ-nitrophenyl fatty acid esters by EstEFH5 esterase (activity against ρ-NP-C3 was taken as 100%).

Table 1: Species-specific and nucleotide sequencing PCR primers used in this studyis

Primer name	Target	Sequence (5′→3′)	References
E9F	7-26*	GAGTTTGATCCTGGCTCAG	Farrelly *et al.*, 1995
U150UR	1490-1512*	GGTTACCTTGTTACGACTT	Farrelly *et al.*, 1995
G1	153-133†	GCCATGGATACTCCAAAAGGA	Whitby *et al.*, 1998
G2	939-958†	TCGGAATCCTGCTGAGAGGC	Whitby *et al.*, 1998
SPR3	969-985§	TCGAAAGAGAACCGGGCG	Whitby *et al.*, 2000
SPR4	939-985§	TCGAAAGAGAACCGATA	Whitby *et al.*, 2000
BCR1(F)	2-20**	TGACCGCCGAGAAGAGCAA	Mahenthiralingam *et al.*, 2000
BCR2(R)	1044-1024**	CTCTTCTTCGTCCATCGCCTC	Mahenthiralingam *et al.*, 2000
EstR1	963-981††	TCAGTGCGCCGCGCGC	This study
EstR2	962-993††	GCAGCGCACCGTCATGTG	This study
Est (N)	1-24††	ATCATACATATGAGCCCGACGCTC CATCTGATC	This study
Est (His)	962-993††	ATTCGCAAGCTTGCAGCGCACCGT CATGTG	This study

* = Position in relation to *E. coli* 16S rRNA gene (GenBank Accession number J10859)

† = Position in relation to *B. cepacia* 16S rRNA gene (GenBank Accession number X16368)

‡= Position in relation to *B. multivorans* 16S rRNA gene (GenBank Accession number Y18703)

§= position in relation to *B. stabilis* 16S rRNA gene (GenBank Accession number DQ118268)

**= Position in relation to *B. multivorans rec*A gene (GenBank Accession number U70431)

††= Position in relation to *B. multivorans EstEFH5* gene (GenBank Accession number AAV97951), restriction sites are underlined

Table 2: BLAST (n) search data for the UWC10 16S rRNA gene sequence.

Strains	**Accession number**	**Score (Bits)**	**Identity (%)**	**E_{value}**
B. multivorans	AF097531	2927	1489/1494 (99%)	0.00
B. multivorans	BMU18703	2919	1476/1478 (99%)	0.00
B. vietnamiensis	AF097534	2896	1485/1494 (99%)	0.00
B. multivorans	AB092606	2882	1466/1771 (99%)	0.00
B. cepacia	AB110089	2872	1479/1490 (99%)	0.00
B. cepacia	AY741359	2870	1478/1489 (99%)	0.00
B. cepacia	AY741345	2866	1464/1471 (99%)	0.00
Burkholderia sp.	AY769904	2866	1464/1471 (99%)	0.00
Burkholderia sp	AY973819	2866	1464/1471 (99%)	0.00
Burkholderia sp.	AF219125	2866	1464/1471 (99%)	0.00

Table 3: Comparison of the amino acid sequence similarities between EstEFH5 and other related proteins

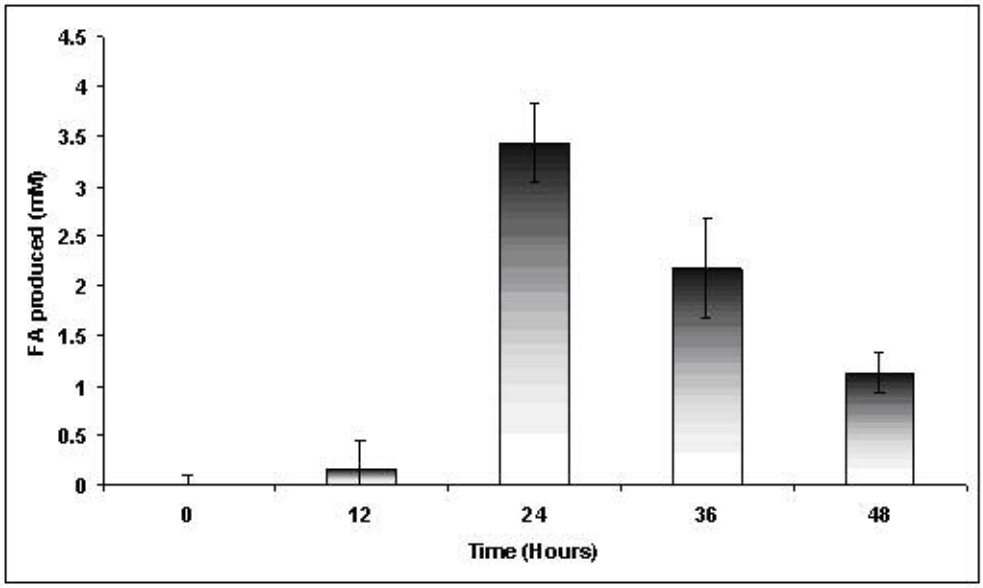
Esterase producing organisms	Accession number	Identity (%)
Lactone hydrolyzing esterases group		
Burkholderia pseudomallei (SS3)	Q63IU6	64.0
Ralstonia mannitolilytica (Est1)	Q6EIRO	61.3
Pseudomonas sp. (EstPF5)	Q9AE76	44.3
Mesorhizobium loti (EstMA)	Q98HN0	36.6
Pseudomonas fluorescens (EstF1)	O87637	46.0

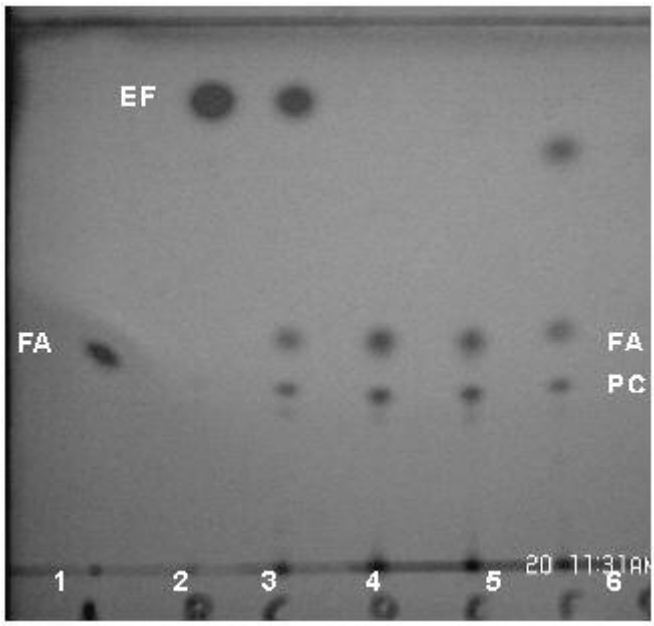
Table 4: Kinetic parameters for hydrolysis of ρ-nitrophenyl esters by EstEFH5 esterase

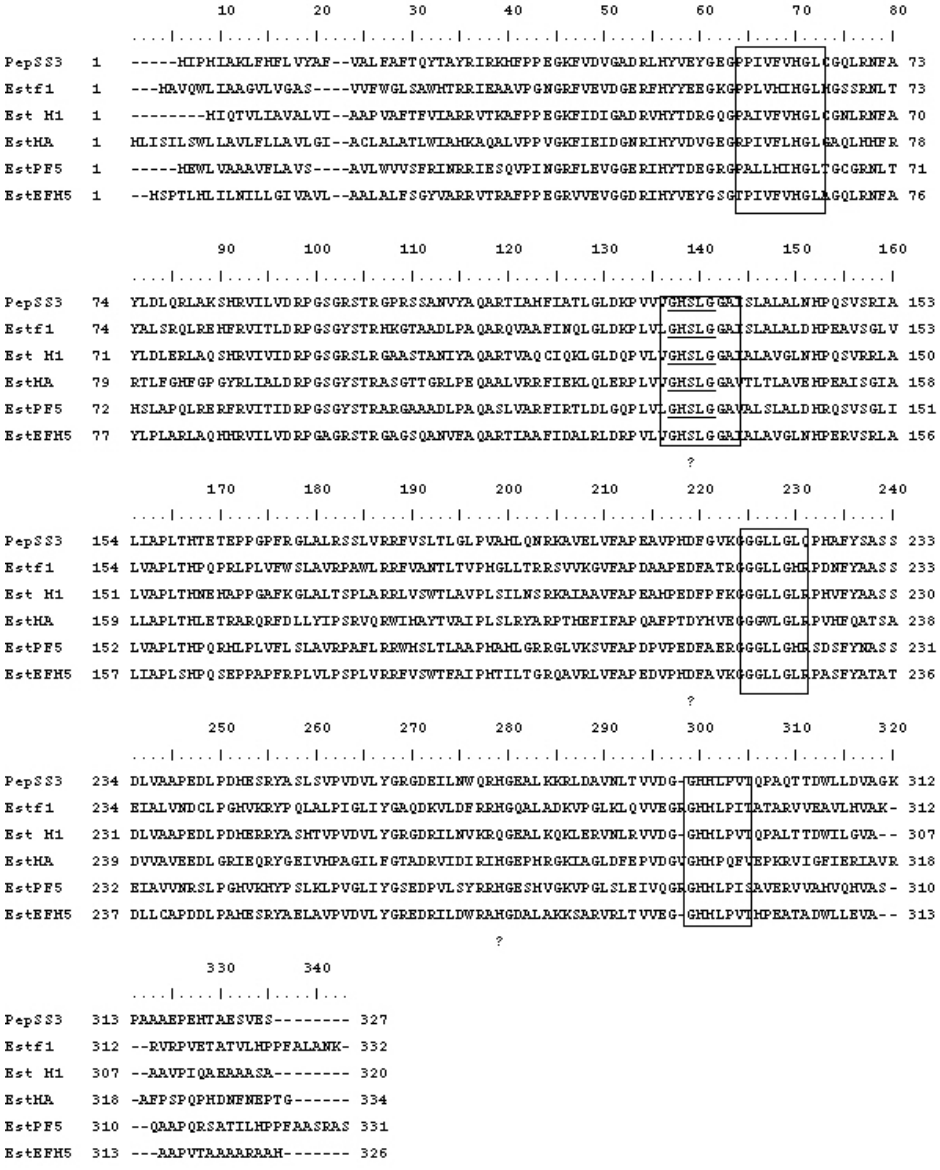
	ρ-NP-C2	ρ-NP-C3
K_M (mM)	9.2 ± 1.2	5.3± .8
V_{max} ($U.mg^{-1}$)	198.9 ± 13.2	125 ± 9.0
k_{cat}(S^{-1})	94.7 ± 6.3	59.5 ± 4.3
k_{cat}/K_M (s^{-1} mM)	10.3	11.2

Enzyme concentration in the assay= 3 µg ml^{-1}

K_{cat} was calculated assuming a molecular weight of 35 kDa as estimated SDS-PAGE analysis, and a single site per monomeric protein.



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Estf1 1 ---MAVQWLIAAGVLVGAS----VVFWGLSAWHTRRIEAAVPGNGRFVEVDGERFHYYEEGKGPPLVHIHGLMGSSRNLT 73
Est H1 1 --------MIQTVLIAVALVI--AAPVAFTFVIARRVTKAFPPEGKFIDIGADRVHYTDRGQGPAIVFVHGLCGNLRNFA 70
EstMA 1 MLISILSWLLAVLFLLAVLGI--ACLALATLWIAMKAQALVPPVGKFIEIDGNRIHYVDVGEGRPIVFLHGLGAQLHHFR 78
EstPF5 1 -----MEWLVAAAVFLAVS----AVLWVVSFRINRRIESQVPINGRFLEVGGERIHYTDEGRGPALLHIHGLTGCGRNLT 71
EstEFH5 1 --MSPTLHLILNILLGIVAVL--AALALFSGYVARRVTRAFPPEGRVVEVGGDRIHYVEYGSGTPIVFVHGLAGQLRNFA 76
PepSS3 74 YLDLQRLAKSHRVILVDRPGSGRSTRGPRSSANVYAQARTIAHFIATLGLDKPVVVGHSLGGAISLALALWHPQSVSRIA 153
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EstMA 79 RTLFGHFGPGYRLIALDRPGSGYSTRASGTTGRLPEQAALVRRFIEKLQLERPLVVGHSLGGAVTLTLAVEHPEAISGIA 158
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EstMA 159 LLAPLTHLETRARQRFDLLYIPSRVQEWIHAYTVAIPLSLRYARPTHEFIFAPQAFPTDYHVEGGGWLGLEPVHFQATSA 238
EstPF5 152 LVAPLTHPQRHLPLVFLSLAVRPAFLRRWHSLTLAAPHAHLGRRGLVKSVFAPDPVPEDFAERGGGLLGHESDSFYMASS 231
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EstEFH5 237 DLLCAPDDLPAHESRYAELAVPVDVLYGREDRILDWRAHGDALAKKSARVRLTVVEGGHHLPVTHPEATADWLLEVA-- 313
PepSS3 313 PAAAEPEHTAESVES-------- 327
Estf1 312 --RVRPVETATVLHPPFALANK- 332
Est H1 307 --AAVPIQAERAASA-------- 320
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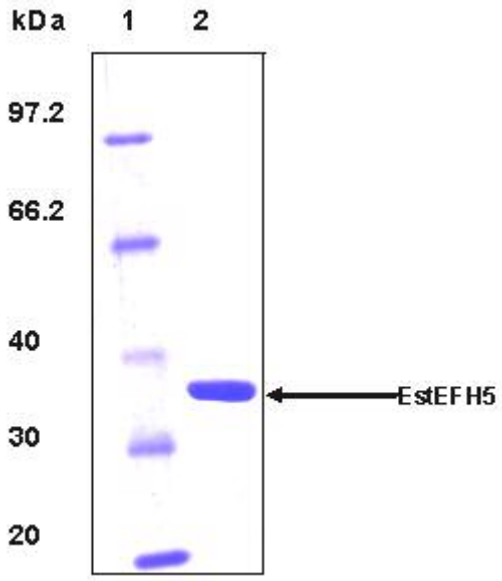
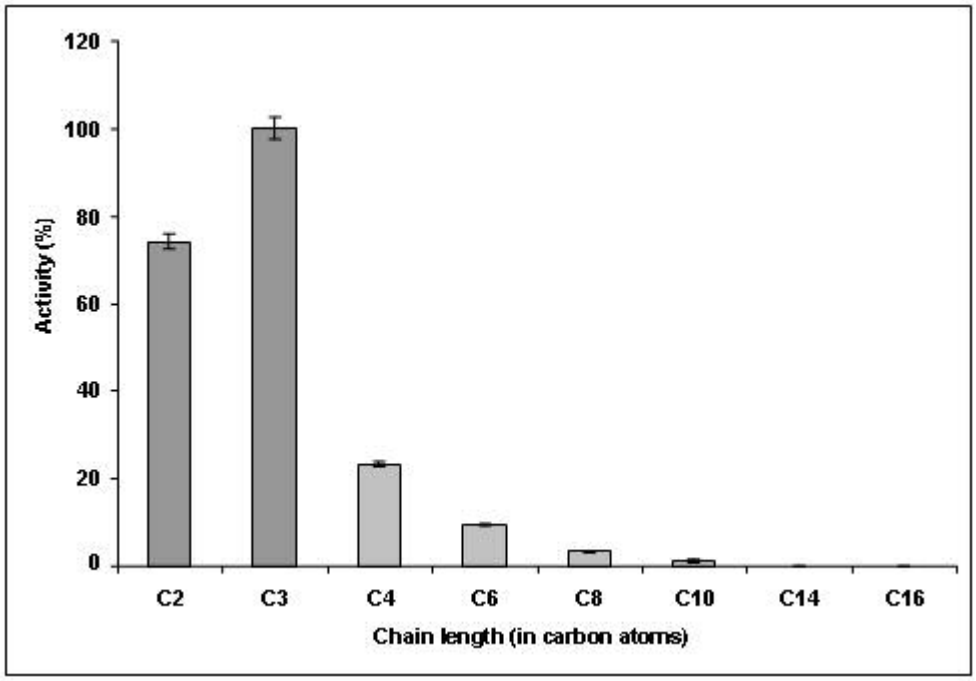
EstEFH5 313 ---AARPVTAAAARAAH------- 326




120
100
80
60
40
20
0
Activity (%)
C2
C3
C4
C6
C8
C10
C14
C16
Chain length (in carbon atoms)