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Full Length Research Paper

Cloning, expression, purification and characterization of the carboxylesterase Yeig from *Escherichia coli* k12

Jie Lu[1]*, Xiao-Lin Jiang[1], Zhen Li[1], Hong-Fen Luan[2], Hong Chen[2], Xiu-Juan Wang[2] and Chi-Bun Ching[2]

[1]School of Chemical and Material Engineering, Jiangnan University, 214122 Wuxi, China.
[2]Division of Chemical and Biomolecular Engineering, School of Chemical and Biomedical Engineering, Nanyang Technological University, 637459, Singapore.

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In this work, enzyme YeiG from *Escherichia coli* K12-MG1655 which has no definitely known biochemical function has been cloned, expressed, purified and characterized. Alignment studies show that YeiG has an alpha/beta-hydrolase fold with catalytic triad formed by Ser145, Asp223 and His256 at active sites and Ser145 is located in the conserved motif Gly–Xaa–Ser–Xaa–Gly. Enzyme assays demonstrate that YeiG has a significant carboxylesterase activity, low enzymatic activities for lipase and epoxide hydrolase and no detectable enzymatic activities for other enzymes selected in our study. Towards the hydrolysis of *p*-nitrophenyl esters of fatty acids, YeiG possesses broad substrate specificity with a preference for short acyl chain esters, and has maximum activity towards C4 ester. The integrating bioinformatics and enzyme assays have suggested that YeiG from *E. coli* K12-MG1655 should be a carboxylesterase.

Key words: YeiG, characterization, carboxylesterase, *Escherichia coli.*

INTRODUCTION

Enzymes widely act as powerful catalysts in the manufacture of fine chemicals, pharmaceuticals, agrochemical intermediates and bioactive materials, particularly in the enantioselective organic synthesis of chiral compounds, because of their excellent properties such as high activity, selectivity and specificity (Bornscheuer, 2002; Koeller and Wong, 2001; Kirk et al., 2002; Straathof et al., 2002; Schmid et al., 2001; Schoemaker et al., 2003; Jaeger, 2004; Panke et al., 2004. As more and more enzymatic reactions are being utilized, a continuously growing demand for new biocatalysts adapted to special needs is generated (Cohen et al., 2001). The screen and engineering for enzymes from biological entities to industrial reactors is thus a very exciting research subject (Wong and Whitesides, 1994).

Native enzymes can be exploited by identifying new genes and isolating from specific microorganisms (Eggert et al., 2004; Cheetham, 1987; Ogawa and Shimizu, 1999; Miller, 2000). Particularly, the search for homologous and paralogous genes in the sequence information derived from genome projects, offers an attractive approach for screening biocatalysts (Lorenz et al., 2002). However, wild-type enzymes do not always show satisfying performance in terms of activity, stability and most importantly enantioselectivity. These properties can be optimized by the alteration of substrates (substrate engineering), reaction system (medium engineering), immobilization or evolution by protein engineering techniques (Petrounia and Arnold, 2000; Cherry and Fidantsef, 2003; Robertson and Steer, 2004; Funke et al., 2005; Olsen et al., 2000; Mateo et al., 2007; Chica et al., 2005; Hult and Berglund, 2003).

The most frequently used esterases and lipases belong to the general class of carboxylic ester hydrolases (EC 3.1.1) that catalyze both the hydrolysis and the synthesis of ester bonds. Both enzymes differ in substrate specificity and type of enzyme kinetics (Schmid and Verger, 1998). Carboxylesterases (EC 3.1.1.1) preferentially hydrolyze water-soluble, ester-containing molecules with short acyl chain (≤10 carbon atoms), whereas lipases (also known as triacylglycerol lipases, EC 3.1.1.3) prefer water-insoluble long-chain acylglycerides (≥10 carbon atoms) and display activation at

*Corresponding author. E-mail: lujie6742@sina.com. Tel: 86-510-85919779. Fax: 86-510-85917763.

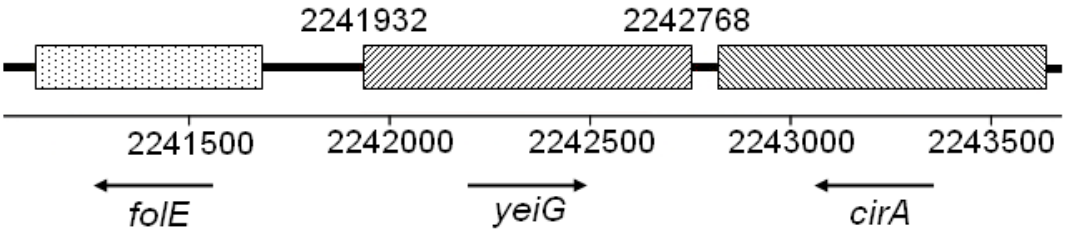

Figure 1. Location of the yeiG gene on the map of the *E. coli* chromosome.

lipid-water interfaces (Kim et al., 2007; Reyes-Duarte et al., 2005). Although lipases and esterases show no general sequence similarity, they share the characteristic α/β hydrolase fold in the three-dimensional structures (Nardini and Dijkstra, 1999; Ollis et al., 1992). That is, most of these enzymes contain a catalytic triad, generally consisting of a serine, a histidine and an asparate (or glutamate), where the serine residue appears in the conserved pentapeptide (Gly-Xaa-Ser-Xaa-Gly) at the active site (Blow, 1990; Fojan et al., 2000; Arpigny and Jaeger, 1999; Sanishvili et al., 2003).

It has previously been shown that the gene yeiG from *Escherichia coli* K12 MG 1655 encodes a protein with 278 amino acid residues. The location of this gene on the map of the *E. coli* chromosome is shown in Figure 1. Kuznetsova et al., 2005 predicted that the enzyme YeiG belonged to putative esterase towards the hydrolysis of palmitoyl-CoA and *p*NP-butyrate. Gonzalez et al., 2006 demonstrated that YeiG had high hydrolytic activity toward S-formylglutathione. They labeled YeiG S-formylglutathione hydrolase with a Ser-His-Asp catalytic triad composed of Ser145, Asp233 and His256. In this work, the cloning, expression, sequencing, purification and biochemical characterization of YeiG from *E. coli* have been conducted. Different from the work of Gonzalez et al., 2006, amino acid sequence alignment suggests that YeiG shall preserve a catalytic triad (Ser145, His256 and Asp223) with a configuration similar to that of the catalytic triad of hydrolases. Meanwhile, a panel of enzyme assays demonstrates that YeiG has a carboxylesterase activity with a preference for short acyl chain substrates.

MATERIALS AND METHODS

General

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA) at the highest purity available. DNA sequencing was conducted using an ABI 373A sequencer (Applied Biosystems, Foster City, CA).

Bacterial strains and plasmid

E. coli strain K12 MG1655 was obtained form American Type Culture Collection (ATCC, Manassas, VA). Cells were cultured according to supplier's instructions. *E. coli* strain BL21 (DE3) was purchased from Novagen (Madison, WI) and employed as the host strain. The expression vector was pET-21b (Novagen, Madison, WI).

Cloning of yeiG gene

The yeiG gene was isolated from *E. coli* K12 MG1655 genomic DNA using polymerase chain reaction (PCR). The forward primer for PCR was 5'-AAAGGATCCTATGGAAATGCTCGAAGAG-3' and the reverse primer was 5'-AAACTCGAGCTTCAGTAAATACTGCGC-3'. PCR was performed over 30 cycles using HotStar Hifidelity DNA Polymerase (Roche Diagnostics, Mannheim, Germany). Each cycle consisted of the following three steps: 94℃ for 45 s, 60℃ for 45 s and 72℃ for 2 min under standard reaction condition as recommended by the supplier.

The PCR product was purified with 'Qiaquick PCR extraction kit' (Qiagen, Valencia, CA). The resulting fragment was digested with BamHI and XhoI respectively and ligated with predigested pET-21b expression vector, which was designed to produce a C-terminal 6×His tagged fusion protein. Quick T4 DNA Ligation kit was from New England BioLabs (Ipswich, MA). The final construct was confirmed by DNA sequencing.

Expression

The constructed plasmid was transformed into *E. coli* BL21 (DE3) cells. The resulting recombinant cells were grown in 100 ml LB media (yeast extract 5 g/l, tryptone 10 g/l and NaCl 5 g/l) containing ampicillin (100 ug/ml) at 37℃. When the optical density at 600 nm reached 0.8, 1 mM IPTG was added to the cell broth. After further growth for 3 h, the cells were harvested by centrifugation at 4℃ (4500 rpm, 20 min), washed twice with 10 ml phosphate-buffered saline (PBS, pH 7.4), resuspended in 10 ml 50 mM Tris-HCl buffer (pH 8.0), and destroyed by sonication. The cell debris was removed by centrifugation (10000 rpm) for 45 min at 4℃. The protein in the supernatant was analyzed by gel electrophoresis and mass spectrometry, and further purified.

Enzyme purification

The supernatant was filtered through 0.45 μm-pore filters (Millipore, Bedford, MA), then loaded onto a Ni^{2+}-chelated (1mL) column (Qiagen, Valencia, CA), equilibrated with buffer containing 500 mM NaCl and 15 mM imidazole. Elution was performed with buffer containing 500 mM NaCl and 50 mM imidazole. The active fractions were gel-filtrated with a Superdex 200 column (1 × 30 cm, GE Healthcare, Waukesha, WI), equilibrated and eluted with buffer containing 150 mM NaCl. Protein concentration was determined by the Bradford and Lowry method (Funke et al., 2005) (Pierce, Rockford, IL).

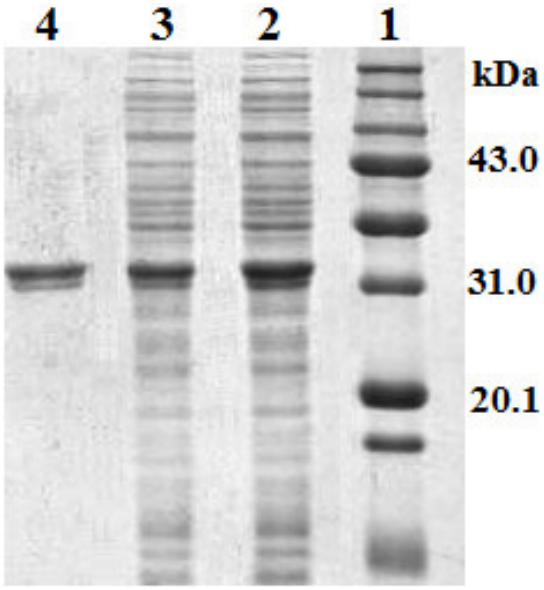

Figure 2. SDS-PAGE analysis of protein fractions, (1) marker; (2) total proteins before purification; (3) after ion exchange; and (4) after gel-filtration.

Gel electrophoresis

Proteins from the crude extract and from various purification steps were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS–PAGE was performed on a 12% separating and a 4% stacking gel as described by Laemmli (Laemmi, 1970). Molecular mass markers were purchased from Bio-Rad (Bio-Rad, Hercules, CA). Proteins were stained by the silver staining procedure of Pierce (Pierce, Rockford, IL).

Mass spectrometry

Protein samples from the crude extract and from various purification steps were also analyzed by iTRAQ-coupled 2D LC-MS/MS system (ABI, Warrington, UK) to detect and quantitate proteins obtained. Proteins were digested by trypsin, labeled with the iTRAQ reagents with one member of the multiplex tagging on individual sample and then these differentially labeled digests were mixed. 2 µL of the combined peptide mixture was initially separated though the strong cation exchange column based on charges and further separated though the reversed phase column by hydrophobicity. In MS/MS, precursor ions are recorded in full-scan mode (all *m/z* values), followed by selective ion isolation and fragmentation for sequence identification. Each MS/MS spectrum was searched against the protein database and protein identifications were accepted based on the ProtScore more than 2.0, which gives the confidence value of 99%.

Relative abundance quantitation and peptide and protein identification were performed using ProteinPilot Software 2.0 (Applied Biosystems, Software Revision 50861). Each MS/MS spectrum was searched against the uniprot _sprot_20070123 database. Parameters such as tryptic cleavage specificity, precursor ion mass accuracy and fragment ion mass accuracy are built-in functions of ProteinPilot software. Relative quantification of proteins using iTRAQ was performed on the MS/MS scans. The ratios of the areas under the peaks at 115 *m*/*z* (representative of treated samples) over the area under 114 *m*/*z* (representative of control sample) denoted the relative amount of a peptide in each treated sample.

Enzyme assays

Rapid screening for enzyme activities were performed using the following procedures: (a) esterase activity was measured using the hydrolysis of the esters of *p*-nitrophenol (*p*-NP). The reaction conditions are: pH 8.0, 37°C, the concentration of substrate solution is 0.1 mM, 10 µL purified protein solution (1.5 g/L) was added into each 2 mL substrate solution. The esterase activity towards *p*-NP esters of different carbon chain length was estimated spectrophotometrically by reading the absorbance of the liberated *p*-NP at 405 nm with the molar extinction coefficient of 18700 $M^{-1}cm^{-1}$. Activity was corresponded to the amount of the liberated *p*-NP per minute per milligram of protein, (b) lipase activity was measured spectrophotometrically at 37°C using *p*-nitrophenyl (*p*-NP) palmitate, (c) aminopeptidase activity was measured using L-leucine p-nitroanilide, (d) trypsin activity was measured using N-benzoyl-L-arginine ethyl ester, (e) phosphatase activity was determined spectrophotometrically using 5 mM *p*-nitrophenyl phosphate in 50 mM HEPES-K (pH 7.5) buffer at 37°C, (f) nitrilase activity was measured using *rac*-mandelonitrile, (g) epoxide hydrolase activity was measured using *rac*-glycidyl butyrate, (h) hydantoinase activity was measured using 5-monosubstituted hydantoin, (i) N-carbamoylase activity was measured using N-carbamoyl-amino acid, (j) peroxidase activity was measured using catalytic oxidization of guaiacol in the presence of hydrogen peroxide and (k) lyase activity was measured using the catalytic dissociation of *rac*-mandelonitrile. All the assays were carried out in triplicate and the mean values estimated.

The enantioselectivity of YeiG was investigated by the esterification of racemic glycidol with butyric acid and the transesterification of racemic 1-phenylethanol with vinyl acetate. In most of the esterification experiments, two hundred microlitres of methylene chloride or chloroform, 360 µmol of *rac*-glycidol and 720 µmol of butyric acid were added to screw-capped vials of 1 ml total capacity. The reaction was started by adding 5 mg of YeiG, and run at 37°C in a water bath with shaking. At regular time intervals, 25 µl aliquots were withdrawn and repeatedly extracted with hexane and 5% sodium bicarbonate solution. The hexane phase was analyzed by HPLC using the chiral column, Chiralcel® OD column (250 × 4.60 mm; Daicel Chemical Industries, Tokyo, Japan) at 225 nm. Hexane/propan-2-ol (97:3) was used as mobile phase at a flow rate of 0.5 ml/min. For the transesterification of racemic 1-phenylethanol, the enzyme reaction was performed in a 1.5 ml tube. Vinyl acetate was used as reactant and solvent. 1-phenylethanol of 80 µL and protein of 3 mg were added into 1 ml of vinyl acetate. The reaction mixture was incubated at 30°C. Small aliquots of reaction mixture were sampled at 12 and 24 h of the reaction, and analyzed on the chiral column, Chiralcel® OD-H column (250 × 4.60 mm; Daicel Chemical Industries, Tokyo, Japan) at 254 and 205 nm. A mixture of 97% (vol/vol) hexane and 3% propan-2-ol was used as mobile phase at a flow rate of 0.5 ml/min. The enantioselectivity was estimated using the method described by Chen et al. (1982).

RESULTS AND DISCUSSION

Purity analysis

As shown in Figure 2, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) shows an estimated 32 kDa-size protein band corresponding to YeiG. Mass spectrometry coupled with liquid chromato-

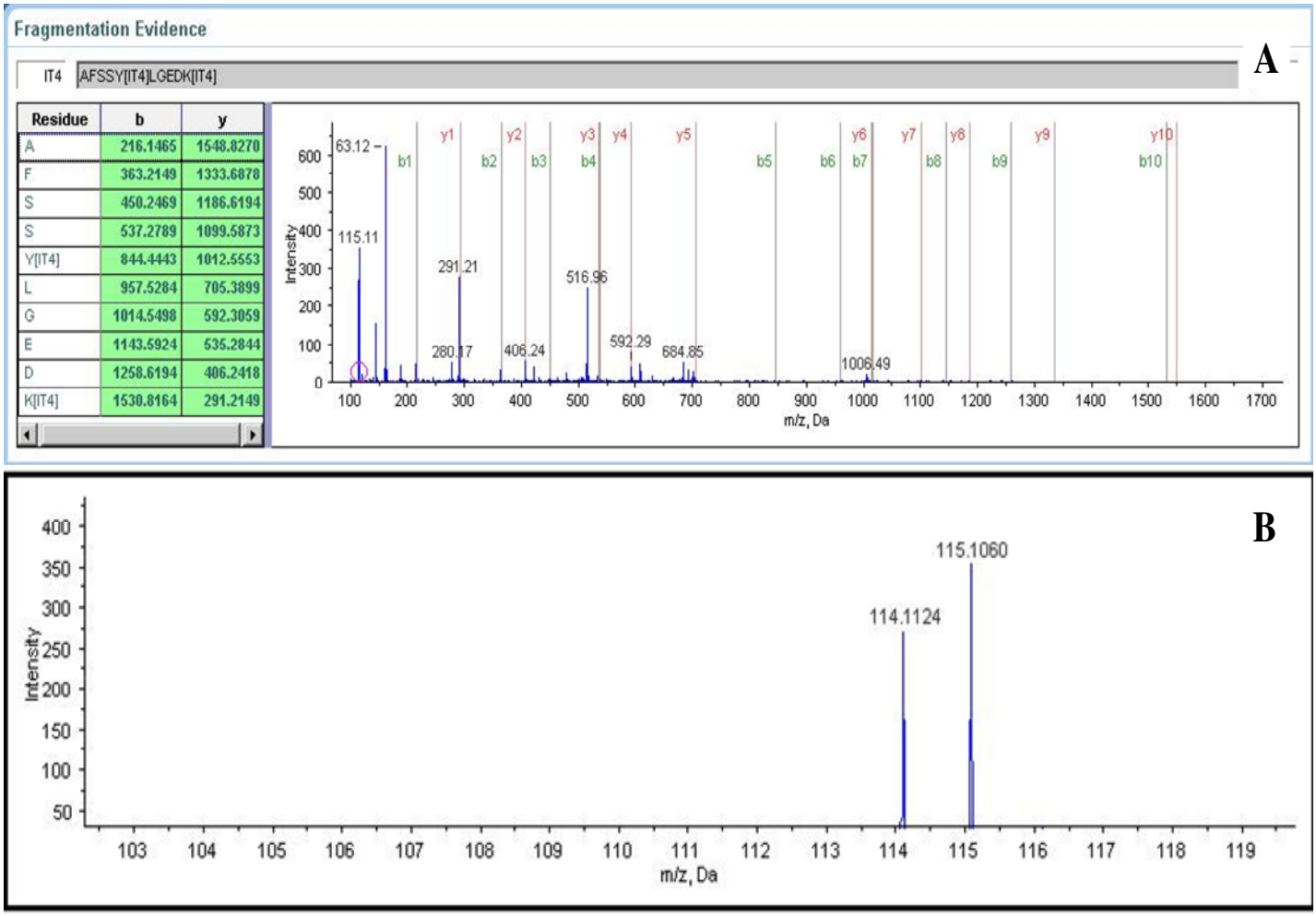

Figure 3. (A) A mass spectrometric representation of the peptide AFSSYLGEDK of YeiG; (B) Displaying the area circled in (A), where report icon 114 denotes the unpurified sample and 115 denotes the purified sample. Both samples contain 100 µg proteins.

Table 1. Mass spectrometry analysis of protein fractions before and after purification.

Names and species	Total[a]	115:114[b]	PVal 115:114[c]	EF 115:114[d]
Esterase YeiG – *E. coli*	68.00	1.30609	7.63E-23	1.053
Aconitate hydratase 2 - *E. coli*	16.75	0.16375	2.44E-06	1.665
Isocitrate dehydrogenase - *E. coli*	12.22	0.14962	2.98E-05	2.109
Beta-galactosidase - *E. coli*	6.88	0.19253	8.39E-06	1.625
Citrate synthase - *E. coli* O6	12.06	0.29926	1.43E-05	1.556
Tryptophanase - *E. coli* O157	32.56	0.17383	4.13E-17	1.298
Cysteine synthase A - *E. coli* O157	4.70	0.11213	8.67E-03	2.417
2-oxoglutarate dehydrogenase - *E. coli* O157	3.13	0.25842	3.33E-03	1.823

[a]: Total indicates the relative significance of the protein in the sample;
[b]: 115:114 is the ratio of protein content in the purified sample relative to that in the unpurified sample;
[c]: PVal 115:114 shows the confidence with the value of less than 0.01 indicating the confidence of higher than 99%;
[d]: Error factor.

graphy has been extensively used with success for the identification and quantification of proteins. From Figure 3 and Table 1, the contents of YeiG in the samples before and after purification are 72.5 and 94.7% respectively.

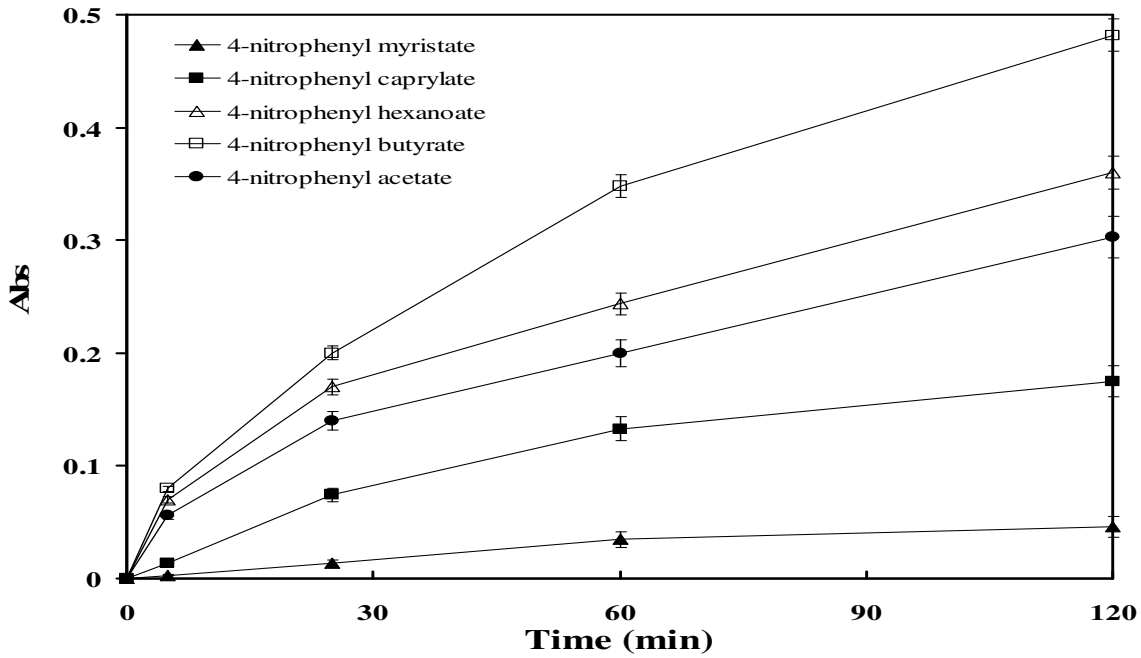

Figure 4. UV absorptions of liberated *p*-NP at 405 nm as a function of reaction time.

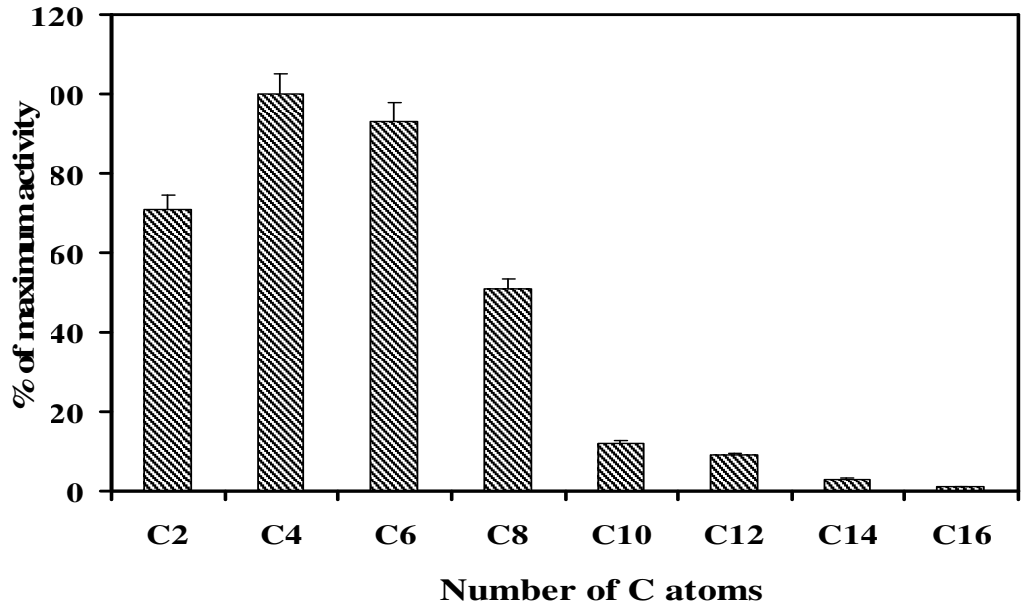

Figure 5. Hydrolysis of *p*-nitrophenyl esters with different aliphatic side chain, ranging from C2 to C16, by YeiG. The assay conditions were described in materials and methods.

Enzyme assays

The progress of the hydrolysis reactions for different substrates is shown in Figure 4. Among *p*-NP esters studied, YeiG displays the highest activity towards *p*-NP butyrate (C4 acyl group), which is fixed as 100%. The typical profile of chain length specificity of this enzyme towards *p*-NP esters is shown in Figure 5. The activity for the esterase towards *p*-NP acetate (C2 acyl group), caproate (C6 acyl group) and caprylate (C8 acyl group) is 71, 93 and 51% of that towards *p*-NP butyrate, respectively. The activity towards *p*-NP myristate (C14 acyl group) and palmitate (C16 acyl group) is significantly decreased. Besides, YeiG shows almost no enantioselec-

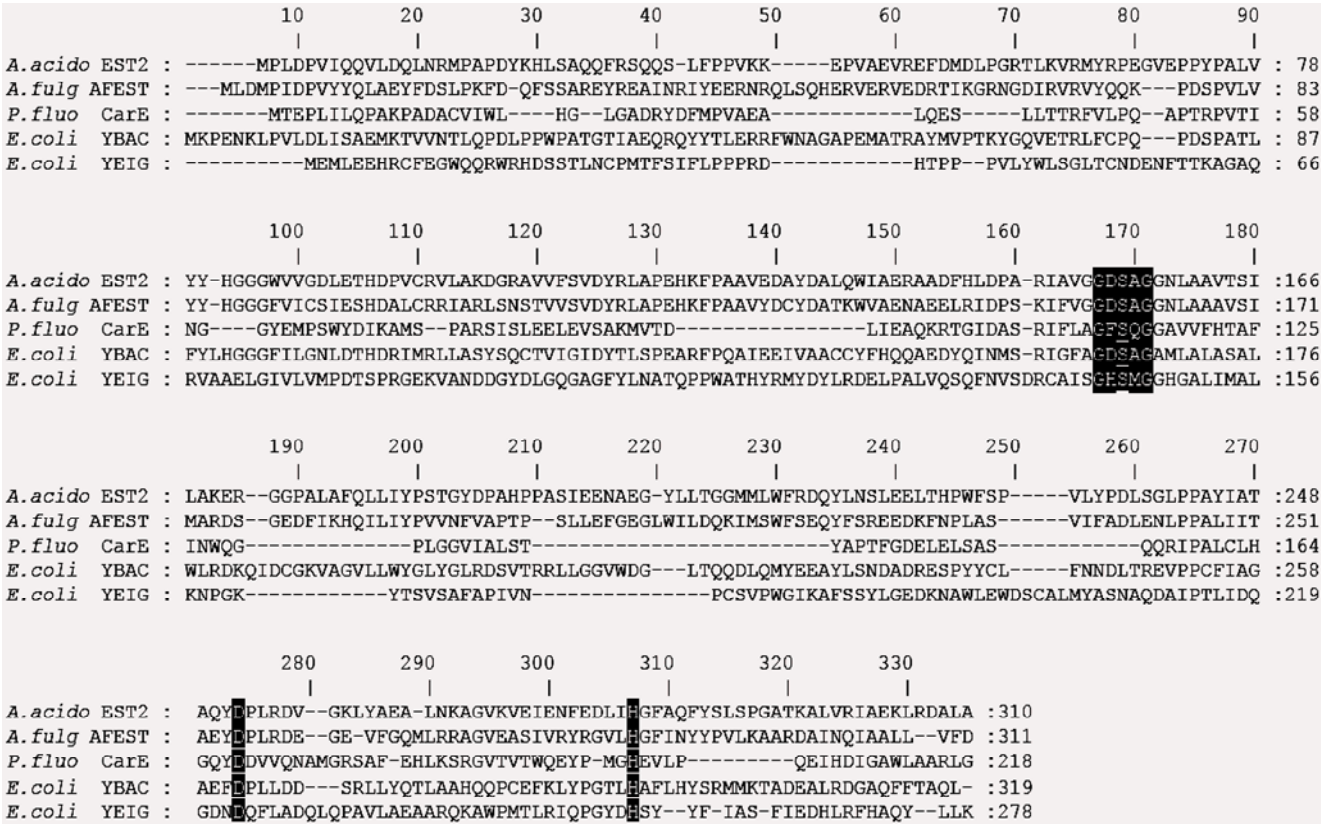

Figure 6. Amino acid sequence alignment of YeiG and other carboxylesterases using CLUSTALW 1.81. The sequences shown are: carboxylesterase EST2 from *A. acidocaldarius*; carboxylesterase AFEST from *A. fulgidus*; carboxylesterase from *P. fluorescens*; carboxylesterase YbaC from *E. coli*; YeiG from *E. coli* (this work). The percent similarity of the YeiG with the other amino acid sequences was 43.3, 46.0, 49.3 and 39.5, respectively. The Gly-Xaa-Ser-Xaa-Gly motif and catalytic triad are in black and underlined.

tivity in the selected reactions (the *ee* values calculated from HPLC analysis below 2%).

Amino acid sequence alignment

Proteins with similar amino acid sequences usually have the same folding conformation, even if their similarities are minimal (Chothia and Lesk, 1986). They are generally related with one another in function as well, and therefore grouped into a family. Occasionally, distant organisms produce proteins that are the members of the same family. In this case, comparison of the properties of these proteins provides valuable information on their evolutionary relationships. In addition, detailed analyses of the structure and function of a given protein may help us to understand a fundamental mechanism for protein function in the family (Bajaj and Blundell, 1984). In some cases, by focusing on the catalytic moieties, functional similarities can be detected even though there is no similarity in sequence, fold, or secondary structure.

The carboxylesterase domain can catalyze hydrolysis of carboxylic esters and includes a catalytic triad: a serine, a glutamate (or aspartate) and a histidine. The sequence around the active site serine is well conserved and can be used as signature pattern (Krejci et al., 1991; Cygler et al., 1993). Such four carboxylesterases as EST2 from *A. acidocaldarius* (Simone et al., 2000), AFEST from *A. fulgidus* (Manco et al., 2000), carboxylesterase from *P. fluorescens* (Kim et al., 1997) and carboxylesterase YbaC from *E. coli* (Kanaya et al., 1998) have been chosen in the amino acid sequence alignment with YeiG. EST2, AFEST and YbaC belong to the hormone-sensitive lipase (HSL) family, which displays a striking amino acid sequence similarity to the mammalian HSL (Hemilä et al., 1994). Meanwhile, *P. fluorescens* carboxylesterase belongs to family VI, which hydrolyses small substrates with a broad specificity and displays no activity towards long-chain triglycerides (Hong et al., 1991). All four carboxylesterases have a classical Ser-Asp-His catalytic triad and the consensus sequence around the active serine (Gly-Xaa-Ser-Xaa-Gly).

The amino acid sequence alignment of YeiG and above four carboxylesterases is shown in Figure 6. The amino acid sequence of YeiG shows the sequence similarity to carboxylesterase EST2 from *A. acidocaldarius* (43.3%), carboxylesterase AFEST from *A. fulgidus* (46.0%), carboxylesterase from *P. fluorescens* (49.3%) and carbo-

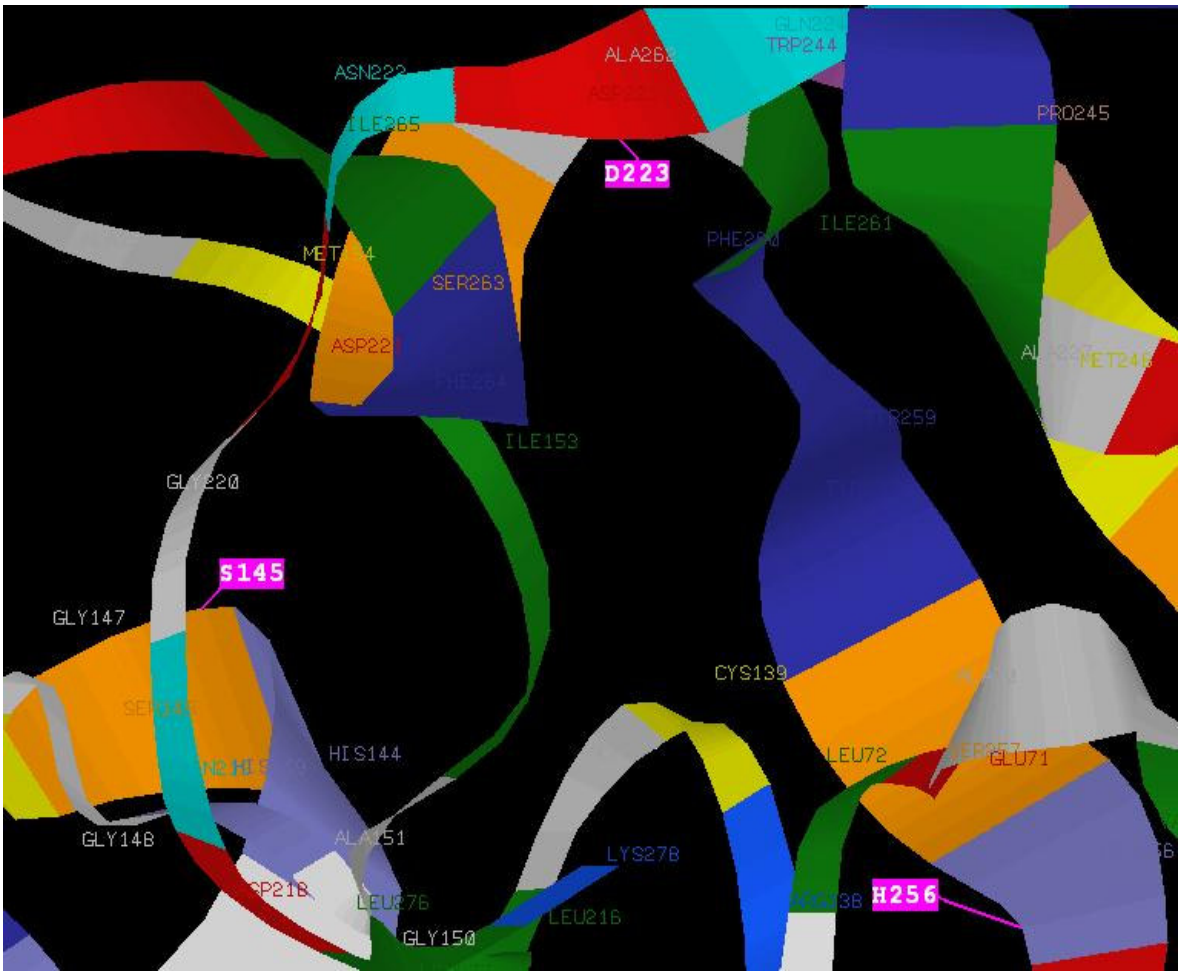

Figure 7. Predicted three-dimensional model of YeiG produced by SWISS-MODEL using the carboxylesterase EstB from *B. gladioli* as the template.

xylesterase YbaC from *E. coli* (39.5%). In particular, sequence alignment clearly reveals that, the characteristic pentapeptide for most lipases and esterases, Gly-Xaa-Ser-Xaa-Gly, is present in YeiG, and residues Ser145, His256 and Asp223 form the catalytic triad. This is similar to the esterase EstB from *Burkholderia gladioli* which belongs to family VIII of esterase, in which some members also have the esterase/lipase consensus sequence Gly-Xaa-Ser-Xaa-Gly [47]. Using the structure of EstB from *Burkholderia gladioli* as a template, the three-dimensional model of YeiG produced by SWISS-MODEL is shown in Figure 7.

YeiG Is a carboxylesterase in *E. coli*

The presence of the catalytic triad Ser-Asp-His suggests that YeiG may possess lipase, protease or esterase activity. However, in the enzyme assays, YeiG showed broad substrate specificity with a preference for short chain substrates (Figure 5), and low enzymatic activities for lipase (using *p*-nitrophenyl palmitate; 31.5 ± 3.3 nmol/min/mg protein), and no detectable enzymatic activity for phosphatase (using *p*-nitrophenyl phosphate as a substrate), aminopeptidase (using L-leucine-*p*-nitroanilide as a substrate; 3.8 nmol/min/mg protein), trypsin (using N-benzoyl-L-arginine ethyl ester as a substrate), nitrilase, lyase, hydantoinase, or peroxidase (guaiacol as a substrate). These results suggest that YeiG represents a kind of carboxylesterase from *E. coli*.

Conclusion and future work

Sequence alignment predicts that YeiG has an α/β hydrolase fold with catalytic triad formed by Ser145, Asp223 and His256 at active sites and Ser145 is located in the conserved motif –Gly–Xaa–Ser–Xaa–Gly–. Enzymatic assays demonstrate that it has a high hydrolytic activity towards substrates with a short acyl chain, whereas it has little hydrolytic activity towards those with an acyl chain length of more than 10. These

results allow us to propose that YeiG has the function of *E. coli* esterase. In future work, we will improve its enantioselectivity using random mutation together with high throughput screening.

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