The Journal of Microbiology, February 2008, p. 100-107 Vol. 46, No. 1
DOI 10.1007/s12275-007-0185-5

Cloning and Characterization of Thermostable Esterase from *Archaeoglobus fulgidus*

Seung-Bum Kim, Wonkyu Lee, and Yeon-Woo Ryu*

Department of Molecular Science and Technology, College of Engineering, Ajou University, Suwon 443-749, Republic of Korea

(Received October 9, 2007 / Accepted December 12, 2007)

Thermostable esterase gene was cloned (Est-AF) from extremophilic microorganisms, *Archaeoglobus fulgidus* DSM 4304. The protein analysis result showed that Est-AF is monomer with total 247 amino acids and molecular weight of estimated 27.5 kDa. It also showed repeating units G-X-S-X-G (GHSLG) (residues 86~90) which is reported as active site of known esterases, and the putative catalytic triad composed of Ser88, Asp198 and His226. The esterase activity test with various acyl chain length of ρ-nitrophenol resulted that Est-AF showed highest specific activity with ρ-nitrophenylbutyrate ($pNPC_4$) and rapidly decrease with ρ-nitrophenyl ester contain more than 8 carbon chain. These results represent that cloned enzyme is verified as a carboxylesterase but not a lipase because esterase activity is decreased with ρ-nitrophenyl ester contains more than 8 carbon chains but lipase activity does not affected with carbon chain length. Optimum temperature of esterase reaction with ρ-nitrophenylbutyrate ($pNPC_4$) was 80°C. When ketoprofen ethyl ester was used as a substrate, activity of Est-AF showed the highest value at 70°C, and 10% of activity still remains after 3 h of incubation at 90°C. This result represents Est-AF has high thermostability with comparison of other esterases that have been reported. However, Est-AF showed low enantioselectivity with ketoprofen ethyl ester. Optimum pH of Est-AF is between pH 7.0 and pH 8.0. K_m value of ketoprofen ethyl ester is 1.6 mM and, V_{max} is 1.7 μmole/mg protein/min. Est-AF showed similar substrate affinity but slower reaction with ketoprofen ethyl ester compare with esterase from mesophilic strain *P. fluorescens*.

***Keywords*:** esterase, extremophile, thermostability, enantioselectivity, *Archaeoglobus fulgidus*

Extremophile, which survive in extreme conditions that are considered inhabitable, are mostly archaea. These microorganisms are one of the most primitive microorganisms and they are important not only in genetics but also their characteristics that can grow at extreme conditions such as high temperature and saturated salt level. These characteristics of extremophile made it researched in various fields (Shinsuke, 2002). These microorganisms are very useful to the bioengineering field because high thermostability of their enzymes have high potential of utilization in industrial process. Representative case would be *Taq* DNA polymerase used in PCR (polymerase chain reaction). Their structural characteristics also lead high resistance against organic solvents as well as resistance to many denaturing conditions (Vieille and Zeikus, 2001). This has been proven by comparative test on various denaturing conditions (Shinsuke *et al*., 2001). Because of thermal stability and strong resistance to organic solvents, thermal stable enzyme can be more ideal than mesophilic enzyme in application to producing process that requires organic solvents and high temperature. Thus finding of new thermostable enzyme for application in industry is highly demanded. Most of the pharmaceuticals which produced by synthetic chemical process are ester compounds that can be hydrolyzed by esterase. Esterase can be utilized in chemical pharmaceutical synthesis, food producing process, kinetic resolution, and other various fields because they do not need any cofactor, are easy to acquire, have activity in hydro liquid as well as organic liquid and have high enantioselectivity and broad substrate range (John and Abraham, 1991; Faber and Franssen, 1993; Roberts, 1999). Esterase is a general name for hydrolase that hydrolysis ester bonds, exists in animals, plants, and microorganisms. Esterase can be categorized into carboxylesterase, thioesterase, lipase, phosphomonoesterase, and phosphodiesterase depends on substrate specificity. However, usually carboxylesterase is defined as an esterase. The most of the α/β-hydrolase is carboxylesterase (Carboxylester hydrolase: EC 3.1.1.1) and lipase (triacylglycerol hydrolase: EC 3.1.1.3). These two enzymes have similar molecular structure and catalytic mechanism. However, esterase can be distinguished with lipase using difference of surface active ability and relatively high activity on short chained acyl derivatives than the long ones. Tributyrine is used to measure standard activity of esterase but triolein is used for lipase (Liu *et al*., 2001).

Ketoprofen [rac-2-(3-Benzoylphenyl) propionic acid] is widely used as drug for rheumatoid arthritis and pain. It is a non-steroidal anti-inflammatory drug (NSAIDs) which is commonly contains 2-aryl-substituted propionic acid chiral center. It also contains benzene ring at one side of the chiral center and methyl or carboxyl group on the other side. (S)-Enantiomer of ketoprofen has better medicine effects and lower side-effects than (R)-ketoprofen which may cause

* To whom correspondence should be addressed.
(Tel) 82-31-219-2455; (Fax) 82-31-216-8777
(E-mail) ywryu@ajou.ac.kr

toxic effects on stomach. Application of esterase has been intensively studied because enantioselectivity of esterase can be utilized as a best way to obtain pure enantiomer profen drug (Margolin, 1993).

In this study, thermostable archaeons that have entire genomic sequence verified were used to obtain thermal stable esterase. Putative esterases from chromosomal DNA of four extremophiles were cloned. This research was carried out to characterize the putative esterases including thermostability, resistance with organic solvents, and enantioselectivity.

Materials and Methods

Chemicals and enzymes

Bacto™ tryptone, Bacto™ yeast extract, and Bacto™ agar for medium preparation were purchased from BD (USA). Rest of chemicals for experiment was purchased from Sigma (USA). Ketoprofen ethyl ester (KetOEt) was synthesized from commercially available ketoprofen (Bornscheuer, 1997). Bio-Rad Protein Assay Dye Reagent Concentrate from Bio-Rad Laboratories (USA) was used for quantitative assay of cloned esterase. *Pfu* polymerase from Intron (Korea) for Polymerase Chain Reaction (PCR) was used. All of oligonucleotides were synthesized from Bionics (Korea). All of restriction enzymes and T4 DNA ligase were purchased from Intron. Cell Lysis Reagent from GenoFocus (Korea) was used for Est-AF purification and activity staining.

Strain isolation and esterase ORF search

Hyperthermophilic archaeons (*Sulfolobus solfataricus* ATCC 35092, *Thermotoga maritima* ATCC 43589, *Pyrococcous horikoshii shinkaj* OT3 ATCC 700860, and *Archaeoglobus fulgidus* DSM 4304) were used for new esterase cloning and entire genetic information was obtained from GenBank (NCBI, http://www.ncbi.nlm.nih.gov/blast). Esterase genes was amplified by PCR with primers prepared based on putative esterase sequences on chromosomal DNA. Chromosomal DNAs of four archaeons were donated from Microbial Genomic and Applications Center (Korea).

Screening

Putative esterase gene PCR product was ligated into expression vector, pQE30 (QIAGEN Inc., USA) and transformed into *Escherichia coli* strain, XL1-blue. Transformants were selected by Urakami and Komagata's activity staining method using α-naphthyl acetate (Urakami and Komagata, 1981) with minor change. Activity staining solution was prepared by adding α-naphthyl acetate (4.6 mg/0.5 ml ethoxyethanol) and Fast Blue B salt (2 mg/ml H_2O) into 50 mM Tris-HCl buffer (pH 8.0) 100 ml with 0.8% agar and poured over colonies. Esterase positive colonies showed brown colored circles around colonies transferred on new LB plate containing ampicillin 100 mg/ml.

Cell Lysis Reagent (GenoFocus, Korea) was overlaid on selected colonies on LB plate containing ampicillin 100 mg/ml and incubated at 80°C for 3 h then performed activity staining to test thermostability of esterase (Alessandra *et al*., 2002). Selected colonies also cultured onto LB plate containing ampicillin 100 mg/ml, ketoprofen ethyl ester and 0.5% triton X-100 and then incubated at 80°C for 1 h to measure activity with ketoprofen ethyl ester (Gong *et al*., 2002).

Purification of Est-AF

E. coli cells with esterase expression were inoculated in 100 ml of LB liquid medium containing 100 mg/ml. When cell OD (A_{600}) reached 0.4∼0.5, 1 mM of IPTG was added and cultured at 37°C for 3 h to induce esterase expression. After cells were washed with 5 ml of 50 mM Tris-HCl buffer (pH 8.0) and stored at -20°C for 2 h. Cells were resuspended in lysis buffer (50 mM phosphate-NaOH; pH 8.0 and 10 mM imidazole) and Cell Lysis Reagent for 30 min and broken by sonication (Cole-Parmer Instrument Co., Ultrasonic Homogenizer 36260 series) three times for 30 sec each with 13∼15 W of intensity. Cell free extract was prepared by incubation at 75°C for 30 min to eliminate thermosensitive proteins and obtained supernatant after centrifugation.

Ni^{+2}-NTA Superflow resin (QIAGEN Co., USA) was used for purification of histidine tagged protein (QIAGEN, 2002). Histidine tagged esterase bound with resin by shaking incubation of 4 ml of cell free extract and 1 ml of 50% resin slurry (20°C, 180 rpm, and 1 h). Esterase and rasin mixture was washed with 5 ml of washing buffer (50 mM phosphate-NaOH; pH 8.0, 20 mM imidazole) twice and elute with elution buffer (50 mM phosphate-NaOH; pH 8.0, 250 mM imidazole).

Measurement of esterase activity with ρ-Nitrophenyl derivatives

For investigate activity with various carbon number of ρ-nitrophenyl compounds [ρ-Nitrophenylacetate ($pNPC_2$), ρ-nitrophenylbutyrate ($pNPC_4$), ρ-nitrophenylhexanoate ($pNPC_6$), ρ-nitrophenylcaprylate ($pNPC_8$), ρ-nitrophenyllaurate ($pNPC_{12}$), ρ-nitrophenyl palmitate ($pNPC_{16}$)] as a substrate were tested. Twenty milimole of substrate stock solution (each ρNP esters in pure acetonitrile) were prepared. 75 μl of each substrate were added in 2,250 μl of 50 mM Tris-HCl buffer (pH 8.0) containing purified esterase 0.1 μg. Amounts of produced ρ-nitrophenol after reaction were measured by UV/VIS spectrophotometer (Lambda 35, Perkin Elmer) at 405 nm every 30 sec for 10 min. ρ-Nitrophenyl ester without esterase was used as a blank. Specific activities with each substrate were measured at 50, 60, 70, 80, and 90°C.

Measurement of esterase activity with ketoprofen ethyl ester

Esterase activities with ketoprofen ethyl ester were measured with different reaction temperatures (35∼95°C). Esterase in 50 mM Tris-HCl buffer (pH 8.0) was reacted with 5 mM ketoprofen ethyl ester as a substrate at various reaction temperatures with 0.3% of Triton X-100 for 1 h. Enzyme reaction was stopped with same amount of 100% ethanol at 70°C for 10 min. One unit of enzyme was defined the amount of enzyme to produce 1 μmole of ketoprofen for 1 min.

The resolution of produced ketoprofen ethyl ester was analyzed with HPLC (Waters Co., USA) system with chiral compound analytical column (Chirex Phase 3005, Phenomenex Co., USA) and UV detector (254 nm). Methanol with 0.03 M of ammonium acetate was used as mobile phase and flow

rate was 0.8 ml/min. Every samples were filtered by fluoropore™ membrane filter (fore size 0.2 μm, Millipore, Ireland) before inject into HPLC system. The purity of enantiomer (ee %: enantiomeric excess) was calculated as below.

$$ee\ \% = \frac{[(S)\text{–}ketoprofen] - [(R)\text{–}ketoprofen]}{[(S)\text{–}ketoprofen] + [(R)\text{–}ketoprofen]} \times 100$$

Results

Esterase cloning and amino acid sequencing

Putative esterases were amplified by PCR with primers which

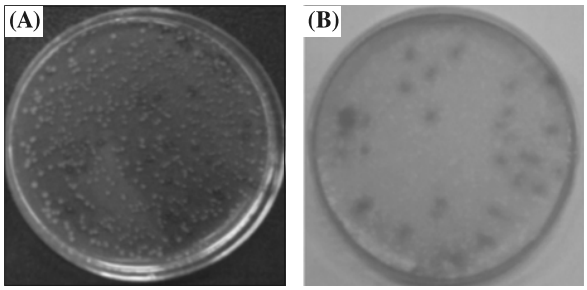

Fig. 1. Transformant with putative esterase screening by activity staining. Positive clones showed dark purple color after agar overlay (A) in room temperature (B) after 2 h of heat-treatment at 75°C.

(A)

```
gi 15639887  47 TSIHPLVRSAFYAGG.[1].HAVLLIH.[3].GTPREMQFLGRALHRDG.[2].VSIPRLPGHG     TNREDFLETG 111
query         6 LEIDALKISVLKGKE     KDVMYIH     GSGCDATLWERQLEDVG     GYAIDLPNHG.[1].SCAAEIRDIG 65
gi 15616116   1 MKLVAPKPFTFEGGT     RAVLLLH.[3].GTTADVRMLGRYLQEKG.[2].CHAPLYKGHG     VPPEELIQTG 64
gi 15926135   1 MRIKTPSPSYLKGTN.[1].HAILLLH.[3].GTNRDVKHLAAELNDQG.[2].CYAPNYPGHG     LLLKDFMTYN 65
gi 15643780   7 KTVKKSLPIFLEGGN     EGVLFIH.[3].GSPHDFEYMAKEVNRAG.[2].VSVPRLPGHG     TCGEDFLTTT 70
                                          *     *              *             **

gi 15639887 112 .[2].DWLRRVCDEYRDLS     AA.[4].SVGGLSMGGVLTALVAARFC     PQKAFFCAPGFA.[1].SDWRIKL 173
query        66      DYAYFVAKTVKKLM.[1].KA     VIVGHSLGGAVAQKIYLEYP.[2].VKALVLVGTGAR.[1].RVMPEIL 124
gi 15616116  65 .[2].DWWEDVEDGYQHLK.[1].QG.[4].AVCGLSLGGVFSLKIGYTLP     VKGIVPMCAPMR.[1].KTDDAIY 127
gi 15926135  66 .[2].DWWEEVEKAYQFLV.[1].EG.[4].SATGVSLGGLMTLKLAQHYP     LKRIAVMSAPKE     KSDDGLI 127
gi 15643780  71 .[2].DWLRRAFDAYYDLK     AI.[4].YVVGLSMGGVIALILASQMN     PPKLVTLAAATH.[1].FDKRIVL 132
                      *          *               * * **

gi 15639887 174 SPLVRWF.[6].DAA     PFYPEQDFNDATKDYRSAH     Y     IAQVAQFYALQRRAIRSLACIRSTLLTILS 239
query       125 TMLKEKP     AEA.[4].SKYAFSNQELAKEFSKVFA.[2].A.[5].DLSLCDRFDLLEDYRSGKVKVDIPTLLIVG 195
gi 15616116 128 KGVLEYA     EEY     KRREKKSDEQIEEEMERFK.[2].P     QTTLFGLKQLIEDVRDHLDHIYAPVFVVQA 189
gi 15926135 128 EHLVYYS     QRM     SNILNLDQQASSAQLAAID.[1].Y     EGEITKFQHFIDDIMTNLNVIKIPANILFG 188
gi 15643780 133 TPILKLF.[6].ENT     EKYEDPDIEYLRKEYWSYN     W     PKQAAELYKLMKLARKSVSKITSATLVVAA 198

gi 15639887 240 RQDPLVPCAAVQKLLDARVRS.[2].QYVVLEHSGHVITDDVE.[1].EQVASCVSAFLRT 293
query       196 EKDALTPVKYSEFFKKHIPSA     EMVVIPEAGHMVMLEKP     DEFNRALKNFIEK 246
gi 15616116 190 RHDEMIDVESANVIHDTVESD.[2].SLKWYEDSTHVITLDKE.[1].EQLHEDVYRFLEG 243
gi 15926135 189 GKDEPSYETSAHFIYEHLGSV.[2].ELNGLKDSHHLMTHGEG.[1].DILEENVIRFFNA 242
gi 15643780 199 KNDNMVPMKAAEFIYNNIRSE.[2].KLLVFEKSGHVLSNDVE.[1].EDVTRAVIEWLKG 252
                 *                              *
```

(B)

```
EST-AF   -MERITLEIDALKISVLKGKEKDVMYIHGSGCDATLWERQLEDVGG----YAIDLPNHGR 55
est-1    --MPYADNGVKIYYEVEDGGEPAIVFVHGWTANMNFWREQREYFKGKHRMLFIDNRGHGK 58
est-2    MLERVFIDVDGVKVSLLKGRERKVFYIHSSGSDATQWVNQLTAIGG----YAIDLPNHGQ 56
                :   :  .: .* *  :.::*.  .: . * .*   . *      **  .**:

EST-AF   SCAAEIRDIGDYAYFVAKT----VKKLMGKAVIVGHSLGGAVAQKIYLEYPKVVKALVLV 111
est-1    SDKPFNRSFYEFDNFVSDLHAAVKDASFDRFVLVGHSFGTMISMRYCVEHPGRVEALVLI 118
est-2    SDTVEVNSVDEYAYYASES----LKKTVGKAVVVGHSLGGAVAQKLYLRNPEICLALVLV 112
         *     ... ::  :.:.      .  ..: *:*****:*  :: :  :. *    ****:
                                                  ▲

EST-AF   GTGARLRVMPEILTMLKEKPA-----EAAELVSKYAFSNQ-ELAKEFSKVFAERAGVLHL 165
est-1    GGGARIQSLHRYGYPIGRLFATLAYGISARIIANMAFGRKAGELRDWGLKEALENTPKHA 178
est-2    GTGARLRVLPEILEGLKKEPE-----KAVDLMLSMAFASKGEEYEKKRREFLDRVDVLHL 167
         * ***:: : .    : .         :. :: . **. :    ..       .    *

EST-AF   DLSLCDRFDLLEDYRSGKVKVDIPTLLIVGEKDALTPVKYSEFFKKHIPSAEMVVIPEAG 225
est-1    ALNTLWTLTTVD-LRDIAREIEKPTLIVVGKEDALLPVSKSEELSRLIKNSKMVIVPDAG 237
est-2    DLSLCDRFDLLEDYRNGKLKIGVPTLVIVGEEDKLTPLKYHEFFHKHIPNSELVVIPGAS 227
          *.    :  ::  *.   ::  ***::**::* * *:.  * : : * .:::*::* *.
                                          ▲

EST-AF   HMVMLEKPDEFNRALKNFIEKL------- 247
est-1    HCVMLEQPEIVNRVLEEFIHTFSAMLIRA 266
est-2    HMVMLEKHVEFNEALEKFLKKVGV----- 251
         * ****:   .*..*::*:...
▲
```

Fig. 2. Amino acid sequence alignment of Est-AF (A) multiple alignment of the partial amino acid sequences containing the conserved motifs of GxSxG, and putative catalytic triad residues of the esterases/lipases from *A. fulgidus* DSM 4304 (query, Est-AF), *Treponema pallidum* subsp. *Pallidum str.* (Gi 15639887), *Bacillus halodurans C-125* (Gi 15616116), *Staphylococcus aureus* subsp. Aureus N315, (Gi 15926135), and *Thermotoga maritime* MSB8 (Gi 15643780) (B) amino acid sequence alignment of Est-AF with other carboxylesterase from *A. fulgidus* DSM 4304. The alignment was performed using the CLUSTAL W method. The arrowheads denote the putative catalytic triad residues. The identical and conserved residues are marked by (*) and (:), respectively.

are forward; 5'-cgggatccgtgctaattcacc-3' containing *Bam*HI and reverse; 5'-aactgcagtcataagaaattctttatg-3' containing *Pst*I for *Sulfolobus solfataricus* ATCC 35092, forward; 5'-acatgcat gcatgaggttctttc-3' containing *Sph*I and reverse; 5'-aactgcagtc attgcgatccc-3' containing *Pst*I for *Thermotoga maritima* ATCC 43589, forward; 5'-cggatccatgccccttatgg-3' containing *Sph*I and reverse; 5'-acgcgtcgactcatctagatattg-3' containing *Sal*I for *Pyrococcous horikoshii shinkaj* OT3 ATCC 700860, forward; 5'-ggatccatggaaagaataactctc-3' containing *Bam*HI and reverse; 5'-gtcgacttacagcttctcaataaaatt-3' containing *Sal*I for *Archaeoglobus fulgidus* DSM 4304. Amplified putative esterases were ligated into expression vector (pQE30) after digestion with appropriate restriction enzymes. Transformations were performed via electroporation and transformants which has purple circle around colony were selected by activity staining method using α-naphthyl acetate and Fast Blue B salt. However, only two *E. coli* strains with putative esterase expression (*T. maritima* ATCC 43589 and *A. fulgidus* DSM 4304) were showed purple circle. Figure 1 showed active staining result of esterase from *A. fulgidus* DSM 4304. Because esterase from *T. maritima* ATCC 43589 showed too low expression in *E. coli* analyzed by SDS-PAGE and Western blotting analysis to proceed further experiment (data not shown), only esterase from *A. fulgidus* DSM 4304 (Est-AF) was studied for further characterization. According to results of SDS-PAGE and amino acid sequence analysis, Est-AF is 27.5 kDa of monomer and composed with total 247 of amino acids. Amino acid analysis showed Est-AF contains repeating units G-X-S-X-G (GHSLG) as known as common esterase active site.

Esterase also has common catalytic triad which is consist of Ser, Asp, and His. BLASTP and PHI-BLAST (Pattern Hit Initiated-BLAST) and PSI-BLAST (Position-Specific Iterated BLAST) analyses for the amino acid sequence comparisons revealed that Est-AF exhibited Ser201, Asp303, and His333 to be the putative catalytic triad (Ollis *et al.*, 1992; Akoh *et al*., 2004) (Fig. 2A). Results that amino acid sequence alignment of other esterases from *A. fulgidus* DSM 4304 also showed Ser201, Asp303, and His333 which are the putative catalytic triad (Fig. 2B).

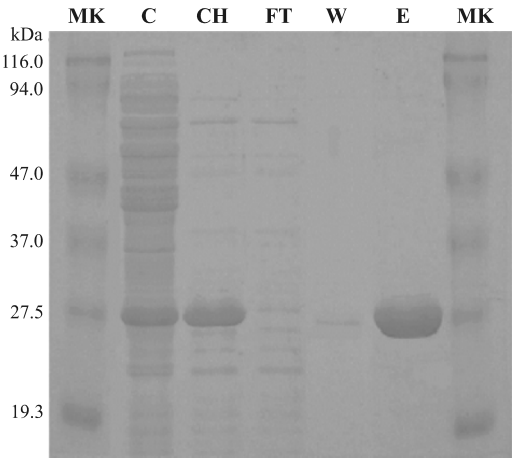

Fig. 3. SDS polyacrylamide gel electroporation of purified Est-AF. (MK, protein size maker; C, cell lysate; CH, cell lysate after at 75°C heat treatment for 30 min; FT, flow-through; W, wash solution; E, eluate)

Purification of Est-AF

Purification of Est-AF was performed for study of enzyme characterization. Est-AF was purified using Ni^{+2}-NTA resin because expressed esterase with pQE30 contains histidine tag in N-terminal. Figure 3 shows SDS PAGE result of samples from each steps of Est-AF purification. FT lane shows no band left because proteins from *E. coli* without histidine tag can not bind with Ni^{+2}-NTA resin and W lane shows washed buffer does not contain any protein. Lane E shows purified Est-AF after elute with elution buffer. Est-AF was expected stable with heat treatment because Est-AF was cloned based of sequence from thermostable strain. Thus Est-AF expressed *E. coli* cell extract was treated at 75°C for 30 min and then remaining protein was analyzed with SDS-PAGE. Lane C of Fig. 3 shows proteins from supernatant of esterase expressed *E. coli* extract before heat treatment. Lane CH shows all of proteins except esterase were disappeared after heat treatment. This result implies that Est-AF is thermostable and this method is easy way to

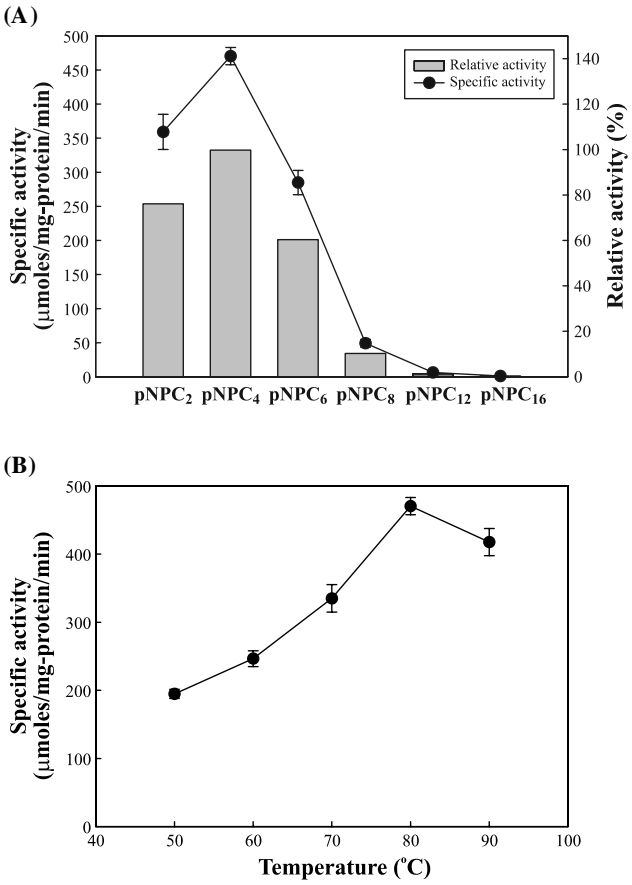

Fig. 4. Activities and optimum temperature of Est-AF (A) activities of Est-AF with various ρ-nitrophenyl derivatives were determined photometrically at 405 nm at 80°C (B) optimum reaction temperature of Est-AF with ρ-nitrophenylbutyrate. Enzyme activities were determined with 0.5 mM ρ-nitrophenylbutyrate in each temperature. Error bars obtained from three independent experiments.

eliminate native *E. coli* proteins. Thus, this technique was applied for Est-AF purification.

Esterase activity with ρ-nitrophenyl derivatives

Esterase is including carboxylesterase, thioesterase, lipase, phosphomonoesterase, and phosphodiesterase depends on substrate specificity (Chasse *et al*., 2001). However carboxylesterase is usually defined as an esterase. Carboxylesterase and lipase are very similar in their characteristics. Two enzymes have to be distinguished using enzyme activity with different substrate because some lipase contains common esterase active site. Lipases are defined that hydrolysis enzymes of acylglycerols contain long carbon chain more than 10. However, carboxylesterase are defines only hydrolyze acylglycerols contain short carbon chain less than 10 (Jaeger *et al*., 1999). In addition, lipase usually shows highest activity with acyl chain of C_8~C_{16}. Esterase has highest activity with acyl chain of C_2 or C_4 and activity decrease with acylglycerols contain longer carbon chain (Liu *et al*., 2001).

To investigate whether Est-AF is carboxylesterase or lipase, activities of Est-AF with ρ-nitrophenyl derivatives contain different length of acyl chains were measured. All of reactions were taken place at 80°C which is optimum temperature for esterase activity with ρ-nitrophenyl derivatives. Among the various ρ-nitrophenyl esters, Est-AF showed highest activity with ρ-nitrophenylbutyrate ($pNPC_4$). Activity with ρ-nitrophenylcaprylate ($pNPC_8$) showed only 10% of activity compared with $pNPC_4$ and no activities observed with ρ-nitrophenyllaurate ($pNPC_{12}$) and ρ-nitrophenylpalmitate ($pNPC_{16}$) (Fig. 4A). These results represent that Est-AF is a carboxylesterase but not a lipase.

To investigate thermostability of Est-AF, purified Est-AF and buffer mixture was pre-treated at various temperatures (50, 60, 70, 80, and 90°C) and time (20, 40, 80, 100, 120, 140, 160, and 180 min) and then add ρ-nitrophenylbutyrate which showed highest esterase activity and then esterase activity was measured every 30 sec for 10 min at 40°C. The highest activity was observed when heat treatment of Est-AF at 80°C and activity at 90°C was decreased as 80% of activity at 80°C (Fig. 4B). These results showed not only optimum temperature of Est-AF is higher than another thermophilic esterase from *Bacillus stearothermophilus* (Henke and Bornscheuer, 2002) but also heat is necessary for activation of Est-AF because Est-AF is not active when pretreated at low temperature.

Esterase activities with ketoprofen ethyl ester

(S)-Enantiomer of ketoprofen showed much higher drug effect and lower side effect than racemic mixture [(S,R)-enantiomer] (Borman, 1990). Thus characterization of Est-AF with ketoprofen ethyl ester including enantioselectivity was investigated.

Optimum reaction temperature of purified Est-AF with ketoprofen ethyl ester was 70°C and more than 90% of activity remains at 65°C and 75°C (Fig. 5A). Even after 3 h incubation at 90°C, 10% of activity was still observed (Fig. 5B). These results are similar with esterase from thermophilic microorganisms, *Archaeoglobus*, *Thermotoga*, *Pyrococcus*, or *Sulfolobus* (Manco *et al*., 2000; Claire *et al*., 2001; Alessandra *et al*., 2002). In addition, it is reported that optimum reaction temperature of esterase from mesophilic strain, *Pseudomonas* sp. is 35°C and no activity observed at 50°C (Choi, 2001). These results allowed us to confirm

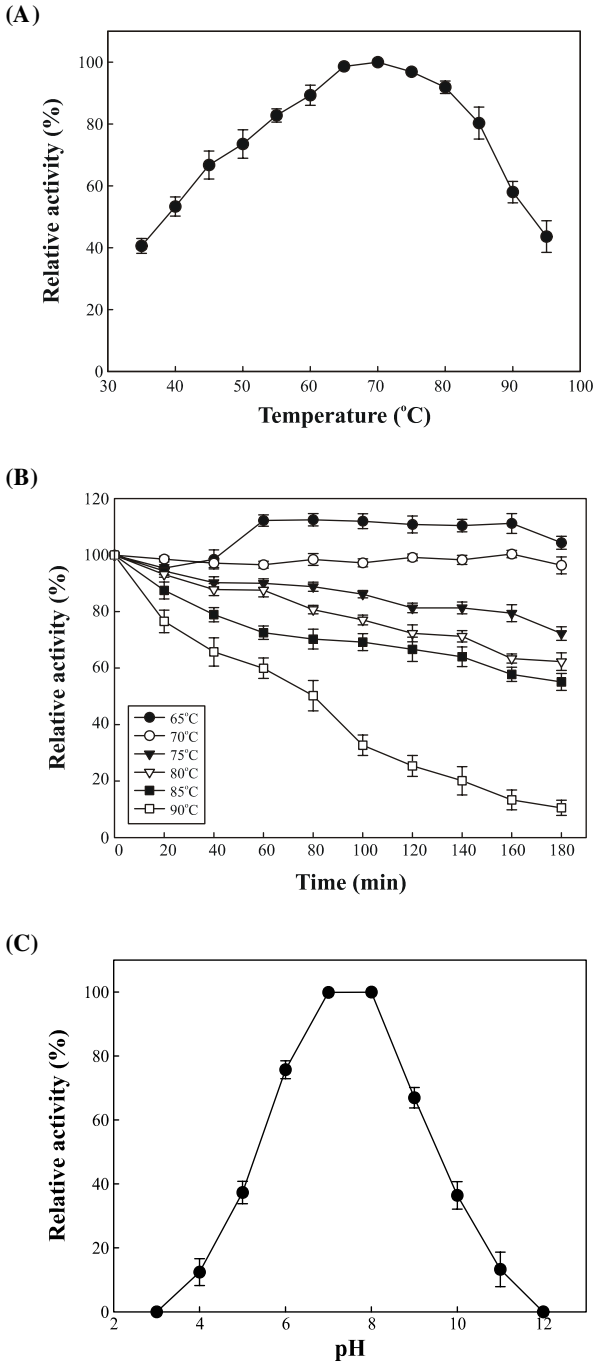

Fig. 5. Activities of Est-AF with ketoprofen ethyl ester at various conditions (A) activities of Est-AF with ketoprofen ethyl ester at various temperatures without heat-heattreatment. (B) Activities of Est-AF with ketoprofen ethyl ester at various temperatures after heat-treatment. The relative activities of Est-AF with 5 mM ketoprofen ethyl ester compare with enzyme activity without heat-treatment was measured after 1 h enzyme reaction at 70°C (C) optimum reaction pH of Est-AF with ketoprofen ethyl ester. The activity was measured with 5 mM ketoprofen ethyl ester as the substrate at 70°C in buffers with different pH for 1 h reaction. Error bars obtained from three independent experiments.

thermostability of Est-AF with ketoprofen ethyl ester. To investigate enantioselectivity of Est-AF, purified Est-AF with (S,R)-ketoprofen ethyl ester as a substrate which was dissolved in buffer contains 0.2% of Triton X-100 was reacted for 1 h then activities and enantioselectivities were measured. Table 1 shows activities and ee values of Est-AF at various reaction temperatures. 100% of ee value represents 100% of (S)-ketoprofen production. ee Values were slightly increased as reaction temperature increased. ee value of Est-AF at 70°C is -47% which means Est-AF prefer (R)-ketoprofen ethyl ester rather than (S)-form. These results represent that Est-AF has high thermostability but not suitable for precise separation of enantiomers. Thus improvement of enantioselectivity of Est-AF will be necessary for further application.

Table 1. Relative activities and enantioselectivities of Est-AF under various reaction temperatures

Temperature (°C)	Relative activity (%)	ee (%)
50	68	-43.9
60	87	-45.2
70	100	-46.4
80	92	-46.9
90	58	-47.3

Table 2. Effects of various surfactants on activities and enantioselectivities of Est-AF

	Relative activity (%)	ee (%)
Control	100	-30.9
Tween 20	1,396	-46.2
Tween 40	1,306	-46.2
Tween 60	1,396	-46.9
Tween 80	1,339	-47.3
Triton X-15	210	-36.2
Triton X-45	662	-32.9
Triton X-100	1,060	-33.5
Triton X-165	1,749	-35.8
Triton X-305	1,628	-29.9

The optimum reaction pH of Est-AF was also investigated. Activities of esterase in various pH were measured after reaction of 5 μg of purified esterase with different buffers [50 mM of citrate buffer (pH 3.0, 4.0, 5.0, and 6.0), phosphate buffer (pH 7.0), Tris-HCl buffer (pH 8.0), glycine-NaOH buffer (pH 9.0 and 10.0), Na_2HPO_4-NaOH buffer (pH 11.0 and 12.0)] containing 5 mM of ketoprofen ethyl ester and 0.1% of Triton X-100. Amount of produced ketoprofen was measured by HPLC. Activities of purified esterase with ketoprofen ethyl ester in different buffers which have different pH were measured. Est-AF showed highest activity at pH 7.0~8.0. However enzyme activity was decreased higher than pH 8.0 and no enzyme activity was observed at pH 3.0 and pH 12.0 (Fig. 5C). This is similar result with esterase from another thermophilic strain *Geobacillus* (Ewis *et al*., 2004).

The effect of surfactants and organic solvents for improvement of enzyme reaction

Because ketoprofen ethyl ester is insoluble in water, it may lead result of decreasing enzyme activity. Thus non-ionic surfactants were added to increase solubility of substrate for improvement of enzyme reaction. Two different kinds of non-ionic surfactant, Triton and Tween type surfactants which are reported no suppression of enzyme activity was added in reaction mixture and esterase activities were measured. Twenty hundreds and fifty microliter of enzyme solution containing 50 mM Tris-HCl buffer (pH 8.0) with 5 μg of purified esterase and surfactants was reacted with 250 μl of substrate solution (0.6% Triton X-100, 50 mM Tris-HCl; pH 8.0, and 10 mM ketoprofen ethyl ester) at 70°C for 1 h. Amount of produced ketoprofen was measured by HPLC. Table 2 showed esterase relative activities when 1% of different surfactants were added in reaction mixture compare with esterase activity without surfactant. Esterase activities with 4 different kinds of Tween type surfactants were similar and all of activities were higher than activities with Triton type surfactants. Esterase activities with 5 different kinds of Triton type surfactant were also tested. The highest esterase activity was observed when Triton X-165 was added and the

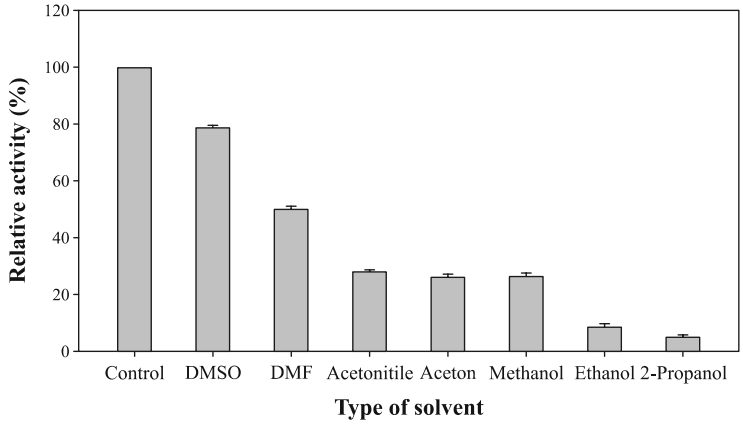

Fig. 6. Effects of various organic solvents on the activity of Est-AF. Error bars obtained from three independent experiments.

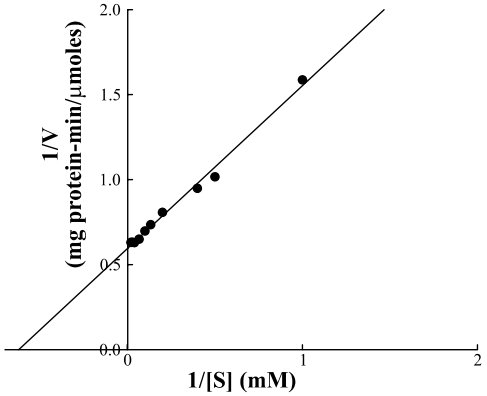

Fig. 7. K_m and V_{max} of Est-AF. The kinetic constants were estimated from a typical Lineweaver-Burk plot.

highest ee value was observed with Triton X-305. The surfactants contain longer carbon chain which leads higher substrate solubility showed higher esterase activity ee value. Organic solvents were often used to increase solubility of substrate because it is reported that esterase activity usually remains in organic solvents (Phytian, 1998; Bornscheuer, 2002). Therefore, organic solvent addition effect of Est-AF was also investigated. Ketoprofen ethyl ester as a substrate was dissolved in buffer which contains 2% of Triton X-100 and purified Est-AF and different kinds of organic solvents [10% of each acetone, acetonitrile, iso-propanol, ethanol, methanol, dimethyl sulfoxide (DMSO) and dimethyl formamide (DMF)] were added and then enzyme activities were measured. All of organic solvents additions were resulted decrease of enzyme activities (Fig. 6). Organic solvents addition is not suitable for improvement of enzyme reaction through increase of substrate solubility.

Determination of kinetic parameter

For more detail characterization of Est-AF, enzyme kinetic parameters were measured. Different concentrations of substrate [(R,S)-ketoprofen ethyl ester] and 1 μg of purified Est-AF were added in 50 mM Tris-HCl (pH 8.0) buffer contains 0.2% of Triton X-100 for enzyme reaction. After measured enzyme activities, kinetic parameters were obtained using Lineweaver-Burk plot (Fig. 6). K_m and V_{max} of Est-AF with (R,S)-ketoprofen ethyl ester are 1.6 mM and 1.7 mole/mg protein/min, respectively. K_m and V_{max} of esterase isolated from mesophilic *P. fluorescens* which is reported activity of ketoprofen ethyl ester were 1.5 mM and 6.2 μmole/mg protein/min, respectively (Kim *et al.,* 2004). Both esterases showed similar affinities to substrate. However, Est-AF showed slower reaction with ketoprofen ethyl ester compare with esterase from *P. fluorescens.*

Discussion

Because application range of enzymes in industry has been expanded, demands of new enzymes development for maintaining their activity in industrial environments have been increased. However, most of enzyme reactions cannot be performed with high temperature or organic solvents which are often needed in industrial processes. Thus, enzymes isolated from microorganisms which can survive at extremely high temperature environment have been gotten attention because of their high thermostability. In this research, thermostable esterase was cloned from extremophilic microorganism which has high thermostability and widely used for chemical synthesis, food process with their high enantioselectivity and wide substrate range.

In this study, esterase genes were cloned using genetic information of putative esterase on extremophile chromosome from GenBank database search and chromosomal DNA donated from Microbial Genomic and Applications Center. Four different esterases were cloned from four different hyperthermophilic archaeons. An esterase from *A. fulgidus* (Est-AF) showed esterase activity but no activities were observed with rest of esterases. Est-AF showed high thermostability but low enantioselectivity with ketoprofen ethyl ester.

In conclusion, Est-AF has higher thermostability as we expected and is considered as heat activated enzyme because it shows strong activity at higher temperature but barely active at lower temperature. Thermostability of Est-AF can help easier enzyme purification because Est-AF can maintain activity after heat treatment to eliminate other thermosensitive proteins. Est-AF showed highest activity with ρ-nitrophenylbutyrate ($pNPC_4$) and activity was decreased with ρ-nitrophenyl derivatives which contain longer carbon chain. Amino acid sequence analysis revealed that Est-AF contains the conserved sequence motifs of esterases/lipases, such as G-X-S-X-G (GHSLG) and the putative catalytic triad composed of Ser88, Asp198, and His226. These results allowed us to consider that Est-AF is a carboxylesterase but not a lipase. Although Est-AF has high thermostability, Est-AF has slow reaction rate and lower enantioselectivity with (R,S)-ketoprofen ethyl ester. For further industrial applications, *in vitro* protein evolution technology such as error prone PCR, site-directed mutagenesis and DNA shuffling should be applied for improvement of esterase has higher enantioselectivity as well as thermostability.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2004-041-D00235).

References

Akoh, C.C., G.-C. Lee, Y.-C. Liaw, T.-H. Huang, and J.-F. Shaw. 2004. GDSL family of serine esterases/lipases. *Prog. Lipid Res*. 43, 534-552.

Alessandra, M., D.P. Natascia, V. Aurilia, R. Mose, and C. Raffaele. 2002. A carboxylesterase from the hyperthermophilic archaeon *Sulfolobus solfataricus*: cloning of the gene, characterization of the protein. *Gene* 283, 107-115.

Borman, S. 1990. Chirality emerges as a key issues in pharmaceutical research. *Chem. Eng. News* 68, 9-14.

Bornscheuer, U.T. 1997. Directed evolution of an esterase for the stereoselective resolution of a key intermediate in the synthesis of epothilones. *Biotechnol. Bioeng.* 58, 554-559.

Bornscheuer, U.T. 2002. Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiol. Rev*. 733, 1-9.

Chasse, G.A., A.M. Rodrigues, M.L. Mak, E. Deretey, A. Perczel, C.P. Sosa, R.D. Enriz, and I.G. Csizmadia. 2001. Peptide and protein folding. *J. Mol. Struct. (Themochem)* 537, 319-361.

Choi, G.-S. 2001. PCR-Cloning and purification of an esterase from *Pseudomonas fluorescens* KCTC 1767 with high activity toward (S)-Ketoprofen ethyl ester. MSc. thesis. Ajou University, Suwon, Korea.

Claire, V., D. Sriprapundh, A. Savchenko, S. Kang, H. Krishnamurthy, and J.G. Zeikus. 2001. Thermostability mechanisms in enzymes from *Thermotoga neapolitana* and *Pyrococcus furious*. *J. Microbiol*. 39, 245-249.

Ewis, H.E., A.T. Abdelal, and C.-D. Lu. 2004. Molecular cloning and characterization of two thermostable carboxyl esterase from *Geobacillus stearothermophilus*. *Gene* 329, 187-195.

Faber, K. and M.C.R. Franssen. 1993. Prospects for the increase application of biocatalysts in organic transformations. *Trends Biotechnol.* 11, 461-470.

Gong, P.-F., H.-Y. Wu, J.-H. Xu, D. Shen, and Y.-Y. Liu. 2002. Biocatalytic preparation of enantiopure (R)-ketoprofen from its racemic ester by a new yeast isolate *Citeromyces matriensis* CGMCC 0573. *Appl. Microbiol. Biotechnol.* 58, 728-734.

Henke, E. and U.T. Bornscheuer. 2002. Esterases from *Bacillus subtilis* and *B. stearothermophilus* share high sequence homology but differ substantially in their properties. *Appl. Microbiol. Biotechnol*. 60, 320-326.

Jaeger, K.E., B.W. Dijkstra, and M.T. Reetz. 1999. Bacterial biocatalysts: molecular biology, three-dimensional structure, and biotechnology applications of lipases. *Annu. Rev. Microbiol.* 53, 315-351.

John, V.T. and G. Abraham. 1991. Lipase catalysis and its application. *In* J.S. Dordick (ed.), Biocatalysts for industry. Plenum press, New York, NY, USA.

Kim, J.-H., G.-S. Choi, S.-B. Kim, W.-H. Kim, J.-Y. Lee, Y.-W. Ryu, and G.-J. Kim. 2004. Enhanced thermostability and tolerance of high substrate concentration of an esterase by directed evolution. *J. Mol. Catal. B: Enzymatic* 27, 169-175.

Liu, A.M.F., N.A. Somers, R.J. Kazlauskas, T.S. Brush, F. Zocher, M.M. Enzelberger, U.T. Bornscheuer, G.P. Horsman, A. Mezzetti, C. Schmidt-Dannert, and R.D. Schmid. 2001. Mapping the substrate selectivity of new hydrolases using colorimetric screening: lipases from *Bacillus thermocatenulatus* and *Ophiostoma piliferum*, esterases from *Pseudomonas fluorescens* and *Streptomyces diastatochromogenes*. *Tetrahedron Asymmetry* 12, 545-556.

Manco, G., E. Giosue, S. D'Auria, P. Herman, G. Carrea, and M. Rossi. 2000. Cloning, overexpression, and properties of a new thermophilic and thermostable esterase with sequence similarity to Hormone-sensitive lipase subfamily from the Archaeon *Archaeoglobus fulgidus*. *Arch. Biochem. Biophys.* 373, 182-192.

Margolin, A.L. 1993. Enzymes in the synthesis of chiral drugs. *Enzyme Microb. Technol.* 15, 266-280.

Ollis, D., E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S. Franken, M. Harel, S. Remington, I. Silman, and J. Schrag. 1992. The alpha/beta hydrolase fold. *Protein Eng*. 5, 197-211.

Phytian, S.J. 1998. Esterases, p. 193-241. *In* D.R. Kelly (ed.), Biotechnology, 2nd ed., Vol. 8s. Wiley-VCH, Weinheim, Germany.

QIAGEN. 2002. A handbook for high-level expression and purification of 6× His-tagged proteins, Fifth edition.

Roberts, S.M. 1999. Biocatalysts for fine chemicals synthesis. John Wiley & Sons, Chichester, UK.

Shinsuke, F. 2002. Extremophiles: Developments of their special functions potential resources. *J. Biosci. Bioeng.* 94, 518-525.

Shinsuke, F., S. Fujiwara, T. Imanaka, and M. Takagi. 2001. Conformational stability of a hyperthermophilic protein in various condition for denaturation. *Electrochemistry* 69, 949-952.

Urakami, T. and K. Komagata. 1981. Electrophoretic comparison of enzymes in the Gram-negative methanol-utilizing bacteria. *J. Gen. Appl. Microbiol*. 27, 381-403.

Vieille, C. and G.J. Zeikus. 2001. Hyperthermophilic enzymes: source, uses, and molecular mechanism for thermostability. *Mol. Biol. Rev.* 65, 1-43.