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# Molecular cloning and characterization of a novel family VIII alkaline esterase from a compost metagenomic library

Yong Ho Kim [a], Eun Ju Kwon [a], Sung Kyum Kim [a], Yu Seok Jeong [a], Jungho Kim [a], Han Dae Yun [b], Hoon Kim [a,*]

[a] Department of Agricultural Chemistry, Sunchon National University, Suncheon 540-742, Republic of Korea
[b] Division of Applied Life Science, Gyeongsang National University, Chinju 660-701, Republic of Korea

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ABSTRACT

A metagenomic library was constructed from completely fermented compost using a fosmid vector. From a total of 23,400 clones, 19 esterase-positive clones were selected on LB plates containing 1% glyceryl tributyrate as the substrate. The esterase gene of an esterase-positive clone, *est*2K, was on an ORF of 1299 bp and encoded a protein of 432 amino acids. Est2K had a SMTK motif and was a family VIII esterase. Unlike most family VIII esterases, Est2K had a signal peptide of 27 amino acids. The molecular mass and p*I* of the mature Est2K was calculated to be 44,668 Da and 4.48, respectively. The amino acid sequence of Est2K showed 72% identity with that of EstC, an esterase of an uncultured bacterium from leachate. The purified Est2K was optimally active at pH 10.0 and 50 °C. Est2K was stable in the presence of 30% methanol and exhibited a 2.4-fold higher activity in the presence of 5% methanol than in the presence of 1% isopropanol. Est2K preferred short to medium length *p*-nitrophenyl esters, especially *p*-nitrophenyl butyrate, as the substrate. Est2K did not hydrolyze β-lactam antibiotics ampicillin and nitrocefin, even though Est2K showed the highest similarity to EstC.



## Introduction

The search for valuable microbial genes has relied upon cultivated cultures, but it has recently become clear that only a small proportion of all the existing microorganisms can be cultivated in laboratories. To secure various useful genes from not easily culturable or unculturable microbes, it is necessary to collect all the genetic material of certain natural environments using culture-independent approaches. Metagenome, a term first used by Handelsman who studied the metagenome of a soil sample [1], refers to all the genetic material in an environmental sample consisting of the genomes of many individual organisms, and is also referred to as eDNA (environmental DNA) [2]. The use of metagenomics for various applications has become possible with the development of techniques for directly obtaining and cloning genetic material from environmental samples without isolating microorganisms [3,4].

Composting lessens environmental contamination and increases agricultural productivity by rapidly transforming organic waste material into an end product that can be used to improve the chemical and physical properties of soil [5,6]. The microbial community in compost includes a myriad of microorganisms and the indigenous microbes of compost produce various enzymes such as cellulase, invertase, alkaline phosphatase, lipase, aminopeptidase, glucosylhydrolase and amidohydrolase [7–9].

Esterases and lipases are carboxylic ester hydrolases (EC 3.1.1) and are grouped into eight families [10]. Esterases (EC 3.1.1.1) hydrolyze ester bonds of short chain fatty acids with less than 10 carbons, and lipases (EC 3.1.1.3) hydrolyze ester bonds of long chain fatty acids with more than 10 carbons. Recently, several esterase/lipase genes have been isolated from metagenomic libraries derived from mud and sediment-rich water in thermal environments [11], oil-contaminated soil [12,13], soil and compost [14], deep-sea sediment [15], arctic sediment [16], leachate [17] and soil [18]. Esterases and lipases are widely used in the detergent, dairy, paper, pesticide and pharmaceutical industries [19]. In this study, we constructed a compost metagenomic library, isolated a novel alkaline esterase gene from the library, and examined some properties of the enzyme.

## Materials and methods

*Preparation of compost metagenome.* Composting was carried out at Yonghyun Nonghyub Compost Factory (Sachon, Korea) from pig manure and mushroom cultural waste using a moving roller system with intermittent mixing and forced aeration [20].

Completely fermented compost was used for metagenomic DNA extraction using a predescribed method with some modifications [4,21]. The primary DNA preparation was electrophoresed for over

* Corresponding author. Fax: +82 61 752 8011.
*E-mail address:* hoon@sunchon.ac.kr (H. Kim).

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15 h at 25 V on a 0.4% low melting point agarose gel (16 × 15 cm, Sigma, St. Louis, MO, USA) overlaid on an 1% agarose gel using λ DNA and λ/HindIII (Fermentas, Ont., Canada) as size markers [22]. The portion of the gel corresponding to DNA with sizes over 35 kb was divided into four fractions by horizontally slicing the gel into pieces 2 mm in width and DNA was extracted from each fraction.

*Construction of the compost metagenomic library.* The metagenomic library was constructed using the CopyControl™ pCC2FOS™ Fosmid Library Production Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. Transformants were selected after incubation of the infected cells for 12 h at 37 °C on LB agar plates containing 12.5 μg $ml^{-1}$ chloramphenicol and fosmids were extracted using the SDS-alkaline lysis method [23]. The quality of the library was tested by BamHI and EcoRI (Promega, Madison, WI, USA) restriction of the prepared fosmids.

*Selection of esterase-positive clones and subcloning of esterase gene.* The transformants were grown for 12 h on LB agar plates containing 1% glyceryl tributyrate (Sigma, St. Louis, MO, USA) as the substrate and the colonies with a clear zone were selected as esterase-positive clones. The esterase gene was subcloned from the fosmids of the esterase-positive clones by the shotgun method.

*Sequence analysis and construction of phylogenetic tree.* The nucleotide sequence was determined by SolGent (Daejeon, Korea). The conserved region of the subcloned esterase gene was identified by analyzing the nucleotide sequence by BlastP of NCBI (http://www.ncbi.nlm.nih.gov) and the signal peptide was predicted by SignalP 3.0 in CBS (http://www.cbs.dtu.dk/services/SignalP/) [24]. A phylogenetic tree of the esterases was constructed using DNA/MAN (Lynnon Biosoft, version 4.11, Que., Canada). Molecular mass and p*I* of the encoded protein were predicted by ExPASy (http://www.expasy.ch/tools/protparam.html) and the active site was predicted with the pfam database [25].

*Amplification of the esterase gene by PCR.* To find out whether the transformants have the same genes, esterase genes of the subclones were amplified by PCR using a primer set containing the sequences of the identified conserved region (forward primer 5′-AATTGCCTGCGGATGGGG-3′, reverse primer 5′-AAGCTGAAGAATGCACAA-3′). Pre-denaturation for 7 min at 95 °C, 35 cycles of denaturation-annealing-extension (45 s at 95 °C, 45 s at 55.5 °C, 90 s at 72 °C, respectively), and post-extension for 7 min at 72 °C were carried out using a PCR machine (Astec, Osaka, Japan).

*Purification of the enzyme.* The transformants were grown in LB broth containing 100 μg $ml^{-1}$ of ampicillin for 12 h at 37 °C with rotary agitation at 200 rpm, harvested by centrifugation and dispersed in 50 mM sodium citrate (pH 5.5) buffer. Crude enzyme solution was prepared by sonication as previously described [26] and dialysis against 50 mM Tris–HCl (pH 8.0) buffer. Purification of the enzyme was carried out by High-Q (1 ml, BIO-RAD, CA, USA), CHT-II (5 ml, BIO-RAD) and *t*-Butyl HIC (5 ml, BIO-RAD) column chromatographies. The progress of the purification was monitored by determining the amount of protein [27] and SDS–PAGE on an 11.5% polyacrylamide gel [28].

*Determination of esterase activity.* Esterase activity was determined by measuring the amount of *p*-nitrophenol generated from *p*-nitrophenyl esters (Sigma, St. Louis, MO, USA). Unless otherwise mentioned, the reaction was carried out for 1 min at 25 °C with 1 mM *p*-nitrophenyl butyrate in 50 mM Tris–HCl (pH 8.0) and the absorbance at 400 nm of the reaction mixture was continuously measured using Photodiode Array Bio-Spectrophotometer (Hitachi, Model U-0080D, Tokyo, Japan). Enzyme activities were calculated from the observed absorbances using the molar extinction coefficients of *p*-nitrophenol (16,400 $M^{-1}$ $cm^{-1}$ at pH 8.0). One unit of esterase activity was defined as the amount of enzyme that generated 1 μmol of *p*-nitrophenol in 1 min under these conditions.

*Characterization of the enzyme.* Thermostability of the enzyme was determined by preincubating the enzyme for up to 60 min in the absence of the substrate at designated temperatures prior to the activity assay. A universal buffer (boric acid/citric acid/trisodium orthophosphate) was used for the determination of optimum pH. The molar extinction coefficients of *p*-nitrophenol were 2440, 11,920, 16,400, 17,880, 18,640, and 19,280 $M^{-1}$ $cm^{-1}$ at pH 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0, respectively. The influences of cations and an inhibitor on enzyme activity were determined in the presence of 5 mM $K^+$, $Na^+$, $Ca^{2+}$, $Cu^{2+}$, $Mn^{2+}$, $Mg^{2+}$ and $Zn^{2+}$, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Substrate specificity of the enzyme was determined by using *p*-nitrophenyl esters, *p*-nitrophenyl acetate (C2), *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl octanoate (C8), *p*-nitrophenyl caprate (C10), *p*-nitrophenyl laurate (C12), *p*-nitrophenyl myristate (C14), and *p*-nitrophenyl palmitate (C16), as substrates. β-Lactamase activity on ampicillin was measured by the method of Avison et al. [29] with some modifications. After adding an appropriate amount of enzyme to 50 mM Tris–HCl (pH 8.0) containing 13.5 μM ampicillin to make a total volume of 1.0 ml, reactions were carried out at 25 °C and the absorbance was continuously measured at 233 nm. β-Lactamase activity on nitrocefin [3-(2,4-dinitrostryl)-(6R,7R)-7-(2-thienylacetamido)-ceph-3-em-4-carboxylic acid, E-isomer] (Calbiochem, Darmstadt, Germany) was measured according to the manufacturer's instructions. The reaction was carried out for 10 min at 25 °C in the presence of 0.1 mM nitrocefin in 100 mM phosphate (pH 7.0) containing 1 mM EDTA and the absorbance was continuously monitored at 486 nm.

*Nucleotide sequence accession number.* The nucleotide sequence of *est*2K has been deposited in the GenBank under the Accession No. GQ426329.

## Results and discussion

### *Preparation of compost metagenomic DNA*

The metagenomic DNA extracted from the completely fermented compost sample was about 40–75 kb in size (data not shown). The purity of the primary DNA preparation was considerably low and the values of A260/A280 and A260/A230 were 1.15 and 0.69, respectively. When the primary DNA preparation was further fractionated and purified by electrophoresis on a 0.4% low melting point agarose gel, all of the purified metagenome fractions consisted of DNA of 35 kb or larger. The values of A260/A280 and A260/A230 for the purified metagenome were 1.52 and 1.36, respectively, indicating that the purification step was quite efficient. The values of A260/A280 and A260/A230 for a DNA sample from a cultured microorganism were 1.89 and 1.57, respectively [21].

### *Construction of compost metagenomic library and cloning of esterase gene*

A compost metagenomic library was constructed with the purified metagenome preparation, and a total of 23,400 clones were obtained. A fosmid vector was used to satisfy the genetic stability and size requirement of the library [30]. BamH1 restriction analysis of the fosmids of the 12 randomly selected clones indicated that all of the fosmids had inserted DNA fragments (data not shown). The sizes of the inserted DNA fragments determined by EcoRI restriction were about 30–45 kb with an average size of 35 kb.

Nineteen esterase-positive clones were obtained from the compost metagenomic library by checking the esterase activities of the transformants on agar plates containing glyceryl tributyrate. The 19 esterase-positive clones were mixed together and grown in an

induction medium, and the DNA extracted from the clones was restricted with Sau3AI and then used for subcloning by shotgun method. Eighteen esterase-positive subclones were identified from about 1000 transformants obtained.

*Characterization of an esterase gene est2K*

The recombinant plasmid of YH-E2, one of the esterase-positive subclones, had an inserted DNA fragment of about 4 kb. An ORF of 1299 bp was identified to be an esterase gene with a domain that showed high similarity to the β-lactamase superfamily, and was named *est*2K. The esterase gene, *est*2K, was expected to encode a protein of 432 amino acid residues and to have a putative signal peptide of 27 amino acid residues. The mature Est2K was calculated to have a molecular mass of 44,668 Da and a theoretical p*I* of 4.48.

The amino acid sequence of Est2K showed the highest identity of 72% to that of EstC, an esterase of an uncultured bacterium from soil (GenBank Accession No. ACH88047), followed by 43% identity to the β-lactamase of *Solibacter usitatus* Ellin6076 (GenBank Accession No. YP_826528), 42% identity to the β-lactamases of *marine gamma proteobacterium* HTCC2143 (GenBank Accession No. ZP_0167296) and *Methylobacterium nodulans* ORS2060 (GenBank Accession No. YP_002501216).

Analysis of the amino acid sequences of the proteins with high identity revealed that the sequence of the 103th to 106th amino acid residues of Est2K was S-M-T-K, a sequence found in most of the β-lactamase superfamily (Supplementary Fig. 1). The Ser103 and Lys106 of the sequence were expected to be the active sites of the proteins by pfam database search. Site-directed mutagenesis

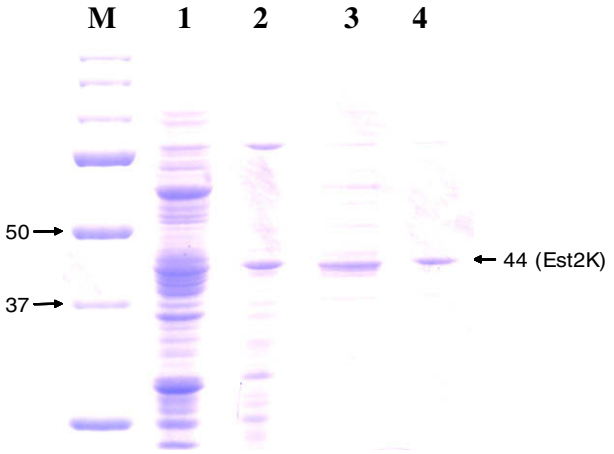


**Fig. 1.** SDS–PAGE of the purified Est2K. M, molecular weight markers; lane 1, crude extract of a subclone YH-E2; lane 2, eluted from High-Q chromatography; lane 3, from CHT- II chromatography; lane 4, from HIC chromatography.

of Ser103 to Ala103 resulted in the complete loss of enzyme activity (data not shown). The S-M-T-K sequence found both in Est2K and EstC [17] corresponds to the S-x-x-K motif, which is conserved in class C β-lactamases [31], penicillin binding proteins (PBPs) [32] and family VIII carboxylesterases [10]. Tyr219 of Est2K was conserved in the esterases except for EstB of *Burkholderia gladioli* when the Est2K was aligned to EstC, four β-lactamases with the highest similarity to the Est2K, a carboxylesterase, and a family VIII carb-

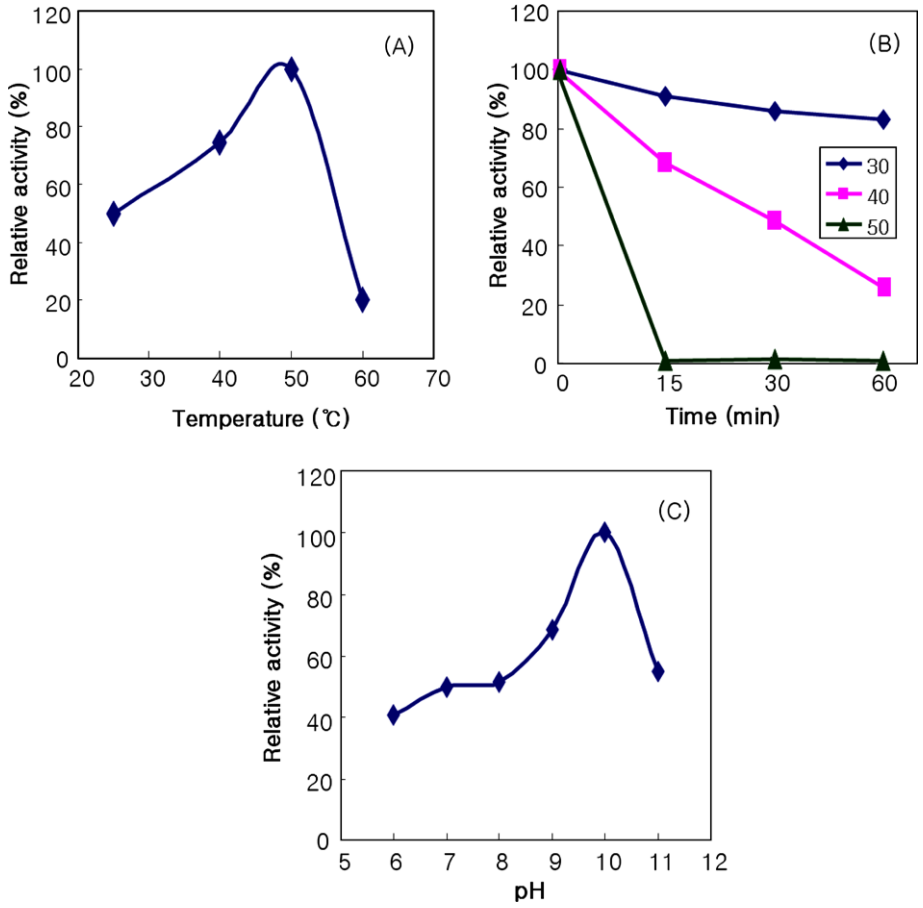


**Fig. 2.** Effect of temperature and pH on enzyme activity of Est2K. (A) Optimum temperature. (B) Thermostability. (C) Optimum pH. Enzyme activities were measured by a continuous method at each designated pH. For more details, refer to Materials and methods.

**Table 1**
Effect of cations, an inhibitor, and organic solvents on the activity of Est2K.

| | Relative activity (%) |
|---|---|
| Control (+1% isopropanol) | 100.0 |
| Cation (5 mM) | |
| $K^+$ | 98.9 |
| $Na^+$ | 93.2 |
| $Ca^{2+}$ | 90.2 |
| $Mg^{2+}$ | 88.3 |
| $Mn^{2+}$ | 86.5 |
| $Zn^{2+}$ | 85.0 |
| $Cu^{2+}$ | 58.6 |
| Inhibitor (1 mM) | |
| PMSF | 5.7 |
| Solvent | |
| Isopropanol (5%) | 150.1 |
| Isopropanol (30%) | 23.5 |
| Methanol (1%) | 134.6 |
| Methanol (5%) | 236.8 |
| Methanol (30%) | 93.7 |
| Acetonitrile (1%) | 23.4 |
| Acetonitrile (5%) | 44.4 |
| Acetonitrile (30%) | 42.2 |

oxylesterase (Supplementary Fig. 1). Tyr219 of EstC was suggested to play a role analogous to that proposed for Tyr105 of the *Citrobacter freundii* class C β-lactamase, activating Ser103 for nucleophilic attack of the ester-carbonyl of the substrate [17].

Est2K has a G-M-S-R-G sequence in its 370th to 374th amino acid residues (Supplementary Fig. 1). The G-x-S-x-G sequence, a reminiscence of the "lipase box", is located near the C-terminus of some members of the family VIII esterases [33–35]. However, the sequence was only partly conserved when a family VIII esterase, EstA from *Arthrobacter nitroguajacolicus*, and two other family VIII esterases, and AmpC were aligned [36]. EstC has a G-M-S-E-G sequence, and the sequence was found to correspond to the typical pentamotif G-x-S-x-G [17]. The typical pentamotif G-x-S-x-G contains a serine residue, which has a catalytic function in other esterase families [37]. However, when Est2K was aligned to the esterase mentioned above, the pentamotif and the serine residue was not conserved in most of them (Supplementary Fig. 1).

In the phylogenetic tree of Est2K and other similar proteins, Est2K was clustered with family VIII esterases (Supplementary Fig. 2). However, Est2K is distinguishable from other family VIII esterases in that it has a putative signal peptide. EstC which is derived from a leachate metagenome library was the only family VIII esterase with a putative signal peptide up to the point [17].

*Purification of Est2K*

Est2K was purified from a subclone with *est*2K by High-Q, CHT-II and *t*-Butyl HIC chromatographies. The recovery rate, purification fold, and the specific activity of the enzyme were 60.6%, 28.1-fold, and 17.1 U $mg^{-1}$ protein, respectively. Purified Est2K appeared as a band on a SDS–PAGE gel, and had a molecular mass of 44 kDa (Fig. 1) that corresponds well with the expected molecular mass of the mature protein and those of other family VIII esterases [13,17,36,38,39].

*Properties of Est2K*

Est2K exerted its maximal activity at 50 °C (Fig. 2A). Est2K retained 68% of the original activity after 15 min at 40 °C, but lost almost all the activity after 15 min of heat-treatment at 50 °C (Fig. 2B). Est2K was found to be an alkaline esterase and showed its maximal activity at pH 10 (Fig. 2C). EstCE1, an alkaline family

**Table 2**
Substrate specificity of Est2K.

| | Specific activity (U $mg^{-1}$) | Relative activity (%) | Km (μM) |
|---|---|---|---|
| PNP-acetate (C2) | 11.4 | 66.9 | 31.0 |
| PNP-butyrate (C4) | 17.1 | 100.0 | 51.5 |
| PNP-octanoate (C8) | 14.0 | 82.0 | 64.2 |
| PNP-caprate (C10) | 6.8 | 39.8 | 34.3 |
| PNP-laurate (C12) | 2.6 | 15.4 | nd |
| PNP-myristate (C14) | 0.3 | 1.9 | nd |
| PNP-palmitate (C16) | 0 | 0 | nd |

nd, not determined.

VIII esterase derived from a soil metagenome, showed only 28% amino acid sequence identity to Est2K [13]. Mono- and divalent cations showed no significant influence on the enzyme activity except that 5 mM $Cu^{2+}$ inhibited 41% of the activity (Table 1). PMSF at a concentration of 1 mM inhibited 94% of the activity (Table 1), indicating that the 103th serine residue is responsible for the catalytic activity. The enzyme activity was higher in the presence of methanol than in the presence of isopropanol, and the activity was much higher, 236.8%, in the presence of 5% methanol than in the presence of 1% isopropanol (Table 1). The enzyme was tolerant to methanol retaining over 90% of its activity in the presence of 30% methanol, and was sensitive to acetonitrile losing more than a half of its activity in the presence of 5% acetonitrile. The enzyme may find its biotechnological applications due to the fact that the enzyme is tolerant to methanol and that the activity is tremendously increased by methanol.

When pNP esters were used as the substrates, purified Est2K efficiently hydrolyzed ester bonds in short chain fatty acids, and the relative activities towards pNP-butyrate (C4), pNP-octanoate (C8), pNP-acetate (C2) and pNP-caprate (C10) were 100%, 82.0%, 66.9% and 39.8%, respectively (Table 2). The ratio of Est2K activity for pNP-C10 to pNP-C4 was 0.4, a value much higher than those of two other family VIII esterases, EstCE1 (0.03) and Est3A (0) [13]. The Km values for pNP-C4, pNP-C8, pNP-C2, and pNP-C10 were 51.5, 64.2, 31.0, and 34.3 μM, respectively (Table 2). The substrate specificity of the enzyme indicates that Est2K is a typical carboxylesterase [40]. Est2K showed no or negligible β-lactamase activity on ampicillin and nitrocefin, though Est2K showed the highest identity to EstC with promiscuous β-lactamase activity on nitrocefin [17]. It has been suggested that the recognition of β-lactam substrates by EstC could have been maintained as EstC evolved from class C β-lactamases [17,38], and that the lack of β-lactam-hydrolyzing activity in family VIII esterases could be due to steric reasons [41].

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.070.

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