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Gene Cloning, Expression, and Characterization of a New Carboxylesterase from *Serratia* sp. SES-01: Comparison with *Escherichia coli* BioHe Enzyme

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The carboxylesterase-encoding gene (*bioHs*) of a newly isolated strain, *Serratia* sp. SES-01, was cloned from the genomic DNA library by detecting formation of transparent halo around the colony on LB-tributyrin agar plates. The amino acid sequence of BioHs was highly similar to the members of the BioH enzyme family involved in the biotin biosynthetic pathway; it showed the highest similarity (91%) with that of *Serratia proteamaculans*. To compare BioHs with other BioH enzymes, the relatively well-known *bioHe* gene of *E. coli* was cloned with PCR. After we achieved high-level expression of soluble BioHs and BioHe through the exploration of different culture conditions, the purified BioHs and BioHe enzymes were characterized in terms of specificity, activity, and stability. BioHe was generally more robust to a change in temperature and pH and an addition of organic solvents than BioHs. The two enzymes exhibited a strong preference for carboxylesterase rather than for thioesterase and were optimal at relatively low temperatures (20–40°C) and alkaline pHs (7.5–9.0). The results in this study strongly suggested that both the BioHs and BioHe enzymes would be potential candidates for use as a carboxylesterase in many industrial applications.

Keywords: Biocatalyst, BioH, biotin biosynthesis, carboxylesterase, *Serratia* sp.

The biosynthetic pathway of biotin, an essential vitamin found in all living cells, has been studied extensively in bacteria. Pimeloyl-coenzyme A (pimeloyl-CoA) is the starting substrate for the consensus stages of the biotin biosynthetic pathway and is converted to biotin *via* 7-keto-8-amino-pelargonic acid, 7,8-diaminopelargonic acid, and dethiobiotin [15]. However, pimeloyl-CoA itself is generated by a number of different routes [15]. Gram-negative bacteria such as *Escherichia coli* and *Serratia marcescens* are most likely to use a modified fatty acid synthetic pathway [5], unlike the condensation of free pimelic acid and CoA by pimeloyl-CoA synthetase in *Bacillus sphaericus* and *Bacillus subtilis* [3, 7]. Although the mechanism of pimeloyl-CoA synthesis in Gram-negative bacteria is still not fully elucidated yet, the combined action of *bioC* and *bioH* gene products was proposed. BioC was presumed to be a specific pimeloyl carrier protein, and BioH could possibly transfer the pimeloyl moiety from the pimeloyl-BioC complex directly to CoA [6, 13]. Therefore, the BioH enzymes were regarded as both thioesterases, hydrolyzing a thioester bond of pimeloyl-BioC, and acyltransferases, transferring a pimeloyl unit to CoA, which was known to be involved exclusively in the biotin synthesis in Gram-negative bacteria.

The *bioH* gene has not always been found in *bioC*-containing bacteria. Various nonorthologous substitutes of *bioH* were also observed in many bacterial genomes; instead of *bioH*, *bioC*-coupled *bioG* and *bioK* were assumed to complement the natural function of *bioH* [8]. Even if the bacteria have *bioH*, the *bioH* gene was separated from the operon organization of the biotin synthetic genes including the *bioC* gene [8]. Recently, the BioH enzyme has been identified as a new carboxylesterase in addition to thioesterase and acyltransferase activities for the biotin synthesis pathway [1, 11, 16]. For example, *E. coli* BioH was investigated using a strategy that integrates the structural and bioinformatics data with the experimental data for several types of enzyme assays [11]. However, focus of the studies on BioH was placed mainly on their role for biotin biosynthesis by performing complementation test and gene mutation in the recombinant bacterial strain [9, 10]. Furthermore, although numerous studies have already been carried out to identify the nature of many different esterases such as carboxylesterases [2], few studies have been conducted to examine the characteristics of BioH enzyme as an esterase that is potentially useful for biocatalytic reactions. Thus, in this study, after identifying the *bioHs* gene from the *Serratia* sp. SES-01 genomic DNA

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library, we examined the enzymatic characteristics of BioHs with emphasis to the potential use as a carboxylesterase in biocatalytic reactions. This examination was also conducted on the PCR-cloned *E. coli bioHe* gene that has been relatively well studied among the BioH enzyme family.

Materials and Methods

Bacterial Strains, Plasmids, and Media

Serratia sp. SES-01, which was isolated from soil samples collected from the rapeseed fields of Jeju Island, Korea, served as the source for genomic DNA. *E. coli* XL-1 Blue (Stratagene) and JM109 (Promega) were used as cloning and expression hosts, respectively. Plasmid pUC19 (Invitrogen) and pQE30 (Qiagen) were used as cloning and expression vectors, respectively. *Serratia* sp. SES-01 and *E. coli* were grown in LB medium (pH 7.2) consisting of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 0.5% (w/v) NaCl. Tributyrin-emulsified indicator plates were prepared by filling each 83-mm petri dish with 25 ml of LB agar containing 1% tributyrin after emulsifying it with sonication.

Isolation of Lipolytic Bacteria from Soil

Soil samples were suspended in the enrichment medium containing 0.5% $(NH_4)_2HPO_4$, 0.1% of KH_2PO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.4% peptone, 0.2% yeast extract, 0.2% NaCl, and 0.2% tributyrin. The mixture was incubated at 28°C for 1 day on a rotary shaker (200 rpm). To isolate the strain based on its ability to produce a transparent halo indicative of lipolytic activity, the broth was spread on LB-tributyrin agar plates supplemented with cycloheximide (35 μg/ml) to control fungal growth. Plates were incubated at 30°C for 3–5 days and the isolates showing the lipolytic activity were purified by streaking single colonies three times on LB-tributyrin agar plates. The isolated strain was confirmed for lipase production in liquid medium supplemented with ampicillin (50 μg/ml), chloramphenicol (34 μg/ml), or kanamycin (10 μg/ml) at 25, 30, and 37°C.

The analysis of 16S rRNA gene sequence was used to identify the isolated strain taxonomically. Primers used for PCR amplification and sequencing of the 16S rRNA gene were 5'-AGAGTTTGATCCTGGCTCAG-3' and 5'-AAGGAGGTGATCCAGCCGCA-3'. The 16S rRNA gene sequences of the isolated strains were subjected to Blast search from the NCBI database. The SES-01 strain was deposited in the MicroBank Center (Daejeon, Korea) and designated as *Serratia* sp. SES-01.

Library Construction and Cloning of the *bioHs* Gene of *Serratia* sp. SES-01

To clone the gene encoding lipolytic enzyme from *Serratia* sp. SES-01, total DNA isolated using a Genomic DNA purification kit (Promega) was partially digested with Sau3AI. Restricted DNA was fractionated by agarose gel electrophoresis. The approximately 4-kb fragments were purified with a QiaexII gel extraction kit (Qiagen) and ligated into plasmid pUC19. The ligation mixture was used to electrotransform *E. coli* XL-1 Blue. Out of about 5,000 colonies, a colony forming a transparent halo around the colony on LB-tributyrin agar plates containing 100 μg/ml ampicillin was selected. Recombinant plasmid designated as pSL35 was purified from the transformant, and the insert DNA sequence was determined on a 310-ABI-PRISM Genetic Analyzer (Perkin-Elmer) using a Big Dye sequencing kit (Applied Biosystems Inc.). The nucleotide sequence from the *Serratia* sp. SES-01 in pSL35 plasmid including the gene encoding esterase activity was deposited in the GenBank sequence database under Accession No. EU414478.

PCR Cloning of the *bioHe* Gene of *E. coli*

Genomic DNA of *E. coli* K strain 12 was isolated from overnight-grown cells in LB media. The *E. coli bioHe* gene was amplified by PCR with a primer set 5-CAGGAGCTCGGTCGCGGATCCGAATTC-3 and 5-GTGGGTACCTCGAGTGCGGCCGCAAGCTTG-3 designed from the *E. coli bioH* sequence (Accession No. AAC76437). PCR was performed with AccuPower *Pfu* Premix (Bioneer, Korea) under the conditions of 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The *bioHe* gene amplified in this study was 100% identical to the known sequence of *E. coli bioH* gene (Accession No. AAC76437).

Expression and Purification of BioHs and BioHe

The *bioHs* gene from *Serratia* sp. SES-01 was amplified from plasmid pSL35 isolated from a lipolytic-activity-producing clone. Restriction enzyme sites for SacI and HindIII were designed in the forward primer and reverse primers (5-ACGGATGAGAATGGAGCTCCAATTACGG-3 and 5-CGAAGCTTTTTTTAGCGCTTTATTGT-3). After gene amplification was performed under the same conditions as in cloning the *E. coli bioH* gene, PCR products were gel-purified and ligated into the SacI- and HindIII-digested pQE30 expression vector (Qiagen). The resulting plasmid pQE-BioHs was transferred by electroporation into *E. coli* JM109 cells. The *E. coli* cells containing pQE-BioHs and pQE-BioHe were grown in LB medium supplemented with 50 μg/ml ampicillin at 25–37°C. Protein expression was induced by the addition of 0.5–1.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when the optical density at 600 nm reached 0.5. After the culture was incubated for an additional 4–6 h, the cells were harvested by centrifugation, washed with phosphate-buffered saline, and resuspended in lysis buffer containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 0.05% Tween 20. The suspension was incubated on ice for 30 min and then sonicated six times for 3 sec. After the cell lysate was centrifuged for 20 min at 4°C and 25,000×*g*, the soluble and insoluble fractions were analyzed on SDS-PAGE gels stained with Coomassie Brilliant Blue R-250. The two BioH enzymes fused with a six-histidine (6× His) affinity tag were purified from soluble fractions under nondenaturing conditions by automated affinity chromatography and a desalting system (Profinia protein purification system; Bio-Rad) with an immobilized metal affinity chromatography (IMAC) column and hydrophilic spherical polyacrylamide beads (Bio-Gel P-6). The concentration of the purified and desalted enzymes was determined by a Bio-Rad protein assay kit with bovine serum albumin as the standard.

Esterase Activity Assay

The carboxylesterase activity was measured by a spectrophotometric method using *p*-nitrophenyl esters (C2-C18) such as *p*-nitrophenyl butyrate (*p*NP-butyrate; C4) and *p*-nitrophenyl palmitate (*p*NP-palmitate; C16) [4]. The *p*-nitrophenyl ester was dissolved in acetonitrile at a concentration of 10 mM. Ethanol and 50 mM Tris-HCl (pH 8.5) were subsequently added to a final ratio of acetonitrile:ethanol:Tris-HCl buffer to 1:4:95 (v/v/v). The reaction was started by adding 50 μl of the purified enzyme in phosphate buffer to 200 μl of reaction mixture at 45°C, and absorbance at 405 nm was monitored for 20 min with a microplate reader (Bio-Rad) to detect the liberated *p*-nitrophenol product. One unit of esterase activity was defined as the amount of enzyme capable of releasing 1 μmol of *p*-nitrophenol per minute.

The thioesterase activity for CoA thioesters was determined spectrophotometrically using acetyl-CoA, malonyl-CoA, and palmitoyl-CoA, as described previously [14].

Effects of Temperature and pH on Activity and Stability of BioH Enzymes

(i) The effect of temperature on enzyme activity was determined by incubating the reaction mixtures at different temperatures and assaying the enzyme by the spectrophotometric method. (ii) The effect of temperature on the stability of BioHs and BioHe was determined by exposure of the enzyme solution to different temperatures (20–80°C) for 30 min. The residual enzyme activity was then measured under the standard assay conditions. (iii) The pH of the reaction mixtures was varied to determine the optimal pH using different buffers, in which the pH ranged from 3.0 to 10.0 (50 mM sodium citrate for pH values of 3.0, 4.0, 5.0, and 6.0; 50 mM sodium phosphate for pH values of 6.0, 7.0, and 8.0; 50 mM Tris-HCl for pH values of 8.0, 9.0, and 10.0). (iv) The pH stability of the enzyme was evaluated by incubating the enzyme solution for 1 h at various pH values at 25°C. The residual enzyme activity was determined under the standard assay conditions.

Effect of Organic Solvents on Stability of BioH Enzymes

The stability against organic solvents was examined by diluting the purified enzyme solution in different final concentrations (10–30%) of organic solvents. After 30 min incubation at 20°C in either methanol, ethanol, isopropanol, 1-butanol, acetonitrile, acetone, or dimethyl sulfoxide (DMSO), the residual activity was assayed with *p*NP-butyrate. In order to correct an abnormal increase of OD values caused by organic solvents added, all experiments were accompanied with each control sample containing only the same amount of organic solvents.

Effect of Metal Ions and Surfactants on Activity of BioH Enzymes

The purified BioH enzymes were preincubated with various metal ions and reagents at a 1, 5, or 10 mM concentration in 50 mM sodium phosphate buffer (pH 7.0) at 20°C for 30 min. The residual esterase activity was assayed with *p*NP-butyrate as a substrate.

RESULTS AND DISCUSSION

Isolation of Bacterial Strain Producing Lipolytic Enzyme

We isolated bacterial strains capable of producing lipolytic enzyme such as lipase and esterase from approximately 20 soil samples. More than 10 strains produced a transparent halo surrounding the colony on LB agar plates containing emulsified 1% tributyrin, indicating that they coded for at least one lipolytic enzyme. Among them, the strain SES-01 showing a relatively higher lipolytic activity was isolated and finally used for further study. The strain was systematically identified by general procedures of Bergey's manual and by 16S rRNA sequencing. Comparative rRNA sequence analysis and its phenotypic characteristics supported a strong relationship between strain SES-01 and members of genus *Serratia*. The 1,434-bp PCR product of the 16S rRNA gene sequence of strain SES-01 (Accession No. EU414474) was almost identical to that of *S. grimesii* strain DSM 30063 (Accession No. AJ233430; 99.93%) [12]. It also showed slightly less similarity to sequences of *S. proteamaculans* strain DSM4543 (Accession No. AJ233434; 99.65%), *S. quinivorans* strain DSM 4597 (Accession No. AJ233435; 99.37%) and several other *Serratia* not identified to the species level. The SES-01 strain was tentatively referred to as a *Serratia* sp. and deposited in the MicroBank Center (Daejeon, Korea) with the collection number SESm01.

Cloning and Sequence Analysis of the *bioHs* Gene of *Serratia* sp. SES-01

The genomic library of *Serratia* sp. SES-01 was constructed by cloning partially Sau3AI-digested chromosomal DNA fragments into plasmid pUC19. The *E. coli* XL1-Blue transformants harboring gel-purified 4–8 kb DNA fragments were screened on LB-tributyrin agar plates. The positive colony showing a transparent halo as indicative of lipolytic activity was found among about 40,000 transformants after incubation for 24 h at 30°C. After the plasmid pSL35 (3,222 bp) was isolated, the sequence of the 3.2-kb genomic insert from *Serratia* sp. SES-01 was deposited in GenBank under Accession No. EU414478.

The ORF (*bioHs* gene) of 258 amino acids conferring the esterase activity was identified using ORF finder (http://www.ncbi.nlm.nih.gov/gorf) and SIB BLAST program (http://www.expasy.org/) (Fig. 1). Three ORFs with unknown function and an ORF encoding ferrous iron transport protein B (*feoB*) were also predicted downstream to *bioHs*. The genomic organization of these genes found in *Serratia* sp. SES-01

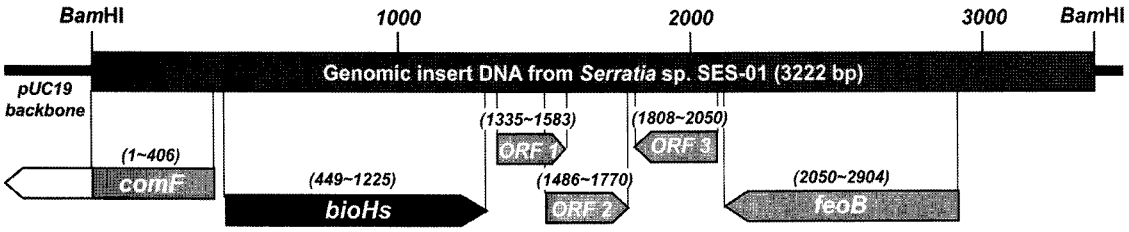

Fig. 1. Schematic diagram of the pSL35 plasmid containing the 3.2-kb genomic DNA fragment from *Serratia* sp. SES-01.
The whole nucleotide and amino acid sequences were analyzed using sequence database searches such as BLAST at NCBI (National Center for Biotechnology Information) and FASTA at EBI (European Bioinformatics Institute). The arrows with the nucleotide number in parentheses indicate the locations and directions of ORFs, with putative genes shaded in grey and *bioHs* gene in black.

Table 1. Comparison of *Serratia* sp. SES-01 BioHs with its closest known relatives.

Microorganism	Accession No.	Length (aa)	MW (kDa)	Similarity to BioHs[a]	Source of sequence information[b]
Serratia sp. SES-01	EU414478	258	28.3	-	GC (this study)
Serratia proteamaculans 568	ABV43725	264	29.0	91	GS
Serratia marcescens	Q8GHL1	255	27.9	82	GC
Erwinia carotovora subsp. *atroseptica*	CAG77029	255	27.9	74	GS
Yersinia pestis	NP991531	258	28.6	70	GS
Yersinia enterocolitica serotype O:8	YP001008141	258	28.6	69	GS
Klebsiella pneumoniae MGH 78578	ABR79169	257	28.2	68	GS
Salmonella typhi	CAD08105	256	28.2	68	GS
Shigella sonnei Ss046	AAZ90100	256	28.5	67	GS
Escherichia coli K12	AAC76437	256	28.5	67	GC (this study)
Enterobacter sakazakii BAA-894	ABU79505	259	28.4	65	GS
Photobacterium profundum 3TCK	EAS42694	254	28.2	53	GS
Vibrio fischeri ATCC700601	AAW84613	257	28.4	52	GS
Aeromonas salmonicida A449	AB092039	254	28.2	50	GS
Moritella sp. PE36	EDM65895	278	30.3	46	GS
Shewanella baltica OS185	ABS10315	264	29.6	43	GS
Buchnera aphidicola	AA027192	265	30.7	41	GS
Idiomarina loihiensis	AAV81089	255	28.4	41	GS
Wigglesworthia glossinidia brevipalpis	BAC24731	259	30.0	41	GS
Alteromonas macleodii	EAR06779	269	29.3	38	GS
Xanthomonas oryzae pv. *oryzae*	BAE66970	253	27.1	38	GS
Psychromonas ingrahamii strain 37	ABM04887	259	28.9	37	GS
Pseudoalteromonas atlantica	ABG42729	258	28.4	37	GS
Nitrosomonas europaea	Q82SL8	252	28.0	37	GS

[a]The percentage similarity scores were calculated using the ClustalW program on the EMBL-EBI Web site (http://www.ebi.ac.uk).
[b]GC and GS, indicate gene cloning and bacterial genome sequencing, respectively.

was very similar to those found in the results of genome sequencing of *S. proteamaculans* (Accession No. ABV43725) and cloning experiment conducted with *S. marcescens* (Accession No. Q8GHL1) [1]. BioHs of *Serratia* sp. SES-01 was highly similar to different members of the BioH enzyme family involved in the biotin synthetic pathway in Gram-negative bacteria (Table 1). The sequence alignment revealed a catalytic triad (Ser169-Asp217-His285 for BioHs), which is common in lipolytic enzymes of the a/b hydrolase superfamily (Fig. 2). In addition, the Gly80(100%)-Trp81(100%)-Ser82(100%)-Leu83(87.5%)-Gly84(100%) sequence around the catalytic Ser residue was very highly conserved among all the BioH enzymes listed in Table 1; the characteristic Gly-X-Ser-X-Gly motif (where X stands for any amino acid) is generally found in lipolytic enzymes.

Expression and Purification of BioHs and BioHe

The enzymatic nature of the BioH family is not yet sufficiently elucidated and there have been few researches reporting that high-level expression of BioH enzymes was achieved. We examined how the expression of *bioHs* genes took place in *E. coli*. In order to compare BioHs with other BioH enzymes, we decided to clone the *bioHe* gene found in *E. coli*. *E. coli* BioHe, which is relatively well studied on the enzymatic activity toward different esters as well as the function in the metabolic pathway of biotin synthesis, was 67% similar to BioHs. This experiment is necessary not only to investigate the biochemical properties of BioHs and BioHe but also to make the recombinant production for their use as new esterase enzymes.

When the genes were subcloned in-frame with the N-terminal His-tag-encoding sequence of pQE30 (pQ-BioHs and pQ-BioHe) and expressed in *E. coli* JM109, the *E. coli* cells gave a positive phenotype of transparent halo formation on LB-tributyrin agar plates. However, the typical attributes of a signal peptide such as a positively charged region, a hydrophobic region, and a signal sequence cleavage site were not predicted (http://www.cbs.dtu.dk/services/SignalP/). Most of the lipolytic enzymes generally produce a transparent halo on LB-tributyrin agar plates, even if they have no secretion signal sequence for *E. coli* and thus are expressed intracellularly. In fact the significantly high levels of BioH enzymes were detected in the total cell fractions, but not in the culture supernatants.

The high solubility of overexpressed enzymes is usually more advantageous in facilitating the biochemical characterization of enzymes as compared with the dissolving and refolding of enzyme aggregates or inclusion bodies.

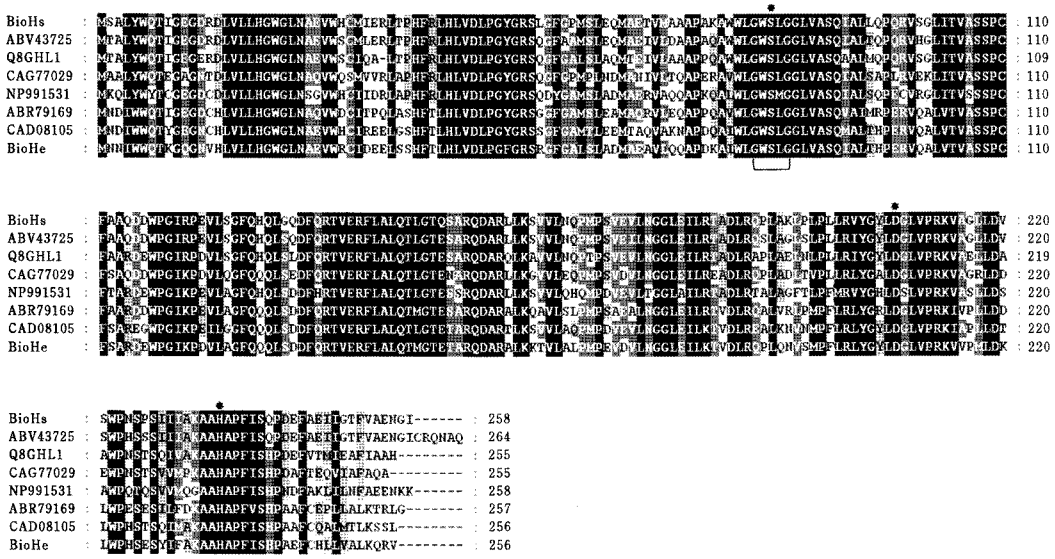

Fig. 2. Multiple sequence alignment of amino acid sequences of members of the BioH enzyme family.
For accession numbers, please refer to Table 1. Identical and similar amino acid residues are indicated on black and grey backgrounds, respectively. Asterisks indicate the putative catalytic triad (Ser/Asp/His). The G-W-S-L-G consensus motif is indicated with the vertical bracket.

To maximize the expression of soluble BioH enzymes, we therefore explored some experimental conditions such as culture temperature, induction time, and IPTG concentration. The highest solubility of BioHs was obtained when *E. coli* containing the *bioHs* gene was cultured at 25°C and induced with 1 mM IPTG for a further 6 h (data not shown). The highest solubility of BioHe was also found in the cultures grown at 30°C and induced with 1 mM IPTG for a further 4 h (data not shown). The 6× His-tagged BioHs and BioHe enzymes from the samples showing the highest solubility were purified and desalted using an automated affinity chromatography system under nondenaturing conditions (Fig. 3). Each BioHs and BioHe in a soluble and enzymatically active form was finally purified about 12.5-fold and 3.5-fold to homogeneity with overall enzyme yield of 25.1% and 33.3%, respectively.

Substrate Specificity

As shown in Table 1, most of the BioH enzymes similar to the BioHs of *Serratia* sp. SES-01 were from whole genome sequencing and their enzymatic nature has not been well understood yet. Only two *bioH* genes of *S. marcescens* [1] and *E. coli* [11] were studied experimentally, but most of the studies focused on their function of pimeloyl-CoA synthesis in the biotin synthetic pathway [5, 6, 13, 15]. In the previous work performed by Sanishvili *et al.* [11], the enzymatic activity of *E. coli* BioH whose sequence was 100% identical to BioHe of this study was investigated with different hydrolase substrates.

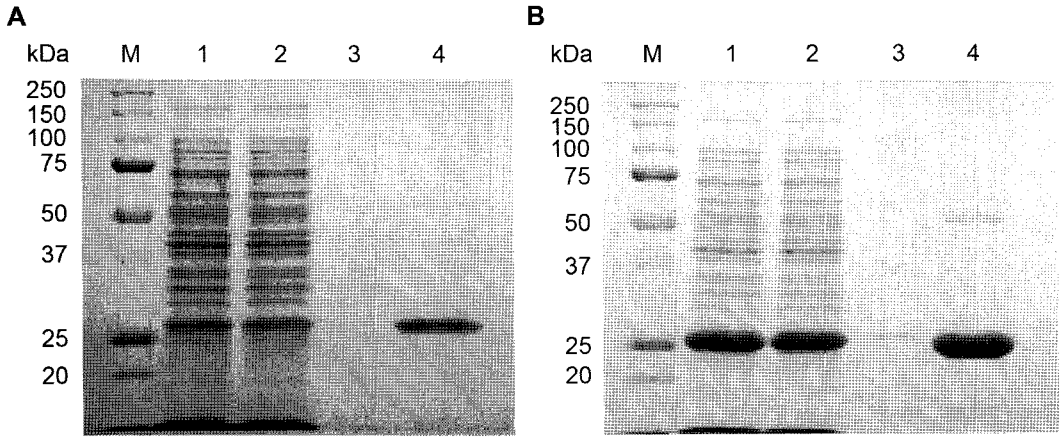

Fig. 3. SDS-PAGE of purified BioHs (**A**) and BioHe (**B**).
Lane M, molecular mass standards (Bio-Rad); lane 1, total homogenate of cells expressing BioH enzymes; lane 2, soluble fraction; lane 3, insoluble fraction; lane 4, BioH enzymes purified with affinity chromatography.

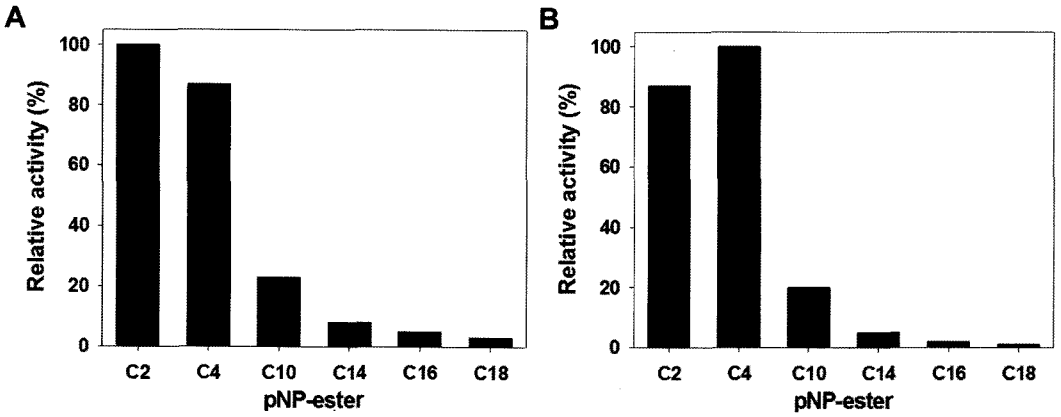

Fig. 4. Activity of BioHs (**A**) and BioHe (**B**) towards *p*-nitrophenyl esters with acyl chains of different lengths.
The carboxylesterase activities of BioH enzymes were expressed as percentages of the highest value of activity for each enzyme. Three independent experiments were carried out, and values whose error ranges were within 5% were averaged. C2, *p*NP-acetate; C4, *p*NP-butyrate; C10, *p*NP-caprate; C14, *p*NP-myristate; C16, *p*NP-palmitate; C18, *p*NP-stearate.

It was revealed that *E. coli* BioHe has significant carboxylesterase activity, with a preference for short acyl chain length of *p*NP esters. It was also demonstrated that *E. coli* BioH showed low enzymatic activities for thioesterase, lipase, and aminopeptidase, but showed no detectable enzymatic activity for phosphatase, trypsin-like endopeptidase, or perhydrolase. Meanwhile, the *bioH* gene of *S. marcescens* was cloned from the genomic DNA library by detecting clear halo around the colony on tributyrin plates, indicating that it encoded an esterase activity acting on some other ester substrates as well as a thioesterase/acyltransferase activity participating in pimeloyl-CoA synthesis [1]. However, to the best of our knowledge, there is no published information that describes the enzymatic characteristics of *S. marcescens* BioH. We attempted here for the first time to examine the newly identified BioHs of *Serratia* sp. SES-01 by assaying the esterase activity under various conditions.

First of all, the enzymatic activities were measured with *p*NP esters and CoA thioesters under the same assay conditions such as pH, temperature, molar concentration, *etc.* The specific activities of the purified BioHs and BioHe were 0.510 and 0.598 U/mg (using *p*NP-butyrate as a substrate) and 0.0153 and 0.0515 U/mg (using malonyl CoA as a substrate), respectively. Under the assay conditions tested, thioesterase activity for CoA thioesters was relatively low compared with carboxylesterase activity for *p*NP esters. More importantly, the ratio of the enzymatic activity for *p*NP ester versus CoA thioester was significantly different from each other (33.3:1 in BioHs; 11.6:1 in BioHe). This ratio was almost the same as those in the experiments using other substrates with a fatty acid moiety of different chain length. These results suggest that BioH enzymes such as BioHe and BioHs can be used as carboxylesterase for *in vitro* biocatalysis, emphasizing that BioHs has the higher possibility to function *in vivo* as a carboxylesterase acting on other substrates besides pimeloyl-CoA.

The substrate specificity of the two BioH enzymes was further investigated by spectrophotometric assay with *p*NP esters of different chain lengths (Fig. 4). The esterase assay was carried out using 0.1 mM of each *p*NP ester in a Tris-HCl buffer (pH 9.0) at 25°C (BioHe) and 35°C (BioHs), respectively, and the OD values at 405 nm were monitored for 10 min. As previously noted by Sanishvili *et al* [11], BioHe showed a preference for short chain substrates such as *p*NP-acetate and *p*NP-butyrate, even though the esterase activity was slightly higher for *p*NP-butyrate than for *p*NP-acetate, unlike the results of the previous report. The BioHs enzyme also showed an evident preference for the short chain *p*NP esters, with the highest activity for *p*NP-acetate. They already explained the preference for the short chain *p*NP esters in agreement with the BioHe crystal structure, emphasizing that the active site was sufficiently large to accommodate short chain substrates with very similar affinities for the range from *p*NP-acetate to *p*NP-caproate [11].

Effects of Temperature and pH

The effects of temperature and pH on the activity and stability of BioHs and BioHe were examined using *p*NP-butyrate as the assay substrate (Fig. 5). The effect of pH on the activity of the two enzymes was very similar to each other and their activities were more than 90% in the pH range from 7 to 9 (Fig. 5C). The BioHe exhibited maximum activity at a significantly lower temperature (20 to 30°C), unlike that the optimum temperature of BioHs was 30 to 40°C (Fig. 5A). Thermostability was analyzed by incubating the purified enzyme at various temperatures for 30 min. Interestingly, more than 95% activity of BioHe remained at 55°C, but the thermostability of BioHs decreased dramatically as the temperature increased; the remaining activity of BioHs was almost diminished at 50°C (Fig. 5B). The effect of pH on enzyme stability was determined by the measurement of the remaining activities at pH 9.0 after incubating the

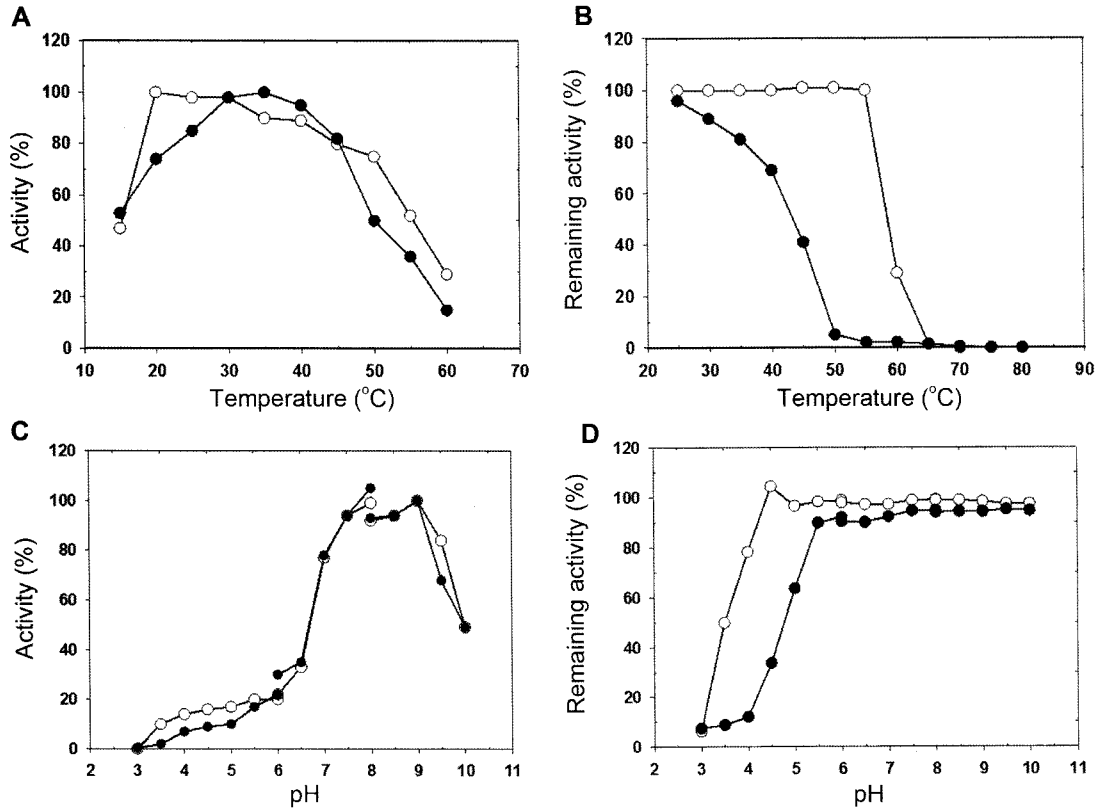

Fig. 5. Effects of temperature and pH on the activity and stability of BioHs and BioHe.
The purified BioH enzymes were assayed at different temperatures (**A**) or pH values (**C**). The remaining activity was measured after incubation of the purified BioH enzymes at different temperatures (**B**) or pH values (**D**). Symbols represent BioHs (closed circle) and BioHe (open circle). Three independent experiments were carried out, and values whose error ranges were within 5% were averaged.

purified enzymes at various pH values for 1 h (Fig. 5D). Both the BioHs and the BioHe exhibited relatively broad stability with retention of more than 90% of maximum activity in the pH range from 5 to 10. BioHe was more stable in the broader range of pH (from 4 to 10) like the assay for thermostability. These results indicate that the biochemical properties of two BioH enzymes whose similarity was 67% can be very different from each other although their function in the biosynthesis of pimeloyl-CoA was nearly identical.

Enzyme Stability in the Presence of Organic Solvents

Tolerance of BioH enzymes in organic solvents, which can be important when using enzymes for synthesis of an ester and in harsh environments, has not been examined yet. When the remaining activity was measured after 30-min incubation of the purified enzymes in different concentrations of organic solvents, BioHe was comparatively more tolerant to organic solvents than BioHs (Table 2). More than 80% activity of BioHe remained after 30-min incubation in 30% methanol, ethanol, acetone, or DMSO solutions, and in particular, DMSO did not seriously affect the enzyme activities of BioHs as well as BioHe. In contrast, BioHs was markedly destabilized in most of the organic solvents tested other than DMSO.

Effects of Metal Ions and Surfactants

We next examined the effect of divalent metal ions on the activity of BioHs and BioHe by adding 1, 5, or 10 mM $CaCl_2$, $CoCl_2$, $CuCl_2$, $FeSO_4$, $MgCl_2$, $MnCl_2$, $NiCl_2$, or $ZnCl_2$ to the

Table 2. Stability of BioH enzymes in the presence of organic solvents.

Organic solvents	Remaining activity (%) at each concentration of organic solvents					
	10%		20%		30%	
	BioHs	BioHe	BioHs	BioHe	BioHs	BioHe
Control	100	100	100	100	100	100
Methanol	93	100	71	91	2	93
Ethanol	92	97	41	91	5	84
Isopropanol	84	91	25	89	4	30
1-Butanol	27	89	14	51	5	38
Acetonitrile	66	93	19	37	3	11
Acetone	88	94	31	92	4	85
DMSO	93	101	90	100	76	100

assay solution. In the case of BioHe, we found that 5 and 10 mM Co^{2+} increased the esterase activity to more than 150% of that of the control and that Cu^{2+} decreased the activity to less than 70% of that of the control. However, the metal ions tested showed no significant effect on the enzyme activity of BioHs besides an increase of about 10% by the addition of 10 mM Co^{2+} and Mn^{2+}. EDTA treatment did not inhibit the activity of the two enzymes at all the concentrations tested (1, 5, and 10 mM), suggesting that it is not a metalloenzyme. Non-ionic detergents such as Triton X-100, Tween 20, and Tween 80 neither enhanced nor inhibited the enzyme activity at the final concentration ranged from 0.2% to 5%, whereas the anionic detergent SDS severely decreased the activity to 48% (BioHe) and less than 1% (BioHs) at the final concentration of 0.2% SDS.

In conclusion, we cloned, expressed, and characterized two *bioH* genes (*bioHe* from *E. coli* and *bioHs* from a soil isolate, *Serratia* sp. SES-01). Although the BioH enzymes from different Gram-negative bacteria have been already well known to function as a pimeloyl-CoA thioesterase/acyltransferase for the biotin synthetic pathway, the biochemical properties of *E. coli* BioHe and *Serratia* sp. BioHs were investigated to facilitate possible applications such as hydrolysis and synthesis of ester compounds. Together with the results of previous studies on *E. coli* BioHe enzyme [11], our results clearly indicate that BioH enzymes such as the BioHs and BioHe in this study can be used as new carboxylesterases acting on many other substrates besides acyl-CoA substrates. The fundamental characteristics were generally similar between *E. coli* BioHe and *Serratia* sp. BioHs. However, they were significantly different in their enzymatic properties such as substrate specificity (the ratio of carboxylesterase versus thioesterase and the chain length specificity) and enzymatic stability on organic solvents, pH, and temperature. We are further investigating the actual application of the two BioH enzymes using a variety of short chain aliphatic and aromatic carboxylic ester compounds as well as the efficient method to produce the catalytically active enzyme in larger quantity.

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