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Identification of the Gene Encoding Esterase, a Homolog of Hormone-Sensitive Lipase, from an Oil-Degrading Bacterium, Strain HD-1[1]

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The gene encoding an esterase (HDE) was cloned from an oil-degrading bacterium, strain HD-1. HDE is a member of the hormone-sensitive lipase family and composed of 317 amino acid residues with a molecular weight of 33,633. The HDE-encoding gene was expressed in *Escherichia coli*, and the recombinant protein was purified and characterized. Amino acid sequence analysis indicated that the methionine residue was removed from its NH_2-terminus. The good agreement of the molecular weights estimated by SDS-PAGE (35,000) and gel filtration (38,000) suggests that it acts in a monomeric form. HDE showed hydrolytic activity towards *p*-nitrophenyl esters of fatty acids with an acyl chain length of 2 to 14 and tributyrin, whereas it showed little hydrolytic activity towards *p*-nitrophenyl oleate (C_{18}), tricaprylin and triolein. Determination of the kinetic parameters for the hydrolyses of the *p*-nitrophenyl substrates from C_2 to C_{14} indicated that HDE shows a relatively broad substrate specificity. However, comparison of the k_{cat}/K_m values indicated that the C_{10}-C_{14} substrates are the most preferred ones. Such a preference for substrates with long acyl chains may be a characteristic of HDE.

Key words: esterase, oil-degrading bacterium, gene cloning, hormone-sensitive lipase, substrate specificity.

We previously isolated an oil-degrading bacterium, strain HD-1, which degrades aliphatic and aromatic hydrocarbons under both aerobic and anaerobic conditions, from an oil spring in Shizuoka (*1, 2*). This mixotrophic bacterium can also grow autotrophically, fixes CO_2, and produces alkanes/alkenes (*3*). The detection of ^{14}C-alkanes/alkenes upon the anaerobic incubation of HD-1 cells in the presence of a ^{14}C-fatty acid or ^{14}C-fatty aldehyde strongly suggests that HD-1 can utilize fatty acids and fatty aldehydes as substrates for alkane/alkene synthesis (*3*). Because esterases and lipases hydrolyze a variety of carboxylesters to produce fatty acids, these enzymes may be involved in the production of fatty acids in HD-1. Therefore, we have decided to clone the gene encoding esterase or lipase from this strain and to characterize the enzymatic properties of the recombinant protein. These studies are part of an effort to elucidate a mechanism by which strain HD-1 produces alkanes/alkenes from CO_2.

Esterases [EC 3.1.1.1] and lipases [EC 3.1.1.3] are widely present in various organisms from bacteria to higher eucaryotes. A characteristic common to these enzymes is that they contain a catalytic triad, composed of Ser, His, and Asp/Glu (*4*). In addition, most of these enzymes have a structural motif, G-X-S-X-G (where X represents any amino acid), which contains the active-site serine residue (*5*). This motif is usually located between a β-strand and an α-helix, and assumes an extremely sharp turn called a *nucleophile elbow* (*6*). Esterases and lipases hydrolyze the substrates through the same mechanism. They hydrolyze ester bonds through the formation of an acylenzyme intermediate in which the hydroxyl group of Ser is acylated by the substrate. However, they are different from each other in substrate specificity. Esterases prefer substrates which are soluble in water, whereas lipases prefer triglycerides with long acyl chains which are not soluble in water. Structural studies revealed that this difference is caused by the absence or presence of a "lid" structure, which is responsible for the interfacial activation of lipase (*7-9*).

Database analyses have shown that mammalian hormone-sensitive lipase (HSL) and several bacterial proteins form a HSL family (*10, 11*). HSL is relatively large in size and composed of an N-terminal domain of unknown function, the catalytic domain, and the regulatory domain (*12, 13*). The bacterial HSL members are only homologous in this catalytic domain of HSL and exhibit either esterase (*14-16*) or lipase (*17*) activity. Of them, the crystal structure of brefeldin A esterase from *Bacillus subtilis*

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Abbreviations: HDE, HD-1 esterase; (k)bp, (kilo)base pair.

(BFAE) has been determined (*18*). Mammalian HSLs hydrolyze triacylglycerols stored in adipose tissue under acute hormonal control and are therefore responsible for the liberation of fatty acids that serve as an energy source (*19*). In contrast, the physiological functions of the bacterial HSL members remain to be determined.

In this study, we have cloned the gene encoding an esterase from strain HD-1 (HDE), which is a member of the HSL family. We have overexpressed it in *Escherichia coli*, purified the recombinant protein, and compared its enzymatic properties with those of other bacterial HSL members. We found that this enzyme hydrolyzes *p*-nitrophenyl esters of fatty acids with long acyl chains more preferably than other esterases in the HSL family do.

MATERIALS AND METHODS

Cells and Plasmids—*E. coli* DH5α [F^-, ϕ80, *lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, hsdR17(r_k^-, m_k^+), *SupE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169, λ^-] and plasmid pBluescript KS(+) were obtained from Toyobo (Kyoto). Cells were grown in Luria-Bertani (LB) medium (*20*) containing 100 mg/liter ampicillin.

Construction and Screening of a HD-1 DNA Library—Genomic DNA of strain HD-1 was prepared from a Sarkosyl lysate as previously described (*21*). This DNA was completely digested with an appropriate restriction enzyme and the resultant DNA fragments were ligated into plasmid pBluescript KS(+). The resultant plasmids were used to transform *E. coli* DH5α. Colonies were grown on a plate of LTB-agar medium (L broth supplemented with 1% tributyrin, 0.1% Tween 80, 100 mg/liter ampicillin, and 1.5% agar) at 37°C. Plasmid DNAs were isolated from colonies which gave clear haloes, and used for further subcloning and sequencing. The DNA sequence was determined by the dideoxy-chain termination method with fluorescent dye terminators using an ABI PRISM™ 310 DNA sequencer (Perkin-Elmer, Tokyo). Nucleotide and amino acid sequence analyses, including the localization of open reading frames, molecular weight determination, and multiple alignment, were performed using DNASIS software (Hitachi Software, Tokyo).

Overproduction and Purification—The plasmid containing the 1.6-kbp *Not*I-*Eco*RI fragment of the genomic DNA of HD-1 was used to overproduce HD-1 esterase (HDE). In this plasmid, the transcription of the HDE-encoding gene is under the control of the *lac* promoter and/or its own promoter. The transformant of *E. coli* DH5α with this plasmid was grown at 37°C overnight. Cells were then harvested by centrifugation and subjected to the following purification procedures.

All purification procedures were carried out at 4°C. Cells from a 1-liter culture were suspended in 25 ml of 40 mM Tris-HCl (pH 7.8) containing 25% sucrose, and then lysed with lysozyme by the method of Nakamura and Yura (*22*). The supernatant (crude extract) was brought to 40% saturation with ammonium sulfate. After stirring for 15 min, the solution was centrifuged at 12,000 × *g* for 15 min. The resultant supernatant was brought to 60% saturation with ammonium sulfate. After stirring for 15 min, the solution was again centrifuged at 12,000 × *g* for 15 min. The precipitate was dissolved in 10 ml of 10 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (TE buffer), and then applied to a column (2 ml) of DE-52 (Whatman, Tokyo) equilibrated with the same buffer. After washing the column, HDE was eluted from the column with a linear gradient of NaCl, from 0 to 0.5 M, in the TE buffer. The enzyme fractions were pooled, concentrated to 2 ml by Centricon 10 centrifugal concentration (Amicon, Tokyo), and then applied to a column (120 ml) of Superdex 200 (Pharmacia Biotech, Piscataway, NJ, USA) equilibrated with the TE-buffer containing 0.1 M NaCl. The enzyme eluted from this column was used for biochemical characterization. For estimation of the molecular weight, bovine serum albumin, ovalbumin, chymotrypsinogen A, and RNase A, with molecular weights of 67,000, 43,000, 25,000, and 13,700, respectively, were individually applied to this column as standard proteins. The level of production of HDE in the cells and the purity of this enzyme were analyzed by SDS-PAGE (*23*).

Enzymatic Activity—The enzymatic activities for the hydrolyses of various *p*-nitrophenyl esters of fatty acids with acyl chain lengths from 2 to 18 were determined in 100 μl of 20 mM sodium phosphate (pH 7.1) containing 20% acetonitrile at 25°C for 15 min. The reaction was terminated by the addition of SDS to the final concentration of 0.2%. The amount of *p*-nitrophenol produced through the reaction was determined from the molar absorption coefficient value of 14,200 $M^{-1} \cdot cm^{-1}$ at 412 nm. One unit of enzymatic activity was defined as the amount of the enzyme that produced 1 μmol *p*-nitrophenol per min at 25°C. For the kinetic analyses, the substrate concentration spanned the K_m value. The hydrolysis of the substrate with the enzyme followed Michaelis-Menten kinetics, and the kinetic parameters, K_m and V_{max}, were determined from a Lineweaver-Burk plot.

The enzymatic activities for the hydrolyses of triglycerides were determined as previously described (*24*) with a slight modification. The reaction mixture, that contained 55 μl of triglyceride in an emulsified state and an appropriate amount of enzyme in 1.5 ml of 25 mM Tris-HCl (pH 8.0), was incubated at 30°C for 30 min with constant shaking (150 oscillations/min). The reaction was terminated by the addition of 5 ml of acetone-ethanol (1:1, v/v), and then the liberated fatty acid was titrated with 2 mM NaOH. One unit of enzymatic activity was defined as the amount of the enzyme that liberated 1 μmol of fatty acid per min.

The protein concentration of HDE was determined from the UV absorption using an $A_{280}^{0.1\%}$ value of 0.59, which was calculated by using 1,576 $M^{-1} \cdot cm^{-1}$ for tyrosine and 5,225 $M^{-1} \cdot cm^{-1}$ for tryptophan at 280 nm (*25*).

Stability against Heat Inactivation—The stability against irreversible heat inactivation was analyzed by incubating the enzyme in 20 mM sodium phosphate (pH 7.1) at 70, 80, and 90°C. The protein concentration was 0.15 mg/ml. At appropriate intervals, aliquots were withdrawn and the enzymatic activity was determined using *p*-nitrophenyl caproate (C_6) as a substrate. The residual activity was calculated by dividing the activity determined after incubation with that determined before incubation.

Amino Acid Sequence Analysis—The NH_2-terminal amino acid sequence of the protein was determined with a pulse-liquid automated sequencing system, Procise 491 (Perkin-Elmer, Tokyo).

RESULTS AND DISCUSSION

Cloning of the Gene Encoding HD-1 Esterase—An LTB-agar plate is often used to examine whether bacteria produce esterases or lipases, because the hydrolysis of tributyrin emulsified in the LTB-agar medium by these enzymes results in the formation of a clear halo. When strain HD-1 was grown on this plate, a weak halo was detected around each colony, suggesting that HD-1 produces an esterase or a lipase. We constructed a plasmid library by ligating the DNA fragments generated on digestion of the HD-1 genome with *Sac*I to plasmid pBluescript KS(+), and transformed *E. coli* DH5α with it. Screening for an *E. coli* transformant that forms a clear halo on an LTB-agar plate indicated that the 6.7-kbp *Sac*I fragment is responsible for the formation of the halo. In the subcloning experiments, a 1.6-kbp *Not*I-*Eco*RI fragment was shown to be sufficient for the formation of a halo. Determination of the DNA sequence suggested that this DNA fragment contains the entire gene encoding HD-1 esterase (HDE) (data not shown). Construction of plasmid libraries using other restriction enzymes, followed by screening for *E. coli* transformants which form clear haloes on LTB-agar plates suggested that the HD-1 genome does not contain additional genes encoding an esterase or lipase. HDE is composed of 317 amino acid residues with a calculated molecular weight of 33,633 and a pI value of 5.2. A potential Shine-Dalgarno (SD) sequence is located six nucleotides upstream of the initiation codon for translation. In addition, possible promoter sites are located in the 5′-noncoding region.

Amino Acid Sequence—Database searches for proteins with similar amino acid sequences to that of HDE indicated that HDE is a member of the HSL family. Several representative members of the HSL family are listed in Table I. Of them, esterases from *Bacillus acidocaldarius* (BaE) (*15*) and *E. coli* (EcE) (*14*), and brefeldin A esterase from *B. subtilis* (BFAE) (*16*) have been shown to exhibit carboxylesterase activity, instead of lipase activity. In contrast, human HSL (hHSL) (*19*) and lipase from *Moraxella* TA144 (Mol2) (*26*) have been shown to exhibit lipase activity. It remains to be determined whether other proteins exhibit esterase or lipase activity. Because hHSL and Mol2 are larger than BaE, EcE, and BFAE in size, hHSL and Mol2 may have a lid or related one which is responsible for the interfacial activation.

The amino acid sequence of HDE is aligned with those of BaE, EcE, BFAE, and hHSL in Fig. 1. A G-D/E-S-X-G motif, which is characteristic of the HSL family and contains the active-site serine residue, as well as the histidine and aspartic acid residues which are presumed to be two other components of a catalytic triad, are conserved in the HDE sequence. In addition, a HGGG motif, which is involved in the formation of the oxyanion hole, is conserved. These results suggest that HDE shares a common three-dimensional structure with other HSL members.

According to the crystal structure, BFAE has a typical α/β hydrolase fold with a unique extended N-terminal subdomain, and is composed of an eight-stranded β-sheet and eleven α-helices (18). The alignment of amino acid sequences shown in Fig. 1 suggests that HDE shares these secondary structures with BFAE, except for the α1-helix, which may be missing in the HDE structure. Ser^{202}, Asp^{308}, and His^{338} have been proposed to form a catalytic triad in BFAE. The previous data indicating that the individual mutations of the corresponding residues in rat HSL (*27, 28*) and EcE (*29*) almost fully inactivate the enzyme support the prediction that these residues form a catalytic triad. Therefore, it seems highly likely that Ser^{160}, Asp^{257}, and His^{287} form a catalytic triad in HDE.

Purification of Recombinant HDE—When the *E. coli* DH5α transformant with the plasmid containing the HDE-encoding gene was grown at 37°C, HDE was constitutively produced and accumulated in the cells as the most abundant protein (Fig. 2). Because the amount of HDE accumulated in the cells did not seriously change upon induction with isopropyl-β-D-thiogalacto-pyranoside (data not shown), the transcription of the HDE-encoding gene is probably under the control of its own promoter. We have not examined whether recombinant HDE accumulated in the cytoplasm or the periplasm of the *E. coli* cells. However, the absence of the typical signal sequence in the HDE sequence suggests that it is localized in the cytoplasm. The production level was estimated to be 30 mg/liter culture from the intensity of the band visualized with Coomassie Brilliant Blue. The protein was recovered in a soluble form from the cells and purified by three purification procedures to give a single band on SDS-PAGE (Fig. 2). The overall purification yield was 13% and approximately 4 mg of the protein was purified from a 1-liter culture. These values were relatively low, because only the fractions that contained the protein with high purity were pooled in each purification step.

Biochemical Characterization—The NH_2-terminal amino acid sequence of recombinant HDE was determined

TABLE I. **List of the major HSL members.**

Abbrev.	Enzyme	Size (no of amino acid residues)	Identity[a] (%)	Origin	Accession number
AeL	Lipase-like enzyme	364	43.8	*Alcaligenes eutrophus*	PIR 139567
BaE	Esterase	310	40.4	*Bacillus acidocaldarius*	GenBank X62835
MtlH	lipH	319	34.7	*Mycobacterium tuberculosis*	EMBL CAB02180
AfE	Esterase (estA)	311	32.8	*Archaeoglobus fulgidus*	GIAAB89533
REE	Heroine esterase	322	29.0	*Rhodococcus* sp.	GIAAC45283
AcE	Carboxylesterase	356	24.6	*Acinetobacter calcoaceticus*	PIR 139510
BFAE	Brefeldin A esterase	372	21.5	*Bacillus subtilis*	GenBank AF056081
Mol2	Lipase 2	433	21.1	*Moraxella* TA144	Swiss-Prot LIP2_MORSP
EcE	Esterase	319	20.2	*E. coli*	Swiss-Prot YBAC_ECOLI
hHSL	Hormone-sensitive lipase	786	17.0	*Homo sapiens*	Swiss-Prot LIPS_HUMA

[a]The amino acid sequence identity between HD-1 esterase and each protein in the region in which the two sequences are aligned.

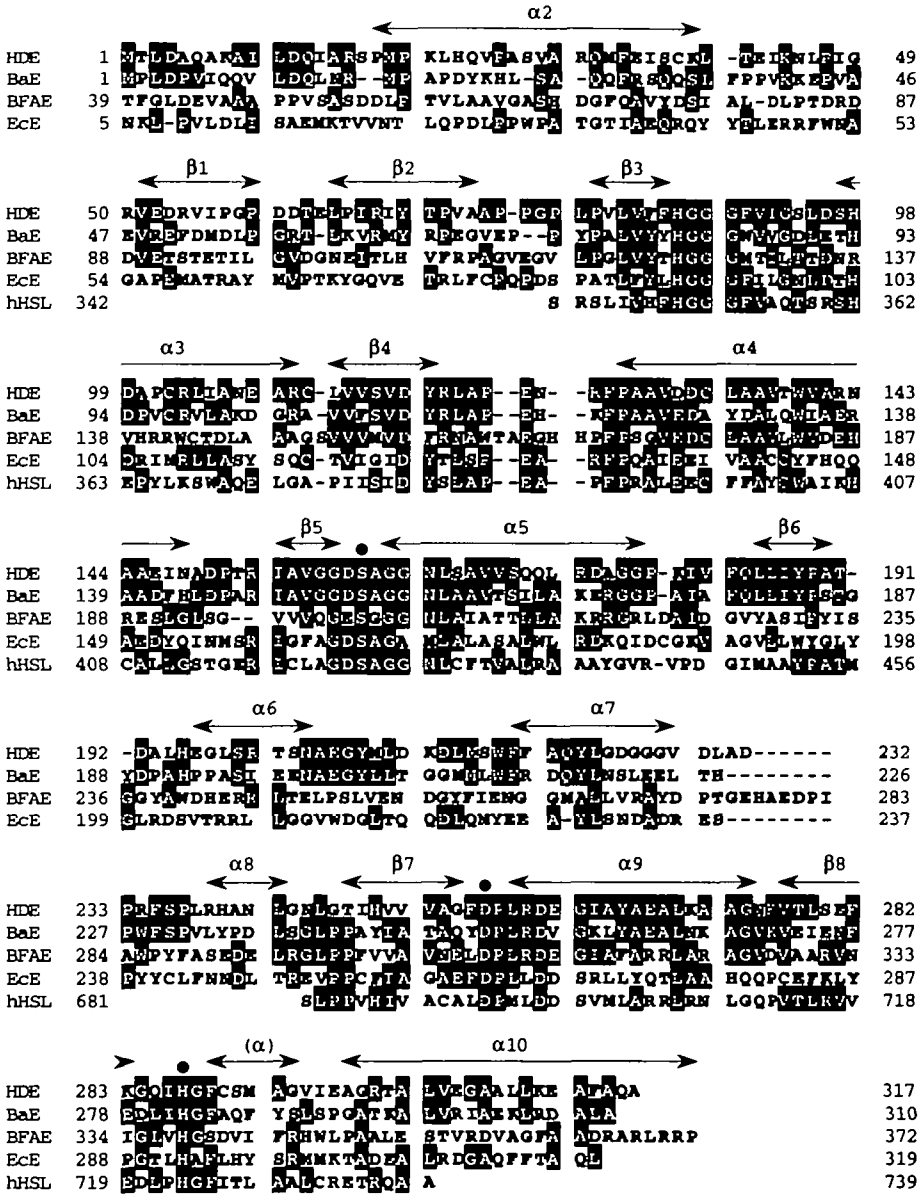

Fig. 1. **Alignment of the amino acid sequences of representative members of the HSL family.** Amino acid residues which are conserved in at least two different proteins are highlighted in black. When two types of amino acid residues were conserved at a given position, the residue conserved in the HDE sequence was selected. Gaps are denoted by dashes. The amino acid residues that are presumed to form a catalytic triad are denoted by solid circles. Numbers represent the positions of the amino acid residues starting from the initiator methionine for each protein. The ranges of the eleven α-helices and the eight β-strands of BFAE (*18*) are shown above the sequences. The abbreviations of the proteins, as well as their accession numbers, except for that of HDE, are listed in Table I. The hHSL sequence is shown only for the regions in which it shows significant similarity to the sequences of bacterial HSL homologs.

to be MTLDAQAK, which was identical with that predicted from the DNA sequence. However, most of the NH_2-terminal methionine residue was post-translationally removed from the recombinant protein. It has been proposed that the second amino acid residue determines whether the NH_2-terminal methionine residue is removed or not (*30*). According to this proposal, the NH_2-terminal methionine residue is removed when the second amino acid is Ala, Ser, Gly, Pro, Thr, or Val. The observation that the NH_2-terminal methionine residue is removed from recombinant HDE supports this proposal, because the second amino acid is Thr for HDE. It remains to be determined whether or not natural HDE has Met at its NH_2-terminus.

The molecular weight of HDE was estimated to be 35,000 on SDS-PAGE, which was nearly identical with that (33,633) calculated from the amino acid sequence. The molecular weight of HDE was estimated to be 38,000 on gel filtration column chromatography as well (data not shown). The good agreement of these values indicates that HDE exists in a monomeric form.

Enzymatic Activity of HDE—The enzymatic activity of HDE was determined by using various *p*-nitrophenyl esters of fatty acids as substrates at pH 7.1 and 25°C. The optimum pH and temperature for the enzyme were determined to be pH 8.5 and 30°C, respectively, using *p*-nitrophenyl caproate (C_6) as a substrate (Fig. 3). However, these conditions were not chosen, because the stability of some substrates, such as *p*-nitrophenyl acetate, decreased as the pH increased beyond 7.5 or the temperature increased beyond 30°C. The enzymatic activity determined at pH 7.1 and 25°C was roughly half as that determined under the optimum conditions. The kinetic parameters of the enzyme for the hydrolysis of the *p*-nitrophenyl esters of fatty acids with chain lengths of 2 to 18 are summarized in Table II. There was a tendency that the K_m value decreased as the aliphatic chain length of the substrate increased beyond C_4, suggesting that the longer the aliphatic chain of the substrate is, the more strongly the enzyme binds to the

substrate. Likewise, there was a tendency that the k_{cat} value decreased as the aliphatic chain length of the substrate increased beyond C_4. Very little enzymatic activity was detected when *p*-nitrophenyl oleate (C_{18}) was used as a substrate. The question as to why the K_m and k_{cat} values for *p*-nitrophenyl acetate (C_2) were decreased as compared to those for *p*-nitrophenyl butyrate (C_4) remains to be answered.

To determine whether or not HDE exhibits lipase activity, we determined the enzymatic activity using tricaprylin (C_8) and triolein (C_{18}) as substrates at 30°C and pH 8.0. The specific activity of the enzyme was 0.6 units/mg for tricaprylin and <0.1 unit/mg for triolein, whereas it was 21.5 units/mg for *p*-nitrophenyl butyrate and ~5 units/mg for tributyrin. These results indicate that HDE cannot hydrolyze lipase substrates. Thus, we conclude that HDE is an esterase, not a lipase.

It was previously shown that EcE hydrolyzes *p*-nitrophenyl fatty acid esters with acyl chain lengths of less than C_8 (*14*). The most preferable substrate for EcE is *p*-nitrophenyl valerate (C_5). BaE showed a similar substrate preference to that of EcE (*15*). The k_{cat}/K_m values of HDE for various *p*-nitrophenyl substrates are compared with those of EcE in Fig. 4. In contrast to EcE and BaE, HDE shows a wide substrate preference but rather prefers substrates with relatively long acyl chains, such as *p*-nitrophenyl caprate (C_{10}), laurate (C_{12}), and myristate (C_{14}). According to the crystal structure of BFAE, Tyr^{233} is located over the putative substrate binding site (*18*). Mutational studies have suggested that the bulky side chain at this position interferes with the interaction between substrates with long acyl chains and the enzyme (*18*). Because the corresponding residue is Ala^{190} in HDE, the absence of a bulky side chain at this position may account for the unique substrate preference of HDE. However, the corresponding residues in BaE and EcE are Ser^{185}, and Gly^{196}, both of which do not have bulky side chains as well. Amino acid substitutions at other positions may be responsible for the difference in substrate preference between HDE and other esterases in the HSL family. It was noted that lipases can hydrolyze fatty acid monoesters as well and show a preference for those with long acyl chains as HDE does. However, our results indicate that enzymes with a preference for fatty acid monoesters with long acyl chains do not necessarily exhibit lipase activity.

The preference for substrates with long acyl chains

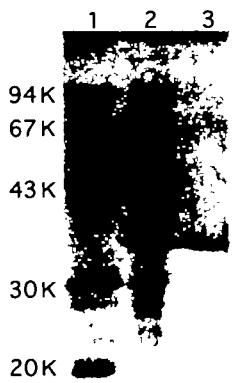

Fig. 2. **Comparison of the purity of HDE by SDS-PAGE.** Samples were subjected to electrophoresis on a 12% polyacrylamide gel in the presence of SDS. After electrophoresis, the gel was stained with Coomassie Brilliant Blue. Lane 1, markers comprising phosphorylase *b* (94K), bovine serum albumin (67K), ovalbumin (43K), carbonic anhydrase (30K), and trypsin inhibitor (20K); lane 2, crude extract of *E. coli* DH5α harboring the plasmid containing the HDE-encoding gene (plasmid containing either the 6.7-kbp *Sac*I fragment or the 1.6-kbp *Not*I-*Eco*RI fragment); lane 3, purified HDE.

TABLE II. **Kinetic parameters of HDE for the hydrolysis of various *p*-nitrophenyl esters of fatty acids.** The hydrolyses of various *p*-nitrophenyl esters of fatty acids with the enzyme were carried out at 25°C for 15 min in 100 μl of 20 mM phosphate buffer (pH 7.1) containing 20% acetonitrile. Kinetic parameters were determined by least-squares fitting of the data obtained from Lineweaver-Burk plots. Errors, which represent the 67% confidence limits, are within 10% of the values reported.

Substrate[a]	Carbon number[b]	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m
Acetate	2	0.48	8.5	17.9
Butyrate	4	1.05	12.6	12.0
Caproate	6	0.54	8.5	15.9
Capryate	8	0.53	7.7	14.5
Caprate	10	0.15	3.7	24.1
Laurate	12	0.09	1.9	21.0
Myristate	14	0.10	2.5	25.2
Oleate	18	–	<0.05	–

[a]*p*-Nitrophenyl esters of fatty acids. [b]The numbers of carbons in the fatty acyl chains.

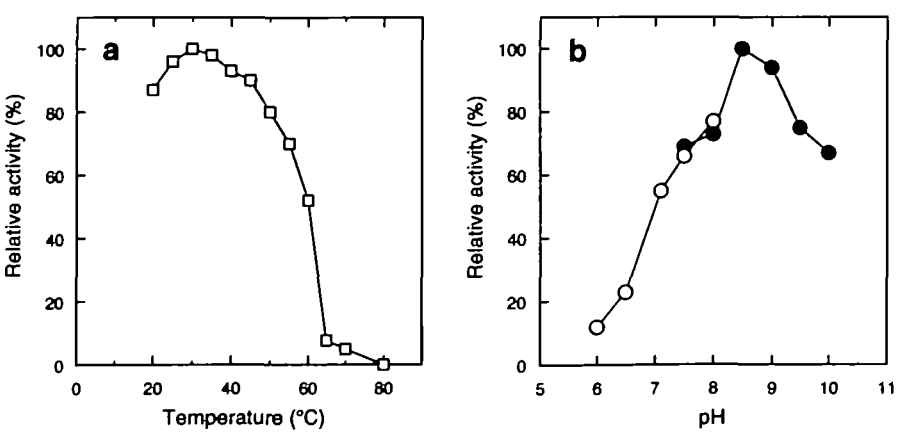

Fig. 3. **Effects of temperature (a) and pH (b) on the enzymatic activity of HDE.** The enzymatic activity was determined using *p*-nitrophenyl caproate (C_6) as a substrate. a: The enzymatic activity was determined at various temperatures in 20 mM sodium phosphate (pH 7.1) containing 20% acetonitrile. The enzyme was incubated for 10 min at the temperature indicated prior to the addition of the substrate. The activity relative to that determined at 30°C is shown as a function of temperature. b: The enzymatic activity was determined at 25°C in either 20 mM sodium phosphate (pH 6.0-8.0) (○) or 20 mM Tris-HCl (pH 7.5-10.0) (●) containing 20% acetonitrile. The activity relative to that determined at pH 8.5 is shown as a function of pH.

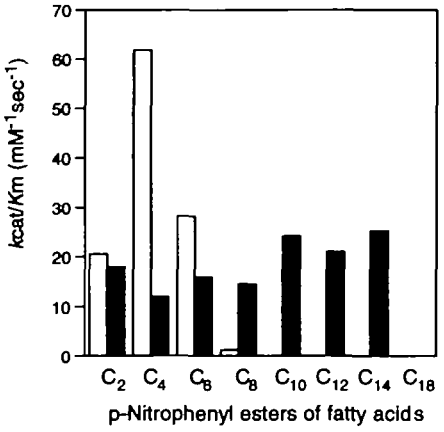

Fig. 4. **Comparison of the substrate specifities of HDE and EcE.** The k_{cat}/K_m values of HDE (solid bars) and EcE (open bars) for the hydrolyses of various *p*-nitrophenyl esters of fatty acids with different acyl chain lengths (C_2-C_{18}) are shown.

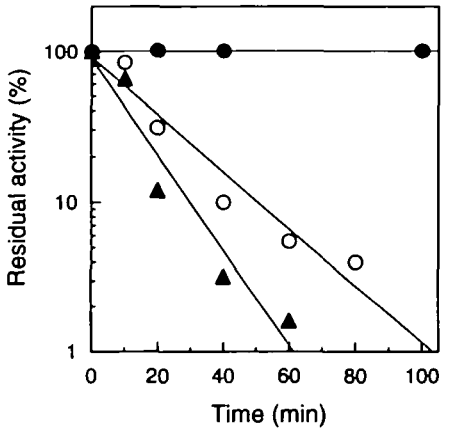

Fig. 5. **Stability against irreversible heat inactivation.** Semi-log plots of the residual activity *versus* the incubation time are shown. HDE was incubated in 20 mM sodium phosphate (pH 7.1) at the concentration of 0.15 mg/ml at 70°C (●), 80°C (○), and 90°C (▲). Aliquots of each sample were withdrawn at the times indicated and the enzymatic activity was determined at 25°C using *p*-nitrophenyl caproate as a substrate. The line was obtained by linear regression of the data.

seems to be a characteristic of HDE. However, it remains to be determined whether or not HDE is involved in a pathway for alkane/alkene synthesis. To answer this question it is necessary to determine whether disruption or amplification of the HDE-encoding gene affects the ability of strain HD-1 to synthesize alkanes/alkenes. We are currently attempting to construct a system that will permit genetic engineering of this strain.

Heat Inactivation—The stability of HDE against irreversible heat inactivation at 70, 80, and 90°C is shown in Fig. 5. The enzyme was stable at 70°C for at least 3 h. In contrast, the enzyme lost roughly half of its activity in 15 min at 80°C and 10 min at 90°C. It has been reported that BaE is stable at 75°C for at least 3 h, and loses half of its activity at 80°C in 30 min and 90°C in 10 min (*15*). Thus, HDE is as stable as BaE against irreversible heat inactivation. However, the optimum temperature for HDE was determined to be 30°C with *p*-nitrophenyl caproate (C_6) as a substrate (Fig. 3). HDE exhibited poor enzymatic activity at 70°C. These results indicated that HDE was thermally denatured at 70°C and fully renatured at 25°C. The optimum temperature of BaE has been reported to be 70°C (*15*), suggesting that BaE is conformationally more stable than HDE. BaE was not significantly more stable than HDE against heat inactivation, probably because it showed poor reversibility against thermal denaturation under the conditions examined.

Because HDE and BaE show relatively high amino acid sequence identity with each other (40.4%), these two proteins would be an ideal protein pair for specify the amino acid substitutions contributing to the thermal stability. Such studies will provide valuable information on the stabilization mechanisms of proteins. When the HDE sequence is compared with the BaE sequence, the proline residues can be seen to be replaced by non-proline residues at nine positions, whereas the non-proline residues are replaced by proline residues at three positions. It has been reported that the substitution of a proline residue for a non-proline residue in the β-sheet or the turn/loop region increases the protein stability due to a decrease in the conformational entropy in the unfolded state (*31, 32*). In fact, thermophilic proteins have more proline residues in the loop regions than mesophilic proteins do (*33*). Therefore, it would be informative to determine whether or not the substitution of a proline residue for a non-proline residue increases the stability of HDE.

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