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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Cloning and characterization of a new cold-active lipase from a deep-sea sediment metagenome

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Abstract To search for new cold-active lipases, a metagenomic library was constructed using cold-sea sediment samples at Edison Seamount and was screened for lipolytic activities by plating on a tricaprylin medium. Subsequently, a fosmid clone was selected, and the whole sequence of 36 kb insert of the fosmid clone was determined by shotgun sequencing. The sequence analysis revealed the presence of 25 open reading frames (ORF), and ORF20 (*EML1*) showed similarities to lipases. Phylogenetic analysis of EML1 suggested that the protein belonged to a new family of esterase/lipase together with LipG. The *EML1* gene was expressed in *Escherichia coli*, and purified by metal-chelating chromatography. The optimum activity of the purified EML1 (rEML1) occurred at pH 8.0 and 25°C, respectively, and rEML1 displayed more than 50% activity at 5°C. The activation energy for the hydrolysis of olive oil was determined to be 3.28 kcal/mol, indicating that EML1 is a cold-active lipase. rEML1 preferentially hydrolyzed triacylglycerols acyl-group chains with long chain lengths of ≥8 carbon atoms and displayed hydrolyzing activities toward various natural oil substrates. rEML1 was resistant to various detergents such as Triton X-100 and Tween 80. This study represents an example which developed a new cold-active lipase from a deep-sea sediment metagenome.

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Introduction

Deep-sea environments cover 63.5% of the Earth's surface and represent the most unexplored marine habitat (Butman and Carlton 1995) and there have been reports that biological communities including a variety of microorganisms are present around the areas of the cold environments that have a constant temperature of 4–5°C below a depth of 1,000 m (Corliss et al. 1979; Kennicutt et al. 1985; Feller and Gerday 2003), considered to be very unique (Gray 2002; Levin et al. 2001).

In spite of this variety, 98.0–99.8% of marine microorganisms are not readily culturable with currently available techniques and therefore not accessible for biotechnology or basic research in a common way of culturing microorganisms (Amann et al. 1995; Streit and Schmitz 2004). The collective genomes of all microorganisms present in a given habitat, the so-called metagenome, has been studied alternatively without culturing microorganisms (Handelsman et al. 1998; Schloss and Handelsman 2003; Steele and Streit 2005) to screen for biocatalysts or molecules with new functions for biotechnological applications and to understand on the microbial ecology and physiology of the studied niches (Handelsman 2004; Streit and Schmitz 2004). Up to date, various industrial enzymes such as esterase/lipase (Elend et al. 2006; Lee et al. 2004, 2006; Rhee et al. 2005; Voget et al. 2003), protease (Gupta et al. 2002), amylase (Yun et al. 2004), nitrilase (Robertson et al. 2004), lyase (Solbak et al. 2005) and so on have been identified by metagenomic approach.

Lipases (triacylglycerol ester hydrolases, EC 3.1.1.3) are ubiquitous enzymes that catalyze the hydrolysis of fats and

oils and form a large group of enzymes that catalyze the hydrolysis of a wide range of carboxyl esters (Schmidt and Verger 1998; Jaeger et al. 1999; Villeneuve et al. 2000; Koeller and Wong 2001; Schmid et al. 2001; Klibanov 2001). A great number of lipases isolated from bacteria, fungi, plants, and higher animals have received a great deal of attention as biocatalysts in numerous industrials (Schmidt and Verger 1998; Jaeger et al. 1999; Villeneuve et al. 2000).

We have been collecting samples from various marine environments, isolating microorganisms followed by a partial identification and systematic deposition to KORDI collection (www.megrc.re.kr) (Kim et al. 2007; Park et al. 2007). Besides the culture dependent approach, we are applying metagenomic approach to develop new bioresources from uncultured microbial communities. In this study, we present cloning, overexpression, and biochemical characterization of a new lipase from deep-sea area of Edison Seamount using metagenomic approach. To screen a cold-active lipase, a fosmid library was constructed using sediment sample of Edison Seamount, where a clam bed community was developed and ambient temperature was below 4°C. A new lipase gene selected by screening lipolytic activity was overexpressed, followed by the biochemical characterization of the purified protein.

Materials and methods

Strains, library construction, and screening *Escherichia coli*

DH5α (Stratagene, LaJolla, CA, USA), EPI300-T1R (Epicentre, Madison, WI, USA), and BL21(DE3) (Novagen, Madison, WI, USA) were used as host strains for cloning and expression. pBluescript SK- (Stratagene), pET-28a(+) vector (Novagen), and fosmid vector (Epicentre) were used as vectors.

Sample collection

Deep sea sediment samples were collected from the southern clam beds area around the summit of Edison Seamount (3°89′Σ, 152°49′E; depth 1,440 m) using TV-guided grab sampler during ‘Condrill’ (Sonne 166) cruise on September 2002. Sediment samples were collected from highly dense *Calyptogena* community area (Lee et al. 2004).

Metagenomic library construction and screening for lipolytic activity

DNA from the sediment samples was extracted based on a method described previously (Hurt et al. 2001) with minor modification. The extracted DNA was further purified by gel electrophoresis in 1% low melting temperature agarose gel (FMC Bioproducts, Rockland, ME, USA) supplemented with 1% polyvinyl polypyrrolidone (PVPP; Sigma, St. Louis, MO, USA). Gel electrophoresis was performed at 35 V for 13 h, and then approximately 40 to 50 kb DNA fragments were isolated from the gel. The isolated DNA was end-repaired with end-repair enzyme mix (Epicentre) resulting in the DNA blunt-ended and 5′-phosphorylated, and then ligated to a copycontrol pCC1FOS vector (Copy-Control Fosmid Library Production Kit; Epicentre). Lambda packaging extracts were added to the ligates and infection of a phage T1-resistant EPI300-T1^R *E. coli* was performed according to the manufacturer’s protocol. The resulting *E. coli* transformants were transferred to 96-well microtiter plates and stored at −80°C. For screening esterases/lipases activity, the transformants were plated onto Luria-Bertani (LB) supplemented with 12.5 μg of chloramphenicol agar plates (Sambrook and Russell 2001) containing 1% tricaprylin as a substrate. Colonies were incubated 1 day at 37°C, followed by incubation for a week at 4°C. Candidates with a clear halo on the plates were selected.

Whole fosmid sequencing and ORF analysis

Among colonies with a clear halo, ES0109D12 harboring the recombinant fosmid, pES0109D12, was selected due to the large halo size on the plate. The insert DNA of pES0109D12 was entirely sequenced as follows. DNA manipulations were carried out according to standard procedures (Sambrook and Russell 2001). pES0109D12 was purified by alkaline lysis method (Birnboim and Doly 1979) and mechanically sheared DNA (1.53 kb) of the fosmid was ligated to pBluescript SK- for shotgun sequencing. DNA sequencing reactions were performed using Big Dye kit (Applied Biosystems) and analyzed using an ABI-3100 automated sequencer (Applied Biosystems). The DNA sequence was assembled using the program ‘Vector NTI Advance’ (InforMax, North Bethesda, MD, USA). After sequence assemblage, two major contigs of >15 kb were obtained. The gap was closed by primer-walking method. Open reading frame (ORF) analysis was performed using ORF Finder of NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). The predicted function of ORFs was annotated using BlastX search toward the NCBI nonredundant protein database (Altschul et al. 1997). Multiple alignments between protein sequences were performed with the CLUSTAL W program (Thompson et al. 1994).

Cloning and expression of EML1

For the overexpression of *EML1* (ORF 20), the full length of the gene was amplified using primers, the forward primer

(5′-*GGATCC*ATGACTTCAACGGCAAA-3′) and the reverse primer (5′-*AAGCTT*CCTTATGAGCAA ATTCCA-3′) using the original pES01029D12 as a template. The primer pairs with restriction enzyme sites (italicized) for *BamH*I and *Hind*III were designed to generate a N-terminal His-tag of the recombinant esterase. The *EML1* gene was cloned into an expression vector, pET-28a(+), and the recombinant plasmid was transformed into *E. coli* BL21 (DE3) cells. When the cell density at 600 nm reached around 0.6, 1 mM IPTG was added for the induction. After 10 h of cultivation at 25°C, the cells were harvested by centrifugation at 5,000×*g* for 20 min, washed with 50 mM Tris–HCl buffer (pH 7.5), resuspended in the same buffer, and disrupted by sonication. Cell debris was removed by centrifugation at 15,000×*g* for 30 min. The resulting supernatant was applied to metal-chelating chromatography using Ni-NTA column with slight modification of Kim et al. (2007).

Lipase assay

The lipase activity of EML1 was measured by titrating free fatty acids released by hydrolysis of olive oil using the pH stat method (Kim et al. 1998). Olive oil emulsion was prepared by emulsifying 1 ml of olive oil in 99 ml of a buffer consisting of 20 mM NaCl, 1 mM $CaCl_2$, and 0.5% (*w*/*v*) gum arabic solution for 2 min at maximum speed in a Waring blender. After the pH of the substrate emulsion (20 ml) was adjusted to 8.0 by 10 mM NaOH, an appropriate amount (10–50 μl) of the enzyme solution was added. The releasing rate of the fatty acid was measured with a pH titrator (718 Stat Titrino, Metrohm, Switzerland) for 5 min at

Table 1 Sequence analysis of ORFs encoded in pES01029D12[a]

No. of ORF	Length (amino acid)	Putative function (most similar homologue)	Putative source organism	Accession number	% Identity/ similarity	*E* value
1	834	Anaerobic dehydrogenases	*Magnetospirillum magnetotacticum* MS-1	ZP_00053617	73/84	0
2	263	Ferredoxin	*M. magnetotacticum* MS-1	ZP_00053618	64/75	$7e^{-9}$
3	315	Ferredoxin-type protein, NapH/MauN family	*Dechloromonas aromatica* RCB	YP_287036	59/73	$1e^{-1}$
4	148	Nitrate reductase cytochrome c-type subunit	*Magnetococcus* sp. MC-1	EAN26848	57/79	$5e^{-2}$
5	214	NapC/NirT cytochrome c, N-terminal	*D. aromatica* RCB	YP_286712	68/79	$2e^{-4}$
6	366	Outer membrane protein/protective antigen OMA87	*Ralstonia eutropha* JMP134	ZP_00166746	36/51	$3e^{-56}$
7	389	Predicted Zn-dependent dipeptidase)	Uncultured marine proteobacterium ANT8C10	AAR05183	49/68	$1e^{-104}$
8	424	Outer membrane efflux protein	*Idiomarina loihiensis* L2TR	YP_155613	32/55	$2e^{-44}$
9	495	Membrane-fusion protein	*Microbulbifer degradans* 2–40	ZP_00316947	42/60	$8e^{-97}$
10	1039	Putative cation efflux system transmembrane protein	*Vibrio parahaemolyticus* RIMD 2210633	NP_799990	64/78	0
11	430	Hypothetical protein ELI2181	*Erythrobacter litoralis* HTCC2594	ZP_00376940	36/55	$1e^{-65}$
12	110	Predicted transcriptional regulators	*R. eutropha* JMP134	ZP_00350661	39/63	$2e^{-5}$
13	183	3,4-Dihydroxy-2-butanone 4-phosphate synthase	*Nitrosomonas eutropha* C71	ZP_00669526	34/54	2.2
14	287	Ice-like protease p20 domain protein	*Colwellia psychrerythraea* 34H	YP_269116	39/60	$2e^{-39}$
15	266	Hypothetical protein CPS_2398	*C. psychrerythraea* 34H	YP_269115	45/61	$4e^{-51}$
16	160	Hypothetical protein CPS_2400	*C. psychrerythraea* 34H	YP_269117	30/57	$3e^{-80}$
17	790	Outer membrane receptor proteins	*Hyphomonas neptunium* ATCC 15444	YP_760906	29/47	$1e^{-18}$
18	248	Lysophospholipase	*Nostoc punctiforme* PCC 73102	ZP_00111337	38/58	$7e^{-49}$
19	470	Hypothetical protein Pflu02002718	*Pseudomonas fluorescens* PfO-1	YP_350945	40/53	$2e^{-49}$
20	304	LipG	uncultured bacterium pFosLip	ABE69172	34/51	$1e^{-11}$
21	409	Membrane-bound lytic transglycolase-related protein	*Shewanella oneidensis* MR-1	NP_717601	51/67	$8e^{-103}$
22	196	Hypothetical protein SbalDRAFT_2838	*S. baltica* OS155	ZP_00581156	37/57	$5e^{-25}$
23	159	PepSY-associated TM helix	*Sphingopyxis alaskensis* RB2256	ZP_00577274	38/57	$2e^{-23}$
24	158	Copper ion-binding	*Burkholderia cenocepacia* HI2424	ZP_00462453	38/53	$2e^{-10}$
25	738	Copper-translocating P-type ATPase	*C. burnetii* RSA 493	NP_820490	48/66	0

25°C. One unit was defined as the amount of enzyme liberating 1 μmol of fatty acid per minute.

Biochemical properties of EML1

The optimum temperature of purified EML1 was determined by assaying the hydrolytic activities of the olive oil emulsions at different temperatures in the range of 5–50°C at 50 mM Tris–HCl (pH 8.0). The thermostability of lipase was determined by preincubating the enzyme for up to 30 min at temperatures ranging between 10°C and 65°C, then measuring the remaining activity at 25°C with the substrate added. To determine the optimum pH of the enzyme, the lipase activities at

(A)

EML1	(69)-IIVAF***RG***IMP	(138)-KRVYLTGH**S**KGGPMAT	(196)-VTRYENHL**D**IVPLVPP	(271)-VMAS**H**DGLVE
ABE69172	(92)-LVVAF***RG***TES	(161)-RALWITGH**S**LGAAIAT	(179)-AYRFENNN**D**IVCKVPP	(281)-AIKD**H**VPVLY
YP_721429	(80)-LCMGF***RG***IDE	(137)-RPLFLTGH**S**LGGSIAT	(193)-FFRFHNNN**D**IITRVPS	(256)-FIAD**H**DMGKY
NP_847303	(65)-IIVAF***RG***TQT	(124)-KKLLATGH**S**LGGALAT	(180)-SFRFVNLF**D**VVPLLPP	(223)-IANN**H**AMITY
YP_897132	(65)-IIVAF***RG***TQT	(124)-KKLLATGH**S**LGGALAT	(180)-SFRFVNLF**D**VVPLLPP	(223)-IANN**H**AMITY
YP_086191	(65)-VIVAF***RG***TQT	(124)-KKLLATGH**S**LGGALAT	(180)-SFRFVNLF**D**VVPLLPP	(223)-ITNN**H**AMITY
ZP_01182938	(65)-IIVAF***RG***TQT	(124)-KKLLATGH**S**LGGALAT	(180)-SFRFVNLF**D**VVPLLPP	(223)-ITNN**H**AMITY
CAG19796	(54)-IIISF***RG***TEG	(125)-QTIWLTGH**S**LGGALAT	(173)-TFRCVNNN**D**VVIRVPP	(195)-HRYL**H**KMVIN
AAZ27340	(131)-IILSF***RG***TEA	(191)-KPLFITGH**S**LGGALAT	(242)-IHRIVNAA**D**SVIMLPP	(335)-LLSD**H**SISNY
ZP_01041937	(105)-LVLSF***RG***TEP	(162)-LPLFITGH**S**LGGALAT	(213)-IYRVVNSS**D**GVIMVPP	(306)-FGID**H**SMSVY
ZP_00551941	(121)-VILAF***RG***TQP	(178)-IPLYITGH**S**LGGALAL	(228)-VYRVVNAA**D**GVAKVPF	(329)-AASD**H**AIRDY
ZP_01694580	(100)-LTLSF***RG***TAG	(167)-RKINITGH**S**QGGALAF	(237)-TVRYENTA**D**LVPLLPP	(384)-LLEA**H**TLQPG
AY527197	(74)-YVIAL***RG***TNP	(166)-AKICVTGH**S**KGGALSS	(228)-CTRIANSL**D**IVPYAWN	(308)-AAYQ**H**VVGYP

(B)

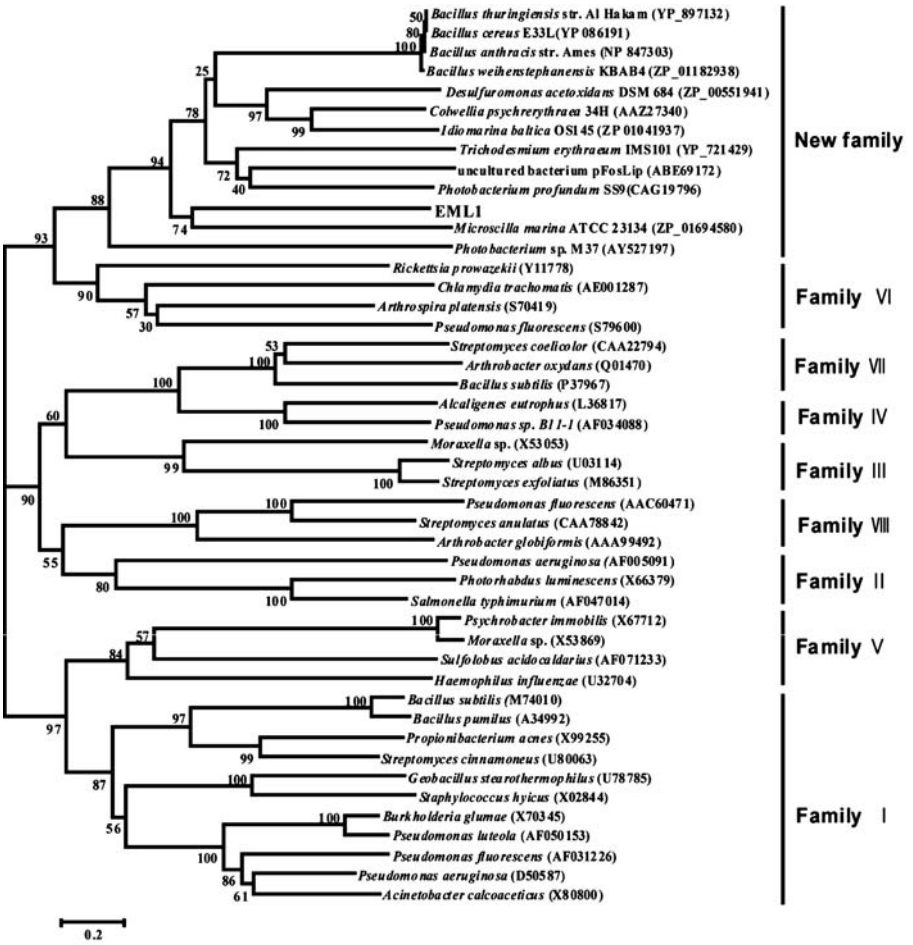

Fig. 1 **a** Multiple sequence alignment of EML1 and other related proteins: ABE69172, probable lipase from uncultured bacterium pFosLip; YP_721429, lipase class 3 from *Trichodesmium erythraeum* IMS101; NP_847303, lipase family protein from *B. anthracis* str. Ames; CAG19796, YP_897132, lipase from *B. thuringiensis* str. Al Hakam; YP_086191, lipase from *B. cereus* E33L; ZP_01182938, Lipase class 3 from *B. weihenstephanensis* KBAB4; Hypothetical protein from *P. profundum* SS9; AAZ27340, lipase family protein from *C. psychrerythraea* 34H; ZP_01041937, lipase family protein from *Idiomarina baltica* OS145; ZP_00551941, Lipase class 3 from *Desulfuromonas acetoxidans* DSM 684; ZP_01694580, lipase family from *Microscilla marina* ATCC 23134; AY527197, lipase M37 from *Photobacterium* sp. M37. Residues consisting of catalytic triad were marked in *bold* and *underlined*, and residues for an oxyanion hole were marked in *bold* and *italicized*. **b** Neighbor joining tree showing the phylogenetic positions of EML1 based on the conserved sequence motifs of bacterial lipolytic enzymes. The phylogenetic tree was constructed by the use of Molecular Evolutionary Genetics Analysis 3.1 software. Bar, 0.2 substitutions per amino acid site. Only bootstrap values higher than 50% were shown

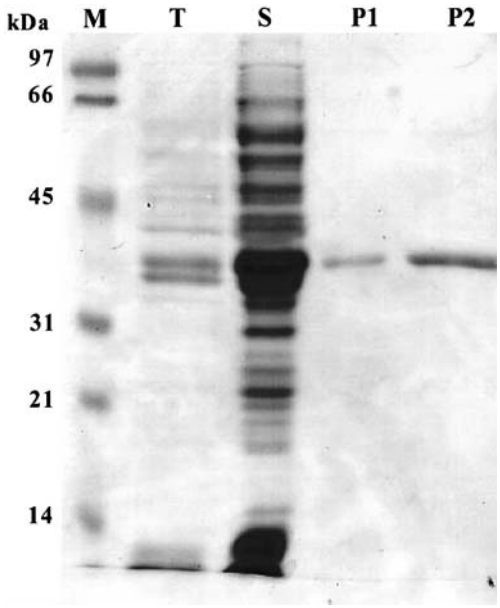

Fig. 2 SDS-PAGE of the purified EML1. *M* Molecular size markers, *T* whole cell extracts, *S* soluble proteins, *P1* the purified EML1 (1 μg), *P2* the purified EML1 (2 μg)

various pH (pH 6–10) were measured at 25°C by pH stat method.

Substrate preference toward *p*-nitrophenyl esters (C2–C18) was determined by measuring the amount of *p*-nitrophenol released by lipase-catalyzed hydrolysis (Lee et al. 1993) and substrate specificities toward various triacylglycerols were determined by using the pH stat method. Triacylglycerols between C2 and C18 were determined using triacetin, tributyrin, tricaporin, tricaprylin, tricaprin, trilaurin, trimyristin, tripalmitin, and triolein. The highest activities of enzyme assay using the substrates (triacylglycerols) were defined as the 100% level. The activities toward natural substrates such as 1% olive oil, cotton seed oil, soybean oil, peanut oil, linseed oil, corn oil, wheatgerm oil, coconut oil, palm oil, and beef tallow were determined by using the pH stat method. The activity toward olive oil was defined as the 100% level.

Various metal ions ($MgCl_2$, $MgSO_4$, $CoSO_4$, $Co(NO_3)_2$, $CuSO_4$, $CaCl_2$, $MnCl_2$, $NiSO_4$, $ZnSO_4$, and $FeSO_4$) and EDTA at final concentrations of 1 mM were added to the enzyme in 20 mM Tris–HCl buffer (pH 8.0), then assayed for lipase activity after preincubation at 25°C for 30 min. Effect of detergents on lipase activity was determined by incubating the enzyme for 30 min at 25°C in 20 mM Tris–HCl buffer (pH 8.0) containing 1% (*w*/*v*) of the detergents SDS, Triton X-100, Tween 20, 40, 60 and 80. Lipase activity was measured at the beginning and end of the

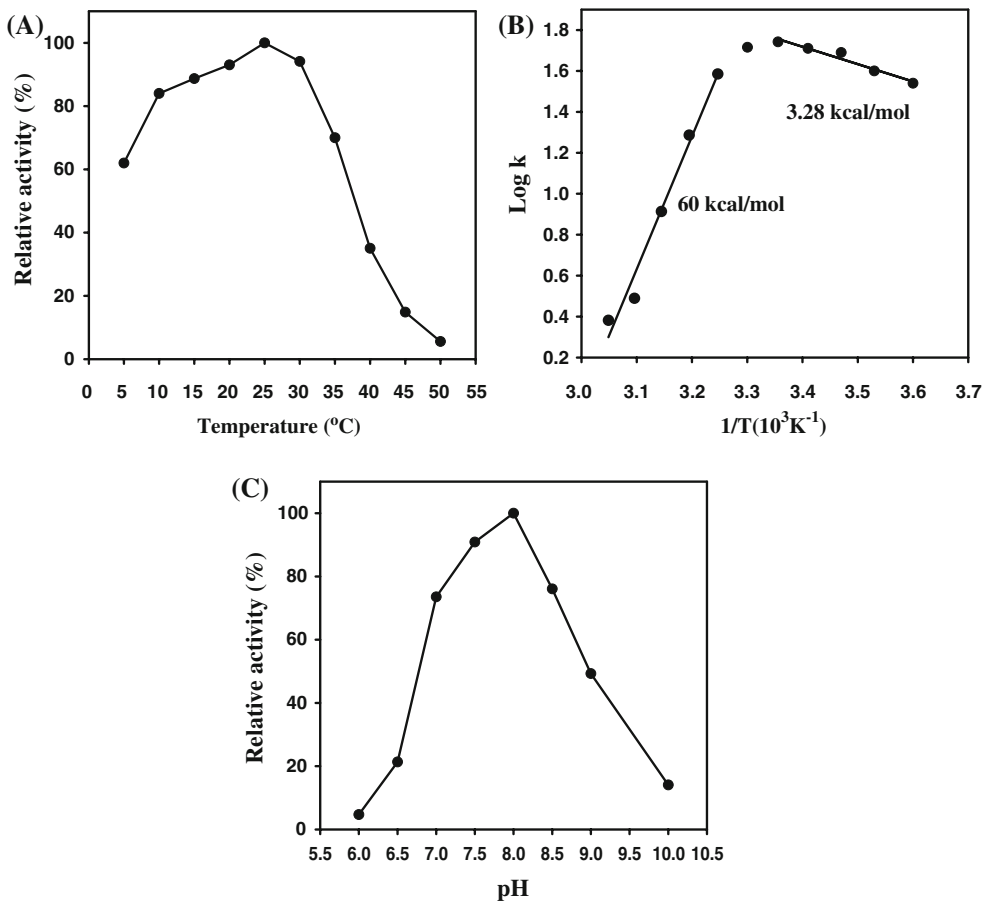

Fig. 3 Effect of temperature and pH on the activity of EML1. **a** The enzyme activity was measured at various temperatures at pH 8.0. The value obtained at 25°C was taken as 100%. **b** The logarithm of the specific activity (V) (in micromoles per milligram per minute) was plotted against the reciprocal of absolute temperature (T). The values shown are activation energy calculated from the linear part of the plot. **c** The enzyme activity was determined various pH at 25°C by using olive oil. The value obtained at pH 8 was taken as 100%

incubation period. The activity of the enzyme preparation in the absence of detergent before incubation was defined as the 100% level.

Nucleotide sequence

The nucleotide sequence of the fosmid ES0109D12 has been assigned GenBank accession number DQ229155.

Results

Construction of metagenomic library and screening

A third small volcanic cone south of Lihir, named "Edison Seamount", is made mainly of phlogopite-rich basalt breccias and is unique in that it hosts hydrothermal system. Near the crest of the volcano, extensive clam beds associated with diffuse and low-temperature vents were discovered at a depth of 1,450 m (Herzig et al. 1994). Considering the average temperature, it was expected that the samples in the deep-sea environment might contain genetic information encoding cold-active enzymes. In this respect, a metagenomic library using a sediment sample from the clam-bed community was constructed as described in "Materials and methods", resulting a metagenomic library (ES01) consisting of 8,823 clones. Restriction analysis of randomly selected 50 fosmid clones (0.5%) showed that the insert sizes of the clones were estimated to be 21 to 40 kb with average insert size of 32.3 kb, covering approximately 284 Mb of the total metagenome DNA. The whole library was screened for lipolytic activities on 1% tricaprylin plates, and consequently, an ES01029D12 clone forming a clear halo on the plate was selected.

To characterize the lipase activity in the clone, the insert DNA of pES01029D12 was sequenced by shotgun sequencing method. The whole sequence of 36,721 base pair in length was determined and the sequence analysis revealed the presence of 25 ORFs longer than 100 amino acids. Eighteen ORFs among those exhibited similarities to genes annotated with predicted functions: five ORFs showed significant similarities to putative cation efflux system transmembrane protein (*Vibrio parahaemolyticus* RIMD 2210633), periplasmic nitrate reductase (*Colwellia psychrerythraea* 34H), copper-translocating P-type ATPase (*Coxiella burnetii* RSA 493), and TonB-dependent receptor (*C. psychrerythraea* 34H) (Table 1). Interestingly, as shown in Table 1, most of ORFs were similar to membrane-associated proteins from γ-*Proteobacteria*, indicating that the DNA insert of pES01029D12 might be originated from a microorganism belonging to γ-*Proteobacteria*. Among the ORFs, a putative lipase (ORF20, designated as *EML1*) could be found, consisting of 915 bp with a deduced molecular mass (Mr) of 33,600. The upstream region from the initiation codon, ATG, at position 1 retained typical transcriptional information such as a putative ribosome binding site (rbs) and −35/−10 promoter sequence. A putative rho-independent terminator (Rosenberg and Court 1979) that consists of dyad symmetry following the T-rich cluster was also present at 29077–29097 nucleotides of the full insert. The deduced amino sequence of *EML1* showed similarities to LipG (ABE69172) from uncultured bacterium pFosLip (34% identity), lipase class 3 (YP_721429) from *Trichodesmium erythraeum* IMS101(34% identity), lipase family protein (NP_847303) from *Bacillus anthracis* str. Ames (33% identity), and lipase (ZP_01694580) from *Microscilla marina* ATCC 23134 (32% identity). Despite the low similarity to proteins characterized to date, the multiple alignment of EML1 (Thompson et al. 1994) revealed the presence of catalytic triad (Asp204, His275, and the catalytic nucleophile Ser146) conserved in lipolytic enzymes of the α/β hydrolase superfamily (Villeneuve et al. 2000; Jaeger et al. 1994). Recently, a new family of bacterial lipases including LigG was proposed (Lee et al. 2006) since it retained Arg-Gly residues as an oxyanion hole in the N-terminal part with unique primary sequence, differentiated from filamentous fungal lipases (Ryu et al. 2006). As shown in Fig. 1, the alignment of EML1, LigG and putative homologues showed that Arg74-Gly75 resi-

Table 2 Effect of various metal ions on EML1 activity

Metal ions (1 mM) or EDTA (1 mM)	Relative activity (%)
None	100
$MgCl_2$	94
$MgSO_4$	113
$CoSO_4$	97
$Co(NO_3)_2$	108
$CuSO_4$	107
$CaCl_2$	35
$MnCl_2$	85
$NiSO_4$	71
$ZnSO_4$	152
$FeSO_4$	109
EDTA	61

Table 3 Effect of various detergents on EML1 activity

Detergent	Relative activity (%)
None	100
SDS	19
Triton X-100	121
Tween 20	81
Tween 40	84
Tween 60	115
Tween 80	142

dues and catalytic triad were well conserved and phylogenetic analysis exhibited that EML1 was grouped into a new branch together with LigG (Arpigny and Jaeger 1999). Taken together, it seems likely that the ORF was responsible for the lipase activity of ES01029D12 clone and belonged to a new family with LigG.

Purification and characterization of EML1

The EML1 gene was expressed as a N-terminal His-tag fusion protein using pET-28a(+) expression system under the control of the T7 *lac* promoter in *E. coli* BL21(DE3). SDS-PAGE analysis of purified EML1 showed a single band corresponding to about 34 kDa which correlated well to the predicted full length of EML1 (Fig. 2). The purified EML1 was active in the range of pH 7.5–8.5, and in the temperature range of 5–35°C, with maximal activity at 25°C and pH 8 (Fig. 3a,c). We determined the activation energy for the olive oil catalyzed by EML1. The activation energy of the enzymes derived from cold-active organisms is usually lower than those from their mesophilic counterparts (Feller et al. 1996). As expected, the activation energy of EML1 was about 3.28 kcal/mol in the range of 5°C to 25°C, but the enzyme activity at temperatures above 40°C declined very rapidly with inactivation energy of 60 kcal/mol (Fig. 3b). The enzyme was highly stable at 10–35°C, with a residual activity greater than 80% of the initial activity. Taken together, EML1 was a cold-active enzyme.

The enzyme was incubated with various metal ions, and the remaining activity was measured at 25°C. In contrast to lipases such as *Aspergillus niger*, *Humicola lanuginose*, *Pseudomonas* sp. Strain B11–1 and KB700A (Choo et al.

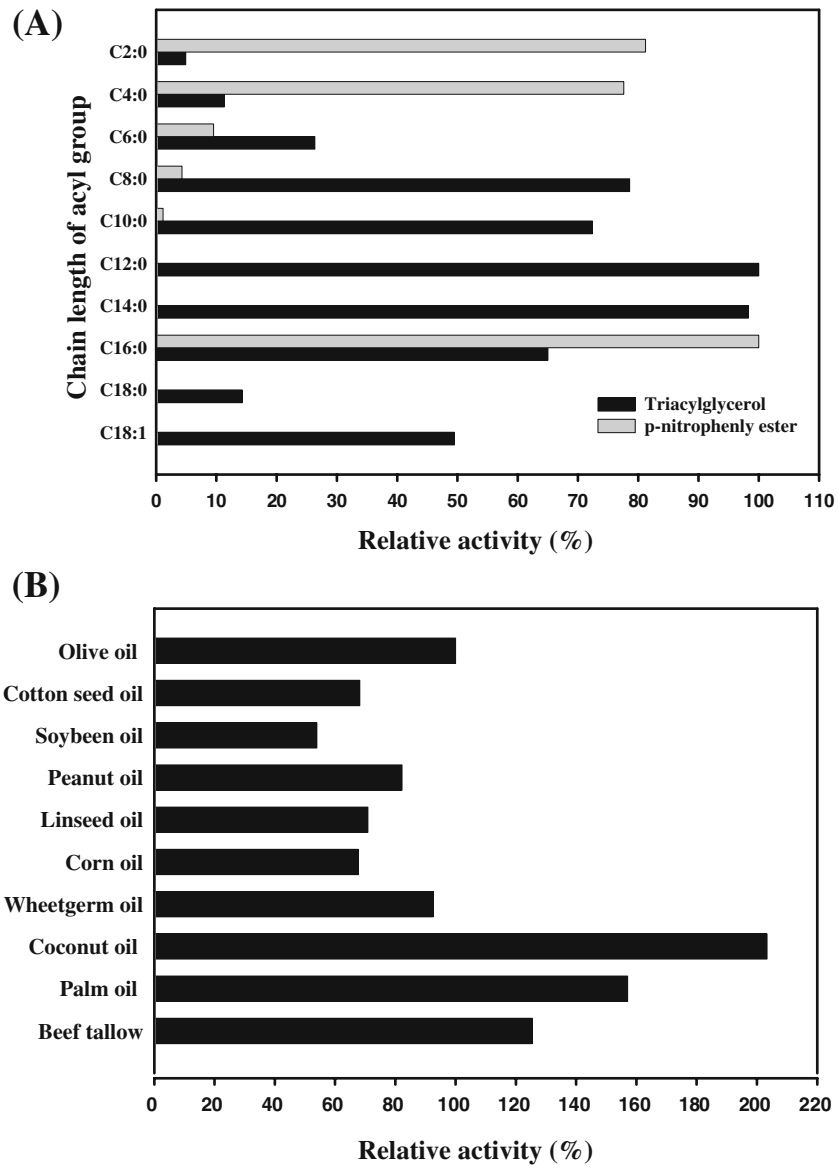

Fig. 4 Substrate specificity of EML1 toward **a** various triacylglycerol and *p*-nitrophenyl esters and **b** various natural products

1998; Naeem et al. 2001; Iwai et al. 1970; Liu et al. 1973), the activity of EML1 was increased by Zn^{2+}, Mg^{2+}, $Cu^{2+}$and Fe^{2+}, and inhibited by Ca^{2+}. EDTA weakly inhibited the lipase activity (Table 2). Various non-ionic detergents such as Triton X-100, Tween 60 and 80 increased enzyme activity (Table 3) while an ionic detergent (SDS) reduced the activity by about 19%.

Substrate specificity of EML1 was determined using *p*-nitrophenyl esters, various triacylglycerols and natural oil substrates. EML1 could hydrolyze preferentially C2, C4, and C16 *p*-nitrophenyl esters while it showed preference toward the medium-chain triglycerides among various triglycerides tested (Fig. 4a). The specific activity of EML1 toward trilaurin (C12) emulsion was 203 U mg^{-1}, eight to 20 times higher than those toward the short-chain triglycerides. It is noteworthy that the activities toward using *p*-nitrophenyl esters and triglycerides may be affected by solubility of substrates. Nonetheless, it seems obvious that EML1 have preference to the medium chain substrates. Furthermore, EML1 showed high activity toward natural substrates such as coconut oil, palm oil, and beef tallow (Fig. 4b).

Discussion

In this study, we characterized a new lipolytic enzyme screened from a metagenomic library constructed from a deep-sea sediment sample. A large number of esterase/lipase from terrestrial organisms have been reported, but the study from marine microorganisms has been limited despite the great potential offering abundant resources for research and development. However, recent advances such as cruise technology on extreme environments, mass sequencing technique, and bioinformatic analysis tools allowed us to develop valuable resources from unexplored communities like deep-sea using metagenome approach. We constructed a metagenomic library from a sediment sample of Edison Seamount with average temperature below 4°C (Herzig et al. 1994) to develop a cold-active enzyme. Among the candidates, EML1 with a big halo in the plate assay was selected even though the size did not necessarily mean the superiority to others, simply resulting from a level of expression in the fosmid vector system. The sequence analysis of the fosmid clone showed that the *EML1* gene retained typical transcriptional information such as a putative ribosome binding site (rbs), −35 and −10 promoter sequences and a putative rho-independent terminator, explaining why the clone displayed a big halo in the plate. In this sense, the less halo represented by other candidate clones on a tributyrin plate may be caused by multiple reasons such as retaining an incomplete transcriptional information to be expressed in a surrogate host, low hydrolyzing activity toward tributyrin, retaining different optimum temperature from our expression conditions and so on. Therefore, those other candidates are still valuable and further studies with those clones are under process.

Based on the data of optimum conditions for the activity and the calculated activation energy, it was suggested that EML1 was cold active, consistent with the origin of the sample (Edison Seamount with average temperature below 4°C), showing an example that metagenome approach using extreme environment samples can provide an opportunity to develop an extreme condition-adapted enzymes. On the other hand, it is not certain whether the cold-active property of EML1 was due to being secreted extracellularly in vivo since it does not have any signal sequence at the N terminus. Considering high activity on a tricaprylin plate and the absence of signal sequence, it seems likely the protein is secreted through a three-component ATP-binding-cassette transporter system previously reported in *Pseudomonas fluorescens* SIK W1 and *Serratia marcescens* (Duong et al. 1994; Rosenau and Jaeger 2000), but it needs further study.

EML1 showed to have the highest activity toward coconut oil among various natural substrates (Fig. 4b). It was thought because the principal fatty acid component of coconut oil was lauric acid (12:0), which was more hydrolysable by the lipase than oleic acid (18:1), the principal fatty acid component of olive oil (Ogino et al. 2000). In conclusion, we identified a new cold-active lipase from deep-sea sediment sample using metagenome approach. It represents an example that the metagenome approach can provide a chance to develop an enzyme adapted to extreme environments with unique primary sequence.

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