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Substitution of Glycine 275 by Glutamate (G275E) in Lipase of *Bacillus stearothermophilus* Affects Its Catalytic Activity and Enantio- and Chain Length Specificity

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Abstract The lipase gene (*lip*) from *Bacillus stearothermophilus* was recombined *in vitro* by utilizing the DNA shuffling technique. After four rounds of shuffling, transformation, and screening based on the initial rate of clear zone formation on a tricaprylin plate, a clone (M10) was isolated, the cell extract of which showed about 2.8-fold increased lipase activity. The DNA sequence of the mutant lipase gene (*m10*) showed 3 base changes, resulting in two cryptic mutations and one amino acid substitution; S113 (AGC→AGT), L252 (TTG→TTA), and G275E (GGA→GAA). SDS-PAGE analysis revealed that the increased enzyme activity observed in M10 was partly caused by high expression of the *m10* lipase gene. The amount of the expressed G275E lipase was estimated to comprise as much as 41% of the total soluble proteins of the cell. The maximum velocity (V_{max}) of the purified mutant enzyme for the hydrolysis of olive oil was measured to be 3,200 U/mg, which was 10% higher than that of the parental (WT) lipase (2,900 U/mg). Its optimum temperature for the hydrolysis of olive oil was 68°C and it showed a typical Ca^{2+}-dependent thermostability, properties of which were the same as those of the WT lipase. However, the mutant enzyme exhibited a high enantiospecificity towards (S)-naproxen compared with the WT lipase. In addition, it showed increased hydrolytic activity towards triolein, tricaprin, tricaprylin, and tricaproin.

Key words: Lipase, *Bacillus stearothermophilus*, molecular evolution, G275E mutant lipase, substrate specificity

Lipases (triacylglycerol acylhydrolases, E.C. 3.1.1.3) are used as versatile biocatalysts in the production of free fatty acids, interesterification of fats, and synthesis of useful esters and peptides [3, 11, 18]. Lipases are widely found in animals, plants, fungi [12], and bacteria. Among them, microbial enzymes are attracting special attention because of many unique characteristics; substrate, regio-, and enantiospecificity [2, 17].

Recently, two new approaches to make an enzyme possessing improved higher activity and stability or to increase the expression level in a heterologous host has been established [15]. One of them is that a mutation can be introduced randomly into the structural gene using an error-prone PCR or a recombinative method such as DNA shuffling [16]. Another one is that many high-throughput screening methods to detect an improved enzyme have been established [10]. The advantage of the 'directed evolution' is that it can be used to evolve a protein into a more efficient one, without knowing its three dimensional X-ray crystal structure.

A lipase gene (*lip*) from *Bacillus stearothermophilus* L1 strain [5, 6] was cloned and sequenced. The lipase enzyme was demonstrated to have an optimum temperature of 68°C for hydrolysis of olive oil emulsion and a Ca^{2+}-dependent thermostability of up to 65°C. This thermostability was also observed independently in the protein unfolding experiment [8]. These properties enabled the lipase enzyme to efficiently hydrolyze some solid lipids such as beef tallow and palm oil at elevated temperatures.

In the present work, we attempted to evolve the L1 lipase to have higher activity and stability by DNA shuffling and TCN plate assay. After four rounds of DNA shuffling, we obtained a mutant lipase with an increased hydrolytic activity. We compared its thermostability and enantio- and substrate specificity with the wild-type (WT) enzyme.

MATERIALS AND METHODS

Chemicals

Triglycerides, isopropyl-D-thio-galactopyranoside (IPTG), and (S)-2-(6-methoxy-2-naphtyl) propionic acid [(S)-naproxen]

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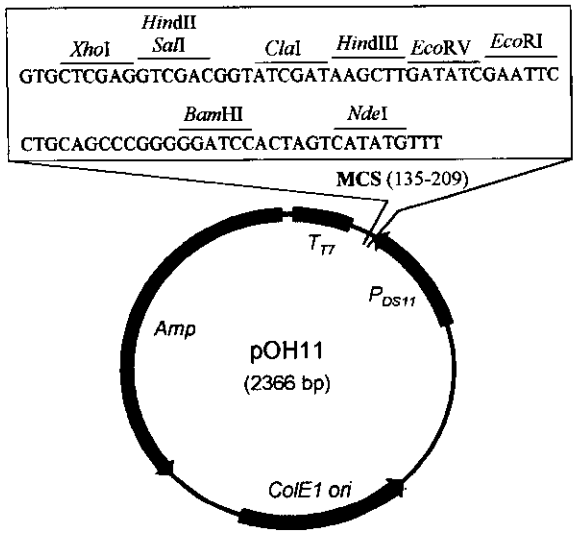

Fig. 1. Physical map of pOH11 vector.
The pOH11 vector was made from a major part (~2 kb) of pBluescript II SK(+) vector and *Bacillus amyloliquifaciens* DS11 phytase promotor (278 bp) and T7 terminator (110 bp). The DS11 promotor located adjacently upstream of multiple cloning sites (MCS).

were purchased from Sigma-Aldrich (St. Louis, U.S.A.). (R,S)-naproxen methyl ester was synthesized by racemization and esterification using (S)-naproxen [1]. (R,S)-Ketoprofen was purchased from Kulk Jeon Medicines Co. (Korea), and (R,S)-Ketoprofen ethyl ester was synthesized by esterification with ethanol [7].

Bacterial Strains and Plasmids

E. coli XL1-Blue [*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*⁻ F' (*proAB*⁺ *lacI*^{q} *lacZ*M15 Tn10(tet^r))] and *E. coli* BL21(DE3) (F*ompT* *hsdS*$_B$($r_B^-m_B^-$) *gal dcm* (DE3)) were grown in LB media at 37°C. Plasmid pOH11, a pBluescript SK derivative containing the DS11 phytase promoter from *Bacillus* sp. [9], was used for cloning (Fig. 1) and plasmid pET-22b(+) (Novagen, Inc.) for the overexpression of an evolved lipase gene.

DNA Shuffling and Screening of the Evolved Enzyme

In vitro recombination was done by the method described by Stemmer [15, 16] with slight modifications. A 1,167 bp *Nde*I and *Bam*HI fragment from pSLE2 [8] was used for DNA shuffling. DNA fragments of 50–200 bp were used for reassembly PCR. The PCR primers for amplification of the reassembled genes were 5'-GAA CAT ATG GCA TCT CCA CGC GCC AAT GAT-3' (forward) and 5'-GCA GGA TCC TTA AGG CCG CAA ACT CGC CAG-3' (reverse).

The amplified gene was digested with *Nde*I and *Bam*HI, and ligated into vector pOH11. The ligation mixture was electroporated into *E. coli* XL1 Blue competent cells and the transformed cells were plated on TCN plates [LB agar plates containing 100 μg/ml ampicillin, 1% (v/v) tricaprylin, 20 mM NaCl, 1 mM $CaCl_2$, and 0.5% (w/v) gum arabic] [4]. The plates were incubated for 12 to 24 h at 37°C. The resulting ampicillin-resistant transformants were visually screened. The colonies with more intense halo than the original one were picked from about 10,000 transformants and their plasmid library was used for the next round of DNA shuffling. The cell-free extracts of the selected clones were assayed with pH stat at 50°C.

Nucleotide Sequence Analysis

DNA sequencing was performed with the Big Dye DNA Sequencing Kit and ABI PRISM sequence analyzer (3.3) (Perkin-Elmer Corp., U.S.A.) according to the manufacturer's instructions, using primers deduced from the nucleotide sequence.

Construction of the Expression Systems

To express the L1 lipase and the mutated genes in *E. coli* cells, the genes digested with *Nde*I and *Bam*HI were ligated with pET-22b(+) vector under the control of the T7 promotor and translational signals. *E. coli* BL21(DE3) cells were transformed with the expression plasmids and grown in 1-l Erlenmeyer flasks containing 0.2 l of LB medium (100 μg/ml ampicillin) at 37°C until OD_{660} reached 0.5–0.6. IPTG (1 mM) was added to the cell culture and the cells were further cultured for 4 h and harvested by centrifugation.

Purification of Enzymes

Cell pellets of the WT lipase and the G275E lipase were suspended in 50 ml of 10 mM phosphate buffer, pH 6.0, disintegrated by ultrasonic treatment (Sonifier 450, Branson) for 30 min, and then centrifuged (10,000 rpm, SS-34) for 1 h at 4°C. The supernatant was put onto a CM-Sepharose column (2.5×18 cm) equilibrated with 10 mM phosphate buffer, pH 6.0, and the column was washed with the same buffer. The bound protein was eluted with an increasing gradient of KCl (0–0.5 M).

Active fractions (0.15–0.25 M KCl fractions) were pooled and its buffer was changed to 20 mM phosphate buffer, pH 7.0, by dialysis. The enzyme solution was applied onto a hydroxyapatite column (2.5×14 cm) equilibrated with the same buffer, and the lipase enzyme was eluted with a phosphate gradient (0–1.0 M).

The purified lipase (0.3–0.5 M phosphate fractions) was concentrated, stored at −70°C, and used for the following characterization.

Lipase Assay and Protein Estimation

Lipase activity was measured by titrating free fatty acids released by hydrolysis of olive oil using the pH-stat method [4]. Olive oil emulsion was prepared by emulsifying 5 ml of olive oil in 495 ml of 20 mM NaCl, 1 mM $CaCl_2$, and 0.5% (w/v) gum arabic solution for 2 min at a maximum speed in a Waring blender. After the pH of the substrate

emulsion (20 ml) was adjusted to 8.0 by adding 10 mM NaOH solution, an appropriate amount (10–50 μl) of the enzyme solution was added. The rate of fatty acid released for 5 min at 50°C was measured with a pH titrator (718 Stat Titrino, Metrohm, Swiss). The activity of the lipase towards various triglycerides, natural fats, and oils was measured at 50°C after the emulsions (1%) were prepared as in the case of the olive oil emulsion. One unit of lipase was defined as the amount of enzyme liberating 1 μmol of fatty acid per min.

The protein concentrations were determined by Bradford protein assays (Bio-Rad Lab., Hercules, U.S.A.).

Enzymatic Hydrolysis of (R,S)-Naproxen and (R,S)-Ketoprofen Esters

Hydrolysis of (R,S)-naproxen methyl ester and (R,S)-ketoprofen ethyl ester was conducted in a microtube (2.0 ml) containing 25 mM substrate, 50 mM Tris-HCl buffer (pH 8.5), and 500 units of enzyme. The reaction was carried out at 50°C for 5 days and terminated by adding 1 ml of methanol.

The formation of (R)- and (S)-forms of naproxen and ketoprofen was monitored by HPLC (Young Lin Co., Korea) using a chiral column (CHIREX Phase 3005, Phenomenex, U.S.A.). The mobile phase was a mixture of 100 mM and 20 mM of ammonium acetate/methanol for naproxen and ketoprofen, respectively, at a flow rate of 1.0 ml/min. Ultraviolet detection (254 nm) was carried out for quantification at the column temperature of 25°C.

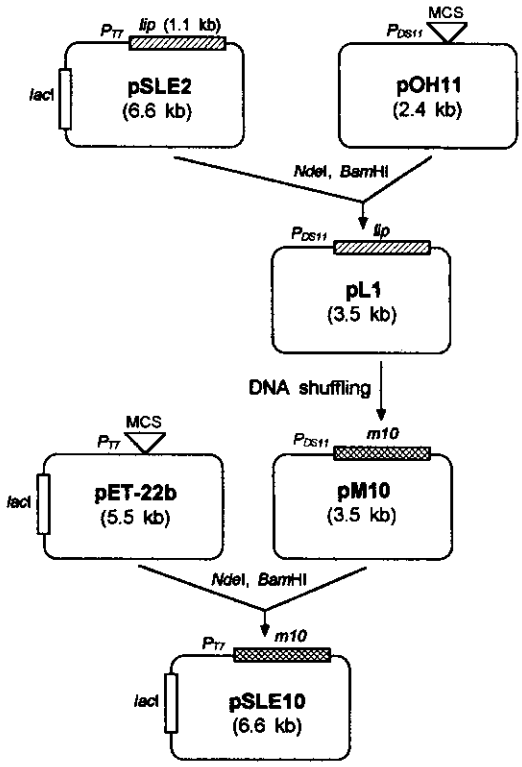

Fig. 2. Schematic representation for the molecular evolution of the *lip* gene.
The mature part of the *lip* gene in the pSLE2 vector was subcloned into the pOH11 vector. After four rounds of DNA shuffling experiments, the *m10* gene was obtained. The *m10* gene was subcloned into the pET-22b vector.

RESULTS

Screening of the Evolved Lipase

Some activity-positive mutants were selected in each round of shuffling based on the size of the clear zone and the initial clear zone-forming rate on the TCN plate. The resulting plasmid libraries were used for the next DNA shuffling. After four rounds of shuffling and screening, a candidate mutant (strain M10) was finally obtained. Its cell-free extract showed about a 2.8-fold higher lipase activity than that of the control strain carrying the pL1 plasmid (Fig. 2, Table 1).

DNA sequencing of the mutant lipase gene (*m10*) revealed that 3 bases were changed (^{339}C→T, ^{756}G→A, ^{825}G→A), resulting in 2 cryptic mutations and 1 amino acid substitution; S113 (AGC→AGT*), L252 (TTG→TTA*), and G275E (GGA→GA*A). This implicated that only one amino acid substitution had occurred at the protein level. Hereafter, we designated the mutant enzyme as a G275E lipase.

Catalytic Activity of G275E Mutant Lipase

To test whether the high lipase activity in the M10 strain was caused by increased catalytic activity of the G275E enzyme, it was necessary to overexpress and purify the mutant enzyme. For the high-level production, an expression plasmid (pSLE10) was constructed with the pET-22b vector (Fig. 2) and was transformed into *E. coli* BL21(DE3). IPTG effectively induced the production of the G275E lipase in the transformed cell at 37°C. The lipase activity in the cell-free extract was measured to be 1,200,000 U/g protein, which corresponded to about a 4.8 times higher activity than those of *E. coli* BL21(DE3)/pSLE2 (Table 1).

SDS-PAGE analysis of cell-free extracts showed that the high lipase activity in the pSLE10-transformant was generated partly by high expression of the *m10* gene (Fig. 3). When the same protein amounts (50, 20, and 10 μg) of

Table 1. Lipase activity in cell-free extracts of *E. coli* transformants.

Plasmid	*E. coli* strain	Lipase activity in cell-free extracts	
		U/l (culture)	U/g (total cell protein)
pLIP1 (WT)	XL1-Blue	12,000	5,800
pSLE2 (WT)	BL21(DE3)	130,000	250,000
pL1 (WT)	XL1-Blue	8,700	31,000
pM10 (G275E)	XL1-Blue	16,000	87,000
pSLE10 (G275E)	BL21(DE3)	530,000	1,200,000

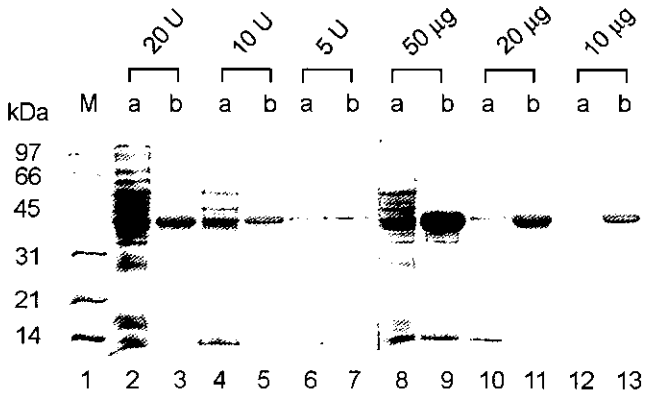

Fig. 3. SDS-PAGE of the cell-free extract of *E. coli* transformants harboring pSLE2 (a) or pSLE10 (b).
SDS-PAGE was done after normalization with lipase activity (lanes 2-7) and protein amount (lanes 8-13). Lane 1 shows the molecular weight marker.

the cell-free extract were loaded, the intensities of G275E protein bands were much higher than those of the WT protein, respectively (lanes 8-13). However, SDS-PAGE analysis also showed that the catalytic activity of G275E lipase was increased in comparison with that of the WT lipase. That is, when the same units (20, 10, and 5 units) of lipase of the cell-free extract were loaded on the gel, the band intensities of the G275E proteins were a little lower than those of the WT protein, respectively (lanes 2-7).

To measure the V_{max} of G275E lipase for the hydrolysis of olive oil, we purified the enzyme from the cell free extract of *E. coli* BL21(DE3)/pSLE10 by CM-Sepharose and hydroxyapatite chromatographies to homogeneity, as evidenced on SDS-PAGE gel. An apparent maximum velocity, $V_{max.app}$, towards olive oil at a standard assay condition (50°C and pH 8.0) was determined to be 3,200 U/mg, which was 10% higher than that of WT lipase (2,900 U/mg) (Fig. 4).

Therefore, the enhanced catalytic activity of G275E lipase along with its enhanced expression seemed to have resulted in the high lipase activity observed in *E. coli* cells carrying the pSLE10 plasmid.

Effects of Temperature on G275E Mutant Lipase

The optimum temperature of the L1 (WT) lipase had been reported to be 68°C when olive oil was used as a substrate [8]. G275E mutant lipase showed a maximum catalytic activity at the same temperature (68°C), while its activity at temperature over 68°C was somewhat higher than that of the WT enzyme (Fig. 5). It showed as much as 30%

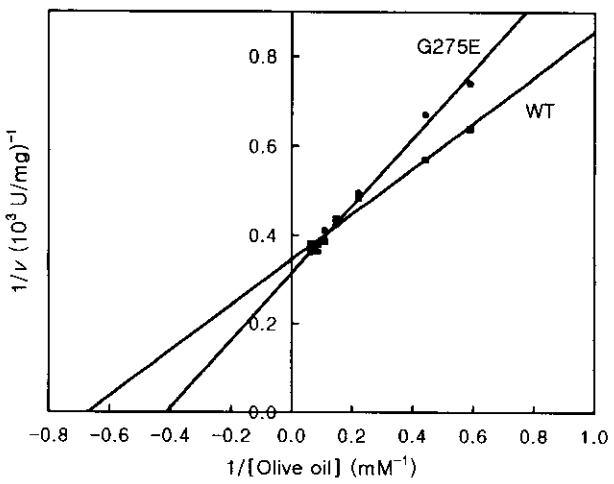

Fig. 4. Lineweaver-Burk plot of WT and G275E lipase.
Hydrolytic activity of WT and G275E lipase was measured with olive oil emulsions. When the molecular weight of one mole of olive oil was assumed to be 885.4 g, the apparent V_{max} values of WT and G275E were 2,900 and 3,200 U/mg, respectively.

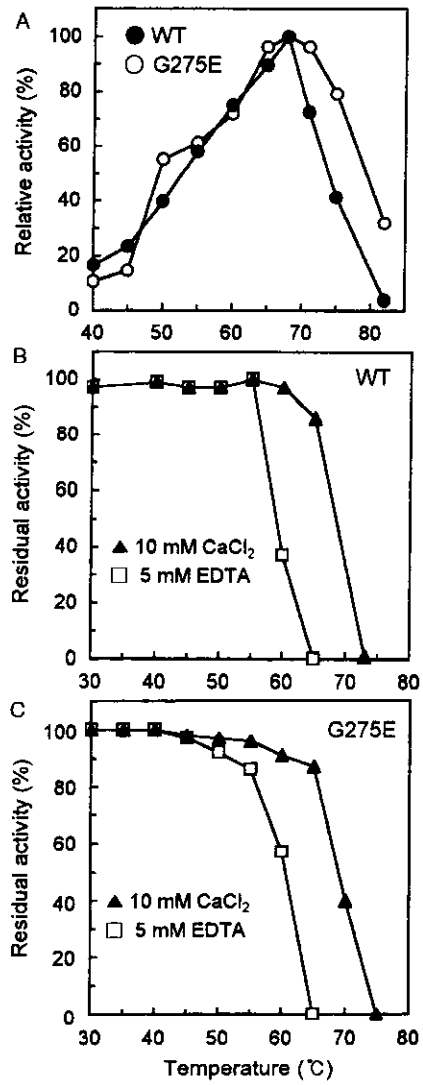

Fig. 5. Effects of temperature on the lipase activity and stability.
A. The hydrolytic activity of WT and G275E lipase was measured at various temperatures with olive oil emulsion. B and C. WT and G275E lipase were incubated at various temperatures for 30 min in the presence of 10 mM Ca^{2+} or 5 mM EDTA and then the residual activity was measured.

Table 2. Hydrolysis of (R,S)-naproxen and -ketoprofen esters.

Enzyme	Naproxen methyl ester			Ketoprofen ethyl ester		
	c (%)	ee_p (%)	E	c (%)	ee_p (%)	E
WT	2.25	21.5	1.55	9.01	33.9	2.09
G275E	5.49	54.2	3.47	16.6	17.8	1.48

c: conversion, ee: enantiomeric excess, E: enantiomeric ratio $ln[1-c(1+ee_p)]/ln[1-c(1-ee_p)]$.

relative activity at 82°C. As a result, it appeared that a substitution of a negatively charged carboxyl group at residue 275 resulted in a slight positive effect on the catalytic site at higher temperatures.

G275E enzyme's thermostability was similar to that of the WT enzyme, since the enzyme was fairly stable up to 65°C for 30 min-incubation in the presence of calcium ions (10 mM), while a steep decrease in the residual activity was observed at higher temperatures. The enzyme thermostability decreased by 10 degrees in the absence of calcium ions (5 mM EDTA). This implied that the Glu275 residue had no inhibitory effects on the calcium-binding as well as calcium-induced structural stabilization of the enzyme.

Enantiospecificity of the G275E Mutant Lipase

To check the change of enantiospecificity of G275E lipase, (R,S)-naproxen and (R,S)-ketoprofen esters were used as model compounds. The same lipase unit (500 units) of WT and G275E enzyme were used for the hydrolysis of those enantiomers. Table 2 showed that G275E enzyme showed a higher enantiospecificity for (S)-naproxen methyl ester but less for (S)-ketoprofen ethyl ester compared to WT lipase. The difference of enantiospecificity between the G275E and WT enzymes suggested that amino acid residue 275 was important for substrate binding and that an introduction of a negatively charged bulky amino acid (Glu) specifically changed the enzyme enantiospecificity.

Hydrolytic Activity of G275E Mutant Lipase Towards Natural Fat and Oils

WT lipase had been reported to have a relatively high hydrolytic activity towards triglycerides containing medium and long-chain saturated fatty acids, and hence was able to efficiently hydrolyze solid lipid (fat) such as beef tallow and palm oil [5].

G275E mutant lipase showed a somewhat different chain length specificity in comparison with WT lipase (Fig. 6). It had a higher specific activity towards medium-chain triglycerides, i. e., triolein ($C_{18:1}$), tricaprin (C_{10}), tricaprylin (C_8), and tricaproin (C_6).

G275E mutant lipase showed overall an increased hydrolytic activity towards most natural fat and oils, compared to WT lipase (Fig. 7). In particular, the hydrolytic activity of G275E enzyme towards beef tallow, palm oil, and coconut oil was remarkably increased. These results also demonstrated that G275E mutation modified the substrate binding site to favor triglycerides containing medium-chain saturated fatty acids.

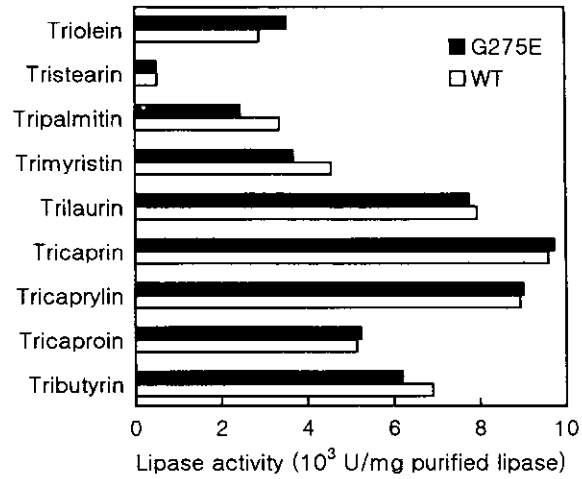

Fig. 6. Chain-length specificity of WT and G275E lipases.

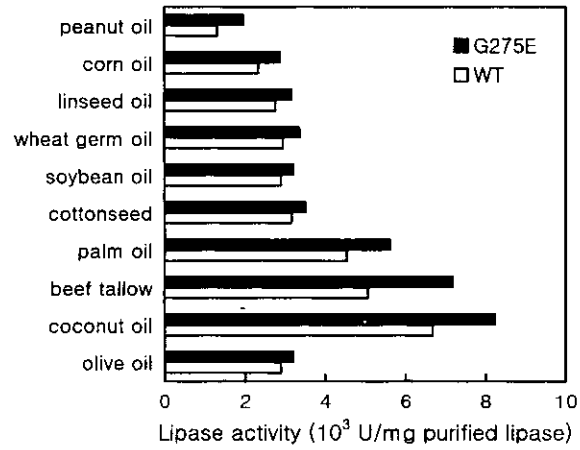

Fig. 7. Hydrolytic activity of WT and G275E lipases toward various natural fats and oils.

DISCUSSION

In the DNA shuffling and screening process, the pOH11 vector was used as a cloning vector for two reasons. Firstly, the vector had appropriate cloning sites (*Nde*I and *Bam*HI) located adjacent downstream of the DS11 promotor, which enabled a simple subcloning of the mature part of the *lip* gene within the correct reading frame. Secondly, the promotor strength was appropriate; the lipase expression level in *E. coli* (31,000 U/g) was suitable to easily discriminate a colony with an increased lipolytic activity from the original one on the TCN plate.

In the context of the pOH11 vector, we found a mutant lipase gene (*m10*) showing a 2.8-fold enhanced lipase activity. Subsequently, when the pET-22b vector was used

as an expression vector, the *m10* gene showed a much higher enhancement (4.8-fold) of lipase activity compared to the wild-type gene (*lip*).

The increased lipase activity of the *m10* gene seemed to be caused by two reasons. Firstly, the catalytic activity of G275E mutant enzyme itself increased by 10% in comparison with WT lipase. Secondly, the expression level of the *m10* gene was much higher than the *lip* gene in the recombinant *E. coli* cells (Fig. 3). The DNA sequence of the *m10* gene had just 3 base changes from the *lip* gene: $^{339}C \rightarrow T$, $^{756}G \rightarrow A$, and $^{825}G \rightarrow A$. All three mutations introduced AT bases in place of GC ones. In particular, the first mutation ($^{339}C \rightarrow T$) seemed to release the regional hairpin structure (^{338}GCC AAG GAG GGC^{349}, ΔG=- 1.9 kcal/mol) of the mRNA molecule. However, a further study is needed to clearly explain the observed expressional difference between the *lip* and *m10* genes.

The three-dimensional structure of the L1 lipase had not yet been elucidated, but Ser113, Asp317, and His385 residues were suggested as its catalytic triad on the basis of primary sequence alignment with *Bacillus thermocatenulatus* lipase (BTL2) [14] and *Pseudomonas glumae* lipase [13], of which the X-ray crystal structure had been elucidated. Therefore, it appeared that the Gly275 residue was located distal from any catalytic sites on the primary sequence. However, G275E mutation enhanced its catalytic activity and distinctly changed its enantio- and substrate specificity. These observations strongly suggested that the Gly275 residue interacted with the catalytic site and/or the substrate binding site on the three-dimensional level.

On the other hand, although naproxen and ketoprofen are racemic compounds widely used as anti-inflammatory drugs, only the (S)-form is active. As a result, the G275E mutant enzyme seemed to be used efficiently in the production of (S)-naproxen after further optimization of the reaction condition.

In addition, it is known that the hydrolysis of solid lipids such as beef tallow and palm oil is difficult compared with that of liquid lipids such as olive oil. However, the G275E enzyme can be used effectively at high temperatures in the hydrolysis of such solid lipids, the main material used for producing fatty acids in the industry, because of its thermostability and preference for long-chain saturated fatty acids.

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