J. Microbiol. Biotechnol. (2000), **10**(5), 656–662

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Staphylococcus haemolyticus Lipase; High-Level Expression in *Escherichia coli* and Activation of Nonionic Detergent

OH, BYUNG-CHUL, HYUNG-KWOUN KIM*, MYUNG-HEE KIM, JUNG-KEE LEE, AND TAE-KWANG OH

Environmental Bioresources Lab., Korea Research Institute of Bioscience and Biotechnology, P.O. Box 115, Yusong, Taejon 305-600, Korea

Received: May 31, 2000
Accepted: August 2, 2000

Abstract A high level of *Staphylococcus haemolyticus* L62 lipase was expressed in an *Escherichia coli* transformant. The expressed lipase activity in the cell-free extract was 70,800 U/l, which corresponded to 30% of the total cellular proteins. Pre-mixing of the L62 lipase with some nonionic detergents enhanced its hydrolytic activity towards olive oil: Tween detergents activated the L62 lipase by 3 fold. Gel filtration chromatography of the Tween-80–L62 lipase mixture demonstrated a polymerized complex (~180 kDa) formed exclusively between Tween-80 and the L62 lipase. The lipase enzyme in the complex showed a higher specific activity towards most triacylglycerols than the intact L62 lipase. The activity enhancement towards each substrate was quite different depending on the acyl chain length; the activity towards tributyrin, trilinolein, and trilinolenin was much more enhanced than that towards the medium and the long-chain saturated triglycerides.

Key words: Lipase, *Staphylococcus haemolyticus*, nonionic detergent

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are a class of enzymes that catalyze the hydrolysis of long-chain triglycerides at an oil-water interface. Many lipases have industrially important properties such as chain length selectivity, regiospecificity, and chiral selectivity. Therefore, the enzymes are widely used for the production of free fatty acids, interesterification of fats and oils, and synthesis of useful esters and peptides [2, 12, 17].

Since long-chain triglycerides have low solubility in water, it is not evenly distributed in an aqueous medium but rather phase-separated or aggregated to form an emulsion with other lipid materials. Although the mode of interaction between the lipase and long-chain triglyceride aggregate is quite complex, the mechanism of emulsion formation, its availability to the enzyme, and the mode of enzyme activation by the hydrophobic interactions have been elucidated to a certain degree.

One of the common kinetic features observed during lipolysis is a lag period before the establishment of steady state hydrolysis. This lag period is not due to diffusional limitation but slow interfacial penetration of the enzyme [19]. During the penetration process, the lipases undergo a coincident conformational change; the 'lid' domain covering the active site peels back to expose the active site to the substrate, which is suggested to be closely related to a lipase-specific phenomenon, called 'interfacial activation' [1].

Lipase binding and activation depend on the 'interfacial quality'. Muderhwa and Brockman [10] demonstrated that the hydrolysis activity of pancreatic lipase towards 1,3-diolein was increased in the presence of some phospholipids, and they suggested that the inclusion of species other than the substrate could enhance lipolysis. Detergent is another example of an interface modulator. In the interaction of lipase-substrate-detergent, the nature of the hydrophilic group on the detergent was demonstrated to determine the hydrolysis efficiency [6].

On the other hand, some detergents bind directly to and activate the lipase proteins. For example, bile salts activate pancreatic lipase by binding to a loop near the active site and stabilizing the open conformation [18]. Bile salts-activated lipases are physiologically important for the absorption of triacylglycerol, cholesterol, and vitamin A in the intestine of adults and milk-fed infants. Recently, the interaction between detergents and microbial lipases has also received attention for their industrial application [6].

Previously, we reported some biochemical properties and nucleotide sequence of the *Staphylococcus haemolyticus*

*Corresponding author
Phone: 82-42-860-4352; Fax: 82-42-860-4595;
E-mail: hkkim@mail.kribb.re.kr

L62 lipase [11]. In this paper, we overexpressed the *S. haemolyticus* lipase in *E. coli* and demonstrated that pretreatment of the lipase with some nonionic detergents strongly enhanced its hydrolytic activity towards various triglyceride substrates.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

E. coli XL1-Blue (*rec*A1 *end*A1 *gyr*A96 *thi hsd*R17(r_k^-, m_k^+) *sup*E44 *rel*A1 *lac* (F *pro*AB$^+$ *lac*I^{q}ΔZ15 ::Tn10(Tet^r)) and *E. coli* BL21(DE3) (F$^-$ *ompT hsd*SB($r_B^-m_B^-$) *gal dcm* (DE3)) were grown on the LB media at 37°C. Plasmid pSHL was used as a template DNA for the polymerase chain reaction (PCR) and cloning of the L62 lipase gene, and plasmid pET-22b(+) (Novagen, Madison, U.S.A.) for the expression of the gene.

Construction of the Expression Systems

To express the L62 lipase gene in *E. coli* cells, we first constructed three expression plasmids (pSHLpre, pSHLpro, and pSHML) carrying the coding region of the L62 lipase gene, as depicted in Fig. 1. pSHLpre was designed to produce its prepro form (711 aa). Two oligomers (primers 1 and 4) and *S. haemolyticus* genomic DNA (in pSHL) were used for PCR to generate the target DNA fragment carrying an *Nde*I restriction site at its 5' end (immediate upstream of the initiation codon ATG of the L62 lipase gene) and *Bam*HI site at its 3' end. After being digested with *Nde*I and *Bam*HI, the DNA fragment was ligated with the pET-22b(+) vector.

In order to prepare pro (651 amino acids) and mature (392 amino acids) forms, PCRs were carried out with other sets of primers (primers 2 and 4 for the pro sequence and primers 3 and 4 for the mature one), the template DNA (pSHL), and generating expression plasmids (pSHLpro and pSHML). The nucleotide sequence of the four primers are as follows: primer 1: 5'-GAACATATGATAAAATTAATT-AACAGT-3'; primer 2: 5'-GAACATATGGCTGAGCAA-CAATCCTCCCAC-3'; primer 3: 5'-GAACATATGGCT-ACTATCAAAAGTAATCAA-3'; primer 4: 5'-GCAGGA-TCCTTAATGTTTTATACCATCTAT-3'

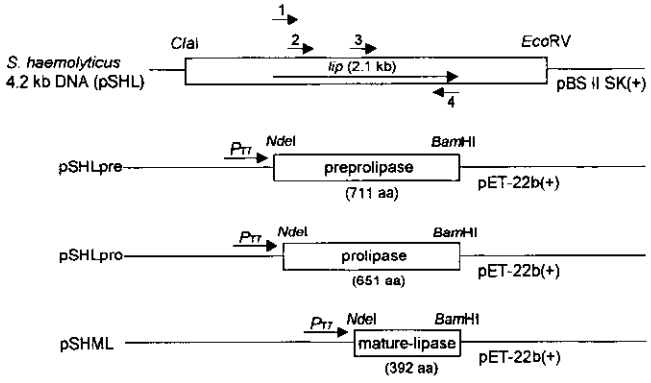

Fig. 1. Construction of expression plasmids for the production of the prepro-, pro-, and mature L62 enzymes.
The L62 lipase gene (*lip*) of *S. haemolyticus* in plasmid pSHL is indicated. Four arrows (1, 2, 3, and 4) on *lip* indicate the location and direction of synthetic oligomers for PCR. Plasmid pSHLpre, pSHLpro, and pSHML were designed for the production of the prepro-, pro-, and mature L62 enzymes, respectively.

Plasmids pSHLpre, pSHLpro, and pSHML are under the control of the T7 promotor and translational signals. *E. coli* BL21(DE3) cells were transformed with the expression plasmids and cultivated at 37°C in the LB medium containing ampicillin (100 μg/ml).

Purification of the Lipase

E. coli BL21(DE3)/pSHML was grown in 1-l Erlenmeyer flasks containing 0.2 l of the LB medium (100 μg/ml ampicillin) at 37°C until OD_{660} reached 0.5–0.6. Isopropyl-β-D-thio-galactopyranoside (IPTG) (1 mM) was added to the cell culture, and the cells were further cultured for 4 h and harvested by centrifugation. Cell pellets were dissolved in 50 ml of 20 mM Tris-HCl (pH 8.0), disintegrated by ultrasonic treatment (Sonifier 450, Branson, U.S.A.) for 20 min, and then centrifuged at 12,000 ×g for 30 min at 4°C. After ammonium sulfate was added to the cell-free lysate to 80% saturation, the precipitate was collected by centrifugation at 12,000 ×g for 20 min, dissolved in a Tris-HCl buffer (20 mM, pH 8.0), and then dialyzed against the same buffer. The dialysate was applied to a Resource™ Q column (Pharmacia, Uppsala, Sweden) pre-equilibrated with the same buffer. The lipase enzyme passed through the column was collected, re-injected to a Resource™ S column (Pharmacia, Uppsala, Sweden) equilibrated with the same buffer and gradient eluted with KCl (0–0.5 M). The purified lipase was concentrated and stored at -70°C.

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 [9]. 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 8.5 by adding 10 mM NaOH solution, an appropriate amount (10–20 μl) of the enzyme solution was added. The rate of the fatty acid released was measured with a pH titrator (718 Stat Titrino, Metrohm, Switzerland) for 5 min at 28°C. One lipase unit is defined as the amount of enzyme liberating 1 μmol of fatty acid per min.

Activity of lipase towards various triacylglycerols was measured by the same pH-stat method. Twenty millimolar

of triacetin, tripropionin, tributyrin, tricaproin, tricaprylin, tricaprin, trilaurin, trimyristin, tripalmitin, tristearin, triolein, trilinolein, and trilinolenin in 20 mM NaCl, 1 mM $CaCl_2$, and 0.5% (w/v) of gum arabic solution were emulsified and used as the substrate solution.

Activity of lipase towards natural fats and oils was measured with the corresponding fat/oil emulsions (1%, v/v) prepared as in the case of the olive oil emulsion.

Lipolytic activity of the protein on the SDS-PAGE gel was detected by using tributyrin (TBN) agar plates. After the electrophoresis, the gel was washed with 50 mM Tris buffer (pH 8.0) containing 1% (v/v) Triton X-100 (TX100) and washed twice with 50 mM Tris buffer. The gel was overlaid on a TBN agar plate prepared with agar (1.5%) and tributyrin emulsion (1% tributyrin, 20 mM NaCl, 1 mM $CaCl_2$, and 0.5% (w/v) gum arabic) and incubated at 28°C until a transparent band appeared (about 1–2 h).

The protein concentration was measured by following the Bradford method using protein assay kits (Bio-Rad Lab., Richmond, U.S.A.) with bovine serum albumin as the standard protein.

Gel Filtration Chromatography

Gel filtration chromatography was performed with a Superose 12HR 10/30 column (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 0.15 M NaCl. The intact lipase and the detergent-treated lipase were applied to the column and eluted at a flow rate of 0.4 ml/min. The K_{av} values for standard proteins were calculated and plotted against the logarithms of their molecular weights.

Results and Discussion

Expression of the L62 Lipase

S. haemolyticus L62 strain produced and secreted a large amount (9,800 U/l) of 45 kDa lipase to the growth medium [11]. However, since the strain also produced a comparably high amount of 34 kDa protease at the same time and this endogenous protease degraded the L62 lipase rapidly, it was difficult to obtain a large amount of the pure L62 lipase.

Previously, the lipase gene (*lip*) was cloned and sequenced. However, the *E. coli* cell transformed with the *lip* gene turned out to have a low lipase activity [11]; although the *E. coli* transformant showed lipase activity on tricaprylin plate, its activity was too low to measure with the pH-stat assay (less than 200 U/l culture).

In this experiment, three expression plasmids were constructed for the high level-production of the L62 lipase (Fig. 1). These plasmids were prepared with pET-22b(+) vector and three PCR products, each coding for the prepro-, pro-, and mature forms of the enzyme.

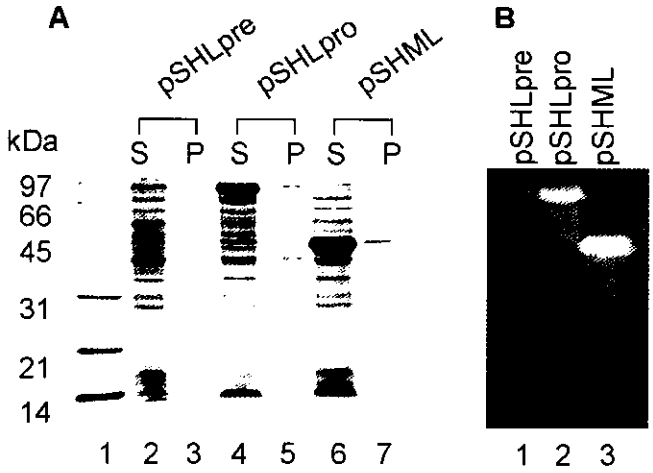

Fig. 2. SDS-PAGE (A) and zymogram (B) of the expressed L62 lipase.

A: The expressed prepro-, pro-, and mature L62 lipases were electrophoresed on a 12% SDS gel and stained with Coomassie Brilliant Blue R-250. Lane 1 is the standard proteins. Lanes 2-7 are the soluble (S) and insoluble (P) fractions obtained by ultrasonic lysis of the *E. coli* transformants. B: Lanes 1, 2, and 3 showed tributyrin-hydrolytic activities of the expressed prepro-, pro-, and mature L62 lipases, respectively.

IPTG effectively induced the production of the mature form of the L62 lipase in the transformant *E. coli* BL21(DE3)/pSHML (Fig. 2). The lipase activity in the cell-free extract was 70,800 U/l, and the specific lipase activity was 180 U/mg, which corresponded to as much as 30% of the total cellular proteins, based on the specific activity (596 U/mg) [11] of the pure L62 lipase (Table 1).

The prolipase was also expressed well in the transformant *E. coli* BL21(DE3)/pSHLpro. The activity of the expressed proenzyme in the cell-free extract was 43,400 U/l, which was about 60% of the expressed mature lipase. The expressed prolipase produced a clear 80 kDa-sized band, but no 45 kDa-sized band on the TBN plate (Fig. 2B), implying that the pro part of the proenzyme was not cut off in the transformed *E. coli* cell.

Table 1. Expression level of the L62 lipase in *S. haemolyticus* L62 and various *E. coli* transformants.

Strain	Enzyme preparation	Expression level[b]	
		U/l (culture)	U/g (protein)
S. haemolyticus L62	Culture Sup	9,800	82,800
E. coli XL1 Blue/pSHL	Sup[a]	ND[c]	ND[c]
E. coli BL21(DE3)/pSHLpre	Sup	15,300	40,100
E. coli BL21(DE3)/pSHLpro	Sup	43,400	133,000
E. coli BL21(DE3)/pSHML	Sup	70,800	180,000

[a]Sup is a soluble fraction of the whole cell lysate obtained after ultrasonic treatment of 4 h-induced cells.

[b]The expression level was measured by using a pH-stat assay with olive oil emulsion as a substrate (28°C and pH 8.5).

[c]ND: not determined.

Table 2. Purification of the L62 lipase from a cell-free extract of *E. coli* BL21(DE3)/pSHML.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Cell-free extract[a]	393.0	70,800	180	1.0	100
$(NH_4)_2SO_4$ precip.	176.0	50,500	287	1.6	71
Resourse Q™	75.1	28,300	377	2.1	40
Resourse S™	34.7	20,700	596	3.3	29

[a]Cell-free extract was derived from one liter of the cell culture.

The high lipase activity in the cell-free lysate of *E. coli* BL21(DE3)/pSHML implies that the mature part is able to fold its active conformation by itself and that the pro-region plays a little role, if any, in bringing the enzyme into an active conformation. This result agreed with a previous report in that the pro-region of *S. hyicus* lipase acted as an intramolecular chaperone facilitating translocation of the cell membrane and it protected the protein from proteolytic degradation rather than acting as a foldase [4].

The preprolipase was also expressed in *E. coli* BL21(DE3)/pSHLpre cell, but the expression level was as low as 15,300 U/l.

Purification of the L62 Lipase

The L62 mature lipase was purified from the *E. coli* BL21(DE3)/pSHML cell free lysate by ammonium sulfate precipitation and two successive ion exchange column chromatographies as described in Materials and Methods. The final purification yield was 29% of the activity in the cell free lysate (Table 2).

Table 3. Effects of detergents-pretreatment on the L62 lipase activity.

Detergent	Conc.	Lipase activity (%)
	(%, w/v)	
None		100
Na-dodecylsulfate	1	0
	5	0
Na-deoxycholate	1	70
	5	76
Na-taurocholate	1	200
	5	164
	(%, v/v)	
Triton X-100	1	175
	5	44
Tween-20	1	281
	5	277
Tween-40	1	305
	5	299
Tween-60	1	254
	5	280
Tween-80	1	282
	5	281

The lipase solution was mixed with various detergents and incubated at 4°C for 30 min. Thereafter, the lipase activities of each mixture were measured by pH stat and their relative activities were calculated based on the non-treated lipase solution (775 U/ml).

Effect of Detergent-Pretreatment on the L62 Lipase Activity

The effect of various detergents on the L62 lipase activity is shown in Table 3. When the L62 lipase was mixed and incubated with sodium dodecyl sulfate, an anionic detergent, it was completely inactivated. Sodium deoxycholate, a bile salt, showed inhibitory effect. But sodium taurocholate did not inhibit the L62 lipase; rather, it stimulated the lipase and obtained a 2-fold higher activity at a 1% (w/v) concentration level. Even at 5% concentration, it stimulated the enzyme by 164%. TX100 also activated the L62 lipase at 1% (v/v) concentration but it inhibited it at 5% concentration.

All Tween detergents strongly activated the L62 lipase 3-fold at both 1% and 5% concentrations. Tween detergents have polyoxyethylene (POE) sorbitan as a common moiety and one varying acyl chain; Tween-20, -40, -60, and -80 have lauryl, myristyl, palmityl, and oleyl groups as their acyl chain, respectively.

As all Tween detergents activated the L62 lipase in spite of their difference in acyl chain, we further studied the Tween-80-pretreated L62 lipase as a model case.

Activation of the L62 Lipase with Tween-80

To determine whether or not the enhancement of the lipase activity was due to the direct interaction of Tween-80 (T80) detergent with the lipase molecule, we premixed T80 (1% or 5%, v/v) with the L62 lipase solution or added it to the reaction mixture at different times (Fig. 3). First, when 10 µl of T80 (1% or 5%) was added to the reaction mixture (20 ml) just after (t=0 min) or at 2 min after the reaction started, the free fatty acid (FFA) was released at the same rate as the T80-nontreated control (Figs. 3A-3C).

However, when the lipase was premixed with the T80 just before the reaction started, its hydrolytic activity increased highly (about 2.5 times) (Fig. 3D). These results clearly showed that T80 interacted directly with the lipase molecule and excluded the possibility that the enhancement of the lipase activity was due to the T80-induced changes in the physical state of the olive oil emulsion.

The lipase activity was enhanced within 30 min of preincubation (Figs. 3D-3F). This time-dependent gradual activation confirmed again the direct interaction of T80 with the lipase.

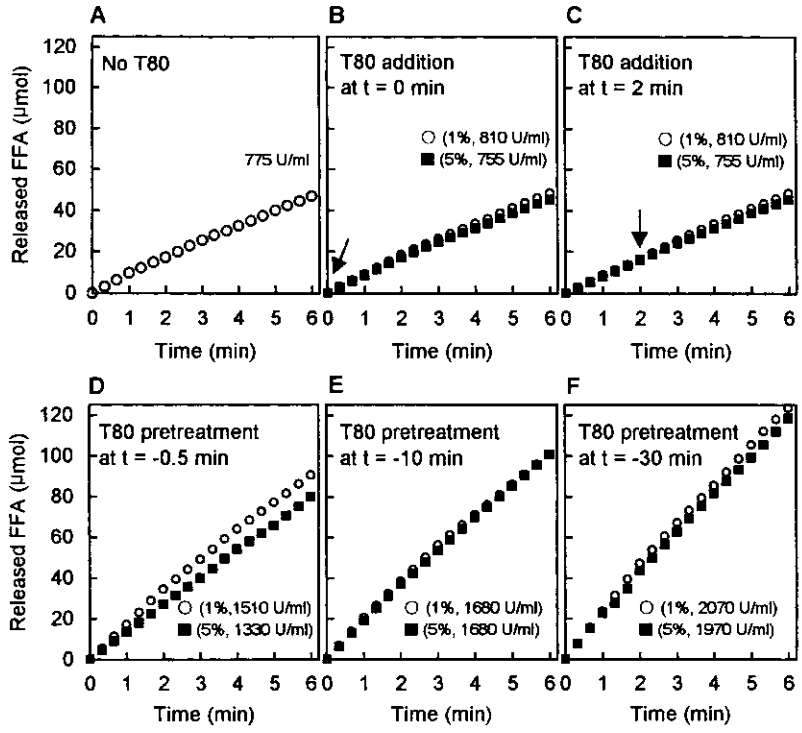

Fig. 3. Enhanced hydrolytic activity of the L62 lipase treated with T80.
The purified lipase (10 μl) and olive oil emulsion (20 ml) were mixed and then the released FFA was measured with a pH stat at 28°C and pH 8.5. The hydrolytic activity of the intact L62 lipase was calculated to be 775 U/ml (A). Ten microliter of T80 (1% or 5%, v/v) was added to the reaction mixture (20 ml) just after (B) and at 2 min (C) after the enzyme was added. Arrows indicate different times of T80-addition. In addition, T80 was premixed directly to the enzyme solution to make up a 1% or 5% concentration. The mixture was incubated for 0.5 min (D), 10 min (E), and 30 min (F) at 4°C and then assayed as described previously.

Tween detergent is different from other detergents in a sense that it can be a substrate of lipases. In fact, the L62 lipase hydrolyzed the acyl group of T80 (1% micelle, v/v) with a specific activity of 14 U/mg enzyme. However, since the value was too low in comparison with that (596 U/mg enzyme) for olive oil, the fatty acid released from T80 did not appear to contribute to the activity-enhancement.

Polymerization of the L62 Lipase by T80

The elution profile of the L62 lipase on Superose 12 HR column is shown in Fig. 4. The intact lipase was eluted with a K_{av} value of 0.322 (MW 45 kDa), whereas the lipase premixed with 1% T80 was eluted with a K_{av} value of 0.208. These results suggested that, during the preincubation period, a high molecular weight (~180 kDa) complex seemed to be formed between T80 and the lipase.

When the active fractions eluted from the column were collected, concentrated, and rechromatographed on the same column, lipase activity was eluted in the protein fraction corresponding to the monomer (MW 45 kDa), suggesting that the aggregation between the L62 lipase and T80 was reversible.

It has been reported that pancreatic cholesterol esterase [7, 16], carboxyl ester lipase [14], and human milk lipase [5] are activated and/or polymerized by bile salts. The bile salt-activation was known to play a nutritionally important role for both adults and milk-fed infants.

Microbial lipases were often secreted into the medium as a complex with lipidic materials and nonionic detergents, which were used frequently to dissociate the bound lipids and to elute the lipase enzyme from hydrophobic columns [3, 15]. In the process, detergents seemed not only to dissociate the bound lipid but also to bind to and sometimes activate the enzyme.

Rollof *et al.* [13] and Jurgens and Huser [8] observed that apparent molecular masses of the lipases of *S. aureus* FN37 and TEN5 in the gel filtration chromatography were 110 kDa and 780 kDa, respectively, although all the monomeric forms were estimated to be 43 kDa according to the SDS-gel electrophoresis. Although further biochemical experiments are needed, it seems that the high molecular weight complex was produced by adding TX100 at a final elution from the octyl-Sepharose column. In addition, the result that the FN37 lipase was activated by 2.6-fold during TX100 elution from the column definitely supports the possibility.

Although no extensive study has yet been made, the nonionic-detergent-induced activation and polymerization

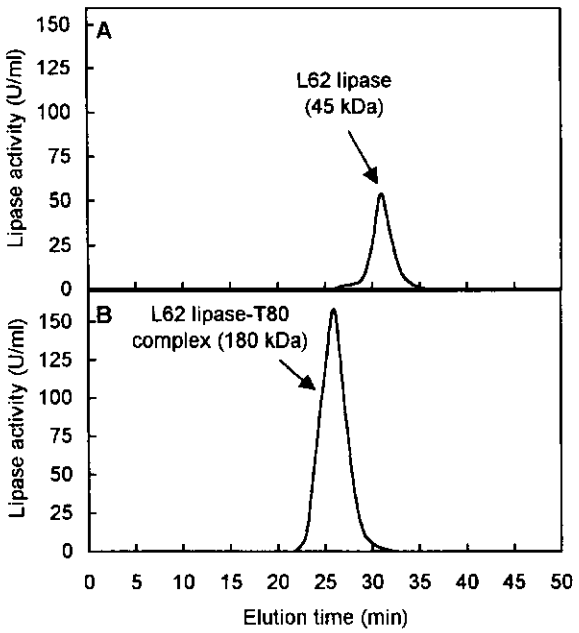

Fig. 4. Gel filtration chromatography of the L62 lipase.
Gel filtration of the intact L62 lipase (A) and the T80-treated L62 lipase (B) was performed with Superose 12 HR 10/30 column connected to a FPLC system (Pharmacia, Uppsala, Sweden). Elution was performed with a 50 mM of Tris buffer (pH 8.0) containing 150 mM of NaCl at a flow rate of 0.4 ml/min. Bed volume (V_t) and void volume (V_o) of the column were 24 ml and 6.88 ml, respectively. The elution volume of the intact and the T80-treated lipase were 12.4 ml and 10.32 ml, respectively. The molecular weights were estimated to be 45 kDa and 180 kDa for the intact and the T80-treated lipases based on the partition coefficients (K_{av}) of 0.322 and 0.201, respectively.

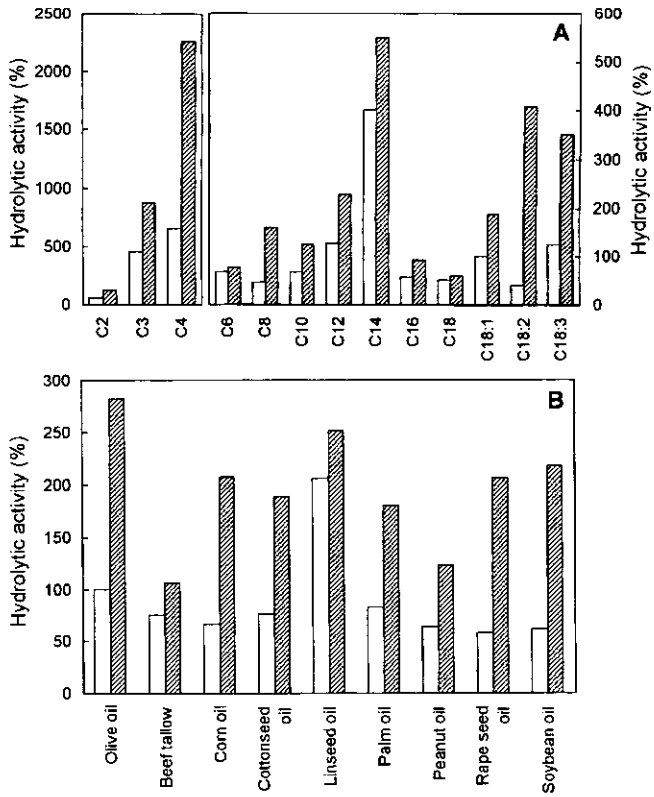

Fig. 5. Hydrolytic activity of the intact and the T80-treated L62 lipase towards various triglycerides (A) and natural fats and oils (B).
Relative hydrolytic activity was expressed as the percentage of those of triolein (600 U/ml) and olive oil (775 U/ml), respectively. Open bars indicate the relative activity of the intact lipase and hatched bars represent the T80-treated enzyme.

do not appear to be a unique phenomenon observed only between T80 and the L62 lipase, but it is a more general property occurring in several lipases.

Hydrolysis of Various Lipids with the Tween-80-Treated Lipase

The intact L62 lipase has been reported to have an acyl chain length-dependent substrate specificity; tributyrin, tripropionin, and trimyristin emulsions are the preferred substrates, whereas change of acyl chain length dramatically reduces its activity [11].

The L62 lipase in the 180 kDa complex showed a much higher hydrolytic activity towards all triglyceride emulsions compared to the intact L62 lipase (Fig. 5A). In addition to the overall increased hydrolytic activity, it showed a differential enhancement of activity towards each substrate; hydrolytic activity for tributyrin, trilinolein, and trilinolenin was much more enhanced than for other chain length triglycerides. The hydrolytic activity towards trilinolein (C18:2) was increased as high as 10 times.

The high activity of the T80 treated lipase towards short chain and unsaturated fatty acids is closely reflected in its hydrolytic activity towards various natural oils and fats (Fig. 5B). The activity towards corn, cottonseed, and soybean oil was increased much more, in which linoleic acid composed up to 50% by weight of their total fatty acids. The increase in the activity towards beef tallow and palm oil was relatively low, which also seemed to be related to their fatty acid compositions; rich in the medium-chain (C10-C14) and long-chain (C16-C18) saturated fatty acids.

We overexpressed the L62 lipase in *E. coli* and found that the treatment of the enzyme with Tween detergent enhanced its hydrolytic activity towards various natural fats/oils. Although further biochemical and biophysical analyses are needed to fully understand the nature of the high molecular weight aggregate (180 kDa) and its activation mechanism, it can be said that the Tween-treated lipase can be easily exploited in the fatty acid production.

REFERENCES

1. Brzozowski, A. M., U. Derewenda, Z. S. Derewenda, G. G. Dodson, D. M. Lawson, J. P. Turkenburg, F. Bjorkling, B. Huge-Jensen, S. A. Patkar, and L. Thim. 1991. A model for

interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature* **351:** 491-494.
2. Dordick, J. S. 1989. Enzymatic catalysis in monophasic organic solvents. *Enzyme Microb. Technol.* **11:** 194-211.
3. Dunhaupt, A., S. Lang, and F. Wagner. 1992. *Pseudomonas cepacia* lipase: Studies on aggregation, purification and on the cleavage of olive oil. *Biotechnol. Lett.* **14:** 953-958.
4. Gotz, F., H. M. Verheij, and R. Rosenstein. 1998. Staphylococcal lipases: Molecular characterisation, secretion, and processing. *Chem. Phys. Lipids* **93:** 15-25.
5. Hernell, O. and T. Olivecrona. 1974. Human milk lipases: II. Bile salt-stimulated lipase. *Biochim. Biophys. Acta* **369:** 234-244.
6. Helisto, P. and T. Korpela. 1998. Effects of detergents on activity of microbial lipases as measured by the nitrophenyl alkanoate esters method. *Enzyme Microb. Technol.* **23:** 113-117.
7. Hyun, J., C. R. Treadwell, and G. V. Vahouny. 1972. Pancreatic juice cholesterol esterase: Studies on molecular weight and bile salt-induced polymerization. *Arch. Biochem. Biophys.* **152:** 233-242.
8. Jurgens, D. and H. Huser. 1981. Large-scale purification of staphylococcal lipase by hydrophobic interaction chromatography. *J. Chromatogr.* **216:** 295-301.
9. Kim, H. K., J. K. Lee, H. Kim, and T. K. Oh. 1996. Characterization of an alkaline lipase from *Proteus vulgaris* K80 and the DNA sequence of the encoding gene. *FEMS Microbiol. Lett.* **135:** 117-121.
10. Muderhwa, J. M. and H. L. Brockman. 1992. Lateral lipid distribution is a major regulator of lipase activity. *J. Biol. Chem.* **267:** 24184-24192.
11. Oh, B. C., H. K. Kim, J. K. Lee, S. C. Kang, and T. K. Oh. 1999. *Staphylococcus haemolyticus* lipase: Biochemical properties, substrate specificity and gene cloning. *FEMS Microbiol. Lett.* **179:** 385-392.
12. Rhee, J. S. and S. J. Kwon. 1998. Water activity control in lipase-catalyzed reaction system. *J. Microbiol. Biotechnol.* **8:** 191-196.
13. Rollof, J., S. A. Hedstrom, and P. Nilsson-Ehle. 1987. Purification and characterization of a lipase from *Staphylococcus aureus*. *Biochim. Biophys. Acta* **921:** 364-369.
14. Rudd, E. A. and H. L. Brockman. 1984. Pancreatic carboxyl ester lipase, pp. 185-204. *In* Borgstrom, B. and H. L. Brockman (eds.), *Lipases.* Elsevier Sciences Publishing, New York, U.S.A.
15. Taipa, M. A., M. R. Aires-Barros, and J. M. S. Cabral. 1992. Purification of lipases. *J. Biotechnol.* **26:** 111-142.
16. Vakos, H. T., G. W. Burton, and H. Kaplan. 1997. Taruocholate-induced dimerization of bovine cholesterol esterase in sodium dodecylsulfate. *Biochim. Biophys. Acta* **1342:** 103-108.
17. Vulfson, E. N. 1994. Industrial applications of lipases, pp. 271-288. *In* Woolley, P. and S. B. Petersen (eds.), *Lipases.* Cambridge Univ. Press.
18. Wang, X., C. Wang, J. Tang, F. Dyda, and X. C. Zhang. 1997. The crystal structure of bovine bile salt activated lipase: Insights into the bile salt activation mechanism. *Structure* **5:** 1209-1218.
19. Wickham, M., M. Garrood, J. Leney, P. D. G. Wilson, and A. Fillery-Travis. 1998. Modification of a phospholipid stabilized emulsion interface by bile salt: Effect on pancreatic lipase activity. *J. Lipid Res.* **39:** 623-632.