Cloning and Nucleotide Sequence of Thermostable Lipase Gene from *Pseudomonas fluorescens* SIK W1

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A gene coding for a thermostable lipase of *Pseudomonas fluorescens* SIK W1 was cloned into *Escherichia coli* JM83 by inserting *Sau*3AI-generated DNA fragments into the *Bam*HI site of pUC19. Twenty colonies with esterase activity on the tributyrin agar plate were isolated by screening the constructed *Pseudomonas fluorescens* genomic library. Only one out of the esterase positive 20 colonies had lipase activity on the agar plate containing olive oil and Rhodamine-B.

The complete nucleotide sequence of the lipase gene was identified. The lipase gene consists of an open reading frame, 1347 bp long, commencing with an ATG start codon encoding a polypeptide of 449 amino acid residues and a TGA stop codon. Comparison of this lipase amino acid sequence with those from another organisms sequenced to date showed the presence of the short homologous region Gly-X-Ser-X-Gly.

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are widely distributed in animals, plants, and microorganisms (fungi and bacteria). Microbial lipases have commercial potentials in lipase-catalyzed triglyceride hydrolysis, interesterification, and other areas of interest such as energy saving process.¹⁾

Recently molecular clonings of some lipase genes have been reported and the primary structures of those lipases were identified.^{2–12)} Cloning and sequence analysis of the lipase gene is expected to allow the understanding of the relationship between the function and the structure of lipase, and mass production of the enzyme. The enzymes that are cloned to date, however, are mostly not thermostable. Thus their industrial usages are somewhat limited.

Thermostable lipases that are produced by a psychrotrophic bacterium, *Pseudomonas fluorescens*, are a major concern during storage of heat-processed, commercially sterile foods.^{13–15)} Andersson *et al.*¹³⁾ reported that the crude lipase from *P. fluorescens* SIK W1

is not only heat stable but also highly stable against chemical denaturants. Though the presence of lipase activity associated with this microorganism has been studied well in the view of heat-sterilization and deactivation of the lipase for long-term storage of foods,^{13–16)} there are few reports which deal with molecular structures of the lipase gene from this microorganism.

We have cloned and sequenced the nucleotides of the thermostable lipase gene from *P. fluorescens* SIK W1, and previously reported the overexpression of the lipase gene in *Escherichia coli* and renaturation into biologically active form.¹⁷⁾

This study deals with the molecular cloning and nucleotide sequence of the lipase gene from *P. fluorescens* SIK W1 in *E. coli*.

Materials and Methods

Bacterial strains, plasmids, and media. *P. fluorescens* SIK W1 was kindly supplied to us by Dr. Andersson (SIK-The

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Swedish Food Institute, Sweden) and used as the source of DNA for cloning. Plasmid pUC19 harboring ampicillin resistance was used as a vector. *E. coli* JM83 was used as the host for recombinant plasmids. *E. coli* was grown in LB medium at 37°C and *P. fluorescens* was grown in nutrient broth at 25°C.

Recombinant DNA techniques. Chromosomal DNA was isolated from *P. fluorescens* by a modification of the method of Marmur¹⁸⁾ and plasmid DNA was prepared from *E. coli* JM83 cells by CsCl-EtBr equilibrium centrifugation of the cleared lysates as described by Maniatis *et al.*¹⁹⁾ For the rapid isolation of plasmids from the bacterial culture, the alkaline lysis method described by Birnboim and Doly was used.²⁰⁾

The genomic library of *P. fluorescens* DNA was constructed by the method described by Choi *et al.*²¹⁾ *E. coli* JM83 competent cells were prepared by the method of Hanahan.²²⁾

Screening for lipase producing *E. coli*. The tributyrin diffusion agar method was used to find the colonies with activities of carboxylic ester hydrolyzing enzyme (EC 3.1.1) as described by Kugimiya *et al.*²⁾ To distinguish the colonies with lipase activity from carboxylester hydrolase activity, olive oil agar containing Rhodamine-B was used by the method of Kouker *et al.*²³⁾ *E. coli* JM83 transformed with a DNA library of *P. fluorescens* was grown on LB-agar plates containing ampicillin (50 µg/ml) and emulsified tributyrin at 37°C for 24 hr. The colonies with clear haloes on the tributyrin agar plate were transferred onto the nutrient agar containing olive oil (1%, v/v) and Rhodamine-B (0.001%, w/v). Lipolytic activity was detected as a fluorescent zone around the colony when the plate was examined on a UV transilluminator.

Nucleotide sequencing. The nucleotides of the DNA fragment containing the cloned lipase gene were sequenced in both directions by the dideoxy chain termination method of Sanger *et al.*²⁴⁾ Recombinant M13mp18 and M13mp19 phage were used for preparation of single stranded DNAs for nucleotide sequencing.²⁵⁾

Chemicals and enzymes. *Sau*3AI and calf intestinal alkaline phosphatase were purchased from Promega Biotech. Co. (U.S.A.) and T4-DNA ligase and all restriction endonucleases were purchased from either KOSCO Biotechnology Labs (Seongnam, Korea) or New England Biolabs, Inc. (Beverly, MA, U.S.A.). Restriction digestions and ligation reactions were done by the manufacture's recommendation. [α -³⁵S]dATP was purchased from Amersham (Buckinghamshire, UK). All other chemicals were of analytical reagent grade.

Results and Discussion

Cloning of the lipase gene from *P. fluorescens*

Chromosomal DNA from *P. fluorescens* was partially digested with the restriction enzyme *Sau*3AI. The digested DNA was isolated by sucrose gradient centrifugation and the fragments of about 4 to 20 kb were used for cloning. The pUC19 plasmid DNA was cut with *Bam*HI and dephosphorylated with calf intestinal alkaline phosphatase. The linearized plasmid DNA was ligated to the *P. fluorescens* DNA fragments and resulting recombinant plasmids were used to transform *E. coli* JM83. To select the colonies with esterase activity including lipase activity, about 12,000 recombinant colonies were screened on tributyrin agar plates. Twenty colonies appeared to show esterase activity, which was proven by a clear halo on tributyrin plates. They could be divided into four groups after mapping the plasmids with various restriction enzymes. To isolate the bacterial clones carrying the lipase gene from these colonies, these were transferred onto the olive oil agar plates containing Rhodamine-B. Only one colony had the lipase activity and formed an orange fluorescent zone around the colony on a UV transilluminator. The plasmid with an insert of a 10.3-kb DNA fragment was isolated from the

Fig. 1. The Screening of *E. coli* for Lipase Activity.

All the esterase positive clones were transferred on nutrient broth agar containing olive oil (1%, v/v) and Rhodamine-B (0.001%, w/v). After incubation for 48 hr at 37°C, the plate was irradiated with UV (350 nm) and photographed. Numbers represent *E. coli* strains carrying the esterase gene from *P. fluorescens*. *P. fluorescens* SIK W1 and a strain (No. 9) of *E. coli* transformants formed orange fluorescent halos around the colonies, indicating production of lipase. CN, control strain *E. coli* JM83 (pUC19); SIK W1, *P. fluorescens* SIK W1.

Fig. 2. Construction of the Recombinant Plasmid pJH92.

The plasmid (pJH9) carrying a 10.3-kb *Sau3AI* fragment originating from *P. fluorescens* was digested by *EcoRI* and *HindIII* and used for a series of subcloning experiments as described in the text. The thick line represents the lipase gene. Abbreviations for the restriction sites are as follows: E, *EcoRI*; H, *HindIII*; P, *PstI*; B, *BamHI*; X, *XhoI*; S, *SalI*; A, *AclI*.

clone and named pJH9. Figure 1 shows that the lipases are produced by both *E. coli* JM83 harboring pJH9 and *P. fluorescens*.

The resultant recombinant plasmid (pJH9) carrying lipase gene has about a 10.3-kb insert of *Pseudomonas* DNA. The insert DNA fragment of pJH9 was partially digested with *Sau3AI* and 1–2 kb DNA fragments were eluted on 1.2% agarose gel. The resultant DNA fragments were ligated into the *BamHI* site of pUC19. When used to transform *E. coli* JM83, we obtained the lipase-positive colonies having the 2.0-kb DNA insert on pUC19. The plasmid was named pJH91, and after deletion of a 400-bp *PstI* fragment of pJH91, the nucleotides of the resultant plasmid designated pJH92 were sequenced. Figure 2 shows the subcloning strategy of the lipase gene.

Primary structure of the lipase

The nucleotide sequence of the inserted DNA of pJH92 was analyzed by Sanger's dideoxy chain termination method. The 1595 base pair nucleotide sequence that contains the entire lipase gene is shown in Fig. 3. Assignment of the open reading frame was done on the basis of N-terminal amino acid sequencing and the molecular weight of the lipase which was measured by SDS-polyacrylamide gel electrophoresis. The molecular weight of the lipase was calculated to be 48,179 (including the initiator, Met) from the predicted amino acid composition. The molecular weight of lipase measured by 9% SDS-polyacrylamide gel electrophoresis was estimated as 52,000, which was slightly different from that by the predicted amino acid composition. An open reading frame (154–1500) commencing with an ATG start codon encoding 449 amino acid residues and a TGA stop codon was found in this nucleotide sequence. A homologous region found in all the lipases sequenced to date appeared at amino acid residues 199–209, which was regarded as a substrate binding site of the lipase and also conformed the correct open reading frame. The N-terminal amino acid sequences of the partial purified lipase which was overproduced from the recombinant *E. coli*¹⁷⁾ were analyzed to obtain the experimental evidence that supports the idea that the open reading frame encodes the lipase. The results of Edman degradation up to 13 cycles (by a protein sequanator, Applied Biosystem, model 471A) agreed with the predicted amino acid sequence of the lipase. Upstream from ATG of the lipase gene, there is a 5'-GAGA-3' sequence that might function as a Shine-Dalgarno (SD) sequence²⁶⁾ and this sequence coincided with that of the *P. fragi* lipase gene.²⁾

The extent of expression of the lipase gene in *E. coli* was dependent on the orientation of the inserted DNA in the vector and controlled by the external vector promoter. Though a putative promoter region that resembles the consensus sequences of *E. coli* promoters could not be found, weak enzyme activity was

1 AAGCTTGCATGCCTGCAGACAAGCCCCCTGTTGCAAAACAAACCGCTGAGGCAAGCCG
HindIII

59 ACCCCCTGTGGCGAGCGGGCTTGCCCGTTGGGCTGCGCAGCAGCAATAAAACCACACAATCTCCGCCAATTCCAAACAACAAGAGAGACAAGACA
SD

154 ATG GGT GTA TTT GAC TAC AAG AAC CTC GGC ACC GAA GCC AGC AAA ACC TTG TTC GCC GAT GCC ACC GCA ATC
1 Met Gly Val Phe Asp Tyr Lys Asn Leu Gly Thr Glu Ala Ser Lys Thr Leu Phe Ala Asp Ala Thr Ala Ile

226 ACG TTG TAT ACC TAT CAC AAC CTG GAT AAC GGC TTC GCA GTC GGC TAC CAG CAA CAT GGC TTG GGG CTC GGC
25 Thr Leu Tyr Thr Tyr His Asn Leu Asp Asn Gly Phe Ala Val Gly Tyr Gln Gln His Gly Leu Gly Leu Gly

298 TGC CGG CAC ACT GGT CGG GGC GTT GCT CGG CAG CAC AGA CTC CCA GGG AGT GAT CCC CCG GCA TTC CCT GGA
49 Cys Arg His Thr Gly Arg Gly Val Ala Arg Gln His Arg Leu Pro Gly Ser Asp Pro Pro Ala Phe Pro Gly

370 ATC CTG ACT CGG AAA AGG CCG CCC TGG ACG CGG TGC ACG CAG CCG GTT GGA CGC CAA TCA AGC GCC AGC GCA
73 Ile Leu Thr Arg Lys Arg Pro Pro Trp Thr Arg Cys Thr Gln Pro Val Gly Arg Gln Ser Ser Ala Ser Ala

442 CTG GGC TAC GGC GGC AAG GTG GAT GCG CGG GGC ACT TTT TTT GGC GAG AAG GCC GGC TAC ACC ACG GCC CAG
97 Leu Gly Tyr Gly Gly Lys Val Asp Ala Arg Gly Thr Phe Phe Gly Glu Lys Ala Gly Tyr Thr Thr Ala Gln

514 GCC GAA GTG CTG GGC AAG TAC GAT GAC GCC GGC AAA CTG CTC GAG ATC GGC ATC GGT TTT CGT GGC ACC TCG
121 Ala Glu Val Leu Gly Lys Tyr Asp Asp Ala Gly Lys Leu Leu, Glu Ile Gly Ile Gly Phe Arg Gly Thr Ser

586 GGC CCT CGG GAA AGC CTG ATT ACG ACT CCA TGC CGA TCT GGT CAG CGA CCT GCT CGC CGC GCT GGG CCC CAA
145 Gly Pro Arg Glu Ser Leu Ile Thr Thr Pro Cys Arg Ser Gly Gln Arg Pro Ala Arg Arg Ala Gly Pro Gln

658 GGA CTA TGC GAA AAA CTA TGC CGG CGA ACG TTT GGC GGC TTG CTC AAG ACG GTG GCC GAC TAT GCC GGC GCC
169 Gly Leu Cys Glu Lys Leu Cys Arg Arg Thr Phe Gly Gly Leu Leu Lys Thr Val Ala Asp Tyr Ala Gly Ala

730 CAT GGC CTG AGT GGC AAG GAT GTG CTG GTC AGC GGC CAC AGC CTG GGC GGC CTG GCG GTC AAC AGC ATG GCC
193 His Gly Leu Ser Gly Lys Asp Val Leu Val Ser Gly His Ser Leu Gly Gly Leu Ala Val Asn Ser Met Ala

802 GAC CTG AGC ACC AGC AAA TGG GCG GGT TTC TAC AAG GAC GCC AAC TAC CTG GCC TAC GCC TCG CCC ACC CAG
217 Asp Leu Ser Thr Ser Lys Trp Ala Gly Phe Tyr Lys Asp Ala Asn Tyr Leu Ala Tyr Ala Ser Pro Thr Gln

874 AGC GCC GGC GAT AAG GTC CTG AAT ATC GGC TAC GAA AAC GAC CCG GTA TTC CGT GCG CTG GAC GGC TCC ACC
241 Ser Ala Gly Asp Lys Val Leu Asn Ile Gly Tyr Glu Asn Asp Pro Val Phe Arg Ala Leu Asp Gly Ser Thr

946 TTC AAC CTG TCG TCC CTC GGC GTG CAT GAC AAG GCC CAC GAG TCG ACC ACC GAC AAC ATC GTC AGC TTC AAC
265 Phe Asn Leu Ser Ser Leu Gly Val His Asp Lys Ala His Glu Ser Thr Thr Asp Asn Ile Val Ser Phe Asn

1018 GAC CAC TAC GCC TCG ACG TTG TGG AAT GTG CTG CCG TTT TCC ATC GCC AAC CTG TCG ACC TGG GTG TCG CAT
289 Asp His Tyr Ala Ser Thr Leu Trp Asn Val Leu Pro Phe Ser Ile Ala Asn Leu Ser Thr Trp Val Ser His

1090 TTG CCC AGC GCT TAC GGC GAC GGC ATG ACG CGT GTG CTG GAA TCG GGG TTC TAC GAG CAA ATG ACC CGT GAC
313 Leu Pro Ser Ala Tyr Gly Asp Gly Met Thr Arg Val Leu Glu Ser Gly Phe Tyr Glu Gln Met Thr Arg Asp

1162 TCG ACG ATT ATC CTG TGC CCA ACC TGG TCC GAC CCG GCG CGC GCC AAC ACC TGG GTC CAG GAC CTC AAC CGC
337 Ser Thr Ile Ile Leu Cys Pro Thr Trp Ser Asp Pro Ala Arg Ala Asn Thr Trp Val Gln Asp Leu Asn Arg

1234 AAT GCC GAG CCG CAC ACA GGC AAT ACC TTC ATC ATC GGC AGC GAC GGC AAT GAC CTG ATC CAG GGC GGC AAG
361 Asn Ala Glu Pro His Thr Gly Asn Thr Phe Ile Ile Gly Ser Asp Gly Asn Asp Leu Ile Gln Gly Gly Lys

1306 GGC GCG GAC TTC ATC GAA GGC GGC AAG GGC AAT GAC ACG ATC CGC GAC AAC AGC GGG CAC AAC ACC TTT TTG
385 Gly Ala Asp Phe Ile Glu Gly Gly Lys Gly Asn Asp Thr Ile Arg Asp Asn Ser Gly His Asn Thr Phe Leu

1378 TTC AGC GGG CAT TTT GGC CAG GAT CGG ATT ATC GGC TAC CAG CCG ACA GGC TGG TGT TCC AGG GCG CCG ACG
409 Phe Ser Gly His Phe Gly Gln Asp Arg Ile Ile Gly Tyr Gln Pro Thr Gly Trp Cys Ser Arg Ala Pro Thr

1450 GCA GCA CCG ACC TGC GCG ACC ACG CGA AGG CCG TGG GGG CCG ATA CCG TGC TGA GTTTTGGCGCCGACTCGGT
433 Ala Ala Pro Thr Cys Ala Thr Thr Arg Arg Pro Trp Gly Pro Ile Arg Cys ***

1523 GACCTGGTGGGGTGGGGCTGGGGCGGCTGTGGAGCGAGGGTGTGCTGATCCCCGGGTACCGAGCTCGAATTC
EcoRI

Fig. 3. The Nucleotide Sequence of the Lipase Gene and the Deduced Amino Acid Sequence of Lipase.

The total DNA sequence of lipase gene is presented and the deduced amino acid sequence is shown below the DNA sequence. A putative ribosome binding site (SD sequence) is underlined. The substrate binding site is shown by the double underline. The deduced N-terminal amino acid sequences, which were confirmed by amino acid sequencing of the lipase (amino acid 1–13), are underlined. The asterisks indicate a stop codon. The putative signal sequence is denoted by the vertical arrow.

detected when the 1.6-kb fragment was inserted opposite direction in the vector. This result suggests that the promoter of this gene may not function well in *E. coli* and agreed with the report of Bagdasarian and Timmis²⁷⁾ that the promoter of *Pseudomonas* did not function well in *E. coli*.

The leader sequence, which was composed of a positively charged amino acid at its N-terminal end followed by a hydrophobic domain is a characteristic structure of secreted proteins. The amino acid sequence deduced from the nucleotide sequence of the lipase had a putative signal sequence containing 23 amino

acids with the classical characteristics of a hydrophilic region followed by hydrophobic amino acid ending in Ala-X-Ala before the mature protein.²⁸⁾

Nucleotide content and codon usage of the lipase

Analysis of the sense strand of the open reading frame for the lipase gene of *P. fluorescens* showed a G+C content of 62.5% which correlated with the value of 60–63% observed from chromosomal DNA of *P. fluorescens*.²⁹⁾ The G+C contents of the *P. fluorescens* lipase gene in the first and second positions of codons were 57.1 and 52.7%, respectively. There was a predominance of codons with G or C in the third position in the *P. fluorescens* gene such that the G+C content in position 3, 77.7%, was even higher than that of the entire gene (Table I). Preference for G or C at the third position has been observed in other sequenced genes of *Pseudomonas*. Minton *et al.*³⁰⁾ reported that the codon bias in the codon usage of *Pseudomonas* forwards codons ending in G or C conferred on the gene

Table I. CODON USAGE FOR THE LIPASE GENE OF *P. fluorescens*

aa	Codon	No.	aa	Codon	No.
Arg	CGU	4	Val	GUU	2
	CGC	6		GUC	6
	CGA	4		GUA	2
	CGG	10		GUG	8
	AGA	1	Lys	AAA	5
Leu	AGG	3		AAG	11
	CUU	0	Asn	AAU	6
	CUC	7		AAC	14
	CUA	2	Gln	CAA	4
	CUG	20		CAG	10
	UUA	0	His	CAU	5
	UUG	7		CAC	8
Ser	UCU	1	Glu	GAA	7
	UCC	5		GAG	5
	UCA	1	Asp	GAU	8
	UCG	9		GAC	19
	AGU	2	Tyr	UAU	3
Thr	AGC	15		UAC	13
	ACU	4	Cys	UGU	1
	ACC	19		UGC	8
	ACA	2	Phe	UUU	8
	ACG	13		UUC	11
Pro	CCU	3	Ile	AUU	3
	CCC	5		AUC	14
	CCA	3	Met	AUA	1
Ala	CCG	12		AUG	4
	GCU	4	Trp	UGG	8
	GCC	21			
	GCA	6			
Gly	GCG	8			
	GGU	5			
	GGC	43			
	GGA	3			
	GGG	7			

Table II. A CONSERVED SEQUENCE AMONG LIPOLYTIC ENZYMES

Lipase	Substrate binding site	References
Microorganisms		
<i>Pseudomonas fragi</i>	⁷⁶ RVN ⁸⁶ LIGHSQGA	2
<i>Staphylococcus aureus</i>	⁴⁰⁵ KVHLV ⁴¹⁵ GHSMGG	3
<i>Staphylococcus hyicus</i>	³⁶² PVHF ³⁷² IGHSMGG	4
<i>Rhizomucor miehei</i>	¹³⁷ KVAVT ¹⁴⁷ GHSLGG	5
<i>Pseudomonas fluorescens</i>	¹⁹⁹ DVLV ²⁰⁹ SGHSLGG	This study
Animals		
Rat hepatic lipase	¹⁴⁰ KVHL ¹⁵⁰ LIGYSLGA	6
Human hepatic lipase	¹³⁸ HVHL ¹⁴⁸ LIGYSLGA	7
Bovine lipoprotein lipase	¹²⁷ NVHL ¹³⁷ LGYSLGA	8
Porcine pancreatic lipase	¹⁴⁵ NVHV ¹⁵⁵ IGHSLGS	9
Human lipoprotein lipase	¹²⁵ NVHL ¹³⁵ LGYSLGA	10

Fig. 4. Hydrophobicity Profile for the Lipase from *P. fluorescens*.

Hydrophobicity profile for the total amino acids of lipase was calculated by the method of Eigenberg³²⁾ with a window of six. The thick bar indicates the substrate-binding region of the lipase.

some properties normally associated with weakly expressing *E. coli* genes and might therefore contribute to the overall low expression of *Pseudomonas* genes in *E. coli*.

Structural features of the *P. fluorescens* lipase

Lipases are characterized by the ability to preferably catalyze the hydrolysis of ester bonds at the interface between the water-insoluble fat phase and the aqueous phase in which the enzyme is soluble. Lipase sequences from several procaryotic²⁻⁵⁾ and eucaryotic⁶⁻¹²⁾ organisms all contain a Gly-X-Ser-X-Gly sequence, which was reported to be a part of the interfacial lipid recognition site by Guidoni *et al.*³¹⁾ Table II shows the conserved sequence among lipases. The homologous region [Asp-Val-Leu-Val-Ser-Gly-His-Ser-Leu-Gly-Gly] was found in *P. fluorescens* lipase, too. A hydrophobicity plot of the lipase, constructed as described by Eigenberg,³²⁾ indicates that this substrate binding site of the lipase is in a highly hydrophobic domain (Fig. 4). All sequenced lipases except those from *P. fragi* and *Rhizomucor miehei* have presence of potential glycosylation sites.³³⁾ The amino acid sequence of the *P. fluorescens* lipase predicted from nucleotide sequence of the lipase gene also contains three potential sites [Asn-X_a-

(Thr/Ser)] for N-linked glycosylation at amino acid residues 266–268, 305–307, and 395–397.

In this paper we described the cloning and nucleotide sequence of the thermostable lipase gene from *P. fluorescens* in *E. coli*. To isolate a specific gene from the gene donor by shot-gun cloning, a screening method for the recombinant colony is very important. An agar plate containing tributyrin or Tween 80 as a substrate is frequently used for screening of lipases. When tributyrin was used for the screening for a lipase producer from the *P. fluorescens* genomic library, twenty colonies with esterase activity were isolated. This implies that substrates such as tributyrin or Tween 80 are not suitable to detect true lipases because they can be hydrolyzed by esterases, too. Therefore for the screening of recombinant colonies with lipase activity we used the method described by Kouker and Jaeger, which is a plate assay method for detection of lipase in a medium containing the fluorescent dye, Rhodamine-B, and olive oil as a true substrate of lipases.

We reported in our previous paper that the thermostable lipase using this cloned lipase gene was overproduced in *E. coli* BL21 as inclusion bodies.¹⁷⁾ They were refolded into a biologically active form and the lipase showed high thermostability; the time required for 90% inactivation of the enzyme was 4 hr at 95°C. The lipase gene had a polypeptide containing nine Cys residues, which might participate in the formation of disulfide bridge. Further research should be done for the elucidation of possible contributions of disulfide bonds to the extreme thermostability of the *P. fluorescens* lipase. Several thermostable lipases from thermostable fungi such as *Humicola lanuginosa*³⁴⁾ and from the genus *Pseudomonas* such as *P. fluorescens*,³⁵⁾ *Pseudomonas* sp. KWI-56,³⁶⁾ and *P. fragi*³⁷⁾ have been reported. The use of the thermostable microbial lipases in the oleochemical industry may provide advantages of decreased fluid viscosity and increased lipid solubility at high temperature. Furthermore, the operation stability of enzyme reactors at high temperatures

may be increased.

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