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Efficient Production of Active Recombinant *Candida rugosa* LIP3 Lipase in *Pichia pastoris* and Biochemical Characterization of the Purified Enzyme

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Candida rugosa lipase (CRL), an important industrial enzyme, possesses several different isoforms encoded by the high-identity *lip* gene family (*lip*1 to *lip*7). In this study, an additional N-terminal peptide in front of the *lip*3 gene was removed by PCR, and the 18 nonuniversal serine codons (CTG) of the *lip*3 gene were converted into universal serine codons (TCT) by means of an overlap extension PCR-based multiple-site-directed mutagenesis to express an active recombinant LIP3 in the yeast *Pichia pastoris*. The regional synthetic DNA fragment (339 bp) is first recombined by primer assembly with 20 overlapping nucleotides, followed by specific overlap extension PCR with outside primers containing restriction enzyme sites for directional cloning into the pGAPZαC vector. The results show that the production yield (0.687 unit/mL) of N-fused *lip*3 (nf*lip*3) has an overall improvement of 69-fold relative to that (0.01 unit/mL) of *lip*3 and of 52-fold (0.47 unit/mL) of codon-optimized *lip*3 (co*lip*3) relative to that (0.01 unit/mL) of non-codon-optimized *lip*3 (*lip*3), with the cultivation time set at 5 days. This finding demonstrates that the reservation of the N terminus and the regional codon optimization of the *lip*3 gene fragment at the 5′ end can greatly increase the expression level of recombinant LIP3 in the *P. pastoris* system. The purified recombinant LIP3 shows distinct biochemical properties compared with other isoforms.

KEYWORDS: ***Candida rugosa* lipase; codon optimization; isoforms; N-terminal peptide; *Pichia pastoris***

INTRODUCTION

Candida rugosa (formerly *Candida cylindracea*) lipase (CRL), in its crude form, is a well-known and widely used enzyme for biotransformation and biocatalytic (acylation and deacylation) reactions to produce valuable materials for food, flavor, fragrance, cosmetic, pharamaceutical, and other industrial applications. However, crude enzyme preparations, which are essentially mixtures of many isozymes obtained from various commercial suppliers, exhibit remarkable variation in their catalytic efficiency, regioselectivity, and stereospecificity. Up to seven closely related lipase genes with high homology (between 60 and 70%), namely, *lip1* to *lip7* which encode LIP 1 to LIP 7, have been identified and sequenced from *C. rugosa* (*1*, *2*). These lipase isoforms are conserved at a catalytic triad, Ser-209, His-449, and Glu-341, with sites (Cys-60/Cys-97 and Cys-268/Cys-277) involved in disulfide bond formation. Some of these lipase isoforms (LIP1, LIP2, and LIP3), consisting of 534 amino acids and an observed molecular mass of 60 kDa, could be isolated from commercial enzyme preparation (*3*). Each of the enzymes differs in their N-glycosylation sites and isoelectric points. Moreover, some local features of their hydrophobicity profiles might cause *C. rugosa* lipase isozymes to possess different substrate specificities and thermal stabilities in biocatalytic applications (*4*−*6*). An effective separation of CRL isoenzymes is thus highly important and desirable in enabling us to better understand the mechanism of lipase catalysis, as well as in finding ways to engineer the lipases to utilize new substrates, increase their substrate specificity, enantioselectivity, stability (temperature, pH, organic solvent, etc.), and specific activities, and find new applications (*7*). However, a high identity in their protein sequences causes similarities in the physical properties of the lipases. These similarities create technical difficulties in the isolation of individual isoenzymes from the cultures of *C. rugosa* by normal chromatographic techniques (*3*, *4*). The recombinant DNA technology combined with manipulating culture conditions in a heterologous expression system is a viable and reasonable

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Table 1. Mutagenic Primers Used for the Multiple-Site-Directed Mutagenesis of the *lip*3 Gene

primer[a]	position of mutated CTG-Ser residue	oligo sequence (5′→3′)[b]
3N		ATACAT ATG **G_{46}CT** CCC ACC GCC AAG CTC GCC AAC
3-1F	55, 59	G_{198} AAG TT**C** AC**T** **TCT** TAC GGC CCG **TCT** TGC ATG CAG
3-2F	209	G_{654} AC**C** ATC TTT GG**C** GAG **TCT** GCG GGC AGC ATG
3-3F	241, 247	C_{756} ATC ATG CAG **TCT** GGA GCC ATG GTG CCG **TCT** GAC CCG GTG
3-4F	282	G_{875}C TTG CGC AGT GCG **TCT** AGC GAC ACC TTG
3-5F	305	T_{946}CG TTG CGG TTG **TCT** TA**T** CT**C** CC**G** CGG C
3-6F	348, 349	T_{1074}TT GG**G** CTC **TCT** **TCT** TTG AAC GTG ACC
3-7F	365	T_{1125}AC TTC AAG CAG **TCT** TTC ATC CAC GCC
3-8F	389	T_{1199}C ACC CAG GG**T** **TCT** CCG TTC GAC AC**G** GG
3-9F	407	G_{1251} TTC AAG AGA ATC **TCT** GCG GTG CTC GGC
3-10F	436, 440	C_{1341} TCG TTC CTC **TCT** AAG CAG CTC **TCT** GGG TTG CCA
3-11F	496, 498	G_{1518} TAC ACC AGC AG**C** **TCT** CAG **TCT** GGC AAC AAC TTG
3-C	530, 531	GAGTCGA CTA CAC AAA GAA **AGA AGA** CGG GTT GGT_{1624}

[a] The primers were used as forward primers except for primer 3-C, which is a reverse primer. The sequences of reverse primers were fully complementary to those of the corresponding forward primers (not shown). [b] The mutated serine codons (TCT) and the mutated nucleotides, which will not result in amino acid substitutions, are in bold. The numbers indicate the locations of the nucleotide in the lipase-encoding sequence. The created restriction sites (*Nde*I in 3-N and *Sal*I in 3-C) are underlined.

approach in the production of different pure CRL isoforms for the characterization and optimization of their biocatalytic properties in an industrial scale.

Unfortunately, despite the general availability of the cloned genes, the non-spore-forming yeast *C. rugosa* utilizes a non-universal codon, that is, the triplet CTG, which is a universal codon for leucine and is read as serine. The CTG triplets encode most of the serine residues, including the catalytic Ser-209, in the lipases. Therefore, the heterologous expression of such genes may result in the production of inactive lipases. To overcome this codon usage obstacle, we utilized a multiple mutagenesis method, which was reported previously (*3*, *9*), to change all nonuniversal serine codons (CTG) into universal TCT serine codons. With this method, we have successfully expressed the recombinant LIP1, LIP2, and LIP4 lipases in *P. pastoris* (*3*, *8*, *9*). The pGAPZαC vector driven by the glyceraldehyde-3-phosphate dehydrogenase (GAP) constitutive promoter (*3*) can avoid the accumulation of formaldehyde and hydrogen peroxide (oxidized products of methanol by alcohol oxidase) in *P. pastoris* (*10*−*12*) and secrete functional recombinant LIP protein into a medium by an N-terminal peptide encoding the *Saccharomyces cerevisiae* α-factor secretion signal (*3*). LIP3 has been reported to be very useful for pitch treatment in the pulp industry due to its ability to catalyze the hydrolysis of both triglycerides and plant steryl esters (*13*). It is also useful for the synthesis of cyclic alcohol esters (*14*). However, the efficient production of active recombinant LIP3 lipase is still not available for industrial applications.

In the present work, we employed PCR to remove an unnecessary linker in order to generate a modified pGAPZαC-LIP3 plasmid and convert the 18 nonuniversal serine codons (CTG) of the LIP3 gene into universal serine codons (TCT) by means of overlap extension PCR-based multiple-site-directed mutagenesis. We designed a regional synthetic gene fragment between *Xho*I and *Not*I near the 5′ end of a *lip*3 transcript optimized for codon usage in *P. pastoris*. Much higher production yields (0.7 unit/mL) within the minimal culture time (5 days) were attained, and the expression levels were affected by different vector constructions. The purified enzyme showed distinct catalytic properties compared with other isoforms (LIP1, LIP2, and LIP4) previously reported. This finding demonstrates that the expression of recombinant LIP3 in the *P. pastoris* system is significantly affected by the N-terminal peptide or codon usage near the 5′ end of the *lip*3 gene.

MATERIALS AND METHODS

Strains and Plasmids. The *P. pastoris* expression vector pGAPZαC (Invitrogen, Carlsbad, CA) was manipulated in an *Escherichia coli* strain, DH5α [F^- *ϕ*80d*lac*ΔM15ZΔ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17(r_k^-, m_k^+) *pho*A *sup*E44*λ*-*thi*-1 *gyr*A96 *rel*A1; Invitrogen], which was used as a host for cloning. The *P. pastoris* strain SMD168H (pep4; Invitrogen), harboring the recombinant plasmids, was used for expressing recombinant nfLIP3 and LIP3. All *P. pastoris* transformants were cultured in a YPD (1% yeast extract, 2% peptone, and 2% dextrose; pH 6.3) broth containing 100 *μ*g/mL of Zeocin (Invitrogen) at 30 °C.

Plasmid Construction for the Expression of Recombinant LIP3. The mature protein-coding sequence of *lip*3 (GenBank accession number X66006) was cloned by RT-PCR, and all CTG-serine codons were replaced with TCT by overlap-extension PCR (**Table 1**) (*3*, *9*). Afterward, the *lip*3 gene was inserted into the pGAPZαC expression vector between the *Kpn*I and *Sal*I sites. There is a short residual peptide linker in front of the mature LIP3 after the cleavage of the N-terminal peptide encoding *S. cerevisiae* α-factor secretion signal (*3*) by Ste13 protease, namely, the pGAPZαC-nf*lip*3 (nf*lip*3) plasmid (**Figure 1A**). We also removed the coding sequence of the N-terminal peptide linker from pGAPZαC-nf*lip*3 by PCR to generate pGAPZαC-*lip*3 (*lip*3) plasmid (**Figure 1B**). In an effort to further scale up the fermentation, the gene fragment between the *Xho*I and *Not*I sites of the mature *lip*3 coding region near the 5′ end was replaced by a synthetic codon-optimized gene between the *Xho*I and *Not*I sites of the mature *lip*3 coding region to generate the pGAPZαC-co*lip*3 plasmid (**Figure 1C**). Several codons of the *lip*3 gene of a 339 bp regional synthetic gene containing *Xho*I and *Not*I sites were changed according to *P. pastoris*'s favorite codon usage from the codon usage database (http://www.kazusa.or.jp/codon), thereby decreasing the G + C content from 61 to 40%. The overlap extension PCR strategy was then employed to change 86 codons of the *lip*3 gene (**Figure 2**). The 339 bp synthetic gene fragment was reassembled using 14 26−60 bp oligonucleotides containing 20 bp overlapping regions, followed by specific overlap extension PCR with outside primers containing restriction enzyme sites for directional cloning into the pGAPZαC vector.

The properly assembled cloning vector, pGAPZαC, was identified and characterized by restriction enzyme analysis, and the entire sequence was reconfirmed by automated sequencing.

Transformation and Expression. The plasmids (10 *μ*g) harboring the engineered *lip*3 and co*lip*3 were linearized with *Avr*II and then were transformed into *P. pastoris* SMD168H by electroporation. High-voltage pulses (1.5 kV) were delivered to 100 *μ*L samples in 0.2 cm electrode gap cuvettes using a Gene Pulser apparatus supplied with the Pulse Controller (Bio-Rad). Transformants were plated on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 2% agar, pH 7.2) plates containing 100 *μ*g/mL Zeocin (Invitrogen) to isolate Zeocin-resistant clones. Individual colonies containing lipase-secreting transformants were picked and patched on 1% tributyrin emulsion YPD

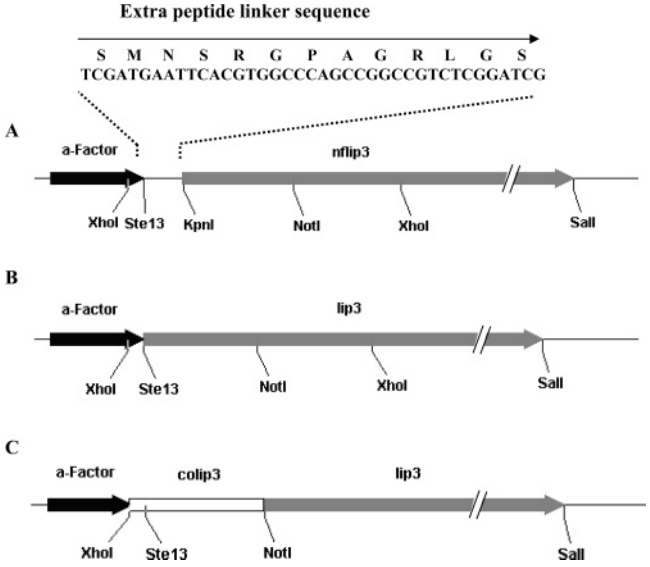

Figure 1. Comparison of the construction of pGAPZαC-nf*lip*3, pGAPZαC-*lip*3, and pGAPZαC-co*lip*3 plasmids: (**A**) the N-fused *lip*3 gene contained an extra peptide linker sequence; (**B**) the *lip*3 gene lacked an N-terminal extra peptide linker, which was due to the cloning positions that we chose on the vector, left after *S. cerevisiae* α-factor secretion signal cleavage; (**C**) the co*lip*3 gene and *XhoI*–*SalI* fragment (gray box) both lacked an N-terminal extra peptide linker and were exchanged with highly used codons in *P. pastoris* to improve the overall expression level of recombinant LIP3. The *lip*3 gene codons without any changes were indicated as white boxes.

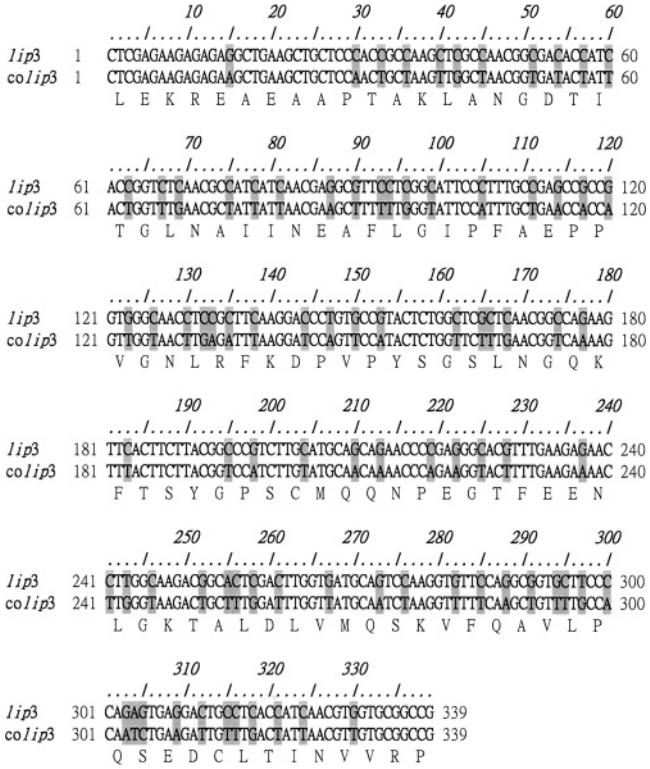

Figure 2. DNA sequence alignment comparison of the *XhoI*–*SalI* fragment of *lip*3 gene (top) and co*lip*3 gene (bottom). Several codons of the *lip*3 sequence were exchanged (shaded) into the new co*lip*3 sequence with highly used codons in *P. pastoris* for heterologous protein expression improvement.

plates. The clear zone on the opaque tributyrin emulsion identified the lipase-secreting transformants. *P. pastoris*, transformed with pGAPZαC and free of any target gene sequence, was used as a negative control.

Protein Concentration Determination. The total protein in the samples was quantified with a Bio-Rad assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard.

Extraction of Total RNA and RT-PCR Analysis. Total RNA extraction was performed using an RNeasy Mini Kit (Qiagen, Hilden, Germany) and was quantified by A_{260} determination. The same amount (1 μg) of RNA sample was used as template using the One-Step RT-PCR kit (Genemark Technology Co., Ltd.). The specific primers (upstream, 5′-GCTTGCGCAGTGCGCTGAGCGACACCTTGC-3′; downstream, 5′-CTACACAAAGAACAGCAGCGGGTTGGT-3′), for common sequences of *lip*3 and co*lip*3 genes, were applied in the PCR analysis. PCR was carried out in an Omnigene thermal cycler (Hybaid, Teddington, U.K.) on the following cycle program: one cycle of 50 °C for 30 min and 94 °C for 2 min for the first strand and cDNA synthesis; 30 cycles of 94 °C for 35 s, 55 °C for 35 s, and 72 °C for 45 s; and a final 7 min extension step at 72 °C. The final products of PCR were detected via agarose gel electrophoresis. DNA band intensity was directly scanned and analyzed by Band Leader software, version 3.00 (Magnitec Ltd.).

Purification of Recombinant LIP3. Selected *P. pastoris* transformants were grown in 500 mL flasks containing 200 mL of the YPD medium with 100 μg/mL Zeocin at 30 °C for 3 days. The culture medium was concentrated by ultrafiltration on the Labscale TFF system with Pellicon XL devices coupling Biomax-50 membranes (Millipore, Bedford, MA) and was applied onto a HiPrep 16/10 octyl FF column (Amersham Pharmacia Biotech Inc., Piscataway, NJ) equilibrated with $(NH_4)_2SO_4$ TE buffer [20 mM Tris-HCl, 2 mM EDTA, 100 mM $(NH_4)_2SO_4$, pH 7.0]. The column was washed with 5 column volumes of TE buffer plus a linear gradient concentration of $(NH_4)_2SO_4$ (100−0 mM). Bound proteins were then eluted with 5 column volumes of the TE buffer containing 10 mM CHAPS. The eluted protein was collected and dialyzed against the TE buffer. The molecular masses of the purified recombinant lipases were determined in denaturing conditions by SDS-PAGE, and the protein concentration was determined using the Bio-Rad assay kit.

Enzyme Characterization. The molecular masses of the recombinant nfLIP3, LIP3, and LIP3 from the co*lip*3 construct were determined by SDS-PAGE analysis using Sigma protein molecular mass markers. The lipase activity was assayed by a Hitachi U-2001 spectrophotometer. The hydrolysis of *p*-nitrophenyl esters was carried out at 37 °C in 500 μL of 50 mM Good's buffer (50 mM each of Bicine, CAPS, sodium acetate, and BisTris propane) at pH 7.0 (*3*), containing 0.24% Triton X-100 and a 0.5 mM solution of the corresponding *p*-nitrophenyl ester. The increase in absorbance was recorded for 10 min at 348 nm (isosbestic point of the *p*-nitrophenol/*p*-nitrophenoxide couple). One unit of activity was defined as the quantity of enzyme necessary to release 1 μmol of *p*-nitrophenol per minute under the assay conditions. The lipolytic activity was evaluated titrimetrically using triacylglycerols with fatty acids of various chain lengths as substrates. The release of nonesterified fatty acids was monitored continuously by titration using 50 mM NaOH with a pH-stat (Radiometer Copenhagen, Bagsvaerd, Denmark). The substrate emulsion was prepared via a modified Sigma quality control test procedure. The emulsification reagent contained NaCl (3 M), sodium taurocholate (1.5%; w/v), gum arabic (10%), and distilled water. Each lipolytic activity assay was carried out in a thermostat reaction vessel containing 5 mL of 20 mM triacylglycerol substrate emulsion, 6 mL of distilled water, 2 mL of 3 M NaCl, 2 mL of 1.5% sodium taurocholate, and 2 μL of the enzyme solution. One unit of lipolytic activity was defined as the amount of lipase necessary to produce 1 μmol of fatty acid per minute under the assay conditions.

Effects of pH and Temperature on Lipase Activity and Stability. The pH effect was assessed using *p*-nitrophenyl butyrate as substrate. The optimum pH was investigated in the pH range of 3.0−10.0 using Good's buffer (50 mM each of Bicine, CAPS, sodium acetate, and BisTris propane) (*3*). The effect of temperature was measured by means of the spectrophotometric method using *p*-nitrophenyl butyrate as a substrate. The optimum temperature for the LIP3 was investigated within the range of 10−90 °C at pH 7.0. To analyze thermal stability, the lipase was incubated for 10 min at various temperatures within the range of 37−90 °C.

Analysis of the Effect of Water-Miscible Solvents, Detergents, and Chemicals on Lipase Activity. The effect of 30% (v/v) water-miscible solvents on lipase activity was determined by means of the spectrophotometric method using *p*-nitrophenyl butyrate as substrate. All reaction mixtures were incubated at 37 °C for 1 h in a 0.1 M Tris-HCl buffer (pH 8.0) with a final solvent concentration of 0.2% (v/v) in the assay. The reaction mixture without the water-miscible solvent was used as control. The detergent effect on the lipase activity was analyzed according to a similar method. All reaction mixtures were incubated at 37 °C for 1.5 h in a 0.1 M Tris-HCl buffer (pH 8.0) containing 1% (w/v) or 0.1% (w/v) detergents. The reaction mixture without detergent was used as control. The influence of various chemicals on the lipase activity was determined by incubating the enzyme at 37 °C for 30 min in a 0.1 M Tris-HCl buffer (pH 8.0) containing 1 or 10 mM of chemicals and then assaying for the lipase activity with *p*-nitrophenyl butyrate as substrate. All measurements were carried out in triplicate.

Cholesterol Esterase Activity Assay. The cholesterol esterase activity assay was performed according to the method of Lee et al. (*3*).

RESULTS

Effect of Additional Peptide and Codon Optimization Located at the N Terminus. The codon-optimized *lip*3 gene with a regional synthetic codon-optimized fragment, which lies between the *Xho*I and *Not*I restriction sites as shown in **Figure 1**, was constructed in the pGAPZαC expression vector to express the recombinant LIP3 in *P. pastoris* (SMD1168H) in secreted form as described previously (*9*). Therefore, the general G + C content, a byproduct of changing the codon bias, decreased from 61 to 40%, and unique restriction sites were strategically positioned throughout the inserted gene. The pGAPZαC plasmid without the *lip*3 gene was used as control.

The effects of the N-terminal peptide and codon optimization were further investigated at the transcriptional level on the RNA samples specifically amplified by the RT-PCR system. The final RT-PCR products from the *Pichia* transformants containing the pGAPZαC, nf*lip*3, *lip*3, and co*lip*3 plasmids were analyzed by agarose gel electrophoresis (**Figure 3A**). The bright bands (775 bp) represented positive *Pichia* transformants containing nf*lip*3, *lip*3, and co*lip*3 constructs, respectively. The negative *Pichia* transformant containing the pGAPZαC vector without a target gene was used as control, and no DNA fragment was observed. After 72 h of cultivation time, the nf*lip*3 construct containing a regional modified gene fragment represented a ≈1.2-fold higher transcription level than both the *lip*3 and co*lip*3 construct, whereas there is no distinct difference from the transcription levels of *lip*3 and co*lip*3. This indicates that the additional N-terminal peptide represented a more important role in transcription levels than that observed by codon optimization.

Unlike in the previous paper, the additional N-terminal peptide, which is an expression obstacle for LIP1 expression, seems to play a distinct role in the expression of recombinant LIP3 in *P. pastoris*. As the additional N-terminal peptide was removed from the nf*lip*3 plasmid, the expression yield of the *lip*3 construct was reduced at least 18-fold as compared to that of the nf*lip*3 construct at 3 days of cultivation time (**Figure 3B**). This finding suggested that the existence of an unnecessary N-terminal peptide does not entirely increase or decrease the expression yield of all *C. rugosa* lipase isozymes; extreme results might occur as a case study.

To further understand the codon optimization effects on a translational level, the recombinant protein expressed from the codon-optimized *lip*3 gene construct, LIP3, rapidly and stably accumulated at a maximum yield of 0.47 unit/mL in 5 days of cultivation time. Additionally, the co*lip*3 construct showed a

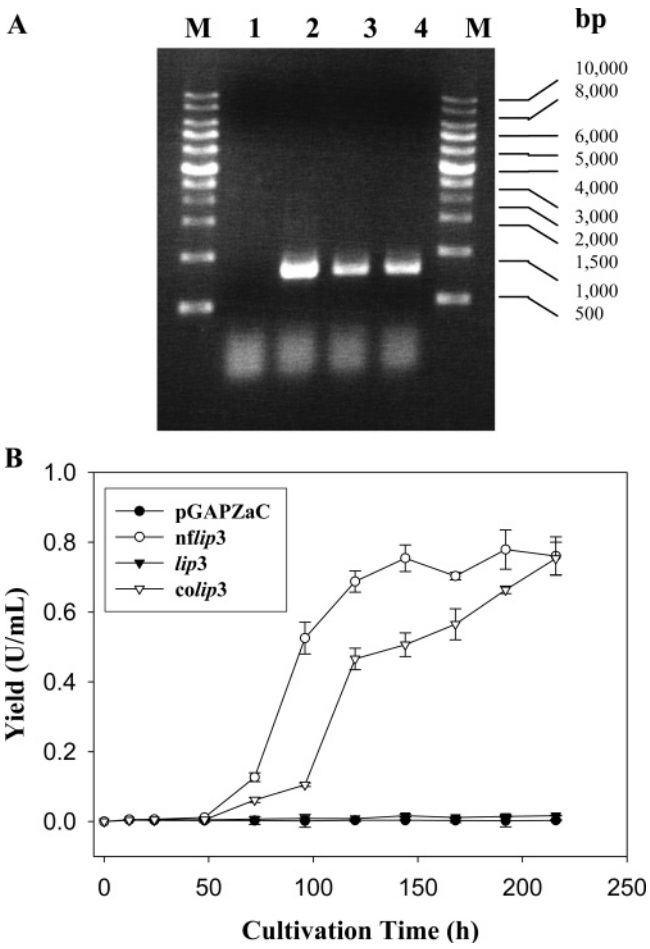

Figure 3. Comparison of transcription level (**A**) and expression yield (**B**) with variant plasmid constructs. (**A**) RT-PCR analysis products (775 bp) of *P. pastoris* (SMD1168H) transformants. Lanes: 1, negative transformant (pGApZαC) at 72 h; 2, pGApZαC-nf*lip*3 transformant at 72 h; 3, pGApZαC-*lip*3 transformant at 72 h; 4, pGApZαC-co*lip*3 transformant at 72 h; M, Bio-1kb DNA size markers. All RT-PCR reactions contained 1 μg of total RNA samples. DNA band intensity was directly scanned and analyzed by Band Leader software, version 3.00 (Magnitec Ltd.). (**B**) All constructive plasmids [pGApZαC (●), pGAPZαC-nf*lip*3 (○), pGApZαC-*lip*3 (▼), and pGApZαC-co*lip*3 (▽)] were transformed into *P. pastoris* hosts and were inoculated in YPD medium at 30 °C. The expression yields of their culture supernatant were measured spectrophotometrically using *p*-nitrophenyl butyrate as substrate at 37 °C and pH 7.0.

52-fold higher production level than that LIP3 (non-codon-optimized *lip*3 construct; 0.01 unit/mL) (**Figure 3B**), indicating that the codon optimization of coding regions toward the codon bias of *P. pastoris* acts as a positive ingredient on protein expression level and provides another functional system for further industrial quantification options as well (*15*).

A simple purification of the recombinant enzyme by ultrafiltration combined with hydrophobic interaction chromatography allowed us to obtain the homogeneous protein (**Table 2**), which had a molecular mass of ≈60 kDa as determined by SDS-PAGE analysis (not shown). This purified protein was used for further biochemical analysis.

Effects of pH and Temperature on the Activity and Stability of the Recombinant LIP3. The pH dependence of LIP3 was studied at 37 °C using *p*-nitrophenyl butyrate as a substrate (**Table 3**). The optimum pH of LIP3 ranged from 4 to 6.0. Upon incubation in Good's buffer with different pH values from 3 to 10.0, the activities were measured spectro-

Table 2. Summary of the Purification of the Recombinant nfLIP3 and LIP3 from *C. rugosa*

purification step of nfLIP3	total vol (mL)	enzyme activity (units mL)	protein concn (mg/mL)	specific activity[a] (units/mg)	purification factor (-fold)	yield (%)
culture medium	1000	0.8	0.19	4.2	1	100
ultrafiltration 50 cutoff	50	1.9	0.29	6.6	1.6	12
octyl-Sepharose	36	2.7	0.04	67.5	16.1	12

purification step of LIP3	total vol (mL)	enzyme activity (units mL)	protein concn (mg/mL)	specific activity[a] (units/mg)	purification factor (-fold)	yield (%)
culture medium	1000	1.2	0.15	8.00	1	100
ultrafiltration 50 cutoff	45	7.8	0.2	39.00	4.9	29
octyl-Sepharose	32	3.47	0.042	82.60	10.3	9

[a] One unit of enzyme will hydrolyze 1.0 μmol of *p*-nitrophenyl butyrate to *p*-nitrophenol and butyric acid per minute at 37 °C and pH 7.0.

Table 3. Effect of pH on the Activity and Stability of Purified Recombinant LIP3[a]

pH	optimal pH (specific activity, units/mg)	pH stability[a] (residual activity, %)
3	66.6 ± 0.7	54 ± 2.9
4	73.8 ± 1.7	66.8 ± 2.4
5	72.8 ± 1.5	96.3 ± 4.1
6	72.7 ± 2.9	79.3 ± 5.4
7	68.1 ± 4.7	99.2 ± 2.4
8	62.7 ± 1.1	100 (203.4 units/mg)[b]
9	27.9 ± 3.8	52.5 ± 4.8
10	0	0.32 ± 0.12

[a] The effect of pH on lipase activity was determined by spectrophotometric method using *p*-nitrophenyl butyrate as substrate. The pH stability of purified LIP3 was incubated at 37 °C for 16 h in Good's buffers with various pH values. Values are means ± SD from three independent experiments. [b] The residual activity was measured by a spectrophotometric method using *p*-nitrophenyl caprate as substrate at 37 °C and pH 7.0. [c] One hundred percent of activity of LIP3 was 203.4 units/mg at 37 °C and pH 8.0.

Table 4. Effect of Temperature on the Activity and Stability of Purified Recombinant LIP3[a]

temperature (°C)	optimal temperature (specific activity, units/mg)	thermostability (residual activity, %)
10	67.4 ± 4	
20	87.3 ± 7.5	
30	82.7 ± 5.2	99.1 ± 6.2
37	85.3 ± 5.2	100 (196.5 units/mg)[b]
40	87.6 ± 3.7	98 ± 3.3
50	80.6 ± 5.2	95.8 ± 4.3
60	77.7 ± 3.7	99.7 ± 4.6
70	80.1 ± 6.3	73.2 ± 6.8
80	34.2 ± 5.5	49.7 ± 3.3
90	24.2 ± 3.8	0.6 ± 0.2

[a]The effect of temperature on purified LIP3 activity was measured by a spectrophotometric method using *p*-nitrophenyl butyrate as substrate at pH 7.0. The thermostability of purified LIP3 was determined by heating the purified enzyme for 10 min at various temperatures. Values are means ± SD from three independent experiments. [b] One hundred percent of activity of purfied LIP3 was 196.5 units/mg at 30 °C and pH 7.0.

photometrically using *p*-nitrophenyl caprate as substrate at 37 °C (**Table 3**). After 16 h of incubation, the lipase retained 85−100% of its activity within a pH range of 5.0−8.0. The effects of temperature on LIP3 activity and stability are given in **Table 4**. The optimum temperature of LIP3 was also investigated using *p*-nitrophenyl butyrate as substrate at pH 7.0. The LIP3 exhibited a broad optimum temperature range from 20 to 50 °C. After incubation at various temperatures from 37 to 90 °C for 10 min at pH 7.0, the lipase was stable in an optimum incubation temperature range (37−60 °C). However, inactivation occurred at temperatures >60 °C.

Table 5. Effect of Detergents on Purified Recombinant LIP3 and nfLIP3[a]

detergent	concn (%, w/v)	rel activity[b] of LIP3 (%)	rel activity[b] of nfLIP3 (%)
control	0	100 (50.7 units/mg)[c]	100 (29.4 units/mg)[c]
Brij	0.1	53 ± 8	42 ± 2
	1	81 ± 3	111 ± 11
Triton X-100	0.1	14 ± 7	9 ± 2
	1	12 ± 1	4 ± 1
CHAPS	0.1	88 ± 6	78 ± 7
	1	0	0
sodium taurocholate	0.1	61 ± 5	122 ± 7
	1	45 ± 5	107 ± 19
Tween 80	0.1	88 ± 1	147 ± 5
	1	68 ± 7	76 ± 6
Tween 20	0.1	53 ± 4	20 ± 4
	1	7 ± 1	6 ± 4
SDS	0.1	0	0
	1	0	0

[a] All experiments were analyzed by incubation of enzyme for 1.5 h at 37 °C in 0.1 M Tris buffer (pH 8.0) with various detergents. [b] The relative activities (percent) were represented as the ratio of LIP3 with different detergents to that without detergent. [c] One unit of esterase activity is the amount of enzyme that hydrolyzes 1.0 μmol of *p*-nitrophenyl ester per minute at 40 °C and pH 8.0.

Effects of Detergents, Water-Miscible Solvents, and Chemicals on LIP3 Activity. The effect of detergents was determined by incubating the LIP3 in a 0.1 M Tris-HCl buffer (pH 8.0) containing 1 and 0.1% (w/v) detergents at 37 °C for 1.5 h (**Table 5**). The presence of 0.1% CHAPS, Tween 80, sodium taurocholate, and Tween 20 slightly reduced the activity of LIP3, whereas its activity further decreased or was eliminated as the concentration rose to 1%. Inversely, Brij increased the activity of LIP3 at a higher concentration (1%) as compared to a lower concentration (0.1%), as shown in **Table 5**. After 1.5 h of incubation, the activity was completely inactivated by SDS and by a higher concentration (1%) of CHAPS. The effect of Triton X-100 showed a nearly 90% inhibitory result at both 0.1 and 1%. Generally, all detergents used in this study represent various degrees of inhibitory effects on LIP3 activity. However, this result differed from those achieved by nfLIP3 (**Table 5**). In particular, adding both concentrations of the sodium taurocholate (0.1 and 1%) facilitated the activity of nfLIP3 with an increase of 7−29%. Moreover, the lipase behaved more actively toward Tween 80 (42−52% increased) and Brij (0.2−22% increased) at concentrations of 0.1 and 1%, respectively.

The effect of water-miscible solvents on LIP3 activity was investigated by incubating the enzyme at 37 °C in a 0.1 M Tris-HCl buffer (pH 8.0) containing 30% (v/v) of solvents for 1 h (**Figure 4A**). Acetone, methanol, and DMSO showed a slight

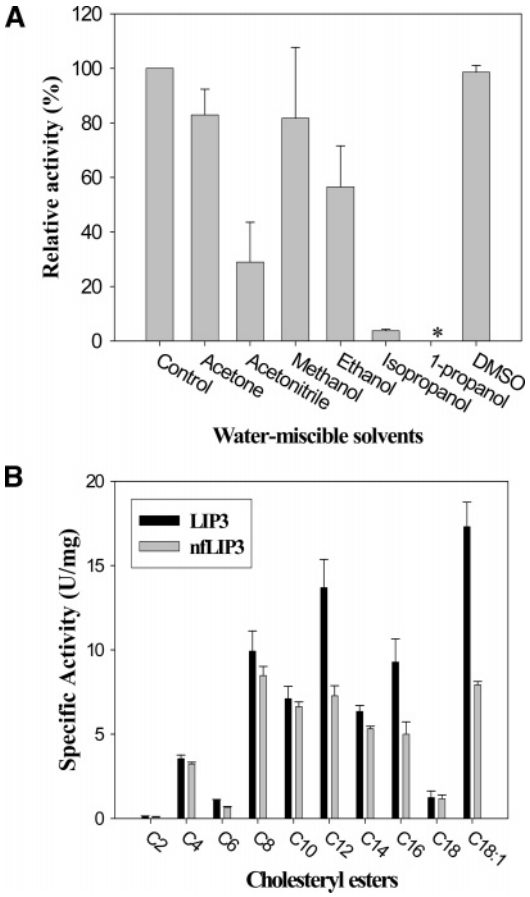

Figure 4. (**A**) Effect of water-miscible solvents on purified recombinant LIP3 activity. Purified lipase was incubated for 1 h at 40 °C in 0.1 M Tris-HCl buffer, pH 8.0, containing 30% (v/v) of various water-miscible solvents. The pure enzyme incubated in 0.1 M Tris-HCl buffer without any water-miscible solvent was used as 100% of activity (85 units/mg). Values are means ± SD from three independent experiments. (**B**) Substrate specificity of purified recombinant LIP3 and nfLIP3 in the hydrolysis of cholesteryl esters containing fatty acids of various chain lengths. One unit of esterase activity is the amount of enzyme necessary to hydrolyze 1.0 μmol of cholesteryl esters per minute at 37 °C and pH 7.0. Values are means ± SD from three independent experiments.

inhibitory effect on LIP3 activity, and the residual activity of LIP3 remained at 83, 82, and 99%, respectively. The addition of ethanol and acetonitrile significantly reduced activity by 45 and 71%, respectively. However, the addition of 2-propanol and 1-propanol almost completely inhibited the lipase activity of LIP3. A similar profile of the effect of water-miscible solvents on the nfLIP3 has also been obtained (data not shown).

For the effect of chemicals (1 and 10 mM), PMSF significantly reduced the lipase activity of LIP3 by at least 15%, whereas the addition of $MgCl_2$, $AgNO_3$, NaN_3, EDTA, $MnCl_2$, KCl, and $(NH_4)_2SO_4$ slightly increased its activity as the concentrations increased. Particularly, 10 mM $MnCl_2$ significantly increased lipase activity by ≈2.3-fold as compared to the control sample. The others—including NaCl, $CaCl_2$, and $CuSO_4$—slightly decreased the activity of LIP3 (<10%) while their concentrations increased (**Table 6**). In general, most chemicals used in this study were reduced or were slightly increased except for $MnCl_2$. Similar results have also been gained by nfLIP3, which differs only in terms of the degree of the effect from those obtained by LIP3 (data not shown).

Substrate Specificity of Recombinant LIP3. Table 7 shows the different preferences of LIP3 and nfLIP3 for *p*-nitrophenyl esters and triacylglycerols containing fatty acids of various chain lengths. For the hydrolysis of *p*-nitrophenyl esters, the most favorable substrate of LIP3 was *p*-nitrophenyl caprate (C_{10}), whereas the nfLIP3 preferred *p*-nitrophenyl caprylate (C_8). Among the nine triacylglycerols tested, the lipolytic activity of both LIP3 and nfLIP3 displayed the highest activity for hydrolysis of the medium-chain triacylglycerol (tricaprylin; C_8).

The hydrolysis of cholesteryl esters containing fatty acids of various chain lengths demonstrated that LIP3 possessed not only unique cholesterol esterase activity but also broad cholesteryl ester preferences, whereas nfLIP3 represented a lower hydrolytic activity toward cholesterol esters (**Figure 4B**). Among 10 different esters, cholesteryl oleate ($C_{18:1}$) was the favorite substrate for LIP3, which differs from those obtained by nfLIP3 (cholesteryl caprylate; C_8). The recombinant LIP3 showed 2−53-fold higher activity than commercial CRL, LIP2, or LIP4 obtained by Lee et al. (*3*).

Table 6. Effect of Various Chemicals on Purified Recombinant LIP3[a]

chemical	concn (mM)	rel activity[b] of LIP3 (%)
control	0	100 (77 units/mg)[c]
$CaCl_2$	1	98 ± 2
$CaCl_2$	10	95 ± 6
EDTA	1	96 ± 5
EDTA	10	96 ± 4
PMSF	1	79 ± 6
PMSF	10	61 ± 15
$MgCl_2$	1	101 ± 3
$MgCl_2$	10	104 ± 7
$AgNO_3$	1	95 ± 5
$AgNO_3$	10	96 ± 3
$MnCl_2$	1	83 ± 18
$MnCl_2$	10	228 ± 11
$(NH4)_2SO_4$	1	99 ± 6
$(NH4)_2SO_4$	10	103 ± 5
NaN_3	1	97 ± 9
NaN_3	10	104 ± 5
KCl	1	90 ± 3
KCl	10	91 ± 5
NaCl	1	102 ± 7
NaCl	10	97 ± 6
$CuSO_4$	1	95 ± 13
$CuSO_4$	10	93 ± 8

[a] All experiments were analyzed by incubation of enzyme for 30 min at 37 °C in 0.1 M Tris buffer (pH 8.0) with various chemicals. [b] The relative activities (percent) were represented as the ratio of LIP3 with different chemicals to that without chemical. [c] One unit of esterase activity is the amount of enzyme that hydrolyzes 1.0 μmol of *p*-nitrophenyl ester per minute at 40 °C and pH 8.0.

DISCUSSION

The difficulty in the heterologous expression of the multigene family coding for *C. rugosa* isozymes was discussed (*3*, *9*) and overcome in the present work by means of overlap extension PCR for the replacement of nonuniversal serine codons. On the basis of our previous paper (*9*), the location of a short N-terminal signal peptide in front of the *lip*1 gene represented an absolutely negative effect on the expression of the LIP1, whereas it brought an opposite result on the recombinant LIP3 expression level obtained in this study. After removal of the N-terminal signal peptide, the recombinant LIP3 expression was readily dropped drastically, whereas its transcriptional level only slightly decreased (0.2%), as shown in **Figure 3**. This result suggested an important role for the additional N terminus in terms of contribution to enzyme production in *P. pastoris*. Recent studies (*16*−*20*) have shown that codon optimization can improve expression levels in various expression systems. Indeed, in the present work, we were able to overcome such a low expression

Table 7. Substrate Specificity of Recombinant LIP3 and nfLIP3 in the Hydrolysis of *p*-Nitrophenyl (p-NP) Esters and Triacylglycerols Containing Fatty Acids of Various Chain Lengths[a]

chain length of acyl group	specific activity (units/mg) of *p*-NP esters[b]		specific activity (units/mg) of triacylglyceride[c]	
	LIP3	nfLIP3	LIP3	nfLIP3
C2	2 ± 0.4 (0.8%)	0.9 ± 0.07 (0.5%)	28 ± 16 (4%)	32 ± 13.4 (13%)
C4	113 ± 1.9 (40%)	75 ± 3.2 (46%)	699 ± 38 (97%)	210 ± 3.5 (86%)
C6	61 ± 1.8 (22%)	35 ± 0.7 (21%)	129 ± 5 (18%)	49 ± 1.9 (20%)
C8	260 ± 7.4 (93%)	164 ± 12 (100%)	723 ± 47 (100%)	243 ± 13.7 (100%)
C10	280 ± 6.5 (100%)	156 ± 2.3 (95%)	267 ± 28 (37%)	116 ± 0.4 (48%)
C12	251 ± 11 (89%)	140 ± 6.3 (85%)	432 ± 34 (60%)	94 ± 15.2 (39%)
C14	150 ± 1.3 (53%)	73 ± 5.9 (45%)	632 ± 33 (87%)	37 ± 6.7 (15%)
C16	111 ± 2.7 (24%)	63 ± 1.7 (38%)	214 ± 17 (30%)	0
C18	68 ± 7.2 (24%)	34 ± 1.9 (21%)	12 ± 1 (1.7%)	20 ± 6.4 (8%)

[a] Hydrolyses of *p*-nitrophenyl esters and triacylglycerols were both measured at pH 7.0 and 37 °C. All values are means ± SD from three independent experiments. Data in parentheses represent the relative activities (percent) of each enzyme with the different substrates. [b] One unit of esterase activity is the amount of enzyme that hydrolyzes 1.0 μmol of *p*-nitrophenyl ester per minute at 37 °C and pH 7.0. [c] One unit of lipolytic activity is the amount of enzyme necessary to produce 1.0 μmol of fatty acid per minute at 37 °C and pH 7.

problem by codon optimization of a gene fragment near the 5′ end of the *lip*3 gene. We were also able to achieve a 52-fold higher expression yield (0.47 unit/mL) of the co*lip*3 construct than that obtained by *lip*3 with *p*-nitrophenyl butyrate as substrate for 5 days of cultivation time. However, the 52-fold expression yield obtained in the co*lip*3 construct relative to the *lip*3 was not supported by an unapparent improvement in transcription level, suggesting a posttranscriptional reason for the expression difference. Regardless of the mechanisms involved, it was suggested that codon optimization near the 5′ end of the coding region toward the bias of *P. pastoris* could have a positive impact on recombinant LIP3 expression levels.

For the properties of purified recombinant LIP3, LIP3 exhibited a broad optimum pH range of 4.0−6.0 (**Table 3**) and retained 85−100% of its activity after incubation over a broad pH range of 5.0−8.0 for 16 h at 37 °C using *p*-nitrophenyl butyrate as substrate (**Table 3**). This differed from the distinct pH stability value (pH 6.1) obtained by isolating the pure native LIP3 from the commercial CRL preparation (*21*). When the pH of the environment was further elevated, our recombinant LIP3 can still possess ≈50% residual activity as illustrated in **Table 3**. This means that the purified recombinant LIP3 is potentially useful under both acidic and alkaline environments.

Among triglycerides and *p*-nitrophenyl esters, the recombinant LIP3 showed the highest specific activity toward tricaprylin (C_8 acyl group) and *p*-nitrophenyl caprate (C_{10} acyl group) at pH 7.0 (**Table 7**). Besides, our enzyme displayed a low hydrolytic activity toward tricaproin and *p*-nitrophenyl caproate, both containing a C_6 acyl group of fatty acid chain length. It is difficult to explain this observation, although similar results for other *C. rugosa* isozymes were also achieved in previous studies (*3*, *9*). In addition, the substrate preferences of our purified recombinant LIP3 differ from those obtained by pure native LIP3 isolated from the commercial CRL preparation (C_4/$C_{18:1}$ triglycerides and C_8 *p*-nitrophenyl esters) (*21*, *22*). The taste and texture of cheese are both altered by the enzymatic conversions taking place during the ripening process, in which lipolytic degradative reactions play a large role (*23*). Also, Arbige et al. (*24*) reported that the ratio of tricaprylin (C_8) to tributyrin (C_4) activity (TCU/TBU) constitutes a good criterion for the usefulness of a lipase esterase in ripening cheese. It is interesting to note that our recombinant LIP3 with a high TCU/TBU ratio (1.0), which is >0.75, revealed potential usefulness in cheese-ripening applications. The purified recombinant LIP3 showed 32-, 2-, 7.4-, and 53-fold higher hydrolysis activities toward cholesteryl laurate (C_{12} acyl group) than that of LIP1 (0.23 ± 0.004 unit/mg), LIP2 (4.02 ± 0.4 units/mg), LIP4 (0.98 ± 0.03 unit/mg), and commercial CRL (0.14 ± 0.5 unit/mg) preparations (*3*, *9*), respectively. Overall, our purified enzyme exhibited the highest cholesterol esterase activity of various *C. rugosa* isozymes and is thus very useful for determining cholesterol levels in clinical and food analyses.

As shown in **Table 4**, our purified recombinant LIP3 is most stable over a broad range of temperatures from 37 to 60 °C for at least 10 min, in which it showed a much better thermostability as compared to the pure native LIP3 isolated from commercial CRL preparation (35.8 °C) (*21*). Our recombinant LIP3 showed distinct biochemical properties from the pure native LIP3 or commercial CRL preparation (*3*, *21*). These findings might be due to the different glycosylation forms, N-terminal sequence deletion, or different combinations of catalytic activities of individual CRL isoforms**,** which might alter some of the biochemical properties of recombinant LIP3.

For industrial applications, the use of hydrolytic lipases in detergents is their most important commercial application. Lipase sales have been estimated to be U.S. $30 million, with detergent enzymes making up 30% in 1995. In one estimate of the demand, 1000 tons of lipases are added to ≈13 billion tons of detergents produced each year (*25*). Generally, all detergents used in this study repressed the activity of LIP3 to various degrees except for Brij, which increased its activity as the concentration increased to 1%. In contrast, nfLIP3 seems to be more active at 0.1% sodium taurocholate, 0.1% Tween 80, and 1% Brij, respectively. Different behaviors of the detergent effects on nfLIP3 may be the cause of the stabilization through the presence of the additional peptide at the N terminus. Therefore, recombinant nfLIP3 was most suitable for use in sodium taurocholate and Tween 80.

It is interesting to note that the enzyme activity apparently increased 2.3-fold in the presence of 10 mM $MnCl_2$, indicating that the Mn^{2+} might possibly combine with the Glu, His, and Cys amino acids by ionic bond, converting the protein configuration into a more active form. The binding site will be further studied to elucidate the activation mechanism of the LIP3 lipase by metal ions.

Most enzymes are easily denatured in highly hydrophilic solvents because of the reduced contact between lipase and lipids, which may inhibit the hydrolysis function of lipase (*26*). Although our recombinant LIP3 was strongly inhibited by 1-propanol, 2-propanol, and acetonitrile, that it was only slightly affected by acetone, DMSO, or methanol was interesting. Our recombinant LIP3 showed a result similar to the pure

native LIP3 isolated from commercial CRL when acetonitrile [>17% (v/v)] was used as a cosolvent. However, it showed greater stability with acetone (*5*). This might be due to some posttranslational modifications (such as glycosylation) that occurred as recombinant LIP3 was expressed in the *Pichia* system.

In summary, the expression efficiency of recombinant LIP3 was functionally enhanced by the additional N-terminal peptide or regional codon optimization for purposes of further quantification. This will provide a more convenient and inexpensive method of lipase production in the *P. pastoris* system on an industrial scale. The distinct biochemical properties established in our study suggest that it might be useful for other industrial applications. Protein engineering and DNA shuffling methods are currently being investigated to further improve the catalytic activity and stability of the enzyme.

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