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Codon Optimization of *Candida rugosa lip*1 Gene for Improving Expression in *Pichia pastoris* and Biochemical Characterization of the Purified Recombinant LIP1 Lipase

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An important industrial enzyme, *Candida rugosa* lipase (CRL) possesses several different isoforms encoded by the *lip* gene family (*lip*1−*lip*7), in which the recombinant LIP1 is the major form of the CRL multigene family. Previously, 19 of the nonuniversal serine codons (CTG) of the *lip*1 gene hav been successfully converted into universal serine codons (TCT) by overlap extension PCR-based multiple-site-directed mutagenesis to express an active recombinant LIP1 in the yeast *Pichia pastoris*. To improve the expression efficiency of recombinant LIP1 in *P. pastoris*, a regional synthetic gene fragment of *lip*1 near the 5′ end of a transcript has been constructed to match *P. pastoris*-preferred codon usage for simple scale-up fermentation. The present results show that the production level (152 mg/L) of coLIP1 (codon-optimized LIP1) has an overall improvement of 4.6-fold relative to that (33 mg/L) of non-codon-optimized LIP1 with only half the cultivation time of *P. pastoris*. This finding demonstrates that the regional codon optimization the *lip*1 gene fragment at the 5′ end can greatly increase the expression level of recombinant LIP1 in the *P. pastoris* system. More distinct biochemical properties of the purified recombinant LIP1 for further industrial applications are also determined and discussed in detail.

KEYWORDS: ***Candida rugosa* lipase; codon optimization; isoforms; *Pichia pastoris***

INTRODUCTION

Candida rugosa (formerly *Candida cylindracea*) lipase (CRL) is a well-known and very important enzyme that has been widely used to catalyze hydrolysis and in the esterification of various triglycerides and fatty acids for industrial biotechnology applications. However, crude enzyme preparations obtained from the various commercial suppliers exhibit remarkable variation in their catalytic efficiency and stereospecificity. Separation of CRL isoenzymes is highly desirable to allow their use under well-defined conditions. However, high identity in their protein sequences causes similarities in the physical properties of the lipases that create technical difficulties in the isolation of individual isoenzymes from cultures of *C. rugosa* on a preparative scale for industrial applications (*1*, *2*). In addition, it possesses stereoselectivity and regioselectivity in the synthesis of pharmaceuticals, glycoderivatives, and carbohydrate esters used in the manufacture of many foods and fragrances (*3*). Previously, several closely related lipase genes with high-identity (between 60 and 70%), namely, *lip*1−*lip*7, encoding LIP1−LIP7, had been identified and sequenced from *C. rugosa* (*4*, *5*). Lipases are mainly conserved at a catalytic triad, Ser-209, His-449, and Glu-341, and the sites (Cys-60/Cys-97 and Cys-268/Cys-277) involved in disulfide bond formation. All of these lipase isoforms, consisting of 534 amino acids and an observed molecular mass of 60 kDa, could be isolated from commercial enzyme preparation. However, the enzymes differ in their N-glycosylation sites and isoelectric points, and some local features of their hydrophobicity profiles might cause *C. rugosa* lipase isozymes to possess different substrate specificities and thermal stabilities in biocatalytic applications (*1*, *6*, *7*). The major component of *C. rugosa* isozymes is encoded by the *lip*1 gene (*8*), which is well characterized and has a known crystallographic structure (*9*).

Unfortunately, despite the general availability of the cloned genes, the non-spore-forming yeast *C. rugosa* utilizes a nonuniversal codon; that is, the triplet CTG, a universal codon for leucine, is read as serine. 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. The transposition of several or even all of the CTG codons into universal serine triplets is

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required for the expression of a functional lipase protein in a heterologous expression system. To overcome the codon usage obstacle, we have successfully overexpressed the recombinant LIP2 lipase by multiple mutagenesis technique in a *Pichia pastoris* fermentation system with a pGAPZαC vector driven by a glyceraldehyde-3-phosphate dehydrogenase (GAP) constitutive promoter (GAPDH) (*2*). The major advantage of the pGAPZαC system is that it avoids the accumulation of formaldehyde and hydrogen peroxide (oxidized products of methanol by alcohol oxidase), which harmed the cell's interior (*10*−*12*). Combination of the pGAPZαC vector and the eukaryotic expression host, *P. pastoris*, was enabled to successfully carry out eukaryotic post-translational modifications such as protein processing, folding, disulfide bond formation, and glycosylation inside the cell, leading to a functional protein that could be secreted into the medium. It is easy to manipulate and has a lower production cost and higher expression level than other eukaryotic expression systems (*13*, *14*). Similarly, a multiple-mutagenesis method was used on the *lip*1 gene, which was inserted into the pGAPZαC vector between the *Kpn*I and *Sal*I sites to express N-fused LIP1 (nfLIP1) in the *P. pastoris* system. As an unnecessary N-terminal peptide linker coding sequence has been removed by PCR from the nfLIP1 plasmid, a higher protein expression level (33 mg/L) has been obtained in the culture medium of *P. pastoris* (*15*). Nevertheless, the expression level is still not high enough for economical industrial applications. Recent reports have shown that AT-rich regions in a native gene may cause early termination of transcription (*16*, *17*), and codon optimization can improve expression levels in various expression systems (*16*, *18*−*21*). It does this by using DNA rebuilding for replacement of rare codons with preferred codons for efficient translation in *P. pastoris*. However, the number of similar studies on the effect codon usage has on the expression of CRL isozymes in *P. pastoris* is limited.

In the present work, we designed a regional synthetic gene fragment between *Pml*I and *Hin*dIII near the 5′ end of a *lip*1 transcript optimized for codon usage in *P. pastoris*. Much higher protein levels (152 mg/L) within the minimal culture time (72 h) were attained, and the expression levels were affected by different vector constructions. The purified enzyme showed distinct catalytic properties compared with LIP2 and LIP4. In comparison to the methanol induction system, the expression of recombinant LIP1, by partial codon optimization near the 5′ end of a transcript, yields more (3-fold increase) than the complete codon-optimized synthetic *lip*1 gene expression reported previously (*22*), in 3 days of cultivation without any induction materials. This finding demonstrated that the expression of recombinant LIP1 in the *P. pastoris* system is significantly affected by the codon usage near the 5′ end of the *lip*1 gene. We also showed that the purified recombinant enzyme possesses some novel properties not previously reported.

MATERIALS AND METHODS

Strains and Plasmids. The *P. pastoris* expression vector pGAPZαC (Invitrogen, Carlsbad, CA) was manipulated in *Escherichia coli* strain TOP10F′ (Invitrogen), which was used as a host for cloning. *P. pastoris* strain KM71 or SMD168H (Invitrogen), harboring the recombinant plasmids, was used for expressing recombinant nfLIP1, LIP1, or codon-optimized LIP1 (coLIP1). All *P. pastoris* transformants were cultured in YPD (1% yeast extract, 2% peptone, and 2% dextrose; pH 6.3) broth containing 100 μg/mL of Zeocin (Invitrogen) at 30 °C.

Plasmid Construction for the Expression of Recombinant LIP1. The mature protein-coding sequence of *lip*1 (GenBank accession number X64703) was cloned by RT-PCR and all CTG-serine codons were replaced with TCT by overlap-extension PCR (*15*). After that, the *lip*1 gene was inserted into the pGAPZαC expression vector between the *Kpn*I and *Eco*RI sites. There is an extra peptide linker in front of the mature LIP1 after the cleavage by Ste13 protease, namely, pGAPZαC−nfLIP1 (nfLIP1) plasmid (**Figure 1A**). We also removed the coding sequence of the N-terminal peptide linker from pGAPZαC−nfLIP1 by PCR to generate pGAPZαC−LIP1 (LIP1) plasmid (**Figure 1B**). In an effort to further scale up fermentation, the gene fragment between the *Pml*I and *Hin*dIII sites of the mature *lip*1 coding region near the 5′ end was replaced by a synthetic codon-optimized gene between the *Pml*I and *Hin*dIII sites of the mature *lip*1 coding region to generate pGAPZαC−coLIP1 plasmid (**Figure 1C**). Several codons of the *lip*1 gene of a 375-bp regional synthetic gene containing *Pml*I and *Hin*dIII sites were changed according to *P. pastoris*'s favorite codon usage from the codon usage database (at Website http://www.kazusa.or.jp/codon), thereby decreasing the G+C content from 63 to 42%. The overlap extension PCR strategy was then employed to change 92 codons of the *lip*1 gene (**Figure 2**). The 375-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 pGAPZαC vector.

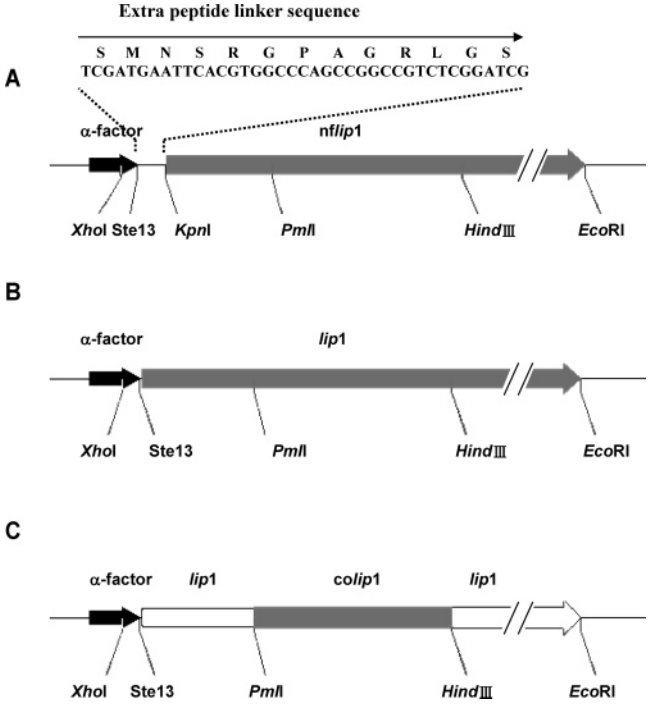

Figure 1. Comparison of the construction of pGAPZαC–nfLIP1, pGAPZαC–LIP1, and pGAPZαC–coLIP1 plasmids. (**A**) The N-fused *lip*1 gene contained an extra peptide linker sequence. (**B**) The *lip*1 gene lacked an N-terminal extra peptide linker, which due to the cloning positions that we chose on the vector, left after *S. cerevisiae* α-factor secretion signal cleavage. (**C**) The co*lip*1 gene and *Pml*I–*Hin*dIII fragment (shaded 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 LIP1. The *lip*1 gene codons without any changes are indicated as white boxes.

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

Transformation and Expression. The plasmids (10 μg) harboring the engineered LIP1 and coLIP1 were linearized with *Rca*I and transformed into *P. pastoris* KM71 or SMD168H by electroporation. High-voltage pulses (1.5 kV) were delivered to 100 μL 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 μg/mL Zeocin (Invitrogen) to isolate Zeocin-resistant clones. Individual colonies containing lipase-secreting

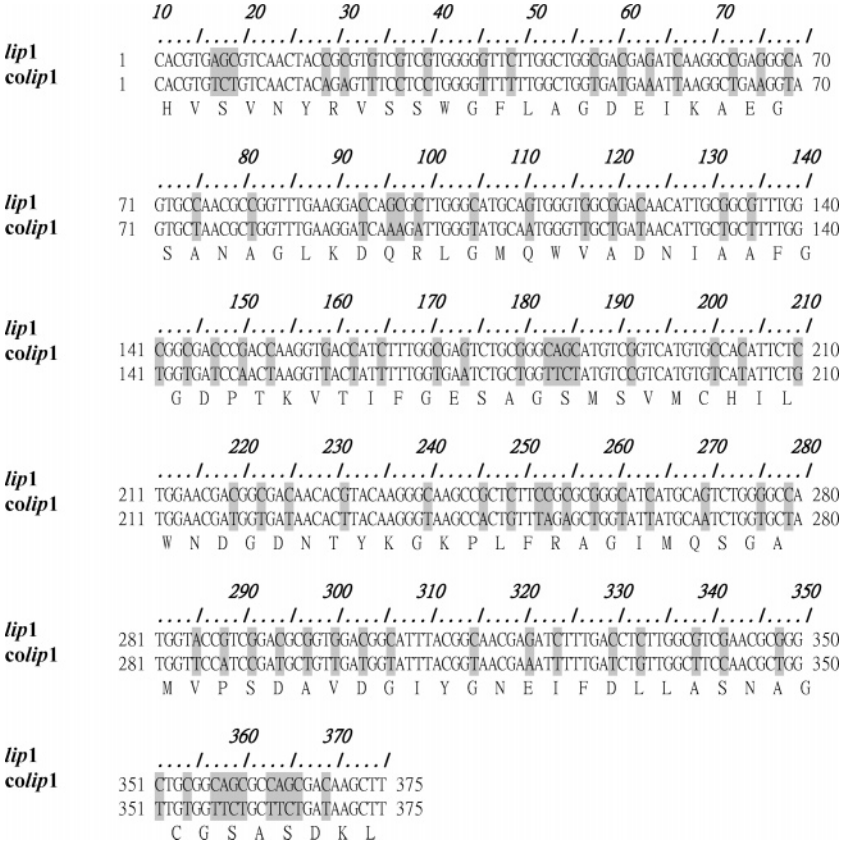

Figure 2. DNA sequence alignment comparison of the *Pml*I–*Hin*dIII fragment of *lip*1 gene (top) and co*lip*1 gene (bottom). Without changing the amino acid sequence, several codons of the *lip*1 sequence were exchanged (shaded) into the new co*lip*1 sequence with highly used codons in *P. pastoris* for heterologous protein expression improvement.

transformants were picked and patched on 1% tributyrin emulsion YPD 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 using a Bio-Rad assay kit (Bio-Rad Laboratories, Hercules, CA). Lipase concentration in the medium was measured by scanning densitometry (UMAX Astra 4700) of the secreted lipase band on SDS-PAGE (**Figure 4A**) and analyzed by using Band Leader software, version 3.00 (Magnitec Ltd.), 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 samples was used as template using the One-Step RT-PCR kit (Genemark Technology Co., Ltd.). The specific primers (upstream, 5′-GCTTGCGCGGTGTGTCTAGCGACACGTTG-3′; downstream, 5′-GGGAATTCTACACAAAGAAAGACGGCGGGTTGGA-3′) for the common sequence of the *lip*1 and co*lip*1 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: 1 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, 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 LIP1. Selected *P. pastoris* transformants were grown in 500-mL flasks containing 200 mL of 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 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 TE buffer containing 10 mM CHAPS. The eluted protein was collected and dialyzed against 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 nfLIP1, LIP1, and coLIP1 were determined by SDS-PAGE analysis using Sigma protein molecular mass markers. 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) (pH 7.0) (*2*), 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 by 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.

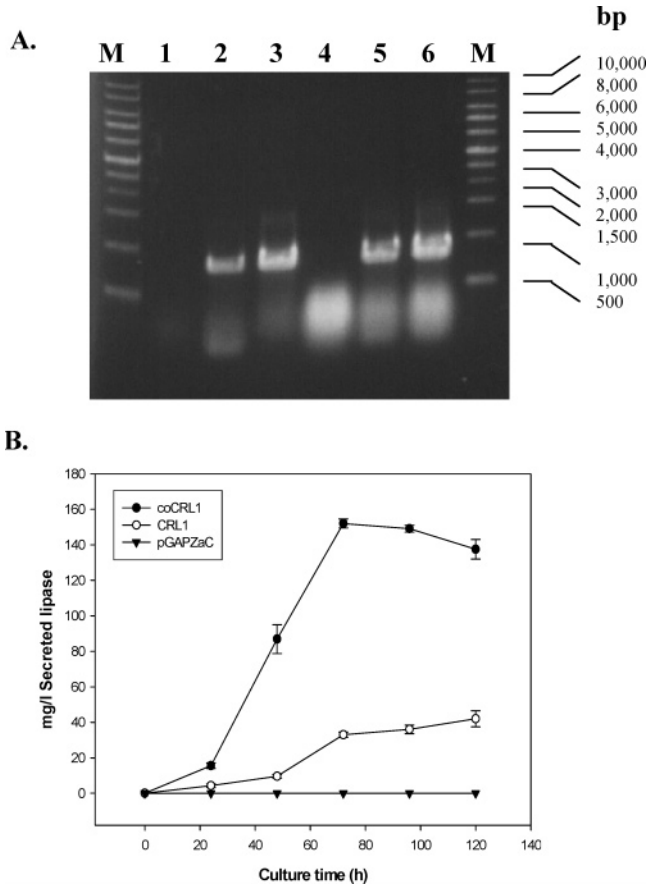

Figure 3. Comparison of transcription level (**A**) and expression yield (**B**) with variant plasmid constructs. (**A**) RT-PCR analysis products (775 bp) of *P. pastoris* transformants: lane 1, negative transformant (pGAPZαC) at 48 h; lane 2, pGAPZαC–LIP1 transformant at 48 h; lane 3, pGApZαC–coLIP1 transformant at 48 h; lane 4, negative transformant (pGAPZαC) at 72 h; lane 5, pGAPZαC–LIP1 transformant at 72 h; lane 6, pGAPZαC–coLIP1 transformant at 72 h; both lanes 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 culture supernatants containing variant plasmid constructs are shown as pGAPZαC (▼), pGAPZαC–LIP1 (○), and pGAPZαC–coLIP1 (●), respectively. All constructive plasmids were transformed into *P. pastoris* cells, and the transformants were inoculated in YPD medium at 30 °C. The expression yields were quantified by Bio-Rad assay kit accompanying scanning densitometer to accurately measure the secreted lipase band on SDS-PAGE.

The optimum pH was investigated in the pH range of 3.0−9.0 using Good's buffer (50 mM each of Bicine, CAPS, sodium acetate, and BisTris propane) (*2*). Temperature effect was measured by the spectrophotometric method using *p*-nitrophenyl caprylate as substrate. The optimum temperature for the LIP1 was investigated in the range of 10−90 °C at pH 7.0. To analyze thermal stability, the lipase was incubated for 10 min at various temperatures in the range of 30−60 °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 according to the spectrophotometric method using *p*-nitrophenyl butyrate as substrate. All reaction mixtures were incubated at 37 °C for 1 h in 0.1 M Tris-HCl buffer (pH 7.0) with a final solvent concentration of 0.2% (v/v) in the assay. The reaction mixture without 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 0.1 M Tris-HCl buffer (pH 7.0) 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 0.1 M Tris-HCl buffer (pH 7.0) 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 method of Lee et al. (*2*).

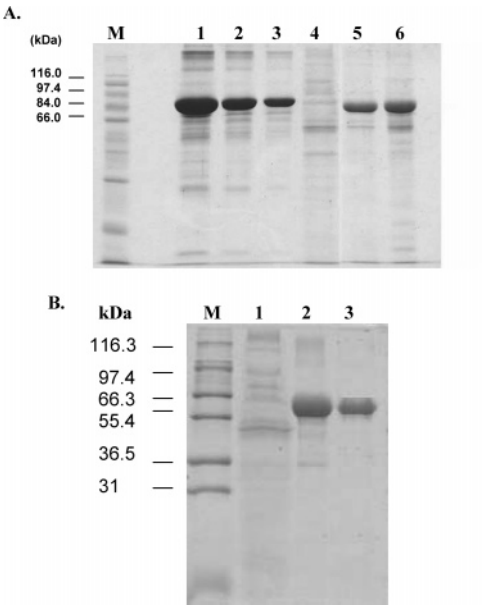

Figure 4. (**A**) SDS-PAGE analysis of different expressed recombinant LIP1. Proteins were stained with Coomassie blue: lane M, molecular mass standards indicated in kDa; lane 1, 10 μg of BSA; lane 2, 4 μg of BSA; lane 3, 2 μg of BSA; lane 4, culture medium of pGAPZαC transformant; lane 5, culture medium of pGAPZαC–LIP1 transformant; lane 6, pGAPZαC–coLIP1 transformant. All transformants were incubated in YPD medium at 30 °C for 72 h. Different protein amounts of BSA weres used as standard to contrast with the recombinant LIP1 expression calculation; 10 mL of total loading sample amount was applied to each lane, respectively. (**B**) SDS-PAGE analysis for the pGAPZαC, crude LIP1, and purified LIP1 lipase: lane M, molecular mass standards indicated in kDa; lane 1, culture medium of pGAPZαC transformant; lane 2, concentrated culture medium of LIP1; lane 3, purified LIP1. Total loading protein amounts of each sample were 0.58 mg (pGAPZαC), 4.6 μg (crude LIP1), and 4 μg (purified LIP1) applied to each lane, respectively.

RESULTS

Effect of Codon Optimization on Protein Expression Levels. The codon-optimized *lip*1 gene with a regional synthetic codon-optimized fragment, which lies between the *Pml*I and *Hin*dIII restriction sites as shown in **Figure 1**, was constructed in the pGAPZαC expression vector for expression of recombinant LIP1 in *P. pastoris* in secreted form as described previously (*15*). Therefore, the general G+C content, a byproduct of changing codon bias, decreased from 63 to 42%, and unique restriction sites were strategically positioned throughout the insert gene. The pGAPZαC plasmid without the *lip*1 gene was used as control.

Without any induction procedure, the majority of LIP1 protein was secreted into the YPD medium, and both of the active recombinant enzymes increased with the cultivation time up to 3 days. The coLIP1, the recombinant protein expressed from the codon-optimized *lip*1 gene construct, rapidly and stably accumulated at a maximum protein concentration of 152 mg/L in 3 days of cultivation time (**Figure 3B**), 4.6-fold higher than the non-codon-optimized LIP1 production level (33 mg/L).

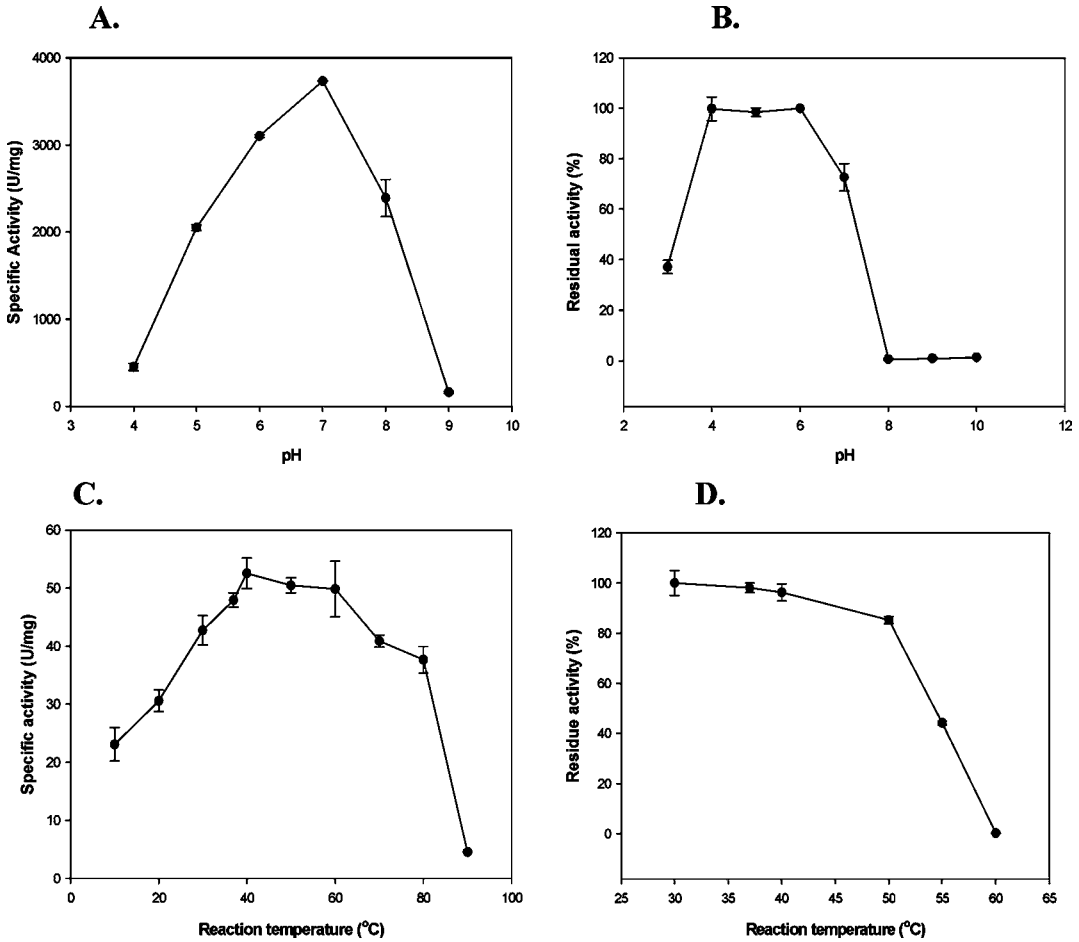

Figure 5. pH effects on the activity (**A**) and stability (**B**) and temperature effects on the activity (**C**) and stability (**D**) of purified recombinant LIP1. (**A**) Effect of pH on lipase activity was determined by the pH-stat assay with tributyrin. (**B**) Purified LIP1 was incubated at 37 °C for 16 h in Good's buffers with various pH values. The residual activity was measured by a spectrophotometric method using *p*-nitrophenyl butyrate as substrate at 37 °C and pH 7.0. One hundred percent activity of LIP1 was 13.2 units/mg at 37 °C and pH 6.0. (**C**) Effect of temperature on LIP1 activity was measured by a spectrophotometric method using *p*-nitrophenyl caprylate as substrate at pH 7.0. (**D**) Thermostability of LIP1 was determined by incubating the purified enzyme for 10 min at various temperatures. One hundred percent activity of LIP1 was 46.7 units/mg at 30 °C and pH 7.0.

Table 1. Summary of the Purification of the Recombinant LIP1 from *C. rugosa*

purifn step	total vol (mL)	enzyme activity (units/mL)	protein concn (mg/mL)	specific activity[a] (units/mg)	purifn factor (fold)	yield (%)
culture medium	1000	12.55	1.9	6.40	1	100
ultrafiltration	50	46.35	0.458	101.00	15.8	19
50 cutoff octyl-Sepharose	35	65.6	0.4	164.00	25.6	18

[a] Activity was measured by photometric assay with *p*-nitrophenyl butyrate as a substrate.

However, as cultivation time further increased, expression levels of the coLIP1 construct declined, whereas the non-codon-optimized LIP1 edged up to 40 mg/L after 5 days.

This may be caused by protease action during the long-term cultivation period. Therefore, the codon optimization significantly increased the secreted protein expression level and shortened the maximum protein production time.

The effect of codon optimization was further investigated at the transcriptional level on RNA samples specific amplified by the RT-PCR system. The final RT-PCR products from *Pichia* transformants containing pGAPZαC, LIP1, and coLIP1 plasmids were analyzed by agarose gel electrophoresis (**Figure 3A**). The bright bands (775 bp) represented positive *Pichia* transformants containing LIP1 and coLIP1 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 48 and 72 h of cultivation time, the coLIP1 construct containing a regional modified gene fragment represented an ∼1.5-fold higher transcription level than the LIP1 construct. Also, transcription levels of both the coLIP1 and LIP1 constructs increased with increasing cultivation time, indicating codon optimization could also have a positive impact on transcription levels.

Simple purification of the recombinant enzyme by ultrafiltration combined with hydrophobic interaction chromatography allowed us to obtain the homogeneous protein (**Table 1**), which had a molecular mass of ∼60 kDa as determined by SDS-PAGE analysis (**Figure 4B**). This purified protein was used for further biochemical analysis.

Effects of pH and Temperature on the Activity and Stability of Recombinant LIP1. The pH dependence of LIP1 was studied at 37 °C using tributyrin as substrate (**Figure 5A**). The optimum pH for LIP1 was 7.0. The activities after incubation in Good's buffer with different pH values from 3 to 10.0 were measured spectrophotometrically using *p*-nitrophenyl butyrate as substrate at 37 °C (**Figure 5B**). After 16 h of incubation, the lipase retained 95−100% of its activity within a pH range from 4.0 to 6.0. The effects of temperature on LIP1

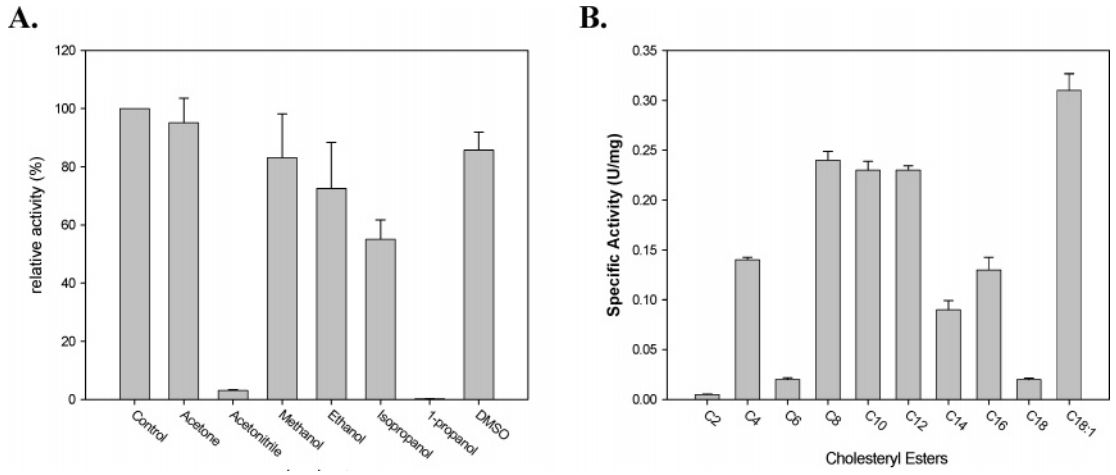

Figure 6. (**A**) Effect of water-miscible solvents on purified recombinant LIP1 activity. Purified lipase was incubated for 30 min at 37 °C in 0.1 M Tris-HCl buffer, pH 7.0, containing 30% (v/v) of various water-miscible solvents. The pure enzyme incubated in 0.1 M Tris-HCl buffer without any water-miscible solvent was used as 100% activity (16.2 units/mg). (**B**) Substrate specificity of purified recombinant LIP1 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.

Table 2. Effect of Detergents on Purified Recombinant LIP1 [All Experiments Were Analyzed by Incubation of Enzyme for 1.5 h at 37 °C in 0.1 M Tris Buffer (pH 7.0) with Various Detergents]

detergent	concn (%, w/v)	rel activity[a] (%)
control	0	100 (15.2 units/mg)[b]
Brij	0.1	116 ± 5
	1	96 ± 17
Triton X-100	0.1	87 ± 3
	1	94 ± 10
CHAPS	0.1	88 ± 3
	1	98 ± 3
sodium taurocholate	0.1	96 ± 10
	1	97 ± 6
Tween 80	0.1	85 ± 5
	1	103 ± 13
Tween 20	0.1	82 ± 15
	1	81 ± 6
SDS	0.1	1.4 ± 0.06
	1	0.6 ±0.7

[a] Relative activities (%) are represented as the ratio of LIP1 with different detergents to that without detergent. [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.

Table 3. Substrate Specificity of Recombinant LIP1 in the Hydrolysis of *p*-Nitrophenyl (p-NP) Esters and Triacylglycerols Containing Fatty Acids of Various Chain Lengths [Hydrolyses of p-NP 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 (%) of Each Enzyme with the Different Substrates]

chain length of acyl group	specific activity (units/mg) of p-NP esters[a]	specific activity (units/mg) of triacylglyceride[b]
C4	18 ± 0.7 (38%)	3160 ± 80 (44%)
C6	6 ± 0.1 (13%)	1340 ± 16 (19%)
C8	48 ± 1.2 (100%)	4573 ± 145 (64%)
C10	47 ± 3.1 (98%)	4302 ± 67 (60%)
C12	31 ± 2.0 (65%)	7200 ± 270 (100%)
C14	13 ± 0.5 (27%)	1635 ± 61 (23%)
C16	10 ± 0.2 (20%)	457 ± 14 (6%)

[a] 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. [b] 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 7.0.

activity and stability are depicted in **Figure 5C**,**D**. The optimum temperature of LIP1 was also investigated using *p*-nitrophenyl caprylate as substrate at pH 7.0, and the LIP1 exhibited higher specific activity at 40 °C. After incubation at various temperatures from 30 to 60 °C for at least 10 minutes at pH 7.0, the lipase was stable in an optimum incubation temperature range (30−40 °C), but inactivation occurred at temperatures >40 °C.

Effects of Detergents, Water-Miscible Solvents, and Chemicals on LIP1 Activity. The effect of detergents was determined by incubating the LIP1 in 0.1 M Tris-HCl buffer (pH 7.0) containing 1 and 0.1% (w/v) detergents at 37 °C for 1.5 h (**Table 2**). Addition of 0.1 or 1% detergents slightly decreased lipase activity except Brij and Tween 80. After 1.5 h of incubation, the activity was completely inactivated by SDS and slightly inactivated by Tween 20 (19−34%), Triton X-100 (6−16%), sodium taurocholate (4−14%), and CHAPS (2−15%). The lipase was more active toward 0.1% Brij (16−21%) and 1% Tween 80 (15−20%), respectively.

The effect of water-miscible solvents on LIP1 activity was investigated by incubating the enzyme at 37 °C in 0.1 M Tris-HCl buffer (pH 7.0) containing 30% (v/v) of solvents for 1 h (**Figure 6A**). Acetone, methanol, ethanol, and DMSO showed a slight inhibitory effect on LIP1 activity, and the residual activity of LIP1 remained 95, 83, 73, and 86%, respectively. The addition of 2-propanol reduced the activity by 45%. However, acetonitrile and 1-propanol almost completely inhibited lipase activity.

For the effect of chemicals (1 and 10 mM), lipase activity decreased as the concentration of $AgNO_3$, $(NH_4)_2SO_4$, and NaCl increased. The others—including $CaCl_2$, EDTA, $MgCl_2$, $MnCl_2$, NaN_3, KCl, and $CuSO_4$—increased the activity of LIP1 while their concentration increased. In particular, 10 mM $CuSO_4$ increased lipase activity significantly, ∼2.5-fold compared with control sample.

Substrate Specificity of Recombinant LIP1. Table 3 shows the different preferences of LIP1 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 LIP1 was *p*-nitrophenyl caprylate (C_8). Among the seven triacylglycerols tested, the lipolytic activity of LIP1 displayed the highest activity for hydrolysis of the medium-chain triacylglycerol (trilaurin; C_{12}).

Hydrolysis of cholesteryl esters containing fatty acids of various chain lengths demonstrated that LIP1 possessed not only unique cholesterol esterase activity but also broad cholesteryl esters preferences (**Figure 6B**). Among 10 different esters, cholesteryl oleate ($C_{18:1}$) was the favorite substrate for LIP1. The recombinant LIP1 showed 2−3-fold higher activity than commercial CRL or LIP4 (*2*).

DISCUSSION

The difficulty in the heterologous expression of the multigene family coding for *C. rugosa* isozymes was discussed and overcome by overlap extension PCR for the replacement of the nonuniversal serine codons in our previous work (*15*). Recent reports (*16*, *18*−*21*) have shown that codon optimization can improve expression levels in various expression systems. Indeed, in the present work, we were able to overcome the low expression problem by codon optimization of a gene fragment near the 5′ end of the *lip*1 gene, and we achieved high-level expression, 152 mg/L (equivalent to 474 units/mL with tributyrin as substrate), for 3 days of cultivation time. Previously, Brocca et al. (*22*) reported the expression of the *lip*1 gene from total synthesis constructed in the pPICZαB vector produced recombinant LIP1 at a level of 150 units/mL with tributyrin as substrate after 12 days of methanol induction (*22*). Our present construct represents ∼3-fold in 3 days higher expression than the previously published results of a methanol induction system (*22*). It appears that the optimization toward the A+T-biased codon choice of many lower eukaryote hosts may affect mRNA secondary structure and increase heterologous protein production (*23*). The 4.6-fold expression yield obtained in the coLIP1 construct relative to the LIP1 was not supported by a similar improvement in transcription level (≈1.5-fold), suggesting a post-transcriptional 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 LIP1 expression levels. Moreover, the pGAPZαC vector was a highly cost-effective expression vector and driven by the constitutive GAPDH promoter (*2*, *15*, *24*), which offers an attractive alternative to the AOX1 promoter for the heterologous expression of some recombinant proteins (*25*). In contrast to the methanol induction system, the pGAPZαC vector's ability to functionally express the target heterologous protein in YPD medium without any induction materials is more economical for industrial production.

For the properties of purified recombinant LIP1, the LIP1 exhibited a 2-fold higher specific activity at pH 7.0 using tributyrin as substrate (**Figure 5A**), which differed from the distinct optimal pH value (pH 5.6) obtained by isolating the pure native LIP1 from the commercial CRL preparation (*26*). It is interesting that the lipase retained 95−100% of its activity after incubation over a broad pH range from 4.0 to 6.0 for 16 h at 37 °C (**Figure 5B**). That means the purified recombinant LIP1 is potentially useful under an acidic environment. When the environment became alkaline, the relative activity of LIP1 plummeted from 73% (pH 7.0) to 0.7% at pH 8.0, which differs from what Brocca et al. reported (*22*).

Among triglycerides and *p*-nitrophenyl esters, the recombinant LIP1 showed the highest specific activity toward trilaurin (C_{12} acyl group) and *p*-nitrophenyl caprylate (C_8 acyl group) at pH 7.0 (**Table 3**). In addition, it preferred long-chain cholesteryl esters ($C_{18:1}$ acyl group) to short- or medium-chain cholesteryl esters (**Figure 6B**). Except for *p*-nitrophenyl esters, the substrate preferences toward triglycerides with the C_8 and C_{10} acyl groups (*26*) of pure native LIP1 isolated from the commercial CRL preparation differed from those of our purified recombinant LIP1. Additionally, the taste and texture of cheese are altered by the enzymatic conversions taking place during the ripening process, and lipolytic degradative reactions play a large role (*27*). Also, Arbige et al. (*28*) reported 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 that our recombinant LIP1 with a high TCU/TBU ratio (1.4), >0.75, revealed potential usefulness in cheese-ripening applications. The cholesteryl ester hydrolysis activity of purified recombinant LIP1, 2-fold greater than the cholesteryl laurate (C_{12} acyl group) hydrolysis activity of the commercial CRL preparation, might be invaluable in determining cholesterol levels in clinical and food analyses.

As shown in **Figure 5C**,**D**, our purified recombinant LIP1 is most active at 40 °C and stable at 30−40 °C for at least 10 min, consistent with previously reported results of recombinant LIP1 (*22*) while slightly differing from the pure native LIP1 isolated from commercial CRL preparation (36.6 °C). Overall, our recombinant LIP1 showed biochemical properties slightly distinct from those of pure native LIP1 or commercial CRL preparation (*2*, *22*, *26*). These findings might be due to N-terminal sequence deletion or different combinations of catalytic activities of individual CRL isoforms, which might alter some of the characterization of recombinant LIP1.

For industrial applications, the hydrolytic lipases in detergent are the most important commercial applications. 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 is added to the ∼13 billion tons of detergents produced each year (*29*). Generally, all detergents slightly affected the activity of LIP1, except for SDS, which almost completely inhibited the lipase activity at any given concentration. In addition, LIP1 was revealed to be more active with Brij and Tween 80, especially at concentrations of 0.1 and 1%, respectively. Therefore, recombinant LIP1 was most suitable for use in Brij and Tween 80 (**Table 2**).

It is interesting that the enzyme activity increased 33%, 35%, and 2.5-fold in the presence of 10 mM $CaCl_2$, $MnCl_2$, and $CuSO_4$, respectively. Mn^{2+}, Cu^{2+}, and Ca^{2+} might possibly combine to 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 LIP1 lipase by the metal ions.

Most enzymes are easily denatured in highly hydrophilic solvents because of the reducing contact between lipase and lipids, which may inhibit the hydrolysis function of lipase (*30*). Although our recombinant LIP1 was strongly inhibited by acetonitrile and 1-propanol, that it was only slightly affected by acetone, DMSO, methanol, or ethanol was interesting. Our recombinant LIP1 showed a result similar to that of the pure native LIP1 isolated from commercial CRL when acetonitrile [>17% (v/v)] was used as a cosolvent, but with acetone it showed much more stability (*6*).

In summary, the expression efficiency of recombinant LIP1 was functionally enhanced by regional codon optimization for purposes of further quantification. Also, a powerful heterologous protein expression system, pGAPZαC vector, was successfully established. This will provide a more convenient and inexpensive

method of lipase production in the *P. pastoris* system on an industrial scale. Our preliminary results showed that the yield for the recombinant LIP1 can reach 4 g/L in a jar fermentor, although the current yield in a shake flask culture was 152 mg/L. A high-density fermentation method is expected to further increase the yield. Protein engineering and DNA shuffling methods are currently underway to further improve the catalytic activity and stability of the enzyme.

Among cholesteryl esters, recombinant LIP1 showed the highest lipolytic activity toward cholesteryl oleate ($C_{18:1}$). The enzyme was greatly inhibited by SDS but little affected by other detergents tested. It retained 95% of its residual activity after incubating in 30% acetone for 16 h. The enzyme activity increased 33%, 35%, and 2.5-fold in the presence of 10 mM $CaCl_2$, $MnCl_2$, and $CuSO_4$, respectively. These distinct biochemical properties suggest it is very useful for further industrial applications.

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