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Engineering the Expression and Biochemical Characteristics of Recombinant *Candida rugosa* LIP2 Lipase by Removing the Additional N-Terminal Peptide and Regional Codon Optimization

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ABSTRACT: *Candida rugosa* lipase (CRL), an important industrial enzyme, has been established, containing several different isoforms which were encoded by the high-identity *lip* gene family (*lip*1 to *lip*7). In this study, we compared the expression and biochemical characterization with three different engineered *lip*2 constructions in the yeast *Pichia pastoris*. Our results showed that *lip*2 (*lip*2) has an overall improvement of 50% higher production yield (1.446 U/mL) relative to that of nf*lip*2 (0.964 U/mL) at 7 days of cultivation time. Codon-optimized *lip*2 (co*lip*2) has a 2.3-fold higher production yield (2.182 U/mL) compared to that of *lip*2 (noncodon-optimized; 1.446 U/mL) and nf*lip*2 (0.964 U/mL), with a cultivation time of 5 days. This finding demonstrated that the removal of the N-terminus and the regional codon optimization of the *lip*2 gene fragment at the 5′ end can greatly increase the expression level of recombinant LIP2 in the *P. pastoris* system. The distinct biochemical properties of our purified recombinant nfLIP2 and LIP2 suggested that they are potentially useful for various industrial applications.

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

INTRODUCTION

Candida rugosa (formerly *Candida cylindracea*) lipase (CRL) is a versatile industrial enzyme which has been widely used to catalyze biotransformation reactions to produce valuable ester compounds for food, flavor, fragrance, cosmetic, pharamaceutical, and other industrial applications.[1−5] Different commercial CRL preparations exhibited remarkable variation in their catalytic efficiency, regioselectivity, and stereospecificity. We have discovered that the variations of those biochemical properties in commercial CRL preparations may be caused by the different combinations of individual CRL isoforms since they are essentially mixtures of many individual lipase isoforms[6,7] which are not isolated and purified before most applications. Therefore, an effective separation of CRL isoenzymes is thus highly important and desirable in enabling us to better understand their catalysis mechanism, 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, to find new applications.[1] However, a high identity in their protein sequences causes similarities in the physical properties of the lipases and creates technical difficulties in the isolation of individual isoenzymes from the cultures of *C. rugosa* by normal chromatographic techniques.[1,6,8] To date, only three purified isoenzymes (LIP1−3) have been isolated[9−13] from commercial CRL preparations, and each enzyme displayed quite different catalytic efficiencies, substrate specificities, and thermostabilities.[9,12,14−17]

In comparison to the traditional purification procedure, the recombinant DNA technology combined with manipulating culture conditions in a heterologous expression system seems to be a viable and reasonable strategy for the characterization of different pure CRL isoforms and optimization of their biocatalytic properties in an industrial scale. For the CRL gene family, the CTG triplets encode most of the serine residues, including the catalytic Ser-209, in five CRL isoforms. Attempts to heterologously express such genes in other hosts, such as *Escherichia coli* and *Pichia pastoris*, may lead to the production of inactive lipases. Thus, the conversion of several or all of the CTG codons into universal TCT serine codons would be the major challenge for an active and functional lipase protein expression in heterologous hosts. For the expression in *P. pastoris* and *Saccharomyces cerevisiae* cells, a complete *C. rugosa lip*1 gene can be synthesized, and CTG Ser codons were replaced with optimized universal Ser codons under the control of the AOX1 promoter in the PICZαB and pYE2 plasmids.[18] Recently, a more efficient multiple site-directed mutagenesis method has been successfully developed[8,19] to convert all nonuniversal serine codons (CTG) into universal TCT serine codons in *lip*1 to *lip*4 genes to express the recombinant LIP1-LIP4 lipases in *P. pastoris*.[8,19−21] The functional recombinant CRL isoforms can be secreted into a medium by an N-terminal *Saccharomyces cerevisiae* α-factor secretion signal peptide driven by the GAP (glyceraldehyde-3-phosphate dehydrogenase) constitutive promoter of the pGAPZαC vector.[8,19,21] Without any methanol induction procedure, the protein toxicity problem by the accumulation of formaldehyde and hydrogen peroxide (oxidized products of methanol by

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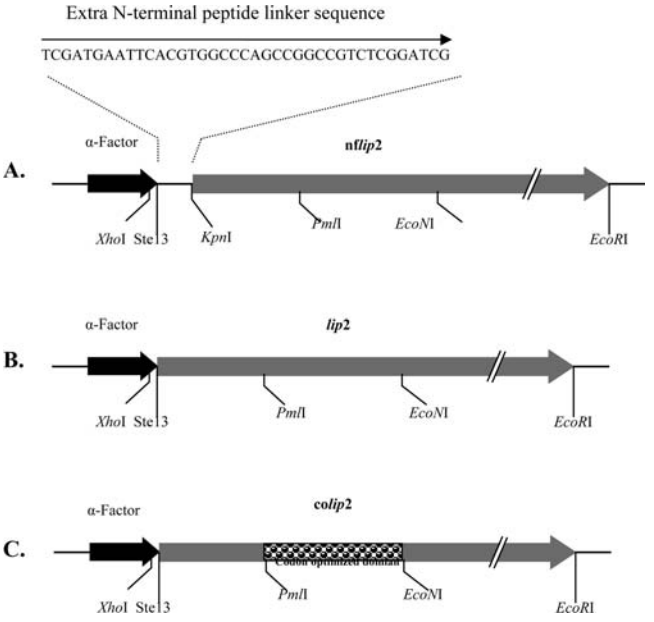

Figure 1. Comparison of the construction of pGAPZαC *lip*2, pGAPZαC-*lip*2, and pGAPZαC-co*lip*2 plasmids: (A) the N-fused *lip*2 gene contained an extra peptide linker sequence; (B) the *lip*2 gene lacked an N-terminal extra peptide linker, which was due to the cloning positions that we chose on the vector, left after *S. cerevisiae* α-factor secretion signal cleavage; (C) the co*lip*2 gene and *Pml*I- *Eco*NI 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 LIP2. The *lip*2 gene codons without any changes are indicated as white boxes.

alcohol oxidase) can be avoided in the *P. pastoris* expression system,[8,19,21−25] and the lipolytic activity of the recombinant LIP1 is 2-fold higher than that achieved by a methanol induction system.[19]

Although a high sequence similarity (84%) and identity (66%) exists across the entire CRL gene family, we have confirmed that individual recombinant CRL isoforms do possess distinct biochemical properties. For example, recombinant LIP1and LIP3 prefer C_8 and C_{10} acyl chains as substrate, but recombinant LIP4 prefers longer acyl chain (C_{16} and C_{18}) substrates.[8,19−21,25] Although we have successfully expressed a recombinant LIP2 with an additional N-terminal fragment in *P. pastoris*, the expression level is low, and it shows quite different properties compared with those of native LIP2.[8,17,25,26]

In the present work, we removed an unnecessary linker from the pGAPZαC-nfLIP2 plasmid using PCR technology to study the effect of the N-terminal peptide on the variant catalytic behavior of recombinant nfLIP2 and LIP2. To maximize the protein expression level, we designed a regional synthetic gene fragment with optimized codon usage of *Pichia pastoris* near the 5′ end of the *lip*2 gene. Such codon optimized strategy was expected to make it possible to obtain sufficient amounts of purified recombinant LIP2 for further studies of detailed biochemical characterization, structural analysis, and industrial applications.

MATERIALS AND METHODS

Strains and Plasmids. The *P. pastoris* expression vector pGAPZαC (Invitrogen, Carlsbad, CA, USA) was manipulated in an *E. 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 nfLIP2 and LIP2. 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 LIP2. The mature protein-coding sequence of *lip*2 (GeneBank accession number X66006) was cloned by RT-PCR, and all CTG-serine codons have been replaced with TCT by overlap-extension PCR as previous described.[8,19] Afterward, the *lip*2 gene was inserted into the pGAPZαC expression vector between the *Kpn*I and *Eco*RI sites. There is a short residual peptide linker in front of the mature LIP2 after the cleavage of the N-terminal peptide encoding the *S. cerevisiae* α-factor secretion signal[8] by Ste13 protease, namely, the pGAPZαC-nf*lip*2 (nf*lip*2) plasmid to express the recombinant nfLIP2 protein (Figure 1A). We also removed the coding sequence of the N-terminal peptide linker from pGAPZαC-nf*lip*2 by PCR to generate the pGAPZαC-*lip*2 (*lip*2) plasmid to express the recombinant LIP2 protein (Figure 1B). In an effort to further scale-up the fermentation, the gene fragment between the *Pml*I and *Eco*NI sites of the mature *lip*2 coding region near the 5′ end was replaced by a synthetic codon-optimized gene between the *Pml*I and *Eco*NI sites of the mature *lip*2 coding region to generate the pGAPZαC-co*lip*2 (co*lip*2) plasmid to express the recombinant coLIP2 protein (Figure 1C). Several codons of the *lip*2 gene of a 391 bp regional synthetic gene containing *Pml*I and *Eco*NI sites were changed according to *P. pastoris*'s favorite codon usage from the codon usage database (Web site: http://www.kazusa.or.jp/codon), thereby decreasing the G + C content from 66% to 41%. The overlap extension PCR strategy was then employed to change 83 codons of the *lip*2 gene (Figure 2). The 391 bp synthetic gene fragment was reassembled using fourteen 30−60 bp oligonucleotides containing 21 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 μg) harboring the engineered nf*lip*2, *lip*2, and co*lip*2 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 μL samples in 0.2 cm electrode gap cuvettes using a Gene Pulser apparatus supplied with 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 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 with a Bio-Rad assay kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (BSA) as standard. Briefly, five standard solutions containing 0.002, 0.004, 0.006, 0.008, and 0.010 mg/mL of BSA and 20% of Bradford reagent were prepared for recording the absorbance of each solution at Vis 595 nm as the standard curve. The OD_{595} of an unknown protein can be determined and its protein concentration calculated according to this curve.

Purification of Recombinant LIP2. Selected *P. pastoris* transformants were grown in 500 mL flasks containing 200 mL of the YPD medium (1% yeast extract, 2% peptone, and 2% dextrose; pH 6.3) with 100 μg/mL Zeocin at 30 °C for three days. During the cultivation

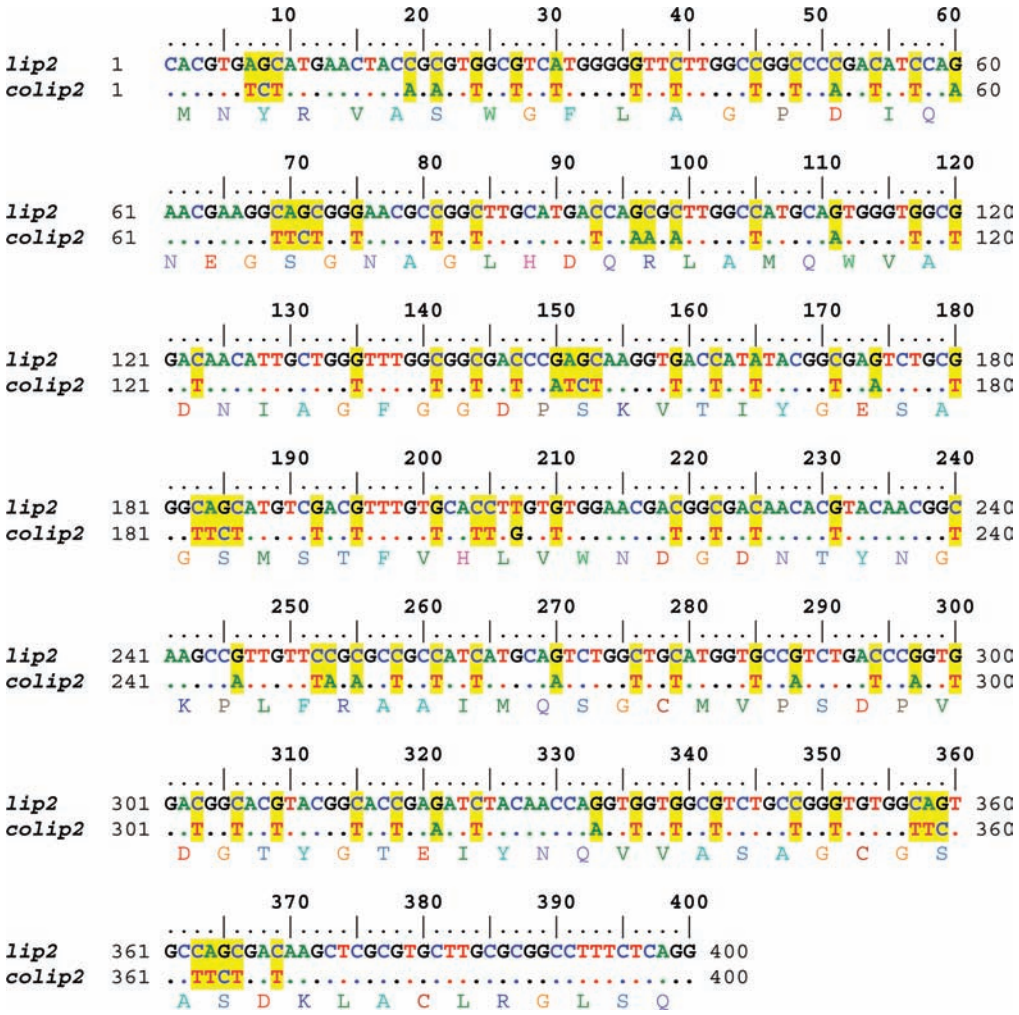

Figure 2. DNA sequence alignment comparison of the *Pml*I-*Eco*NI fragment of the *lip*2 gene (top) and co*lip*2 gene (bottom). Several codons of the *lip*2 sequence were exchanged (shaded) into the new co*lip*2 sequence with highly used codons in *P. pastoris* for heterologous protein expression improvement.

period, the shaker speed was fixed at 200 rpm to make a high aeration environment and minimize the influence of dissolved oxygen for cell growth. The culture medium was concentrated by ultrafiltration on the Labscale TFF system with Pellicon XL Devices coupling Biomax-50 membranes (Millipore, Bedford, MA, USA) and was applied on to a HiPrep 16/10 Octyl FF column (Amersham Pharmacia Biotech Inc., Piscataway, NJ, USA.) equilibrated with $(NH_4)_2SO_4$ TE buffer (20 mM Tris/HCl, 2 mM EDTA, and 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.

Biochemical Characterization for Various *lip*2 Constructs. The expression amount of the recombinant nfLIP2, LIP2, and LIP2 from the co*lip*2 construct was determined by SDS−PAGE analysis. The analysis of lipase activity was carried out spectrophotometrically at 37 °C in 50 mM Good's buffer containing Bicine, CAPS, sodium acetate, and BisTris propane (pH 7.0),[8] containing 0.24% Triton X-100 and a 0.5 mM solution of corresponding *p*-nitrophenyl ester. The hydrolysis of *p*-nitrophenyl esters will result in releasing *p*-nitrophenol to increase the absorbance 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 min under specific reaction conditions. The lipolytic activity was evaluated titrimetrically, and the release of fatty acids was monitored continuously by titration using 50 mM NaOH and a pH-stat (Radiometer Copenhagen, Bagsvaerd, Denmark). All reactions used the triacylglycerols with fatty acids of various chain lengths as substrates. The substrate emulsion reagents were prepared using a modified Sigma Quality Control Test procedure. Each assay reaction mixture contained 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 2.5 μL of the enzyme solution in a thermostat reaction vessel. One unit of lipolytic activity was defined as the amount of lipase necessary to produce 1 μmol of fatty acid per min under the assay conditions.

pH and Temperature Effects on Lipase Activity and Stability. All enzyme activities were assessed using *p*-nitrophenyl palmitate 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).[8] At the same pH range, the stability was analyzed by incubating the lipase for 16 h and then assaying at 37 °C for residual activity. For the optimum temperature effect, the enzyme activity was measured within the range of 30−90 °C at pH 7.0. The

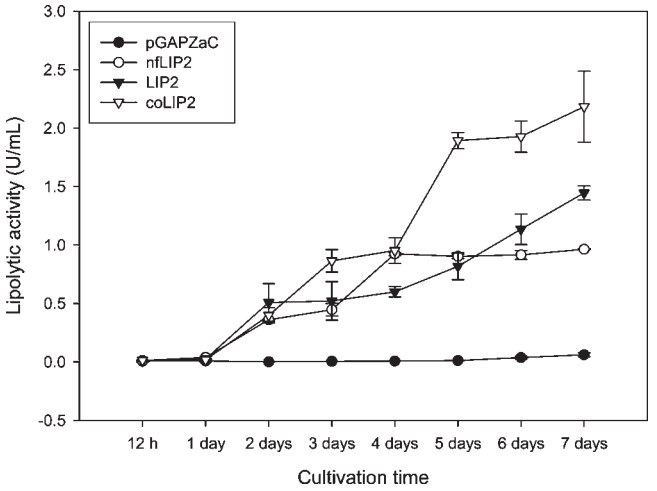

Figure 3. Comparison of expression yield with variant plasmid constructs. All constructive plasmids [pGAPZαC, pGAPZαC-nf LIP2, pGAPZαC-LIP2, and pGAPZαC-coLIP2] were transformed into *P. pastoris* hosts and were inoculated in YPD medium at 30 °C. The expression yields of their culture supernatant were measured spectrophotometrically using *p*-nitrophenyl butyrate as substrate at 27 °C and pH 7.0.

stability of lipase was analyzed by incubating for 10 min at a temperature range of 30−90 °C and then assaying at 37 °C for residual activity.[8,21,25]

Effects of Water-Miscible Solvents, Detergents, and Chemicals on Lipase Activity. The effect of 30% (v/v) water-miscible solvents on lipase activity was spectrophotometrically determined by using *p*-nitrophenyl palmitate as substrate. All reaction mixtures were incubated at 37 °C for 1 h in a 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 the water-miscible solvent was used as the control. The effect of various detergents on lipase activity was analyzed by a similar method. All reaction mixtures were incubated at 37 °C for 1.5 h in a 0.1 M Tris/HCl buffer (pH 7.0) containing 1% (w/v) or 0.1% (w/v) detergents. The reaction mixture without detergent addition was used as the control. The influence of various chemicals on lipase activity was determined by incubating the enzyme at 37 °C for 30 min in a 0.1 M Tris/HCl buffer (pH 7.0) containing 1 mM or 10 mM of chemicals. All measurements were assayed by using *p*-nitrophenyl palmitate as substrate and carried out in triplicate.

Cholesterol Esterase Activity Assay. Cholesterol esterase activity assay was performed according to a previous method described by Lee et al.[8]

RESULTS

Effect of Additional N-Teriminal Signal Peptide and Codon Optimization on Protein Expression Level. Several literatures indicated that translational inefficiencies could be limiting protein production in the higher eukaryotes system, despite the high-level transcription of the appropriate transgene.[27−30] Therefore, the analysis of translation rate would be focused on and discussed in this study. As the additional N-terminal peptide was removed from the n*flip*2 plasmid, the expression yield of the *lip*2 construct was increased up to 50% as compared to the n*flip*2 construct at 7 days of cultivation time (Figure 3). This finding suggested that the existence of additional N-terminal peptide seems to act as an expression obstacle for the *lip*2 gene expression in *Pichia pastoris*, which is similar to the *lip*1 gene as observed in the previous report.[21]

To understand the effect of codon optimization on translation rate, we replaced a regional codon-optimized fragment between the *Pml*I and *Eco*NI restriction sites of the *lip*2 gene (Figure 1), and the general G + C content of the chimeric gene was therefore decreased from 66% to 41%, and unique restriction sites were strategically positioned throughout the inserted gene.

After the long-term cultivation time period, the recombinant LIP2 expressed from the partial codon-optimized *lip*2 gene construct, stably accumulated to 1.89 U/mL of the translation level, showed a 2.3-fold higher production level than that observed from the noncodon-optimized *lip*2 construct (0.82 U/mL) in 5 days of cultivation time (Figure 3). Both the active recombinant enzymes of LIP2 and coLIP2 constructs increased with the cultivation time up to 7 days, and there was only 50% higher lipolytic activity (2.18 U/mL) of the coLIP2 construct than those obtained by the LIP2 construct (1.45 U/mL). However, the cell densities were reduced on day 5 (data not shown), which might be because the cell growth and enzyme activities were independent, and these transformants are highly stable in the absence of selective pressure.[19,20] For industrial applications, longer cultivation time might not be economical to save time and energy. Our observation demonstrated that the codon optimization of N-terminal coding regions toward the preferential codon usage of *P. pastoris* could act as a tool for increasing the protein expression level with shorter cultivation time. Such an engineered transformant would be more suitable for application in industrial fermentation.[19,20]

For further biochemical characterization, both recombinant nfLIP2 and LIP2 can be simply purified by ultrafiltration combined with hydrophobic interaction chromatography to obtain purified enzymes. Both enzymes do not have the same purification efficiency, and the purification factor of recombinant nfLIP2 was 3-fold higher than that of LIP2 obtained by the octyl-sepharose step as shown in Table 1. This suggested the existence of N-terminal peptide might increase the nonpolar exposure area to enhance the protein binding strength as the octyl-sepharose was used as the solid stationary phase. The lower recovery yield of both enzymes might be because of the enzyme retained in the membrane in the first step of purification (Table 1).

Effects of pH and Temperature on Recombinant nfLIP2 and LIP2. The pH dependence of nfLIP2 and LIP2 were studied at 37 °C using *p*-nitrophenyl palmitate as substrate (Figure 4A). Both nfLIP2 and LIP2 represented similar behaviors under different pH environments, and their highest hydrolytic activities were obtained at pH 7.0. When examining enzyme stability with different pH values from 3.0 to 10.0, LIP2 exhibited a distinct behavior compared to nfLIP2 after 16 h of incubation time. Recombinant nfLIP2 retained 90%−100% of its activity within a pH range of 6.0−7.0, whereas LIP2 was more stable at pH 7.0 (Figure 4B).

The temperature effect on both activity and stability of purified nfLIP2 and LIP2 was also estimated by using *p*-nitrophenyl palmitate as substrate at pH 7.0. Similar behavior between the recombinant nfLIP2 and LIP2 was obtained, and both of them exhibited a broad optimum temperature range from 30 to 50 °C. After incubation at various temperatures from 30 to 90 °C for 10 min at pH 7.0, both nfLIP2 and LIP2 were stable in a broad temperature range from 30 to 70 °C, whereas both enzymes were inactivated rapidly at higher temperature over 80 °C.

Effects of Detergents, Water-Miscible Solvents, and Chemicals. Lipases bind at the oil−water interface, resulting partly from a conformational change, to place the lipase close to

Table 1. Summary of the Purification of the Recombinant nfLIP2 and LIP2 from *Candida rugosa*

purification step of nfLIP2	total volume (mL)	enzyme activity (U/mL)	Protein concentration (mg/mL)	specific activity[a] (U/mg)	purification factor (fold)	yield (%)
culture medium	1000	0.27	0.06	4.6	1	100
ultrafiltration 50 cutoff	10	7.66	0.45	16.9	3.67	28.48
octyl-sepharose	5	8.03	0.03	247	53.7	14.92
purification step of LIP2	total volume (mL)	enzyme activity (U/mL)	protein concentration (mg/mL)	specific activity[a] (U/mg)	purification factor (fold)	yield (%)
culture medium	1000	0.83	0.07	12.68	1	100
ultra filtration 50 cutoff	10	15.96	0.79	20.30	1.57	19.23
octyl-sepharose	5	8.32	0.04	216	17.03	5.01

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

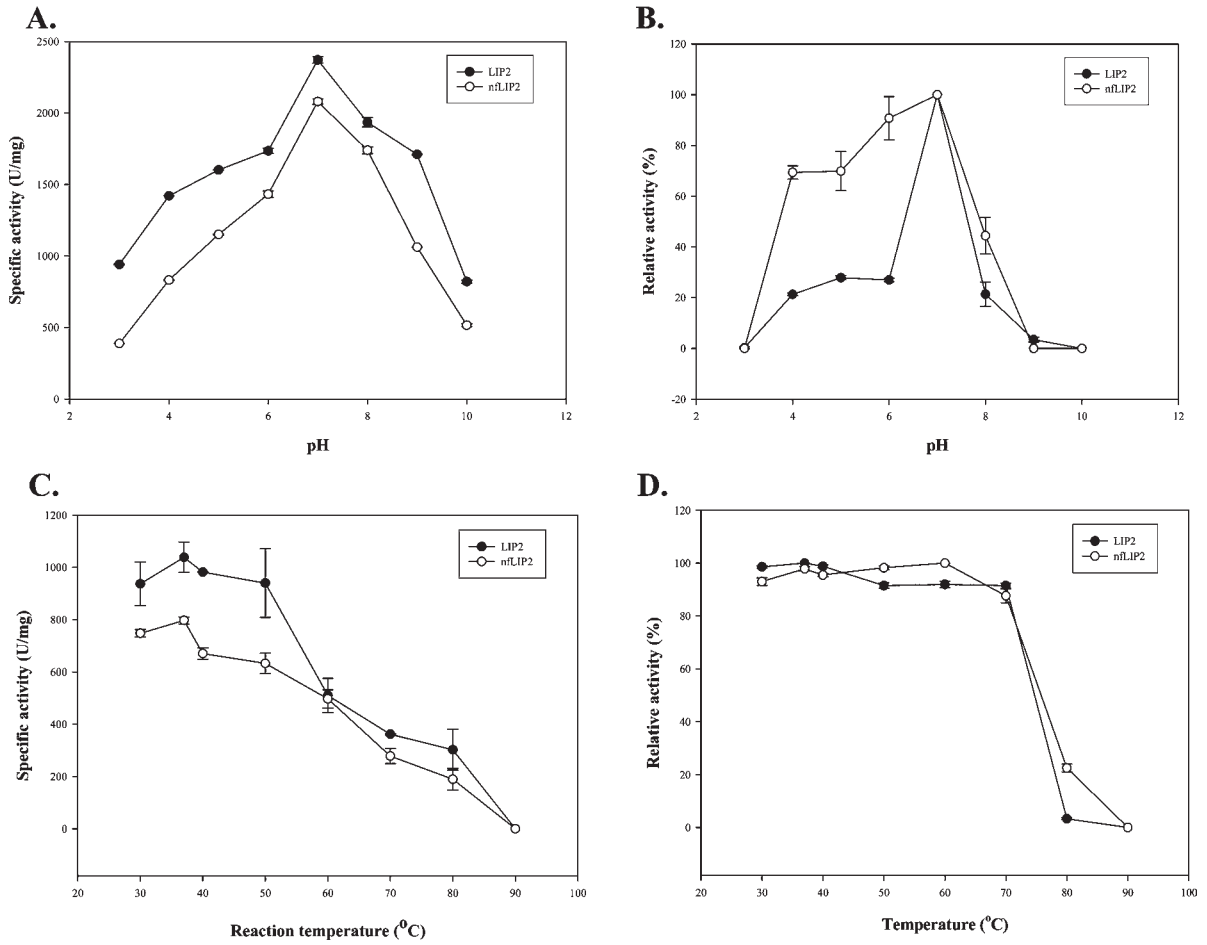

Figure 4. pH effects on the activity (A) and stability (B), and temperature effects on the activity (C) and stability (D) of purified recombinant LIP2 and nfLIP2. (A) Effect of pH on lipase activity was determined by the spectrophotometric method using *p*-nitrophenyl palmitate as substrate at 37 °C. (B) For the pH stability of recombinant LIP2 and nfLIP2, the hydrolysis activity of all purified enzymes were measured spectrophotometrically using *p*-nitrophenyl palmitate as substrate at 37 °C and pH 7.0 after incubating with Good's buffers of various pH values. The 100% relative activity of LIP2 and nfLIP2 was calculated as 1034.40 U/mg and 312.19 U/mg, taking the assay at 37 °C and 0.1 M Tris-HCl buffer (pH 7.0), respectively. (C) Effect of temperature on LIP2 and nfLIP2 activities was measured by a spectrophotometric method using *p*-nitrophenyl palmitate as substrate at pH 7.0. (D) Thermostability of LIP2 and nfLIP2 was determined by incubating the purified enzyme at various temperatures for 10 min. The 100% relative activity of LIP2 and nfLIP2 was calculated as 1022.24 U/mg and 593.78 U/mg, taking the assay at 37 °C and pH 7.0, respectively. Values are the means ± SD from three independent experiments.

the substrate to increase their activity during a catalysis reaction, referred to as interfacial activation. The effect of detergents on enzyme activity would be related to the increase of the interfacial area of substrate−enzyme interaction and was determined by incubating purified nfLIP2 and LIP2 in 0.1 M Tris/HCl buffer (pH 7.0) containing 1% and 0.1% (w/v) detergents at 37 °C for

Table 2. Effect of Detergents on Purified Recombinant LIP2 and nfLIP2[a]

detergents	concentration (%, w/v)	relative activity[b] of LIP2 (%)	relative activity[b] of nfLIP2 (%)
control	0	100 (634.6 U/mg)[c]	100 (475.2 U/mg)[c]
Brij	0.1	99.7 ± 0.3	71.6 ± 1.3
	1	81.6 ± 0.6	94.6 ± 2.7
Triton X-100	0.1	118.5 ± 0.05	77.6 ± 0.2
	1	85.4 ± 1.8	77.3 ± 1.2
CHAPS	0.1	68.3 ± 3.4	81.0 ± 1.4
	1	64.2 ± 2.3	57.0 ± 0.9
sodium taurocholate	0.1	89.5 ± 1.6	98.4 ± 1.8
	1	44.9 ± 1.9	82.2 ± 4.1
Tween 80	0.1	127.8 ± 3.0	90.4 ± 2.0
	1	85.4 ± 2.1	81.6 ± 1.0
Tween 20	0.1	126.7 ± 2.1	81.0 ± 2.9
	1	74.3 ± 0.1	79.4 ± 0.9
SDS	0.1	0	0.6 ± 0.8
	1	0	0

[a] All experiments were analyzed by the incubation of the enzyme for 1.5 h at 37 °C in 0.1 M Tris-buffer (pH 7.0) with various detergents. [b] The relative activities (%) were represented as the ratio of LIP2 and nfLIP2 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 min at 37 °C and pH 7.0.

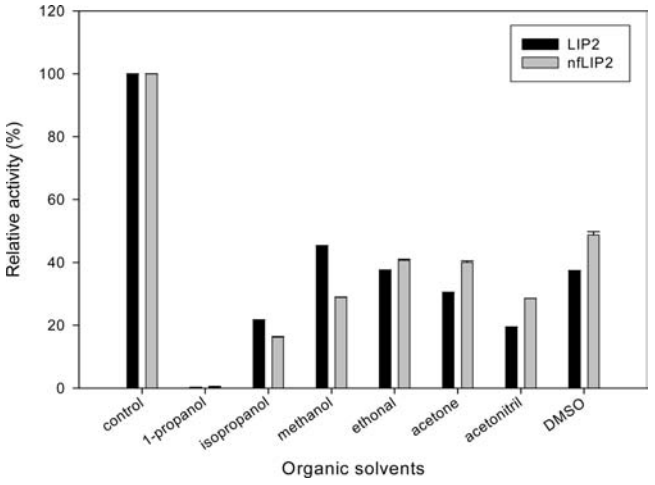

Figure 5. Effect of water-miscible solvents (30%) on purified recombinant LIP2 and nfLIP2 activity. The relative activities of recombinant LIP2 and nfLIP2 after 1 h of incubation at 37 °C in 0.1 M Tris-HCl buffer (pH 7.0) containing 30% (v/v) of various water-miscible solvents were measured spectrophotometrically using *p*-nitrophenyl palmitate as substrate at 37 °C and pH 7.0. The percentages represent relative activities, taking the assay at 37 °C without any water-miscible solvent addition, and were used as 100% of activity. The 100% relative activity of LIP2 and nfLIP2 were calculated as 1864.19 U/mg and 980.44 U/mg, respectively. Values are the means ± SD from three independent experiments.

1.5 h (Table 2). It is interesting to find that the presence of 0.1% Triton X-100, Tween 80, and Tween 20 significantly improved the activity of recombinant LIP2 with an increase of 18.5−27.8% activity, suggesting that this conformational change increases the

Table 3. Effect of Various Chemicals on Purified Recombinant LIP2 and nfLIP2[a]

chemicals	concentration (mM)	relative activity[b] of LIP2 (%)	relative activity[b] of nfLIP2 (%)
control	0	100 (1892.9 U/mg)[c]	100 (1516.7 U/mg)[c]
$CaCl_2$	1	95.4 ± 0.3	63.6 ± 0.7
$CaCl_2$	10	80.3 ± 0.2	22.1 ± 0.3
EDTA	1	103.1 ± 0.7	71.0 ± 0.4
EDTA	10	97.0 ± 0.9	63.1 ± 0.1
$MgCl_2$	1	94.0 ± 0.5	57.7 ± 0.3
$MgCl_2$	10	110.0 ± 0.6	14.6 ± 0.2
$AgNO_3$	1	96.8 ± 0.2	75.0 ± 0.7
$AgNO_3$	10	125.5 ± 0.1	71.4 ± 0.3
$MnCl_2$	1	106.9 ± 0.5	76.4 ± 0.8
$MnCl_2$	10	96.0 ± 0.3	19.4 ± 0.02
$(NH4)_2SO_4$	1	96.9 ± 0.1	68.2 ± 0.07
$(NH4)_2SO_4$	10	89.6 ± 0.003	60.0 ± 0.67
NaN_3	1	97.1 ± 0.5	94.5 ± 0.7
NaN_3	10	91.7 ± 0.4	40.4 ± 0.2
KCl	1	101.6 ± 0.08	57.6 ± 0.4
KCl	10	96.3 ± 0.2	21.3 ± 0.4
NaCl	1	97.2 ± 0.4	96.2 ± 0.1
NaCl	10	93.2 ± 0.005	24.4 ± 0.03
$CuSO_4$	1	99.0 ± 0.2	98.8 ± 0.6
$CuSO_4$	10	65.2 ± 0.3	23.3 ± 0.3

[a] All experiments were analyzed by the incubation of the enzyme for 30 min at 37 °C in 0.1 M Tris-buffer (pH 7.0) with various chemicals. [b] The relative activities (%) were represented as the ratio of LIP2 and nfLIP2 with different chemicals to that without chemicals. [c] One unit of esterase activity is the amount of enzyme that hydrolyzes 1.0 μmol of *p*-nitrophenyl ester per min at 37 °C and pH 7.0.

hydrophobicity of the enzyme surface in the vicinity of the active site, enhancing lipase interfacial binding in this region.[31] All other detergents used in this study seem to act as inhibitors for both recombinant enzymes whose activity was reduced by 9.6−22.4% or completely inactivated as the concentration was increased from 0.1% to 1% and suggested that the proteins might be hydrolyzed or be denatured chemically by those detergents. Thus, LIP2 appeared to be more stable than nfLIP2 toward various detergents, except sodium taurocholate, suggesting LIP2 is more suitable as an additive in the detergent industry.

The effect of water-miscible solvents on purified nfLIP2 and LIP2 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 5). The activities of both nfLIP2 and LIP2 were significantly decreased or completely inactivated by various organic solvents used in this study, although they were different in susceptibility. The recombinant LIP2 showed 5.3% and 16.4% higher relative activity than that of nfLIP2 by incubating for 1 h at 30% isopropanol and methanol, respectively.

For the effects of various chemicals, we found that recombinant LIP2 was quite stable at 1 mM or 10 mM of concentration, whereas the activities of recombinant nfLIP2 were significantly decreased as the concentration of chemicals were increased (Table 3). It is interesting that several chemicals such as $MgCl_2$ and $AgNO_3$ increased the hydrolytic activity of the recombinant LIP2 by 10 to 25% as compared to that of the control sample. The distinct effects of various chemicals on nfLIP2 and LIP2

Table 4. Substrate Specificity of Recombinant LIP2 and nfLIP2 in the Hydrolysis of *p*-Nitrophenyl (p-NP) Esters and Triacylglycerides Containing Fatty Acids of Various Chain Lengths[a]

	specific activity (U/mg) of *p*-NP esters[b]		specific activity (U/mg) of triacylglyceride[c]	
chain length of acyl group	LIP2	nfLIP2	LIP2	nfLIP2
C2	0.7 ± 0.0 (0.03%)	1.4 ± 0.3 (0.07%)	84.7 ± 1.5 (14.7%)	106.7 ± 11.6 (13.9%)
C4	216.7 ± 3.6 (8.9%)	247.3 ± 8.5 (11.7%)	523.1 ± 12.3 (91.0%)	703.0 ± 7.9 (91.9%)
C6	31.3 ± 0.3 (1.3%)	32.7 ± 6.5 (1.5%)	123.9 ± 6.8 (21.5%)	182.9 ± 6.8 (23.9%)
C8	765.1 ± 7.0 (31.5%)	493.9 ± 6.0 (23.3%)	383.0 ± 14.2 (66.6%)	505.3 ± 3.3 (66.0%)
C10	194.0 ± 1.6 (8.0%)	572.1 ± 11.8 (27.0%)	233.6 ± 17.4 (40.6%)	349.8 ± 12.0 (45.7%)
C12	1702.4 ± 15.2 (70.0%)	907.0 ± 9.9 (42.7%)	575.0 ± 29.7 (100%)	765.4 ± 39.3 (100%)
C14	2432.4 ± 11.3 (100%)	1992.4 ± 9.3 (94.0%)	98.1 ± 5.0 (17.1%)	224.4 ± 12.0 (29.3%)
C16	2325.9 ± 6.8 (95.6%)	2121.7 ± 8.4 (100%)	9.9 ± 1.0 (1.7%)	22.5 ± 1.6 (2.9%)
C18	335.5 ± 5.8 (13.8%)	197.0 ± 8.0 (9.3%)	81.2 ± 1.2 (14.1%)	172.8 ± 7.4 (22.6%)

[a] Hydrolysis of *p*-nitrophenyl esters and triacylglycerols were both measured at pH 7.0 and 37 °C. All values are the means ± SD from three independent experiments. Data in parentheses represent the relative activities (%) 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 min 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 min at 37 °C and pH 7.

demonstrated that the existence of an additional N-terminal peptide really affected their stability by various chemicals which were similar to those obtained by detergent effects. Moreover, the higher chemical stability and specific activity of recombinant LIP2 would enable it to be more suitable for further industrial applications.

Substrate Specificity of Recombinant nfLIP2 and LIP2. Table 4 shows the different preferences of LIP2 and nfLIP2 for *p*-nitrophenyl esters and triacylglycerides containing fatty acids with various chain lengths. For the hydrolysis of *p*-nitrophenyl esters, both recombinant nfLIP2 and LIP2 preferred *p*-nitrophenyl myristate (C_{14}) and *p*-nitrophenyl palmitate, whereas LIP2 showed higher specific activity than that of nfLIP2. In particular, we found that the purified recombinant LIP2 (2432.4 ± 11.3 U/mg) revealed 187- and 16-fold higher hydrolytic activity than that of recombinant LIP1 (13 ± 0.5 U/mg) and LIP3 (150 ± 1.3 U/mg) with *p*-nitrophenyl myristate (C_{14}) as substrate at 37 °C[8,21,25]. For all triacylglyceride tests, both recombinant nfLIP2 and LIP2 displayed a higher lipolytic activity for short-chain (tributyrin; C_4) and medium-chain triacylglycerides (trilaurin; C_{12}) at 37 °C. However, unlike other substrates, it is unusual to get 34% and 33% higher hydrolytic activity of the purified nfLIP2 than LIP2 toward tributyrin (C_4) and trilaurin (C_{12}), respectively. According to a previous report,[32] the recombinant r-Lip2 showed the maximum specificity for triacylglycerols toward medium-chain esters (C-8), which differ from our observation with purified nfLIP2 and LIP2 in this study. The recombinant r-Lip2 was obtained by a partial purification process which might cause more residual protein to be mixed with the recombinant enzyme and to result in different behavior for their biochemical characterization such as substrate specificity.[32] In comparison with other CRL isoforms, we found that our recombinant nfLIP2 (765.4 ± 39.3 U/mg) showed a 77% higher hydrolytic activity than recombinant LIP3 (432 ± 34 U/mg) toward trilaurin (C_{12}), whereas it displayed 9.4- and 4.5-fold lower hydrolytic activity than recombinant LIP1 toward tributyrin (C_4; 3160 ± 80 U/mg) and trilaurin (C_{12}; 7200 ± 270 U/mg), respectively.[8,21,25]

In this study, we also examined the hydrolytic ability of nfLIP2 and LIP2 on cholesteryl esters containing fatty acids with various chain lengths. The result shows that recombinant LIP2 exhibited

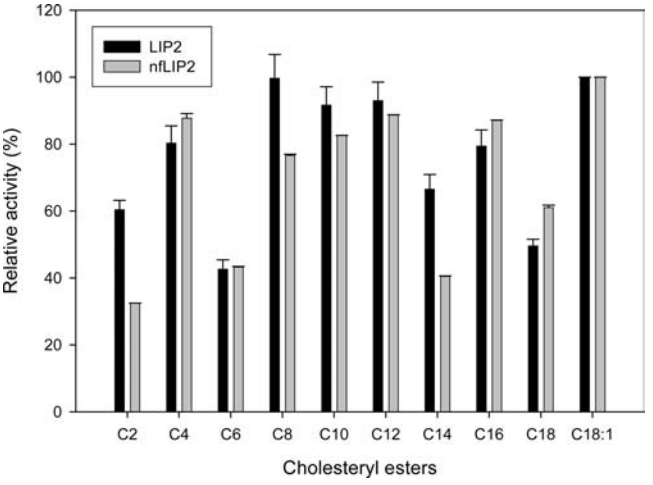

Figure 6. Relative activity of recombinant LIP2 and nfLIP2 in the hydrolysis of cholesteryl esters containing fatty acids of various chain lengths. The specific activity of purified LIP2 and nfLIP2 was determined by the hydrolysis 1.0 μmol of fatty acid per min at 37 °C. The 100% of activity of purified LIP2 and nfLIP2 was calculated as 0.15 U/mg and 0.14 U/mg, respectively. Values are the means ± SD from three independent experiments.

7−90% higher hydrolytic activity toward C_2 and C_8−C_{14} of cholesteryl esters which behaved differently from those obtained by nfLIP2 (Figure 6). For all cholesteryl ester tests, cholesteryl caprylate (C_8) and cholesteryl oleate ($C_{18:1}$) were the favorite substrate of LIP2, which showed a wider substrate specificity and higher specific activity than nfLIP2. In comparison with our previous results, the purified recombinant LIP2 (0.15 ± 9.7 U/mg) showed 2- and 115-fold lower hydrolysis activity than that of the purified LIP1 (0.31 ± 0.02 U/mg) and LIP3 (17.3 ± 1.5 U/mg) by using cholesteryl oleate ($C_{18:1}$) as substrate at 37 °C[8,19,21,25]. This suggested that the recombinant LIP3 was still the most efficient CRL isoenzyme for determining cholesterol levels in clinical and food analyses, but the recombinant LIP2 would be more useful for other environmentally friendly product applications such as cleaning agents, cosmetics, drugs, flavors, or structured lipids.

DISCUSSION

The difficulty in the heterologous expression of the multigene family coding for *C. rugosa* isozymes has been discussed and overcome by means of overlap extension PCR for the replacement of nonuniversal serine codons.[8,19,21,25] On the basis of our previous report,[25] the existence of a short N-terminus signal peptide in front of the *lip*1 gene represented an absolutely negative effect on the expression of LIP1, whereas it acted as an activator on the recombinant LIP3 expression level as shown in our previous work.[21]

For the effect of the additional N-terminus on the biochemical properties of purified recombinant LIP2, we found that the existence of an additional N-terminal peptide did not affect its pH preference including the same optimal pH at 7.0, whereas the recombinant LIP2 showed a higher hydrolytic activity than nfLIP2 as shown in Figure 4A. This suggested that the N-terminal 13 amino acids of the recombinant nfLIP2 contain 53% of hydrophilic residues which might induce a conformational change to reduce the hydrophobicity of the enzyme surface in the vicinity of the active site, preventing lipase interfacial binding in this region. Further, it is interesting to find that the additional N-terminal peptide might contribute to the stabilization of the native structure of recombinant LIP2 to remain at 70−100% of its activity over a broad pH range of 4.0−7.0 by incubating for 16 h at 37 °C (Figure 4B). Regardless of the additional N-terminal peptide was removed, the pH stability of our recombinant enzymes differed from those obtained by the native LIP2 purified from the commercial CRL preparation with maximum stability at pH 5.0 for 1 h of incubation time.[17] In comparison with recombinant LIP2, the recombinant nfLIP2 showed approximately 20% higher residual activity than LIP2 as the pH was increased (Figure 4B). That means the purified recombinant nfLIP2, containing an additional N-terminus, was more stable under both acidic and alkaline environments and therefore could be useful for certain industrial applications. As shown in Figure 4C,D, purified recombinant LIP2 was more active at 30−50 °C and showed about 50% higher hydrolytic activity than that of purified nfLIP2, whereas both of them are stable over a broad range of temperatures from 30 to 70 °C for at least 10 min. In comparison with recombinant LIP1, LIP3, and LIP4, our purified recombinant LIP2 showed more stability with 8-, 6-, and 1.7-fold higher hydrolytic activity at 70 °C, respectively.[8,21,25] This suggest that recombinant LIP2 has a unique biochemical property which should have different industrial uses; nfLIP2 and the N-terminal peptide could be useful tools for protein engineering to alter the biochemical properties for various applications.

For the hydrolysis of *p*-nitrophenyl esters, both recombinant nfLIP2 and LIP2 displayed low hydrolytic activity toward *p*-nitrophenyl esters containing short-chain (C_2−C_6) and long-chain (C_{18}) fatty acid groups. For triacylglycerides, both recombinant nfLIP2 and LIP2 showed low hydrolytic activity toward triacetin (C_2 acyl group) and tripalmitin (C_{16} acyl group) at 37 °C. In contrast, the pure native LIP2 isolated from the commercial CRL preparation prefers shorter and longer acyl chain triacylglycerides (C_4 and $C_{18:1}$) and medium acyl chain *p*-nitrophenyl esters (C_{12}).[1,17,26] The taste and texture of cheese are both altered by the lipase conversions during the ripening process.[33] Also, Arbige et al.[34] 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 in ripening cheese. It is interesting to note that the TCU/TBU ratio of our recombinant nfLIP2 and LIP2 is similar to those obtained by milk lipase (>0.70), which was useful in cheese ripening applications. For cholesterol level determination, the purified recombinant nfLIP2 showed 4.3-fold and 70% higher hydrolytic activity toward cholesteryl butyrate (C_4 acyl group) and cholesteryl stearate (C_{18} acyl group) than that of recombinant LIP1 (0.02 ± 0.0015 U/mg) and commercial CRL preparation (0.065 ± 0.007 U/mg) as shown in Figure 6,[8,19] respectively. Overall, the purified recombinant LIP2 exhibited 7−90% higher hydrolytic activity toward most of the cholesteryl esters (such as C_2, C_8, C_{10}, C_{12}, C_{14}, $C_{18:1}$, etc.) than those of purified nfLIP2 (Figure 6), indicating that the existence of an additional N-terminal peptide in front of recombinant LIP2 might change the conformation of the substrate binding site to reduce its hydrolysis ability toward cholesteryl esters containing C_2 and C_8−C_{14} fatty acid groups.

For industrial applications, lipase sales have been estimated to be $30 million (US), with detergent enzymes making up 30% in 1995, and 1,000 tons of lipases are added to approximately 13 billion tons of detergents produced each year.[35] Generally, all detergents used in this study inhibited the activity of our purified recombinant nfLIP2 in various degrees. In contrast, recombinant LIP2 seems to be more active by increasing 18.5%, 27.8%, and 26.7% of relative activity at 0.1% Triton X-100, Tween 80, and Tween 20, respectively. Different behaviors of the detergent effects between recombinant nfLIP2 and LIP2 may be due to the higher structure stabilization with the removal of the additional peptide at the N-terminus from the nfLIP2 protein. Our recombinant LIP2 was therefore potentially useful in industries which use Triton X-100, Tween 80, and Tween 20 as detergents.

Interestingly, we found that only the recombinant LIP2 displayed higher stability by increasing 10% and 25.5% of the relative activity in the presence of 10 mM $MgCl_2$ and $AgNO_3$, possibly due to the interaction of Mg^{2+} and Ag^{+} with the Glu, His, and Cys amino acids by an ionic bond, which converts the protein conformation into a more active form. In contrast, the presence of the additional N-terminal peptide might block the ionic interaction between amino acids and metal ions to reduce the stabilization of our recombinant nfLIP2 in various chemical solutions. Protein crystallization and X-ray diffraction are currently underway to elucidate the activation mechanism.

Most lipases are interfacial-activated in alternative conformational states with different activity by the presence of a water/lipid interface.[36] Highly hydrophilic solvents might result in lower enzyme activity due to the shift of enzyme to the closed conformation, and its active site will be covered by an amphipathic lid structure to reduce the entrance of the hydrophobic substrates through the catalytic tunnel.[36,37] Indeed, our recombinant nfLIP2 and LIP2 were significantly inhibited or completely inactivated by most water-miscible solvents used in this study. Overall, the purified recombinant nfLIP2 showed higher tolerance in 30% of ethanol, acetone, acetonitrile, and DMSO than that of LIP2, whereas the specific activity of the recombinant LIP2 was 1.9-fold higher than that of nfLIP2. However, it is interesting that the purified recombinant LIP2 showed 5.3% and 16.4% higher relative activity than recombinant nfLIP2 by incubating at 30% of methanol and isopropanol, respectively. For enzymatic FAME/biodiesel production, high methanol stability of the biocatalyst is essential to improve the FAME/biodiesel conversion. Protein engineering and DNA shuffling methods are currently underway to further improve the recombinant LIP2 for FAME/biodiesel production.

In conclusion, the expression efficiency of recombinant LIP2 was greatly enhanced by the additional N-terminal peptide deletion and regional codon optimization. Such a strategy will provide a more convenient and inexpensive method for large scale lipase production in the *P. pastoris* system. The existence of an additional N-terminus in front of the *C. rugosa lip*2 gene might significantly affect its pH stability and tolerance toward various concentrations of chemicals and detergents. After removing the N-terminus, recombinant LIP2 showed more stability toward various detergents and wider substrate specificity of cholesteryl esters, suggesting that it would be a potentially useful biocatalyst for specific applications, especially in the cleaning agent industry. To conform to the more specific requirements from various industries, protein engineering and DNA shuffling methods would be functional tools to improve their stability, catalytic activity, and specificity for further biotechnological applications.

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