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Characterization of Codon-Optimized Recombinant *Candida rugosa* Lipase 5 (LIP5)

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ABSTRACT: Recombinant *Candida rugosa* lipase 5 (LIP5) has been functionally expressed along with other isoforms in our laboratory. However, the characterization and codon optimization of LIP5 have not been done. In this work, we characterized, codon-optimized and compared LIP5 with commercial lipase. LIP5 activity on hydrolysis of *p*-nitrophenyl (*p*-NP) butyrate was optimal at 55 °C as compared with 37 °C of the commercial lipase. Several assays were also performed to determine the substrate specificity of LIP5. *p*-NP butyrate (C_4), butyryl-CoA (C_4), cholesteryl laurate (C_{12}), and *N*-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester (L-NBTNPE) were found as preferred substrates of LIP5. Interestingly, LIP5 specificity on hydrolysis of amino acid-derivative substrates was shown to be the highest among any lipase isoforms, but it had very weak preference on hydrolyzing triacylglycerol substrates. LIP5 also displays a pH-dependent maximum activity of a lipase but an esterase substrate preference in general. The characterization of LIP5 along with that of LIP1−LIP4 previously identified shows that each lipase isoform has a distinct substrate preference and catalytic activity.

KEYWORDS: *Candida rugosa* lipase 5 (LIP5), substrate, specificity, hydrolysis

INTRODUCTION

The importance of *Candida rugosa* lipase in industrial processes began after its purification and succeeding discoveries of its catalytic and hydrolytic activity in fats and oils.[1] Commercial dry lipase (from *C. rugosa* formerly *Candida cylindracea*) became available for industrial uses and has been exploited in several research studies for deeper understanding of the enzyme. Early publications on the improved lipase production in the fermentation process,[2,3] immobilization of lipase to improve its reusability, and substrate preferences were reported. At the dawn of recombinant DNA technology *C. rugosa* lipase was identified to have 5 isoforms (LIP1−5) by colony hybridization.[4,5]

C. rugosa lipase (CRL) isoforms have been identified and characterized for their catalytic activity and substrate specificity in different conditions. Successful demonstration of multiple extracellular lipases (LIP1, LIP2, LIP3, LIP4, and LIP5) to quantify the expression of lip genes was done by competitive reverse transcription-PCR.[6] Since then our laboratory has been exploring ways to optimize and improve the catalytic activity of these isoforms. LIP1 was codon-optimized through multiple site-directed mutagenesis to improve its optimum expression in *Pichia pastoris*,[7,8] and it was reported that it can catalyze transesterification to produce human milk fat substitute.[9] Similarly, LIP2, LIP3 and LIP4 were also codon-optimized and mutated to enhance and improve their catalytic activities and substrate specificities.[10−13] But in the case of LIP5 little is known about this isoform. Expression of LIP5 in culture media with olive oil or oleic acid was promoted even in the presence of glucose as reported.[14] The significant induction of LIP5 gene by oleic acid was supported by its putative transcriptional elements such as oleate response element (ORE) and upstream activation sequence 1 (UAS1).[15] It has been proposed that LIP5, similar to LIP1 and LIP3, has three glycosylation and 16 nonuniversal (CTG) serine codons. LIP5 has 534 amino acid residues with 15 amino acids as putative signal peptides. In a swap-flap study of chimera Trx-LIP4/lid5, a thioredoxin fused LIP4 with modified lid, the hydrolysis of tributyrin decreased by 20% as compared with the native nonchimeric Trx-LIP4 with unmodified lid.[16] However, the characteristics of LIP5 isoform are still unexplored. In this present work, we codon-optimized and characterized the catalytic performance of recombinant LIP5 in various substrates and at vaying pH and temperature, and determined the rate of hydrolysis.

MATERIALS AND METHODS

Chemicals and Biological Materials. Substrates such as *p*-nitrophenyl (*p*-NP) esters, triacylglycerols (TAG), thioesters, amino acid derivatives, and cholesteryl esters were purchased from Sigma Aldrich. Cholesterol oxidase, 500 units/L (Roche Diagnostic, Germany), Thesit,

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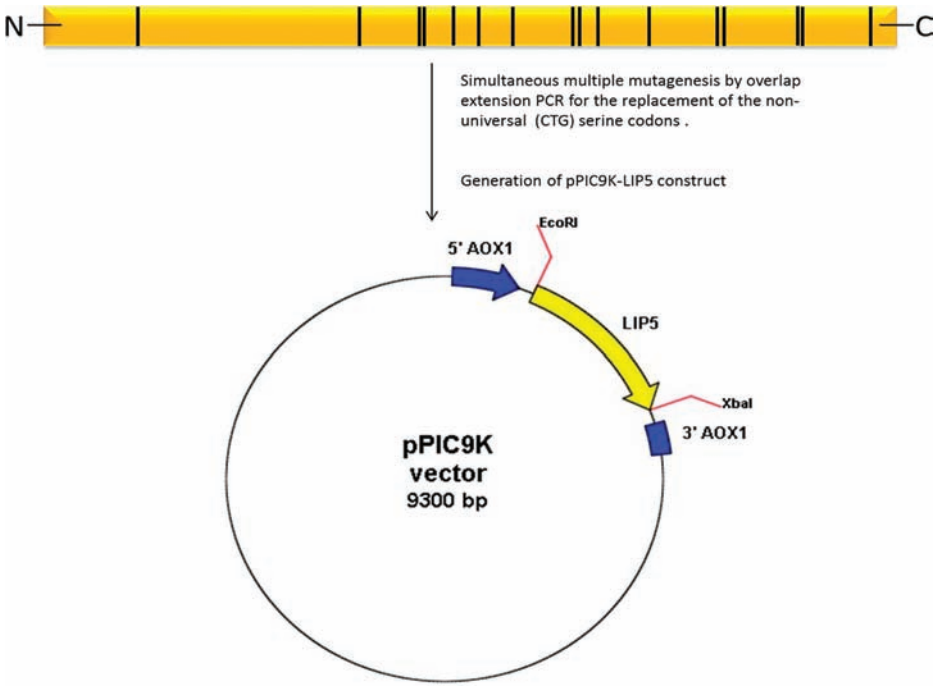

Figure 1. Codon optimization and pPIC9K-LIP5 construct. This is a schematic distribution of 16 codon triplets, CTG, encoding the serine residues in *C. rugosa* lipase LIP5 and pIC9K-LIP5 construct. Simultaneous multiple mutagenesis by overlap extension PCR and plasmid generation were performed as previously described.[17].

3 g/L (Boehringer Mannheim, Germany) and peroxidase, 400 units/L (Roche Diagnostic, Germany) were used for cholesterol esterase activity assay. Plasmid and DNA extraction system kits were supplied by Viogene (Taiwan). *Escherichia coli* TOP10F, *Pichia pastoris* GS115, expression vector pIC9K, and Zeocin were from Invitrogen. All buffers and reagents used in this study were of analytical grade.

Expression Vector, Growth Condition and Crude LIP5. The *lip5* was codon-optimized to improve expression in *P. pastoris* as previously described.[8] Plasmid pIC9K-LIP5 (Figure 1) containing *lip5* coding sequence in pIC9K vector was constructed, transformed into *E. coli* TOP10F as a host for cloning, and grown in low-salt Luria–Bertani (LB) broth with Zeocin (25 mg/mL) overnight. The plasmids were extracted, purified and transformed to *P. pastoris* GS115 by electroporation.[13] Transformants were incubated on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, and 2% agar, pH 7.2) plates containing Zeocin (100 μg/mL). Selected colonies were cultured in a 5000 mL YPD medium with Zeocin (500 μg/mL) in a shaking flask at 30 °C for 3 days. Addition of 0.5% (v/v) methanol was done every 12 h to induce the expression of recombinant LIP5. The culture medium was collected and concentrated by ultrafiltration on the Labscale TFF System with Pellicon XL devices coupling Biomax-30 membranes (Millipore, Bedford, MA). The filtrate was considered as LIP5 and used in all investigations.

Homogeneity, Molecular Weight Determination, and Native In-Gel Lipase Assay. Protein concentration was determined using the Bio-Rad protein assay kit. To check the expression of LIP5 and determine molecular weight, the crude enzyme was analyzed using denaturing condition SDS–PAGE on 12% separating gel and 5% stacking gel according to the procedure of Laemmli (1970). After running in SDS–PAGE, gel was stained and destained and the bands were compared to the standard protein ladder. In in-gel assay, the gel after the SDS–PAGE run was immersed in a solution with Fast Blue RR salt in 25 mL of 25 mM NaPi buffer, 25 μL of α-naphthyl butyrate, and 3 mL of acetone in the dark for 15 min and washed with distilled water.

Temperature and pH Optima and Thermal Stability. The effect of temperature and pH on LIP5 was determined by measuring the hydrolysis of *p*-NP butyrate as a substrate. In the effect of temperature on LIP5 activity, the reaction in Good's buffer, pH 7.0 (50 mM each of Bicine, CAPS, sodium acetate, and BisTris propane), was carried out over the temperature range of 10 to 90 °C. The optimal pH was investigated in the pH range of 3.0–9.0 using Good's buffer, and activity was checked at 37 °C under the standard test conditions. To analyze thermal stability, the LIP5 was incubated for 10 min at temperature range from 37 °C to 90 °C. Residual activity was determined spectrophotometrically at 37 °C, pH 7.0, using *p*-NP butyrate as a substrate.

Esterase Activity Assay. The substrate solution was 5 mM *p*-nitrophenyl esters, acetate (C_2), butyrate (C_4), caproate (C_6), and caprylate (C_8), 2.5 mM *p*-nitrophenyl esters, caprate (C_{10}), laurate (C_{12}), myristate (C_{14}), palmitate (C_{16}) and stearate (C_{18}) dissolved in a 50 mM sodium phosphate buffer with a pH level of 8.0 containing 2.1% (v/v) Triton X-100. The enzyme reactions were measured at room temperature, and a 1 h reaction was initiated by adding 2.0 μL of enzyme solution into 200 μL of substrate solution. Enzyme activity was determined by changes in absorbance at 405 nm. The molar extinction coefficient of *p*-nitrophenol in this buffer system was 1,725 M^{-1} cm^{-1}.

Cholesteryl Esterase Activity. The cholesteryl esterase activity of lipase was analyzed using a peroxidase/cholesterol oxidase coupled system to measure the formation of cholesterol during the hydrolysis of a cholesteryl ester. The hydrolytic reaction was carried out at room temperature in a mixture of 200 μL of reagent solution [100 mM Tris/HCl, pH 7.0, 50 mM $MgCl_2$, 6 mM phenol, 1 mM 4-aminoantipyrine, 4 mM 3,4-dichlorophenol, 10 mM sodium cholate, 3 g/L Thesit (Boehringer Mannheim, Mannheim, Germany), 500 units/L cholesterol oxidase (Roche Diagnostics, Mannheim, Germany), 400 units/L peroxidase (Roche Diagnostics)] and 100 μL of 100 g/L Thesit solution containing 10 mM substrate for 1 h. The absorbance was recorded at 500 nm. One unit of cholesterol esterase activity was defined as the amount of lipase necessary to hydrolyze 1.0 μmol of ester per min.

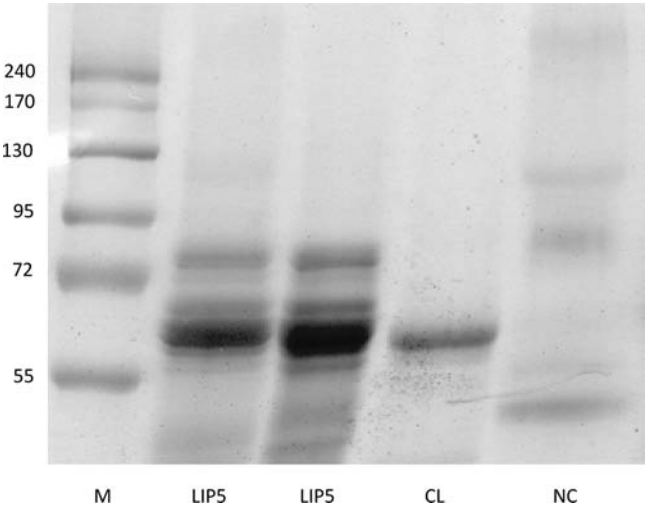

Figure 2. SDS−PAGE (12%) of the LIP5 obtained from pIC9K-LIP5 in GS115 after purification. Proteins were stained with Coomassie blue. Lane 1: M (Xpert Prestained Protein Marker) marker proteins with relative molecular masses in kDa indicated on the right. Lane 2: LIP5 (5 μL loading volume). Lane 3: LIP5 (10 μL loading volume). Lane 4: CL (commercial lipase). Lane 5: NC (negative control).

Thioesterase Activity Assay. The enzyme substrates acetyl-CoA, butyryl-CoA, lauroyl-CoA, stearoyl-CoA, and oleoyl-CoA were separately prepared in a reaction mixture containing 0.10 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 80 μg/mL bovine serum albumin (BSA), and 50 mM potassium phosphate buffer, pH 8.0. The enzyme activity was monitored at 37 °C by the increase of absorbance at 412 nm for 1 6 min.

Amino Acid Derivative Substrate Assay. Stock solutions (2.0 mM) of the amino acid derivative substrates, *N*-carbobenzoxy-L-tyrosine *p*-nitrophyl ester (L-NBTNPE), *N*-carbobenzoxy-L-phenylalanine *p*-nitrophyl ester (L-NBPNPE), or *N*-carbobenzoxy-D-phenylalanine *p*-nitrophyl ester (D-NBPNPE), were prepared in 1.00 mL of dioxane. Before the enzyme reaction was initiated, a 10.0 μL aliquot of substrate stock was freshly diluted into the reaction solution made up of 0.50 mL of 0.10 M sodium phosphate buffer with pH 7 at 37 °C. The initial rate was measured by monitoring the changes in absorbance at 400 nm. One unit is defined as catalyzing the hydrolysis of 1 μmol of substrate to produce 1 μmol of *p*-nitrophenol per min. The molar extinction coefficient was 3,454 M^{-1} cm^{-1} at this condition.

Enzyme Kinetic Analysis of LIP5. The kinetic studies were performed at room temperature, and the enzyme activities were measured spectrophotometrically. For the esterase activity assay, five concentrations of *p*-NP butyrate (10, 5, 2.5, 1.25, and 0.125 mM), L-NBTNPE (40, 20, 10, 5, and 2.5 μM) and butyryl CoA (14, 7, 3.5, 1.75, and 0.875 μM) were prepared by dissolving *p*-NP butyrate, L-NBTNPE and butyryl-CoA in esterase, thioesterase and amino acid derivative buffers. The initial rate was measured by monitoring the changes in absorbance at 400 nm.

RESULTS

Purification and Native In-Gel Lipase Activity of LIP5. The extracellular expression of LIP5 produced was partially purified from the supernatant of the cell culture by ultrafiltration. Molecular weight was identified by 12% SDS−PAGE along with commercial lipase (Figure 2). LIP5 lanes showed similar molecular weight to commercial lipase approximately at 60 kDa. In addition, LIP5 was also confirmed active by in-gel lipase activity on hydrolyzing *p*-NP butyrate (data not shown).

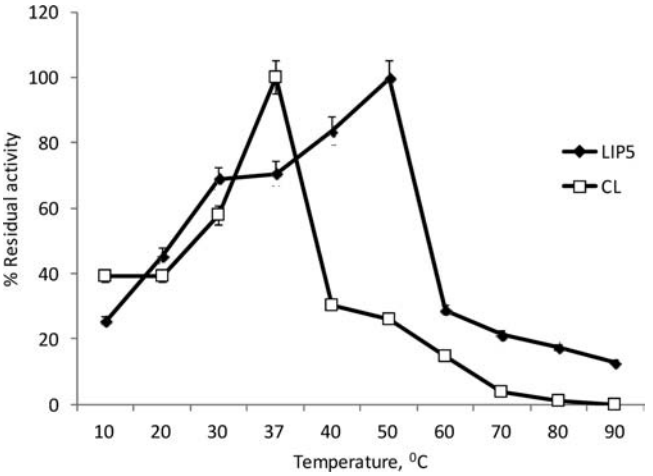

Figure 3. Temperature effect on LIP5 and CL. The activities of recombinant LIP5 (◆) and CL (□) were measured by a spectrophotometric method using *p*-nitrophenyl butyrate as substrate at pH 7.0. The percentages represent residual activities, taking that in the highest activity as 100%. The residual activities of LIP5 (50 °C) and CL (37 °C) in the assay were 1472 and 6635 units/mg respectively. Values are means ± SD from three independent experiments and error bars at 5% value.

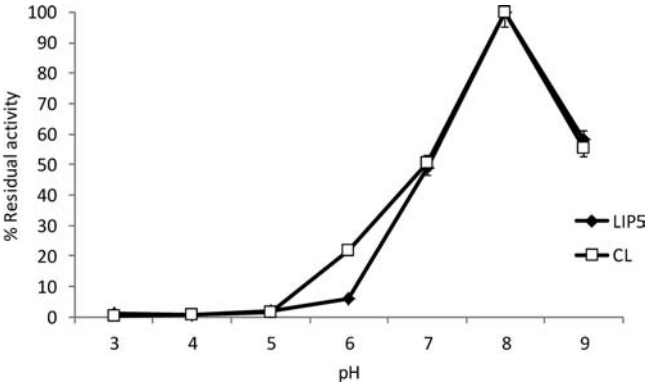

Figure 4. pH effect on LIP5 and CL. The specific activities of recombinant LIP5 (◆) and CL (□) were measured spectrophotometrically using *p*-nitrophenyl butyrate as substrate at 37 °C. The percentages represent residual activities, taking that in the highest activity as 100%. The residual activities of LIP5 and CL at pH 8.0 assay were 1518 and 6543 units/mg respectively. 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). Values are means ± SD from three independent experiments and error bars at 5% value.

Temperature and pH Optima, and Thermal Stability of LIP5. Temperature and pH are two of the factors that affect the enzyme catalytic activity. The effect of temperature on LIP5 is shown in Figure 3. LIP5 showed highest catalytic activity on *p*-NP butyrate at 55 °C as compared with commercial lipase. Most recombinant *C. rugosa* lipase isoforms have an optimum temperature around 37 to 40 °C. The pH dependence of LIP5 and commercial lipase showed no difference in Figure 4. Both have optimal pH values at 8.0, a similar optimum pH to other lipase isoforms as reported. The residual activity of LIP5 after heating for 10 min at various temperatures was assayed (Figure 5). Overall, the residual activity of LIP5 was similar to that of the commercial lipase. As the temperature increases, the residual activity

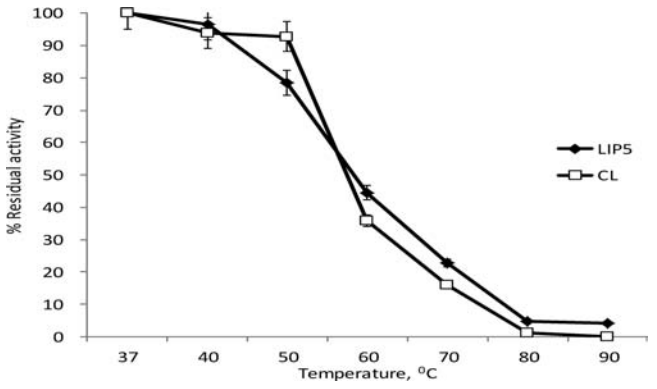

Figure 5. Thermal stability of LIP5 and CL. The residual activities of recombinant LIP5 (◆) and CL (□) after 10 min of heating at different temperatures were measured spectrophotometrically using *p*-nitrophenyl butyrate as substrate at 37 °C and pH 7.0. The percentages represent relative activities, taking that in the assay at 37 °C as 100%. The residual activities of LIP5 and CL in the 37 °C assay were 1284 and 2960 units/mg respectively. Values are means ± SD from three independent experiments and error bars at 5% value.

Table 1. Substrate Specificity of LIP5 and CL in the Hydrolysis of *p*-Nitrophenyl (*p*-NP) Esters Containing Fatty Acids of Various Chain Lengths

	sp act.[a] (units/mg)	
substrate	LIP5	CL
p-NP acetate (C_2)	606 ± 17.3 (50%)	573 ± 310.1 (10%)
p-NP butyrate (C_4)	1211 ± 82.2 (100%)	4496 ± 310.1 (79%)
p-NP caproate (C_6)	501 ± 34.5 (41%)	2246 ± 252.9 (40%)
p-NP caprylate (C_8)	461 ± 51.4 (38%)	5668 ± 122.4 (100%)
p-NP caprate (C_{10})	255 ± 3.7 (21%)	4323 ± 357.1 (76%)
p-NP laurate (C_{12})	1032 ± 34.5 (85%)	4767 ± 442.8 (84%)
p-NP myristate (C_{14})	352 ± 18.6 (29%)	3994 ± 275.8 (70%)
p-NP palmitate (C_{16})	381 ± 11.2 (31%)	4653 ± 11.4(82%)
p-NP stearate (C_{18})	268 ± 9.5 (22%)	931 ± 138.8 (16%)

[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. Values are means ± SD from three independent experiments. Data in parentheses represent the relative activities (%) of each enzyme with the different substrates. The highest activity of each enzyme is denoted as 100%.

of the enzymes starts to drop, and both LIP5 and commercial lipase showed the same curve pattern.

Substrate Specificity Assays. *Esterase Activity of LIP5.* Esterase activity of LIP5 was measured through hydrolysis of *p*-NP esters containing fatty acids of various chain lengths, and the results are tabulated in Table 1. LIP5 showed preference on hydrolyzing *p*-NP butyrate (C_4), a short chain length ester substrate. This preference is unique for LIP5 since LIP1−4 and commercial lipase all preferred medium to long chain length ester substrates as previously reported.[8,12,17]

Cholesteryl Esterase Activity of LIP5. Formation of cholesterol during the hydrolysis of a cholesteryl ester on cholesteryl substrates with various chain lengths of fatty acid by LIP5 was analyzed using a peroxidase/cholesterol oxidase coupled system, and the results are shown in Table 2. LIP5 showed a preference

Table 2. Substrate Specificity of LIP5 and CL in the Hydrolysis of Cholesteryl Esters Containing Fatty Acids of Various Chain Lengths

	sp act.[a] (units/mg)	
substrate	LIP5	CL
cholesteryl *n*-butyrate (C_4)	2.2 ± 1.45 (38%)	9.27 ± 0.923 (43%)
cholesteryl *n*-octanoate (C_8)	1.7 ± 0.83 (29%)	14.06 ± 1.047 (65%)
cholesteryl laurate (C_{12})	5.8 ± 3.16 (100%)	11.19 ± 1.29 (52%)
cholesteryl *n*-palmitate (C_{16})	2.7 ± 0.42 (46%)	14.06 ± 2.031 (100%)
cholesteryl stearate (C_{18})	0.3 ± 0.008 (5%)	0.03 ± 0.003 (0.1%)
cholesteryl *n*-oleate ($C_{18:1}$)	0 (0%)	14.81 ± 0.55 (69%)

[a] One unit of esterase activity is the amount of enzyme necessary to hydrolyze 1.0 μmol of acid per min at 37 °C. Values are means ± SD from three independent experiments. Data in parentheses represent the relative activities (%) of each enzyme with the different substrates. The highest activity of each enzyme is denoted as 100%.

Table 3. Substrate Specificity of the LIP5 and CL with Thioesters

	sp act.[a] (units/mg)	
substrate	LIP5	CL
acetyl-CoA ($C_{2:0}$)	21 ± 0.4 (43%)	2 ± 0.1 (8.9%)
butyryl-CoA ($C_{4:0}$)	48 ± 3.8 (100%)	3 ± 0.18 (18.6%)
lauroyl-CoA ($C_{12:0}$)	14 ± 0.4 (28%)	18 ± 1.2 (100%)
stearoyl-CoA ($C_{18:0}$)	5 ± 0.9 (9.8%)	5 ± 1.5 (24.7%)
oleoyl-CoA ($C_{18:1}$)	7 ± 3.4 (15%)	2 ± 0.46 (9.8%)

[a] One unit of activity is the amount of enzyme necessary to hydrolyze 1.0 μmol of ester bond per minute at room temperature and pH 8.0. Values are means ± SD from three independent experiments.

Table 4. Substrate Specificity of the LIP5 and CL with Amino Acid Derivatives

	sp act.[a] (units/mg)	
substrate	LIP5	CL
L-NBPNPE[b]	114 ± 19.6 (14%)	19 ± 4.2 (75%)
D-NBPNPE[b]	150 ± 8.7 (18%)	14.6 ± 4.5 (55%)
L-NBTNPE[b]	841 ± 72 (100%)	25 ± 4.8 (100%)

[a] One unit of activity is the amount of enzyme necessary to hydrolyze 1.0 μmol of ester bond per minute at room temperature and pH 8.0. Values are means ± SD from three independent experiments. [b] Substrates of amino acid derived esters: *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (L-NBTNPE), *N*-carbobenzoxy-L-phenylalanine *p*-nitrophenyl ester (L-NBPNPE), and *N*-carbobenzoxy-D-phenylalanine *p*-nitrophenyl ester (D-NBPNPE).

on hydrolyzing cholesteryl laurate (C_{12}), medium chain length substrate. This preference is similar to LIP2 and LIP4,[17] whereas LIP1[8] and LIP3[12]preferred long chain length substrate.

Thioesterase Activity of LIP5. Hydrolysis of thioester bond on acyl-CoA substrates was performed to determine the catalytic activity and preference of LIP5. Table 3 showed that LIP5 preferred to hydrolyze butyryl-CoA (C_4), a short-chain substrate. This is likely similar to LIP2, LIP3, and LIP4 as all preferred

acetyl-CoA (C_2). However, the catalytic activity of LIP5 on hydrolyzing short-chain thioester substrate is not high as compared with LIP2 and LIP4.[17] In addition, commercial lipase and LIP1 showed a preference on lauroyl-CoA (C_{12}).

Amino Acid Derivative Substrate Activity of LIP5. Hydrolysis of ester bond between *p*-nitrophenyl and amino acid derivative substrates was assayed. LIP1-LIP5 including commercial lipase showed a specific activity on different amino acid derivative substrates. Interestingly, LIP5 was shown to be the highest among lipases on hydrolyzing L-NBTNPE as shown in Table 4.

Kinetic Analysis. All the kinetic studies were performed at 37 °C. LIP5 was analyzed to determine substrate-binding affinity (K_m), turnover number (k_{cat}) and catalytic efficiency (k_{cat}/K_m) values. Three of the substrates with higher preference of recombinant LIP5, *p*-NP butyrate, L-NBTNPE, and butyryl-CoA ($C_{4:0}$), were selected to represent simple ester, thioester, and amino acid-derived substrates, respectively. Catalytic efficiency of LIP5 is highest in *p*-NP butyrate by almost 2-fold compared to L-NBTNPE and 5-fold compared to butyryl-CoA. However, the K_m value of butyryl CoA is the smallest among the 3 substrates; thus, recombinant LIP5 affinity is highest in this substrate.

DISCUSSION

The level of gene expression of LIP isoforms from highest to lowest order, *LIP1*, *LIP3*, *LIP2*, *LIP5* and *LIP4,* had been reported for almost 20 years in our laboratory.[6] Since then, the isoforms underwent several characterizations and genetic engineering manipulations in order to enhance their catalytic performance. However, LIP5 was left uncharacterized; thus, in this paper we reported the characterized recombinant LIP5. By overlap extension PCR-based multiple-site-directed mutagenesis we codon-optimized wild type *lip5* from *C. rugosa* before we characterized.[8] The purified extracellular recombinant LIP5 from *P. pastoris GS115* showed a molecular weight similar to that of the commercial lipase, and its in-gel lipase assay against *p*-NP butyrate showed that it is active and hydrolyzes the substrate.

LIP5 isoform has 534 amino acids and an observed molecular weight of 60 kDa similar to other CRL isoforms. The isoform genes shared high identity between 60 and 70% and high similarity about 84% in amino acid sequence across the entire protein.[8,16] However, results from characterization of individual isoforms showed a distinct preference for substrates, catalytic performance, rate of hydrolysis, and behavior in varying pH and temperature as reported from previous publications. The optimum temperature of LIP5 is 55 °C where its catalytic activity is the highest. This optimum temperature of LIP5 is higher than that of recombinant LIP1, LIP2, LIP3, LIP4 and commercial lipase as previously reported.[8,12,17] Moreover, after LIP5 was incubated at 55 °C for 10 min and cooled down to 37 °C, the residual activity was similar to the residual activity of LIP5 when measured at 37 °C as seen in Figure 3 and Figure 5. The optimum temperature of LIP5 could be attributed to conformational rigidity and flexibility,[18] and its amino acid sequence variation with other isoforms.[19] It is hypothesized that enzyme stability is related to its specific amino acid sequence. For the optimum pH and thermal stability, LIP5 is similar to the commercial lipase and other isoforms reported.

To understand clearly the characteristics of recombinant LIP5, different substrates were tested for hydrolytic activity. These substrates contain an ester bond in which recombinant LIP5 can hydrolyze and consequently release the quantifiable or observable product after hydrolysis through spectrophotometry. As shown in Table 1, esterase activity of recombinant LIP5 is highest in hydrolyzing *p*-NP butyrate (C_4), a short chain length ester substrate, and *p*-NP laurate (C_{12}), a medium chain length ester substrate, at pH 7.0. In addition, recombinant LIP5 also prefers to hydrolyze cholesteryl laurate (C_{12}) among cholesteryl ester substrates. However, the overall hydrolytic performance of recombinant LIP5 in hydrolyzing *p*-NP and cholesteryl ester substrates is inferior as compared with commercial lipase. These observations are also reported in LIP2 and LIP4[17] and can be explained in the hydrophobicity of the substrate and its interaction in the binding sites. In contrast, recombinant LIP5 hydrolytic performance in thioester and amino acid derivative substrates is higher than that of the commercial lipase. Recombinant LIP5 prefers to hydrolyze butyryl-CoA ($C_{4:0}$) and L-NBTNPE as compared with commercial lipase by 16- and 34-fold respectively. But the direct evidence for the preference of substrates for recombinant LIP5 and other isoforms needs further investigation.

Table 5. Enzyme Kinetic Analysis of LIP5

substrate	parameter[a]	LIP5
p-NP butyrate	k_{cat} (s^{-1})	385.57 ± 40.12
	K_m (mM)	0.75 ± 0.08
	k_{cat}/K_m	513.54 (100%)
L-NBTNPE	k_{cat} (s^{-1})	268.08 ± 27.14
	K_m (μM)	4.111 ± 0.54
	k_{cat}/K_m	65.21 (100%)
butyryl CoA (C4:0)	k_{cat} (s^{-1})	18.02 ± 3.71
	K_m (μM)	1.34 ± 0.14
	k_{cat}/K_m	13.48 (100%)

[a] The kinetic parameters were calculated from the Michaelis−Menten equation with nonlinear regression by using the substrates (*p*-NP butyrate, L-NBTNPE, and butyryl-CoA).

To further understand the preference of recombinant LIP5 to *p*-NP butyrate (C_4), butyryl CoA ($C_{4:0}$), and L-NBTNPE substrates, kinetic analyses were performed (Table 5). The estimated K_m values using *p*-NP butyrate (C_4), butyryl-CoA ($C_{4:0}$), and L-NBTNPE as substrates were 0.75 mM, 1.34 μM, and 4.11 μM and the k_{cat} values were 386, 18, and 268 s^{-1} respectively. These findings show that recombinant LIP5 prefers to hydrolyze the ester bond in the substrate with amino acid derivative and thioester. Recombinant LIP5 preference on substrate resembles the *E. coli* thioesterase I/protease I/lysophospholipase L_1 (TAP) that prefers substrates with aromatic group(s) such as amino acid derivatives and *p*-nitrophenyl esters.[20]

Lipases and esterases belong to the same class of enzymes: hydrolases. Both enzymes hydrolyze ester bonds and share a common α/β hydrolase fold. However, esterases and lipases differ in several ways such as substrate preference, working pH in which the activity is maximum, and protein structure like presence of lid-like structure in lipases. Shorter chain fatty acids are preferred substrates of esterases, whereas lipases preferred longer chain fatty acids, insoluble or aggregated state substrates. The two enzymes also differ in their working pH condition for maximum activity: esterases favor pH 6, whereas lipases work well in pH 8 or above.[21] In this study, pH-dependent maximum activity of LIP5 supports that it is a lipase. However, the characteristics of LIP5 on hydrolyzing substrates with shorter chain length of *p*-NP ester and cholesteryl ester substrates resemble an esterase substrate preference. Another interesting observation on LIP5 is that

it prefers C_{12} of *p*-NP and cholesteryl ester substrates and not C_{12} thioester substrate. These substrates contain a ring structure group aside from the C_{12} fatty acid. The specific preference of LIP5 to C_{12} substrates could be influenced by the ring structure group; however the mechanism of this is still unknown and needs further investigation.

The rate acceleration and substrate specificity are two distinct features of enzyme-catalyzed reactions. These are a few of the criteria used in the selection of an industrial enzyme. CRL, for example, has been used in industry for its hydrolytic action. Its industrial applications span to lipid stain removal for detergent, cheese flavor for food, dough stability and conditioning for baking, pitch and contaminant control for pulp and paper, transesterification for fats and oils, resolution of chiral alcohols and amides for organic synthesis and depickling for leather.[22] However, CRL in most of these applications is a mixture of lipase isoforms; thus, purification, codon optimization and characterization of recombinant LIP5 and other isoforms previously reported are so important for future use. Overall, recombinant LIP5 is a promising lipase isoform for its ability to perform hydrolysis at high temperature as compared with LIP1−LIP4. This thermotolerant characteristic of recombinant LIP5 is good for hot extrusion processes or elevated temperature vats in industrial productions. Another interesting feature of recombinant LIP5 is the preference for a substrate containing an aromatic ring or rings. This preference of recombinant LIP5 can be a good enzyme candidate for a stereospecific, regiospecific and enantioselective organic synthesis. Hence, the characterization of recombinant LIP5 completes the LIP1−LIP5 previously identified in the lab and shows that each lipase isoform has a distinct substrate preference and catalytic activity.

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