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C-Terminal Region of *Candida rugosa* Lipases Affects Enzyme Activity and Interfacial Activation

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Supporting Information

ABSTRACT: *Candida rugosa* contains several lipase (CRLs) genes, and CRLs show diverse enzyme activity despite being highly homologous across their entire protein family. Previous studies found that LIP4 has a high esterase activity and a low lipolytic activity and lacks interfacial activation. To investigate whether the C-terminal region of the CRLs mediates enzymatic activity, chimeras were generated in which the C-terminus of LIP4 from either residue 374, 396, 417, or 444 to residue 534 was swapped with the corresponding peptide from the isoform LIP1. A chimeric lipase containing the C-terminus from 396 to 534 of LIP1 on a LIP4 scaffold showed activity similar to that of commercial CRL on triolein, and lipolytic activity increased 2−6-fold over that of LIP4. Moreover, interfacial activation was also observed in the chimeric lipase. To improve its enzymatic properties, a novel glycosylation site was added at residue 314. The new glycosylated lipase showed improved thermostability and enhancement in enzymatic activity, indicating its potential for use in further application.

KEYWORDS: *Candida rugosa* lipase, alcohol binding site, interfacial activation, C-terminal region, glycosylation

INTRODUCTION

Lipases (EC 3.1.1.3) catalyze both the hydrolysis and the synthesis of ester groups in triglycerides of soluble or insoluble substrates.[1] Extracellular lipases from *Candida rugosa* (CRLs) are important in various bioindustries.[2] CRLs recognize a broad spectrum of substrates: lipids and esters of various carbon chain lengths, various forms of carbon bond saturation in the acyl group, aromatic rings, and cholesterol ring, etc.[3−5] CRLs are versatile biocatalysts to catalyze nonspecific or stereospecific hydrolysis, alcoholysis, esterification, transesterification, and interesterification.[6] They are widely applied in a variety of biotechnological applications for the production of sterols and sitostanol, synthesis of n-3 polyunsaturated fatty acids,[7] reduction of pitch during paper manufacture,[8] enantioselective hydrolysis for anti-inflammatory drugs,[9,10] and synthesis of biodiesel.[11,12]

The genome of *C. rugosa* contains *LIP1*−*LIP7* genes that consist of 534 amino acids with 84% similarity and 66% identity in sequence across the entire protein family.[6] The family of CRLs conserves both a catalytic triad (Ser209, His449, and Glu341) and residues involving disulfide bond formation (Cys60−Cys97 and Cys268−Cys277).[5,13] However, CRLs differ in N-glycosylation sites, isoelectric points, and some local features in their hydrophobic profiles. In addition, CRLs have been reported to show diverse enzyme activities, despite the highly homologous protein sequences.[5]

In previous studies, LIP1 was found to exert its highest activity for medium-chain substrates in the hydrolysis of both triglycerides and methyl esters.[14,15] LIP2 shows its highest lipolytic activity for long-chain fatty acids and also demonstrates cholesterol esterase activity.[16,17] LIP3 is characterized by its preference for short-chain soluble substrates and by its ability to hydrolyze cholesterol esters of long-chain fatty acids.[18,19] LIP4 prefers long-chain soluble substrates (C16−C18) but has low lipolytic activity with insoluble substrates.[20] The diverse substrate specificities of CRLs related to their sequences and structures are noteworthy.

CRLs have an α/β-hydrolase structure with a catalytic triad and a lid that covers the active site.[21] The lid region in CRLs, between amino acids 65 and 92, interacts with the substrate, leading to an open form that participates in substrate binding and recognition.[6] By swapping the lid domain in LIP1 with that from LIP3, this chimera has demonstrated cholesterol esterase activity, observed through the absence of a specific shift on the chain-length specificity.[22,23] The three-dimensional structures of CRLs show two well-defined binding sites for the scissile fatty acid and the alcohol moieties.[24] To accommodate the acyl group of the substrate, the binding sites form an L-shape tunnel conformation that may contribute to the broad range of fatty acid chain lengths catalyzed by CRLs. The interaction of the scissile fatty acid binding sites and triacylglyceride may determine substrate specificity of CRLs.[13,25,26] Using molecular modeling and site-directed mutagenesis for studying the LIP1 and LIP4 tunnels, mutations at different locations inside the LIP1 tunnel (Pro246Phe, Leu413Phe, Leu410Trp, Leu410Phe/Ser300Glu, and Leu410Phe/Ser365Leu) or LIP4 tunnel (Ala296Ile, Val344Arg, and Val344His) have been investigated.[27,28] On the basis of the mutants of LIP1, residues that mediate chain length

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specificity were replaced by bulkier amino acids, and the resulting mutants may block hydrolyzing chain lengths longer than C8.[27] In a study on LIP4, residues 296 and 344 located at the mouth of the tunnel were found to be responsible for the different substrate specificities of LIP4.[28] Thus, although studies on lid, active site, tunnel, and glycosylation of CRLs have been performed, a better understanding of the sequence−structure relationship is needed to develop further applications of CRLs.

In our previous studies, LIP4 was demonstrated to have a higher esterase activity toward long acyl-chain esters and lower lipase activity toward triglycerides.[20,29] To improve LIP4 enzymatic activity and evaluate whether the C-terminus of the lipase may mediate enzyme activity and specificity, we created chimeras in which the C-terminus of the LIP4 from residue 374, 396, 417, or 444 to residue 534 was replaced with the corresponding peptides from isoform LIP1. Compared with the enzymatic activity of LIP4, the chimeric lipase showed higher lipase activity. To improve the lipase enzymatic properties, Phe314 in the lipase was replaced with Asn to create a new glycosylation site. The engineered lipase-increased glycosylation not only promotes esterase and lipase activity but also improves the thermostability of the enzyme. By swapping the C-terminal residues from 396 to 534 of the LIP1 on the LIP4 scaffold and adding the glycosylation at residue 314, the novel chimeric lipase demonstrated an even greater activity and stability for further applications.

MATERIALS AND METHODS

Materials and Strains. Commercial CRL (CL) and the substrates for esterase and lipase activity assays were obtained from Sigma (St. Louis, MO). Peptone, tryptone, and yeast extract was purchased from BD Diagnostic Systems (Sparks, MD). Other chemicals were obtained from Merck (Darmstadt, Germany). For molecular c1oning, restriction enzymes and ligase were obtained from Promega (Madison, WI). *Escherichia coli* cloning vector pGEM-T was from Promega. *Pichia pastoris* expression vector pGAPZαC, Zeocin, yeast strains *P. pastoris* X-33 and *E. coli* TOP10F′ were purchased from Invitrogen (Carlsbad, CA). For protein purification, DEAE Sepharose CL-6B and Butyl Sepharose CL-6B for hydrophobic interaction chromatography (HIC) were supported from GE Health (Piscataway, NJ).

Overlapping PCR To Construct Chimeric Lipases. Because *C. rugosa* employs the nonuniversal codon CUG-Leu for Ser, CUG sites in LIP1 and LIP4 were used to replace Ser codons by the multiple-site-directed mutagenesis method.[30] To fuse LIP1 and LIP4 to form chimeric lipases, the chimeric lipase genes were constructed by overlapping PCR (see the Supporting Information, Figure S-1). PCR products were ligated into the pGEM-T vector and transformed into the cloning host *E. coli* TOP10F′. Transformants were selected, and the recombinant genes were confirmed by sequencing. The pGEM-T vector harboring chimeric lipase was digested by *Kpn*I and *Sal*I, and the gene was ligated into the *P. pastoris* expression vector pGAPZαC. To add a glycosylation site at residue 314, residue Phe314 in L4-1C395 was used to replace the Asn by site-directed mutagenesis with overlapping PCR method. After confirmation by sequencing, the mutated gene, termed L4-1C395-N, was cloned into pGAPZαC vector.

Transformation of *P. pastoris*. The pGAPZαC vector containing the chimeric lipase gene was linearized by *Eco*RV for transformation. A DNA sample was mixed with *P. pastoris* X-33 competent cells, which were prepared by 1.0 M ice-cold sorbitol according to the manual of Bio-Rad Laboratories (Hercules, CA). Electroporation was performed using a voltage of 1.5 kV in a 0.2 cm electrode cuvette by MicroPulser (Bio-Rad Laboratories). After electroporation, cells were cultured on YPDS plates (1% yeast extract, 2% peptone, 2% glucose, 1.0 M sorbitol, and 2% agar) containing 100 μg/mL zeocin for selection. Colonies picked from YPDS zeocin plates were cultured on YPD plats (1% yeast extract, 2% peptone, and 2% glucose) containing 1% (v/v) tributyrin. Colonies showing a clear zone indicated enzyme activity.

Purification of the Chimeric Lipases. Transformants of *P. pastoris* X-33 harboring chimeric lipase L4-1C395 or L4-1C395-N were incubated in 10 mL of YPD with 100 μg/mL zeocin at 30 °C. The culture was added to 1000 mL of YPD in a 2000 mL flask for 3 days of incubation at 30 °C. The culture medium was then concentrated by ultrafiltration with Pellicon XL 10 cutoff Biomax-10 membranes (Millipore, Bedford, MA). The chimeric lipases were purified with DEAE and butyl HIC columns. The concentrated medium was loaded onto a DEAE Sepharose CL-6B column equilibrated with TE buffer (20 mM Tris-HCl, 2 mM EDTA, pH 8.0). The protein was eluted using 5 column bed volumes of TE buffer containing 350 mM $(NH_4)_2SO_4$. The fractions containing lipase activity were collected and processed by a Butyl Sepharose CL-6B HIC column equilibrated with TE buffer containing 350 mM $(NH_4)_2SO_4$. The column was washed with TE buffer, and the bound-protein portion was eluted by 30 mM CHAPS. The enzyme activity was measured using *p*-nitrophenyl laurate as a substrate. Protein concentration was determined according to the Bradford method using bovine serum albumin (BSA) for a standard. The molecular weights were estimated by SDS-PAGE.

Enzyme Characterization. The esterase activity was determined spectrophotometrically using *p*-nitrophenyl esters as substrates. Hydrolysis was performed with 3 mM substrate dissolved in 50 mM sodium phosphate buffer (pH 7.0) containing 2% Triton X-100. The increase in absorbance at 410 nm was determined at 37 °C for 3 min. One unit of activity was defined as 1 μmol of nitrophenol hydrolyzed from substrate by the amount of enzyme per minute under the above conditions. The lipolytic activity was determined titrimetrically using triacylglycerides with acyl esters of various chain lengths as substrates.[31] The different triacylglyceride substrates at the concentration of 3 mM were emulsified in 1 L of an emulsification reagent (pH 8.0) containing 17.9 g of NaCl, 0.41 g of KH_2PO_4, 540 mL of glycerol, and 6 g of gum arabic. Lipase was added to 15 mL of emulsion substrate at 37 °C. The free fatty acids released were titrated continuously by 10 mM NaOH using a pH-stat (Radiometer Copenhagen, Bagsvaerd, Denmark). One unit of lipase activity was defined as 1 μmol of fatty acid released from the lipid substrate hydrolyzed by the amount of enzyme per minute under the assay conditions. Interfacial activation was detected using triolein in a concentration range of 0.1−3.0 mM. To detect thermostability, lipases were incubated at 37, 45, 55, and 65 °C for 10 min. After incubation, residual enzymatic activity was measured using *p*-nitrophenyl laurate as the substrate.

RESULTS

Construction of Chimeric Lipases. Previous papers have indicated that LIP4, with respect to LIP1, has higher esterase activity and lower lipolytic activity.[20,29] In addition, LIP4 does not show the interfacial activation that implies that hydrophobic substrates recruit lipase into lipid micelles to activate the enzyme activity.[20] To investigate whether the C-terminus of CRLs is involved in enzymatic activity, novel chimeric lipases were constructed by swapping the C-terminus of LIP4 with the corresponding fragment of LIP1 as depicted in Figure 1A. The chimeric lipases were termed L4-1C373, L4-1C395, L4-1C416, and L4-1C443, referencing the fusion site of LIP4. In Figure 1B, the protein sequences from residues 355−534 of LIP1 and LIP4 are aligned, and the numbers of different amino acids of the chimeric lipases are available as Supporting Information (Supplementary Table 2). There is only a two amino acid difference between L4-1C373 and L4-1C395, and L4-1C416 has four different amino acids compared with L4-1C443.

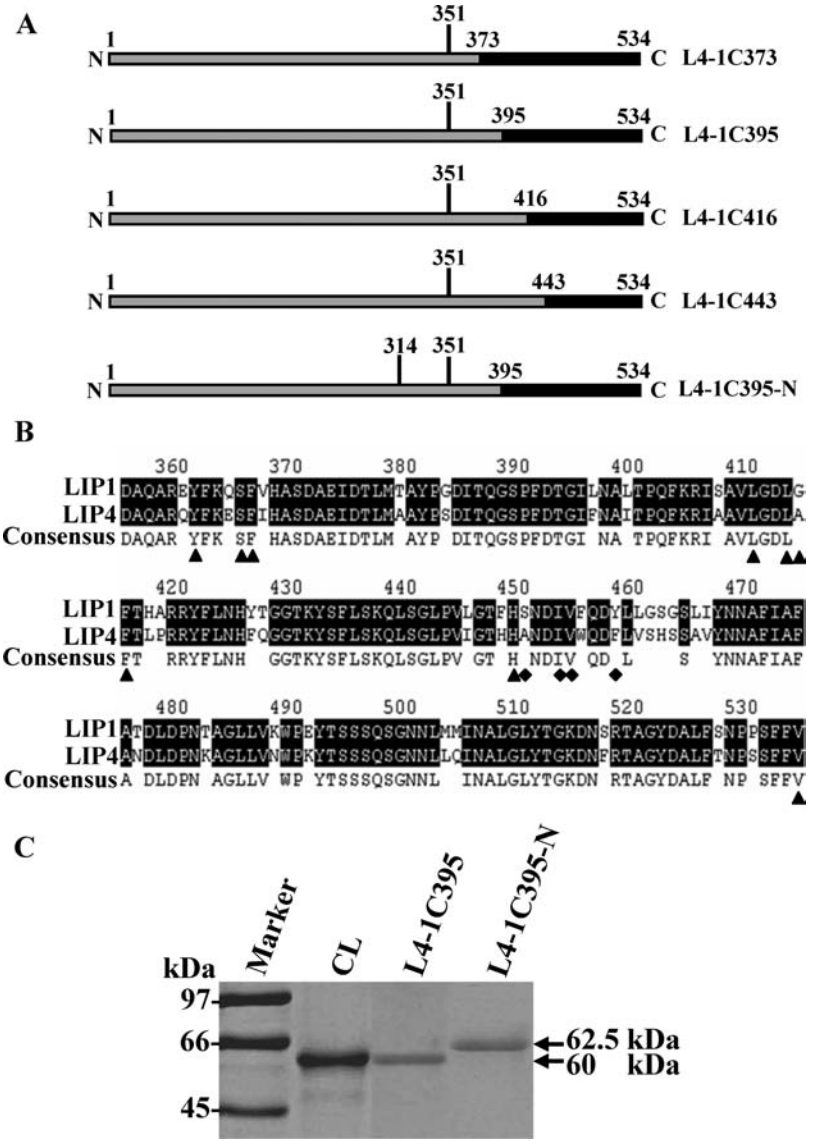

Figure 1. (A) Chimeric lipases contain fragments from swapping the C-terminus of LIP4 (gray) with the corresponding peptide of LIP1 (black). The number indicates the fusion site, and N-glycosylation residues are shown as long rods. (B) Sequence alignment of the C-terminal region from residue 355 to 534 of LIP1 and LIP4. The fatty acid binding sites and the alcohol binding sites are labeled by black triangles and diamonds, respectively. (C) Molecular masses of L4-1C395 and L4-1C395-N. Proteins were analyzed by SDS-PAGE and stained with Coomassie blue. Marker, molecular weight marker; CL, commercial CRL. The molecular weights of purified L4-1C395 and L4-1C395-N are 60 and 62.5 kDa, respectively.

Production of Chimeric Lipases. To detect lipase activity, the chimeric lipases were transformed into *P. pastoris*, and the transformants were analyzed by tributyrin plate. Both L4-1C395 and L4-1C443 showed a clear zone on the plate, whereas L4-1C373 and L4-1C416 did not display enzymatic activity (see the Supporting Information, Figure S-2). For characterization of enzymatic activity, L4-1C395 and L4-1C443 were produced and purified from *P. pastoris*. However, the L4-1C443 was stuck in various HICs, creating an obstacle for further analysis. Therefore, we focused on characterizing the enzymatic activity of L4-1C395.

Glycosylation and Enzymatic Properties. LIP4 and L4-1C395 contain only one glycosylation site at Asn351, but LIP1 contains three: Asn291, Asn314, and Asn351. Because replacement of Asn291 into Gln in LIP1 did not lead to changes in enzymatic activity,[32] residue 314 was chosen to replace the residue into Asn, and the novel lipase was termed L4-1C395-N. By SDS-PAGE analysis, the molecular weight of L4-1C395-N was 62.5 kDa (Figure 1C), which is larger than those of CL and L4-1C395. This result indicates that L4-1C395-N performs glycosylation, leading to an increase in molecular weight. To investigate thermostability of novel lipases, these lipases were exposed to 37, 45, 55, and 65 °C for 10 min. At 55 °C, the activity of L4-1C395 was notably reduced to about 53%, whereas LIP4 and CL retained 82 and 95% activities, respectively, indicating that L4-1C395 is less stable (Figure 2). Significantly, L4-1C395-N was more stable than L4-1C395 and as stable as LIP4 at 55 °C. This result shows that the increase in glycosylation at residue Asn314 in L4-1C395 improves thermostability.

Hydrolytic Activity of the Chimeric Lipases. To determine substrate specificity, the chimeric lipases were used to hydrolyze *p*-nitrophenyl esters containing fatty acids of various chain lengths: *p*-nitrophenyl butyrate (C4), *p*-nitrophenyl caprylate

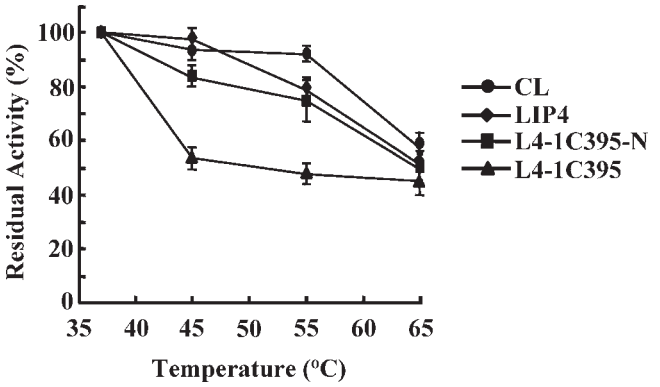

Figure 2. Thermal stability of the chimeric lipases: squares, L4-1C395-N; triangles, L4-1C395; diamonds, LIP4; circles, CL. The enzyme was incubated at 37, 45, 55, or 65 °C for 10 min. Enzyme activity was measured using *p*-nitrophenol laurate as substrate at 37 °C. The residual esterase activity was calculated in comparison to untreated enzyme. Values are the mean ± SD from three experiments.

(C8), *p*-nitrophenyl laurate (C12), and *p*-nitrophenyl palmitate (C16). Specific activities of the chimeric lipases on the soluble substrates was 2−6-fold higher than that of CL but less than that of LIP4 (Figure 3A). The most favorable substrate for L4-1C395 and L4-1C395-N was *p*-nitrophenyl laurate (C12). Toward medium and long chain lengths (C8−C16), the chimeric lipases displayed a higher activity, showing a 2−5-fold increase in activity over short chain lengths. Moreover, L4-1C395-N was more active than L4-1C395 toward medium to long acyl chain lengths, showing that increased glycosylation in the chimeric lipase may enhance the esterase activity.

Lipase activity was measured by the pH-stat assay to determine the lipolytic activity of the chimeric lipases for triglycerides of various chain lengths: the saturated fatty acids tributyrin (C4:0), tricaprylin (C8:0), and tristearin (C18:0); and the unsaturated fatty acid triolein (C18:1). Specific activities of the chimeric lipases were 3−6-fold higher than that of LIP4 toward insoluble substrates. Remarkably, L4-1C395 and L4-1C395-N had activities similar to that of CL toward triolein (Figure 3B). In addition, L4-1C395 and L4-1C395-N showed 2−4-fold more activity than LIP4. Therefore, our results demonstrated the lipolytic activity of LIP4 was improved by swapping its C-terminus with that from LIP1 from amino acids 396−543 in addition to enhancing lipolytic activity by glycosylating Asn314.

Interfacial Activation. Hydrolysis of insoluble lipids by lipases may take place during interfacial activation, and specific activity of the enzymes shows a sigmoidal curve against substrate concentrations; however, this hydrolysis does not obey Michaelis−Menten kinetics. Additionally, interfacial activation was not observed in LIP4 but is present in commercially available CRL. Because LIP1, LIP2, and LIP3 generally show interfacial activation,[17] a key question is whether the chimeric lipases have interfacial activation. To address this issue, the hydrolysis rate of enzyme by LIP4, CL, L4-1C395, and L4-1C395-N was determined using triolein as substrate. CL clearly showed interfacial activation when the concentration of triolein was >0.2 mM, whereas LIP4 did not, as expected (Figure 4). Remarkably, the chimeric lipases followed the same pattern as CL. This finding indicates that L4-1C395 and L4-1C395-N demonstrate properties similar to CL for the substrate specificity and interfacial activation.

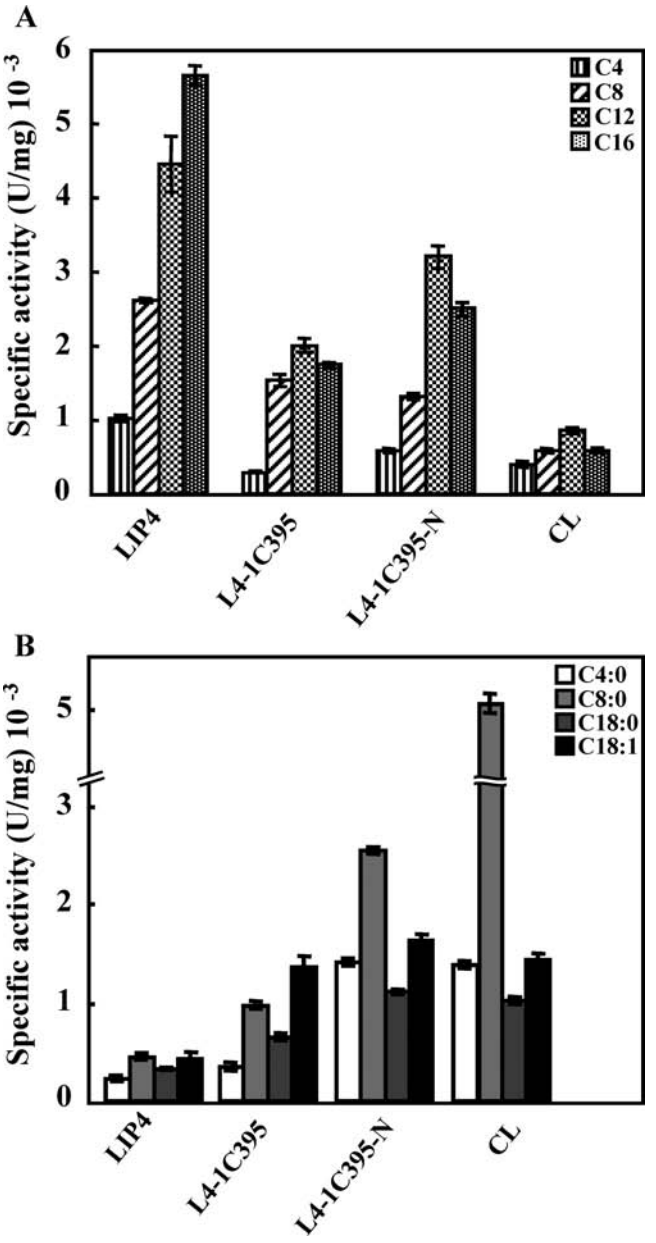

Figure 3. (A) Substrate specificity of the chimeric lipases in the hydrolysis of *p*-nitrophenyl esters with different chain lengths. One unit of enzyme activity is defined as the amount of enzyme that hydrolyzes 1 μmol of *p*-nitrophenol ester to *p*-nitrophenol per minute at 37 °C. C4, *p*-nitrophenyl butyrate; C8, *p*-nitrophenyl caprylate; C12, *p*-nitrophenyl laurate; C16, *p*-nitrophenyl palmitate. (B) Substrate specificity of the chimeric lipases in the hydrolysis of triacylglycerol substrates tributyrin (C4:0), tricaprylin (C8:0), tristearin (C18:0), and triolein (C18:1). One unit of the lipase activity is defined as the amount of enzyme that produces 1 μmol of fatty acid by hydrolyzing lipid substrate per minute at 37 °C. For both panels, values are the mean ± SD from three experiments.

DISCUSSION

Here we describe the enzymatic activity of LIP4, which was improved by replacing the C-terminal residues of LIP4 with the corresponding peptide residues of LIP1. The lipase L4-1C395 showed increased lipolytic activity and interfacial activation. Although mutants engineered from LIP1 and LIP4 in the lid

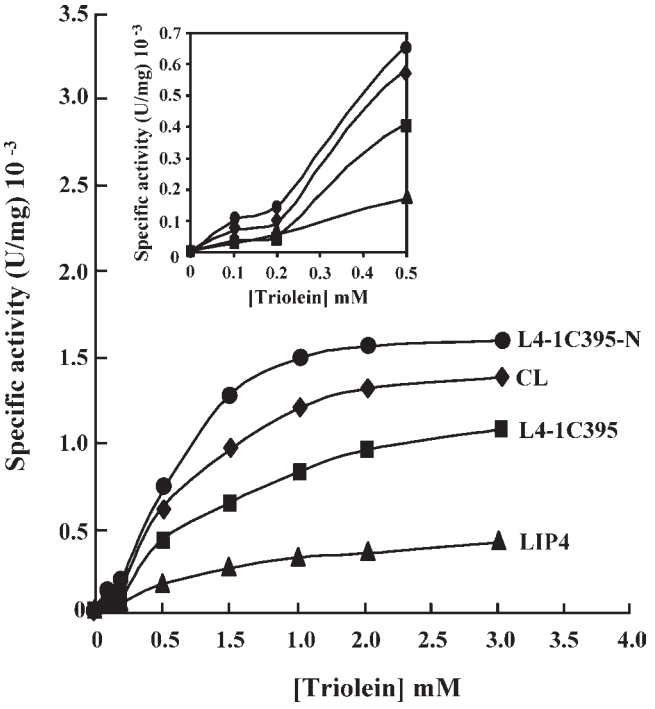

Figure 4. Interfacial activation of the chimeric lipases. Specific activities of L4-1C395, L4-1C395-N, LIP4, and CL were measured using different concentrations of triolein as indicated. CL (diamonds), L4-1C395 (squares), and L4-1C395-N (circles) show interfacial activation, but LIP4 (triangles) does not display this phenomenon.

and tunnel regions have been investigated,[5,6] it is of interest to unravel the substrate specificity by re-engineering the alcohol binding sites. Our findings indicate that the alcohol binding residues in the C-terminus of CRLs implicate the enzymatic characteristics of CRLs. This demonstrates that modification of the alcohol binding sites in LIP4 may cause a highly lipolytic activity on long-length fatty acids. Moreover, the increase in lipase and esterase activity in L4-1C395-N shows that glycosylation at residue 314 may improve enzymatic activity of the chimeric lipase.

Lee and co-workers proposed that residues 296 and 344 located at a hydrophobic pocket near the entrance of the hydrophobic tunnel are involved in the initial recognition and transport of hydrophobic substrates into the binding sites.[28] All three re-engineered LIP4 improved the hydrolytic activity toward short-chain triacylglycerides.[28] The chimeric lipase, which differs in only a few amino acid residues, has the same contents at the lid, tunnel, and functional regions. However, it showed a significant increase in lipolytic activity on the long-chain triacylglycerides. These results show that alcohol binding sites may contribute to substrate recognition.

The substitutions of Ala450 and Phe458 in LIP4 by Ser450 and Tyr458 in L4-1C395, respectively, are perhaps the most remarkable changes because hydrophobic residues are replaced with hydrophilic residues (see the Supporting Information, Supplementary Table 3). These changes may increase the lipolytic activity or promote the interaction of substrates with enzyme in the insoluble lipid substrates. By three-dimensional structure modeling (see Supporting Information, Figure S-3), Ser450, located in the substrate binding sites at the mouth of the tunnel, may play an important role in lipolytic activity of the chimeric lipases. This finding was also supported by replacing the Gly450 in LIP2.[33] This finding was also demonstrated by a previous report that substitutions of Ala296Ile, Val344Arg, or Val344His resulted in shifting the preference of LIP4 from long-chain substrates to medium- or short-chain substrates.[28] The reasons of L4-1C373 and L4-1C416 did not have lipolytic activity can be explained by the studies of Brocca et al.[22] and Yen et al.[33] They demonstrated that the enzymatic activity of CRL is affected by mutation on specific amino acids and proposed that these results may due to inactivation, decreased enzymatic stability, or blocked protein secretion.

LIP4 contains one glycosylation site at Asn351, but LIP1 has three glycosylation sites at Asn291, Asn314, and Asn351. Asn351 is conserved in all CRLs.[34] On the basis of crystallographic analysis of the native LIP1, two ordered sugar residues are linked at positions 351 and 314, whereas residue 291 is free.[32] In LIP1, when Asn314 or Asn351 is replaced with Gln, the enzymatic activity of the lipase was abolished.[32] However, both glycosylated LIP4 and unglycosylated LIP4 show the same substrate specificity and enzymatic activity, whereas unglycosylated LIP4 is decreased in its thermostability.[20] Using a tributyrin plate, L4-1C443 in *E. coli* as well as L4-1C395 and L4-1C443 in *P. pastoris* all showed enzyme activity (see the Supporting Information, Figure S-2). This finding shows that glycosylation is essential for L4-1C395 but not for L4-1C443 and LIP4. The carbohydrates linked to Asn351 are located at the LIP1 surface in the proximity of the lid structure, leading to stabilization of the enzyme conformation.[35] However, because the position of Asn314 is far from the surface-interacting residues and active sites, it does not directly mediate the catalytic reaction. Remarkably, L4-1C395-N has lipolytic and esterasic activities higher than those of L4-1C395. The mechanism involved in the increased enzyme activity of L4-1C395-N by Asn 314 glycosylation remains to be examined.

In conclusion, this study characterizes a novel chimeric lipase constructed by swapping the C-terminus of LIP4 with the corresponding peptide of LIP1 and by adding a new glycosylation site at residue 314. The new lipase is more active toward triglycerides and has increased thermostability, suggesting that it might be useful for bioindustrial applications.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Additional procedural experiment details and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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