Catalytic Characteristics of a *sn*-1(3) Regioselective Lipase from *Cordyceps militaris*

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A total of 39 agricultural products were screened for natural sources of lipases with distinctive positional specificity. Based on this, Cordyceps militaris lipase (CML) was selected and subsequently purified by sequential chromatography involving anion-exchange, hydrophobic-interaction, and gel-permeation columns. As a result of the overall purification procedure, a remarkable increase in the specific activity of the CML (4.733 U/mg protein) was achieved, with a yield of 2.47% (purification fold of 94.54). The purified CML has a monomeric structure with a molecular mass of approximately 62 kDa. It was further identified as a putative extracellular lipase from C. militaris by the partial sequence analysis using ESI-Q-TOF MS. In a kinetic study of the CML-catalyzed hydrolysis, the values of V_{max}, K_m, and k_{cat} were determined to be 4.86 μmol·min^{-1}·mg^{-1}, 0.07 mM, and 0.29 min^{-1}, respectively. In particular, the relatively low K_m value indicated that CML has a high affinity for its substrate. With regard to positional specificity, CML selectively cleaved triolein at the sn-1 or 3 positions of glycerol backbone, releasing 1,2(2,3)-diolein as the major products. Therefore, CML can be considered a distinctive biocatalyst with sn-1(3) regioselectivity. © 2018 American Institute of Chemical Engineers *Biotechnol. Prog.*, 35: e2744, 2019.
Keywords: triacylglycerol hydrolase, positional specificity, sn-1(3) regioselectivity, Cordyceps militaris, enzyme kinetic study

Introduction

Lipases (triacylglycerol acylhydrolase, EC 3.1.1.3) are a class of enzymes that catalyze the hydrolysis of long-chain triacylglycerols, which results in the liberation of free fatty acids from glycerol backbone.[1] The reverse reaction, on the other hand, is also possible in micro-aqueous or nonaqueous media, leading to esterification of fatty acids with various hydroxyl groups.[2,3] Because of this versatility, lipases are among the most studied enzymes and have been used extensively in biotechnological applications for lipid modifications,[4] synthesis of bioactive esters,[5] and production of food additives and biopolymers.[6,7]

As the biocatalyst in the aforementioned biotechnological applications, numerous lipases have been discovered from various sources including plants, animals, and microorganisms, and genetically engineered for desired biocatalytic characteristics.[8,9] Especially with regard to substrate specificity, lipases can be classified according to their positional specificity

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(i.e., *sn*-regioselectivity), which has been the focus of recent research.[10,11] There has been an increasing interest in the development of novel sources of lipases with distinguishing positional specificity, which could be applied to the selective synthesis of valuable intermediates in lipid hydrolysis and/or esterification.[7,10]

Our research groups have been investigating on lipase-catalyzed bioconversions including esterification for multifunctional additives (e.g., erythorbyl laurate) and food emulsifiers (e.g., 1-monocaprin).[12–14] In a bid to make the enzymatic bioconversions more desirable, one goal of our research is to discover a novel lipase with distinctive positional specificity to be utilized for selective production of lipid derivatives. Hence, a total of 39 agricultural products were screened for natural sources of lipases with distinctive positional specificity, and crude enzyme extract from *Cordyceps militaris* exhibited remarkable catalytic performance in terms of both total and specific activities.

C. militaris, belonging to the Ascomycetes, generally parasitizes the larvae or pupae of lepidopteran insects, and it has been used in traditional medicines and tonics (especially in East Asia).[15] Biologically active compounds (e.g., cordycepin and cordymin) have been isolated from this fungus and extensively investigated for a variety of pharmacological effects, such as anti-cancer, anti-inflammatory, and/or immune-enhancing activities.[16,17]

Based on its background, *C. militaris* was selected as a potential source of lipases, and *C. militaris* lipase (*CML*) was purified to characterize its catalytic performance, including enzyme kinetics, optimum conditions, and positional specificity. Moreover, partial peptide sequencing of purified *CML* was performed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) and the resulting sequences matched the genome sequence of *C. militaris* in a BLAST search of the NCBI database, providing significant information for further molecular cloning and enzyme engineering of *CML*.

Materials and Methods

Materials

Trizma® base (≥99.9%), *p*-nitrophenyl palmitate (*p*-NPP), lipid standards composed of triolein (i.e., glyceryl trioleate), 1,3-diolein, 1,2(2,3)-diolein (i.e., 1,2-dioleoyl-*rac*-glycerol), and monoolein (i.e., glyceryl monooleate) in an equal weight ratio, glyceryl trioleate (≥99.0%), and calcium chloride (≥99.9%) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Triton X-100, agar, ammonium sulfate (≥99.5%), and sodium chloride (≥99.5%) were purchased from Junsei Chemical Co., Ltd. (Tokyo, Japan). Boric acid, potassium chloride, and oleic acid were obtained from Duksan Pure Chemicals Co., Ltd. (Ansan, Gyeonggi-do, Korea). Hydrochloric acid (35–37%) and acetone (99.5%) were obtained from Daejung Chemicals & Metals Co., Ltd. (Shihueng, Gyeonggi-do, Korea). HPLC-grade acetic acid and chloroform were purchased from J.T. Baker Co. (Philipsburg, NJ) and Samchun Pure Chemical Co., Ltd (Pyeongtaek, Gyeonggi-do, Korea), respectively. All other chemicals were of analytical grade and were used without further purification.

Screening of lipase activity from agricultural products

Sample Preparation. A total of 39 agricultural products cultivated in the Republic of Korea, including vegetables, fruits, mushrooms, and cereals, were purchased from local markets. Supporting Information Data S1 shows the samples we selected to screen for lipase activity. Each sample (10 g) was homogenized in 100 mL 50 mM Tris–HCl buffer (pH 7.0) and 0.1% Triton X-100 at 4°C for 3 h. The mixture was centrifuged at 20,000g for 30 min, and the resulting supernatant was filtered through 0.45 μm filter paper. The crude extract was fractionated by ammonium sulfate precipitation at 80% saturation. The pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.0), and the suspension was used as the crude enzyme solution for analysis of lipase activity.

Lipase Assay of the Crude Enzyme Extracts from Agricultural Products. Lipase activity was measured using a spectrophotometric method[18] with slight modifications. The substrate solution was prepared by dissolving 0.0317 g *p*-NPP in 100 mL distilled water with 0.1 g Triton X-100 and 17.0 mg sodium dodecyl sulfate (SDS). The substrate solution (0.5 mL) was added to an equal volume of Tris–HCl buffer (pH 8.2), and the mixture was preincubated in a water bath at 37°C for 10 min. Lipase-catalyzed hydrolysis was initiated by adding 0.1 mL of the crude enzyme solution to the substrate solution, followed by magnetic stirring at 400 rpm. During enzymatic hydrolysis, the absorbance at 400 nm was monitored to quantitate the *p*-nitrophenol liberated from *p*-NPP. One unit of activity was defined as the amount of enzyme liberating 1 μmol *p*-nitrophenol per minute.

Isolation and purification of the lipase from C. militaris

Lyophilized *C. militaris* (500 g) were homogenized in a blender using 0.1% Triton X-100 dissolved in 50 mM Tris–HCl buffer (pH 7.0) at 4°C for 10 min. The homogenate was centrifuged at 14,000g for 30 min, and the resulting supernatant was filtered through a 0.45 μm membrane filter. Ammonium sulfate precipitation of the supernatant was performed at 40–80% saturation. Precipitated proteins were collected by centrifugation at 10,000g for 15 min, and the resulting pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.0). The suspension was dialyzed overnight against the same buffer using dialysis tubing (molecular weight cut-off [MWCO] 12–14 kDa, Fisherbrand®, Fisher Scientific, Pittsburgh, PA). The protein concentration of the enzyme solution was determined by the Bradford protein assay,[19] using bovine serum albumin as an external standard.

Purification steps were performed using a fast protein liquid chromatography (FPLC) system (GE Healthcare, Uppsala, Sweden) equipped with a UV detector to measure the absorbance at 280 nm. The suspension was applied to a HiTrap™ DEAE-Sepharose Fast Flow column (0.7 × 2.5, GE Healthcare) previously equilibrated with 50 mM Tris–HCl buffer (pH 7.0, buffer A), and unbound proteins were washed off the column using the same buffer. The elution was performed using a step gradient with buffer A containing 1.0 M sodium chloride (buffer B) at a flow rate of 1.0 mL/min, and the gradient conditions were as follows: 0% buffer B for 20 min, 5% for 90 min, 10% for 90 min, 15% for 60 min, and 100% for 40 min. The fractions (10.0 mL) from the FPLC system were analyzed for lipase activity and protein concentration as above described. The fraction containing the target protein were collected and concentrated by ultrafiltration (MWCO 10 kDa, Millipore, Billerica, MA).

The concentrated fraction containing the target protein obtained from the DEAE-Sepharose column was adjusted to

1.0 M ammonium sulfate and applied to a HiTrap™ phenyl-Sepharose 6 Fast Flow column (high substitution, 0.7 × 2.5 cm, GE Healthcare) pre-equilibrated with 50 mM Tris–HCl buffer (pH 7.0) containing 1.0 M ammonium sulfate (buffer C). The loosely associated proteins were removed by washing the column with buffer C at a flow rate of 0.8 mL/min, and proteins were further eluted by a step gradient of ammonium sulfate using buffers C and D (50 mM Tris–HCl buffer, pH 7.0). The gradient conditions were as follows: 0% buffer D for 30 min, 25% for 70 min, 50% for 70 min, 75% for 50 min, and 100% for 50 min. Fractions (10.0 mL) were collected, and lipase activity and protein concentration were determined. The fractions with lipolytic activity were concentrated as above described and subsequently loaded onto a HiPrep™ Sephacryl S-100 HR column (1.6 × 60 cm, GE Healthcare) pre-equilibrated with buffer D containing 0.15 M sodium chloride. The flow rate of washing and elution buffer in this step was 0.4 mL/min. Finally, the targeted fraction with lipolytic activity was re-loaded onto the Sephacryl S-100 column to confirm a single peak, indicating the purity of *CML.*

Identification of CML

Lipase Activity Staining. Lipase activity was detected and visualized on chromogenic plates.[20] Chromogenic substrate plates were prepared using 0.01% phenol red, as an indicator, containing 1% triolein, 10 mM $CaCl_2$, and 2% agarose. The pH was adjusted to 7.3–7.4 using 0.1 N NaOH. *CML* was impregnated onto a small disc of filter paper (diameter 5.0 mm) and placed on the chromogenic substrate plate at 37°C. *Chromobacterium viscosum* lipase (*CVL*) and thermally inactivated *CML* were used as positive and negative controls, respectively.

SDS-Polyacrylamide Gel Electrophoresis (PAGE) and Molecular Mass Determination. The purity and subunit structure of *CML* was determined by SDS-PAGE using the method by Laemmli.[21] Purified *CML* was diluted in Laemmli sample buffer and boiled for 3 min before loading onto a 12% polyacrylamide gel. A protein marker (Bio-Rad, Hercules, CA) was used as a broad range (10–250 kDa) protein standard to estimate the molecular weight of the proteins. The protein sample was isolated using the Hoefer SE 250 mini-gel system (GE Healthcare) at 20 mA and room temperature. The proteins were stained with Coomassie Brilliant Blue R-250.[22]

The precise molecular mass of *CML* was determined by FPLC equipped with a gel permeation chromatography column (Sephacryl S-100). The column was pre-equilibrated with 50 mM Tris–HCl containing 0.15 mM NaCl (pH 7.0) at a flow rate of 0.4 mL/min. A calibration curve (i.e., retention volume vs. molecular mass of the globular protein) was plotted for the following standard proteins of known molecular mass: transferrin (81 kDa), ovalbumin (44 kDa), carbonic anhydrase (29 kDa), and ribonuclease A (13.7 kDa). The void volume was determined using blue dextran (2000 kDa).

In-Gel Digestion. Proteins were subjected to in-gel trypsin digestion according to standard protocol with slight modification.[23] Protein spots were excised from gels and destained with 100 μL destaining solution (50% methanol/distilled water) by shaking for 5 min. After removal of the solution, gel spots were incubated in 200 mM ammonium bicarbonate for 20 min. The gel pieces were dehydrated with 100 μL acetonitrile and dried by vacuum centrifugation. The dried gel pieces were rehydrated with 20 μL 50 mM ammonium bicarbonate containing 0.2 μg modified trypsin (Promega, Madison, WI) for 45 min on ice. After removal of the solution, 70 μL 50 mM ammonium bicarbonate was added, and digestion was conducted overnight at 37°C.

Desalting and Concentration. Custom-made chromatographic columns were used for desalting and concentrating the peptide mixture prior to mass spectrometric analysis. A column consisting of 100–300 nL POROS™ reverse phase (20–30 μm bead size, Applied Biosystems™, Foster City, CA) was packed in a constricted gel loader tip (Eppendorf, Hamburg, Germany). A hypodermic syringe was used to force liquid through the column by applying gentle air pressure. The peptide mixture (30 μL) from the digestion supernatant was diluted with 30 μL 5% formic acid, loaded onto the column, and washed with 30 μL 5% formic acid. For ESI-MS/MS analyses, the peptides were eluted with 1.5 μL methanol/distilled water/formic acid (50:49:1, v/v/v) and added directly to a pre-coated borosilicate nano-electrospray needle (EconoTip™, New Objective, Woburn, MA).

Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS). ESI-MS/MS analyses of peptides generated by in-gel digestion were performed using nano-ESI on a quadrupole-time of flight (Q-TOF2) mass spectrometer (AB Sciex Instruments, Redwood City, CA). A potential of 1 kV was applied to the pre-coated borosilicate nano-electrospray needles (EconoTip™) as the ion source combined with a nitrogen back-pressure of 0–5 psi to produce a stable flow rate (10–30 nL/min). The cone voltage was 40 V, and a quadrupole analyzer was used to select precursor ions for fragmentation in the hexapole collision cell. Argon was used as the collision gas at a pressure of $6^{-7} \times 10^{-5}$ mbar, and the collision energy was 25–40 V. Product ions were analyzed using an orthogonal TOF analyzer, which was fitted with a reflector, a micro-channel plate detector, and a time-to-digital converter. The samples were ionized in the positive ion mode and their MS data were acquired.

Positional specificity of CML

Thin Layer Chromatography. The positional specificity of *CML* was assessed by thin layer chromatography (TLC) analysis of the lipolytic products of triolein[24] using a silica gel plate (150 Å, Whatman). A reaction mixture (2 mL) of 50 mM Clark and Lubs buffer (pH 9.0) containing *CML* and 20 mM triolein was incubated with orbital shaking (500 rpm) at 40°C for 40 h. After termination of the enzymatic reaction, the reactant was extracted with 0.2 mL chloroform and applied to a TLC plate. Chloroform/acetone/acetic acid (96:4:1, v/v/v) was used as the developing solvent. Lipid standards was used as a reference marker, and the developed spots were visualized using iodine vapor.

High Performance Liquid Chromatography. The hydrolyzed products in the reaction mixture were analyzed using an high performance liquid chromatography (HPLC) system equipped with a Gemini C_{18} column (5 μm, 250 × 4.6 mm), Waters 600 controller pump (Waters Corp., Milford, MA), and Waters 717 plus auto sampler (Waters Corp.). The HPLC

system was equipped with an evaporative light-scattering detector (ELSD 2000, Alltech Corp., Deerfield, MA). Aliquots (150 μL) of the enzymatic reaction mixture were collected and extracted with 0.3 mL diethyl ether, and then the supernatant (20 μL) was injected into the HPLC system. The mobile phase was eluted using a gradient of acetonitrile/acetic acid and dichloromethane at 1.5 mL/min according to a previous report[25] with slight modifications (Supporting Information Data S2). The column temperature was fixed at 40°C, the drift pipe temperature at 70°C, and the nitrogen flow rate at 1.5 mL/min.

Results and Discussion

Screening of lipases and purification of CML

A total of 39 agricultural products were screened for potential candidate natural sources of lipases. The results of lipase activity screening are summarized in Supporting Information Data S1. Among the agricultural samples evaluated, the crude enzyme extract from *C. militaris* exhibited remarkable catalytic performance in terms of both total (12.477 U) and specific (0.497 U/mg protein) activities. Because there are no previous reports on the lipolytic activity of *C. militaris*, *C. militaris* was selected as a potential source of lipases based on these results, and *CML* was purified to characterize its catalytic performance.

Table 1 displays the comprehensive results obtained from *CML* purification. In the initial purification step, the crude extract was precipitated using ammonium sulfate at 40–80% saturation, and the activity was increased 1.86-fold with a 51.96% yield. Subsequently, a stepwise procedure employing a FPLC system using DEAE-Sepharose, phenyl-Sepharose, and Sephacryl S-100 columns was conducted. The ammonium sulfate fraction (40–80% saturation) was loaded onto a DEAE-Sepharose column (i.e., ion exchange chromatography), and the target protein possessing lipolytic activity was eluted with 50–100 mM sodium chloride (Supporting Information Data S3a). This fraction showed a specific activity and yield of 0.864 U/mg protein and 25.03%, respectively. The fraction containing the target protein was applied to a phenyl-Sepharose column (i.e., hydrophobic interaction chromatography) and eluted using a stepwise gradient of ammonium sulfate; next, the lipolytic proteins were eluted with 0.25–0.00 M ammonium sulfate (Supporting Information Data S3b). At this stage of the purification, the specific activity of the target protein fraction was 2.288 U/mg protein, indicating a 45.76-fold increase in purification. Finally, the fraction was subjected to gel permeation chromatography employing a Sephacryl S-100 column, and the lipolytic protein was eluted in an approximately 50 mL volume (Supporting Information Data S3c). The active fraction was loaded again onto the Sephacryl S-100 column and exhibited a single peak (Supporting Information Data S3d). A remarkable increase in specific activity (4.733 U/mg protein, 94.54-fold increase in purification) and a yield of 2.47% were achieved in the overall purification procedure.

Identification of purified CML

Purity, Subunit Structure, Molecular Mass, and Lipolytic Activity. The purity and subunit structure of *CML* was analyzed by SDS-PAGE, and the purified *CML* exhibited a single band with a relative molecular mass of 50–75 kDa, implying a monomeric structure (Figure 1a). The precise molecular mass of *CML* was determined by FPLC and the results of FPLC revealed a molecular mass of approximately 62 kDa for the purified *CML* (Figure 1b). The molecular mass and monomeric structure of *CML* were similar to those of a *Pseudomonas* sp. lipase (66 kDa).[26]

To confirm the actual lipolytic activity of the purified *CML*, in-gel activity staining was carried out using triolein as a natural substrate. As seen in Figure 2, a change in color from pink to yellow was observed in discs impregnated with active enzymes from *C. militaris* and *Chromobacterium viscosum* (a positive control). Contrary to this, thermally inactivated enzymes of both *C. militaris* and *Ch. viscosum* (negative controls) and the blank (50 mM Tris–HCl buffer, pH 7.3) did not exhibit any change in color even after 18 h. The in-gel activity staining revealed that purified *CML* had actual lipolytic activity against triolein.

Partial Protein Sequence Analysis. ESI-MS/MS analysis by electrospray ionization quadrupole-time of flight mass spectrometry (ESI-Q-TOF MS) was employed to identify the partial protein sequence of the purified *CML*. The peptide mixture obtained from proteolytic digestion was separated (Supporting Information Data S4). Three peptides with multiple charge states, the $[M + 2H]^{2+}$ ion of *m/z* 664.9, $[M + 2H]^{2+}$ ion of *m/z* 739.9, and $[M + 3H]^{3+}$ ion of *m/z* 898.8, were observed, and MS/MS spectrum was acquired for each fragmented peptide (Supporting Information Data S5). To validate the protein identification obtained by MS/MS analysis, *de novo* sequencing was performed. The sequences of the identified peptides are shown in Supporting Information Data S6. A sequence database search (NCBI nonredundant database) indicated a high degree of homology with a putative extracellular lipase from *C. militaris*, which was previously not characterized at the protein level (Mascot server, http://www.matrixscience.com).

Table 1. Purification Profile of Lipase from *C. militaris*

Purification steps	Protein (mg/mL)	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Yield (%)	Purification fold
Crude extract	5.06	329.03	6625.30	0.050	100	1
40–80% $(NH_4)_2SO_4$	3.03	170.95	1838.18	0.093	51.96	1.86
Anion exchange chromatography on DEAE-Sepharose	0.72	82.37	95.33	0.864	25.03	17.28
Hydrophobic interaction chromatography on phenyl-Sepharose	0.14	28.13	12.29	2.288	8.55	45.76
Gel permeation chromatography on Sephacryl S-100	0.02	8.14	1.72	4.733	2.47	94.54

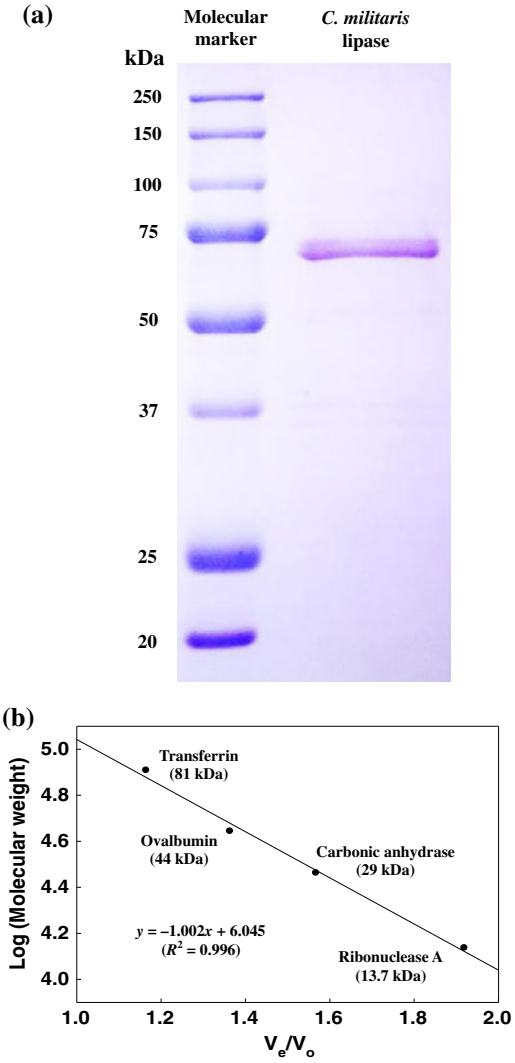

Figure 1. SDS-PAGE of purified *C. militaris* lipase (*CML*) (a) and calibration curve for the molecular mass determination of *CML* by Sephacryl S-100 gel permeation chromatography (b) V_e and V_o represent the elution and void volumes of each protein, respectively.

Based on this result, cDNA from the identified *CML* was generated by cloning and expression of the lipase using the primers M13F/R. The *CML* gene was found to consist of 1743 bp and one open reading frame (ORF). The ORF of *CML* encodes of 579 amino acids (data not shown, ongoing research).

Optimum pH and temperature for CML lipase activity

Optimum pH and pH Stability. The optimal pH for lipase activity of *CML* was determined by evaluating various pH levels from 6.0 to 11.0 using Britton–Robinson buffer containing 50 mM boric acid (H_3BO_3), phosphoric acid (H_3PO_4), and acetic acid (CH_3COOH). As shown in Figure 3a, *CML* exhibited relatively higher activity at neutral to basic pH, and the optimum pH was found to be 9.0, similar to lipases from palm oil fruit and *Carica papaya* latex.[27,28] In addition, the stability of *CML* at different pH levels was evaluated by measuring the residual activity after incubation at different pH levels for 24 h (Figure 3b). Interestingly, *CML* was highly stable over a broad pH range (3.0–12.0), retaining over 80% of its original activity. At pH 2.0, however, the residual activity of *CML* decreased rapidly in a time-dependent manner, with a half-life ($t_{1/2}$) and deactivation constant (k_d) of 17.09 min and 0.04 min^{-1}, respectively. The decrease in catalytic activity at extremely acidic pH may be related to structural changes and unfolding of the lipase.[29]

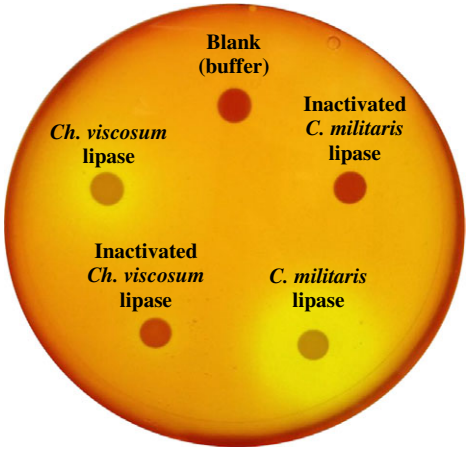

Figure 2. Detection of lipolytic activity on a chromogenic plate containing triolein as the natural substrate. Discs were impregnated with samples before incubation for 18 h at 40°C.

Optimum Temperature and Thermal Stability. The optimum temperature for *CML* activity was determined by measuring its catalytic activity at various temperatures (20–60°C) and found to be 40°C (Figure 4a), similar to lipases from French peanut and coconut seed.[30,31] The thermal stability of *CML* was also evaluated by measuring the residual activity after preincubation at various temperatures (Figure 4b). *CML* was relatively stable below 50°C, while the residual activity decreased sharply at 60°C.

Kinetic parameters for CML hydrolysis

With regard to the enzyme kinetics of *CML*, kinetic parameters including V_{max}, K_m, k_{cat}, and k_{cat}/K_m were determined using a Hanes–Woolf plot. A lipase assay was carried out at the optimal conditions (at pH 9 and temperature 40°C) and varying *p*-NPP concentrations (0.05–0.8 mM). The V_{max}, K_m, and k_{cat} values obtained from *CML*-catalyzed hydrolysis were 4.86 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, 0.07 mM, and 0.29 min^{-1}, respectively (Figure 5). The catalytic efficiency (i.e., k_{cat}/K_m) of the lipase was calculated as the value of 4.11 $min^{-1} \cdot mM^{-1}$. In enzyme kinetics, K_m is defined as the concentration of substrate at which the reaction reaches half of the V_{max}, thus, theoretically, an enzyme with a high K_m requires a greater concentration of substrate to achieve V_{max}. It is worthy of attention that the K_m value of *CML* was relatively lower than those of lipases from *Ralstonia* sp. (2.73 mM)[7] and *Bacillus stearothermophilus* (0.33 mM),[32] indicating that *CML* has a high affinity for its substrate.

Effects of additives on the catalytic performance of CML

Metal Ions. The effect of metal ions on *CML* activity was investigated by measuring relative activity in the presence of 20 mM metal ions (Table 2). The relative activity of *CML* was drastically increased by the stimulation of Ca^{2+} ions (172.4% of the original activity without Ca^{2+}). Conversely,

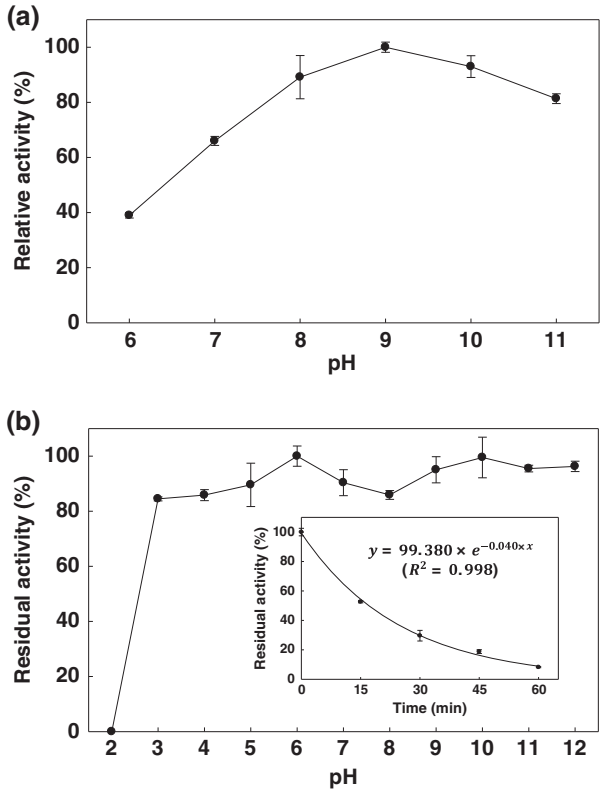

Figure 3. Effect of pH on the activity (a) and stability (b) of *CML*. The inset represents the deactivation profile of *CML* at pH 2.0. Half-life ($t_{1/2}$) refers to the time required for a residual activity to reduce to half and deactivation constant (k_d) was calculated from a gradient of fitting curve.

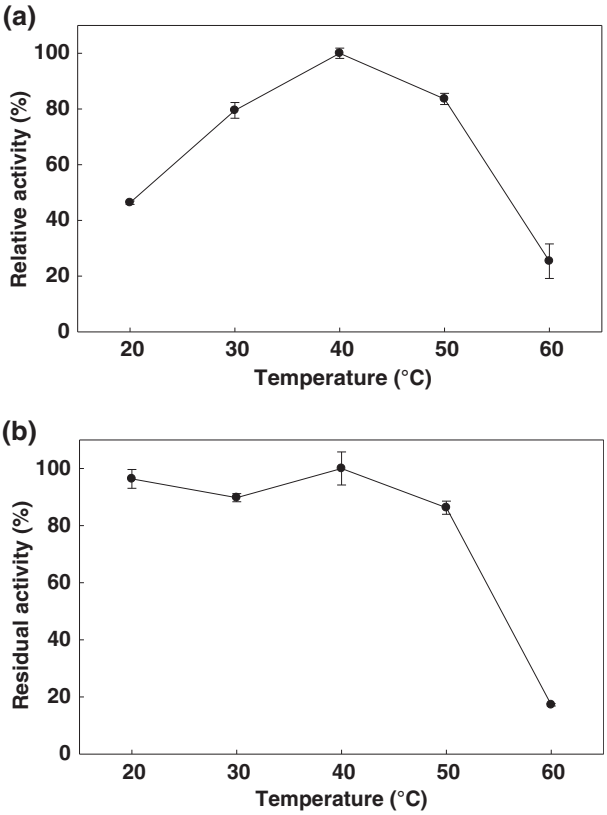

Figure 4. Effect of temperature on the activity (a) and stability (b) of *CML*. The reaction mixture was preincubated for 10 min at each temperature.

CML was completely inactivated in the presence of Fe^{3+} ions (0.0%), and Mg^{2+} and Li^{+} also had an inhibitory effect on *CML*-catalyzed hydrolysis (69.9% and 80.2% of the activity without Mg^{2+} and Li^{+}, respectively). It has been well-documented that metal ions hae different roles in influencing the structure and function of lipases.[9] Generally, metal ions have an inhibitory effect on lipases, which has been explained by the following interaction models. First, most metal ions significantly decrease lipase activity by binding to amino acids within the catalytic site. Further, several metal ions have been reported to hinder the interaction between the amino acid side chains of the lipase, thereby inducing changes within the active sites.[33] On the contrary to this, Ca^{2+} ions may help stabilize the structure of lipases by binding to amino acids such as histidine in the catalytic triad, thereby typically exerting a positive effect on lipase activity.[34]

Surfactants. Regarding the effect of surfactants on *CML* activity, relative lipase activity was assessed in the presence of Triton X-100, polysorbate 20 (Tween 20), SDS, and cetyltrimethyl ammonium bromide (CTAB). The addition of Triton X-100 and Tween 20, typical non-ionic surfactants, decreased lipase activity by 9.7% and 16.2%, respectively. Meanwhile, the inhibitory effect of ionic surfactants was more conspicuous than that of non-ionic surfactants, and SDS (anionic surfactant) and CTAB (cationic surfactant) decreased lipase activity by 24.7% and 25.3%, respectively. This inhibitory effect could be attributed to the interaction between the enzyme and surfactant. According to a previous report, the interactions between nonionic surfactants and enzymes are mainly based on hydrophobic interaction, while ionic surfactants are associated with electrostatic one.[35] Although a hydrophobic interaction might alter the hydrophobicity of the enzyme surface, an electrostatic interaction with an ionic surfactant could lead to complete unfolding of the tertiary structure of the enzyme as a result of an additional hydrophobic interaction.

Positional specificity

Lipases have various positional specificities distinct from those other enzymes, facilitating their specific application in

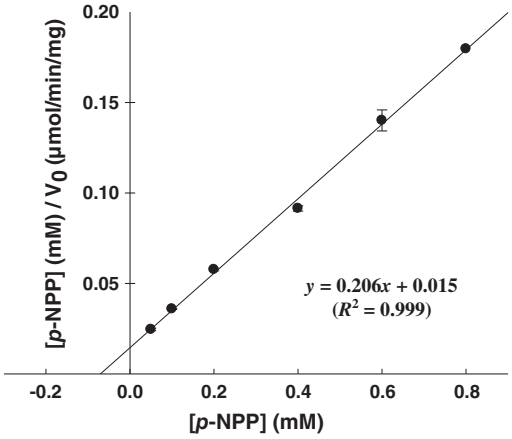

Figure 5. Hanes-Woolf plot for the determination of kinetic parameters in *CML*-catalyzed hydrolysis. Initial velocities (V_0) were calculated by fitting the experimental data to the Michaelis–Menten equation using nonlinear regression.

Table 2. Effects of Various Additives on the Catalytic Efficiency of Lipase from *C. militaris*

Additives		Concentration	Relative activity (%)
Metal ions	LiCl	20 mM	80.2 ± 8.47
	$MgCl_2$	20 mM	69.9 ± 4.48
	$CaCl_2$	20 mM	172.4 ± 5.22
	$FeCl_3$	20 mM	0.0 ± 0.00
Surfactants	Triton X-100	1.0% (w/v)	90.3 ± 1.83
	Sodium dodecyl sulfate (SDS)	1.0% (w/v)	75.3 ± 1.02
	Polysorbate 20 (Tween 20)	1.0% (w/v)	83.8 ± 4.12
	Cetyltrimethyl ammonium bromide (CTAB)	1.0% (w/v)	74.7 ± 4.61
Oxidants	Hydrogen peroxide (H_2O_2)	1.0% (v/v)	85.3 ± 2.61
	Sodium hypochlorite (NaOCl)	0.1% (v/v)	28.9 ± 5.26

diverse industries, such as the food, detergent, pharmaceutical, textile, and cosmetic industries.[36] In particular, lipases have significant specificity for the *sn* position (i.e., regioselectivity), which can be classified into two typical categories. One type of lipases acts preferentially on ester bonds at the *sn*-1 and *sn*-3 position of triacylglycerols, and the other on ester bonds at all positions (i.e., random position). Interestingly, there are few lipases that act at only a single position.[36]

To determine positional specificity, the *CML*-catalyzed hydrolysates, using triolein as the sole substrate, were investigated by TLC (Figure 6a). Reference standards for lipid and *CVL*-catalyzed hydrolysates were used as the control group. First, the *CVL*-catalyzed hydrolysates appeared yellow in all spots: oleic acid, 1,2(2,3)-diolein (major product), 1,3-diolein, and monoolein. These results show that *CVL* acts preferentially on ester bonds not only at the *sn*-1, 3 position of triolein (triacylglycerol) but also at the *sn*-2 position, which has been reported previously.[37,38] Conversely, *CML* released oleic acid, 1,2(2,3)-diolein (major product), and 1,3-diolein (minor product) from triolein, but not monoolein, indicating that *CML* has positional specificity for ester bonds at the *sn*-1 or *sn*-3 position only (i.e., *sn*-1(3) regioselectivity).

To clarify the positional specificity of *CML*, HPLC was performed to detect the *CML*-catalyzed hydrolysates more precisely. The composition of hydrolysates formed by *CML* over time (0–7 days) and the chromatogram obtained on Day 7 are shown in Supporting Information Data S7 and Figure 6b, respectively. As shown in Supporting Information Data S7, oleic acid and 1,2(2,3)-diolein, but not 1,3-diolein or monoolein, were detected in the initial stage of *CML*-catalyzed hydrolysis. By extending the reaction time, the amount of 1,3-diolein increased as a consequence of long incubation at elevated temperature and following acyl migration reaction,[39] while monoolein was still barely detected. This result indicated that *CML* selectively catalyzed the hydrolysis of ester bonds at the *sn*-1 or *sn*-3 position in triacylglycerol, but did not recognize diacylglycerol as its substrates. It was still unknown which *sn*-position can be definitely hydrolyzed by *CML*; however, *CML* was considered to possess distinctive *sn*-1(3) regioselectivity compared with any other lipases.

As mentioned previously, lipases with regioselectivity can be exploited in diverse industries, which involves the enzymatic production of high-value modified oils (e.g., cocoa butter equivalents, human milk fat substitutes, and other specific-structured lipids).[4] Several lipases with *sn*-1(3) regioselectivity, such as microbial lipases from *Pseudomonas fluorescens*[40] and *Yarrowia lipolytica,*[41] have been employed in the industrial applications, producing both 2-monoacylglycerol and 2,3(1,2)-diacylglycerol as major products from substrates. As distinct from these microbial lipases, a novel lipase from *C. militaris* possesses unique *sn*-1(3) regioselectivity to release only diacylglycerols without the subsequent conversion into monoacylglycerols. Thus, it can be utilized for more specific bioconversions such as selective synthesis of diacylglycerols and selective interesterification at *sn*-1(3) position.

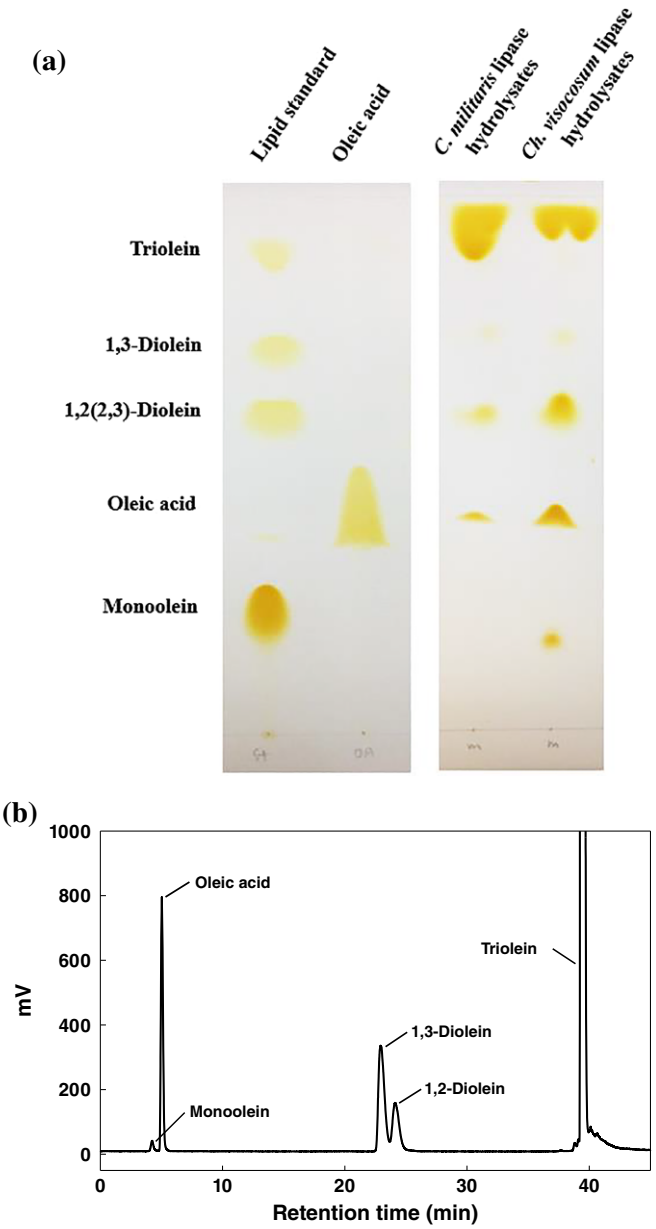

Figure 6. TLC separation (a) and HPLC chromatogram (b) of the products from *CML*-catalyzed hydrolysis to determine the positional specificity of *CML*.

Conclusions

A novel lipase with distinctive positional specificity was purified from *C. militaris*, a natural source for traditional medicines and tonics in East Asia including China, Japan, and Korea. The purified *CML* showed high selectivity for the ester bond at *sn*-1(3) position of triacylglycerols (i.e., *sn*-1(3) regioselectivity), thereby releasing only 2,3(1,2)-diacylglycerol as major products without the subsequent conversion into monoacylglycerols. In addition, the *CML* exhibited remarkable catalytic performance in terms of both high stabilities at a broad pH-range and relatively low K_m value (i.e., high affinity for its substrate). In academic fields, *sn*-1(3) regioselective lipase is essential to identify the fatty acids esterified at *sn*-1(3) position of glycerol backbone, facilitating positional analysis for various lipid derivatives. In the food industry, *sn*-1(3) regioselective lipase can be utilized for the specific production of structured lipids interesterified at *sn*-1(3) position. Hence, the further study of distinctive *sn*-1(3) regioselectivity of *CML* will facilitate its application in both academic and industrial fields.

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