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Cold-active extracellular lipase: Expression in Sf9 insect cells, purification, and catalysis

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ABSTRACT

Cold-active lipases are gaining special attention nowadays as they are increasingly used in various industries such as fine chemical synthesis, food processing, and washer detergent. In the present study, an extracellular lipase gene from *Yarrowia lipolytica* (*LIPY8*) was cloned and expressed by baculovirus expression system. The recombinant lipase (LipY8p) was purified using chromatographic techniques, resulting in a purification factor of 25.7-fold with a specific activity of 1102.9U/mg toward olive oil. The apparent molecular mass of purified LipY8p was 40 kDa. The enzyme was most active at pH 7.5 and 17 °C. It exhibited maximum activity toward medium chain (C10) esters. The presence of transition metals such as Zn^{2+}, Cu^{2+}, and Ni^{2+} strongly inhibited the enzyme activity, which was enhanced by EDTA. The lipase activity was affected by detergents and was elevated by various organic solvents at 10% (v/v). These enzymatic properties make this lipase of considerable potential for biotechnological applications.

1. Introduction

Lipase (EC 3.1.1.3) enzymes are able to hydrolyze triacylglycerol to glycerol and long-chain fatty acids, in addition to the reverse reaction of ester synthesis using a broad range of unnatural substrates. The amount of water in the reaction medium can influence lipase behavior [1,2]. As a consequence of their useful features, such as independence from cofactors, broad range of substrate specificity, chemoselectivity, regioselectivity, stereoselectivity and stability in organic solvents, they have been used in various biotechnological applications, including organic synthesis, detergent manufacturing, food processing, biodiesel production, the chemical industry and biomedical sciences [3–6].

Lipases from different sources have been characterized and commercialized for industrial utilities. However, with the intensification of global warming and the energy crisis, the development of cold-active lipases has attracted increased attention. Cold-adapted lipases possess relatively high catalytic activities at a low-temperature range between 0 and 30 °C whereas normal lipases exhibit dramatically reduced or no catalytic activities [6,7]. Thus, cold-active lipases are desirable in many areas for their lower energy costs, reduced microbial contamination in industrial processes, reduced chemical side-reactions and product stabilization [8–10]. Cold-active lipases primarily originate from psychrophilic and psychrotrophic microorganisms, which exist in low-temperature environments such as deep seawater and Antarctic/polar regions [11–16].

In a previous study, we isolated and characterized the *LipY* lipase from a psychrotrophic *Yarrowia lipolytica* (Bohaisea-9145), which exhibited high catalytic activity at low temperatures [17]. We also cloned the *LIPY8* lipase gene from this strain, which was previously reported by Song et al. [18]. Preliminary experiments indicated that the cold-active feature of the encoded extracellular lipase LipY8p has not been fully characterized. In this paper, we heterologously overexpressed the *LIPY8* gene in a baculovirus expression system, followed by purification and careful characterization of the recombinant lipase, with the aim of facilitating the industrial utility of this cold-active lipase.

Abbreviations: $C_{12}E_8$, octaethylene glycol monododecyl ether; DMF, Dimethylformamide; *p*NPA, *p*-nitro phenyl acetate; *p*NPB, *p*-nitro phenyl butyrate; *p*NPD, *p*-nitro phenyl decanoate; *p*NPL, *p*-nitro phenyl dodecanoate; *p*NPM, *p*-nitro phenyl myristate; *p*NPP, *p*-nitro phenyl palmitate; P_H, polyhedrin; RhB, rhodamine B; RhB-OOe, RhB-olive oil; β-DDM, n-Dodecyl-β-D-Maltoside; β-ME, β-mercaptoethanol; β-OG, n-octyl-β-D-glucoside.

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2. Materials and methods

2.1. Materials

Plasmid pUC57-LipY8 containing the *LIPY8* gene (GenBank accession number DQ200800) without the N-terminal signal peptide coding sequence was obtained from Dr. Sun's laboratory. Enzymes used for manipulating DNA, such as Pfu polymerase, T4 DNA ligase, *Eco*RI and *Not*I were purchased from NEB (Canada). All primers were synthesized by IDT-DNA (Canada). The Bac-to-Bac Baculovirus Expression System kit, which includes the pFastBac1 vector, the *E. coli* competent cell DH10Bac and Cellfectin II reagent was from Invitrogen (Canada). *Spodoptera frugiperda* insect cell line Sf9, Sf-900 III SFM serum-free media and Trypan blue stain (0.4%) were purchased from ThermoFisher Scientific (Canada). I-MAX serum-free media was from Wisent (Canada). T25, T75 cell culture flask and TC 150 × 20 mm cell culture dish was from Sarstedt (Canada). Ni-NTA agarose resin was from ThermoFisher Scientific (Canada). Mono Q HR 5/5 columns were obtained from GE Healthcare (USA). The different lipase substrates were purchased from Sigma and Alfa Aesar. All reagents were of analytical grade. All curve fitting was performed using GraphPad Prism version 7 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. This protein sequence alignment figure was generated with MEGA 7 software [19].

2.2. Construction of pFastBacSP6His vector

The original pFastBac1 vector from Invitrogen does not have a signal peptide and is unsuitable for secreted protein expression. Based on pFastBac1, the signal peptide coding sequence (MGGLLLAAFLALVSVPRAQA) from human lipocalin-6 (NCBI code NM_198946) was added downstream of the polyhedrin (P_H) promoter, followed with a 6His purification tag. This reconstructed vector was named pFastBacSP6His.

2.3. Construction of recombinant transfer vector

The *LIPY8* gene was amplified from pUC57-LipY8 using a primer pair designed for the pFastBacSP6His vector. The signal peptide coding sequence of the *LIPY8* gene was deleted from this construct. The sequence of the forward primer (F) was 5′-GCGCGAATTCGCGGGCGTGAGCCAGGGT -3′, the added *Eco*RI restriction site is underlined. The reverse primer (R) was 5′-GCGCCTCGAGTTATGCGGCCGCGTTTTC -3′ bearing an *Xho*I restriction site (underlined). The PCR was performed using 32 cycles of denaturation at 94 °C for 30 s, an annealing step at 63 °C for 30 s, extension at 72 °C for 1.5 min followed by a 5-min final extension at 72 °C. The amplified product separated on a 1% agarose gel, purified by gel-extraction kit (Qiagen, Canada) and digested with *Eco*RI and *Xho*I, was ligated into the *Eco*RI-*Xho*I sites of the pFastBacSP6His vector (Fig. S1). The recombinant vector pFastBacSP6His–LipY8 was transformed into competent *E.coli* DH5α cells. The integrity of the recovered plasmid was confirmed by restriction endonuclease digestion with *Eco*RI and *Xho*I, and sequencing (service provided by the genome sequencing and genotyping platform of the research center of University Laval) using the primers described above. The recombinant pFastBacSP6His–LipY8 plasmid was extracted from DH5α cells and transformed into competent *E.coli* DH10Bac cells. The cells were spread on blue/white selective LB agar plates containing 50 μg/ml kanamycin, 7 μg/ml gentamicin, 10 μg/ml tetracycline, 100 μg/ml Bluo-gal and 40 μg/ml IPTG, and incubated overnight at 37 °C. Recombinant Bacmid-LipY8 DNA was isolated and integration of the target gene into the Bacmid DNA was detected by PCR using the pUC/M13 forward and pUC/M13 reverse primers as described by the Bac-to-Bac Baculovirus Expression System kit user manual.

2.4. Cell culture and virus preparation

In the present study, Sf9 cells were all adherently cultured at 27 °C unless otherwise stated. Cells grown in T25 flasks with Sf-900 III SFM medium were used for P1 viral stock generation, cells in T75 flasks with Sf-900 III SFM medium were used for P2 viral stock preparation and cell propagation, and cells in TC 150 × 20 mm Petri dishes with I-MAX serum-free medium were used for protein expression. Passage of Sf9 cells was performed as described in the Insect Cell Lines, version K 2002 (Invitrogen). Briefly, when cells reached confluence (usually 3 days) in a T75 flask, they were dislodged by tapping the flask. Cells from six T75 flasks were pooled and pelleted by centrifugation at 500×g for 5 min. After removing the excess medium, the cell pellet was resuspended in 30 ml of the remaining medium (around 4×10^6 cells/ml). Thereafter, the cells were aliquoted into six new T75 flasks (2 ml per flask) and three TC 150 × 20 mm dishes (6 ml per dish) to which 10 ml and 24 ml fresh medium were added, respectively.

The P1 viral stock preparation was performed in a T25 flask containing 5 ml of Sf-900 III SFM medium. Sf9 cells were placed at 70% confluence (approximately 2.5×10^6 cells) 24 h before the transfection. The monolayer of Sf9 cells was transfected with Cellfectin II reagent according to the manufacturer protocol using purified recombinant Bacmid DNA. Four days after transfection, the virus from the cell culture medium was harvested by centrifugation at 500×g for 5 min. The obtained P1 viral stock was then used to generate high titer P2 viral stocks through an infection of Sf9 cells in T75 flasks using a multiplicity of infection (MOI) of 0.1. The titer of the baculoviral stocks was determined by the plaque assay described in Bac-to-Bac Baculovirus Expression System, version E 2009 (Invitrogen). Two percent (v/v) fetal bovine serum was added to all viral stocks, which were stored at 4 °C and protected from light. The negative control viral stocks were prepared by the same procedures using the wild-type Bacmid DNA

2.5. Expression of recombinant LipY8p lipase

To monitor the time course of LipY8p expression and cell viability, Sf9 cells were placed in two six-well plates, and 8×10^5 cells were seeded in each well. Virus from the P2 stock was added into each well at an MOI of 15, and the negative control virus was added in one well. Every 24 h in the 7-day expression, cells in one well were gently detached by blowing with a pipette. The viability of the cells was obtained by counting live and dead cells using trypan blue as a dye. In addition, 1 ml of the medium was collected and centrifuged at 500×g for 5 min, in which the supernatant was retained for lipase activity assay. The supernatant of the medium in the negative control well was collected on the fourth day in the same manner and used as a negative control in the following experiment.

In the large-scale expression of LipY8p, six TC 150 × 20 mm dishes were used on each batch. Cells were plated as described above and infected with P2 stock virus after 24 h of incubation using an MOI of 15. The medium was harvested 7 days post infection.

2.6. Purification of recombinant LipY8p lipase

All purification performances were carried out at 4 °C unless otherwise stated. The medium from the TC 150 × 20 mm dishes were transferred to 50 ml tubes, and the detached cells and debris were precipitated by centrifugation at 500×*g* for 10 min. The supernatant (180 ml in total) were combined and Tris buffer pH 8.0 was added to

a final concentration of 50 mM. In order to reduce the working volume, ammonium sulfate precipitation was used as the first step of purification. The ammonium sulfate powder was gradually added to 75% saturation under constant stirring over a 2 h period. The protein pellet was collected by centrifugation at 3200 × g for 30 min and resuspended in 50 ml of buffer A (20 mM Tris−HCl, pH 8.0). The solution was then centrifuged at 32,000 rpm for 30 min and the supernatant was retained for further purification.

For Ni-NTA affinity chromatography, 50 ml of the lipase solution was applied to a Ni-NTA column (10 ml, 1.6 × 5 cm) equilibrated with buffer A. The lipase was eluted by a stepwise imidazole gradient with increasing concentrations in buffer A. The eluted fraction was collected and the solution buffer was changed to buffer B (50 mM Tris pH 7.5, 20% (v/v) glycerol, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA)) by repeated concentration and dilution with a Centricon filtration unit (EMD Millipore, Canada) [20].

Ion-exchange chromatography was performed on an AKTA Explorer FPLC system (GE, USA) with a Mono Q HR 5/5 column. The lipase solution was loaded onto the column equilibrated with buffer B and eluted with a linear salt gradient using 1 M NaCl (pH 7.5).

2.7. SDS-PAGE and protein concentration determination

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12% polyacrylamide gel on a vertical mini-gel apparatus (Bio-Rad, Canada). Molecular mass markers were obtained from Bio-Rad. Proteins were stained with Coomassie Brilliant Blue R250 (Bio-Rad, Canada).

The concentration of LipY8p was determined by the Bradford assay [21], from the absorption at 595 nm, using bovine serum albumin as a standard. The optical density was measured with a UV/Vis spectrophotometer (UV70, Beckman Coulter, USA).

2.8. Lipase assay

Lipase activity was measured spectrophotometrically (410 nm) using *p*-nitro phenyl dodecanoate (*p*NPL) as substrate by the method described by Winkler and Stuckmann [22]. In brief, 100 μl of substrate stock solution (0.3% (w/v) *p*NPL) was added to 1 ml standard reaction buffer (50 mM Na_2HPO_4 pH 7.5, 0.2% (w/v) Na deoxycholate, 0.1% (w/v) gum arabic) and incubated in a water bath with constant shaking at 200 rpm at 22 °C for 5 min. The reaction was initiated by the addition of 2 μl of enzymes and terminated by the addition of 1.2 ml acetone-ethanol (1:1) solution. The reaction duration was 2 min, and the release of *p*-nitro phenyl (*p*NP) was recorded at 410 nm using the UV70 spectrophotometer. Enzyme activity was calculated by constructing a standard curve with *p*NP under the same buffer conditions as the reaction. One unit (U) of lipase activity was defined as the amount of enzyme that liberated 1 μmol of *p*NP per min under standard assay conditions.

Lipase activity was also measured by the fluorescence-based rhodamine B (RhB) assay using olive oil emulsion [23] with some modifications. An RhB-olive oil emulsion mixture (RhB−OOe) containing 50 mM Na_2HPO_4 (pH 7.5), 1% (w/v) gum arabic, 0.001% (w/v) RhB, and 2% (v/v) olive oil was emulsified with a DrinkMaster for 5 min, and then the pH was adjusted. The enzymatic assays were performed in a 45 mm × 12.5 mm quartz cuvette with magnetic stirring at pH 7.5 and 22 °C using a fluorescence spectrofluorometer (HORIBA Fluorolog, USA). The enzymatic reactions were initiated by the addition of 2 μl of enzyme solution to 1 ml of emulsion. The liberated fatty acids were calculated from the fluorescence emitted at 580 nm (excitation wavelength is 350 nm). The reaction emulsion with heat-denatured enzyme solution was measured in the same way and used as a blank control. A standard curve for oleic acid in the presence of RhB and gum arabic was prepared, and a linear regression was performed allowing the calculation of lipase activity. The fluorescence emission changes were converted into the hydrolysis rate using polynomial equations. One unit (U) of lipase activity was defined as the amount of enzyme releasing 1 μmol of fatty acid per min under the assay conditions.

2.9. Effect of temperature and pH on lipase activity and stability

The activity of the lipase at different temperatures and pH was determined by a *p*NP release assay using *p*NPL as substrate. To investigate temperature stability, the lipase solution was incubated for 1 h at different temperatures ranging from 0 to 45 °C. For pH stability, the lipase solution was incubated for 1 h at different pH at 22 °C. Buffers used for different pH values included 50 mM sodium phosphate buffer (pH 6–8), 50 mM Tris−HCl (pH 8.5, 9), 50 mM CHES (pH 9.5), and 50 mM CAPS (pH 10). Residual activity was measured by *p*NP release assay using *p*NPL as substrate.

2.10. Substrate specificity

For the determination of substrate specificity, several *p*-Nitro phenyl esters including *p*NP-acetate (*p*NPA, C2), *p*NP-butyrate (*p*NPB, C4), *p*NP-decanoate (*p*NPD, C10), *p*NP-dodecanoate (*p*NPL, C12), *p*NP-myristate (*p*NPM, C14), and *p*NP-palmitate (*p*NPP, C16) were used as substrates.

2.11. Effect of metal ions and inhibitors on lipase activity

The *p*NP release assay was used to determine the effect of metal ions and inhibitors on lipase activity. The reaction buffer was preloaded with different chemicals at the desired final concentrations.

2.12. Effect of detergents on lipase activity and stability

The effects of detergents on enzyme activity and stability were evaluated by *p*NP release assay using *p*NPL as substrate. For the effect on lipase activity, different detergents were pre-loaded into the reaction buffer. To determine lipase stability, the purified enzyme was pre-incubated with various detergents for 2–72 h at 22 °C and the residual activity was determined by standard assay. Several detergents were used in this study including SDS, Triton-X100, Tween 20, NP40, n-Dodecyl-β-D-Maltoside (β-DDM), n-octyl-β-D-glucoside (β-OG) and octaethylene glycol monododecyl ether ($C_{12}E_8$).

2.13. Effect of organic solvents on lipase activity and stability

Seven different organic solvents including methanol, ethanol, isopropanol, acetone, Dimethyl sulfoxide (DMSO), Dimethylformamide (DMF) and ethyl ether were used to determine their effects on lipase activity and stability. The lipase residual activity was measured by *p*NP release assay using *p*NPL as substrate. To determine the effects on enzyme activity, the standard reaction buffer was prepared with the addition of different solvents to yield the desired final solvent concentrations (10 or 20% v/v). For the lipase stability test, the enzymes were incubated with different solvents (20% v/v) for 2 h at 22 °C, and the residual activity was measured.

3. Results and discussion

3.1. Protein sequence analysis

LipY8p contains 371 amino acids (AAs) with a 28-AAs signal sequence, resulting in a 343-AAs mature protein. The lipase

engineering database search indicated that the lipase belongs to the abH23 superfamily with a highly conserved GX pattern in the amino acid sequence [24]. Sequence alignment between LipY8 and the closely related *Y. lipolytica* lipase genes exhibited 99.2% identity with LipY (UniProt: E0Z5H2), 99.2% with Lip8 (UniProt: Q872L3), 78.1% with Lip7 (UniProt: Q872L4) and 40.9% with Lip2 (UniProt: Q9P8F7). Blast analysis with the UniProt database revealed homology of LipY8 to several yeast lipases such as those from *Candida galli* (CgLIP8, 91.3%; CgLIP7, 77.5%), *Candida deformans* (CdLIP3, 90.7%; CdLIP2, 71%), and *Candida alimentaria* (CaLIP7, 66%) (Fig. 1). The conserved GHSLG(G/A)A motif characteristic of the triacylglycerol hydrolases, shared by the filamentous fungi lipase family [25], was found at position ~190. The serine, aspartic acid, and histidine residues of the lipase catalytic triad were located in the conserved positions. Eight highly conserved Cys residues were also found at conserved positions in all of these lipases and are hypothesized to form disulfide bridges (Fig. 1).

3.2. Cloning and expression of LipY8p lipase

Pichia Pastoris has historically been the first choice for over expression of yeast genes [26]. However, in the present study, LipY8p was over-expressed as a secreted protein in baculovirus-infected insect cells, providing sufficient post-translational modification. The recombinant baculovirus, which contains the *LIPY8* gene, was prepared as described in Materials and Methods. The P1 viral stock typically had a plaque forming units (pfu) of 2×10^6 per ml, which was then amplified to about 3.7×10^8 pfu/ml in the P2 viral stock.

The Sf9 cells were infected with *LIPY8* recombinant virus or with wild-type recombinant virus. The time course of recombinant

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                                                                                                   80
LIPY8  : MVSLSARIKD FFSVLLLGAA TITP------ ------STQT AGVSQGFYDF ARDFAHLSNI AYCVNAPITP LNPDFTCGNS
LIPY   : MVSLSARIKD FFSVLLLGAA TITP------ ------STQT AGVSQGFYDF ARDFAHLSNI AYCVNAPITP LNPDFTCGNS
LIP8   : MVSLSARIKD FFSVLLLGAA TITP------ ------STQT AGVSQGFYDF ARDFAHLSNI AYCVNAPITP LNPDFTCGNS
CgLIP8 : MVSLSARIKD FFSVLLLGAA EITP------ ------STQT AGVSQGFYDF ARDFAHLSNV AYCVDAPITP LKADFTCGNA
CdLIP3 : MVTLSARLKD FFSVLLLGAA QITP------ ------STQT AGVSQGFYDF ARDFAHLSNI AYCVDAPITP LNPDFTCGNS
LIP7   : MVSFGARIKD FFSVLLFGAA STSS------ ------STKT ALVSQGFYDA ALDFSHLSNI AYCVNAPITP LKSDFSCGQS
CgLIP7 : MVNFGARVKD FFSVLLFGAA STTS------ ------STKT ALVSQGFYDA ALDFSHLSNI AYCVNAPITP LKDDFSCGQS
CaLIP7 : MVQIG-KFTE WLSVTLWGAA ATTS------ ------STAT SSITQNTYDF VRTFSHLSNV AYCVKAPIKS LDDNFQCGNA
LIP2   : MKLSTILFTA CATLAAALPS PITPSEAAVL QKRVYTSTET SHIDQESYNF FEKYARLANI GYCVGPGTKI FKP-FNCGLQ
CdLIP2 : MVNFGARIKD FFSVLLFGAA STSS------ ------SSKT ALVSQGFYDA ALDFSHLSNV AYCVHAPITP LKDDFSCGQS

                                                                                                  160
LIPY8  : CKHFPDIELV KTFGGNFFKT SITGYLAVDH VKKEKYVVFR GTFSLADAIT DMQFLLSPFL VDVPALNTFS ANDTTAEAQT
LIPY   : CKHFPDIDLV NTFGGNFFNT SITGYLAVDH VKKDKYVVFR GTFSLADAIT DMQFLLSPFL VDVPALNTFS ANDTTADAQT
LIP8   : CKHFPEIELV KTFGGNFFKT SITGYLAVDH VKKEKYVVFR GTFSLADAIT DMQFQLSPFL VDVPALNTFS ANDTTAEAQT
CgLIP8 : CKHFPDIELV KTFGGDFFDT SITGFLAVDH VKKEKYVVFR GTFSLADAIT DMQFQQSPFL VDVPAMNTFS ANDTAAEAQT
CdLIP3 : CKYFPDIELV KTFGGDFFET SITGYLAVDH VKKEKYVVFR GTFSLGDAIT DFQFQQSPFL VDVPALNTFS ANDTSAEAQI
LIP7   : CVHFPDIELV HIFGGDFFST SITGYLALDH VKKEKYVVFR GTFSIADAIT DIQFQQSSFL VNVPALNTFT ANDTAPEAQI
CgLIP7 : CVHFPDIELV HTFGGDFFST SITGYLALDH VKKEKYVVFR GTFSIADAIT DIQFQQSSFL VNVPALNTFT ANDTSAEAQI
CaLIP7 : CKNFPNMELV TTFGGDFFQT SITGFLALDH VKKEKYVVYR GTFSIADVIT DLQFQQSGFL VDAPALNSLK ANDTSESAKI
LIP2   : CAHFPNVELI EEFHDPRLIF DVSGYLAVDH ASKQIYLVIR GTHSLEDVIT DIRIMQAP-L TNFDLAANIS STAT------
CdLIP2 : CVHFPDMELV TTFGGDQFST SITGYLALDH VKKEKYVVFR GTFSIPDAIT DIRFQQSSFL VNVPALNTFA PNDPSGEAQI

                                                                                                  240
LIPY8  : HCEGCKIHDG FSKAFTETWG NIGEDLQKHL DANPDYQLYV TGHSLGAAVA LLGATSIKLK GYDPILINYG QPRVGNKPFA
LIPY   : HCEGCKIHDG FSKAFTETWG NIGEDLQKHL DANPDYQLYV TGHSLGAAVA LLGATSIKLK GYDPILINYG QPRVGNKPFA
LIP8   : HCEGCKIHDG FSKAFTETWG NIGEDLQKHL DANPDYQLYV TGHSLGAAMA LLGATSIKLK GYDPILINYG QPRVGNKPFA
CgLIP8 : QCEGCKIHDG FSKAFTETWG EIGEDLHKHL DSNPDYQLFV TGHSLGAAMA LLGATSIKLK GYDPILINYG QPRVGNKPFS
CdLIP3 : QCEGCKIHDG FSKAFTETWG NIGEDLKKHL DSNPDYQLYV SGHSLGAAMS LLGATSFKLK GYDPILINYG QPRVGNKPFS
LIP7   : DCKQCKIHDG FSKAFTETWH NIGDLLEQHL DSYPDYQLYV TGHSLGAAMA LLGATSIKLR GYDPILINYG QPRVGNKAFA
CgLIP7 : DCKHCKIHDG FSKAFTETLH NIGPQLKQHL DSYPDYQLYV TGHSLGAAMA LLAGTSIKLK GYDPILINYG QPRVGNKAFA
CaLIP7 : DCKDCKIHDG FKKANTETMT NIGDDLKKHL DSYPDYKLYV TGHSLGAAQA LLSAISIKLQ GYDPTLINFG QPRVGNAAFA
LIP2   : -CDDCLVHNG FIQSYNNTYN QIGPKLDSVI EQYPDYQIAV TGHSLGGAAA LLFGINLKVN GHDPLVVTLG QPIVGNAGFA
CdLIP2 : DCKECKIHDG FSRAFTETLH NIGPVLQQHL DSYPEYQLYV TGHSLGAAMA LLAGTSIKLQ GYDPIVINYG QPRVGNRAFA

                                                                                                  320
LIPY8  : EFINKLWFGE GNG--LEITP ERKLYRMTHW NDIFVGLPNW EGYTHSNGEV YINNRFINPP LKDVISCAGG ENSKCYRSSF
LIPY   : EFINKLWFGE GNG--LEITP ERKLYRMTHW NDIFVGLPNW EGYTHSNGEV YINNRFINPP LKDVISCAGG ENSKCYRSSF
LIP8   : EFINKLWFGE GNG--LEITP ERKLYRMTHW NDIFVGLPNW EGYTHSNGEV YINNRFINPP LKDVISCAGG ENSKCYRSSF
CgLIP8 : EFINKLWFGD DNG--LEIKP ERRLYRMTHW NDIFVGLPNW EGYTHSNGEV YINNRFINPP LKDVISCAGG ENSQCYRSSF
CdLIP3 : EFINKLWFGD GNG--LEITP ERRLYRMTHW NDIFVGLPNW EGYTHSNGEV YIKNRFINPP VSDVISCAGG ENSQCYRSSF
LIP7   : DYISALWFGN GDG--LEINQ QRRLYRMTHW NDVFVGLPNW DGYTHSNGEV YIKGKYVNPP LKDVFSCAGG ENSKCYRSEF
CgLIP7 : DYISTLWFGK GDG--LEINK DRRLYRMTHW NDVFVGLPNW DGYTHSNGEV YIKGKYVNPP LKDLMSCAGG ENSKCYRSTF
CaLIP7 : NYVDRLFFGE DAG--LSVTS DRKLYRLTHW NDVFVGLPNW DGYQHNVGEV FIDWRFTNPP LQYVKSCEGG ENPKCYRKDF
LIP2   : NWVDKLFFGQ ENPDVSKVSK DRKLYRITHR GDIVPQVPFW DGYQHCSGEV FIDWPLIHPP LSNVVMCQGQ SNKQCS-AGN
CdLIP2 : DYISTLWFGN GDG--LEINR QRRMYRMTHW NDVFVGLPNW DGYTHSSGEV YIKGKWVNPA LRDVFSCAGG ENPECYRSTF

                                                                                     389
LIPY8  : SLLSQINLLQ NHLAYIDYIG YCALNIGRRE LA----DQEH YTGPYYYGHR SEEDFKKLGL ELSTPQVEN
LIPY   : SLLSQINLLQ NHLAYIDYIG YCALNIGRRE LA----DQEH YTGPYYYGHR SEEDFKKLGL ELSTPQVEN
LIP8   : SLLSQINLLQ NHLAYIDYIG YCALNIGRRE LA----DQEH YTGPYYYGHR SEEDFKKLGL ELSTPQVEN
CgLIP8 : NLLSQINLLQ NHLAYIDYIG YCALNIGRRE LA----DQPH YKGNYFYAHR TEEDFKKLGL ELSTRTKQ-
CdLIP3 : NILSQINLLQ NHLAYIDYIG YCALNIGRRE LA----DQKK YTGNYYYAHR TEEDFKKLGL ELGPRAAKQ
LIP7   : NLLAQINLLQ NHLCYIDYIG FCALNVGRRE LN----DLPH YNGPYKYGHK TEEQFIAEGL ELSN-----
CgLIP7 : NLLAQINLLQ NHLCYIDYIG FCALNVGRRE LN----DMPH YTGPYTYGHK TEEDFVAEGL ELSN-----
CaLIP7 : NLLAQINLLQ NHLAYIDYIG YCTLNIGRRA QM----NLPR YTGPNTYAHK TEDD-----V ELALY----
LIP2   : TLLQQVNVIG NHLQYFVTEG VCGI------ ---------- ---------- ---------- ---------
CdLIP2 : NLLAQINLLQ NHLCYIDYIG FCALNVGRRE VNELQTDLPS YTGPYRYGNK TEEDFVREGL ELAQ-----
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Fig. 1. Multiple sequence alignments between LipY8p and highly homologous lipases from *Y. lipolytica* (LIPY, E0Z5H2; LIP8, Q872L3; LIP7, Q872L4; LIP2, Q9P8F7), *Candida galli* (CgLIP8, A0A078BRV6; CgLIP7, A0A078BNS3), *Candida deformans* (CdLIP3, Q875G8; CdLIP2, Q875G9) and *Candida alimentaria* (CaLIP7, A0A078BMP3). Cysteine residues are marked in gray and conserved residues of the active site including serine, aspartic acid and histidine are marked in black.

extracellular lipase production was monitored by analyzing the activity of the culture medium every 24 h for up to 7 days (Fig. 2A). Meanwhile, the cell count and cell viability versus time were also measured (Fig. 2A and B). The initial lipase activity resulted from the enzyme activity with the introduction of the virus from the viral stock. Maximum lipase activity was attained 3 days post infection when cell viability decreased to around 75%. Thereafter, lipase activity stabilized until at least 7 days post-infection, indicating strong resistance to protein degradation. The maximal value of lipase specific activity in culture medium reached 17.37 U/mg by *p*NP release assay at 3 days post-infection. No activity was detected in the wild-type recombinant virus-infected cell group.

3.3. Purification of recombinant LipY8p lipase

Lipase purification was performed using ammonium sulfate precipitation followed by Ni-NTA affinity and Mono Q anion exchange chromatography as described in Materials and Methods. The ammonium sulfate precipitation step slightly increased the specific activity of LipY8p by 1.46 times. However, further purification was required according to the result from SDS-PAGE (Lane 3 in Fig. 3C). In the Ni-NTA affinity chromatography, Peak 3 had the highest lipase activity (Fig. 3A) and SDS-PAGE showed the accumulation of the band at 40 kDa (Lane 2 in Fig. 3C). The active fractions from Ni column were then pooled and applied to a Mono Q HR 5/5 column. The LipY8p was eluted at about 10 mS/cm conductivity, resulting in a clear band in SDS-PAGE (Fig. 3B and Lane 1 in Fig. 3C). The purification process resulted in an approximate 25.7-fold purification factor and a final recovery of 23.2% of the enzyme protein with a molecular mass of 40 kDa and specific activity of 446.85 U/mg by *p*NP release assay (Table 1).

3.4. Effect of temperature and pH on lipase activity and stability

The insect cells expressing LipY8p lipase exhibited an extraordinary cold-active property that was not observed in the previous report [18]. Cold-active lipases show optimal reaction temperatures at lower than 30 °C [27]. LipY8p had optimal activity at a temperature of 17 °C and retained 70.6% of the highest activity at 8 °C, which is similar to the reported cold-active lipases from *P. lynferdii* NRRL Y-7723, *Geotrichum* sp. SYBC WU-3 and *Candida albicans* [7,27,28]. The optimal temperature of LipY8p is lower than many reported cold-active lipases [10,12,14,29–34], but higher than the lipase from *Microbacterium luteolum* [35]. Moreover, similar to these reported cold-active lipases [7,27], the activity of LipY8p drastically declined as the temperature rose above 25 °C and approached inactivition at temperatures above 45 °C (Fig. 4A). However, LipY8p showed less thermostability than the cold-active lipases from *P. lynferdii* NRRL Y-7723, *Geotrichum* sp. SYBC WU-3 and *Candida albicans* [7,27,28]. Its activity was essentially maintained from 0 to 30 °C temperature, whereas a sharp decrease in stability was observed as temperatures rose above 35 °C (Fig. 4A).

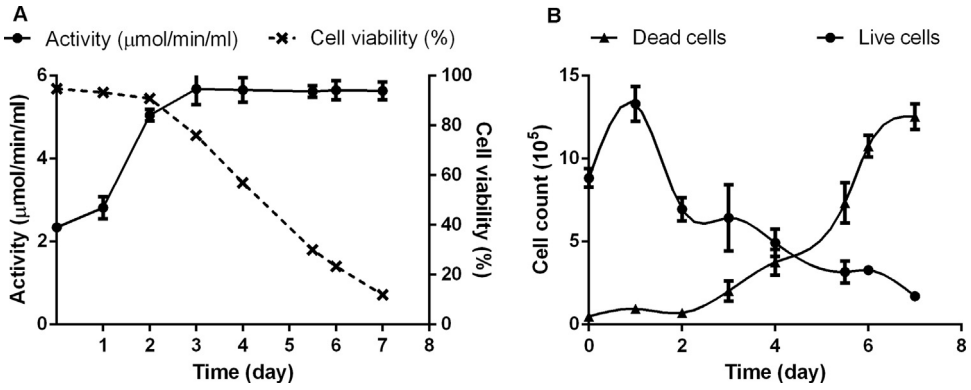

Fig. 2. Time course of LipY8p expression. (A) The plot of lipase activity and cell viability against time. (B) The plot of the cell count versus time.

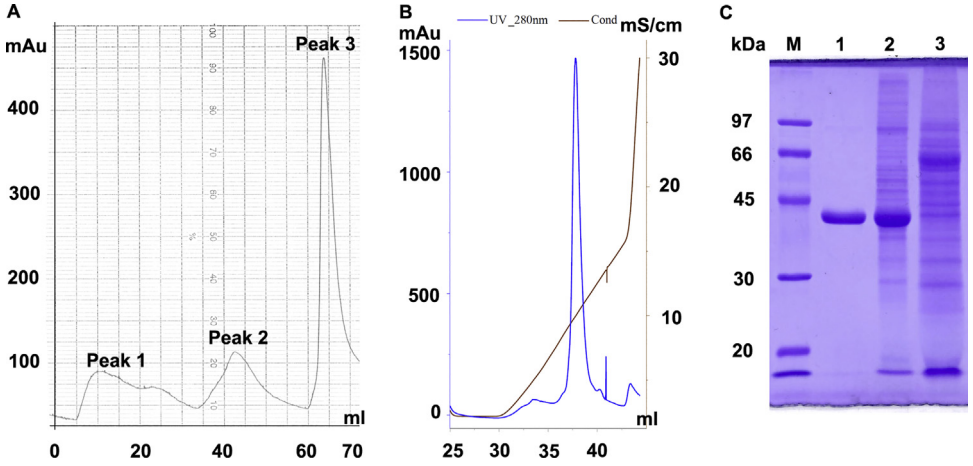

Fig. 3. Purification of the recombinant LipY8p lipase. (A) Ni-NTA affinity chromatography. Peak1, peak2, and peak3 were eluted by buffer A containing 5 mM, 20 mM and 150 mM imidazole, respectively; (B) Mono Q anion exchange. LipY8p was eluted at around 10 mS/cm conductivity. (C) The SDS-PAGE analysis of purified LipY8p. M, protein marker; 1, Mono Q anion exchange chromatography; 2, Peak 3 of Ni-NTA affinity chromatography; 3, ammonium sulfate precipitation.

Table 1
A summary of LipY8p lipase purification.

Purification steps	Protein (mg)	Lipase activity (kU[a])	Specific activity (U/mg)	Yield (%)	Purification (fold)
Culture medium	104.37	1.81	17.37	100	1
Ammonium sulfate	61.93	1.57	25.36	86.63	1.46
Ni-NTA column	3.61	0.64	177.08	35.30	10.19
Mono Q column	0.94	0.42	446.85	23.19	25.72

[a] Activity test was carried out by spectrophotometer in phosphate buffer pH 7.5 at 17 °C, using *p*NPL as substrate. One unit (U) of enzyme activity was defined as the amount of enzyme required for the liberation of 1.0 μmol *p*-nitrophenol per min under the assay conditions.

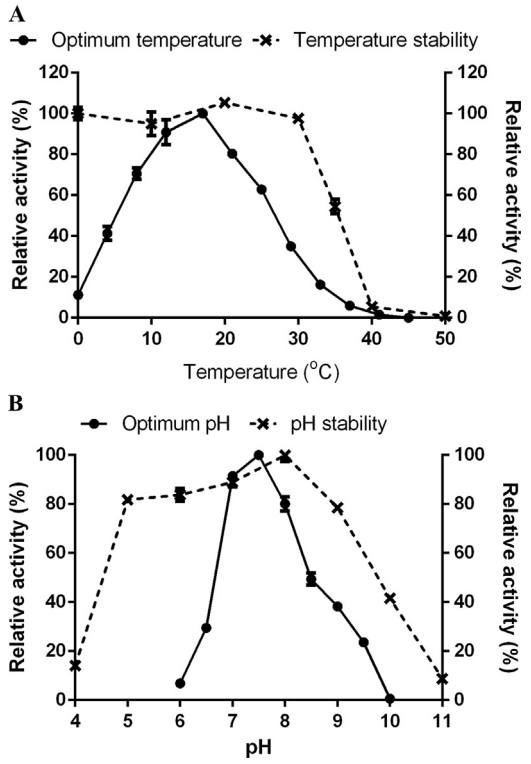

Fig. 4. Optimum temperature (A) and optimum pH (B) on the activity and stability of LipY8p lipase.

The majority of cold-active microbial lipases exhibit optimal activity at near neutral or alkaline conditions [32]. LipY8p showed considerable stability over the pH ranges 5–9 with optimal activity at pH 7.5 (Fig. 4B), which is similar to the lipases from *Rhizomucor endophyticus* [32] and *Candida zeylanoides* [29]. The wide range of stability of the lipase indicated its potential use in both acidic and alkaline conditions.

3.5. Substrate specificity of lipase

To investigate the substrate specificity of LipY8p, various lengths of *p*-Nitro phenyl esters were used as the substrates. The lipase showed the highest specific activity toward *p*-nitro phenyl decanoate (*p*NPD) (C10) (relative activity of 155.0%) at 791.3 ± 9.5 U/mg. *p*-nitro phenyl palmitate (*p*NPP) (C16), *p*-nitro phenyl myristate (*p*NPM) (C14) and *p*-nitro phenyl butyrate (*p*NPB) (C4) were equally utilized as substrates. The shorter carbon chain ester (C2) was poorly hydrolyzed (Fig. 5). This indicated that LipY8p preferred medium chain esters [18], which is a typical property of the GX class lipase [24]. Similar results were reported for cold-active lipase from *Pseudomonas proteolytica* (GBPI_Hb61) [10] and *Pseudomonas* sp. strain KB700A [36]. However LipY8p lipase exhibited much higher hydrolysis activity toward olive oil with a specific activity of 1102.9 U/mg (Fig. 6), which was much higher than the AMS8 lipase (394.43U/mg) from Antarctic *Pseudomonas* sp. [33].

3.6. Effect of metal ions and inhibitors on lipase activity

Lipase activity was assayed in the presence of various metal ions at 1 mM concentrations (Table 2). Remarkable inhibition of the enzyme activity was observed in the presence of various transition metals such as Zn^{2+}, Cu^{2+}, as well as Ni^{2+}. Similarly, cold-active lipases from *Psychrobacter cryohalolentis* $K5^T$ [34] were reported to be inhibited by these three metals, and lipases from Antarctic *Pseudomonas* (AMS8 lipase) [33] and *Pseudomonas* sp. Strain B11-1 [12] were inhibited by Zn^{2+}, Cu^{2+}, and Fe^{2+}. The lipase activities were fairly stable in the presence of Mg^{2+} and Ca^{2+} and activated by K^+ (118.4 ± 5.8%). In contrast, the presence of EDTA (1 mM) resulted in a considerable stimulation of lipase activity (136.1 ± 4.5%), and the inhibitory effect of Ni^{2+} was eliminated by the addition of EDTA, indicating that the lipase was not a metalloenzyme. Similar results were reported for the YlLip2 lipase from *Yarrowia lipolytica* [37] and the lipase from *Psychrobacter cryohalolentis* $K5^T$ [34]. In contrast, certain cold-active lipases require metal ions as the enzyme cofactor [36]. Of interest are the failed attempts to inhibit lipase activity through reduction of disulfide bonds in the protein despite sequence analysis revealing that the protein may contain several conserved disulfide bonds (Fig. 1). The addition of different concentrations of β-mercaptoethanol (β-ME) to the reaction buffer gave rise to significant activation of lipase activity. However, the simultaneous addition of 1 mM β-ME and 0.1% (w/v) n-octyl-β-D-glucoside (β-OG) led to a marked inactivation of the enzyme (77.2 ± 2.1%). This observation indicated that additional destabilizing factors, such as a detergent, were necessary for the reductant to gain access to the disulfide bond [38]. The activation effect of β-ME on enzyme activity was also reported with lipases from the *P. aeruginosa* mutant [39] and *S. bambergiensis* OC 25-4 [40] where lipase activity was enhanced by 19.6% and 8%, respectively, after treatment with a concentration of

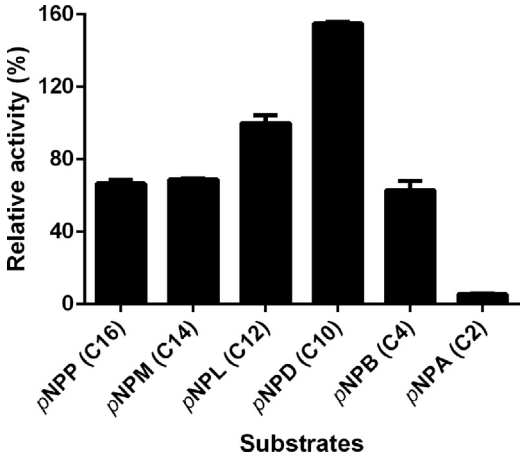

Fig. 5. Substrate specificity of LipY8p lipase against different chain length *p*NP esters. Activity of *p*NP dodecanoate (*p*NPL) was considered as 100%. *p*NPP, *p*NP palmitate; *p*NPM, *p*NP myristate; *p*NPD, *p*NP decanoate; *p*NPB, *p*NP butyrate; *p*NPA, *p*NP acetate.

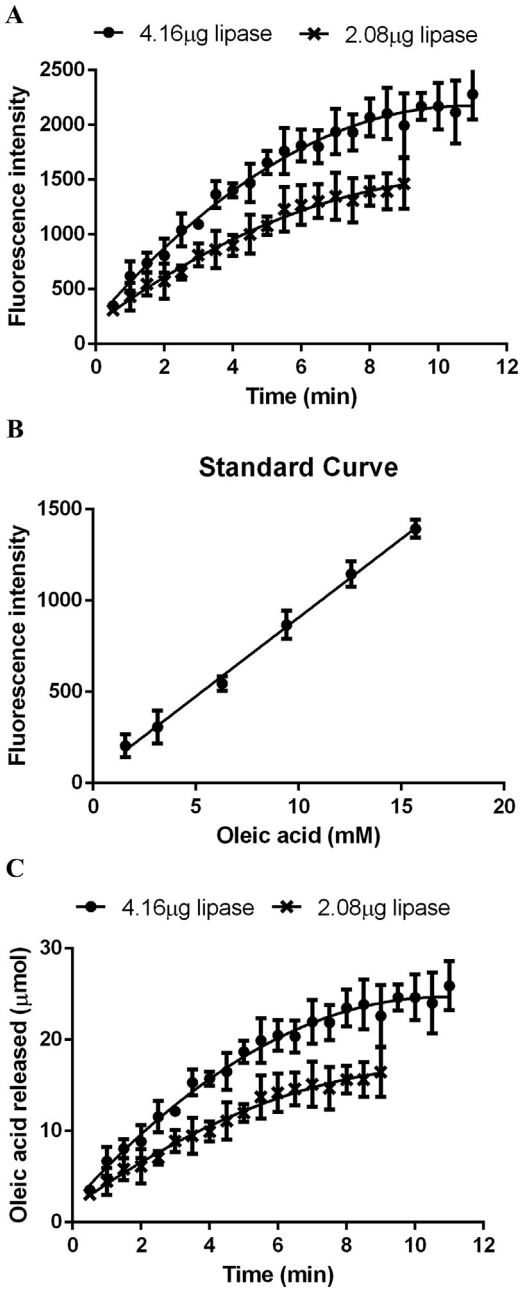

Fig. 6. Quantification of fatty acid released by LipY8p hydrolysis of olive oil. (A) Hydrolysis of olive oil in RhB-OOe leads to fluorescence emission. (B) A standard curve prepared with RhB-OOe using 3–18 mM oleic acid. (C) Quantification of fatty acid released by LipY8p hydrolysis of olive oil. The excitation wavelength was set to 350 nm, and fluorescence emission was recorded at 580 nm. Each measurement was performed three times, and standard deviations were indicated.

0.1% (v/v) β-ME. This can be explained by the requirement for sulfhydryl groups for lipase activity [39]. As a serine protease inhibitor, phenylmethylsulfonyl fluoride (PMSF) (1 mM and 4 mM) showed significant inhibitory effects with 80.1% and 47.6% residual activities, respectively, demonstrating that the lipase is of the serine hydrolase type [41].

3.7. Effect of detergents on lipase activity and stability

Detergents such as Tween-20 and Triton-X100 are commonly used as emulsifying agents to improve the emulsion of substrates, thereby making the substrate more accessible. However, the presence of detergents in the reaction system may affect the catalytic activity of lipase depending on the concentration used. At a concentration of 0.1% (v/v or w/v), the detergents SDS, Triton-X100, NP40, Tween-20 and β-DDM strongly inhibited LipY8p activity. β-OG had a mild positive effect at 0.1% w/v (105.5 ± 2.2%), but significantly inhibited lipase activity as the concentration increased to 0.3% w/v (2.2 ± 0.1%). The inhibitory effect was also observed with $C_{12}E_8$ at 0.001% w/v (63.1 ± 5.2%) and 0.002% w/v (7.9 ± 1.4%) (Table 3). These results can likely be attributed to the hydrophobic property of the long chains of these detergents making them act as substrates and therefore competitive inhibitors of the enzyme [42].

Table 2
Effect of metal ions and inhibitors on lipase activity.

Compounds	Concentration (mM)	Residual activity (%)
Control	none	100 ± 1.18
β-ME	1	117.6 ± 1.22
	5	129.8 ± 8.32
	10	125.8 ± 1.69
PMSF	1	80.1 ± 2.22
	4	47.6 ± 3.92
KCl	1	118.4 ± 5.83
$CaCl_2$	1	96.3 ± 3.99
$MgCl_2$	1	96.8 ± 1.50
$ZnSO_4$	1	1.2 ± 0.10
$CuCl_2$	1	12.5 ± 0.02
$NiSO_4$	1	51.8 ± 2.11
EDTA	1	136.1 ± 4.49

Although most of the tested detergents have an inhibitory effect on LipY8p activity when present in the reaction buffer system, almost all of them exhibited an activating effect on the enzyme activity when being added into the enzyme stock for pre-incubation. When LipY8p was pre-incubated with 0.1% (v/v or w/v) Triton-X100 (128.1 ± 4.1%), β-OG (147.1 ± 0.3%), or $C_{12}E_8$ (144.2 ± 5.5%) for 2 h at 22 °C, we observed a strong activation of lipase activity (Table 4). This positive effect on lipase activity was retained for up to 72 h for Triton-X100 and longer for β-OG and $C_{12}E_8$ (Table 4). Thus, this indicates that the detergents were able to weaken the hydrophobic interaction within the lipase protein, resulting in disaggregation and stabilization of the enzyme [43]. However, as the incubation time increased, the denaturation effects of these detergents became dominant and the enzyme activity decreased. Pre-incubating of lipase with 0.1% (w/v) β-DDM produced a sharp decrease in lipase activity (39.9 ± 3.3%), and the destabilization effect was more pronounced with SDS, NP40, and Tween-20 at the same concentration (Table 4). This suggested that the lipase showed greater sensitivity to these detergents, which may have induced inappropriate conformational changes and denaturation of the protein [44].

3.8. Effect of organic solvents on lipase activity and stability

Enzymes could be used to perform reactions in organic solvents that are not possible in aqueous systems. However, the activity and stability of enzymes in organic solvents show a strong dependence on the nature of the enzymes [45]. As proteins, enzymes tend to lose their activity in solutions containing higher than 10–20% organic co-solvents [46]. Thus, reaction buffers containing 10% or 20% (v/v) various organic solvents were used to examine their effects on lipase activity and stability. LipY8p activity was dramatically increased by the presence of 20% (v/v) DMSO (416.9 ± 22.7%) during the reaction. A similar phenomenon was also observed for *P. fluorescens* lipase whose activity increased up to 4.0-fold in the presence of 50% (v/v) DMSO [47]. This significant activation of lipase activity may be attributed to a conformational change and increased flexibility of the protein caused by the

Table 3
Effect of detergents on lipase activity when present in reaction buffer.

Detergents	Concentration (v/v or w/v)	Residual activity (%)
Control	none	100 ± 1.18
SDS	0.1%	0.89 ± 0.16
Triton-X100	0.1%	–
NP40	0.1%	–
Tween-20	0.1%	0.14 ± 0.02
β-OG	0.1%	105.5 ± 2.18
	0.3%	2.2 ± 0.10
β-DDM	0.1%	–
$C_{12}E_8$	0.001%	63.1 ± 5.16
	0.002%	7.9 ± 1.38

-, Activity undetectable.

solvent. Activation of lipase activity was also observed with 20% (v/v) methanol (180.3 ± 1.7%). Similar effects were recorded for ethanol (266.7 ± 5.4%), acetone (361.5 ± 11.3%) as well as isopropanol (174.4 ± 5.3%) at concentrations of 10% (v/v). These became inhibitory as the concentration increased to 20% (v/v) (Table 5). The effects of organic solvents on lipase stability are recorded in Table 6. The enzyme lost almost 90% activity after exposure to 20% (v/v) ethyl ether or Dimethylformamide (DMF) at 22 °C for 2 h, and activity was virtually eliminated following the addition of acetone, ethanol or isopropanol. However, LipY8p exhibited relatively higher stability in methanol and DMSO retaining 72.8 ± 1.4% and 88.1 ± 4.6% residual activity, respectively, after treatment. These results suggest that longer chain length alcohols have a stronger inhibitory effect. Binding of a thin layer of water molecules to the surface is essential for the enzyme protein to maintain its native conformation [37]. Water is a particular solvent type that shows lower affinity toward the protein surface in comparison to water-miscible organic solvents [48]. Water patches on the protein surface are formed by a limited number of directly-bound water molecules and also by water–water interactions. Thus, the presence of water-miscible organic solvents deprives the enzyme of bound water leading to enzyme inactivation. Lipases show diversity in their tolerance to water-miscible organic solvents [42]. The cold-active lipase from *Pseudomonas proteolytica* (GBPI_Hb61) showed decreased stability after a 30-min exposure to various water-miscible organic solvents, with the exception of methanol (103.5%) [10].

Table 5
Effect of organic solvents on lipase activity.

Solvents	Concentration (v/v)	Residual activity (%)
Control	none	100 ± 7.15
Iso-propanol	10%	174.4 ± 5.27
	20%	5.2 ± 0.29
Methanol	20%	180.3 ± 1.71
Ethanol	10%	266.7 ± 5.4
	20%	50.1 ± 3.51
Acetone	10%	361.5 ± 11.3
	20%	38.7 ± 2.09
DMSO	20%	416.9 ± 22.7

Table 6
Stability of lipase in different organic solvents.

Solvents	Concentration (v/v)	Residual activity (%)
Control	none	100 ± 2.24
Methanol	20%	72.81 ± 1.37
Ethanol	20%	0.90 ± 0.03
Acetone	20%	1.38 ± 0.04
Iso-propanol	20%	–
DMSO	20%	88.05 ± 4.64
DMF	20%	13.21 ± 0.13
Ethyl Ether	20%	10.86 ± 0.16

-, Activity undetectable.

4. Conclusions

In the present work, we report the cloning and expression of the *LIPY8* gene by the baculovirus expression system, as well as purification and characterization of the enzyme. The results from this study revealed that the purified recombinant enzyme was highly active in cold temperatures ranging from 8 to 21 °C with maximal activity at 17 °C. The lipase showed high stability over a wide range of pH values from 5 to 9 with optimal activity at 7.5. The enzyme also exhibited stability in the presence of a selection of inhibitors, metal ions, detergents, and organic solvents. It is particularly interesting that the LipY8p expressed by insect cells showed a marked difference in enzymatic characterization with regard to optimal pH values and temperatures to that expressed by *Pichia Pastoris* reported by Song et al. These differences also exist between LipY8p and LipY despite both originating from marine *Y.*

Table 4
Effect of detergents on lipase activity and stability when present in enzyme stock solution.

Detergents	Concentration (v/v or w/v)	Residual activity (%)	Incubation time (h)
Control	none	100 ± 1.85	2
SDS	0.1%	–	2
Triton-X100	0.1%	128.1 ± 4.05	2
	0.1%	140.2 ± 4.15	24
	0.1%	110.4 ± 2.75	48
	0.1%	96.4 ± 4.44	72
NP40	0.1%	–	2
Tween-20	0.1%	–	2
β-OG	0.1%	147.1 ± 0.25	2
	0.1%	124.7 ± 4.19	24
	0.1%	119.2 ± 2.83	48
	0.5%	143.9 ± 4.12	2
	0.5%	127.7 ± 1.58	24
	0.5%	121.6 ± 2.49	48
β-DDM	0.1%	39.9 ± 3.29	2
$C_{12}E_8$	0.01%	140.0 ± 2.85	2
	0.1%	144.2 ± 5.50	2
	0.1%	140.8 ± 3.56	24
	0.1%	130.0 ± 1.88	48

-, Activity undetectable.

lipolytica and sharing high sequence identity. To the best of our knowledge, with regard to closely related lipases of LipY8p, only the 3D structure of *Y. lipolytica* Lip2 lipase (40.9% identity) has been solved. Thus, solving the 3D structure of LipY8p will shed light on the enzyme structure and function, and also contribute to the understanding of enzymatic activities at low temperatures as well as their optimization for biotechnological applications.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2018.e00295.

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