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Separation, characterization and catalytic properties of Lip2 isoforms from *Candida* sp. 99-125

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ABSTRACT

Lipase (EC.3.1.1.3) from *Candida* sp. 99-125 was separated into four isoforms (isoform A, isoform B, isoform C, and isoform D) by two steps of ion exchange chromatography. As analyzed on SDS- and non-denaturing PAGE, the four isoforms were homogenous and had the same molecular weight of approximate 38 kDa. MALDI-TOF peptide mass fingerprinting maps and circular dichroism spectra showed the isoforms had similar peptide patterns belonging to the same protein encoded by the *YLlip2* gene and different secondary structures. The isoforms had a little distinct optimum temperature in the range of 20–35 °C, and the same optimum pH (8.0). They remained to be active in methanol, ethanol and ethylene glycol at the concentration of 10% and 20% (v/v) and acetone at the concentration of 10% (v/v), and sensitive to EDTA. Triton X-100, Sodium cholate and CHAPS slightly increased their activities. The metal ion Ca^{2+} and Mg^{2+} had mild effect on lipase activity. The isoforms showed a preference for long chain fatty acid triglyceride (triolein and olive). The lipase purified by one step of ion exchange chromatography and isoforms were less active than crude enzyme to catalyze cetyl alcohol and oleic acid in *n*-hexane, whereas the presence of small concentration of added water dramatically activated crude lipase but less the purified preparations.

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1. Introduction

Lipases (triacylglycerol acylhydrolases, E.C.3.1.1.3) catalyze the hydrolysis of triglycerides to glycerol and free fatty acids at an oil–water interface. Interest in lipases has increased markedly in the last 2 decades owing to their potential industrial applications. A large number of lipases have been screened for applications in various industries including those for pharmaceuticals, foods and detergents [1]. Lipases can also perform the reverse reaction of synthesis of triacylglycerols from free fatty acids and glycerol. This property is extensively used in trans- and inter-esterification reactions in organic solvents to produce useful acylglycerols [1–6]. Each application requires unique properties with respect to specificity, stability, temperature and pH dependence or ability to catalyze synthetic ester reactions in organic solvents.

Lipases secreted by fungi or yeast cells are intensively investigated as potential catalysts for biotransformations, since they display useful properties related to their stability and ease of purification from the culture media. The yeast from *Yarrowia* genus is one of the most extensively studied non-conventioal yeasts. So far, there have been many papers dealing with lipases from this family and identifying different genes [7–13]. Our laboratory previously isolated a stable and high producing mutant strain *Candida* sp. 99-125 (belonging to *Yarrowia* genus) and reported the purification and characterization of extracellular lipase. SDS–PAGE showed that the molecular weight of this lipase was about 38 kDa. N-terminal amino acid sequencing and MADLI-TOF mass spectral analysis revealed the lipase was encoded by gene *YLlip2*, and contained about 12% sugar [14]. However, we found that the isoenzyme LIP2 was not homogeneous and comprised of four active forms (isoform A, isoform B, isoform C and isoform D). This paper describes the separation, characterization and catalytic properties of the four isoforms of *Candida* sp. 99-125.

2. Materials and methods

2.1. Materials

Mono Q 5/50 GL column and ion exchange media were from Amersham Biosciences (Uppsala, Sweden). Endoglycosidase H_f (Endo H_f) was from New England Biolabs. Triton X-100, Trishydroxymethylaminomethane (Tris), olive oil, and polyvinyl alcohol (PVA) were obtained from Sanbo Biotech (Beijing, China). Molecular weight markers for electrophoresis were obtained from Bio-Rad (Richmond, USA). All other chemicals used were of reagent grade quality.

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2.2. Enzyme activity

Lipase activity was measured by titrimetric assay according to an olive oil emulsion [14] with some modifications. Olive oil (5%, v/v) was emulsified in distilled water containing 2% (w/v) of PVA in a homogenizer for 6 min. Then the enzyme solution (1 ml), pure or diluted, depending on the quantity of lipase, was added to 5 ml of substrate emulsion and 4 ml of 100 mM phosphate buffer, pH 8.0 (K_2HPO_4–KH_2PO_4). Samples were incubated for 10 min at 35 °C. The reaction was stopped by adding 15 ml of ethanol. Enzyme activity was determined by titration of the fatty acid released with 50 mM NaOH. One activity unit of lipase was defined as the amount of enzyme which released 1 μmol of fatty acid per minute under assay conditions.

2.3. Culture conditions

Candida sp. 99-125 strain was isolated by our laboratory. Lipase was obtained from our previous work [15] operating at 400 rpm, 1 vvm in 30 L bioreactor. Seventeen liters of culture broth were tested.

2.4. Purification of isoforms

All purification steps were carried out at room temperature except when stated otherwise.

2.4.1. Triton X-100 treatment and acetone precipitation

Triton X-100 was added to culture broth, until the final concentration of 0.2% (w/v) was reached. After being stirred for 30 min, the broth was centrifuged (4000 rpm, 15 min) to remove culture medium and cells. Three volumes of ice-cold acetone were slowly added to the supernatant under constant stirring during the addition of acetone and for 10 min afterwards. The precipitate was collected by filtration and dried in the room temperature.

Five grams of lipase powder was dissolved in 40 ml of 25 mM Tris–HCl buffer (pH 8.0). The insoluble material was discarded by centrifugation (12,000 rpm, 20 min, 4 °C). The supernatant was desalted by Sephadex G 25. Then active fraction was collected as crude lipase solution.

2.4.2. Anion exchange chromatography

All chromatographic steps were run on ÄKTA basic 100 (Amersham biosciences). The crude lipase solution was loaded on a Q Sepharose Fast Flow column (6 × 2.5 cm) previously equilibrated with 25 mM Tris–HCl buffer (pH 8.0). The column was eluted by a 0–200 mM NaCl linear gradient in the same buffer at a flow rate of 5.0 ml/min. The active fractions were collected, then concentrated and desalted against 25 mM Tris–HCl buffer (pH 8.0) by ultrafiltration with an Amicon cell using a PM 10 membrane. The concentrated solution (0.2 ml) was loaded onto a Mono Q 5/50 GL column (1 ml) previously equilibrated with 25 mM Tris–HCl (pH 8.0). After washing out unbound protein with the same buffer, elution was performed by a 0–150 mM NaCl linear gradient in the same buffer at a flow rate of 1 ml/min.

2.5. Electrophoresis

Electrophoresis was run on a Bio-Rad Mini-Protean II cell according to the manufacturer's instructions. A 12% separating gel and a 4% stacking gel for SDS–PAGE were used. The non-denaturing electrophoresis was performed as described in SDS–PAGE analysis, but in the absence of SDS and β-mercaptoethanol, and without boiling the samples. Isoelectric focusing (IEF) was performed at 4 °C using a PROTEAN IEF cell (Bio-Rad, USA) according to the manufacturer's recommendations using Readystrip IPG strips (Bio-Rad) with a pH range of 3–10. The gels were stained for protein detection with Coomassie Blue R-250 following standard procedures.

For detection of hydrolytic activity, isoelectric focusing gel was placed onto a 2% agar plate containing 3% olive oil and 0.001% Rhodamine B. After incubation for 6 h at 25 °C, lipase activity was visualized as a fluorescent band under 350 nm UV light [16].

2.6. Temperature and pH effects on lipase activity

The optimum temperature of the isoforms was measured at pH 8.0 by assaying the hydrolytic activities to olive oil emulsions at various temperatures (15–50 °C). Also, to find out the optimum pH of the isoforms, the lipase activities at various pHs (6.0–10.0) were measured at 35 °C.

To examine the thermostability of the isoforms, they were incubated at various temperatures (15–60 °C) for 15 min, respectively. The residual activities were measured at 35 °C and pH 8.0. The pH stability of the isoforms was studied by measuring residual activity at 35 °C and pH 8.0 after 15 min of incubation at pH ranging from 7.0 to 9.5.

2.7. Effects of organic solvents, detergents, reductors, and metal ions

The isoforms were added to various effectors, including organic solvents, metal ions, detergents and reductors, respectively. The samples were incubated at room temperature for 15 min, then withdrawn and measured for residual activity by titrimetric assay.

2.8. MALDI-TOF mass spectrometry

The mass spectra were noted by using a time-of-flight delayed extraction MALDI mass spectrometer (Bruker Autoflex). The samples were mixed in an Eppendorf tube with the same volume of the matrix solution of cyano hydroxycinnamic acid (CHCA). The matrix solution was prepared at a concentration of 15 mg/ml in 2:1 (v/v) ACN/0.1%TFA. The mixtures were applied to a gold-plated sample holder and introduced into the mass spectrometer after drying. The spectra were gotten in the reflectron mode by summing 200 laser shots with an ion source voltage 1 of 19 kV, ion source 2 of 16.27 kV, 100 ns delay and the low mass gate at m/z 600. Monoisotopic peptide masses obtained from MALDI-TOF were queried against entries for protein databases in NCBI by a protein search program, Mascot (Matrix Science Ltd., London) [14].

2.9. Circular dichroism (CD) spectra

Circular dichroism spectra were measured in 25 mM Tris–HCl buffer (pH 8.0) and recorded on a JASCO J-810 spectropolarimeter (JASCO) at 25 °C. The protein concentration and optical path length were 0.2 mg/ml and 5 mm, respectively. The secondary structure was analyzed using four component model (Helix, Beta, Turn, and random coil) reference spectra.

2.10. Protein analysis

Protein concentration was determined according to the enhanced method of Brodfold using bovine serum albumin as a standard [17].

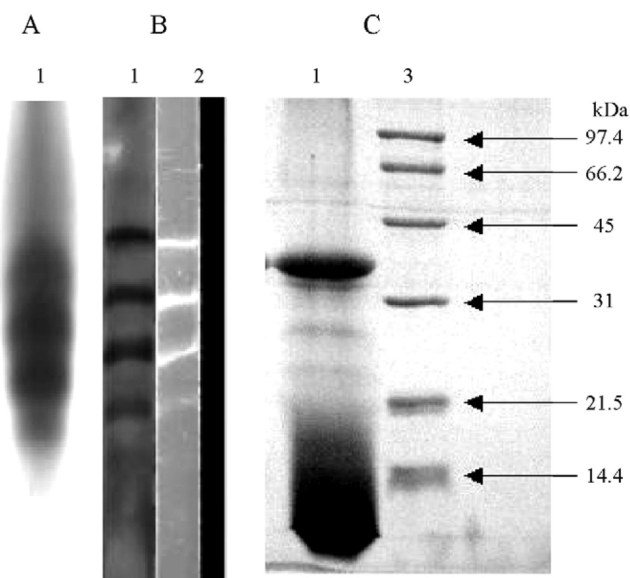

Fig. 1. (A) Non-denaturing PAGE, (B) isoelectrofocusing and (C) SDS–PAGE of crude lipase. (1) Stained by Comassic blue; (2) stained by activity; (3) molecular weight marker.

2.11. Esterification

The reaction content was *n*-hexane (3 ml), cetyl alcohol (4 mmol), oleic acid (4 mmol) and lipase (400 U) with silica gel as water absorbent. The mixtures were incubated at 40 °C with continuous shaking at 150 rpm for 24 h. The reaction liquids were determined by GC-2010 gas chromatography (Shimadzu Japan) using a DB-1ht capillary column (30 m × 0.25 mm; J&W Scientific, USA) and a flame ionizing detector (FID).

3. Results and discussion

3.1. Identification of the major proteins in the crude lipase

The crude solution was initially characterized by electrophoresis (Fig. 1). One major band was visualized with an estimated molecular weight of 38 kDa on SDS–PAGE, whereas there were four major bands on non-denaturing PAGE. The predicted p*I* for LIP2 is 5.4. By IEF and active staining, the result showed crude lipase was comprised of four protein hydrolytic active bands to olive oil with p*I* of 5–6.

3.2. Purification

The culture broth included a number of culture medium, residual oil and cells, and was emulsified badly. There were two causes: one was the yeast was capable of assimilating hydrocarbons containing a variety of water-immiscible carbon substrates and also

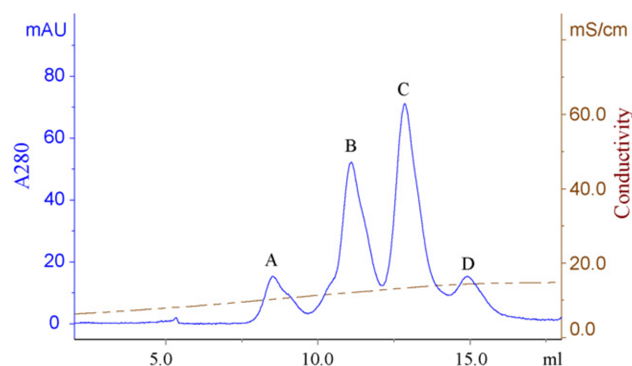

Fig. 2. Chromatography on Mono Q 5/50 GL. The partially purified lipase from Q Sepharose Fast Flow was loaded on a Mono Q 5/50 GL column equilibrated in Tris–HCl 25 mM (pH 8.0). Elution was carried out by a gradient of 0–150 mM NaCl. Flow rate, 0.8 ml/min.

capable of emulsifying these hydrocarbons during the substrate degradation process, and the other was high-molecular-weight polysaccharides with emulsifying properties were co-secreted with lipases [18,19]. Only 25% activity was recovered by centrifugation. And the recovery of lipase could not be increased greatly by washing precipitate with a number of phosphate buffer [9] and Brush's method [20]. Through experiments, 0.2% (w/v) Triton X-100 can increased the recovery of lipase to 82% by centrifugation. Then, acetone was used to precipitate the lipase and remove residual oil and other components. The crude enzyme powder obtained had the lipase activity of about 40,000 U/g. The dried powder was not stable and easy to lose its activity. The presence of 0.5% (w/w) PEG 6000 could increase the storage time to 20 months at 4 °C.

Lipase was of strong hydrophobicity and easy to form aggregation of high molecular mass with itself or lipid [14,21,22]. The aggregation was disrupted by ammonium sulfate precipitation, detergent and organic solvent [23,24]. The activity from crude lipase solution was mostly precipitated by 60% saturation of ammonium sulphate. The result indicated that the lipase was released from oil by treatment of Triton X-100 and participating of acetone.

In previous work [14], although after Q Sepharose and Butyl Sepharose chromatography the LIP2 was purified, it was not homogenous on non-denaturing PAGE and IEF. Because LIP2 was of strong hydrophobicity and could be too strongly absorbed by the hydrophobic matrix, IEX chromatography was possibly more efficient to isolate isoforms than HIC chromatography [25,26]. By Q Sepharose column and Mono Q 5/50 GL column, four distinct isoforms were obtained and named as isoform A, B, C and D (Fig. 2). The protein ratio isoform A:isoform B:isoform C:isoform D was 1:4.7:6.6:2.2 (Table 1). It was not unexpected to detect more than one form. A number of microorganisms, such as *Yarrowia lipolytica* [22], *Aspergillus niger* [21], *Ophiostoma piliferum* [20] and *Candida ruaosa* [23,24], produce multiple lipase forms.

Table 1
Purification of lipase from crude supernatant

Purification step	Total activity ($\times 10^3$ U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Culture broth	260	496	524	100	1
Enzyme powder	200	233	858	76.9	1.64
Q Sepharose FF	160	31.8	5030	61.5	9.60
Mono Q GL					
Isoform A	10.4	0.912	11400	4.00	21.8
Isoform B	24.1	2.53	9540	9.27	18.2
Isoform C	50.4	6.25	8070	19.4	15.4
Isoform D	23.4	2.09	11200	9.00	21.4

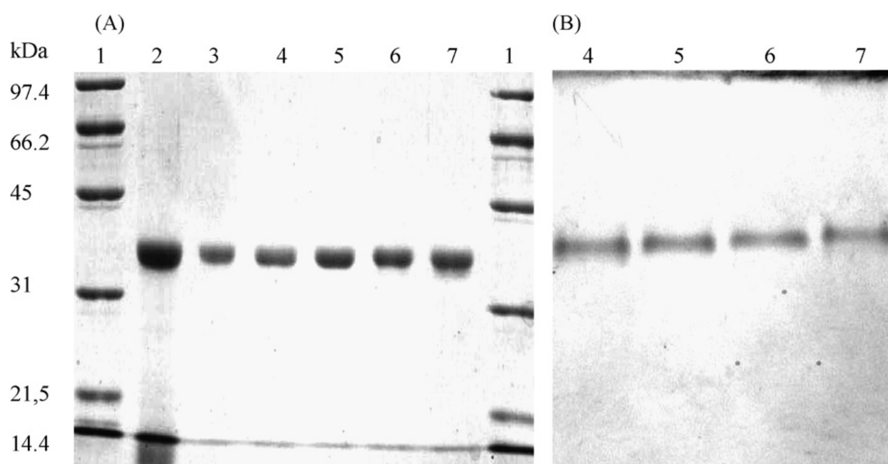

Fig. 3. (A) SDS–PAGE and (B) non-denaturing PAGE analysis of lipase. (1) Molecular mass marker; (2) crude lipase; (3) collected lipase from Q Sepharose; (4) isoform A; (5) isoform B; (6) isoform C; (7) isoform D.

3.3. Physical characterization, MALDI-TOF and circular dichroism analysis of the isoforms

SDS- and non-denaturing PAGE (Fig. 3) of purified isoforms both showed they were homogeneous. The molecular weights of the isoforms were estimated to be approximate 38 kDa. Based on the nearly identical spectra from MALDI-TOF mass analysis of the four isoforms (Fig. 4), the data indicated that the isoforms were the protein encoded by the same gene. Compared with theoretical maps of some protein, the maps were the most similar to that of the LIP2 from *Y. lipolytica*, which indicated the isoforms were encoded by the same gene LIP2. These masses were all found to be larger than the calculated value for the mature non-glycosylated lipase (33.4 kDa). The molecular weight of these isoforms decreased after they were previously treated with endoglycosidases [14]. The result showed that all of the isoforms were glycosylated.

The isoforms were subjected to CD and their far UV spectra 190–240 nm was obtained (Fig. 5). The secondary structure estimation of the isoforms was performed by the JASCO Secondary Structure Estimation Program. The distribution of secondary structure of isoform B, C and D was similar and slightly different from isoform A (Table 2). Bacterial lipases have a characteristic α/β hydrolase fold [26]. McCabe et al. [27] reported that under the optimum pH 7.0, the distribution of secondary structure of *C. antarctica* lipase B was 38% for α-helix, 20% for β-sheet, 18% for β-turn and 24% for other. Secondary structure did not appear to change significantly at the extremes of the pH range of 4.2–9.0 except for slight change of increase for other and decrease for turns.

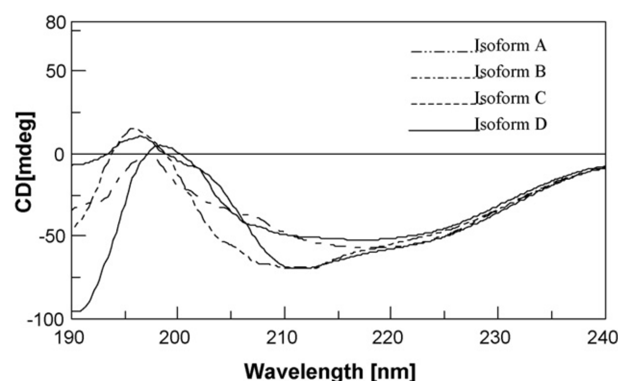

Fig. 5. Far-UV circular dichroism spectra of isoforms from YLlip2.

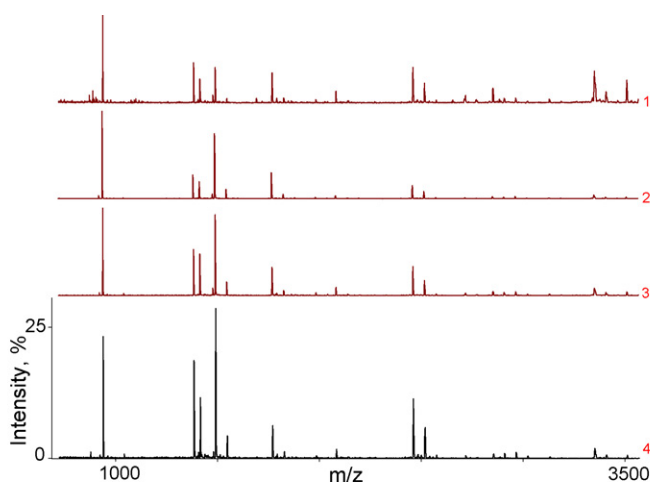

Fig. 4. MALDI-TOF peptide mass fingerprinting maps of isoforms. (1) Isoform A; (2) isoform B; (3) isoform C; (4) isoform D.

3.4. Effect of temperature, pH, organic solvents and other chemical reagents on activity of isoforms

A number of lipases from fungi have the maximum activity at temperature of 30–40 °C and at pH 6.0–8.0 [28–33]. The optimal temperature of isoforms was 30 °C for isoform A, 35 °C for isoform B and C, and 20 °C for isoform D, which was slightly lower than ordinary lipases. The four isoforms all showed over 80% of their maximum activity in the temperature range of 20–35 °C. Their activity dropped off rapidly above 40 °C with only 2–20% relative

Table 2
Isoforms secondary structure estimation from CD spectrometry

Model	Ratio (%)			
	Isoform A	Isoform B	Isoform C	Isoform D
α-Helix	14	18	17	22
β-Sheet	38	23	21	19
β-Turn	18	24	30	26
Other	30	35	32	34
Total	100	100	100	100

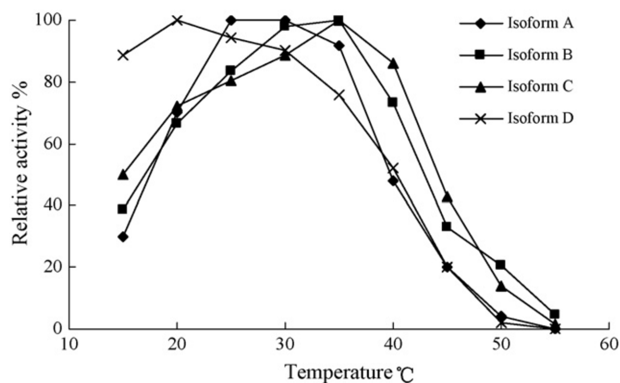

Fig. 6. Effect of temperature on isoforms. The maximal activitiy of each isoform was set as 100%. All measurements were repeated three times.

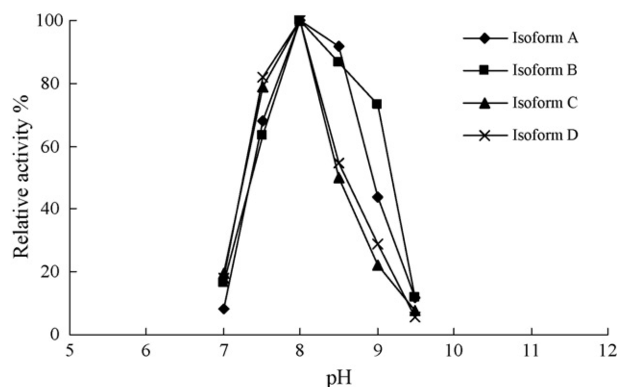

Fig. 8. Effect of pH on isoforms. The maximal activity of each isoform was set as 100%. All measurements were repeated three times.

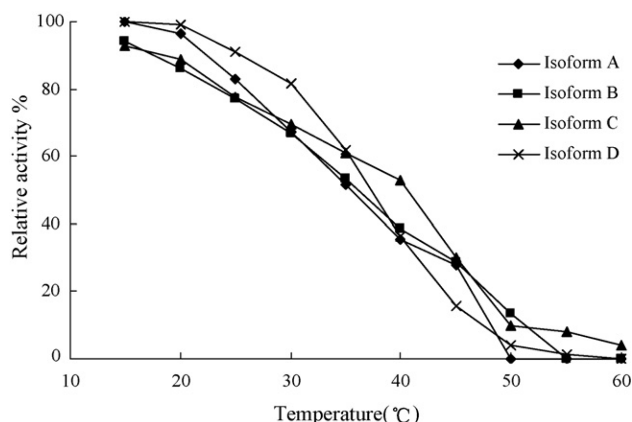

Fig. 7. Thermostability of isoforms. Residual activity was measured at 35 °C by titrimetric assay. The activity of each isoform at 35 °C was set as 100%. All measurements were repeated three times.

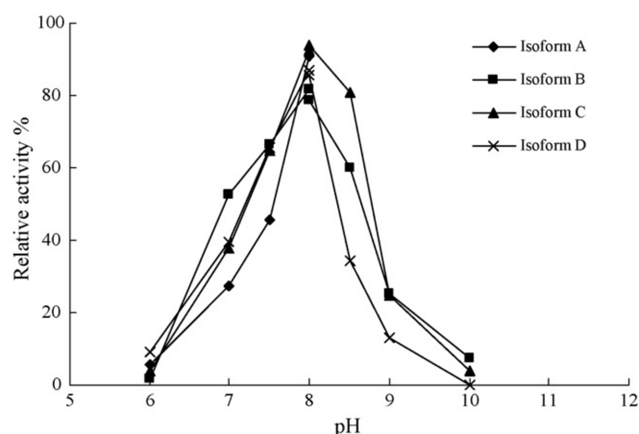

Fig. 9. pH stability of isoforms. Residual activity was measured at pH 8.0 by titrimetric assay. The activity of each isoform at pH 8.0 was set as 100%. All measurements were repeated three times.

activity at 50 °C (Fig. 6). The isoforms were stable below 30 °C for 15 min and retained at least 65% activity (Fig. 7). The optimal pH for every isoform was 8.0 (Fig. 8). The activity was reduced drastically at pH below 7.0 and over 9.0, and the loss of activity was at least 80% of the maximal activity. In a pH stability test, the isoforms showed to be stable after 15 min at pH 7.5–8.5 and the most stability in pH 8.0 (Fig. 9).

The isoforms remained to be active in shorter chain alcohol at the concentration of 10% and 20% (v/v). In particular, they remained approximate 90% and 70% relative activity in methanol at the concentration of 10% and 20%, respectively (Table 3). It is potential to the use of lipase for transesterification and synthesis of esters which are allowed to occur in media containing water-miscible organic

Table 3
Effect of organic solvents on isoforms activity

Compounds	Concentration (%)	Relative activity (%)			
		Isoform A	Isoform B	Isoform C	Isoform D
Control	–	100 ± 1.45	100 ± 1.60	100 ± 1.58	100 ± 1.52
Methanol	10	91.7 ± 1.78	97.4 ± 1.89	91.2 ± 1.92	89.0 ± 1.86
	20	72.0 ± 1.88	81.6 ± 2.02	82.5 ± 2.32	70.0 ± 2.23
Ethanol	10	76.0 ± 1.83	81.8 ± 1.92	77.3 ± 2.01	78.0 ± 2.21
	20	68.0 ± 1.92	69.7 ± 1.83	75.0 ± 1.99	68.0 ± 2.32
1-Propanol	10	80.0 ± 1.68	53.3 ± 3.98	55.8 ± 3.45	43.0 ± 4.03
	20	8.00 ± 6.45	0	2.27 ± 5.67	8.82 ± 5.43
1-Butanol	10	38.0 ± 3.45	31.1 ± 3.62	27.9 ± 3.78	44.4 ± 2.99
	20	0	0	9.31 ± 8.55	8.82 ± 9.03
Isopropanol	10	76.0 ± 3.11	75.6 ± 2.97	75.6 ± 3.22	55.6 ± 3.02
	20	40.0 ± 4.01	35.6 ± 3.99	65.1 ± 4.30	52.2 ± 4.39
Ethylene glycol	10	66.7 ± 2.87	65.0 ± 3.25	65.0 ± 2.93	65.4 ± 3.23
	20	83.3 ± 2.36	86.0 ± 3.16	90.9 ± 2.48	88.2 ± 2.38
Acetone	10	96.0 ± 2.03	70.0 ± 2.49	65.0 ± 2.87	69.2 ± 2.26

Table 4
Effect of detergents, enzyme inhibitors and metal ions on isoforms activity

Compounds	Relative activity (%)			
	Isoform A	Isoform B	Isoform C	Isoform D
Control	100 ± 1.45	100 ± 1.60	100 ± 1.58	100 ± 1.52
DTT	87.5 ± 2.47	76.3 ± 2.67	86.0 ± 2.31	95.0 ± 1.99
β-Mercapoethanl	117 ± 1.75	97.4 ± 1.54	91.2 ± 1.61	92.5 ± 1.87
EDTA	25.0 ± 3.05	36.8 ± 3.12	38.6 ± 3.53	22.5 ± 3.54
Sodium cholate	117 ± 1.70	108 ± 1.65	107 ± 1.59	103 ± 1.75
CHAPS	113 ± 1.66	103 ± 1.64	102 ± 1.58	90.0 ± 2.32
Triton X-100	101 ± 2.87	100 ± 3.25	101 ± 4.39	100 ± 3.43
$CaCl_2$	97.5 ± 2.36	101 ± 3.14	100 ± 2.73	102 ± 2.21
$MgCl_2$	94.2 ± 1.82	97.9 ± 1.94	90.4 ± 2.06	93.5 ± 2.49
$FeCl_3$	76.7 ± 2.58	70.5 ± 3.04	74.9 ± 2.45	70.5 ± 1.93

solvents. 20% ethylene glycol could increase relative activity by 20% than 10% ethylene glycol. In addition, the isoforms remained to be active in 10% acetone among which isoform A was more stable than the other isoforms. The isoforms showed high sensitivity to the metal chelator, EDTA. In the presence of 1 mM metal ions, Ca^{2+}, Fe^{2+} and Mg^{2+} caused moderate inactivation (11–30%). Sodium cholate and CHAPS slightly increased the lipase activity. Triton X-100 did not increase the activity. The isoform A was more stable than the others in these agents (Table 4).

3.5. *Chain length selectivity*

The relative activity of each isoform towards triolein, tributyrin and triacetin was shown in Fig. 10. The assays were carried out at 35 °C in 50 mM phosphate buffer (pH 8.0). Under the experimental conditions tested, the four isoforms all displayed the highest activity on the hydrolysis of triolein and no activity towards triacetin (Fig. 10). The isoforms hydrolyzed the triolein approximately 6× faster than tributyrin. The isoform B had higher relative activity to these triglycerides than the other three isoforms. Although the lipases from fungi prefer long chain triglycerides, some lipases have high activity towards medium chain [34,35] and short chain [24] triglycerides.

3.6. *Esterification*

An investigation of the esterification capacity of the crude and purified preparations was performed. The ester conversion was very low in non-aqueous organic media after long reaction time (24 h). And the crude lipase was more active than the purified preparations. Low water will favour esterification [6]. In order to study the effect of low water presence in the reaction system on the esterification, the water content was all reached to 2% (by weight of oleic acid). Ester production increased at presence of low water. The ester conversion catalyzed by crude lipase even increased from 16.7% to 97.3%. However, the water did not increase efficiently the ester reaction catalyzed by purified preparations (Table 5). It is not clear that why the crude preparation was activated more than the purified. However, it is possible that the presence of materials in the crude preparation can remain lipase activity and make it easier to distribute crude lipase over reaction medium. It was opposite that the purified lipase preparation from *Candida rugosa* was less active than the crude enzyme in dry *n*-heptane, whereas the presence of small concentrations of added water dramatically activated the purified enzyme but not the crude enzyme [36].

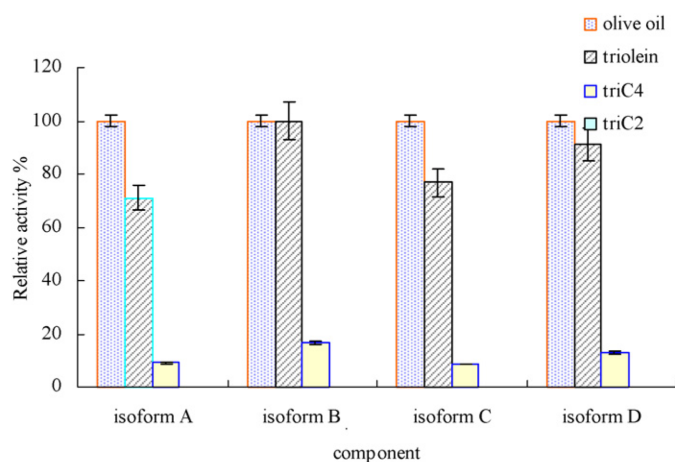

Fig. 10. Length selectivity of four isoforms. Activity of the isoforms towards olive oil triolein, tributyrin and triacetin was determined at 35 °C in 0.05 mM K_2HPO_4–KH_2PO_4 (pH 8.0) by titrimetric assay. The activity of each isoform towards olive oil was set as 100%. All measurements were repeated three times.

Table 5
Esterification of cetyl alcohol and oleic acid catalyzed by lipase preparations

Catalyzer	Ester conversion of (%)	
	In non-aqueous system	In low aqueous system
Crude lipase	16.7	97.3
Lipase partly purified by a Q Sepharose FF column	3.6	15.3
Isoform A	0	9.3
Isoform B	4.0	17.5
Isoform C	2.4	14.5
Isoform D	4.5	9.6

4. Conclusions

In this study, we separated four lipase isoforms from *Candida* sp. 99-125 and charactered them, respectively. MALDI-TOF mass spectra analysis showed that they had identical peptide mass fingerprinting maps and were all encoded by gene LIP2. The distribution of secondary structure of isoform A was a little different from that of the other three. The isoforms were stable in methanol, ethanol, ethylene glycol and acetone. Isoforms activity were slightly stimulated by CHAPS, and sodium cholate, and heavily inhibited by EDTA. Isoform A was more stable than the others in these agents. The lipase showed a preference for long chain triglyceride, triolein and olive oil. The esterification capacity of the crude preparation was higher than that of the purified. At the presence of low water, the crude lipase was activated sharply in *n*-heptane.

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