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Purification and characterization of lipase from *Spirulina platensis*

Burcu Saygıdeğer Demir[a,*], S. Seyhan Tükel[b]

[a] University of Osmaniye Korkut Ata, Faculty of Arts & Sciences, Department of Chemistry, 80020 Osmaniye, Turkey
[b] University of Cukurova, Faculty of Arts & Sciences, Department of Chemistry, 01330 Adana, Turkey

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ABSTRACT

A lipase from photosynthetic cyanobacterium *Spirulina platensis* (*Arthrospira*) was purified by sequential operation of ammonium sulphate precipitation, dialysis, DEAE-Sepharose anion exchange chromatography, and Sepharose-6B gel filtration chromatography for the first time. This purification procedure resulted in 375-fold purification of lipase with 29.35% final yield. The purified lipase showed a prominent single band on SDS-PAGE. It is a monomeric protein of 45 kDa molecular weight and its isoelectric point is 5.9. The purified lipase exhibited maximal hydrolytic activity at a temperature of 45 °C and pH of 6.5. The values of K_m and V_{max} calculated from the Lineweaver–Burk plot using *p*-nitrophenyl palmitate (*p*-NPP) as hydrolysis substrate were 0.02 mM and 38.9 μmol min^{-1} mg^{-1}, respectively. The catalytic efficiency (k_{cat}/K_m) of purified lipase was determined as 1.5×10^6 M^{-1} s^{-1}. The remaining activity of the lipase was about 95% of its original activity at 25 °C for 24 h of preincubation. However, the remaining activity was about 26% of the original activity at 45 °C for 24 h. The purified lipase appears to be unique since it cleaved triolein at only 3-position releasing 1,2-diolein. Lipase activity was stimulated by Ca^{2+}, Mg^{2+}, Zn^{2+}, Triton X-100 and SDS, and inhibited by Li^+, Fe^{2+}, Mn^{2+}, EDTA and PMSF.

1. Introduction

Lipases (EC 3.1.1.3) catalyze the hydrolysis of ester bonds in triacylglycerols and can differ considerably in their positional and fatty acids specificity [1–3]. Hydrolytic reaction is reversible and lipase also catalyses the synthesis of esters and transesterification in microaqueous conditions [3–7]. These reactions usually proceed with exquisite chemoselectivity, regioselectivity and stereoselectivity, making lipases an important group of biocatalysts in biotechnology [3,4]. Lipases are widely used in food processing, in production of biodegredable polymers, in synthesis of fine chemicals, in production of biodiesel, in detergents, in leather industry, in pulp and paper manufacture, etc. [8–10]. In view of the variety in applications, there has been a renewed interest in the development of sources of lipases. The purification and biochemical characterization of lipases obtained from different organisms such as fungi [1,3,8], bacteria [2,9], plants [11] and animals (pancreatic) [12,13] were studied previously. In the literature, there is no data about the purification and characterization of lipase from photosynthetic cyanobacterium *Spirulina platensis*. *S. platensis* has found worldwide interest as photosynthetic planktonic organism suitable for mass production. It is a microscopic and filamentous cyanobacterium (blue-green algae) that has a long history of use as food due to its high protein content, high digestibility and specific amino acid content [14–16]. In addition to rich content of protein, *S. platensis* has much amounts of vitamins, essential amino acids, minerals, essential fatty acids, and enzymes [17,18].

In this study *S. platensis* was used as lipase source for the first time and *S. platensis* lipase was purified and characterized in terms of molecular weight, p*I*, optimal temperature, optimal pH and buffer concentration, kinetic constants, thermal and storage stabilities, positional and substrate specificity. The effects of organic solvents, metal ions, detergents and enzyme inhibitors on lipase activity were also investigated.

2. Materials and methods

2.1. Materials

Reference proteins for molecular mass determination were purchased from Fermentas and isoelectro focusing (IEF) standards were purchased from Bio-Rad. DEAE-Sepharose, Sepharose 6B, *p*-NPP and all other chemicals were purchased from Sigma. Bandelin sonopuls (HD 2200) sonicator was used for cell digestion. *S. platensis* was obtained from Cukurova University, Faculty of Fisheries,

* Corresponding author at: University of Osmaniye Korkut Ata, Faculty of Arts & Sciences, Department of Chemistry, 80020 Osmaniye, Turkey.
Tel.: +90 322 3386081/26; fax: +90 322 3386070.
E-mail addresses: burcusaygidemir@yahoo.com.tr (B.S. Demir), stukel@cu.edu.tr (S.S. Tükel).

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and was maintained and grown in inorganic medium as reported by Ihena [19].

2.2. Lipase activity assay

Lipase activity was measured with a modified spectrophotometric assay with *p*-NPP as substrate [20]. The substrate solution (0.1 mM) was prepared by dissolving *p*-NPP in ethanol. 50 mM phosphate buffer (pH 6.5) was used as reaction buffer. 0.5 ml of enzyme or blank solution (50 mM phosphate buffer pH 6.5) was added into 0.5 ml of reaction buffer and then 1 ml of substrate solution was added to the reaction medium. The enzyme–substrate mixture or blank–substrate mixture was incubated at 45 °C for 5 min with stirring. The reaction was stopped by adding 2 ml of 0.25 M Na_2CO_3 solution. The molar extinction coefficient of *p*-nitrophenol (*p*-NP) was estimated as 5.5×10^3 cm^2 mol^{-1} from the absorbance measured at 410 nm of standard solutions of *p*-NP. One enzyme unit (U) was defined as the lipase activity that liberated 1 mol equivalent of *p*-NP per mililiter per minute under the standard assay conditions.

2.3. Purification of lipase

2.3.1. Step 1: preparation of cell free extract and ammonium sulphate precipitation

Before ammonium sulphate precipitation, the harvested cells were washed three times with 10 mM pH 7.0 phosphate buffer at room temperature, and 15 g wet sample suspended in 45 ml of sonicating buffer (0.01 M pH 7.0 phosphate buffer containing 0.1 mM EDTA). Cell free extracts were prepared by sonicating the suspension at 149 μm amplitude using cycles of 20 s on and 10 s off. The sonicate was centrifuged at 12,000 rpm for 30 min, at 4 °C and the supernatant was used for lipase as crude extract.

The ammonium sulphate precipitation of cell free extract was carried out at a range of 20–90% (w/v) saturation at 4 °C [18]. Precipitation was allowed for 1 h at 4 °C and followed by centrifugation at 12,000 rpm for 30 min. The precipitate was dissolved in 50 mM pH 7.0 phosphate buffer. Dialysis of this sample was carried out using Sigma cellulose tubing (mol.wt. cut off: 12–14 kDa) for 24 h with three changes in the buffer at 4 °C.

2.3.2. Step 2: DEAE-Sepharose column chromatography

The clear sample obtained in the previous step was loaded on a DEAE-Sepharose column (1.2 cm × 10 cm) previously equilibrated with 50 mM phosphate buffer (pH 7). After washing with two bed volumes of the equilibration buffer, elution was performed with a linear gradient of 0–1.0 M NaCl in the same buffer at a flow rate of 60 ml/min [3]. Fractions showing lipase activity were pooled and analyzed.

2.3.3. Step 3: Sepharose-6B chromatography

The partially purified enzyme was applied to a Sepharose-6B fast flow column (1.2 cm × 20 cm) which was pre-equilibrated with 50 mM phosphate buffer (pH 7) and eluted with the same buffer at a flow rate of 30 ml/min [21]. The fractions were collected and analyzed.

2.4. Gel electrophoresis

Purity was analyzed by SDS-PAGE and native polyacrylamide gel electrophoresis (native PAGE) using the method of Laemmli [22] on 12.5% polyacrylamide gel on a vertical mini gel apparatus (Bio-Rad) at 150 V for 1 h. Reference proteins for molecular mass determination (Fermentas SM0431) were lysozyme (14.4 kDa), β-lactoglobulin (18.4 kDa), REase Bsp98I (25 kDa), lactate dehydrogenase (35 kDa), ovalbumin (45 kDa), bovine serum albumin (66.2 kDa), β-galactosidase (116 kDa). Protein bands were visualized by silver staining.

2.5. Isoelectric focusing

Isoelectric focusing was performed in a Protean IEF cell system (Bio-Rad) following the manufacturer's instructions. Isoelectric points of reference proteins for determination of p*I* were phycocyanin (4.45, 4.65, 4.75), β-lactoglobulin B (5.1), bovine carbonic anhydrase (6.0), human carbonic anhydrase (6.5), equine myoglobin (6.8, 7.0), human hemoglobin A (7.1), human hemoglobin C (7.5), lentil lectin (7.8, 8.0, 8.2) and cytochrome c (9.6). Gels were stained with Coomassie Brilliant Blue R250.

2.6. Determination of protein

Protein was assayed by the method of Lowry et al. [23].

2.7. Determination of optimal pH, temperature, buffer concentration, and kinetic constants

The optimal pH of the lipase was determined by measuring the activity in a pH range of 5–8 in the following buffers: 50 mM sodium acetate buffer (5.0–5.5), 50 mM sodium citrate buffer (pH 6.0), 50 mM potassium phosphate buffer (6.5–8.0). Lipase activity of samples in each buffer was measured using the standard assay as described previously, and buffer concentrations of 25, 50, 75 and 100 mM at pH 6.5 were also tested.

The optimal temperature for the purified lipase was determined by measuring the activity at various temperatures in the range of 25–70 °C at pH 6.5 in 50 mM phosphate buffer.

The activity assays were carried out in different *p*-NPP concentrations (0.066–0.331 mM) to determine maximum reaction rate (V_{max}) and Michaelis–Menten constant (K_m) of *S. platensis* lipase. Turnover numbers (k_{cat}) of lipase was calculated from the equation:

$$k_{cat} = \frac{V_{max}}{[E]_T}$$

where $[E]_T$ is the total amount of enzyme in reaction medium. Catalytic efficiency (k_{cat}/K_m) of lipase was also calculated. The activation energy (E_a) was estimated by using the Arrhenius equation.

2.8. Determination of thermal and storage stabilities

An estimation of the thermal stability was performed by measuring the residual activity of purified lipase exposed to temperatures of 25, 35, 45 and 55 °C. Samples were taken at 2, 8, 16,

Table 1
Summary of the results of purification procedures of the lipase from *S. platensis*.

Purification steps	Volume (ml)	Activity (U/ml)	Protein (mg/ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg prot)	Yield (%)	Fold purification
Crude enzyme solution	60	0.23	2.03	121.80	13.80	0.12	100.00	1
$(NH_4)_2SO_4$ precipitation	14	0.92	0.13	1.82	12.88	7.08	93.33	59
DEAE-Sepharose	48	0.26	0.02	0.96	12.48	13.00	90.43	108
Sepharose-6B	9	0.45	0.01	0.09	4.05	45.00	29.35	375

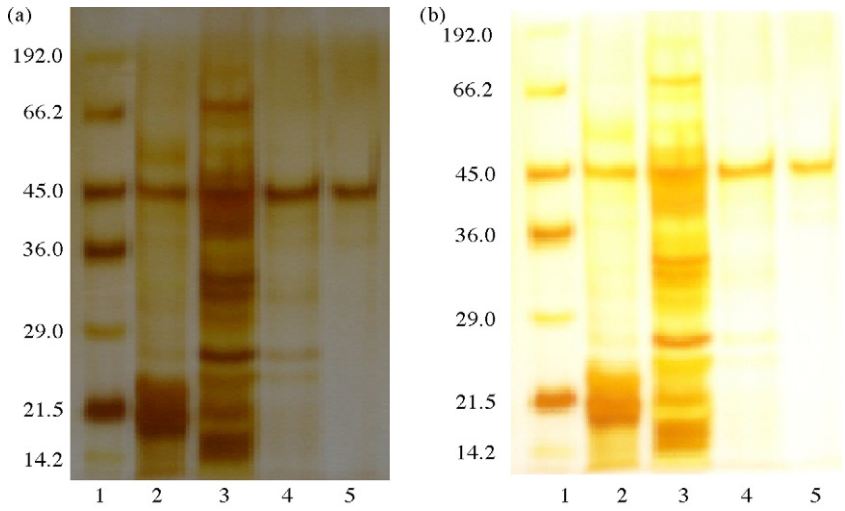

Fig. 1. SDS-PAGE pattern of purified lipase from *S. platensis* (a), Native PAGE pattern of purified lipase from *S. platensis* (b). SDS-PAGE was conducted in 12.5% gel. Lane 1: molecular weight markers; Lane 2: crude enzyme; Lane 3: 80% ammonium sulfate precipitate; Lane 4: Partially purified lipase (after DEAE-Sepharose column chromatography); Lane 5: Purified lipase (after Sepharose-6B column chromatography).

24 h time intervals during incubation and the residual activities were measured.

For the determination of storage stability, purified lipase was stored at room temperature and 5 °C as a solution in 50 mM pH 6.5 phosphate buffer and the residual activity of each sample was measured.

2.9. Determination of positional specificity

The positional specificity of the lipase was determined by analyzing lipolytic products of triolein by thin-layer chromatography (TLC) on silica gel G-60 plate (Merc, Germany) [24]. Emulsions (1 ml) of 20 mM triolein were hydrolyzed with 3 ml 1.35 U of purified lipase, pH 6.5 at 45 °C for 12 h. The reaction was stopped by the addition of 0.1 ml 3 M HCl, followed by extraction with 1 ml of chloroform. Chloroform layer was analyzed by TLC. TLC plate was developed with a solvent mixture of chloroform/acetone/acetic acid (96:4:1, by vol.). Pure trioleylglycerol, 1,3-dioleylglycerol, 1,2-dioleylglycerol and 2-monooleyl glycerol were used as reference glycerols esters. The spots were visualized with iodine vapour.

2.10. Determination of substrate specificity

Lipase activity on different major and minor oil (Olive oil, Sunflower seed oil, Laurel oil, Soybean oil, Canola oil, Peanut oil, Palm oil, Cottonseed oil, Mustard oil, Castor oil) was studied using titrimetry (Sigma enzymatic assay of lipase) for determine the substrate specificities of the purified lipase.

2.11. Effects of metal ions, organic solvents, detergents and enzyme inhibitors on lipase activity

Various effectors, including metal ions (Li^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, Fe^{3+}, Fe^{2+}, Cu^{2+} and Mn^{2+}), organic solvents (methanol, ethanol, acetone, isopropanol, n-propanol, n-butanol, chloroform diethylether, benzene and hexane), detergents (SDS, Tween 20 and Triton X-100) and enzyme inhibitors (EDTA and PMSF), were added to the enzyme solution and incubated at room temperature for 30 min. Residual activity was measured by the titrimetric assay.

3. Result and discussion

3.1. Purification of S. platensis lipase

The summary of a typical purification of lipase is shown in Table 1. Using the lipase activity assay described in the materials and methods section, the supernatant had a hydrolytic activity against *p*-NPP of 0.23 U/ml. The ammonium sulphate precipitation of the cell free extract was carried out at a range of 20–90 saturation percent. Salt precipitation with ammonium sulphate with saturation percent of 0–50% resulted in green colored precipitates due to presence of excessive amount of pigments (phcocyanin, chlorophyl-A, carotinoids). The enzyme activity in these fractions was not completely separable from the pigments and were therefore not characterized. Precipitates obtained at 60–90% saturation were free of pigments. 90% fraction did not show any activity. The

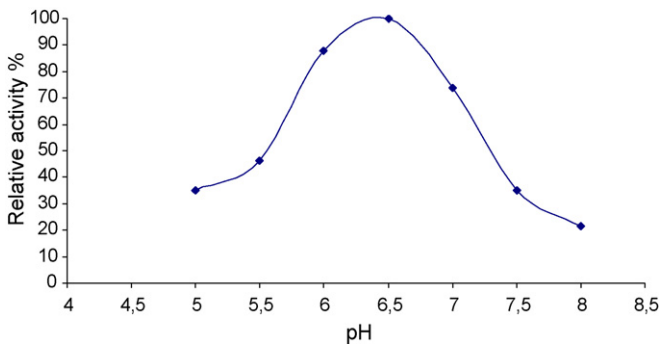

Fig. 2. Effect of pH on the activity of purified lipase.

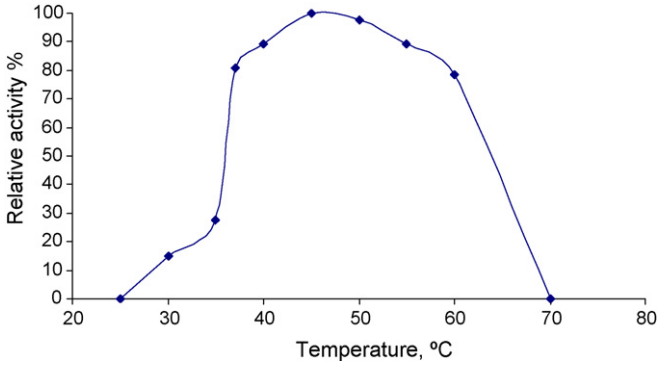

Fig. 3. Effect of temperature on the activity of purified lipase.

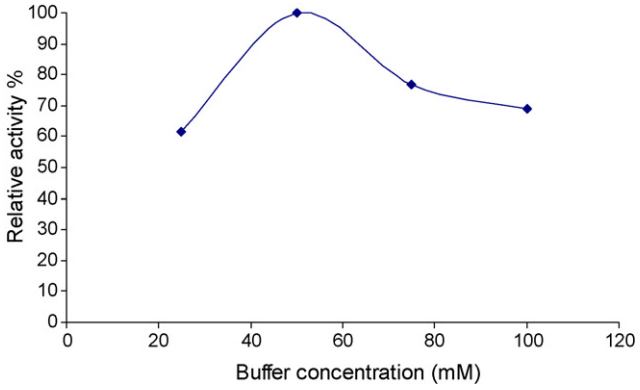

Fig. 4. Effect of buffer concentration on the activity of purified lipase.

60–80% fraction showed the maximum specific activity, was subsequently used for further purification. After sequential application of salt precipitation, DEAE-Sepharose fast flow column chromatography and Sepharose-6B gel filtration chromatography, the lipase was finally purified to 375-fold with a yield of 29.35% (Table 1).

The purified lipase was homogenous when tested with native PAGE and SDS-PAGE (Fig. 1). Molecular weight of the *S. platensis* lipase was 45 kDa. The enzyme p*I* value was estimated as 5.9. The specific activity of the purified lipase was found 45 U/mg protein.

3.2. Effects of pH, temperature and buffer concentration on enzyme activity

The purified lipase could tolerate pH 5.0–8.0 (Fig. 2). Maximum lipase activity was obtained at pH 6.5. Lipase activity was 35% of its maximum value when pH was 5.0. The activity decreased significantly when pH was increased from 6.5 to 8.0 and about 22% residual activity was obtained at pH 8.0. Low pH optima for lipase activity have been reported in *Penicillium cyclopium* (6.0) [25], *Penicillium camaembertii*-U150 (5.6) [26], *Candida rugosa* (6.5–7.5) [27]. However, high pH optima for lipase activity have also been reported in *P. camaembertii* Thom PG-3 (8.0) [4], Rice bran (11.0) [11], *Penicillium expansum* (9.4) [25] and *Humicola lanuginosa* (11.0–12.0) [27].

The purified lipase was active in the temperature range 37–60 °C, with maximal activity at 45 °C (Fig. 3). The activity dropped off rapidly and no activity was detected at 70 °C. *S. platensis* lipase is moderate thermophile enzyme. The result was similar to those of *Antrodia cinnamomea* (45 °C) [1], *P. camaembertii* Thom PG-3 (48 °C) [4], *Yarrowia lipolytica* (40 °C) [7] and *Aspergillus niger* F044 (45 °C) [8].

The effect of buffer concentration (25–100 mM) on the activity of purified lipase was determined at optimum pH and temperature values and results were presented in Fig. 4. Purified lipase showed maximum activity at 50 mM buffer concentration and at 100 mM buffer concentration lipase retained about 70% of its maximum activity.

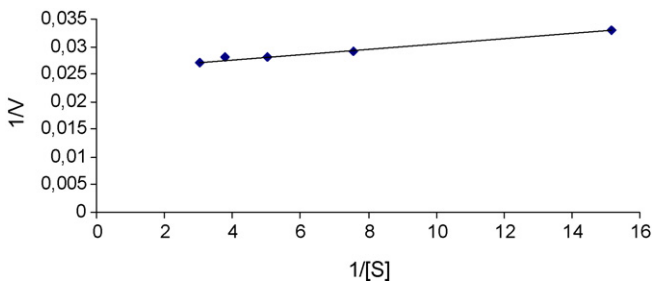

Fig. 5. Lineweaver-Burk plot of the purified lipase.

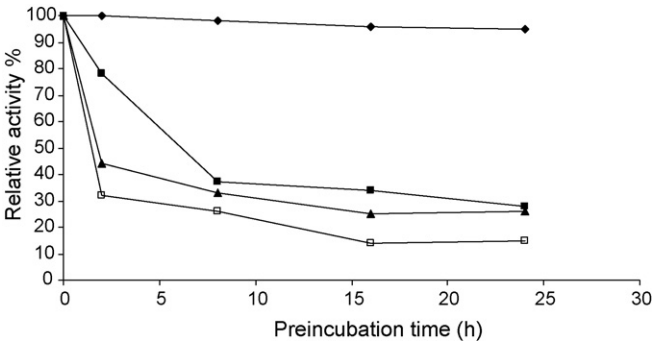

Fig. 6. Thermal stability of purified lipase. The lipase was incubated at 25 °C (♦), 35 °C (■), 45 °C (▲) and 55 °C (□) for up to 24 h in 50 mM pH 6.5 phosphate buffer.

3.3. Determination of kinetic constants

The values of K_m and V_{max} of the purified lipase, as calculated from the Lineweaver–Burk plot, were 0.02 mM and 38.91 μmol min^{-1} mg^{-1}, respectively (Fig. 5). The catalytic efficiency (k_{cat}/K_m) of lipase was found to be 1.5×10^6 M^{-1} s^{-1}. These results showed that K_m value of the lipase (*p*-NPP as substrate) from *S. platensis* was appreciably lower than K_m values of lipase from other sources such as *A. niger* F044 (7.37 mM) [8], *Bacillus stearothermophilus* MC 7 (0.33 mM) [28] and *Bacillus* sp. J33 (2.5 mM) [29]. The activation energy of lipase was calculated by using Arrhenius equation and it was found as 146.34 kJ/mol.

3.4. Determination of thermal and storage stabilities of purified enzyme

The thermal stability of purified lipase was investigated at 25–55 °C. The residual activities of purified lipase were 95%, 45%, 33% and 25% of its initial activity at 25, 35, 45 and 55 °C, respectively for 24 h preincubation (Fig. 6). These results showed that purified lipase showed the highest thermal stability at 25 °C for 24 h preincubation.

As shown in Fig. 7, the stabilities of lipase were almost same at room temperature and 5 °C upon 5 days storage. However, lipase retained 51% of its initial activity after 30 days storage at room temperature and 38% of its initial activity after 30 days storage at 5 °C.

3.5. Determination of positional specificity

We have investigated the positional specificity of purified lipase using triolein as substrate. The result of the thin-layer chromatography analysis of the hydrolysis products of triolein is shown in Fig. 8. The purified lipase hydrolysed triolein producing only 1,2-

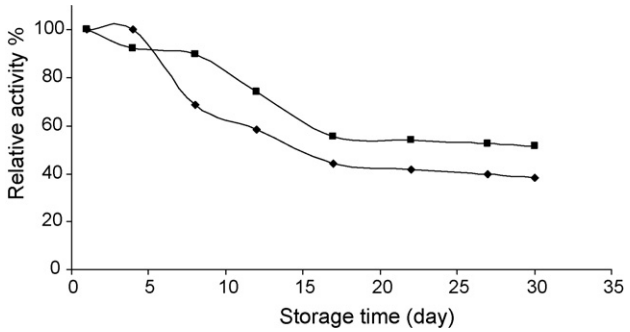

Fig. 7. Storage stability of purified lipase. The lipase was incubated at room temperature (■) and 5.0 °C (♦) for up to 30 days in 50 mM pH 6.5 phosphate buffer.

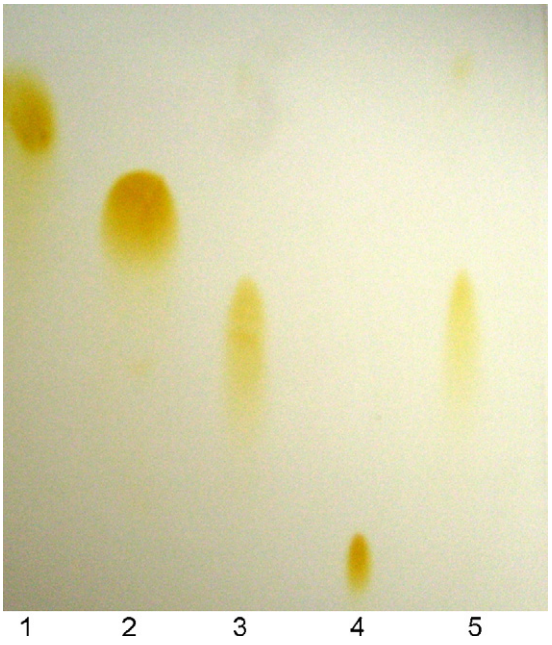

Fig. 8. TLC analysis of hydrolysis products after incubation of purified lipase on triolein as a substrate at 55 °C for 24 h. Lane 1: triolein; Lane 2: 1,3-diolein; lane 3: 1,2-diolein; Lane 4: 2-monoolein; Lane 5: Prufied Lipase.

diolein as and product even after 12 h indicating that the enzyme has 3-position specificity. Most of the lipases such as lipases from *Aspergillus carneus* [3], *Geotrichum* sp. [30], *P. camembertii Thom* PG-3 [4], *Rhizopus delimar*, *Rhizopus miehei*, *Mucor javanicus* and *Yerrowia lipolytica* [31] show 1,3-positional specificity releasing 2-monoacylglycerol and 1,2- and 2,3-diacylglycerol as products from the substrate. Few lipases such as Lipase C produced by *Geotrichum* sp. F0401B [32] show selectivity at 2-position of triacylglycerol. *Bacillus* sp. J33 [29], *Streptomyces rimosus* [24], BTID-B from *Bacillus thermoleovorans* ID-1 [5] are good examples of nonspecific lipases. Lipase from *S. platensis* cleaved triolein at only 3-position realising 1,2-diolein as main product such as lipase from *A. niger* NCIM 1207 [33].

3.6. Determination of substrate specificity

S. platensis lipase was active on wide range of natural lipids like Olive oil, Sunflower seed oil, Laurel oil, Soybean oil, Canola oil, Peanut oil, Palm oil, Cottonseed oil, Mustard oil and Castor oil (Fig. 9). Since an oil contains heterogeneous triacylglycerols, strict parallelism with the hydrolysis of triacylglycerols consisting of only one type of fatty acid, cannot be expected. It was difficult to directly measure the amount of substrates. Instead, degree of hydrolysis was quantitively measured for olive oil using triolein, a major triglyceride in olive oil. *S. platensis* lipase had high hydrolysis efficiency to olive oil, sunflower seed oil, soybean oil, palm oil and mustard oil but it had low hydrolysis efficiency to canola oil, peanut oil, cottonseed oil and castor oil. No obvious substrate specificity was observed.

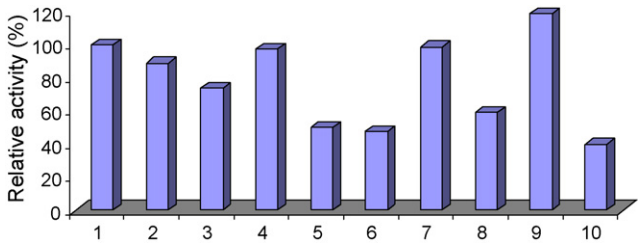

Fig. 9. Substrate specificity of *S. platensis* lipase toward natural lipids. Columns: 1, Olive oil; 2, Sunflower seed oil; 3, Laurel oil; 4, Soybean oil; 5, Canola oil; 6, Peanut oil; 7, Palm oil; 8, Cottonseed oil; 9, Mustard oil; 10, Castor oil.

Table 2
Stability of purified *S. platensis* lipase in organic solvents.

Organic solvent	Concentration (%)	Relative activity (%)
Control	–	100
Methanol	20	72
Ethanol	20	67
Acetone	20	11
Isopropanol	20	89
n-Propanol	20	33
n-Butanol	20	44
Chloroform	20	–
Diethyl ether	20	67
Benzene	20	–
Hexane	20	44

Activity without organic solvent was set as 100%. All measurements were repeated three times.

Table 3
Effect of metal ions on lipase activity.

Compounds	Concentration (mM)	Relative activity (%)
Control	–	100
LiCl	0.5	56
$MgCl_2$	0.5	155
$CaCl_2$	0.5	155
$ZnCl_2$	0.5	111
$FeCl_3$	0.5	89
$FeSO_4$	0.5	11
$CuCl_2$	0.5	89
$MnSO_4$	0.5	55

Activity without metal ions was set as 100%. All measurements were repeated three times.

Table 4
Effect of detergents and enzyme inhibitors on lipase activity.

Compounds	Concentration	Relative activity (%)
Control	–	100
SDS	0.5 mM	103
Triton X-100	0.1%	121
Tween 20	0.1%	91
EDTA	0.1%	88
PMSF	0.1%	93

Activity without detergents and enzyme inhibitors was set as 100%. All measurements were repeated three times.

3.7. Effects of organic solvents, metal ions, detergents and enzyme inhibitors on lipase activity

The effects of organic solvents on lipase activity are depicted in Table 2. The lipase was more stable in isopropanol than the other tested organic solvents and was completely inactivated in chloroform and benzene. The effects of metal ions on the lipase activity were evaluated and the results are given in Table 3. 0.5 mM of Ca^{2+}, Mg^{2+}, Zn^{2+} ions were found to enhance the lipase activity, whereas Li^{+}, Fe^{2+}, Mn^{2+} ions strongly inhibited the lipase activity. The lipase was highly active in the presence of 0.1% Triton X-100 or 0.5 mM SDS (Table 4). 0.1% EDTA or 0.1%PMSF showed inhibitory effect on the lipase activity.

4. Conclusion

In this study, the *S. platensis* lipase was purified and characterized for the first time. The lipase was purified about 375-fold with

a specific activity of 45 U/mg protein. The molecular weight and p*I* of purified lipase were found as 45 kDa and 5.9, respectively. *S. platensis* lipase is monomeric and specific for 3-position in the ester bond.

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