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Purification and Properties of Extracellular Lipases with Transesterification Activity and 1,3-Regioselectivity from *Rhizomucor miehei* and *Rhizopus oryzae*

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Rhizomucor miehei NRRL 5282 and *Rhizopus oryzae* NRRL 1526 can produce lipases with high synthetic activities in wheat bran-based solid-state culture. In this study, the purification and biochemical characterization of the lipolytic activities of these lipases are presented. SDS-PAGE indicated a molecular mass of about 55 and 35 kDa for the purified *R. miehei* and *Rh. oryzae* enzymes, respectively. *p*-Nitrophenyl palmitate (*p*NPP) hydrolysis was maximal at 40°C and pH 7.0 for the *R. miehei* lipase, and at 30°C and pH 5.2 for the *Rh. oryzae* enzyme. The enzymes showed almost equal affinity to *p*NPP, but the V_{max} of the *Rh. oryzae* lipase was about 1.13 times higher than that determined for *R. miehei* using the same substrate. For both enzymes, a dramatic loss of activity was observed in the presence of 5 mM Hg^{2+}, Zn^{2+}, or Mn^{2+}, 10 mM *N*-bromosuccinimide or sodium dodecyl sulfate, and 5–10% (v/v) of hexanol or butanol. At the same time, they proved to be extraordinarily stable in the presence of *n*-hexane, cyclohexane, *n*-heptane, and isooctane. Moreover, isopentanol up to 10% (v/v) and propionic acid in 1 mM concentrations increased the *p*NPP hydrolyzing activity of *R. miehei* lipase. Both enzymes had 1,3-regioselectivity, and efficiently hydrolyzed *p*-nitrophenyl (*p*NP) esters with C8–C16 acids, exhibiting maximum activity towards *p*NP-caprylate (*R. miehei*) and *p*NP-dodecanoate (*Rh. oryzae*). The purified lipases are promising candidates for various biotechnological applications.

Keywords: Zygomycetes, extracellular lipase, enzyme purification, lipolysis characterization, synthetic activity

Introduction

Lipases (glycerol ester hydrolases; E.C. 3.1.1.3) find promising applications in a wide range of biotechnological and industrial processes, including flavor enhancement in the food industry, biodiesel production, and pharmaceutical processing [1, 2]. These enzymes hydrolyze the triacylglycerols to fatty acids, glycerol, and partial acylglycerols. This reaction is reversible; thus, lipases also catalyze the formation of acylglycerols from glycerol and free fatty acids via esterification. Other valuable properties of most lipases are the ability of catalyzing enzymatic interesterification reactions rearranging a triglyceride molecule, and transesterification between oil compounds, alkyl or aryl esters, and alcohols [3]. Lipases can also be used to accelerate the degradation of fatty waste and polyurethane [4, 5].

Many species of the fungal subphylum Mucoromycotina (a representative group of the former class Zygomycetes) have successfully been used in several areas of biotechnological applications, including biotransformation of steroid compounds, production of organic acids, and other valuable bioactive metabolites (*e.g.*, carotenoids, polyunsaturated fatty acids), as well as production of extracellular enzymes [6]. Some strains, especially those

belonging to the genera *Mucor*, *Rhizomucor*, and *Rhizopus*, are well known from a food-industrial point of view, in consequence of their effective enzyme production [7].

Industrial application of fungal lipases has spread out in the last decades, replacing the expensive and time-consuming chemical processes [8]. Therefore, the need for new producer strains and enzymes with industrially useful properties has increased, and current research studies are mainly focused on enzymes potentially utilizable for catalysis of specific reactions, such as alkyl and phenyl ester synthesis. There are excellent lipase producers among Mucoromycotina fungi, and some enzymes have been isolated and characterized from this group [9, 10]. The most frequently used isolates to obtain lipase preparations with outstanding practical interest are from *Rhizomucor*, *Rhizopus*, and *Mucor* species [7]. Some of these biocatalysts expose high synthetic activity and thus can be utilizable for production of eco-friendly biofuels [11].

In the frame of our recent study, the lipolytic activity of several Mucoromycotina strains was tested by liquid and solid-state fermentation methods. Some thermophilic *Rhizomucor* and *Rhizopus* isolates showed intensive extracellular lipase activity, and the enzyme production was maximal when wheat bran was used as the carbon source [12]. The transesterification capacity of their crude lipase extracts was investigated in non-aqueous conditions, in which the *Rhizomucor miehei* NRRL 5282 and *Rhizopus oryzae* NRRL 1526 enzymes efficiently catalyzed the formation of ethyl palmitate ester at 40°C [13].

The present study describes the isolation of the *R. miehei* NRRL 5282 and *Rh. oryzae* NRRL 1526 lipases and biochemical characterization of their hydrolytic activities, including temperature and pH optimum assays, stability studies in the presence of various solvents and metal salts, and examinations for their substrate specificity and regioselectivity. Transesterification activity of the purified lipases has also been identified.

Materials and Methods

Solid-State Fermentation

For the induction of lipase production, 10^6 spores of *R. miehei* NRRL 5282 or *Rh. oryzae* NRRL 1526 were transferred to 3 L Erlenmeyer flasks containing 130 g of wheat bran moisturized with 130 ml of mineral salt medium and 1.5% (w/v) olive oil [12]. The cultures were incubated at 37°C for 6 days.

Purification of the Enzymes

The wheat bran-fungal mycelia ferment was extracted with 800 ml of 100 mM acetate buffer (pH 6.0) at 4°C for 24 h. After filtration on gauze and Whatman No. 1 filter paper, the crude extract was centrifuged at 5,040 ×*g* for 15 min. The protein of the supernatant was precipitated by ammonium sulfate, and precipitates from the fractions of 50% to 85% saturation were collected by centrifugation (5,040 ×*g*, 15 min). Then, the concentrates were re-dissolved in the smallest possible volume of 100 mM acetate buffer (pH 6.0). Precipitates of the fractions having saturation between 50% and 65% (in the case of *Rh. oryzae*) and 75% and 85% (in the case of *R. miehei*) showed the highest lipolytic activity. These concentrated enzyme solutions were loaded onto a Sephadex G-75 (Sigma-Aldrich, Germany; exclusion range 3 to 80 kDa) column (16 × 325 mm) equilibrated with 50 mM acetate buffer (pH 6.0). Elution was carried out at a flow rate of 0.5 ml/min using the same buffer. Fractions having lipolytic activity were collected, and fractions from *R. miehei* were applied to a Macro-Prep High Q (12.6 × 40 mm; Bio-Rad, USA), whereas fractions from *Rh. oryzae* to a Uno Q-1 (7 × 35 mm; Bio-Rad, USA) anion-exchange column. The columns were equilibrated with 50 mM acetate buffer (pH 6.0) and eluted with a linear gradient of NaCl from 0 to 1 M at a flow rate of 1 ml/min. In the case of *R. miehei* lipase, a polishing step was applied on a Sephacryl S-200 HR column (exclusion range 5 to 250 kDa; 16 × 60 mm; GE Healthcare, Sweden) equilibrated with 50 mM acetate buffer (pH 6.0) containing 150 mM NaCl, and eluted with the same buffer at a flow rate of 0.5 ml/min.

Protein Determination

During the chromatography procedures, the protein content was monitored by measuring the absorbance at 280 nm. The total protein content in the fractions having the highest lipolytic activity was determined by using a Qubit Fluorometer (Invitrogen, USA) and the Quant-iT Protein Assay Kit (Invitrogen, USA).

Protein Electrophoresis and Zymography

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 4−12% NuPage Bis-Tris gel (Invitrogen, USA) according to the manufacturer's instructions. Protein bands were detected by staining the gels with 0.0025% Coomassie Brilliant Blue R-250.

For zymogram analysis, enzyme proteins were separated on 3−12% polyacrylamide gel (native-PAGE) at 150 V. After electrophoresis, the gels were washed with sodium phosphate buffer (50 mM, pH 6.8) for 30 min at room temperature. Activity bands were developed by incubating the gels in 50 ml of sodium phosphate buffer (50 mM, pH 6.8) containing 200 μM 4-methylumbelliferyl nonanoate (Sigma-Aldrich, USA), or 1 mM α-naphthyl acetate (Sigma-Aldrich, USA) and 25 mg Fast Red, at 37°C for 30 min or 2 h, respectively.

Enzyme Activity Assays

Standard determination of lipolytic activity was performed by using *p*-nitrophenyl palmitate (*p*NPP; Sigma-Aldrich, USA) as the substrate. A 3 mM *p*NPP stock solution was prepared in dimethyl sulfoxide, and an equal volume of sodium phosphate buffer (0.1 M,

pH 6.8) was added. Unless otherwise stated, 50 µl of buffered *p*NPP solution was added to 50 µl of diluted extract, and incubated for 30 min at 37°C. The reaction was stopped by 25 µl of 0.1 M sodium carbonate, and the *p*-nitrophenol release was measured at 405 nm using an Asys Jupiter HD (ASYS Hitech, UK) microplate reader. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 µM of *p*-nitrophenol per minute. All enzyme activity measurements were carried out in triplicates.

Transesterification activities were studied following a standard *p*NPP-based spectrophotometric assay described previously [13].

Effects of Temperature and pH

The optimum temperature of the purified lipases was studied by assaying lipolytic activities in 0.1 M sodium phosphate buffer (pH 6.8) containing 0.75 mM *p*NPP in the range from 10°C to 80°C for 30 min. Thermal stability was ascertained by incubating the enzyme for 4 h at the desired temperature, and then the residual activity was estimated at the optimal temperature of the enzymes using 0.75 mM *p*NPP as a substrate. The pH optimum of the purified lipolytic activities was determined at the appropriate optimal temperatures for 30 min in the range from pH 2.2 to 8.0 by using 50 mM McIlvaine buffer supplemented with 0.75 mM *p*NPP. The pH stability was established by pre-incubating the purified lipases in the same buffer for 24 h at 4°C, and then the residual activity was evaluated by incubation for 30 min at the optimal temperature of the enzymes using 0.75 mM *p*NPP as a substrate.

Enzyme Kinetics

The apparent kinetic parameters K_m and V_{max} for the purified lipases were estimated from Lineweaver-Burk plots. The assays were carried out in sodium phosphate buffer (0.1 M, pH 6.8) with *p*NPP substrate in concentrations that varied from 0.05 to 3.2 mM at 37°C for 30 min.

Hydrolysis of Various Aryl Esters

Hydrolysis of aryl esters with different chain-length acids was studied by incubating the purified enzymes (10 U/ml) in 0.1 M sodium phosphate buffer (pH 6.8) containing 0.75 mM aryl-esters (*p*NP-acetate, *p*NP-propionate, *p*NP-butyrate, *p*NP-valerate, *p*NP-caproate, *p*NP-caprylate, *p*NP-decanoate, *p*NP-dodecanoate, and *p*NPP; Sigma-Aldrich, USA) at the optimal temperature of the lipolytic activity for 30 min. Then, the activities were examined by measuring the liberated *p*-nitrophenol at 405 nm. The relative rate of hydrolysis was determined as percentages of the initial rate of hydrolysis obtained with *p*NPP.

Effects of Metal Ions and Reagents

The effects of metal ions and chemical reagents on the lipolytic activity were assayed by incubating the purified enzymes under standard assay conditions in the presence of 5 mM $CoCl_2$, $HgCl_2$, $CuSO_4$, $ZnCl_2$, $MnCl_2$, $CaCl_2$, $MgSO_4$, NaCl, or KCl, or 10 mM N-bromosuccinimide (NBS), ethylenediaminetetraacetic acid (EDTA), or sodium dodecyl sulfate (SDS).

Effects of Various Organic Solvents and Fatty Acids

The effects of alkanes and alcohols on the *p*NPP-hydrolyzing activity of the purified enzymes were studied at concentrations ranging from 5% to 20% (v/v). The effects of fatty acids on the enzyme activity were investigated in the range from 1 to 5 mM. The *p*NPP hydrolysis was measured at standard assay condition in the presence of solvents or fatty acids relative to the background control (without solvent or fatty acid; 100% residual activity).

Regioselectivity Studies

Positional specificity of the purified lipases was examined through thin-layer chromatography (TLC) of the products obtained from the hydrolysis of triolein (99%; Sigma-Aldrich, USA). The reaction medium consisted of 5 mg/ml triolein, 795 µl of 0.1 M sodium phosphate buffer (pH 6.8), and 200 µl of purified enzyme solution (10 U/ml). After incubating the mixtures in an orbital shaker (200 rpm) at 37°C for 2 h, the products were extracted with diethyl ether in a volumetric ratio of 1:1. Then, a 30 µl sample from the organic phase was subjected to TLC on a silica gel 60 plate (Merck, Germany) using *n*-hexane/diethyl ether/acetic acid (59:40:1 (v/v)) as a mobile phase. Triolein, oleic acid, 1,3-diolein, (±)-1,2-diolein, and monoolein were purchased from Sigma-Aldrich and used as reference standards. Reaction products and the standards were visualized using a saturated iodine chamber. A control reaction mixture contained 0.1 M sodium phosphate buffer (pH 6.8) instead of the enzyme solution.

Results and Discussion

Purification of Lipases

Previous studies revealed high lipolytic activities in the crude enzyme extracts of strains *R. miehei* NRRL 5282 and *Rh. oryzae* NRRL 1526 cultivated in wheat bran-based solid-state medium [12]. Additionally, it has recently been proven that these extracts contain organic solvent-tolerant lipases with significant transesterification activities [13]. In this study, we successfully purified lipases from these *R. miehei* and *Rh. oryzae* crude enzyme extracts through ammonium sulfate precipitation followed by three or two steps of chromatographic separation, respectively. The purification data are summarized in Table 1. The *R. miehei* and *Rh. oryzae* lipases were purified 107.3- and 94.7-fold, and the recovery rate was 2.3% and 0.7%, respectively. The relatively low yield can be attributed to the fractionated desalting of the proteins, which caused some activity losses during the steps, and/or the partial aggregation of the enzymes with olive oil added to the solid fermentation medium as a lipase inductor compound (see reference [12]). This lipase-lipid complex may negatively affect the enzyme purification process [14].

The molecular mass of the *R. miehei* and *Rh. oryzae* enzymes

Table 1. Purification of lipases from *R. miehei* NRRL 5282 and *Rh. oryzae* NRRL 1526.

	Total protein (mg)	Total activity (μmol/min)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Rhizomucor miehei NRRL 5282					
Crude extract	3,222.4	65,489.2	20.3	1	100
Ammonium sulfate (75–85%)	98.7	25,364.5	257	12.7	38.7
Sephadex G-75	44	23,213	527.6	25.9	35.4
Macro-Prep HQ	4.1	3,957.9	970	47.7	6
Sephacryl S200HR	0.7	1,513	2,181.4	107.3	2.3
Rhizopus oryzae NRRL 1526					
Crude extract	5,662.8	143,028.6	25.26	1	100
Ammonium sulfate (50–65%)	102.2	57,865.5	566.2	22.4	40.5
Sephadex G-75	11.7	8,147.9	694.9	27.5	5.7
Uno Q-1	0.42	1,002	2,392.6	94.7	0.7

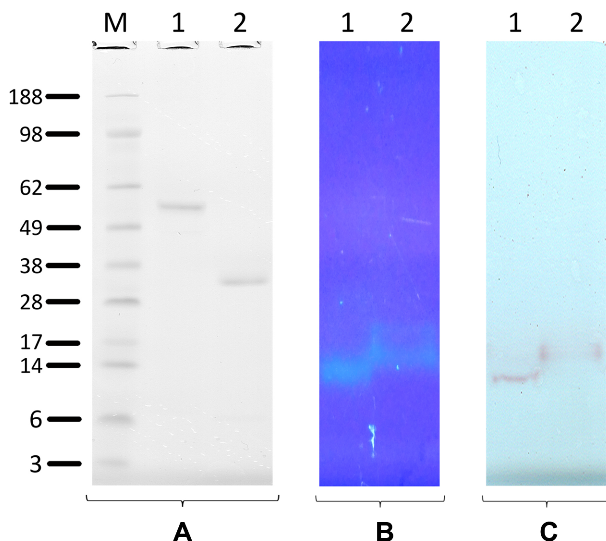

Fig. 1. SDS-PAGE (**A**) and zymogram (**B** and **C**) analysis of the purified lipases.

Lane 1 and lane 2 are *R. miehei* NRRL 5282 and *Rh. oryzae* NRRL 1526 lipases, respectively. Lipolytic activity staining was performed after native-PAGE by using 4-methylumbelliferyl nonanoate (**B**) and α-naphthyl acetate (**C**). "M" indicates the SDS-PAGE molecular weight (kDa) standards (SeeBlue Plus2; Invitrogen, USA).

was estimated to be approximately 55 and 35 kDa, respectively, by SDS-PAGE (Fig. 1A). In the case of filamentous fungi, the molecular mass for lipases generally varies between 25 and 70 kDa [10]. Biochemical properties of some *Rhizomucor* and *Rhizopus* lipases are summarized in Table 2. It shows that the molecular masses of the lipases isolated in the current study are slightly higher than those identified previously by most *Rhizomucor* and *Rhizopus* strains.

Zymogram analysis of the purified enzymes showed active lipases stained with 4-methylumbelliferyl nonanoate and α-naphthyl acetate (Figs. 1B and 1C). Moreover, both purified enzymes catalyzed the ethyl palmitate formation from ethanol and *p*NPP as a result of their transesterification activities. In these assays, the specific activities of 15.7 and 20.9 U/mg were detected for the *R. miehei* and *Rh. oryzae* lipases, respectively.

Temperature Optimum and Stability

The temperature optimum for maximal lipolytic activity was 40°C and 30°C for *R. miehei* and *Rh. oryzae* enzymes, respectively (Fig. 2). The *p*NPP hydrolysis by *R. miehei* lipase decreased significantly at temperatures above 52°C, and the enzyme exhibited only 21% of its original activity at 60°C. However, this enzyme can be considered as thermotolerant because it retained its initial activity at 50°C after 4-h incubation (Fig. 2A). As mentioned above, a significant decrease in the enzymatic activity was observed above this temperature, which may be caused by the formation of aggregates instead of protein denaturation [28]. The thermal stability of many lipases, however, can be further enhanced by entrapping it to a suitable carrier [29]. Lipases with a high temperature optimum and stability have been identified and investigated from many other filamentous fungal sources [30, 31]. Such lipases can be utilized in the detergent and chemical industries, including biodiesel production [1]. The *Rh. oryzae* lipase was less stable at temperatures above 25°C as compared with *R. miehei* enzyme (Fig. 2B). It lost about 10–15% of its activity at room temperature and was completely inactivated above 40°C after 4-h pre-incubation. Both enzymes were stable at 37°C; therefore, we used this condition for further analyses.

Table 2. Molecular mass, temperature, and pH optimum of lipases isolated from various *Rhizomucor* and *Rhizopus* strains.

Microorganism	Molecular mass (kDa)	Temperature optimum (°C)	pH optimum	Reference
Rhizopus arrhizus	67	n.r.	n.r.	[15]
Rhizopus chinensis	28.4	37	5.5	[16]
Rhizopus chinensis				
Lip 1	60	n.r.	n.r.	[17]
Lip 2	33	40	8.0–8.5	[18]
Rhizopus chinensis				
SSF lipase	62	40	8.0	[19]
SmF lipase	40	40	8.0	
Rhizopus delemar	30.3	30	8.0	[20]
Rhizopus japonicus (NR 400)	30	n.r.	5.0	[21]
Rhizopus niveus				
Lipase I	34	35	6.0–6.5	[22]
Lipase II	30	40	6.0	
Rhizopus oryzae	32	35	7.5	[23]
Rhizopus oryzae	17	40	7.0	[24]
Rhizopus oryzae WPG	29	37	8.0	[25]
Rhizopus homothallicus				
SmF lipase	32	30	7.5	[14]
SSF lipase	32	40	7.5	
Rhizomucor miehei	31.6	37	8.0	[26]
Rhizomucor miehei (UzLT-3)				
Lipase A	43	55	8.7–8.8	[27]
Lipase B	40	45	8.2–8.3	
Rhizopus oryzae	35	30	6.8–7.4	This work
Rhizomucor miehei	55	40	5.0–5.4	This work

n.r.: not reported.

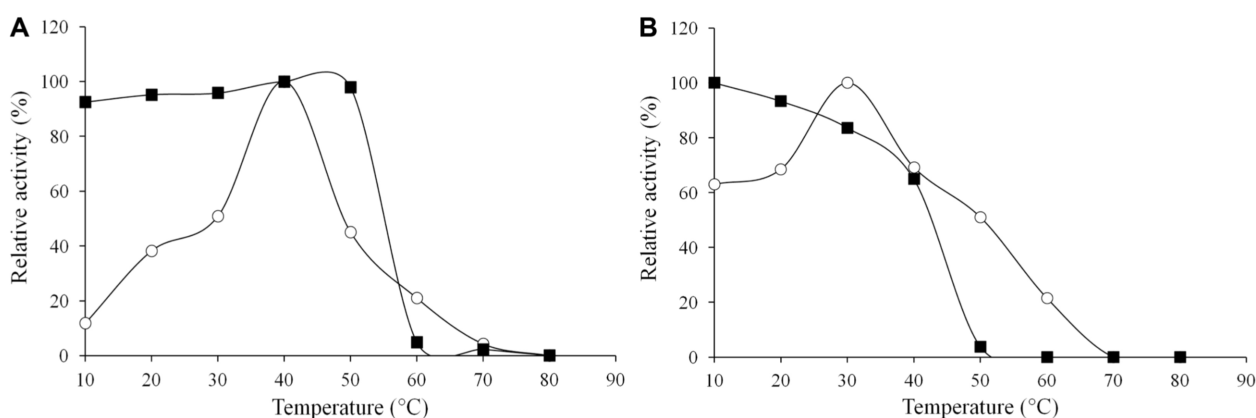

Fig. 2. Effect of temperature on the *p*NPP-hydrolyzing activity (-○-) and stability (-■-) of purified *R. miehei* NRRL 5282 (**A**) and *Rh. oryzae* NRRL 1526 (**B**) lipases.
See Materials and Methods for experimental conditions.

pH Optimum and Stability

The purified *R. miehei* lipase had a pH optimum between 6.8 and 7.4 (Fig. 3A), whereas that of the *Rh. oryzae* enzyme was found to be between pH 5.0 and 5.4 (Fig. 3B) after incubating at their optimal temperature conditions for 30 min. This corresponds to the range documented for most fungal lipases [9]. Unlike the *Rhizomucor* lipase, the *Rhizopus* enzyme had a slightly wider pH optimum range,

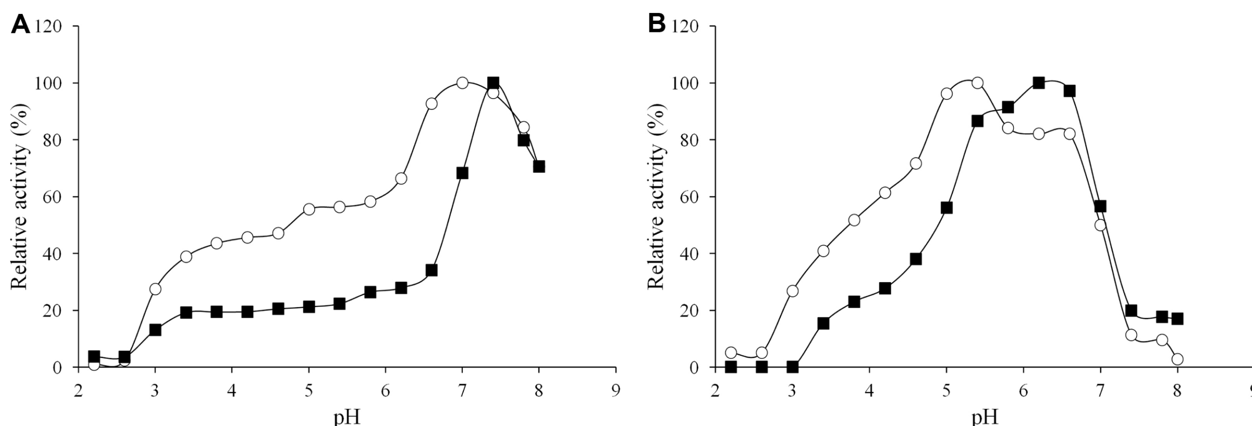

Fig. 3. Effect of pH on the *p*NPP-hydrolyzing activity (-○-) and stability (-■-) of purified *R. miehei* NRRL 5282 (**A**) and *Rh. oryzae* NRRL 1526 (**B**) lipases.
See Materials and Methods for experimental conditions.

presenting more than 70% residual activity from pH 4.6 to 6.8. Fungal lipases with a pH optimum of about 5.0 have also been described from *Aspergillus niger* [31], *Rhizopus japonicus* NR 400 [21], and *Penicillium camamberti* (Amano Lipase G) isolates. Both enzymes retained about 30% of their original activity at pH 3.0, which indicates good tolerance to acidic working conditions. One of the most common applications of acid active lipases is in the leather industry where they are used for treating the animal skins stored in a pickled state [8].

The pH stability of the purified lipases was also investigated by measuring the residual activity after 24-h incubation at 4°C at pH values ranging from 2.2 to 8.0. Since the *R. miehei* lipase was fairly stable and retained more than 70% of its activity in a pH range from 7.0 to 8.0 (Fig. 3A), it can be considered as an alkaline-tolerant enzyme. It is interesting to note that only 20−30% of its original activity was detected in the pH range from 3.4 to 6.2. Wu *et al.* [26] reported a similar pH stability profile for a commercial *R. miehei* lipase preparation using olive oil as a substrate. The *Rh. oryzae* lipase had a wider pH stability range than the *Rhizomucor* enzyme, and retained 60−100% of its original activity between pH 5.4 and 6.8. However, a significant decrease in its stability could be observed after incubation in buffers above pH 7.0 (Fig. 3B). Similarly, reduced pH stability was detected by the lipase from *Rh. oryzae* isolated from palm fruit in alkaline pH [23], but it was more stable under acidic conditions than the *Rh. oryzae* enzyme investigated in this study. Since both tested enzymes proved to be stable at pH 6.8, further analyses were carried out under this condition.

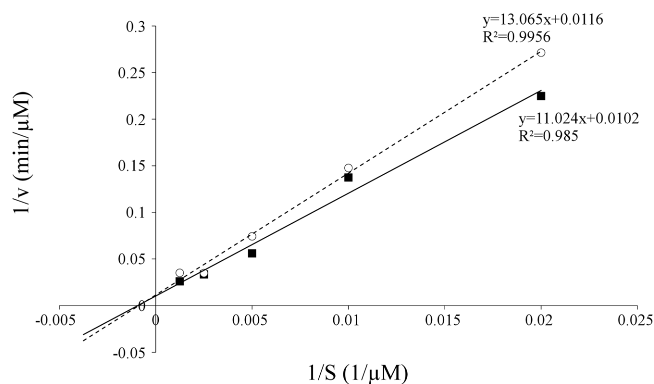

Fig. 4. Lineweaver-Burk plots for *p*NPP with the *R. miehei* NRRL 5282 (-○-, dotted line) and *Rh. oryzae* NRRL 1526 (-■-) lipases.

Kinetic Studies

Kinetic parameters were determined from Lineweaver-Burk plots using different concentrations of *p*NPP substrate. The estimated K_m and V_{max} values were 1.13 mM and 86.2 µM/min for the *R. miehei* lipase, and 1.08 mM and 98.1 µM/min for the *Rh. oryzae* enzyme, respectively (Fig. 4). These results show that both purified enzymes have almost equal affinity to the substrate, but the *Rh. oryzae* lipase-catalyzed *p*NPP hydrolysis was about 1.13 times faster than that determined for the *R. miehei* enzyme. In previous studies, *p*NPP substrate was also used to determine the kinetic parameters of different zygomycete lipases. For *Mucor hiemalis* f. *corticola* IDM11B lipase [32], K_m and V_{max} values were found to be 1.327 mM and 91.11 µM/min, respectively, which are very close to those obtained during

our assays for the two enzymes. Yu *et al.* [33] reported K_m and V_{max} values of 0.304 mM and 30.76 U/mg, respectively, for the recombinant *Rhizopus chinensis* (r27RCL) lipase. These latter values indicate a more stable enzyme-substrate complex but similar V_{max}/K_m ratio to those found for our purified *R. miehei* and *Rh. oryzae* lipases.

Hydrolysis of Various Aryl Esters

p-Nitrophenyl monoesters with C2–C16 acids were used to evaluate the chain-length specificity of the purified lipases. As can be seen in Fig. 5, both enzymes showed similar lipolysis profiles: they were most active against substrates with medium- and long-chain (C8–C16) acids, and showed low activities on short-chain (C2–C3) aryl esters. Moderate enzyme activities could be detected in the case of *p*NP-butyrate (C4) and *p*NP-valerate (C5). More importantly, the initial hydrolysis exhibited by the purified *R. miehei* enzyme was about 1.2 to 1.8 times higher on *p*NP-caproate (C6), -caprylate (C8), -decanoate (C10), and -dodecanoate (C12) than that obtained on *p*NP-palmitate (C16). For the *Rh. oryzae* enzyme, the highest activities were detected against aryl esters with C8 to C12 acids. In the case of lipases from filamentous fungi, chain-length specificity generally varies between C8 and C18 [14, 34]. Most zygomycete lipases present maximal activity for medium- or long-chain acids, but some of them have high reaction specificity for C2 to C6 acids as well [33, 35].

Effects of Metal Ions and Reagents on Lipase Activity

The effects of various metal ions (5 mM) and reagents (10 mM) on *R. miehei* and *Rh. oryzae* lipase activity were investigated. As shown in Table 3, significant inactivation was observed with Hg^{2+}, NBS, and SDS. The effect of Hg^{2+} suggests that thiol groups are required for the adequate function of the enzyme. The inhibition effect of NBS suggests that tryptophan may be involved in the active site of the enzyme [36]. The enzyme activity was enhanced about 10% by the addition of Mg^{2+} to the reaction mixture; furthermore, K^+ had no significant effect on the enzyme activity. It was interesting to observe that, under optimal conditions (40°C, pH 6.8, and 30 min), *p*NPP hydrolysis by *R. miehei* lipase was stimulated about 37% in the presence of 5 mM Na^+. Similar stimulative effects of Na^+ have been described for lipases from *Rhizopus oligosporus* var. *microsporus* [37] and *Geotrichum marinum* [38]. Based on literature data, it can be assumed that the Na salt stabilizes the enzyme conformation, which enhances the enzyme activity through strengthening the integrity of the active site [39].

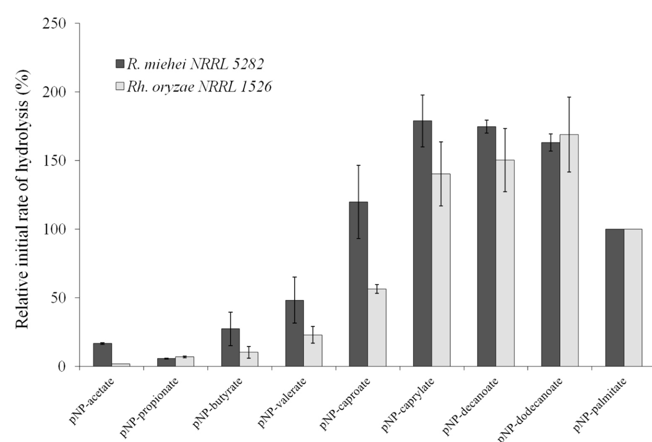

Fig. 5. Relative initial rate of hydrolysis of various aryl esters by the lipases of *R. miehei* NRRL 5282 and *Rh. oryzae* NRRL 1526.

The lipolytic activity value detected during *p*NPP hydrolysis was considered to be 100%.

Effects of Solvents on the Enzyme Activity

Lipase activities are widely utilized in the organic synthesis processes, which normally require enzymes to be stable and active in most organic solvents. Such well-known application is biodiesel production, which primarily uses methanol, but other alcohols such as ethanol, isopropanol, or isobutanol are also appropriate for efficient transesterification

Table 3. Influence of metal ions and chemical reagents on the *p*NPP-hydrolyzing activity of *R. miehei* NRRL 5282 and *Rh. oryzae* NRRL 1526 lipases.

Compounds	Relative activity (%)	
	R. miehei	*Rh. oryzae*
Control	100	100
$HgCl_2$	10	12
$CuSO_4$	77	91
$ZnCl_2$	76	44
$MnCl_2$	60	63
$CaCl_2$	84	38
$MgSO_4$	110	107
NaCl	137	76
KCl	98	93
$CoCl_2$	79	80
NBS	18	8
EDTA	65	74
SDS	1	4

Metal ions and reagents were used in 5 mM and 10 mM final concentration, respectively. Activity measured without any additive was considered to be 100%.

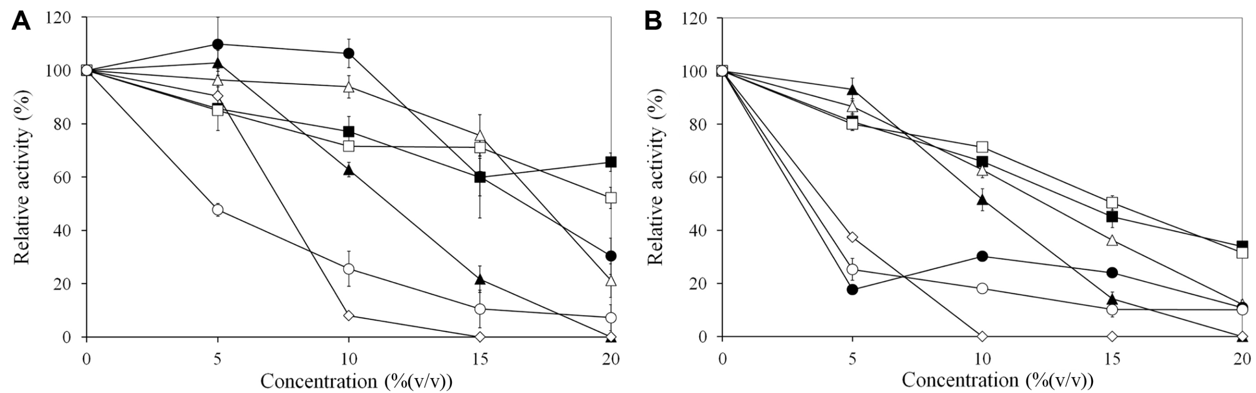

Fig. 6. Effects of alcohols (-■- methanol, -□- ethanol, -▲- propanol, -△- isopropanol, -◇- butanol, -●- isopentanol, -○- hexanol) on the *p*NPP-hydrolyzing activity of lipases from *R. miehei* NRRL 5282 (**A**) and *Rh. oryzae* NRRL 1526 (**B**).

Values are the average of assays performed in triplicates; error bars represent the standard deviation.

of fatty acids [40]. Here, we investigated the lipolytic activity of the purified *R. miehei* and *Rh. oryzae* lipases in the presence of various primary and secondary alcohols and alkanes. Fig. 6 shows that the activities of both enzymes decreased in the presence of 5–10% (v/v) hexanol and butanol, and low concentrations of methanol, ethanol, propanol, and isopropanol had no considerable effect on the *p*NPP hydrolysis. The *Rh. oryzae* enzyme proved to be sensitive for the presence of isopentanol as well (Fig. 6B). However, interestingly, a slight increase in the *p*NPP hydrolysis could be observed by the *R. miehei* lipase at up to 10% (v/v) of isopentanol (Fig. 6A). Overall, the enzymes were more stable in methanol and ethanol at higher concentrations (20% (v/v)) than the other alcohols tested, which is similar to that documented for the lipase from *M. hiemalis* f. *corticola* IDM11B [32]. Both enzymes retained their initial activity at high concentrations of *n*-hexane, cyclohexane, *n*-heptane, and isooctane (Fig. 7). In a similar study, a *Rh. oryzae* strain isolated from palm fruit was also stable in alkanes, but it denatured in the presence of short-chain alcohols [23]. The changes in the enzyme activity due to the effect of various organic solvents can be attributed to the influences of various factors, such as the chemical structure of the solvent, its physical parameters, and the overall structure of the enzyme [41].

Effects of Fatty Acids on the Activity

Lipases produce free fatty acids that have different effects on the original enzyme activity: they may act as a competitive inhibitor or can stimulate the initial activity in low concentrations [42, 43]. Although lipase-catalyzed esterification and modification of various free fatty acids have extensively been investigated in the literature, as we know, the effect of these compounds on the hydrolytic

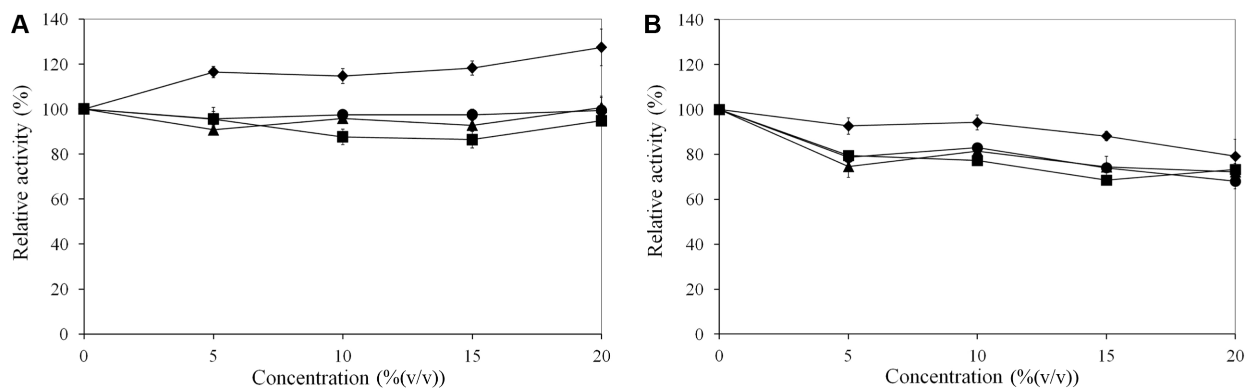

Fig. 7. Effects of alkanes (-■- *n*-hexane, -▲- cyclohexane, -●- *n*-heptane, -◆- isooctane) on the *p*NPP-hydrolyzing activity of lipases from *R. miehei* NRRL 5282 (**A**) and *Rh. oryzae* NRRL 1526 (**B**).

Values are the average of assays performed in triplicates; error bars represent the standard deviation.

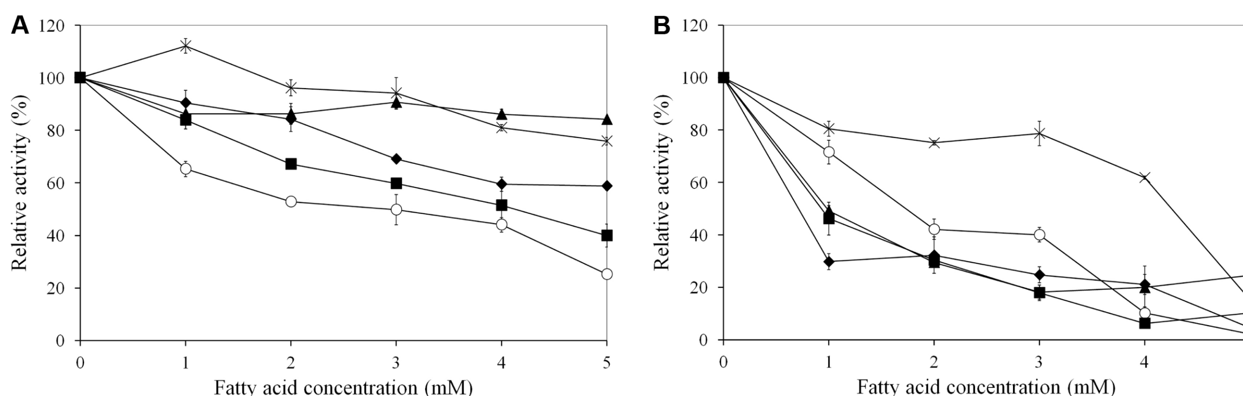

Fig. 8. Effects of fatty acids (-×- propionic acid, -◆- myristic acid, -○- palmitic acid, -▲- stearic acid, -■- linoleic acid) on *R. miehei* NRRL 5282 (**A**) and *Rh. oryzae* NRRL 1526 (**B**) lipases.

Values are averages calculated from the data of three independent measurements; error bars indicate the standard deviations.

activity of *R. miehei* and *Rh. oryzae* lipases was still unknown. Here, we have examined the fatty acid tolerance of the purified *R. miehei* NRRL 5282 and *Rh. oryzae* NRRL 1526 lipases in the presence of propionic (C3), myristic (C14), palmitic (C16), stearic (C18), and linoleic (C18:2) acids in concentrations ranging from 1 to 5 mM. Relative activity data in Fig. 8 show the effect of fatty acid concentrations on the *p*NPP hydrolysis. Overall, the long-chain fatty acids generally decreased the enzyme activities in a dose-dependent manner. These fatty acids are closer to the optimal substrate of both enzymes (see Fig. 5), which suggests a competition with the *p*NPP substrate for the active site. However, the *R. miehei* enzyme showed moderate activation (112% of the initial activity) at 1 mM propionic acid (Fig. 8A). As reported for other microbial lipases [43], this activation raises a specific or cooperative binding of the fatty acid to the protein surface. The *Rh. oryzae* lipase was more sensitive to all fatty acids than the *R. miehei* enzyme. Propionic acid exhibited the lowest inhibitory effect towards the *Rh. oryzae* lipase too (Fig. 8B), which also proves the above suggestions. Interestingly, the *R. miehei* enzyme showed a moderate inhibition in the presence of 5 mM stearic acid, the longest carbon chain fatty acid involved in the analysis (Fig. 8A). Although these results cannot be extrapolated directly from one enzyme to another, they would also provide useful data for industries that utilize *Rhizomucor* and *Rhizopus* commercial lipases as biocatalysts.

Regioselectivity

Positional specificity during the hydrolysis of triacylglycerol is an important practical feature of lipases. It influences not only the nature of diglycerides formed as a result of the hydrolysis but also reflects the potential use of the enzyme for the catalysis of unique esterification reactions. According to their preference for acyl groups on the triacylglycerol, lipases can be categorized as 1,3-specific, 2-specific, and nonspecific. This property can be investigated by TLC of the products formed after the enzymatic hydrolysis of triolein substrate. Fig. 9 shows the mode of reaction of the purified *Rh. oryzae* (lane 7) and *R. miehei* (lane 8) lipases. After 2 h of incubation, both enzymes cleaved only the 1- and 3-positioned ester bonds, since the obtained products were oleic acid, (±)-1,2- diglyceride, and monoolein. 1,3-Diglyceride was not seen; thus, they do not act on the 2-positioned ester bond of triacylglycerols. This kind of position-specific property has been documented for some fungal lipases, such as enzymes from *Rhizopus oryzae* ATCC 96382 and *Rh. rhizopodiformis* [44], *Aspergillus oryzae* and *A. niger* NCIM 1207 [45, 46], and for many other bacterial enzymes [47–49]. It is also known that the 1- or 3-regioselectivity is typical of several lipases that can catalyze transesterification reactions with high conversion yield [50, 51]. The enzymes investigated in our study also possess such synthetic activities (see the above section “Purification of Lipases” and [13]).

This study reported the purification and biochemical characterization of *R. miehei* NRRL 5282 and *Rh. oryzae* NRRL 1526 lipases from crude enzyme extracts, which exhibited high esterification and transesterification activities [13]. The purified enzymes had a molecular mass of 55 and 35 kDa on SDS-PAGE, respectively, and catalyzed transesterification in organic solvents. The *Rhizomucor* enzyme presented maximal *p*NPP hydrolysis at 40°C and pH 5.2, whereas these features proved to be 30°C and pH 7.0 for the *Rhizopus* lipase. The *R. miehei* enzyme had short-term stability at 50°C, and it remained active in the

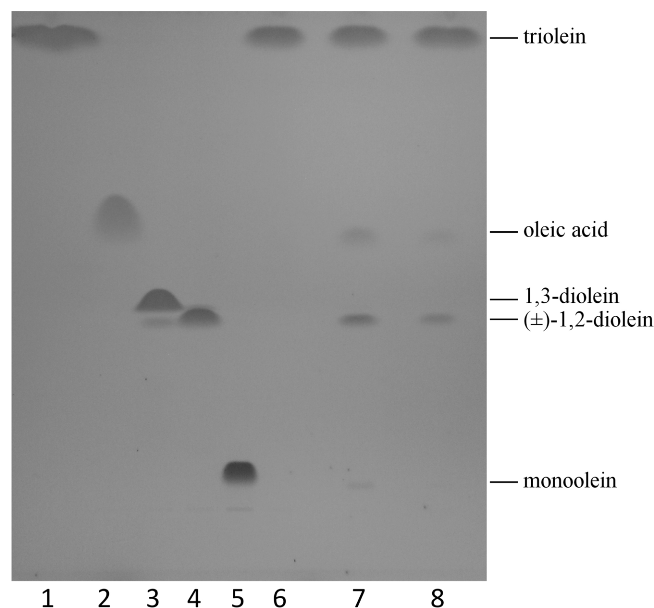

Fig. 9. Regioselectivity of the lipases from *Rh. oryzae* NRRL 1526 and *R. miehei* NRRL 5282.

Lane 1: triolein; Lane 2: oleic acid; Lane 3: 1,3-diolein; Lane 4: (±)-1,2-diolein; Lane 5: monoolein; Lane 6: control (without addition of enzyme); Lane 7: hydrolysis products by *Rh. oryzae* NRRL 1526; Lane 8: hydrolysis products by *R. miehei* NRRL 5282. The lower spot in lane 3 may be impurity.

presence of a low concentration of free fatty acids; moreover, 1 mM propionic acid positively affected the *p*NPP hydrolysis. Both purified lipases displayed high affinity to the *p*NPP substrate, wide substrate specificity (C8-C16 acids), 1,3-positional specificity, and stability in most common solvents used for enzymatic reactions in organic media. These results suggest that *R. miehei* NRRL 5282 and *Rh. oryzae* NRRL 1526 lipases possess properties that can be valuable for future industrial applications.

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