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Production, purification and characterization of an extracellular lipase from *Mucor hiemalis f. hiemalis*

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Abstract

Mucor hiemalis f. hiemalis is a major contaminant of cameroonian palm fruit and produces an inducible extracellular lipase in batch fermentation. Rape oil was the best inducer for enzyme production, with the highest activity being achieved after 6 days of incubation. The enzyme was purified 2200-fold by ultrafiltration, ammonium sulfate fractionation, Sephadex G75 chromatography, Q-Sepharose chromatography, and Sephacryl S-200 chromatography. The purified enzyme showed a prominent polypeptide band in polyacrylamide gel electrophoresis, associated with esterase activity according to activity staining. Molecular weight of the lipase was estimated to be 49 kDa using gel filtration on Sephadex G75, and 49 kDa by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The enzyme was identified as a glycoprotein with pI of 4.6. The N-terminal amino acid sequence data (19 residues) and the amino acid composition were determined. The optimum pH and temperature for activity of the enzyme were 7.0 and 40°C, respectively. The lipase was stable in the pH range of 4–9 and at 45°C for 15 min. It hydrolyzed both synthetic and natural triglycerides with optimal activities recorded on tricaprylin and rape oil, respectively. Ca^{2+}, Mg^{2+}, Co^{2+}, Mn^{2+}, and Na^{+}-enhanced lipase activity, whereas Fe^{2+}, Cu^{2+}, Ba^{2+}, and surfactants—such as taurocholic acid, triton X-100, and Tween 20—strongly reduced lipase activity. The enzyme activity was not affected by EDTA (ethylenediaminetetraacetic acid disodium dihydrate), PMSF (phenylmethylsulfonylfluoride), (p-chloromercuribenzoic), and Benzamidine.

Keywords: Mucor hiemalis f. hiemalis; Palm fruit; Extracellular lipase; Inducible; Purification; Enzyme characterization

1. Introduction

Lipases (EC 3.1.1.3) act as fat-water interfaces to catalyze the hydrolysis of triglycerides to fatty acid and glycerol [1]. An important characteristic of lipases is their ability not only to hydrolyze the ester bonds, trans-esterify triglycerides, and resolve racemic mixture but also to synthesize ester bonds in non-aqueous media [2–4]. Therefore lipases have become industrially useful for the modification of fats and oils. The deterioration during the storage of palm fruit and oils which are obtained from them has been attributed to fungi [5–7]. However, further studies concerning isolation and biochemical features of lipases are required to relate the presence of fungi on palm fruit to their involvement in the deterioration processes. During our search for microbial lipases involved in the release of fatty acid from palm oil, we screened four lipolytic strains on Rhodamine B-olive oil plate: *Mucor hiemalis f. hiemalis*, *Rhizopus oryzea*, *Aspergillus sydowi*, *Fusarium verticillioides. Mucor hiemalis f. hiemalis*, a major contaminant of Cameroonian palm fruit, produces an inducible extracellular lipase in batch fermentation. For several reasons including high production, stability, and certain specificities, lipases are obtained from microbial fermentation. Lipases from *Pseudomona* [8], *Geotrichum* [9], *Candida rugosa* [10], *Aspergillus* [11], *Rhizopus* [12] and *Penicillium* [13,14] have been isolated and characterized. This report described for the first time the isolation, purification, and characterization of an extracellular lipase from *Mucor hiemalis f. hiemalis*.

2. Materials and methods

2.1. Micro-organisms

Mucor hiemalis f. hiemalis was isolated in our laboratory from Cameroonian palm fruit. The fungus was identified by

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the Institute of Hygiene and Epidemiology Mycology of Brussels, Belgium.

Palm fruit were obtained from CIRAD (Centre de Coopération Internationale en Recherche Agronomique pour le Développement—Montpellier, France). Peptone from casein, pancreatically digested, free from sulfonamide antagonists and Sabouraud-4% malt agar were obtained from E. Merck. Yeast extract was purchased from Difco and Biomérieux. Q-Sepharose, Sephadex G75, Sephacryl S-200, and reagents for electrophoresis were obtained from Pharmacia. Polyvinylidene difluoride (PVDF) membrane and pI standards were obtained from Bio-Rad.

Culture medium: (w/v) 5% Polypeptone, 1% yeast extract, 1.4% KH2PO4, 0.24% Na2PO4, 0.04% MgSO4, and 0.5% inducer. The initial pH medium was adjusted to 6.0, using 1 N NaOH.

2.2. Growth conditions

The fungus was cultivated at 24°C in 1 l erlenmeyer flasks containing 100 ml of culture medium. Inoculations were made with 1 ml of blended mycelium from 3-day-old culture in Sabouraud-4% malt agar. At 24 h intervals, the culture was filtered, and the medium harvested was used for the necessary growth studies. The cell-free filtrate was defatted in the cold with chilled n-hexane and used as a source of extracellular lipase. The results were triplicate determinations.

2.3. Growth studies

After filtration, the mycelium was washed three times with water and three times with acetone. Mycelia mass was expressed as dry weight after drying at 105°C for 12 h. Values were the mean of three sets of experiments run simultaneously.

2.4. Determination of lipase activity

Activity determination was carried out titrimetrically as described previously [15], using a VIT 90 Video titrator (Radiometer, Copenhagen, Denmark) and 0.05 N NaOH as titrant. The reaction medium contained 20 ml of 2.5 mM Tris–HCl pH 7, 0.1 M NaCl, 10 mM CaCl2, and 200 mM tricaprylin as substrate. Unless stated otherwise, incubations were carried out at 35°C with a set point pH 7 under nitrogen atmosphere. The enzymatic reaction was initiated by addition of enzyme to the emulsion. Controls were carried out as above, but the enzyme solution was inactivated by boiling for 15 min. Kinetics of hydrolysis were zero-order within the first 5 min of the reaction. Activity again long-chain triacylglycerol or oil was determinated with 20 ml of substrate emulsion prepared in a blender (Sofraca, Turmix, Vitrolles, France) by mixing 40 ml of oil with 400 ml of a 5% solution of Arabic gum. One unit of lipase activity was defined as the release of 1 μmol of fatty acid per min under these conditions. The specific activity is the number of lipase units per mg protein.

2.5. A rapid assay of lipase activity on Rhodamine B-olive oil agar plates

The assay was adopted from Samad et al. [16]. Lipase activity was detected upon UV irradiation as orange–red fluorescent halos.

2.6. Esterase assay

The esterase activity was determined spectrophotometrically using *p*-nitrophenylacetate (p-NPA) as substrate according to Erlanson [17]. One hundred μmol of p-NPA was dissolved in 1 ml of methanol and added with a submerged pipette to 99 ml of 0.05 N Na-acetate buffer pH 5.0. The incubation mixture contained 1 ml of the substrate solution as described above, 1 ml of 0.5 M Tris-HCl buffer pH 7.4, and 100 or 250 μl of enzymatic source. The incubations were performed at room temperature and the released p-nitrophenol was estimated during 5 min by reading the solutions in a spectrophotometer (Ultraspec III Pharmacia Biotech associated with a PC/ Software Kinetic) at 400 nm against a blank prepared with heat-inactived enzyme.

2.7. Determination of temperature and pH effects on the lipolytic activity

The optimal temperature for enzyme activity was determined by incubating the reaction mixture at 20, 25, 30, 35, 40, 45, or 50°C. Controls were performed with boiled enzyme. The optimal pH was determined by incubating of the enzyme-substrate at various pH from 5 to 9 in a suitable buffer. The effect of storage pH on lipase activity was measured by preincubating the enzyme at the desired pH and assaying residual activity at pH 7.

2.8. Assay protein content

Protein determination was performed according to Lowry et al. [18] using bovine serum albumin as standard. In chromatography experiments, the protein of fractions was routinely estimated by measuring absorbance at 280 nm.

2.9. Enzyme purification

All operations were performed at 4°C unless otherwise mentioned. Culture supernatant was concentrated by ultrafiltration with an Amicon stirred cell with a PM-10 membrane, until a 20-fold concentration was achieved. The enzyme was precipitated in two steps. The first step was the addition of ammonium sulfate up to 40% (w/v) saturation at 0°C and centrifugation at 10 000 $\times$ g for 45 min. The precipitate was then discarded. As a second step, ammonium sulfate was added to the supernatant to a final con-

centration of 75% (w/v). After standing in the ammonium sulfate solution for 5 h at 0°C, the precipitate was collected by centrifugation at 10 000 × *g* for 15 min and dissolved in 20 mM Tris–HCl buffer pH 6.8 containing 0.2 mM PMSF, 2 mM EDTA, 2 mM benzamidine and 0.02% (w/v) sodium azide (buffer A). Undissolved materials were removed by centrifugation as described above. The enzyme solution was applied to a Sephadex G75 column (2.6 × 70 cm) previously equilibrated with buffer A containing 0.15 NaCl. The enzyme was eluted with the same buffer at a flow rate of 18 ml/h (3 ml fraction). The active fractions were pooled, concentrated by ultrafiltration and dialyzed against buffer A. The enzyme was loaded on a Q-Sepharose column (2.6 × 10 cm) equilibrated previously with buffer A. The column was washed sequentially with 50 ml buffer A containing 0.05, 0.1, 0.2, 0.3, 0.5, and 1 M NaCl. The highest lipase activity was found in the fractions eluted with 0.1 M NaCl solution. Active fractions from Q-Sepharose were concentrated by ultrafiltration and applied to a Sephacryl S-200 column (2.6 × 70 cm) previously equilibrated with buffer A containing 0.15 M NaCl. The enzyme was eluted with the same buffer at a flow rate of 15 ml/h. The purified enzyme was concentrated by ultrafiltration.

2.10. Gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing were run on a Bio-Rad Mini-Protean II DUAL SLAB cell according to manufacturer's instructions. SDS-PAGE was performed as described Laemmli et al. [19] in a 12.5% polyacrylamide gel slab. Phosphorylase B (94 kDa), bovine serum albumin (68 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa) were used as molecular markers. Samples and controls were boiled for 3 min in the sample buffer, which contained 5% β-mercaptoethanol. Proteins were stained with Coomassie brillant blue R-250 (Bio-Rad). Native electrophoresis was carried out in 7.5% polyacrylamide gel in Tris-glycine buffer (pH 8.3). Lipase activity was detected by placing the gel onto a solid lipase detection medium containing olive oil and Rhodamine B. The incubation was run at room temperature for 60 min and examined under UV light for bright pink bands.

2.11. Electrotransfer on PVDF membrane

After the SDS-PAGE, purified lipase was electrotransfered onto a PVDF membrane with the Mini Trans-Blot electrophoretic Transfer cell from Bio-Rad according to manufacturer's instruction. The PVDF membrane was rinsed with water and then stained by Coomassie brillant blue R-250. The stained component was used for the N-terminal sequencing.

Amino acid analysis was performed in duplicate with an HPLC column coupled with and absorbance detector (254 nm) using the picotap procedure. The protein was hydrolyzed with 6 N HCl for 24 h (110°C) and derivatized with PITC before being injected on a 30 cm picotac column. The amino acids were eluted using an acetonitrile linear concentration gradient in 50 mM sodium acetate buffer.

2.12. Amino acid sequence determination

N-terminal sequence analysis was performed by automated Edman degradation using instrumentation protocols from Applied Biosystems. A model 470A gas-phase sequencer equipped with online 120A PTH-analyzer and a model 900A control/data analysis module were employed [20].

2.13. Analytical RP-HPLC

The purified lipase preparation was analyzed under conditions previously described [21]. Elution was achieved with a linear gradient (total volume: 70 ml) from 2 to 72% acetonitrile in 0.1% trifluoroacetic acid in water at a rate flow of 1 ml/min. Proteins were detected at 230 nm.

2.14. Analytical gel filtration

Purified lipase (1 ml) was loaded on to a Sephadex G75 (2.6 × 30 cm) column. Bovine serum albumin (68 kDa), ovalbumin (45 kDa), chymotrypsinogen A (25 kDa), and cytochrome C (12.5 kDa) were used as protein standards.

2.15. Sugar content

The phenol-sulfuric acid method [22] was used to determine the degree of glycosylation of the purified lipase, using mannose as reference standard.

2.16. The effect of various effectors

The purified lipase (15 U) was incubated with various compounds at 30°C for 1 h, and various concentrations of compounds were assayed. The remaining activity was measured using the standard assay system.

2.17. Stability in organic solvents

The enzyme was incubated in the presence of various solvents as described by Rolf Schmid et al. [23], and the residual activity was measured by using the standard assay system.

3. Results and discussion

3.1. Enzyme production and purification

Mucor hiemalis f. hiemalis produced an inducible extracellular lipase in buffered medium consisting of polypep-

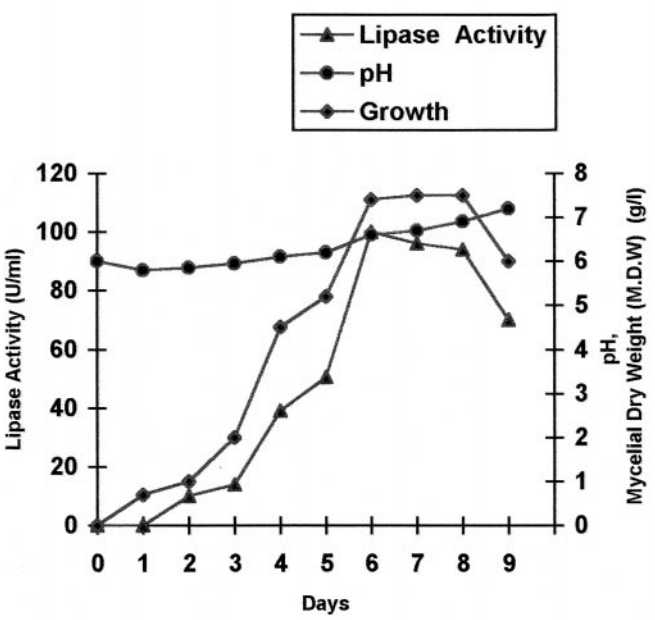

Fig. 1. Growth, final pH of culture medium, and lipolytic activity of *Mucor hiemalis f. hiemalis*. Cultivation was performed with (w/v) 0.5% rape oil, 1% yeast extract, and 5% peptone at 24°C.

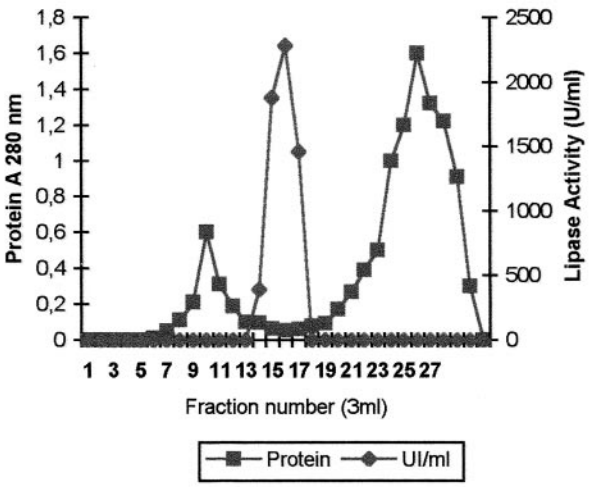

Fig. 2. Chromatography of lipase from *Mucor hiemalis f. hiemalis* on Sephadex G75. The enzyme solution after ammonium sulfate precipitation was loaded onto a Sephadex G75 column (2.6 × 70 cm) equilibrated in buffer A containing 0.15 M NaCl. The flow rate was adjusted to 25 ml/h, and 3 ml fractions were collected with the same buffer.

tone, yeast extract, mineral salt, and oil as inducer. Highest lipase production (97 U/ml) was observed with at the late logarithmic phase growth (Fig. 1). Rape oil was the best inducer. The production of enzyme was highly dependent on carbon/nitrogen (C:N) ratio. At high C:N ratios, the level of enzyme production decreased slowly. The highest lipase production was obtained with C:N about 0.1 (data not shown). A *Mucor hiemalis* strain has been reported to produce an inducible lipase [24]. However only 1.97 U/ml was obtained, and no attempt to purify this enzyme was investigated. The culture supernatant solution was defatted with cold *n*-hexane without losing activity. This process was nonetheless necessary, as with no-hexane delipidation, the recovery of the lipase of subsequent gel filtration chromatography was drastically reduced. Although no direct evidence of lipids binding to the lipase have been reported, the drop in lipase activity might be due to the enzyme aggregation. Furthermore, upon Sephadex G75 gel filtration, undefatted enzyme was eluted with the void volume, indicating a molecular mass of 80 kDa or more (data not shown). Disadvantages of the presence of lipids during purification process of lipases produced by fermentation from media containing lipids have been reported with lipase from *Rhizopus delemar* [25] or *Penicillium* sp. lipase [26]. The crude enzyme was concentrated 20-fold by ultrafiltration and centrifuged. This resulted in a 3.5-fold higher specific activity in comparison to crude enzyme. After the two steps ammonium precipitation and subsequent centrifugation, the specific activity increased 17-fold with 80% recovery of lipase activity. The enzyme was dissolved in buffer A described above and loaded on a Sephadex G75 (Fig. 2). The active fractions were pooled and exhibited 192.5-fold higher specific activity in comparison to the culture filtrate. The enzyme was applied onto the Q-Sepharose column with two fractions with lipase activity. The major activity was eluted with buffer A containing 0.1 M NaCl and was purified further to the lipase described in this paper. Unsuccessful attempts have been made to pursue the complete purification of the other activity probably due to the low yield. However the MW of the second lipase activity was low (about 20 kDa) compare to the purified lipase from *Mucor hiemalis f. hiemalis* (49 kDa) in native electrophoresis gel. A 434-fold increase in specific activity was obtained by Q-Sepharose chromatography purification step. However, the enzyme was not completely pure. After concentration by ultrafiltration, further purification was achieved using a Sephacryl S-200 gel filtration. The active fractions were pooled concentrated and exhibited 6153 specific activity with a total recovery of 18.1%. A summary of the purification procedure is shown in Table 1. The final specific activity obtained here was high compared to some fungal lipases, e.g. 63 U/mg protein for *Pythium ultimum* lipase [27], 193 U/mg protein for *Penicillium cyclopium III* [28], and 3485 U/mg protein for *Mucor miehei lipase A* [29]. However *Rhizopus niveus* lipase II exhibited 7638 U/mg protein [30].

3.2. Enzyme characterization

Purified lipase was used for gel filtration chromatography as described in Section 2. A single peak of lipase activity was eluted and corresponded to a native molecular mass of 49 kDa. With SDS-PAGE (Fig. 3); a single band around 49 kDa was found. These data indicate that the enzyme was a monomer. The purified lipase contained approximately 7.5% carbohydrate by weight, estimated by the

Table 1
Summary of the purification of lipase from *Mucor hiemalis f. hiemalis*

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Culture filtrate	88000	31428	2.8	1	100
Ultrafiltration	86500	8650	10	3.5	98
Ammonium sulfate	72051	1533	47	17	82
Sephadex G-75	53920	100	539	192.5	61
Q Sepharose	24308	20	1215	434	27.6
Sephacryl S-200	16000	2.6	6153	2200	18.1

method of Dubois using mannose as reference standard. The isoelectric point of the enzyme was determined to be 4.6 by isoelectric focusing (data not shown).

3.3. The amino acid composition and N-Terminal sequence

The amino acid composition of purified lipase is presented in Table 2. The enzyme exhibited a low content (77.51 mol/mol) of apolar amino acid, A, V, L, and I despite that its natural substrates are very hydrophobic. Similar finding have been reported with *Rh. delemar* [31] lipase as indicated in Table 2 above. It was suggested that its affinity to interfaces might arise from the tertiary structure of the protein giving to the molecule an amphipathic structure such as found with detergent. The low content of apolar amino acids of this lipase was confirmed by RP-HPLC analysis. The *Mucor hiemalis f. hiemalis* lipase was eluted one major protein peak 9% acetonitrile (data not shown). The acetonitrile concentration required for lipase elution is indicative of the strong affinity of the lipase for hydrophobic phase. Actually, under the same chromatographic conditions, *P. cyclopium* is eluted with 55% acetonitrile [32].

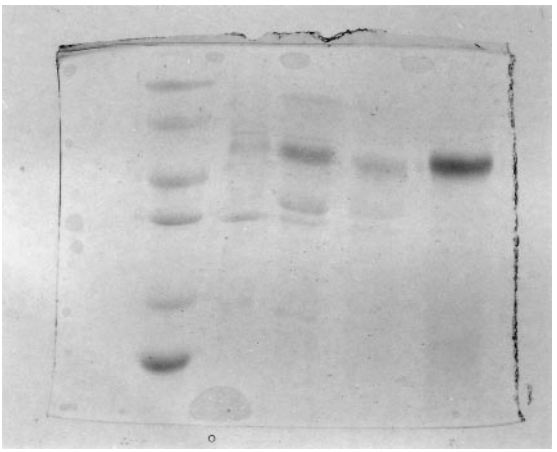

Fig. 3. SDS-PAGE of *Mucor hiemalis f. hiemalis* lipase at various stages of purification. Lane 1, molecular weight markers; lane 2, Sephadex G75 pool (3 μg); lane 3, fraction concentrated by ultrafiltration after Sephadex G75 (5 μg); lane 4, Q-Sepharose pool (3 μg); lane 5, purified lipase (10 μg).

Even though all Asx and Glx are present as Asp and Glu, these amino acids (29 mol/mol enzyme) are outnumbered by Lys (43 mol/mol). This contradicts with the acidic pI value found for the enzyme. Also the level of Gly is high. With 448 residues, the purified lipase indicated a minimum molecular mass of about 49 kDa. This agreed with the value determined by SDS-PAGE and gel filtration. The N-Terminal amino acid sequence of the enzyme was determined to be AAGTCISGSSGKNNGTDYY. The background of contaminating PTH-amino acids was very low during all sequence analyses. This showed that the enzyme resolved by SDS-PAGE was pure. As shown in Table 3, N-Terminal sequence of lipase from *Mucor hiemalis f. hiemalis* in distinct from all known fungal lipases. Only a very limited sequence homology was observed with the lipase of *P. simplicissimum*. A search of the SWISS-PROT protein database [35] revealed that N-Terminal sequence of lipase from *Mucor hiemalis f. hiemalis* [1–15] was homologous to the sequence found in *Rhizopus delemar* lipase starting at

Table 2
Amino acid composition of the *Mucor hiemalis f. hiemalis* lipase

Amino acid	Mucor *hiemalis f. hiemalis* lipase (mol/mol)[a]	*Rh. delemar* lipase (mol/mol)[b]
Asx	15.21	28
Glx	14.23	24
Ser	2.64	22
Gly	153.17	21
His	1.13	8
Arg	1.08	10
Thr	65.68	21
Ala	33.93	19
Pro	47.81	16
Tyr	15.38	11
Val	24.43	19
Met	0.76	2
Cys	n.d	8
Ile	8.86	14
Trp	n.d	n.d
Leu	10.29	17
Phe	10.78	14
Lys	42.62	18

[a] Measured in purified lipase mol/mol as described in Section 2.
[b] Determined on the purified lipase from *Rh. delemar* [31].

Table 3
N-Terminal sequence of some fungal lipases

AAGTCISGSSGKNNGTDYY	*Mucor hiemalis f. hiemalis*
AVAAFPDLXRAAKLSSAVY	*Penicillium expansum* [33]
SDGGKVVAATTAQIQEFTKY	*Rhizopus delemar* [31]
SIDGGIRAAT	*Mucor miehei* [34]

position 29–38. However there is no functional significance related to this similarity. Cloning and expression are underway and should soon provide a complete amino acid sequence for further investigations such as folding pattern or three dimensional structure.

3.4. Effect of various compounds on purified lipase

The effect of various compounds on the purified enzyme was examined by adding each compound to the standard reaction mixture (Table 4). It can be observed that Ca^{2+} strongly enhanced lipase activity. Calcium ion have been reported to form complexes with ionized fatty acids, changing their solubility and behaviors at interfaces [26]. This enzyme activity was also lightly enhanced by Na^{+}, Mg^{2+}, Co^{2+}, and Mn^{2+} but was inhibited by Fe^{2+}, Cu^{2+}, and Ba^{2+}. The chelating agent EDTA did not affect the activity of the enzyme, and this suggested that it was not a metalloenzyme. In contrast with lipase from *Rh. miehei*, taurocholic acid and other surfactants such as triton X-100, SDS, and Tween 20 reduced lipase activity. The serine specific inhibitor PMSF, benzamidine, and P-chloromercuribenzoic acid (PCMB) had no significant effect on the enzyme activity.

Table 4
Effect of various compounds on the activity of purified lipase A

Compound	Concentration (mM)	Relative activity (%)
none	—	100
$CaCl_2$	10	121
$CaCl_2$	25	176
$MgCl_2$	1	105
$FeSO_4$	1	22
NaCl	100	107
$CuSO_4$	1	32
$CoCl_2$	1	103
NaN_3	1	100
$BaCl_2$	1	53
$MnCl_2$	1	109
PMSF	0.2	101
PCMB	1	98
EDTA	10	102
Benzamidine	2	104

[a] The activities are expressed as the mean and SD of three determinations.

Table 5
Stability of *Mucor hiemalis f. hiemalis* lipase in various organic solvents

Organic solvent	Relative activity
Methanol	0
Ethanol	22
Propanol	52
buthanol	78
Acetone	31
n-Hexane	100
n-Heptane	100
Isooctane	107
Tris–HCl buffer	100

3.5. Stability in organic solvent

The stability of lipase in organic solvents offers advantages for enzyme precipitation and for synthesis. As shown in Table 5 the enzyme had a good stability in water-immiscible organic solvents hexane, heptane, and isooctane. Acetonitrile at a concentration of 5% (v/v) slightly activated the enzyme activity (data not shown). *Candida rugosa* lipase is also known to be activated by this solvent. It was suggested that the solvent keeps the enzyme in the open conformation; the lid of the enzyme does not cover the active site crevice, thus keeping a flexible conformation [10].

3.6. Substrate specificity

Except triacetin, the purified lipase hydrolyzed all triacylglycerol tested, and highest activity was obtained with tricaprylin in terms of chain length (Table 6). Olive oil and palm oil were also hydrolyzed by the lipase, but their reaction rate was not higher than that obtained with rape oil. Physical factors such as surface pressure and lipid distribution are important determinants of lipase activity [37]. Thus, differences in the substrate specificity could arise not only from specificity substrate-binding interaction but also from differences in the activation of the enzyme by the interface.

Table 6
Specificity of lipase from *Mucor hiemalis f. hiemalis* in respect of various lipids

Substrate	Relative activity (%)
Triacetin	0
Tributyrin	37
Tricaproin	72
Tricaprylin	100
Tricaprin	66
Triolein	46
Palm oil	69
Olive oil	64
Rape oil	127
Tween 80	0
Span 80	0

The activity was assayed titrimetrically at pH 7 as described in Section 2.

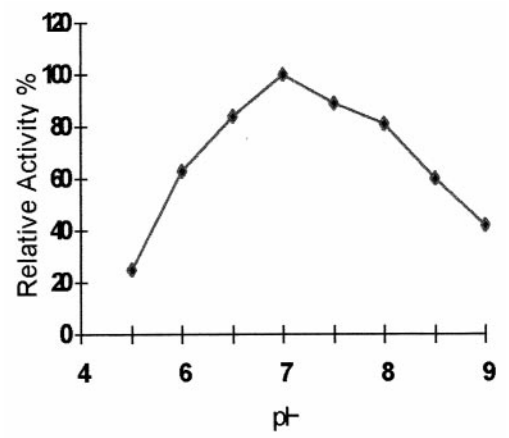

Fig. 4. The effect of pH on the activity of the purified lipase. The activity was assayed titrimetrically at various pH values as described above using glycerine–HCl (pH 3.0–4.0), phosphate buffer (pH 5.0–7.0), and Tris–HCl buffer (pH 7.0–9.0). Activities are expressed relative to that of the most active sample.

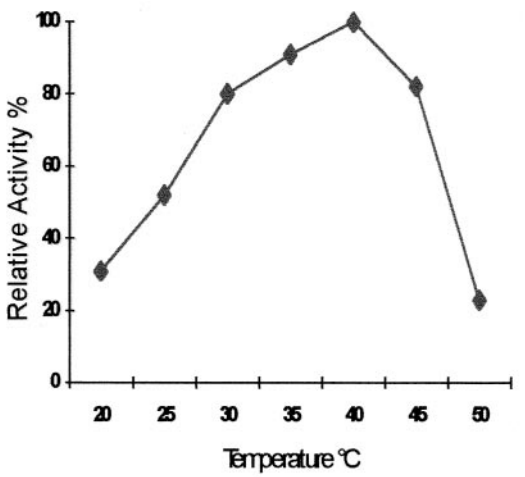

Fig. 5. Dependence of lipase activity upon temperature. The activity was assayed at the indicated temperatures, using water jackets as described above. Activities are given as % of the most active sample.

No activity was detected with monoesters such as Tween 80 and Span 80, although the enzyme hydrolyzed *p*-nitrophenyl esters of fatty acids with short and long chains (C2, C4, and C18). Apparent K_m and V_{max} values obtained for lipase with p-nitrophenyl acetate as substrate were 0.62 mM and 861 μmol/min/mg protein, respectively. Using mono-, di-, and triolein the positional specificity of lipase from *Mucor hiemalis f. hiemalis* was studied by thin-layer chromatography. The result (data not shown) showed that the lipase hydrolyzes each of the three bonds of the triacylglycerol although a certain preference for primary ester bonds was observed.

Physical properties of extracellular lipase from *Mucor hiemalis f. hiemalis* were investigated. Fig. 4 shows that the activity of the *Mucor hiemalis f. hiemalis* lipase exhibited 60% or more of maximum activity between pH 6 and 8.5, with optimal activity at pH 7. In addition when the enzyme was incubated at various pH values at 4°C for 24 h, and the activity was measured at pH 7, we observed that the lipase was stable over the range pH 4–9. Hydrolytic activity was detected between 20 and 50°C, with an optimum at 40°C (Fig. 5). At temperatures between 30 and 45°C the enzyme had activity over the range 80 and 100%. The thermal stability of the lipase is shown in Fig. 6. At 45°C, the residual activity of the purified lipase was almost 80%, but at 55°C this activity fell to 30%. Above 60°C, the enzyme was completely inactivated.

4. Conclusion

The main criteria for assessing the quality of palm oil is the level of free fatty acids. Although the biodeterioration of palm oil has been attributed to fungi, there are few or no results about the molecular characterization of the enzymes suspected. The strain isolated from palm fruit in this study identified as *Mucor hiemalis f. hiemalis*, produced an extracellular lipase. Many significant conditions of fermentation were optimized for high production of this lipase. The enzyme was purified to homogeneity, as evidenced by the acquisition of amino terminal sequence data. The specific activity was particularly high, and the activity was stable to treatment with organic solvents. This enzyme, therefore, is a good candidate for esterification reactions in organic media. Since the N-terminal amino sequence of this enzyme is quite different from known lipases, it may be considered as a new lipase. Experimentation is currently being performed in order to determine the complete sequence of this lipase. In the view of its relative high activity on emulsified palm oil, this fungal lipase may play a significant part in the biodeterioration of palm oil. Further studies will focus on investigating the direct evidence of its involvement in the spoilage of palm oil.

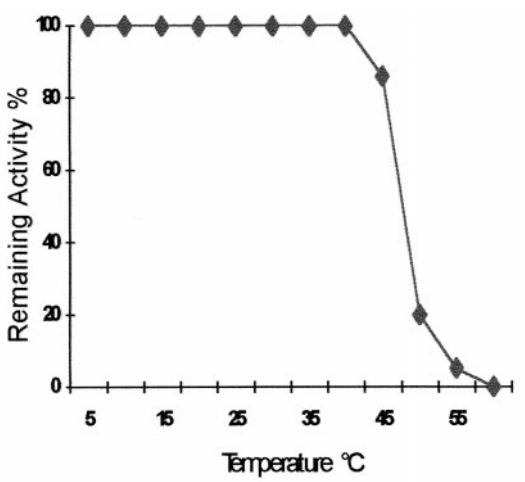

Fig. 6. Thermal stability of the lipase. Purified lipase solutions were incubated for 15 min at the indicated temperatures. Residual activity was assayed at 40°C as described.

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