# Purification and Characterization of a Lipase from *Neurospora* sp. TT-241

**Shuen-Fuh Lin[a,b,*], Jane-Chyi Lee[b], and Chien-Ming Chiou[b]**
[a]Department of Bioengineering, [b]Institute of Chemical Engineering,
Tatung Institute of Technology, Taipei, Taiwan, Republic of China

**ABSTRACT:** An extracellular lipase, which is produced by the *Neurospora* sp. TT-241 strain, grown on wheat bran at 30°C for 4 d, was purified 370-fold with an overall yield of 16%. The molecular weight was determined to be 55 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The optimal pH at 30°C and optimal temperature at pH 6.5 were 7 and 45°C, respectively. The lipase was stable in the pH range of 5 to 8, and it was temperature-sensitive. It was active on a wide range of natural substrates of either vegetable or animal origins and toward *p*-nitrophenyl esters, greatly favoring those containing $C_4$ acyl groups. It cleaved all of the ester bonds of triolein; however, the 1- or 3-ester bond was the preferred target. A complete inhibition by diisopropyl fluorophosphate suggested the presence of a serine residue at the active site. Partial inhibition was shown by either $Hg^{2+}$ or chloramine T. Enzyme activity persisted in nonionic surfactants, a water-miscible solvent (dimethylsulfoxide), and a water-immiscible solvent (hexane).
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**KEY WORDS:** Alcohol ester synthesis, detergent stability, enzyme purification, esterolysis, extracellular lipase, *Neurospora*, nonpositional specificity, solid-state fermentation, solvent stability, wheat bran.

Lipases [E.C. 3.1.1.3, triacylglycerol acylhydrolase] mainly catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. They also catalyze the synthesis of partially substituted glycerides (1–4). Because of their activity in both aqueous and nonaqueous solvent systems (5–7), it has become evident that lipases have considerable applications in industry and medicine (8–13). Lipases have been obtained from several mycelial fungi, including *Rhizopus delemar* (14), *Geotrichum candidum* (15), *Aspergillus oryzae* (16), *Penicillium roqueforti* (17), *Pythium ultimum* (18), and *Fusarium heterosporum* (19). Recently, we have implemented a program of screening filamentous microorganisms for lipase production. An isolated *Neurospora* sp., strain TT-241, was found to secrete a lipase capable of withstanding solvents and detergent treatment and to exhibit low positional specificity. This enzyme should prove useful in the synthesis of both primary and secondary alcohol esters. This paper presents the purification and characterization of this novel lipase from *Neurospora* sp. TT-241.

*To whom correspondence should be addressed.

## EXPERIMENTAL PROCEDURES

*Materials.* Sephadex G-100, isoelectric focusing calibration kit, and ampholines were from Pharmacia LKB (Uppsala, Sweden); Toyopearl Phenyl-650M was from Toyo Soda Manufacturing (Tokyo, Japan); Ultrogel-HA was from IBF Biotechnics (Paris, France); sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) molecular weight standards and protein assay dye were from Bio-Rad (Richmond, CA); polyoxyethylene octyl phenyl ester (Triton X-100) and silica gel 60 were from Merck (Darmstadt, Germany); and *p*-nitrophenyl fatty acid, SDS, polyoxyethylene sorbitan monooleate (Tween 80), sorbitan monooleate (Span 80), cholic acid, and oils or fats were from Sigma Chemical (St. Louis, MO). Sodium dodecyl benzene sulfonate (DBS) and sodium alkylbenzene sulfonate (ABS) were from Hayashi Pure Chemical Industries (Osaka, Japan). Sodium tripolyphosphate (STPP) was from Ishizu Pharmaceutical Co. (Osaka, Japan). Wheat bran was from a local market. All other chemicals were of analytic reagent grade.

*Microorganism and culture conditions.* A fungal strain, TT-241, was isolated from soil and kindly identified as *Neurospora* sp. by CBS (Centraalbureau Voor Schimmelcultures, The Netherlands). For enzyme production, strain TT-241 was inoculated from a slant culture into a solid medium that consisted of 5 g of wheat bran and 5 mL water in a 250-mL flask. After 5 d of cultivation at 30°C, the entire culture was used for enzyme preparation.

*Protein assay.* Protein concentration was determined according to the method of Bradford (20). Protein assay dye was from Bio-Rad Laboratories. Bovine serum albumin was used as a standard.

*Enzyme assay. Spectrophotometric method.* During the purification process, *p*-nitrophenyl butyrate was used as the substrate to trace lipase activity (21). The assay mixture contained 0.5 mL of 100 mM Tris-HCl buffer (pH 8.5) and 0.5 mL of substrate solution (0.08% *p*-nitrophenyl butyrate and 1% Triton X-100 in 5 mM sodium acetate buffer, pH 5.0) and an appropriately diluted enzyme solution. The liberated *p*-nitrophenol was measured at 410 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of *p*-nitrophenol/min.

*Cupric acetate method.* The cupric acetate method (22) was used to characterize the lipase. The cupric–pyridine

reagent was prepared as follows: a 5% (w/w) aqueous solution of cupric acetate was prepared and filtered, and the pH adjusted to 6.0 with pyridine. To monitor the enzyme reaction in an emulsion system, 0.5 mL olive oil and 0.5 mL buffer were mixed vigorously for 90 s until the water–oil solution became emulsified; the enzyme was subsequently added to the emulsion mixture. After incubation at 37°C for 1 h, the reaction was stopped by adding 1 mL of 6N HCl. After 2 mL isooctane was added and mixed, 1 mL of the isooctane layer containing fatty acids was drawn off and mixed with 1 mL cupric acetate–pyridine reagent; the two phases thus formed were mixed vigorously for 90 s in a vortex mixer. After centrifugation at 4000 rpm for 10 min, the absorbance at 715 nm of the water layer was measured.

*Enzyme purification.* A typical purification scheme of the lipase from the wheat bran culture is described as follows. All of the operations were carried out at 4°C.

*Preparation of crude extract.* Wheat bran culture (50 g) of strain TT-241 was soaked in 600 mL of 50 mM phosphate buffer (pH 7.0, buffer A) for 30 min at 4°C and squeezed through a cloth. The aqueous extract was centrifuged to remove particles.

*Ammonium sulfate fractionation.* Solid ammonium sulfate was added to the clarified aqueous extract up to 60% saturation. The resulting precipitate was collected by centrifugation at 12,000 × *g* for 30 min and was dissolved in a minimal volume of buffer A. The enzyme solution was dialyzed overnight against a 50-fold excess of the same buffer.

*Sephadex G-100 gel-filtration chromatography.* Enzyme solution (70 mL) was applied to a Sephadex G-100 column (4 × 120 cm) which was eluted with buffer A at a flow rate of 40 mL/h, and 8-mL fractions were collected.

*Toyopearl phenyl-650M column chromatography.* Ammonium sulfate was added to the pooled active fractions from gel filtration to a final concentration of 1 M. The enzyme solution was then applied to a Toyopearl phenyl-650M column (2.0 × 18 cm), which had been preequilibrated with buffer A containing 1 M ammonium sulfate. After the column was washed with 300 mL of buffer A with 1 M ammonium sulfate, it was eluted with 1 L of a linear gradient from 1 to 0 M ammonium sulfate in buffer A. The flow rate was 60 mL/h, and 4-mL fractions were collected. The time period for the gradient elution was 16 h.

*Ultrogel-HA hydroxyapatite column chromatography.* The pooled active fractions from the Toyopearl column were further purified by passage through an Ultrogel-HA column (3.0 × 9 cm), which was preequilibrated with 10 mM phosphate buffer (pH 7.0). After washing with 10 mM phosphate buffer, the column was eluted with a 1-L linear gradient from 10 to 500 mM phosphate buffer. The flow rate was 50 mL/h, and 5-mL fractions were collected. The time period for the gradient elution was 15 h. The active fractions were pooled and stored at –20°C.

*SDS–PAGE.* Discontinuous SDS–PAGE was performed on 1.5-mm-thick slab gels (pH 8.8), which contained 12% polyacrylamide (wt/vol), and a stacking gel (pH 6.8) of 4% polyacrylamide under the conditions developed by Laemmli (23). The sample (20 μL) was boiled for 5 min in the presence of 20 μL of loading buffer [Tris (0.5 M, pH 6.8), 1.2 mL; glycerol, 2.5 mL; SDS (10% wt/vol), 2 mL; Bromophenol Blue (0.05%), 2 mL; mercaptoethanol, 0.5 mL; water, 2 mL]. The presence of protein bands was detected by Coomassie brilliant blue staining.

*Denaturing polyacrylamide gel isoelectric focusing.* Isoelectric focusing (IEF) was performed by the method of Bollag and Edelsstein (24). An 8% polyacrylamide gel, containing 8 M urea and 2.4% Ampholine of pH range 3.5–10, was used. The anode and cathode solutions were 10 mM phosphoric acid and 20 mM sodium hydroxide, respectively. The pH gradient was determined by the migration of pI markers.

*Thin-layer chromatography.* Thin-layer chromatography of the enzyme reaction products, obtained with pure triolein as the substrate, was conducted to examine positional specificity of the lipase. A reaction mixture, composed of 0.05 g of triolein, 0.5 mL of 10% gum arabic solution, 1 mL of 50 mM $Na_2CO_3$-$NaHCO_3$ (pH 9.5), and 10 units of the enzyme, was incubated at 30°C for 30 min. After the incubation, 3 mL ethyl ether was added to extract the reaction products. Aliquots of the ether layer were applied to a silica gel 60 plate (Merck), which was then developed with a hexane/diethyl ether/acetic acid mixture of 75:25:1 (vol/vol/vol). Pure monoolein and diolein were used as reference glycerides. Spots were visualized by spraying the plate with 50% $H_2SO_4$ in water (vol/vol), and then heating it in an oven at 160°C until charring occurred.

## RESULTS AND DISCUSSION

*Lipase production.* The extracellular lipase from *Neurospora* sp.TT-241 was produced in solid-state fermentation but not in submerged culture. Compared with rice bran, corn flour, and soy meals, wheat bran was the most suitable medium for enzyme production. There was no improvement in enzyme productivity by adding other carbon (such as glucose, olive oil, glycerol, starch, and cellulose) or nitrogen sources (urea, pepton, yeast extract, and soymeal). The optimum fermentation medium was comprised of wheat bran and water in the ratio of 1:1 (wt/vol).

*Purification of lipase.* Table 1 summarizes the results of the purification of lipase. Both the 370-fold purification and the 16% recovery of activity were reproducible. The specific activity of the purified enzyme was 8,200 units/mg of protein. The final enzyme preparation was judged by SDS–PAGE (Fig. 1) and IEF PAGE (data not shown) to be homogeneous.

*Molecular weight.* The apparent molecular weight of the purified enzyme was 55 kDa by SDS–PAGE (Fig. 1). This molecular weight was within the range of other fungal extracellular lipases (19–64 kDa). However, this enzyme was eluted near the void volumn of a Sephadex G-100 column (Fig. 2), which suggests that it has a strong tendency to aggregate. The same phenomena were observed in both native PAGE and IEF analysis. In the native PAGE experiment (pH

**TABLE 1**
**Purification of Lipase from *Neurospora* sp. TT-241 Strain**

| Step | Total protein (mg) | Total activity (unit) | Specific activity (unit/mg) | Purification (fold) | Yield (%) |
|---|---|---|---|---|---|
| Crude extract | 1380.0 | 30,300 | 22 | 1.0 | 100 |
| Ammonium sulfate precipitation | 720.0 | 27,890 | 39 | 1.8 | 93 |
| G-100[a] gel filtration | 180.0 | 14,700 | 82 | 3.7 | 49 |
| Toyopearl phenyl-650 M[b] | 32.0 | 10,727 | 339 | 15.4 | 36 |
| Ultrogel-HA[c] | 0.6 | 4920 | 8203 | 371.0 | 16 |

[a]From Pharmacia LKB (Uppsala, Sweden).
[b]From Toyo Soda Manufacturing (Tokyo, Japan).
[c]From IBF Biotechnics (Paris, France).

8.3), a major part of the purified lipase failed to penetrate into the separating gel. Similarly, the enzyme aggregated when subjected to native IEF in the absence of 8 M urea.

*Isoelectric point.* The pI value of this enzyme was estimated to be about 5.1 by denaturing polyacrylamide IEF (data not shown).

*Optimal-reaction pH and pH stability.* Judging from the bell-shaped pH vs. activity profile, shown in Figure 3A, the optimal-reaction pH of this enzyme was between 6 and 7. The enzyme was stable in the pH range from 5 to 8, as shown in Figure 3B.

*Optimal-reaction temperature and thermal stability.* As shown in Figure 4A, the enzyme activity reached a maximum at about 45°C, as determined by the cupric acetate assay. Thermal stability was investigated by incubating the enzyme in buffer A at various temperatures for 1 h. The residual activity was determined as shown in Figure 4B. The enzyme lost its activity at temperatures above 40°C.

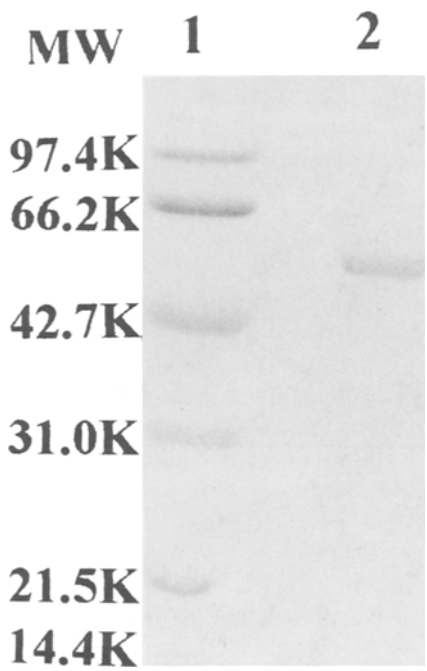


**FIG. 1.** Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of lipase. The gel concentration was 12%. Lane 1, molecular mass markers: phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa), and lysozyme (14.4 kDa). Lane 2, 5 µg purified enzyme. MW, molecular weight.

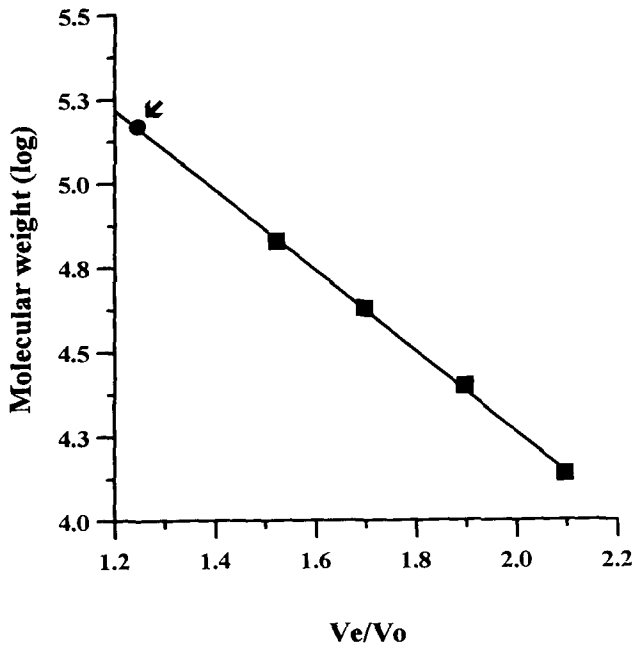


**FIG. 2.** Estimation of molecular weight of lipase by Sephadex G-100 gel filtration (Pharmacia LKB, Uppsala, Sweden) chromatography. The molecular weight markers used were bovine serum albumin( 67 kDa), ovalbumin (43 kDa), chymotrypsinogen A (25 kDa), and ribonuclease (13.7 kDa). The position of the lipase is indicated by the arrow. The protein that eluted was monitored at 280 nm.

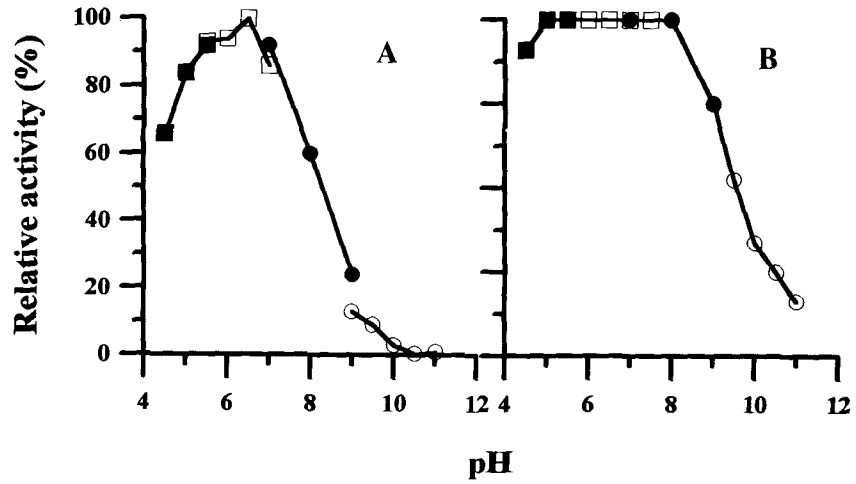


**FIG. 3.** Effect of pH on (A) lipase activity and (B) stability. (A) Enzyme activity was assayed in various buffers at the pH values indicated by the cupric–acetate method as described in the text. (B) Enzyme was incubated in various buffers at the pH values indicated. After incubation at 30°C for 1 h, the residual activities were estimated at pH 8.5 by spectrophotometric method as described in the text. The buffer systems (50 mM) used were $CH_3COOH$-$CH_3COONa$ (pH 4.5–5.5) (■), $KH_2PO_4$-$Na_2HPO_4$ (pH 5.5–8.0) (□), Tris-HCl (pH 7.0–9.0) (●) and $Na_2CO_3$-$NaHCO_3$ (pH 9.0–11.0) (○).

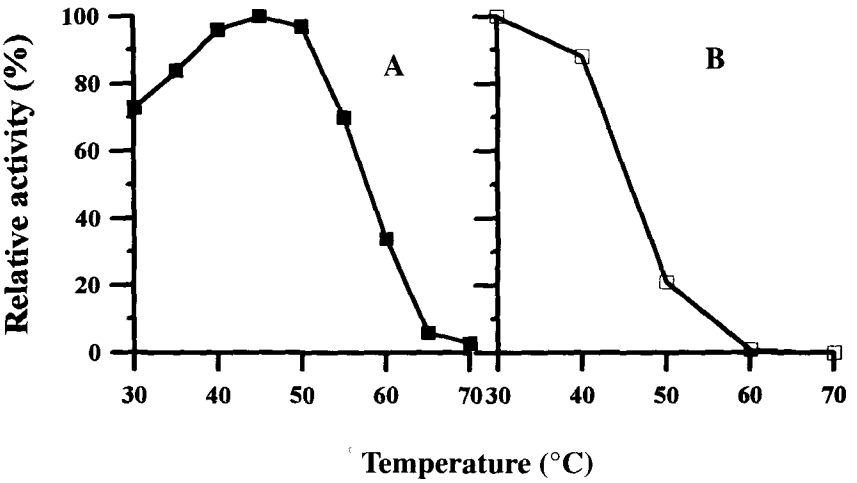


**FIG. 4.** Effect of temperature on (A) lipase activity and (B) stability. (A) Enzyme activity was assayed by the cupric–acetate method as described in the text at various temperatures in buffer A. (B) Enzyme, in buffer A, was incubated at the temperature indicated for 1 h, and the remaining activity was estimated at 30°C by spectrophotometric method as described in the text.

*Substrate specificity.* The enzyme was active on a wide range of natural substrates of either vegetable or animal origin. If the hydrolysis activity for peanut oil is taken as 100%, the relative activity was 94% for olive oil and sunflower oil, 88% for corn oil, 85% for soybean oil, 73% for sesame oil, 61% for fish oil, 45% for lard oil, and only 7% for safflower oil, which contains higher amounts of polyunsaturated fatty acids rich in $C_{18:2}$ and $C_{18:3}$.

The activities of the lipase toward various *p*-nitrophenyl esters were investigated (Table 2). The ester of butyrate was most actively hydrolyzed. The enzyme showed low lipolytic activity to long-chain fatty acid esters, such as laurate, myristate, palmitate, and stearate.

*Positional specificity.* Figure 5 shows a thin-layer chromatogram of the lipase reaction products with triolein as the substrate. Spontaneous isomerization of the reaction products

**TABLE 2**
**Substrate Specificity of *Neurospora* sp. TT-241 Lipase Toward Several *p*-Nitrophenyl Esters[a]**

| Substrate | Relative activity (%) |
|---|---|
| *p*-Nitrophenyl acetate | 7 |
| *p*-Nitrophenyl propionate | 90 |
| *p*-Nitrophenyl butyrate | 100 |
| *p*-Nitrophenyl valerate | 69 |
| *p*-Nitrophenyl caproate | 30 |
| *p*-Nitrophenyl octanoate | 30 |
| *p*-Nitrophenyl caprate | 22 |
| *p*-Nitrophenyl laurate | 15 |
| *p*-Nitrophenyl myristate | 16 |
| *p*-Nitrophenyl palmitate | 14 |
| *p*-Nitrophenyl stearate | 7 |

[a]The enzyme activity was measured by the spectrophotometric method as described in the text.

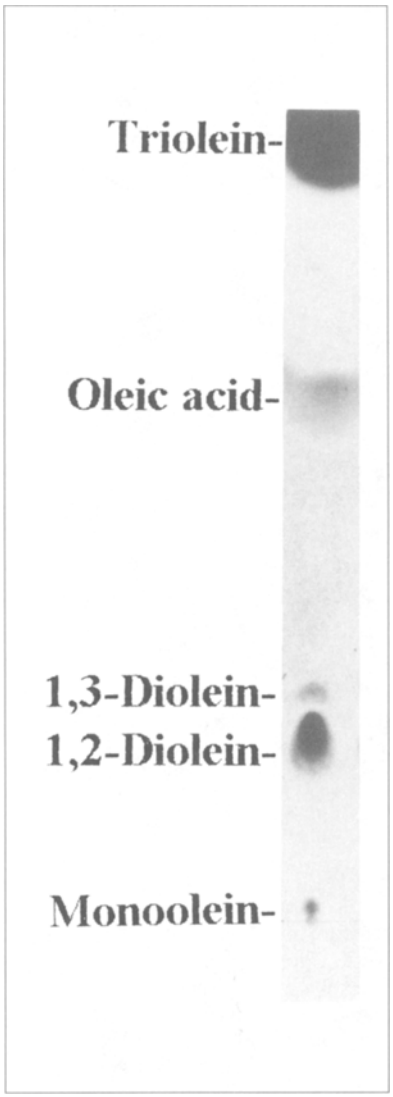


**FIG. 5.** Thin-layer chromatography of enzyme reaction products obtained with triolein as the substrate. Experimental conditions are given in the Experimental Procedures section.

was considered negligible because of the short reaction times (30 min). Judging from the amounts of the 1,2(2,3)-dioleyl glycerol and 1,3-dioleyl glycerol of reaction products formed, the enzyme cleaved the 1(3)-position ester bond faster than the 2-position ester bond. These results suggest that the positional specificity of the TT-241 lipase is similar to that of *G. candidum* (25), but unlike those of the lipase of *Py. ultimum* (18), *P. cyclopium* (26), and *F. heterosporum* (19), which show 1,3-positional specificity.

*Effect of organic solvents on stability.* Attempts have been made to utilize lipase action in hydrolysis, ester synthesis, and transesterification in reaction mixtures with solvents. To investigate the effect of organic solvents on its stability, the remaining activity was measured after incubation at 30°C for 1 h in 50 mM phosphate buffer (pH 6.5) that contained 20% concentrations of various solvents (Table 3). The present lipase was stable in most of the water-miscible solvents, such as methanol, ethanol, and 2-propanol, and stable even in water-immiscible solvents, such as isooctane. However, dimethylsulfoxide and hexane either stimulated or stabilized enzyme activity.

*Effects of metals and reagents.* The effects of various metal ions on the enzyme activity are shown in Table 4. The enzyme activity was significantly inhibited by 1 mM $Hg^{2+}$, but slightly inhibited by $Ca^{2+}$, $Zn^{2+}$, $Cd^{2+}$, and $Fe^{3+}$. Metal ions generally form complexes with ionized fatty acids, changing their solubility and behavior at the interface. However, the inhibition might involve the catalytic site directly, although alteration of the properties of the interface must also be considered. The activity of $Hg^{2+}$ might be attributed to its binding of the thiol group of the enzyme or to sulfhydryl groups that may be present in the active center of the enzyme. But the thiol effectors, iodoacetamide, and *p*-chloromercuribenzoate did not significantly affect the activity, suggesting that cysteine residues do not participate in the expression of activity. However, the possibility that mercury compounds do not react with the cysteine residues involved in the expression of activity cannot be excluded.

**TABLE 3**
**Effect of Solvents on Lipase Activity[a]**

| Chemical | Relative activity (%) |
|---|---|
| None | 100 |
| Methanol | 118 |
| Ethanol | 113 |
| 2-Propanol | 96 |
| 1-Butanol | 39 |
| Acetonitrile | 50 |
| Acetone | 72 |
| Dimethylsulfoxide | 172 |
| Dioxane | 30 |
| Hexane | 148 |
| Isooctane | 108 |
| Benzene | 21 |
| Toluene | 13 |

[a]Enzyme was incubated in buffer A containing various solvents for 60 min at 30°C. The residual activities were assayed by the spectrophotometric method as described in the text.

**TABLE 4**
**Effect of Metal Ions and Reagents on Lipase Activity[a]**

| Chemical (1 mM) | Relative activity (%) | Chemical (1 mM) | Relative activity (%) |
|---|---|---|---|
| None | 100 | $HgCl_2$ | 29 |
| $CoCl_2$ | 109 | Ethylenediaminetetraacetic acid | 102 |
| $MnCl_2$ | 107 | Iodoacetamide | 100 |
| $MgCl_2$ | 101 | Iodoacetic acid | 97 |
| $CuCl_2$ | 99 | *O*-Phenanthroline | 90 |
| $PbCl_2$ | 97 | *p*-Chloromercuribenzoate | 80 |
| $FeCl_2$ | 89 | Tetranitromethane | 64 |
| $CaCl_2$ | 84 | Chloramine T | 38 |
| $CdCl_2$ | 83 | Diisopropylfluorophosphate | 0 |
| $ZnCl_2$ | 77 | | |

[a]Enzyme was incubated in buffer A containing various metal ions and reagents for 30 min at 30°C. The residual activities were assayed by the spectrophotometric method as described in the text.

The presence of tetranitromethane and chloroamine T in the reaction mixture reduced activity by 64 and 38% respectively; therefore, it was deduced that tyrosine residues might be involved in the catalysis reaction. Tyrosine residues involved in active centers of lipase are rarely seen in other reports. Metal-chelating agents (ethylenediaminetetraacetic acid and *o*-phenanthroline) did not show a significant inhibition effect on the enzyme activity. Lipolysis was potently inhibited by 1 mM DFP, indicating that an essential serine residue might be involved in the catalysis.

*Effects of detergents.* The effects of various detergents on the lipase activity were tested at concentrations of 0.1% (wt/vol). The results are shown in Table 5. The anionic surfactants tested, such as ABS, DBS, and SDS, were potent inhibitors. On the other hand, STPP and the nonionic surface active agents, such as Triton X-100, Tween 80, and Span 80, showed activating effects. Furthermore, cholic acid, which stimulates pancreatic lipase, also stimulated enzyme activity. Whether the stimulation of lipase activity was due to a direct interaction of the surfactants with the enzyme or to an alteration of emulsion properties, which in turn affected activity, is not known. Similar results have been reported by others for lipase from different sources (18,27).

In spite of the increasing interest in microbial lipases, few studies have been done on *Neurospora* sp. lipases. The purified extracellular lipase from the TT-241 strain was different from the lipase of *Neurospora crassa* (28) in molecular weight, and positional and substrate specificity. The molecular weight of the lipase of this study was greater than that of the lipase from *N. crassa* (molecular weight 27,000 by SDS–PAGE). With regard to fatty acid specificity, the *N. crassa* lipase hydrolyzed longer-chain fatty acids, but the TT-241 lipase preferred shorter-chain fatty acids. Furthermore, the positional specificity of *N. crassa* lipase was 1,3-specific, whereas the TT-241 lipase was nonspecific. These results suggest that *Neurospora* TT-241 lipase is a different protein than that from *N. crassa*.

In summary, the lipase described here differs from those reported to date in at least one of the following aspects: molecular weight, pI, chemical stability, substrate specificity, and positional specificity. All of these results strongly suggest that *Neurospora* sp. TT-241 produces a novel lipase. Given its resistance to nonionic detergents, and solvents, and its low positional specificity, it should prove useful in the synthesis of both primary and secondary alcohol esters (3).

**TABLE 5**
**Effect of Detergents on Lipolytic Activity[a]**

| Chemical (0.1%) | Relative activity (%) |
|---|---|
| None | 100 |
| Sodium dodecyl sulfate | 0 |
| Sodium dodecyl benzene sulfonate | 0 |
| Sodium alkylbenzene sulfonate | 0 |
| Sodium tripolyphosphate | 140 |
| Polyoxyethylene octyl phenyl ester (Triton X-100)[b] | |
| Polyoxyethylene sorbitan monooleate (Tween 80)[c] | 138 |
| Sorbitan monooleate (Span 80)[c] | 129 |
| Cholic acid | 142 |

[a]Enzyme was incubated in buffer A containing various detergents for 60 min at 30°C. The residual activities were assayed by the spectreophotometric method as described in the text.

[b]From Merck (Darmstadt, Germany).

[c]From Sigma (St. Louis, MO).

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