

Purification, Characterization, and Crystallization of Two Types of Lipase from *Rhizopus niveus*

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The purification and some properties of two types of lipase (Lipase I and Lipase II) from *Rhizopus niveus* are described. The enzymes were purified to homogeneity by column chromatographies on DEAE-Toyopearl (1 pass) and CM-Toyopearl (2 passes). Lipase I consists of two polypeptide chains [a small peptide with sugar moiety (A-chain) and a large peptide of molecular weight 34,000 (B-chain)]. Lipase II has a molecular weight of 30,000 consisting of a single polypeptide chain. Lipase I appeared to be converted to Lipase II by limited proteolysis by a specific protease a small amount of which is in the culture supernatant from *Rh. niveus*, because one of the peptides formed has the same N-terminal sequence and C-terminal amino acid as Lipase II, as well as the molecular mass estimated by SDS-PAGE. Lipase I had a pH optimum of 6.0–6.5 and a temperature optimum of 35°C, while, for Lipase II these values were pH 6.0 and 40°C. Both enzymes were obtained in the crystalline state using the hanging drop method of vapor diffusion and PEG as the precipitating agents.

Lipase [EC 3.1.1.3] catalyzes the hydrolysis of the ester bonds of triacylglycerols and under certain conditions, the synthesis of ester bonds *via* transesterification. Therefore, lipases are useful for industry. It is necessary to improve their properties, such as optimal reaction conditions and substrate specificities, and to understand their structure–function relationships for more extended use.

Although the physical properties and biochemical features of lipases have been investigated extensively, little structural information is available to them. Recently, three tertiary structures of lipases at high resolution^{1–5)} as well as the structure of a lipase/inhibitor complex from *Rhizomucor miehei*^{6,7)} and the pancreatic lipase/procolipase complex^{8–10)} have been published.

Although many *Rhizopus* lipases with potential for industrial use have been purified and characterized,¹¹⁾ their primary and three-dimensional structures are not generally available. The primary structure of a lipase from *Rhizopus delemar* (based on the nucleotide sequence of a cloned cDNA) has been reported.¹²⁾

Lipases from *Rhizopus niveus* have 1.3 positional specificity and are useful in producing cocoa butter substitutes.¹³⁾ We have cloned the complementary DNA encoding such a lipase.¹⁴⁾

In this paper, we report the purification, characterization, and crystallization of two types of lipase from *Rhizopus niveus*. In addition, we report that Lipase II is produced from Lipase I by limited proteolysis due to the action of a serine protease present in a small amount in the culture supernatant from *Rh. niveus*.

Materials and Methods

Strain and media. *Rhizopus niveus* IFO 4759 was obtained from the Institute for Fermentation, Osaka. A peptone medium was used, which contained 5% peptone, 2% glucose, 0.02% yeast extract, 0.1% KH₂PO₄, and 0.05% MgSO₄ at pH 6.5.

Purification of lipases. Lipases from *Rh. niveus* were isolated as described in our previous paper¹⁴⁾ with some modifications. *Rh. niveus* was cultured

in the above peptone medium and 27°C for 4 days with shaking. The culture filtrate was adjusted to pH 6.0 and a concentration of 10 mM sodium phosphate by adding 0.1 M sodium phosphate buffer (pH 6.0). The resulting solution was kept at 4°C for 1 h, and the precipitate was removed by centrifugation. The supernatant was placed on a column of DEAE-Toyopearl (5.0 × 35.0 cm, Tosoh) previously equilibrated with 10 mM sodium phosphate buffer (pH 6.0). The lipase activity was found in unadsorbed fractions and these fractions were collected and put on a column of CM-Toyopearl (5.0 × 35.0 cm, Tosoh) previously equilibrated with the same buffer. After the column was washed with the same buffer, substances on the column were eluted with a linear NaCl gradient (0–0.5 M). Two fractions with lipase activity (Lipase I and Lipase II) were collected and precipitated with (NH₄)₂SO₄ (0.7 saturation).

The precipitated Lipase I was dissolved in deionized water, and the solution was desalted with a Sephadex G-25 column (2.0 × 120 cm, Pharmacia LKB). To the desalted solutions, 0.1 M sodium phosphate buffer (pH 6.0) was added to a concentration of 10 mM. This solution was placed on a CM-Toyopearl column equilibrated with the same buffer. Elution was done by increasing the pH of 10 mM sodium phosphate buffer stepwise from pH 6.0 to 7.0. The fraction containing the lipase activity was eluted at pH 6.8 (10 mM sodium phosphate buffer).

Lipase II was placed on a CM-Toyopearl column equilibrated with 10 mM sodium phosphate buffer (pH 6.8), after it was desalted by the same method as was used for Lipase I. After the column was washed with the same buffer, Lipase II was eluted with a linear NaCl gradient (0–0.3 M).

Lipase assay. The activity was measured by the olive oil–polyvinyl alcohol emulsion method of Yamada and Machida.¹⁵⁾ The reaction mixture consisting of 5 ml of the emulsion, 4 ml of 0.2 M sodium phosphate buffer (pH 7.0) and 1 ml of the enzyme solution was incubated for 30 min at 37°C. The enzyme reaction was stopped by the addition of 10 ml of an acetone–ethanol mixture (1:1, v/v). The liberated free fatty acids were titrated with a 0.05 N HCl solution using a titrating pH meter after the addition of 10 ml of 0.02 N NaOH solution. One unit of lipase activity was defined as the amount of enzyme that liberated 1 μmol of acid per min at 37°C.

The effects of pH on the activities were assayed by the standard method using 0.2 M McIlvaine buffer (pH 4.5–7.5) instead of sodium phosphate buffer (pH 7.0). The optimal temperatures for lipase activities were measured by incubation over the temperature range from 20 to 60°C in a thermostatically controlled water bath. Thermal stabilities were measured by incubating the lipase solutions at 0–70°C for 30 min and assaying residual activities by the standard method at 37°C.

Electrophoresis method. SDS-PAGE (10–20% polyacrylamide gradient gel) was done described by Laemmli.¹⁶⁾ Tricine-SDS-PAGE (16.5%

acrylamide and 6 M urea gel) was done by the method of Schagger and von Jagow.¹⁷⁾ Molecular mass markers were obtained from Daiichi Pure Chemicals Co. and Pharmacia LKB. Protein was stained with Coomassie brilliant blue R-250.

Electrotransfer on PVDF membranes. After the SDS-PAGE, to sequence the N-terminus of the component that appeared from Lipase I, electrotransfer onto a PVDF membrane (Immobilon-P, Millipore) was done as described by Ploug *et al.*¹⁸⁾

The PVDF membrane was rinsed with water and then stained by Coomassie brilliant blue R-250. The stained component was used for the N-terminal sequencing.

Purification of A-chain and B-chain of Lipase I by HPLC. To separate the A- and B-chains of Lipase I, the purified Lipase I was analysed by reverse-phase HPLC (column; Wakosil C₁₈-200, 4.6 × 150 mm, Wako Chem. Co.) using a linear gradient of acetonitrile (10–90%) containing 0.1% trifluoroacetic acid.

Inhibition of limited proteolysis of Lipase I. 10 µl of Lipase I solution (0.2 mg/ml) and 2 µl of PMSF solution (1.5 mg/ml EtOH) were mixed and incubated at 37°C. After incubation, the mixed solution was treated with the sample buffer for SDS PAGE. The conditions of SDS PAGE were the same as described above.

Amino acid analysis. The amino acid composition of Lipase II was analyzed with an automated amino acid analyzer (Model L-8500, Hitachi Co.) after hydrolysis of Lipase II in 6 N HCl containing 0.05% β-mercaptoethanol at 110°C for 24, 48, and 72 h. The N-termini of Lipase I, Lipase II, and the component that appeared from Lipase I after several days at 37°C were sequenced with a gas-phase protein sequencer (Model 473A, Applied Biosystems). The C-terminal amino acid of Lipase II was analyzed by hydrazinolysis.¹⁹⁾

Crystallization experiments. All experiments were done by the hanging drop method²⁰⁾ under a wide range of experimental conditions. One ml of reservoirs at a variety of pH, buffer, and precipitant agents were put in 24-well tissue culture plates (Falcon No. 3047, Becton Dickinson Co.). Drops consisted of protein solution (6 µl) at various concentrations and reservoirs (6 µl). The experimental plates were settled in an incubator controlled at 4°C or 20°C. The growth of crystals were observed by a microscope.

Results and Discussion

Purification of lipases

The two lipases were partially separated by the passage through a column of DEAE-Toyopearl and the column chromatography of CM-Toyopearl at pH 6.0 as shown in Fig. 1. At this point, neither Lipase I (the early emerging component) nor Lipase II (the later emerging component) were completely pure. Lipase I was further purified on a CM-Toyopearl column using pH stepwise elution as described by Aibara *et al.*²¹⁾ It eluted at a fixed and low concentration of sodium phosphate buffer (10 mM) and the pH was increased stepwise (pH 6.0–7.0) instead of varying the concentration. Under these conditions, Lipase I eluted as a single peak at pH 6.8. Lipase II was purified using a NaCl gradient. Purified Lipase I and Lipase II showed single bands by the native disk polyacrylamide gel electrophoresis (data not shown).

The results of the purification protocols are summarized in Table I. Specific activities were increased 54.4-fold (Lipase I) and 67.9-fold (Lipase II), respectively from the crude enzyme solution. Specific activities were 5000 U/mg for Lipase I and 6200 U/mg for Lipase II. It is known that specific activities of *Rhizopus* lipase increase in the presence of calcium ions.²²⁾ Those of Lipase I and Lipase II also increased by about 15% to 5700 U/mg (Lipase I) and 7250 U/mg (Lipase II) in the presence of 15 mM CaCl₂. The

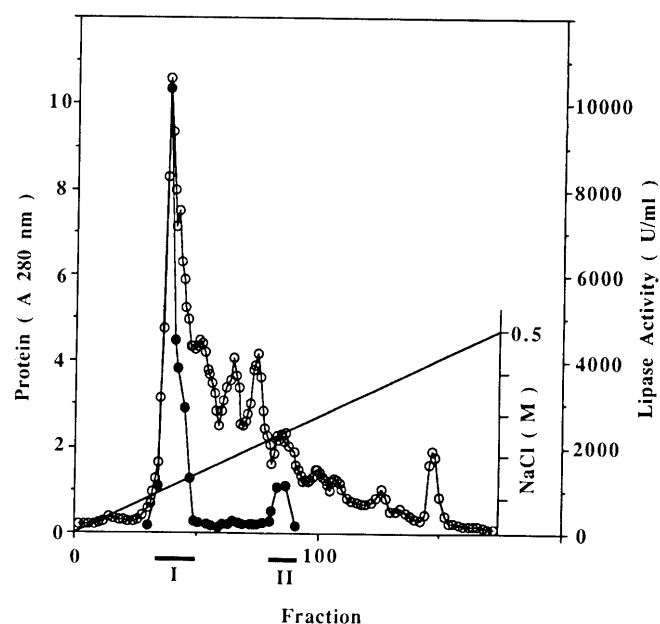


Fig. 1. CM-Toyopearl Chromatography of the Partially Purified Lipase. It was obtained from an initial fractionation on DEAE-Toyopearl showing lipase activity (●) and total protein distribution (○). Fractions 30–49 (I) and 75–91 (II) were collected.

Table I. Summary of Purification of Lipase I and Lipase II

Step of purification	Activity (U)	Protein (mg)	Specific activity (U/mg)	Purification fold	Recovery (%)
Crude filtrate	4.79×10^6	51.2×10^3	91.3	1	100
DEAE-Toyopearl	4.54×10^6	27.7×10^3	163.9	1.75	99.3
Lipase I					
1st CM-Toyopearl	3.12×10^6	2.92×10^3	1069	11.4	65.1
2nd CM-Toyopearl	1.49×10^6	0.30×10^3	4966	54.4	31.1
Lipase II					
1st CM-Toyopearl	0.34×10^6	0.13×10^3	2667	29.2	7.1
2nd CM-Toyopearl	0.15×10^6	24.2	6198	67.9	3.1

specific activity of Lipase II is nearly the same as that reported for *Rh. delemar* lipase in the presence of calcium ion.²³⁾

Structure of Lipase I

Lipase I was purified to a single band by the native disc electrophoresis. However, the analysis of the N-terminal amino acid sequence of Lipase I showed that Lipase I consisted of two polypeptides as shown in Table II. One sequence agreed with the N-terminal sequence of mature RNL predicted from lipase cDNA of *Rh. niveus* in reference 14. Moreover, by the analysis of tricine-SDS-PAGE, Lipase I consisted of three components of molecular weights 5500, 7500, and 34,000 (Fig. 2). To separate these components, Lipase I was treated by reverse-phase HPLC. Two main peaks were detected as shown in Fig. 3. The fractions were designated A-fraction and B-fraction, respectively. The molecular weight of the polypeptide from the B-fraction (B-chain) was 34,000 as shown in Fig. 2 and its N-terminal sequence agreed with those of mature RNL (Table II).¹⁴⁾ While the A-fraction (A-chain) contained two bands of molecular weight 5500 and 7500 by the tricine-SDS-PAGE (Fig. 2), the N-terminal sequence of A-chain was single

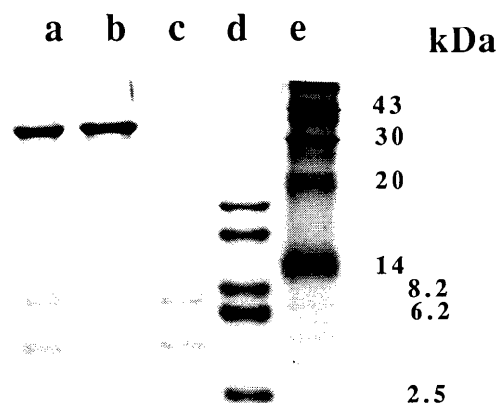
Table II. N-Terminal Sequence of Lipase I

Lipase	N-Terminal amino acid sequence
	I 10
Lipase I ^a	Asp-Asn-Ala-Ala-Leu-Pro-Pro-Leu-Ile-Ser- Asp-Asp-Asn-Leu-Val-Gly-Gly-Met-Thr-Leu-
A-Chain ^b	Asp-Asn-Ala-Ala-Leu-Pro-Pro-Leu-Ile-Ser-
B-Chain ^b	Asp-Asp-Asn-Leu-Val-Gly-Gly-Met-Thr-Leu-
Mature RNL ^c	Asp-Asp-Asn-Leu-Val-Gly-Gly-Met-Thr-Leu-

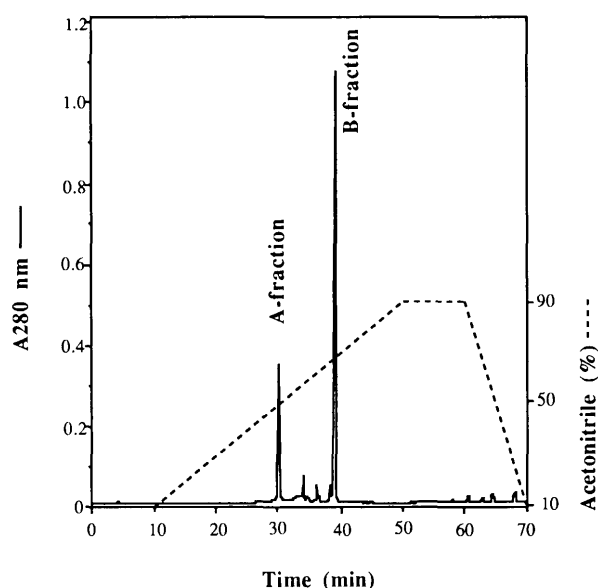
^a Lipase I. The purified Lipase I by ion exchange chromatographies as Materials and Methods.

^b A-chain and B-chain, the separated A-chain and B-chain of Lipase I by reverse-phase HPLC.

^c Mature RNL, the mature *Rh. niveus* lipase sequence that was predicted from cDNA as described in ref. 14.

**Fig. 2.** Tricine SDS PAGE of Lipase I.

Lane a, 5 μ g of pure Lipase I; lane b, 5 μ g of B-fraction; lane c, 5 μ g of A-fraction; lane d and e, molecular mass markers. The mass of the molecular mass marker proteins (kDa) are indicated in the right margin.

**Fig. 3.** Isolation of A-Chain and B-Chain by Reverse-phase HPLC.

The purified Lipase I (12 μ g) was dissolved with 10% acetonitrile containing 0.1% trifluoroacetic acid and treated by a reverse-phase HPLC (see Materials and Methods). Flow rate was 0.5 ml/min.

Table III. Amino Acid Compositions of the *Rh. niveus* Lipase II and *Rh. delemar* Lipase^a

Amino acid	Lipase II		<i>Rh. delemar</i> lipase ^d
	Measured ^b	Predicted ^c	
Asx	23	23	23
Thr	21	22	22
Ser	24	24	24
Glx	25	22	22
Gly	23	21	21
Ala	15	15	15
Cys	n.d. ^e	6	6
Val	31	26	26
Mel	1	1	1
Ile	17	17	17
Leu	18	16	16
Phe	16	15	15
Lys	16	15	15
His	8	7	7
Arg	10	9	9
Pro	18	15	15
Tyr	12	12	12
Trp	n.d. ^e	3	3

^a Expressed as number of amino acid residues per lipase molecule.

^b Measured in the purified Lipase II as described in Materials and Methods.

^c Predicted by deducing amino acids of A-chain and N-terminal 28 amino acids of B-chain from those of Lipase I.

^d Predicted the nucleotide sequence of the LIP cDNA for amino acid +1-+269.¹²⁾

^e n.d., not determined.

(Table II). A-chain is a glycopeptide of sugar contents around 25% by the phenolsulfonic acid method.²⁴⁾ So we suggest that a difference of molecular weight of A-chain is due to the heterogeneity of sugar chains binding to it. A-chain and B-chain of Lipase I are thought to bind each other noncovalently, because they are easily separated by the tricine-SDS-PAGE or the reverse-phase HPLC.

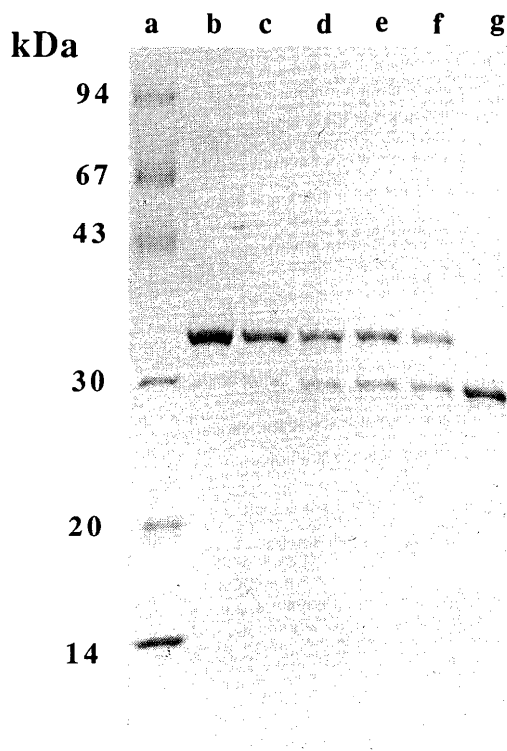
In comparison with the several reports about lipase from *Rhizopus* species, Lipase I from *Rh. niveus* is similar to the lipases from *Rh. arrhizus* and *Rh. delemar*. The lipase from *Rh. arrhizus* has two polypeptide chains which bind each other noncovalently.^{25,26)} One peptide is a glycopeptide of molecular weight around 8500. The other one has a catalytic function of molecular weight around 34,500. The lipase gene sequence of *Rh. niveus* is the same as that of *Rh. delemar*.^{12,27)} The c-lipase from *Rh. delemar* is similar to Lipase I from *Rh. niveus* with respect to its molecular weight (41,000) and properties.^{11,22,28)} Also, the proenzyme from *Rh. delemar* is thought to be a component with 366 amino acid residues (molecular weight 39,500) by expression of the *Rh. delemar* lipase gene in *E. coli*.²⁹⁾ So we suggest that Lipase I closely resembles the c-lipase and the proenzyme from *Rh. delemar*.

Structure of Lipase II

Lipase II is a single peptide by analysis with SDS-PAGE and reverse-phase HPLC (data not shown). The amino acid composition, the N-terminal amino acid sequence (10 amino acids), and the C-terminal amino acid of Lipase II were identified (Tables III and IV). In a comparison of these results with those of Lipase I, it was found that Lipase II

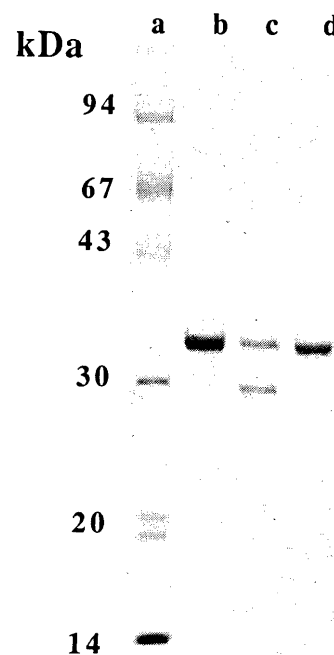
Table IV. N-Terminal Sequence and C-Terminal Amino Acid of Lipase II and Similar Lipases

Lipase	N-Terminal Amino acid sequence	C-Term ^a
	1	10 269
R. N. Lip. II ^b	Ser-Asp-Gly-Gly-Lys-Val-Val-Ala-Ala-Thr-	-Leu
Proteolytic Lip. I ^c	Ser-Asp-Gly-Gly-Lys-Val-Val-Ala-Ala-Thr-	n.d. ^f
R. D. Lip. ^d	Ser-Asp-Gly-Gly-Lys-Val-Val-Ala-Ala-Thr-	-Leu
Rm. M. Lip. ^e	Ser-Ile-Asp-Gly-Gly-Ile-Arg-Ala-Ala-Thr-	-Thr

^a C-term., C-terminal amino acid.^b R. N. Lip. II, *Rh. niveus* Lipase II; Measured in the purified Lipase II as described in Materials and Methods.^c Proteolytic Lip. I, proteolytic Lipase I; the limited proteolytic component (MW. 30,000) by treatment with the sample buffer for SDS-PAGE of Lipase I (Fig. 3).^d R. D. Lip., *Rh. delemar* lipase.^{12,29)}^e Rm. M. Lip., *Rhizomucor miehei* Lipase.³⁰⁾^f n.d., not determined.**Fig. 4.** SDS-PAGE of Lipases from *Rh. niveus*.

Lane a, molecular mass markers; lane b, 2 μ g of pure Lipase I; lanes c-f, 2 μ g Lipase I left at 37°C for 1, 3, 5, and 7 days, respectively; lane g, 2 μ g of pure Lipase II. The mass of the molecular mass marker proteins (kDa) are indicated in the left margin.

lacked the A-chain and 28 N-terminal amino acid residues of B-chain from Lipase I. Fifty percent of the purified Lipase I gradually shifted to a component having a molecular weight of 30,000 after several days at 37°C (Fig. 4). This component was electrotransferred on the PVDF membrane and its N-terminus was sequenced. The N-terminal sequence of this component agreed with that of Lipase II as shown in Table IV. So, it was found that Lipase I shifted to Lipase II gradually. The shift from Lipase I to Lipase II is done by a specific serine protease which exists in a small amount in the purified Lipase I solution, because the shift was inhibited by the addition of specific serine protease

**Fig. 5.** The Effects of PMSF on the Shift of Lipase I to Lipase II.

Lane a, molecular mass markers; lane b, 2 μ g of pure Lipase I; lane c, 2 μ g Lipase I after incubation for 7 days at 37°C; lane d, 2 μ g Lipase I left for 7 days at 37°C with PMSF. The numbers in the left margin are the same as Fig. 2.

inhibitors such as PMSF (Fig. 5). So, it is suggested that Lipase II, which was purified at the same time, is produced from Lipase I by a limited proteolysis due to the action of the specific serine protease which exists in a small amount in the purified Lipase I solution as described above.

Lipase II might have the same amino acid sequence as the mature lipase from *Rh. delemar*, because of the same N-terminal sequence and C-terminal amino acid as shown in Tables III and IV. The primary structure of Lipase II is highly similar to that of *Rhizomucor miehei* lipase (60% homology)³⁰⁾ and the N-terminal amino acid sequence of this lipase strongly resembles that of Lipase II (Table IV). The results suggest that the site of Ala28-Ser29 of B-chain of Lipase I of *Rh. niveus* or the site of the prolipase to the mature lipase of *Rh. delemar* is very susceptible to proteolysis by the processing enzyme of *Rhizopus* sp.

Properties of lipases

The pH optima were between pH 6.0 and 6.5 for Lipase I and pH 6.0 for Lipase II (Fig. 6). The small differences in pH optima between Lipase I and Lipase II may due to differences in net charge. In fact, elution patterns of two lipases eluting in the 1st cation exchange chromatography step (CM-Toyopearl column) (Fig. 1) showed that Lipase I and Lipase II have different charges on their surfaces.

The optimal temperature activities for Lipase I and Lipase II were 35°C (Lipase I) and 40°C (Lipase II) (Fig. 7). Lipase I was more temperature sensitive than Lipase II. At temperatures between 28°C and 38°C, Lipase I had activity between 80% and 100%. Lipase II had this level of activity between 23–43°C. However, Lipase II had 0% activity at 60°C, while Lipase I had 10%.

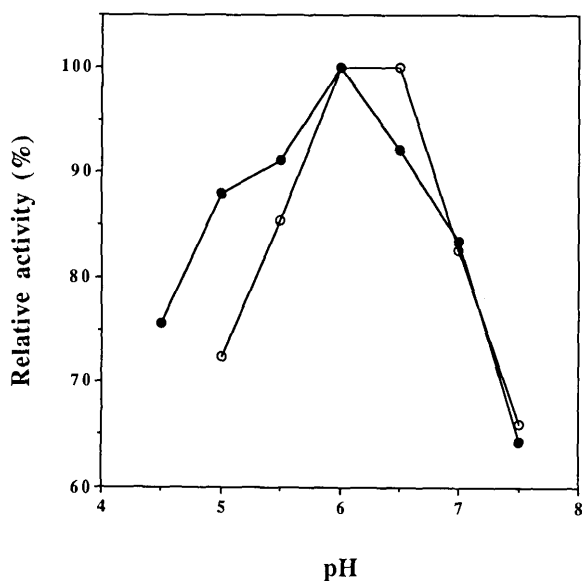


Fig. 6. The Effects of pH on the Activity of Lipase I and Lipase II.

The activity was assayed as described in Materials and Methods and indicated as the relative activity to that of the most active sample. Shown are Lipase I (○) and Lipase II (●).

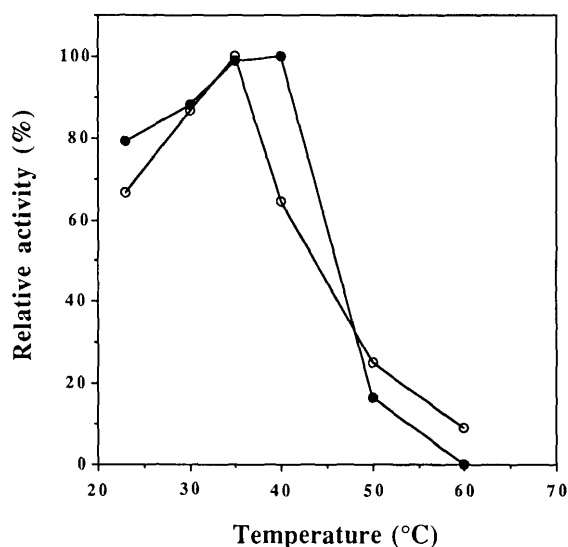


Fig. 7. Dependence of Lipase Activity on Temperature.

Shown are Lipase I (○) and Lipase II (●). The activity was assayed at the indicated temperatures, using water jackets as described in Materials and Methods. Activities are given as % of the most active sample.

The thermal stabilities (30 min) of the two lipases are shown in Fig. 8. Lipase I is more stable than Lipase II. At 40°C, the residual activity of Lipase I was almost 80% but that of Lipase II was only about 50% against the residual activity at 0°C. At 50°C, Lipase II was barely active while, about 50% of the activity of Lipase I remained.

The differences of Lipase I and Lipase II in optimal temperature and thermal stability are due to the sugar moiety that links to the A-chain of Lipase I. It is known that sugar chains of glycoproteins block the aggregation and denaturation of the proteins.

Crystallization of lipases

All crystals of Lipase I and Lipase II were obtained using the hanging drop method. For crystallization of Lipase I, both drop and reservoir, contained 0.1 M MES or HEPES

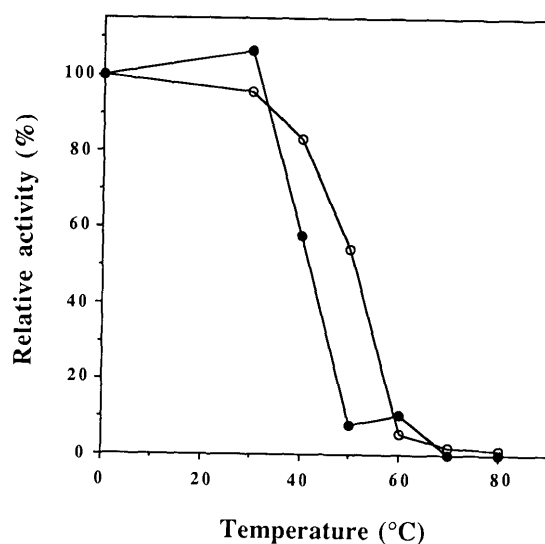


Fig. 8. Thermal Stability of Lipase I and Lipase II.

Purified lipase solutions were incubated for 30 min. Residual activity was measured at 37°C as described in Materials and Methods. Activities give as % of sample being incubated at 0°C of Lipase I (○) and Lipase II (●).

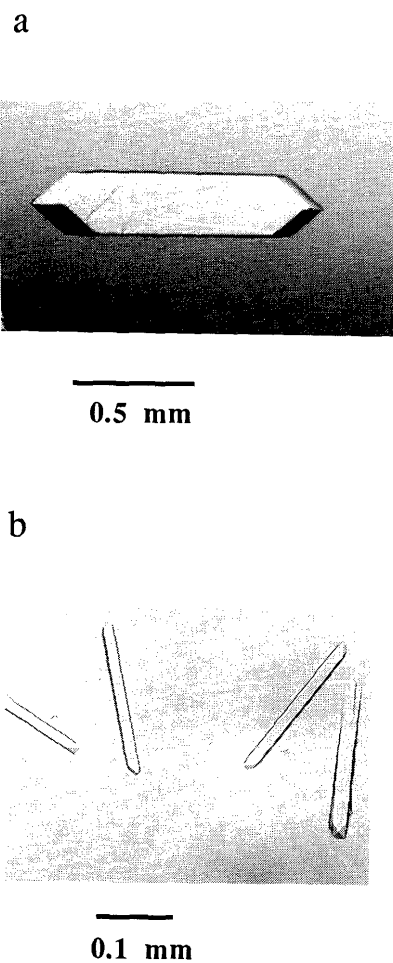


Fig. 9. Microscopic Photographs of Lipase I and Lipase II Crystals.

Lipase I (a); The size of the crystal was 1.2 mm by 0.3 mm by 0.3 mm. The crystal was grown by the hanging drop method and photographed after 2 weeks at 20°C. Crystallization conditions were 15% PEG 8000 as precipitant (reservoir) and 0.1 M MES or HEPES buffer, pH 6.0 to 7.0. Lipase II (b); The size of the crystal was 0.2 mm by 0.03 mm by 0.03 mm. Crystals were grown by the same method and photographed after 20 days at 4°C. Crystallization conditions were 14% PEG 2000 as precipitant (reservoir) and 0.1 M acetate buffer, pH 5.5.

buffer, pH 6.0 to 7.0. PEG 8000 was used as a precipitant (drop, 7 to 8% (w/v); reservoir, 14 to 16% (w/v)). Protein concentration was 18 mg/ml. Chunky rod-shaped crystals appeared after about two weeks at 20°C, and grew to a size of 1.2 mm by 0.3 mm by 0.3 mm (Fig. 9a).

The following conditions were used for the crystallization of Lipase II. Both drop and reservoir contained 0.1 M sodium acetate buffer pH 5.5. The precipitant was PEG 2000 (drop, 6 to 8% (w/v); reservoir, 12 to 16% (w/v)), and the protein concentration was 14 mg/ml. The crystals grew to a size of 0.2 mm × 0.03 mm × 0.03 mm after 20 days at 4°C (Fig. 9b).

Although many investigations of *Rhizopus* lipase have been reported, this is the first report that a *Rhizopus* lipase has been crystallized, and this is the first description of the crystallization of a lipase in which two polypeptide chains associate noncovalently except for the pancreatic lipase/procolipase complex.^{8–10)} The tertiary structure of *Rhizomucor miehei* lipase was solved at high resolution.⁴⁾ This lipase has a “lid” which moves according to the presence of its substrates. Lipase II is expected to have a tertiary structure that resembles that of *Rhizomucor miehei* lipase. Furthermore, Lipase I has only slight differences from Lipase II in its properties. The physiological role of the A-chain of Lipase I is uncertain. A preliminary X-ray study of Lipase I crystals is described in another paper.³¹⁾ Future studies on the three-dimensional structure of this lipase may elucidate the role of A-chain.

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