Purification and Some Properties of a Fungal Lipase Preparation Used for Milk Flavouring

By Susumu OI, Osamu YAMAZAKI, Akira SAWADA*
and Yukio SATOMURA

Faculty of Science, Osaka City University

Received August 30, 1968

Intracellular lipase of a strain of *Rhizopus* fungus which is effective for producing a milk flavor was purified and fractionated into two components, I and II, by DEAE Sephadex A-50 column chromatography. They both proved homogeneous by electrophoresis and ultracentrifugal analysis. The sedimentation coefficient was respectively calculated to be 5.8×10^{-13} for lipase I, and to be 2.2×10^{-13} for lipase II. From substrate specificity, it was found that lipase I was an ordinary lipase hydrolyzing olive oil and tributyrin favourably, while, II, rather, a special lipase having a high affinity towards tricaprylin. They, also, respectively had an apparent phospholipase activity on soy-lecithin and, clearing activity on chylomicron prepared from olive oil and human serum. Their mode of action, and the effect of metals and emulsifying agents on their activity are also presented.

It is known that certain lipolytic enzymes are available to improve the milk flavour which is of great significance on quality of dairy products, by changing the constitution of free fatty acids in milk.¹⁾ There must be a close relation between the effectiveness of such lipase on producing a pleasant milk smell and the substrate specificity of the enzyme. Recently, it has been made clear that the substrate specificities of microbial lipases are different from each other little by little with the origins.^{2~7)} Some lipases from microbial and animal origins were found to have an

activity on substrate other than glyceride such as Tween, phospholipid and lipoprotein.^{8,9)} It is of interest to investigate and compare the substrate specificities and properties of these lipase, with referring to the effect on milk flavouring. In this paper, the purification and characterization of a commercial *Rhizopus* lipase preparation which is at present used as flavouring enzyme on milk products are dealt.

METHOD AND MATERIALS

Crude enzyme preparation. A commercial *Rhizopus* lipase preparation** which is the extract from the mycelium of submerged culture of a *Rhizopus* fungus (unidentified strain) was extracted twice with a dilute diammoniumhydrogen-phosphate solution. The extract was salted out with 0.9 saturation of ammonium sulfate. The precipitate was collected by centrifuga-

* Osaka Saikin Kenkyūsho Ltd., Taisha-chyō, 9-55, Nishinomiya City.

1) United States Patent No. 2794743.

2) S. Tatsuoka, A. Miyake, S. Wada and C. Matsumura, *J. Biochem.*, **46**, 575 (1959).

3) J. Fukumoto, M. Iwai and Y. Tsujisaka, *J. Gen. Appl. Microbiol.*, **9**, 353 (1963).

4) J. Fukumoto, M. Iwai and Y. Tsujisaka, *ibid.*, **10**, 257 (1964).

5) Y. Satomura, S. Oi, A. Sawada and J. Fukumoto, *Agr. Biol. Chem.*, **24**, 329 (1960).

6) S. Oi, A. Sawada and Y. Satomura, *ibid.*, **31**, 1357 (1967).

7) J. A. Alford, D. A. Pierce and F. G. Suggo, *J. Lipid Res.*, **5**, 390 (1964).

8) Y. Satomura, M. Ono, S. Oi and J. Fukumoto, *Agr. Biol. Chem.*, **25**, 15 (1961).

9) K. Arima, T. Narasaki, Y. Nakamura and G. Tamura, *ibid.*, **31**, 924 (1967).

** Products of Osaka Saikin Kenkyūsho Ltd.

tion at 4000 rpm for 20 min, dissolved in a small volume of water, and dialyzed against distilled water. The dialyzed solution was used as crude enzyme preparation.

Measurement of activity. Reaction mixture containing 4 ml of olive oil emulsion (0.25 g olive oil/1 ml of 1.5% polyvinylalcohol), 2 ml of 0.1 M McIlvaine buffer (pH 6.0), 1 ml of enzyme solution, 1 ml of 0.2 M calcium chloride solution and 2 ml of distilled water, was usually incubated at 37°C for 120 min in a test tube (1.5×12.5 cm) plugged with a rubber stopper in stationary condition, unless otherwise stated. Then, the mixture was poured into a flask with 30 ml of 95% ethylalcohol, and after warming at 55°C for 20 min, free fatty acid liberated was titrated against 0.05 N NaOH using a potentiometer. One unit of the lipase activity was defined as that amount of enzyme which produces 1 μ mole of free fatty acid under the condition. Specific activity was expressed by units per mg protein, and protein quantity was determined spectrophotometrically at 280 m μ applying the factor $E_{1\text{cm}}^{1\%}=14.5$ which was estimated from the dry weight of the purified preparation.

Gas-liquid chromatography. The constitution of free fatty acids in the hydrolysates by enzyme was analyzed by a gas-liquid chromatography using Yanagimoto model 550T gas chromatograph. The hydrolysates were developed on silica gel thin-layer, and the location of free fatty acids was detected by spraying 75% sulfuric acid and heating. The bands corresponding to the free fatty acids on thin-layer chromatogram were scraped up by a spoon, and extracted with petroleum ether. To convert the fatty acids in the extract to methyl esters, the extract was evaporated to a small volume, mixed with 4 ml of 0.7 N anhydrous methanolic hydrochloric acid, and refluxed for 2 hr at 80°~100°C. After cooling and adding 1 ml of water, the mixture was extracted three times with 3 ml of petroleum ether. The extracts were combined and evaporated to about 1 ml under a stream of nitrogen. A portion of the concentrate (weighing about 5~25 μ g) was sampled, and developed on silicone SE-30 with hydrogen gas at 210°C.

Clearing activity. Clearing activity on chylomicron was measured by using a spectrophotometer, turbidimetrically. Chylomicron was prepared as the following; 1 ml of olive oil emulsion (1 g olive oil/3.0 ml of 2% polyvinylalcohol) was incubated with

9 ml of human serum for 1 hr at 37°C.

Hemolytic effect. Hemolytic effect of enzyme was tested on human erythrocytes according to the Slotta's method.¹⁰⁾

Ultracentrifugal analyses. These were carried out at Faculty of Medicine of this university using a Hitachi Analytical Ultracentrifuge UCA -1. The sedimentation coefficient was calculated as described by Schachman,¹¹⁾ and was corrected to 20°C in water, according to the method described by Svedberg.¹²⁾

Electrophoresis. Electrophoretic experiments were carried out using a Hitachi Tiselius Electrophoretic Apparatus HT-B.

EXPERIMENTAL AND RESULTS

Purification of lipase

The purification procedure is summarized in Table I. The crude enzyme solution diluted with 0.025 M Tris-HCl buffer (pH 9.1) was passed through DEAE cellulose column (2.2×60 cm) which had been equilibrated with 0.025 M Tris-HCl buffer (pH 9.1), whereby the lipase was adsorbed on the column. The column was eluted with a linear gradient pH change of Tris-HCl buffer from 9.1 to 7.2 by using the mixing chamber containing 2000 ml of 0.025 M Tris-HCl buffer (pH 9.1) and the reservoir containing 2000 ml of 0.025 M Tris-HCl buffer (pH 7.2). The main lipase which has a high specific activity was eluted at pH 8.8. Then, the lipase fraction obtained was brought to 0.9 saturation with ammonium sulfate, and allowed to stand over night at 4°C. The resulting precipitates were collected by centrifugation, dissolved in a small volume of distilled water, and dialyzed against distilled water. After adjusting pH to 7.0 with 0.1 M phosphate buffer the dialyzed solution was applied on the Sephadex G-200 column

10) K. H. Slotta and G. Szyzka, *Ber.*, **71**, 258 (1938).

11) H. K. Schachman, "Methods in Enzymology," Academic Press INC., New York, (1957) p. 32.

12) T. Svedberg and D. K. Pederson, "The Ultracentrifuge," Clarendon Press, Oxford (1940) p. 445.

TABLE I. PURIFICATION OF *Rhizopus* LIPASE I AND II

Procedure	Vol. ml	Total activity units	Specific activity units/mg protein
Crude enzyme solution	3600	900,000	50
↓ Adsorbed on DEAE cellulose column equilibrated with 0.025 M Tris-HCl buffer at pH 9.1			
↓ Eluted by changing pH of Tris-HCl buffer, linearly from 9.1 to 7.2			
↓ Eluate	1000	150,000	175
↓ Ppt. with 0.9 satur. of $(\text{NH}_4)_2\text{SO}_4$ and dialyzed against water	200	140,000	200
↓ Chromatography on Sephadex G-200 column equilibrated with 0.1 M phosphate buffer at pH 7.0			
↓ Eluate	120	60,000	500
↓ Ppt. with 0.9 satur. of $(\text{NH}_4)_2\text{SO}_4$ and dialyzed against water	40	55,000	575
↓ Adsorbed on DEAE Sephadex A-50 column equilibrated with 0.02 M veronal buffer at pH 8.0			
↓ Eluted by changing mol. concn. of NaCl			
↓ Eluate			
↓ Lipase II	120	15,400	425
↓ Lipase I	120	17,000	500
↓ Ppt. with 0.9 satur. of $(\text{NH}_4)_2\text{SO}_4$ and dialyzed against water	30	15,000	600
↓ Adsorbed on CM-Sephadex C-50 column equilibrated with 0.02 M phosphate buffer at pH 6.0			
↓ Eluted by changing pH of phosphate buffer, linearly from 6.0 to 7.5			
↓ Eluate	36	13,000	625

(3.0×120 cm) which had been equilibrated with 0.1 M phosphate buffer (pH 7.0). The column was eluted with the same buffer and the eluate at tube No. 99 to 110 was collected, as presented in Fig. 1. Then, the lipase fraction collected was brought to 0.9 saturation with ammonium sulfate, and allowed to stand over night at 4°C. The resulting precipitates were collected by centrifugation, dissolved in a small volume of distilled water, and dialyzed against distilled water. After adjusting pH at 8.0 with 0.02 M veronal buffer the dialyzed solution was adsorbed on the DEAE Sephadex A-50 column

(2.2×56 cm) which had been equilibrated with 0.02 M veronal buffer (pH 8.0), whereby the enzyme was again adsorbed on the column. The column was eluted with a linear gradient change of the concentration of NaCl from 0 to 1.5 M by using the mixing chamber containing 500 ml of 0.02 M veronal buffer (pH 8.0) and the reservoir containing 500 ml of the same buffer to which NaCl was added in 1.5 M concentration, as presented in Fig. 2. The lipase I (peak I) and II (peak II) are respectively eluted at tube No. 10 and No. 75, where the concentration of NaCl was respectively 0.17 and 1.0 M. Then, after adjust-

ing pH to 6.0 with 0.02 M acetate buffer the lipase I fraction was again adsorbed on CM Sephadex C-50 column which had been equilibrated with 0.02 M acetate buffer (pH 6.0), and eluted at pH 6.5 with a linear gradient pH change of acetate buffer 6.0 to 7.5, as seen in Fig. 3. In this way, the activity of lipase I was attained to 12.5 fold per mg protein, and that of lipase II attained to 8.5 fold.

FIG. 1. Chromatography on Sephadex G-200 Column.

Column size: 3.0 × 120 cm.
Flow rate : About 6 ml/hr.
— : OD value.
- - - : Lipase activity.
· · · : Specific activity.

FIG. 2. Chromatography on DEAE-Sephadex A-50.

Column size: 2.4 × 56 cm.
— : OD value.
- - - : Lipase activity.

Homogeneity of purified lipase

a) *Gel-filtration.* As presented in Fig. 4, the gel-filtration of lipase I and II on Sephadex

FIG. 3. Chromatography of Lipase I on CM-Sephadex C-50.

Column size: 2.4 × 56 cm.
— : OD value.
- - - : Lipase activity.

FIG. 4. Gel-Filtration of Lipase I and II on a Sephadex G-100 Column.

Column size: 2.4 × 58 cm.
Buffer : Phosphate buffer at pH 7.0.

FIG. 5. Electrophoretic Patterns of Lipase I and II.

I: 0.6% of lipase I in 0.02 M veronal buffer (pH 9.20).

II: 1.0% of lipase II in 0.02 M phosphate buffer (pH 7.5).

The electrophoresis was carried out at 40 volts, 6 mamp., and 18°C, adjusting the ionic strength to 0.2 with NaCl.

G-100 column which was equilibrated with 0.1 M phosphate buffer (pH 7.0) showed a symmetric elution pattern, respectively, and proved their homogeneity.

b) Electrophoresis and ultracentrifugal analysis.

As seen in Fig. 5, these lipases were also homogeneous electrophoretically. Ultracentrifugal analysis was operated at 20.2°C in a standard 12 mm, 4° sector cell. The sedimentation coefficient $s_{20,w}$ was calculated from the sedimentation velocity runs shown in Fig. 6, and a value of 5.8×10^{-13} and 2.2×10^{-13} was obtained respectively for lipase I and II. Also, the single peak shown in the sedimentation pattern again revealed the homogeneity of these enzymes.

Characterization of lipase I and II

i. Enzymatic properties

a) Optimum pH and stability. As presented in Fig. 7, although optimum pH of the crude enzyme was 6.0, that of purified lipase I and II was rather shifted to near 3.5, where a high activity on a wide pH range 3.5 to 7.0 appeared, and pH-activity curves of these two enzymes almost completely coincided. While, when 0.1 M of NaCl and 10 mg of bile salt

were added to the reaction mixture at that time, the optimum pH of lipase I returned to 6.5. Also, when reaction mixture was emulsified with 10 mg of pectin, optimum pH of lipase I was near 6.0. As presented in Fig. 8, pH stability of these lipase were stable at 30°C between pH 4.0 and 7.0 for 20 hr. As presented in Fig. 9, these enzyme were thermostable at pH 6.0 and did not lose activity by heating the enzyme at 47°C for 15 min.

ii. Substrate specificity

As shown in Table II, lipase I hydrolyzed olive oil most efficiently, and tributyrin was the second most favorable substrate. On the other hand, lipase II hydrolyzed tricaprylin much more efficiently than olive oil. The relative rate of hydrolysis by lipase I was the following, *i.e.* [Olive oil>Tributyrin>Trilaurin>Tricaprylin>Triacetin] and that of lipase II, *i.e.* [Tricaprylin>Trilaurin>Olive oil>Tributyrin]. Besides, these lipase both had a high activity on soy-lecithin, a tolerable activity on phosphatidylcholine, and a little activity on phosphatidylinositol. Also, lipase II could hydrolyze Tween 20 and methylbutyrate under stationary condition, and Span 20 under shak-

FIG. 6. Sedimentation Patterns of Lipase I and II.

I: 0.4% of lipase I in 0.02 M veronal buffer (pH 8.75).

II: 0.6% of lipase II in the same buffer as I. The ionic strength was adjusted to 0.2 with NaCl. The photographs were taken at 30 min and 60 min after reaching full speed.

ing condition. While lipase I only hydrolyzed Tween 20 under shaking condition.

iii. Mode of action

The hydrolysis rate of olive oil by lipase I (530 unit) reached to about 30% after 72 hr, but that by lipase II (450 unit) reached to 18% as presented in Fig. 10-a. The ether extract of the hydrolysates by lipase II was concentrated and developed on thin-layer chromatogram. As seen in Fig. 10-b, spots of oleic acid, diglyceride, monoglyceride and triglyceride were detected. The hydrolysis of olive oil by lipase II was thus proved to proceed via di- and mono- glyceride.

FIG. 7. pH-Activity Curve of Lipase I and II.

- (i) 0.1 M of NaCl and 10 mg of bile salt was added to reaction mixture. (Purified lipase I)
- (ii) Reaction mixture was emulsified with 10 mg of pectin. (Purified lipase I)
- (iii) Crude enzyme.

FIG. 8. pH Stability of Lipase I and II.

Incubated at 30°C for 22 hr.

pH 3.0~6.0: McIlvaine buffer.

pH 6.0~7.5: Phosphate buffer.

pH 7.5~9.0: $\text{NH}_3\text{-NH}_4\text{Cl}$ buffer.

The hydrolysis rate of soy-lecithin by lipase I and II reached respectively to only 10% after 24 hr, as shown in Fig. 10-a. The chloroform extract of the hydrolysate by lipase II was concentrated and subjected to thin-layer chromatography. Lysolecithin was finely detected, as shown in Fig. 10-c. The lysolecithin was extracted with chloroform,

FIG. 9. Thermo-Stability of Lipase I and II. Incubated at pH 6.0 for 15 min.

FIG. 10-a. Hydrolysis Curves of Olive oil and Soy-lecithin by Lipase I and II.

— Olive oil.
--- Soy-lecithin.

Solvent system:
Chloroform:Aceton =
96:4.
Detection with conc.
 H_2SO_4 - $K_2Cr_2O_7$ at $160^\circ C$,
for 15 min.

FIG. 10-b. TLC of Hydrolysate of Olive oil by Lipase II on Silica Gel GF+Boric acid 2%.

TG: Triglyceride.
DG: Diglyceride.
MG: Monoglyceride.
FA: Free fatty acid.

Solvent system:
Chloroform:
Methanol: H_2O =
60:25:4.
Detection with
Dragendorff
reagent.

FIG. 10-c. TLC of Hydrolysate of Soy-lecithin by Lipase II on Silica Gel G.

and saponified. The fatty acids liberated from lysolecithin was analyzed by gas-chromatography, as described above. As presented in Fig. 11-b, palmitic and oleic methyl ester were mainly detected in the ratio of 1:2. It indicated that the lysolecithin may be α -form, because lecithin usually contains the fatty acids in the ratio of 1.5:2, and palmitic acid is mainly located at α -position.

After 23% hydrolysis of lard by lipase II, the free fatty acids liberated was also analyzed by gas-chromatography as described above. (The lard used was purified by the silica gel column chromatography).

As seen in Fig. 11-a, palmitic and oleic methyl ester were detected in the ratio of 1:2.7. It indicated that lipase II mainly hy-

drolyzed the ester bond of α -position in lard, because lard contains the fatty acids in the ratio of 1:2, and oleic acid is mainly located at α -position.

iv. Hemolytic effect

As shown in Table III, lipase I and II had a hemolytic effect on human erythrocytes. It may be due to the phospholipase activity.

v. Clearing activity

As shown in Fig. 12, lipase I and II had a clearing activity on chylomicron prepared as the above. However, the activity was rather lower than that of pancreatic lipase, but higher than that of crude enzyme.

vi. Effect of metals and emulsifiers

As shown in Table IV, the activity of lipase

a. Free fatty acids in lard hydrolysate. b. Fatty acid components of lysolecithin.

FIG. 11. Gas Chromatograms of Methyl Esters of Fatty Acids in Lard Hydrolysate and Lysolecithin.

Stationary phase: SE 30 10% on Chromosorb W.
Column length : 210 cm.
Temp. : 210°C.
Carrier gas : H_2 .
Flow rate : 17 ml/min.

TABLE II. SUBSTRATE SPECIFICITY OF LIPASE I AND II

Substrate	Activity of			
	Lipase-I, under conditions as below		Lipase-II	
	Stationary	Shaking	Stationary	Shaking
Olive oil	90	60	55	35
Trilaurin	—	33	—	79
Tricaprylin	—	24	—	179
Tributyrin (added β -globulin and $CaCl_2$ M/50)	60	42	20	35
Triacetin	0	12	10	8
Tween 20	0	13	20	0
Span 20	0	0	0	20
Methyl- <i>n</i> -butyrate	0	0	31	0
Soy-lecithin (added albumin and $CaCl_2$ M/50)	115	115	140	65
Phosphatidylcholine (from egg)	—	40	—	30
Phosphatidylinositol (from soy-lecithin)	—	10	—	15

(Shaking condition: 180 times oscillation/min in 3 cm amplitude).

I and II was increased by bimetallic ions such as Ca^{2+} , Fe^{3+} and Mg^{2+} , and also by Na^+ ion, but inhibited by Co^{2+} , Zn^{2+} and Fe^{2+} ion.

TABLE III. HEMOLYTIC EFFECT OF PURIFIED LIPASE

Enzyme added	Hemolytic effect time in min				
	0	30	60	90	120
Lipase I (100 units)	—	—	±	++	+++
Heated at 100°C for 30 min	—	—	—	—	—
Lipase II (200 units)	—	±	+	++	+++
Heated at 100°C for 30 min	—	—	—	—	—

One ml of 4% human erythrocytes suspension (0.9% saline), 4 ml of 0.9% saline and 0.1 ml of enzyme solution were mixed and incubated at 37°C for 2 hr.

+, ++: Partial hemolysis.

+++ : Complete hemolysis.

TABLE IV. EFFECT OF METALS ON ACTIVITY OF LIPASE I AND II

Addition	Concn. (M)	Relative activity on olive oil	
		Lipase I	Lipase II
None	—	100	100
CaCl_2	2×10^{-2}	210	236
$\text{Fe}_2(\text{SO}_4)_3$	"	210	237
NaCl	"	130	146
MgCl_2	"	130	146
ZnCl_2	"	50	64
CoCl_2	"	50	46
FeSO_4	"	36	36

As shown in Table V, although the activity of crude enzyme was increased by several emulsifying agents such as polyvinylalcohol, globulin, heparin, bile salt, chondroitin, agar-agar and pectin, that of purified lipase I and II was only promoted by polyvinylalcohol and globulin.

FIG. 12. Clearing Activity of Lipase I, II and Pancreatic Lipase on Serum-Olive Oil Chylomicron.

Enzymes; Panc.: A crude pancreatic lipase preparation (2 mg/ml), I: Lipase I, II: Lipase II, III: Crude *Rhizopus* lipase.

Reaction mixture; 1 ml of enzyme solution, 2 ml of 0.3 M $\text{NH}_4\text{OH-NH}_4\text{Cl}$ buffer (pH 8.3), 2 ml of 0.2% olive oil chylomicron and 1 ml of water.

TABLE V. EFFECT OF EMULSIFIERS ON LIPASE ACTIVITY

Emulsifier added	Conc.	Relative activity on olive oil		
		Lipase I	Lipase II	(Crude enzyme)
None	—	100	100	(100)
β -Globulin Fract. III	20 mg	150	150	(350)
α -Globulin Fract. IV	"	120	125	(224)
Heparin—Na	"	100	120	(350)
Chondroitin sulfate	"	100	100	(200)
Agar-agar	10 mg	110	110	(194)
Pectin	"	110	110	(171 at pH 4.0)
Polyvinyl alcohol	20 mg	220	230	(350)
Linoleic acid	"	100	100	(210 at pH 4.0)
Lecithin	"	100	100	(140)
Cholesterin	"	100	100	(150)
Bile salt	"	110	110	(250)

DISCUSSION

From the substrate specificity of lipase I and II shown above, it is suggested that lipase II has an important role on milk flavoring. Among the free fatty acids contained in milk, the fatty acids having a chain length of $C_6 \sim C_{14}$ are said to give a pleasant milk smell.¹⁾ It is of interest that lipase II specifically liberate the caprylic acid, as described above. Enzymatic properties of lipase I and II are very similar each other though, molecular weight of lipase II is shown to be smaller than that of lipase I from the above experiments. The difference of the substrate specificity between lipase I and II may be in part attributed to the difference in the physico-chemical properties of the enzyme proteins. Recently, it has been found that two types of *Penicillium* lipase are convertible under an appropriate condition.⁶⁾ It is possible that *Rhizopus* lipase I is converted to lipase II,

intracellularly.

According to Haas *et al.*¹³⁾ a highly purified pancreatic lipase can hydrolyze lecithin into lysolecithin, releasing mainly a saturated fatty acid. The *Rhizopus* lipase I and II also hydrolyze lecithin in the same manner, and have a hemolytic effect too, as shown in the above experiments. With the lipoprotein lipase activity, clearing effect of the *Rhizopus* lipases appears to be rather low when compared with other microbial enzymes which has been reported as lipoprotein lipase.⁹⁾ It is of interest that optimum pH and the effect of emulsifiers on activity of *Rhizopus* lipase were different with the degree of purification of enzyme. It is conceivable that although there are various types of lipase in nature, the type of *Rhizopus* lipase II is novel.

13) G. H. De Haas, L. Sarda and J. Roger, *Biochem. Biophys. Acta*, **106**, 638 (1965).