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Studies on Bile-sensitive Lipase. V.[1] Purification and Properties of Lipase from *Mucor javanicus*[2]

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It had been found that in one of lipase [EC 3.1.1.3] from *Mucor javanicus* the hydrolysis of fats was accelerated by bile salts. The lipase was purified about 20-fold from the original powder (about 100-fold from the culture broth) by ammonium sulfate precipitation, chromatography on Sephadex G-75, CM-cellulose, and Sephadex G-200. Recovery of the activity was about 18%. The purified enzyme was homogeneous on electrophoresis. Optimum pH for hydrolysis of olive oil was 7.0 by the assay method using a PVA system and 7.5 by that using a shaken system. Optimum temperature was 40°. The enzyme was stable below 30°. It was strongly inhibited by Ag^{+}, Hg^{2+}, and N-bromosuccinimide (NBS). The apparent I_{50} value of NBS was 1.4×10^{-4}M, that of both iodine and sodium lauryl sulfate were 2.4×10^{-3}M. The lipase hydrolyzed tricaprylin most efficiently, and next to it tricaprin and trilaurin. The Michaelis constant (Km) for triglycerides agreed closely with the substrate specificity.

Recently many kinds of lipase [glycerol ester hydrolase EC 3.1.1.3.] from microorganisms were purified and their enzymological properties have been made clear.[4]

The lipolytic action of lipase is chiefly conducted at the duodenum and upper part of the jejunum, mixing with bile juice. Therefore, lipase whose action is accelerated by bile juice should be used as a digestive for fats. It has been known for a long time that pancreatic lipase was activated by bile juice or bile salts.[5] However, most of lipase from microorganisms was inhibited by the salts,[6] so that lipolysis of microbial lipase as a digestive enzyme seems less than we expected.

Sugiura, *et al.* found that in one of lipases from *Mucor javanicus*, lipolytic action was accelerated by bile salts in polyvinyl alcohol–emulsified system (PVA system).[6b] A part of results on the accelerating effect of bile salts has already been reported.[6b] To study proteochemical and enzymological properties of the lipase, especially to clarify the mechanism of the bile-sensitive action, this enzyme was purified.

In this report, the purification procedure and some properties of the purified lipase are described.

Materials and Methods

Enzyme Source——The lipase preparation from *Mucor javanicus* was used for purification. The enzyme preparation had been obtained by EtOH precipitation of the culture broth and purified about 5-fold from the broth.[7] Some data for the lipase production by *Mucor javanicus* were reported previously.[7]

1) Part IV: M. Sugiura, T. Ogiso, Y. Naka, and K. Nagase, *Yakuzaigaku*, **27**, 314 (1967).
2) This forms Part XXXX of "Studies on Enzymes" by M. Sugiura. This work was presented at the 88th Meeting of the Pharmaceutical Society of Japan, Tokyo, 1968.
3) Location: *Mitahora, Gifu.*
4) *a*) J. Fukumoto, M. Iwai, and Y. Tsujisaka, *J. Gen. Appl. Microbiol.* (Tokyo), **9**, 353 (1963); **10**, 13 (1964); *b*) N. Tomizuka, Y. Ota, and K. Yamada, *Agr. Biol. Chem.* (Tokyo), **30**, 1090 (1966); *c*) S. Oi, A. Sawada, and Y. Satomura, *Agr. Biol. Chem.* (Tokyo), **31**, 1357 (1967).
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7) M. Sugiura, T. Ogiso, Y. Naka, and H. Asano, *Eisei Kagaku*, **13**, 265 (1967).

Materials——Polyvinyl alcohol (PVA)-117 (Kurashiki Poval-117), which had a mean degree of polymerization of 1725—1750, Saponification degree of 98—99 molar %, and the viscosity of 28—32 cps (4%, 20°), and PVA-210 (Kurashiki Poval-210), which had the values of 1000—1100, 87—89 molar %, and 9—13 cps, respectively, were obtained from Kurashiki Rayon Co., Ltd. Sephadex G-75 and G-200 (particle size, 40—120 μ) were products obtained from Pharmacia Uppsala, Sweden. CM-cellulose (0.60 meq/g) and DEAE-cellulose (0.89 meq/g) were the products obtained from Brown Co., Ltd. Duolite A-2, Duolite CS-101, and Amberlite IRA- 93 were obtained from Sasaki Shokai and the Japan Organo Co., Ltd., respectively. Olive oil and various triglycerides were obtained from Iwaki Seiyaku Co., Ltd., and Tokyo Kasei Kogyo Co., Ltd., respectively. The other chemicals were of special or reagent grade.

Assay Procedure

Lipase Activity——Throughout the experimental work the assay of lipase activity was made by one of the following methods. However, for the sake of consistency, all lipase units were determined by the first method (Method A).

1) **Assay Method using a PVA System**——An emulsion was prepared by stirring 75 ml of aqueous PVA solution (2% solution of a 9:1 mixture of PVA-117 and PVA-210) and 25 ml of olive oil for 10 min in a homogenizer cooled with ice to below 10°, 0.1M triglyceride emulsion was prepared by 2% aqueous PVA solution described above, cooled below 10° for liquid triglycerides at normal temperature and heated at about 5° higher than the melting point for solid triglycerides.

Method A——The method of Yamada and Machida was used with slight modification.[8] Four ml (3 ml when other chemicals were added to the reaction mixture) of McIlvaine buffer (0.2M Na_2HPO_4 and 0.1M citric acid, pH 7.0) and 5 ml of emulsion were mixed sufficiently in a test tube (3×13 cm). After preheating at 37° for 10 min, 1 ml of the enzyme solution was added, and the reaction mixture was shaken at intervals. After incubation at 37° for 20 or 30 min, 20 ml of a mixture of equal volumes of acetone and EtOH was used to stop the reaction, and 10 ml of 0.05N NaOH solution was added. The mixture was titrated with 0.05N HCl solution, stirring with N_2 gas and using phenolphthalein as an indicator.

The initial reaction rate was proportional to enzyme concentration till the titration value to 2 ml.

Method B——This method is an adaptation of method of Dole.[9]

One ml of the emulsion and 1 ml (0.5 ml when other solution was added to the reaction mixture) of McIlvaine buffer (pH 7.0) were mixed in a test tube (1.8×18 cm). After preheating at 37° for 10 min, 0.5 ml of the enzyme solution was added and the mixture was shaken at intervals for 20 min. The reaction was stopped by the addition of 5 ml a solution consisting of 800 ml of iso-PrOH, 200 ml of heptane, and 20 ml of 2N H_2SO_4. The liberated fatty acids were extracted with 3 ml of heptane, which was titrated with 0.01N ethanolic KOH solution, using thymol blue as an indicator.

The initial reaction rate was proportional to enzyme concentration till the titration value to 0.4 ml.

One unit of lipase was defined as the amount of enzyme which was able to liberate 1 μmole of free fatty acid per min under the conditions tested. The specific acitivity of lipase was expressed in units per mg of enzyme.

2) **Assay Method using a Shaken System without an Emulsifier (Shaken System)**——In a L-shaped flask, the reaction mixture consisting of 2 ml of olive oil, 5 ml of Michaelis buffer (barbital sodium–sodium acetate–HCl) or Menzel buffer (0.05M Na_2CO_3 and 0.1M $NaHCO_3$), and 1 ml of 0.1M $CaCl_2$ solution was preheated at 37° for 10 min and then 1 ml of the enzyme solution was added. The incubation was carried out at 37° with constant shaking (110 oscillations/min in 4 cm amplitude), using a Monod-type shaking machine. After 30 min, the reaction was stopped with 20 ml of a 1:1 mixture of acetone and EtOH. The mixture was titrated with 0.05N aqueous NaOH solution, stirring with N_2 gas and using phenolphthalein as an indicator.

Other Enzyme Activity——Other enzyme activities were estimated by the following methods: Blue value method for α-amylase activity,[10] the method of Sumner for cellulase activity,[11] and a modification of the method of Anson for proteinase activity.[12]

Determination of Protein Concentration——Protein concentration of each fraction obtained during purification was determined from its absorbancy at 280 mμ.

Determination of Carbohydrate——The phenol–H_2SO_4 method according to Dubois, *et al.* was used for the determination of carbohydrate concentration in the lipase preparation[13] and glucose was used as a standard.

8) K. Yamada and Y. Ota, *Nippon Nogeikagaku Zasshi*, **36**, 858 (1962).
9) V.P. Dole, *J. Clin. Invest.*, **35**, 150 (1956).
10) S. Akabori(ed.), "Koso Kenkyu-ho," Vol. II, Asakura Shoten, Tokyo, 1956, pp. 108—110.
11) J.B. Sumner, *J. Biol. Chem.*, **47**, 5 (1951); **65**, 393 (1925).
12) M.L. Anson, *J. Gen. Physiol.*, **22**, 79(1938); S. Akabori(ed.), "Koso Kenkyu-ho," Vol. II, Asakura Shoten Tokyo, 1956, pp. 237—246.
13) M. Dubois, K. Gilles, J.K. Hamilton, P.A. Rebers and F. Smith, *Anal. Chem.*, **28**, 350 (1956); *Nature*, **168**, 167 (1951).

Physical Measurements and Apparatus——Absorbancy was determined with Hitachi 101 type spectrophotometer. Electrophoresis was carried out with a Tiselius-type apparatus (Model HTD-1) of Hitachi Co., Ltd., and polyacrylamide electrophoresis with a Disc-type apparatus made by MS Kiki Co., Ltd.

Results

Purification Procedure

Extraction with Water: A solution of 20 g of original powder of the enzyme mixed with 200 ml of pure water for 20 min at 4—6° was centrifuged at 12500×*g* for 15 min at 2° and the supernatant was pooled.

Ammonium Sulfate Precipitation: The supernatant was brought from 0.1—0.4 Osborne's saturation with $(NH_4)_2SO_4$[14] with constant slow stirring. After standing overnight at 3° the mixture was centrifuged at 12500×*g* for 15 min. The precipitate was dissolved in 20 ml of pure water, and allowed to dialyse against cold water for 1 day. The dialyzed solution was centrifuged at 12500×*g* for 15 min to remove the precipitate.

Fractionation on Sephadex G-75 Column: The dialyzed solution obtained previously was applied on Sephadex G-75 column (5.0×80 cm) at 4—6°, 0.01M phosphate buffer (KH_2PO_4 and Na_2HPO_4, pH 6.0) was constantly fed into the column and the rate of elution was maintained at 60 ml/hr. Fractions of 10 ml were collected and analyzed for protein content and lipase activity. The lipase fractions thus obtained were centrifuged at 12500×*g* for 15 min.

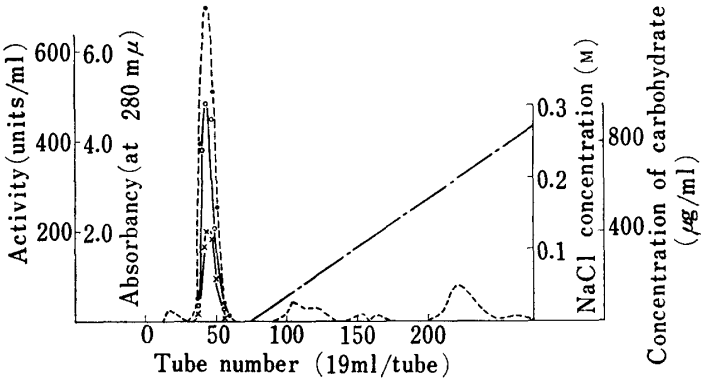


Fig. 1. Chromatography of *Mucor* Lipase on a Column of CM-cellulose

A column (5×40 cm) of CM-cellulose had been equilibrated at pH 5.2 with 0.01M phosphate buffer. A flow rate of 0.01M phosphate buffer (pH 5.0) was 100 ml/hr.

—●—: ultraviolet absorption at 280 mμ
—○—: lipase activity
— - —: concentration of Nacl
—×—: concentration of carbohydrate

CM-cellulose Column Chromatography: The supernatant obtained above was placed on a column (5×40 cm) of CM-cellulose which had been equilibrated with 0.01M phosphate buffer (pH 5.0). The column was washed with 1100 ml of the same buffer at a flow rate of 100 ml/hr and fractions of 19.0 ml were collected. As shown in Fig. 1, lipase was not adsorbed on the CM-cellulose column, but proteinase and amylase in the enzyme solution were adsorbed. The enzymically active fractions were combined and centrifuged at 12500×*g* for 15 min to remove the precipitate.

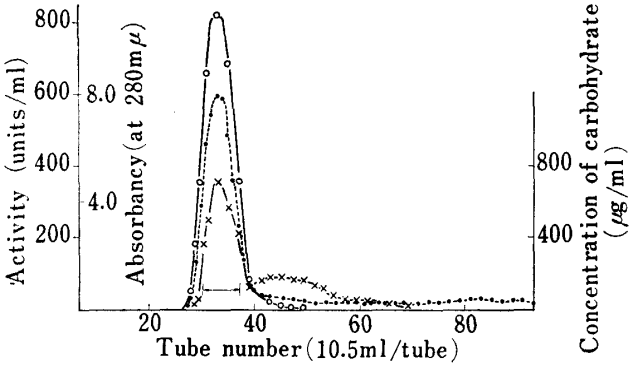


Fig. 2. Chromatography of *Mucor* Lipase on a Column of Sephadex G-200

The ellution was carried with the solution of 0.01M phosphate buffer at 3°, column size, 5.0×61 cm and flow rate, 20 ml/hr.

—●—: ultraviolet absorption at 280 mμ
—○—: lipase activity
—×—: concentration of carbohydrate

Fractionation on Sephadex G-200 Column: The supernatant was

14) S. Akabori(ed.), "Koso Kenkyu-ho," Vol. I, Asakura Shoten, Tokyo, 1961, pp. 133—134.

divided into two parts and fractionated on a column (5×61 cm) of Sephadex G-200 using 0.01 M phosphate buffer (pH 6.0) as eluent and the rate of elution was maintained at 30 ml/hr at 4—6°. As shown in Fig. 2, one protein peak was obtained and the peak showed lipase activity. The fractions, having lipolytic activity were combined and cleared by centrifugation at 12500×*g*. The supernatant was subjected to rechromatography on a column (5×61 cm) of Sephadex G-200 under the same condition as above. The fractions having lipolytic activity was dialysed against cold water and lyophylized. The content of carbohydrate of each fraction paralleled to the protein content as shown in Fig. 2.

The results of the purification procedure are summarized in Table I. The final enzyme preparation was purified about 20-fold from the original powder (about 100-fold from the culture broth). The recovery of the activity was about 18% from the original powder.

Amylase, cellulase and proteinase activity were not detected in the enzyme preparation.

Table I. Purification Procedure for Lipase

	Total enzyme (units)	Specific activity (units/mg)
Original powder	338800	8.3
↓ extracted with water and centrifuged		
Supernatant	159000	7.8
↓ precipitated with ammonium sulfate (0.1—0.4 satn.) and after standing overnight centrifuged		
Precipitate	123300	19.4
↓ dissolved in water, dialyzed against water and fractionated with Sephadex G-75		
Effluent	105000	80.0
↓ centrifuged and passed CM-cellulose column equilibrated with 0.01 M phosphate buffer at pH 5.0		
Effluent	98300	86.0
↓ centrifuged and fractionated with Sephadex G-200		
Effluent	68000	158.9
↓ centrifuged and fractionated with Sephadex G-200		
Purified lipase	60500	165.8
yield of activity: 17.9%		

Homogeneity

Electrophoretic Analysis: Homogeneity of the purified lipase preparation was tested by free boundary electrophoresis carried out under the conditions given in the legend to Fig. 3. Before using, the sample solution was dialyzed against acetate buffer (pH 3.8) or phsophate buffer (7.0), ionic strength of 0.05, at 3° for 24 hr. The patterns obtained after migration for 90 min are shown in Fig. 3. Only a single component was observed at each pH and mobility of the lipase was approximately zero at pH 3.8. The purified enzyme was subjected to polyacrylamide electrophoresis under the standard conditions described in detail by Ornstein and Davis (80—100 V/cm at 3° for 3 hr, 2 mA/tube).[15] Polyacrylamide electrophoretic patterns of the enzyme, as shown in Fig. 4, gave only a single component.

Both analyses strongly indicate homogeneity of the lipase preparation.

15) L. Ornstein, *Ann. N. Y. Acad. Sci.*, **121**, Art. 2, 321 (1964); B.J. Davis, *ibid.*, **121**, Art. 2, 404 (1964).

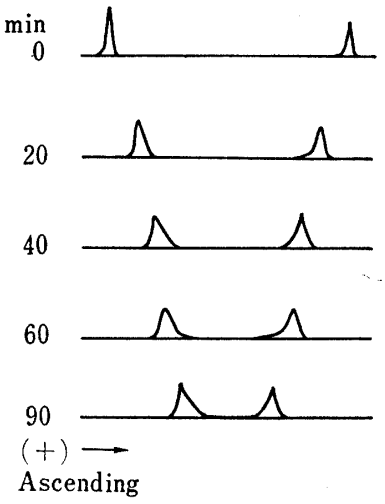


Fig. 3. Electrophoretic Patterns of Purified Lipase with a Tiselius–Type Apparatus

7mA 120 V
phosphate buffer pH 7.0
ionic strength 0.05

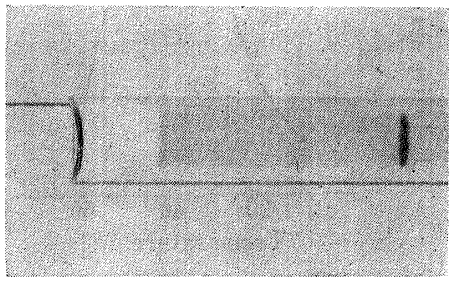


Fig. 4. Electrophoretic Patterns of Purified Lipase by Disc Electrophoresis

Tris-glycine buffer, pH 9.4 (pH 8.3-gel)
80—100 V/cm at 3° for 3 hr 2 mA/tube

Properties of Purified Enzyme

Effect of pH on Enzyme Activity and Stability: The optimum pH for the lipase activity was determined in various buffer. As shown in Fig. 5, the optimum pH was present at nearly 7.0 in the PVA system and 7.5 in the shaken system without a macromolecular emulsifier, and the enzyme exhibited a high activity between pH 6.0 and 8.0. In Fig. 6, effect of pH on the lipase stability is presented. The lipase activity in buffers (McIlvaine and Menzel buffer) was comparatively stable in the pH range from 5.0—7.0.

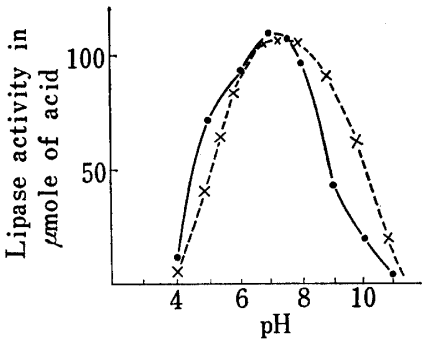


Fig. 5. Effect of pH on Activity

Buffer was as follows; pH 2.5—8.0 in PVA system: McIlvaine buffer, pH 3.0—10.0 in shaken system: barbital sodium–sodium acetate–HCl, pH 9—11: Menzel buffer.
—●—: in PVA system (method A)
---×---: in shaken system with a Monod-type shaking machine (110 oscillation/min)

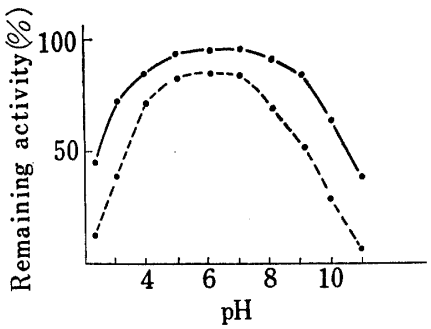
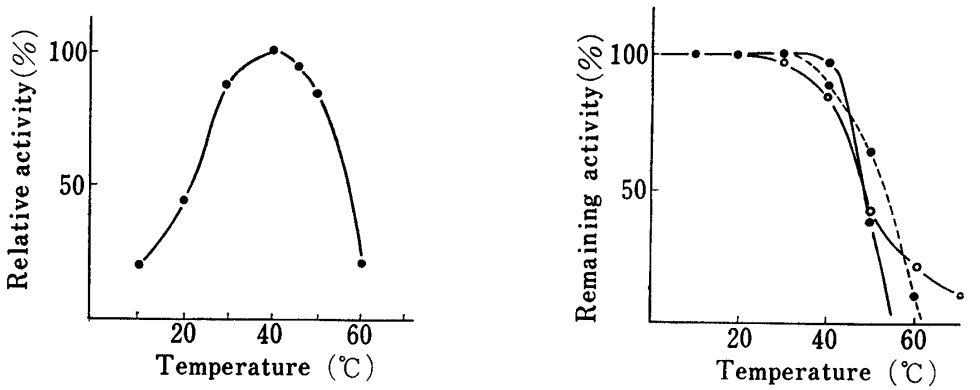


Fig. 6. Effect of pH on Stability

Equal volumes of the enzyme solution and buffer solution were mixed, after standing for 3 and 24 hr at 3° the residual activity was measured in PVA system (method A). Buffer was as follows; pH 2.5—8.0: McIlvaine buffer, pH 9—11: Menzel buffer.
—●—: after 3 hr
—○—: after 24 hr

Thermal Activity and Heat Stability: Thermal activity and heat stability were measured at pH 7.0 and 6.0, respectively, in the PVA system. As seen in Fig. 7, the optimum temperature was about 40° and the lipase was stable up to 30°, but very unstable over that temperature. It completely lost the activity at 60° within 15 min. By the addition of a very small amount of olive oil to the enzyme solution, stability of the lipase was enhanced at 40°, and by adding Ca^{2+} the heat stability increased slightly at higher temperature of 60°.



Thermal activity — Heat stability

Fig. 7. Thermal Activity and Heat Stability of Lipase

The activity was measured in PVA system at various temperature (method A).

18 ml of the enzyme solution was incubated with 50 μl of olive oil, 2 ml of 0.1M $CaCl_2$ or without them at pH 6.0 at various temperature, respectively, and the activity was measured in PVA system (method A).

---●---: enzyme alone
—●—: enzyme+olive oil
—○—: enzyme+$CaCl_2$

Effect of Metal Ions: Effect of metal ions on the lipase activity was examined. As shown in Table II, the activity was not increased by such bivalent ions as Ca^{2+} and Mg^{2+}. The enzyme was strongly inhibited by Ag^{+} and Hg^{2+}, the activity was completely lost at concentration of 5×10^{-2}M of these ions in enzyme system. Other heavy metal ions, Fe^{3+} and Cu^{2+} at 5×10^{-2}M in enzyme system, also inhibited the lipase activity. The activity of metal-inactivated enzyme was not restored by the addition of 5×10^{-2}M of EDTA.

Table II. Effect of Metal Ions on Lipase Activity

Metal ions	Residual activity (%) 5×10^{-3} a)	5×10^{-2} a)
NaCl	100	95
$CaCl_2$	96	107
$MgCl_2$	99	114
$BaCl_2$	105	95
$ZnCl_2$	101	86
$CuSO_4$	100	57
$CoCl_2$	99	95
$FeCl_3$	94	36
$AgNO_3$	39	0
$HgCl_2$	9	0

a) mole concentration of metal ions in enzyme system
Two ml of lipase solution was incubated with 2 ml of each reagent solution at pH 5—7, 37° for 15 min and after 1 ml of each mixed solution was diluted to 10 ml with cold water the activity was measured in PVA system (method A).

Effect of Various Compounds: Effect of various compounds on the lipase was examined at the concentration of 10^{-3}M in enzyme system in the pH range of 5.5—7.5. The results shown in Table III indicate that thiol reagents (*p*-chloromercuribenzoic acid and iodoacetic acid), reducing agents ($Na_2S_2O_3$ and $NaHSO_3$), metal-chelating agents (EDTA, 8-hydroxyquinoline and *o*-phenanthroline), H_2O_2, $K_3Fe(CN)_6$, NaF, formaldehyde, and urea had no effect on the lipase activity. While, N-bromosuccinimide (NBS), iodine, and sodium laurylsulfate (SLS) inhibited olive oil hydrolysis, especially NBS completely inhibited hydrolysis under these conditions. Iodine and SLS caused 28% and 26% inhibition, respectively.

Table III. Effect of Various Compounds on Lipase Activity

Compounds[a]	Activity (%)	Compounds	Activity (%)
None	100	Iodine	72
$K_3Fe(CN)_6$	101	PCMB[c]	99
$NaHSO_3$	98	Iodoacetic acid	99
$Na_2S_2O_3$	99	*n*-Bromosuccinimide	0
Sod. thioglycollate	99	NaF	101
l-Cysteine	102	Sodium lauryl sulfate	84
KCN	98	Lauric acid	104
Hydroquinone	98	Myristic acid	106
EDTA[b]	102	Formaldehyde	100
8-Hydroxyquinoline	101	Guanidine-HCl	99
o-Phenanthroline	95	Urea	100
H_2O_2	100		

a) Concentration of various compounds was 10^{-3}M in enzyme system.
b) disodium ethylenediamine tetraacetate
c) *p*-chloromercuribenzoic acid
Equal volume of the enzyme solution and various compounds solution were mixed, after standing for 15 min at 2°, 1 ml of the mixed solution was diluted to 50 ml with cold water and the activity was measured in PVA system (method B).

The apparent I_{50} values for these inhibitors were measured at 20°, and results obtained are shown in Fig. 8 and Table IV. Among the inhibitors tested, NBS was the most potent inhibitor, with I_{50} of 1.4×10^{-4}M, and the inhibition by NBS would occur at about 1×10^{-4}M in enzyme system. $AgNO_3$, whose the apparent I_{50} value was 1.5×10^{-3}M, was found to give an inhibitory effect similar to $HgCl_2$ with I_{50} of 7.1×10^{-4}M on the enzyme as shown in Fig. 8. Formaldehyde was found to exert a markedly less inhibitory effect on the enzyme, which was inhibited only by a concentration higher than 1M of formaldehyde.

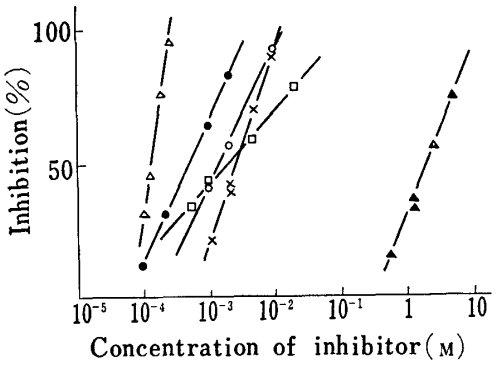


Fig. 8. Measurement of Apparent I_{50} Value of Various Inhibitors

Equal volume of the enzyme solution and inhibitor solution of appropriate concentration were mixed, after preheating at 20° for 15 min 1 ml of each mixed solution was diluted to 50 ml with cold water and the lipase activity was measured in PVA system (method B).
—○—: $AgNO_3$ —●—: $HgCl_2$
—△—: N-bromosuccinimide
—▲—: formaldehyde
—×—: sodium lauryl sulfate —□—: iodine

Table IV. I_{50} Values of Several Inhibitors

Inhibitors	I_{50} values (M)
$AgNO_3$	1.5×10^{-3}
$HgCl_2$	7.1×10^{-4}
NBS[a]	1.4×10^{-4}
SLS[b]	2.4×10^{-3}
Iodine	2.4×10^{-3}
Formaldehyde	2.6

a) *n*-bromosuccinimide
b) sodium lauryl sulfate
I_{50} values of inhibitors were measured in the same way as described for Fig. 8.

Substrate Specificity: In Table V, amount (in μmoles) of fatty acid liberated from 0.1M triglyceride emulsion for 20 min/unit is presented. Tricaprylin, followed by tricaprin and trilaurin, was hydrolyzed most efficiently, while the lipase had little or no activity on triacetin

16) I_{50} value is the inhibitor concentration causing 50% inhibition.

and tristearin . The substrate specificity of the lipase decreased in the order of tricaprylin, tricaprin, trilaurin, trimyristin, triolein, tributyrin, tricaproin, and tripalmitin in PVA emulsion.

Michaelis Constants: The Michaelis constants (Km) for olive oil and tricaprylin are shown in Fig. 9. Constant value of Km was found when the emulsion prepared under the conditions previously described was used for the measurement of Km. Km values were found to be 404±19 mg for olive oil and $8.9\pm1.4\times10^{-5}$M for tricaprylin. Km values for various triglycerides[17] by Lineweaver-Burk plot[18] are shown in Table V. Km values of tricaprin and trilaurin were $9.4\pm1.2\times10^{-5}$ and $9.8\pm1.5\times10^{-5}$M, respectively. On the other hand, the value for tristearin was very high.

The values of Km agreed closely with the substrate specificity of the lipase previously described.

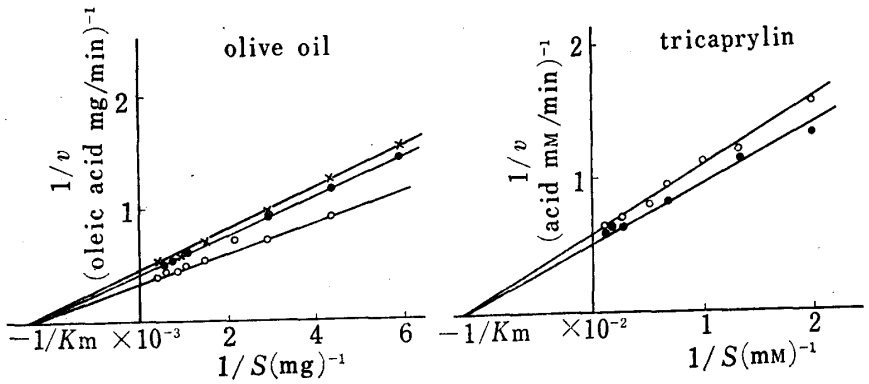


Fig. 9. Lineweaver–Burk Plots of Lipase Activity

Lipase (units)	K_m (mg)
—○—: 3.1	417
—●—: 3.4	400
—×—: 4.1	396

Lipase (units)	K_m (M)
—●—: 2.5	8.6×10^{-5}
—○—: 2.8	9.3×10^{-5}

Table V. Substrate Specificity and Michaelis Constant

Triglyceride	Activity[a]	Michaelis constant $(K_m)^{b}$ ($\times10^{-5}$M)
Triacetin	0	—
Tributyrin	4.4	17.1± 3.2
Tricaproin	4.0	19.0± 3.0
Tricaprylin	27.7	8.9± 1.4
Tricaprin	15.9	9.4± 1.2
Trilaurin	15.8	9.8± 1.5
Trimyristin	11.0	12.4± 1.1
Tripalmitin	2.1	22.8± 4.1
Tristearin	0.4	42.7±10.0
Triolein	7.8	15.6± 1.7

a) μmoles of acid liberated for 20 min/unit
b) K_m was measured by Lineweaver–Burk's method.[18] mean ± S.E. of each 4 samples
The activity was measured in PVA system (method A).

17) Concentration of triglyceride in the emulsion was 0.1M.
18) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).

Discussion

In order to study proteochemical and enzymological properties of the lipase, the enzyme was purified. For purification, the enzyme was applied on various ion exchangers, Duolite CS-101, Duolite A-2, Amberlite IRA-93, CM-cellulose, and DEAE-cellulose. The lipase was adsorbed on DEAE-cellulose and was not eluted with NaCl gradient, while it passed through cationic exchangers such as Duolite CS-101 and CM-cellulose. The enzyme becomes slowly insoluble in aqueous solution and this is probably due to association of the enzyme molecules.

Electrophoresis indicated that this purified lipase is highly pure and homogeneous. Behavior of the enzyme on electrophoresis demonstrates that the isoelectric point is about pH 3.8. This result indicates that the enzyme is an acidic protein.

The purified lipase showed a typical chromatographic pattern on a column of Bio-Gel A-5m (Bio·Rad Laboratories) equilibrated with 0.02M phosphate buffer (pH 6.5), and the content of the carbohydrate of each fraction paralleled to the protein content. Since this purified lipase contains an unknown carbohydrate, it is suggested that the enzyme is a glycoprotein like the lipase from *Candida cylindracea*.[4b]

The enzyme was found to be comparatively unstable as compared with the lipase from *Penicillium crustosum*[4c] and *Aspergillus niger*,[4a] which were stable below 45° and 50°, respectively, and the range of stable pH was considerably narrower than those from *Candida cylindracea*[4b] (pH 2.0—8.5), *Penicillium crustosum*[4c] (pH 6.0—9.0), and *Aspergillus niger*[4a] (pH 2.2—6.8).

As shown in the experiment for the substrate specificity, the lipase has high activity on medium triglyceride which is an important nutrient. Therefore, it seems that the enzyme is good for digestion of fats containing medium triglyceride.

It had been reported that if the area of the interface of the insoluble substrates was used instead of the weight for the measurement of *K*m in emulsion system, accurate *K*m values would be obtained.[19] However, when preparation of the emulsion as substrate was carried out under a constant condition as described above, almost invariable and reproducible *K*m values are easily obtained. Thus, *K*m values for triglycerides agreed closely with the substrate specificity of the lipase.

Though most of lipase was generally activated with Ca^{2+},[4c,20] the enzyme was little or not activated by it. One of the reasons will be the difference of emulsifying agents as reported previously.[21] As compared with PVA-117 emulsion, this PVA emulsion has many particles less than 2 μ that the physical state of the emulsion will not become better by the addition of the bivalent ions. Therefore, the rate of lipolysis by the lipase will be the same as that in the absence of these ions.

As shown in Table III, the results of inhibitor experiments in which thiol reagents, reducing agents, metal-chelating agents, and H_2O_2 had no influence on the lipase, indicate that sulfhydryl groups are not essential for the activity, and the inhibitory effect of NBS and iodine on the enzyme suggests that the aromatic amino acid residue, *e.g.* tyrosine, in enzyme molecule may have a significance in the activity of the lipase.

19) G. Benzonana and P. Desnuelle, *Biochim. Biophys. Acta*, **105**, 121 (1965).
20) Y. Ota and K. Yamada, *Agr. Biol. Chem.* (Tokyo), **30**, 1030 (1966).
21) M. Sugiura, T. Ogiso, Y. Naka, and Y. Nagase, *Yakuzaigaku*, **28**, 240 (1968).