Studies on Bile-sensitive Lipase. X.[1] Preparation and Properties of Carrier-bound Mucor Lipase[2]

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Mucor lipase purified was transformed into water-insoluble forms by attaching the enzyme to insoluble carriers covalently and with ionic bond, and some characteristics and kinetics behavior of DEAE-cellulose-lipase were studied in comparing to the soluble form. There was very little difference between the free and bound forms of the lipase with respect to optimum pH, optimum temperature, substrate specificity, action pattern, Km value and the energy of activation, indicating that the immobilization did not alter the substantial properties of the lipase. However, a highly increase in pH and heat stability, a decrease in inhibitory effect of n-bromosuccinimide and iodine, and of the susceptibility towards the action of some proteases were found by the immobilization. The lipolytic activity of the bound lipase was enhanced 4—6 times higher at lower concentration of bile salts, whereas the activity was inhibited at higher concentration. The bound lipase little released from the carrier during lipolysis at 37° for 12—48 hr, although the inactivation caused on it.

Water-insoluble derivatives of enzymes prepared from their soluble state have been the subject of markedly increased interest during recent years. Easy removal of insoluble enzyme prepatations from their digestion mixture without heat or other denaturing conditions and availability of insoluble preparations for the study of the kinetics of enzyme in biphasic systems provides great technical advantages. Water-insoluble derivatives of many purified enzymes may be more close to their naturally occurring forms than the corresponding soluble preparations, because many enzymes actually perform their biological missions *in vivo* in a form of attachment to insoluble cell material.

Inosoluble preparations of enzymes can readily be prepared from the soluble forms. Four different principles are used for the preparation of the insoluble derivatives: a) covalent linkage to insoluble carrier,[4] b) binding to carrier with ionic bond,[4b,5] c) physical adsorption,[6]

1) Part IX: T. Ogiso and M. Sugiura, *Chem. Pharm. Bull.* (Tokyo), **19**, 2457 (1971).
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3) Location: a) *Mitahora 492-36, Gifu*; b) *Ueno Sakuragi, Taitoku, Tokyo.*
4) a) N. Grubhofer and L. Schleith, *Hoppe-Seyler's Z. physiol. Chem.*, **297**, 108 (1954); b) H. Brandenberger, *Rev. fermentation et inds. aliment.*, **11**, 237 (1956); c) J.J. Cerva, D. Givol, H.J. Silman, and E. Katchalski, *J. Biol. Chem.*, **236**, 1720 (1961); d) M.A. Mitz and L.J. Summaria, *Nature*, **189**, 576 (1961); e) A.N. Glazer, A. Bar-Eli, and E. Katchalski, *J. Biol. Chem.*, **237**, 1832 (1962); f) A. Bar-Eli and E. Katchalski, *J. Biol. Chem.*, **238**, 1960 (1963); g) A. Rimon, M. Gutman, and S. Rimon, *Biochim. Biophys. Acta*, **73**, 301 (1963); h) E. Reisel and E. Katchalski, *J. Biol. Chem.*, **239**, 1521 (1964); i) J. Kirimura and T. Yoshida, *Jap. Pat.*, 27492 (1964); j) Y. Levin, M. Pecht. L. Goldstein and E. Katchalski, *Biochemistry*, **3**, 1905 (1964); k) A. Rimon, B. Alexander, and E. Katchalski, *Biochemistry*, **5**, 792 (1966); l) R. Axén and J. Porath, *Nature*, **210**, 367 (1966); m) R. Axén, J. Porath, and S. Ernback, *Nature*, **214**, 1302 (1967); n) R. Haynes and K.A. Walsh, *Federation Proc.*, **28**, 534 (1969); o) R. Axén and S. Ernback, *Eur. J. Biochem.*, **18**, 351 (1971).
5) a) L.B. Barnett and H.B. Bull, *Biochim. Biophys. Acta*, **36**, 244 (1959); b) M.A. Mitz and R.J. Schlueter, *J. Am. Chem. Soc.*, **81**, 4024 (1959); c) T. Tosa, T. Mori, N. Fuse, and I. Chibata, *Enzymologia*, **31**, 214 (1966); *idem, ibid.*, **31**, 225 (1966); d) T. Tosa, T. Mori, N. Fuse, and I. Chibata, *Biotech. Bioeng.*, **9**, 603 (1967); e) T. Tosa, T. Mori, N. Fuse, and I. Chibata, *Agr. Biol. Chem.* (Tokyo), **30**, 807 (1966); *idem, ibid.*, **33**, 1047 (1969); f) T. Tosa, T. Mori, N. Fuse, and I. Chibata, *Enzymologia*, **32**, 153 (1967); g) T. Tosa, T. Mori, and I. Chibata, *Agr. Biol. Chem.* (Tokyo), **33**, 1053 (1969).
6) a) A.D. McLaren, *Science*, **125**, 697 (1957); b) S. Usami and N. Taketomi, *J. Fermentation Assoc.*, **23**, 267 (1965).

d) embedding into a highly cross-linked, insoluble polymer,[7] *i.e.* polyacrylamide, and into a microcapsule prepared with polystyren, ethylcellulose and silicon derivatives.[8]

Many investigators have studied on the characteristics of the insoluble derivatives of enzymes and application of them for the continuous enzyme reaction.[4-8] However, detailed studies on the insoluble lipase preparations have been unreported.[4b,8] To study enzymological properties of the insoluble derivatives of lipase and clarify the specific properties of lipase which is capable of functioning efficiently at an oil-water interface, purified lipase from *Mucor javanicus* was transformed into insoluble derivatives by binding or coupling its native form to insoluble carriers, such as cellulose derivatives and Agarose. One of the insoluble lipase preparations, *i.e.* DEAE-cellulose-lipase, was chosen for the study on the properties because of having the highest activity. The present work deals with some characteristics and kinetics behavior of DEAE-cellulose-lipase in comparing to the soluble form of the same enzyme.

Experimental

Preparation of Enzyme——The lipase from *Mucor javanicus* was purified according to the method described in the previous paper.[9]

Materials——DEAE-cellulose (0.89 meq/g) and aminoethyl (AE)-cellulose were the products from Brown Co., Ltd., and Tokyo Kasei Kogyo Co., Ltd., respectively. Agarose (Bio-gel A-5 m, Agarose 6%) and diethyl-2-hydroxypropyl aminoethyl (QAE)-Sephadex A-25 were obtained from Bio-Rad Laboratories and Pharmacia, respectively. Polyvinyl alcohol (PVA)-117 and 210 were obtained from Kurashiki Rayon Co., Ltd. Bile salts were identical with that employed for the previous study.[10] Trypsin (2 × crystallized) and neutral protease (BNP, 1 × crystallized) from *Bacillus subtilis* were the products from Worthington Biochemical Corp. and Seikagaku Kogyo Co., Ltd., respectively.

Preparation of Insoluble Derivatives of Lipase——AE-cellulose-lipase and Agarose-lipase were prepared by the method of Habeeb[11] and of Porath,[12] respectively. DEAE-cellulose-lipase was prepared as follows: a DEAE-cellulose column (1.8 × 12 cm) was equilibrated with 0.01M phosphate buffer (pH 6.0) and to it the lipase (16000—17000 units) dissolved in the same buffer was applied. The column was successively washed with the same buffer, 300 ml of 1M NaCl and pure water at 4—5°. The resulted complex was suspended in water and lyophilized. On washing the column with 1M NaCl approximately two-third of the enzyme adsorbed on the cellulose was elutriated out. QAE-Sephadex-lipase was also prepared by the same procedure as in DEAE-cellulose-lipase.

Assay Procedure——In a L-shaped flask, the reaction mixture consisted of 1 ml of olive oil emulsion (2% PVA emulsion),[9] 1 ml of McIlvaine buffer (0.2M Na_2HPO_4 and 0.1M citric acid, pH 7.0) and 0.5 ml of additives or water was preincubated at 37° for 10 min and then 0.5 ml of the insoluble lipase preparation (0.4—0.5 unit/ml) suspended in water by vigorous stirring or free (soluble) lipase 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 (Toyo Kagaku, M-100). After 20 min, the reaction was stopped by the addition of 5 ml of a solution consisting of 80 ml of iso-PrOH, 20 ml of *n*-heptane and 2 ml of 2N H_2SO_4. The liberated fatty acids were extracted by the method of Dole[13] and titrated with 0.01N ethanolic KOH solution, using thymol blue as an indicator.

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.

Estimation of Enzyme Concentration in Insoluble Complex——Approximately 200 mg of insoluble enzyme preparation was hydrolyzed in tridistilled HCl for 24 hr at 105° in closed vessels. The tyrosine content in the mixture neutralized with 10N NaOH solution was determined by spectrophotometry.[14] Enzyme concentration in insoluble complex was calculated on a base of tyrosine content of free enzyme (9.71 μmole tyrosine per 100 mg of enzyme). As blanks for determination of enzyme content, 200 mg of

7) *a*) P. Bernfeld and J. Wan, *Science*, **142**, 678 (1963); *b*) P. Bernfeld, R.E. Bieber, and P.C. MacDonnell, *Federation Proc.*, **27**, 782 (1968); *c*) P. Bernfeld, R.E. Bieber, and D.M. Watson, *Biochim. Biophys. Acta*, **191**, 570 (1969).
8) M. Kitajima, S. Miyano, and A. Kondo, *Kogyo Kagaku Zasshi*, **72**, 493 (1969).
9) T. Ogiso and M. Sugiura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1025 (1969).
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11) A.F.S.A. Habeeb, *Arch. Biochem. Biophys.*, **119**, 264 (1967).
12) J. Porath, R. Axén, and Ernback, *Nature*, **215**, 1492 (1967).
13) V.P. Dole, *J. Clin. Invest.*, **35**, 150 (1956).
14) S. Udenfriend and J.R. Cooper, *J. Biol. Chem.*, **196**, 227 (1952).

each insoluble carrier was hydrolyzed in the same conditions. A part of the estimation was done by the method of Lowry et al.[15] after liberating enzyme from the carrier with 0.2N NaOH solution.

Thin-Layer Chromatography (TLC)——TLC was done with the same method as described in previous paper.[1]

Result

Activity and Enzyme Content of Insoluble Lipase Preparations

In order to obtained the insoluble derivatives of the lipase with high activity, two different method, attaching the free enzyme to insoluble carriers covalently and with ionic bond, were used. The enzyme content, activity towards olive oil emulsion and specific activity of the insoluble preparations obtained are shown in Table I. The enzyme content of all the preparations was very low, only below 2.6%. In AE-cellulose-lipase and Agarose-lipase prepared by covalent fixation, only 1.4 and 2.3% of total weight consisted of enzyme, respectively, and that in almost inactive form. On the other hand, QAE-Sephadex-lipase obtained

TABLE I. Activity and Enzyme Content of Water-insoluble Lipase Preparations

Derivative	Activity (units/g of prep.)	% enzyme by wt.	Specific activity (units/mg of enzyme)
Free (soluble)	331600	100	331.6
AE-cellulose-lipase[a]	1.2	2.3	0.05
Agarose-lipase[b]	24.1	1.4	1.7
DEAE-cellulose-lipase[c]	250—285	2.2—2.6	11.4—11.0
QAE-Sephadex-lipase	5.9	1.3	2.4

a) aminoethyl-cellulose b) Bio-gel A-5m (Agarose 6%)
c) diethylaminoethyl-cellulose d) diethyl-2-hydroxypropyl-aminoethyl-sephadex
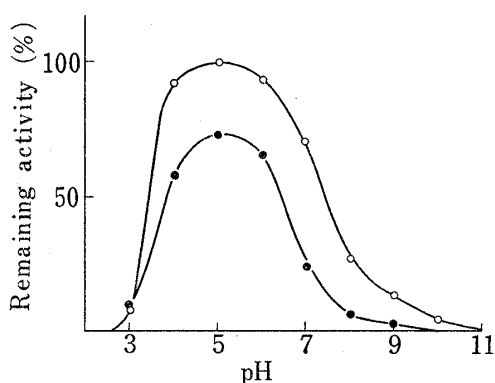

Fig. 1. Effect of pH on Stability of Free and DEAE-cellulose-lipase

Equal volume of the enzyme mixture (6 units/ml) and buffer solution (pH 2.5—8: McIlvaine buffer, pH 9—11: Menzel buffer ($0.05M$ Na_2CO_3 and $0.1M$ $NaHCO_3$)) were mixed, after standing at 37° for 1 hr, the mixture was diluted to 10-fold with McIlvaine buffer, pH 7.0, and assayed.
—●—: free
—○—: DEAE-cellulose-lipase

by means of ionic bond also showed low activity. DEAE-cellulose-lipase had the highest activity out of four insoluble lipase preparations, however, the specific activity (units/mg of enzyme) was approximately one-thirtieth of free lipase, indicating that most of lipase lost the activity on converting into insoluble preparations and washing with buffer or 1M NaCl. The extremely low enzyme contents in AE-cellulose-lipase and Agarose-lipase may be due to using a low concentration of enzyme solution, approximately 0.5 %, for the preparation of the insoluble complexes. Consequently, DEAE-cellulose-lipase, having the highest activity, was chosen for the following experiments.

Properties of DEAE-cellulose-Lipase

Effect of pH on Enzyme Activity and Stability——The optimum pH for DEAE-cellulose-lipase activity was determined at various pH values. As the result, the optimum pH was present at nearly 7.0 and the pH curves were identical with free lipase.[9] The stability of the bound lipase to pH in the range from pH 2.5 to 11 was com-

15) O.H. Lowry, N.J. Rosenbrough, A.L. Farr, and R.T. Randall, J. Biol. Chem., 193, 265 (1951).

pared with that of free enzyme at 37°. As shown in Fig. 1, the bound lipase was more stable than free lipase in the pH range from 4 to 8.

Thermal Activity and Heat Stability——The optimum temperature of DEAE-cellulose-lipase was at 37—40° and agreed with that of free lipase.[9] However, heat stability of the bound lipase was enhanced at relatively higher temperature as shown in Table II. The velocity constants (k) of heat denature for the bound lipase markedly reduced, 7.05×10^{-2} (min^{-1}) for free lipase to 1.21×10^{-2} for DEAE-cellulose-lipase at 50°, indicating that heat stability of the lipase was enhanced by conversion into the bound form.

TABLE II. Process and Velocity Constants of Heat-denaturation of Free and DEAE-cellulose-lipase

Condition		Free lipase		DEAE-cellulose-lipase	
Temperature	Time (min)	Residual activity (%)	Velocity constant (min^{-1}, $\times 10^{-2}$)	Residual activity (%)	Velocity constant (min^{-1}, $\times 10^{-2}$)
40°	10	68.4	1.44	99.8	0.24
	15	64.0		96.5	
	20	58.8		95.8	
	25	55.2		93.9	
50°	10	42.9	7.05	90.7	1.21
	15	35.4		83.2	
	20	31.6		75.4	
	25	26.9		68.3	
60°	10	20.1	14.28	53.8	5.44
	15	15.4		44.6	
	20	12.4		38.9	

0.5 ml (0.3 unit) of enzyme mixture containing McIlvaine buffer, pH 5.0, was incubated for 10—25 min at various temperature. To the mixture 2 ml of olive oil emulsion diluted 2-fold with McIlvaine buffer, pH 7.0, preheated at 37°, was added and assayed.

Effect of Metal Ions and Inhibitors——
The effect of metal ions on lipolysis by the bound lipase was investigated at the concentration of 10^{-3}M and 10^{-2}M under the standard assay condition. DEAE-cellulose-lipase was inhibited by heavy metal ions as well as the free form. On the other hand, the effect of n-bromosuccinimide (NBS) and iodine, which were known as the inhibitor,[9] on the bound lipase activity was studied at about pH 6. The results are shown in Fig. 2. It was found that the inhibitory effect of the chemicals on DEAE-cellulose-lipase was less as compared with that on free lipase. This suggests that the immobilized lipase will be protected from some conformational changes in the protein structure that may occur by modification with the chemicals. The view is strongly supported by the result that the inhibitory effect by Fe^{3+} was the same for both free and bound forms of the enzyme as shown in Fig. 2.
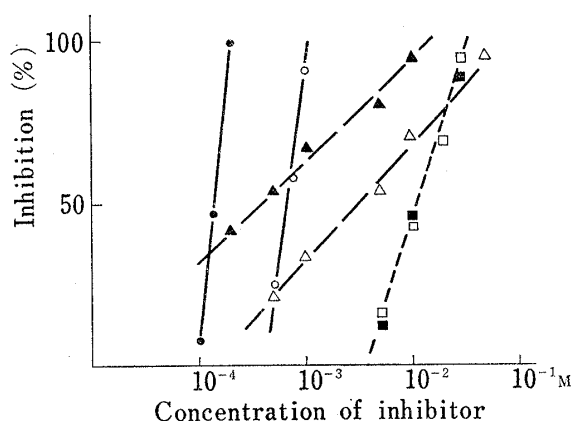

Fig. 2. Effect of Various Inhibitors on Free and DEAE-cellulose-lipase Activity

Equal volume of the enzyme mixture (9.6 units/ml) and inhibitor solution of appropriate concentration were mixed, after preheating at 20° for 15 min 1 ml of the mixed solution was diluted to 8 ml with water and assayed. Open symbols represent activity of DEAE-cellulose-lipase, and closed symbols free lipase.

○,●: n-bromosuccinimide △,▲: iodine □,■: FeCl$_3$

Substrate Specificity and Action Pattern——Substrate specificity and action pattern of the bound lipase were studied on triglycerides with a chain length of 6—18 carbons and olive oil, respectively. The distribution rate of fatty acids with the chain length below 12 carbons to n-heptane and iso-PrOH mixture (1:4) was determined, using individual free acid and the results obtained were used for the correction for the amount of acids. As the result, the substrate specificity of the bound lipase was the same as the soluble form.[1] The action pattern of lipolysis of olive oil by the bound form, studied by TLC technique, was also quite the same as by free lipase.[1]

Effect of Bile Salts on Lipase Activity——As shown in previous paper,[10] free lipase activity was highly accelerated by various bile salts. It will be interesting to know whether the accelerating effect changes or not by immobilization of the lipase. Accordingly, the effect of bile salts on DEAE-cellulose-lipase activity was tested in concentrations verying from 0.05 to 4%. The results are shown in Fig. 3. There was a marked increase in lipolysis at lower concentration of all the salts, however, inhibition at higher concentration. The lipolytic action of the bound lipase in the presence of lower concentration of bile salts, such as glyco-cholate, taurocholate and cholate, was enhanced 4—6 times higher than that in their absence, whereas free lipase was accelerated 1.8—2.2 times higher under the same conditions.[10] The highly acceleration of lipolytic activity of the bound lipase may be due to increasing amount of the molecules adsorbed at oil-water interface of the substrate with the aid of electrostatic binding of the carrier to oil particles having a weak negative charge. An inhibitory effect above 1% of bile salts may be ascribed to release of the enzyme molecules, contained inactive form, from DEAE-cellulose. To clarify the expectation, the release of the enzyme from the cellulose and binding of bile acid to it were studied. As shown in Fig. 4, the levels of lipase molecules released and bile acid bound to DEAE-cellulose increased with rising concentration of taurocholate, and reached to the maximum release at approximately 1% of the salt. The activity of lipase released from the carrier, assayed in the presence of 1.3% tauro-cholate, was approximately 40% as active as the insoluble lipase preparation. Since the
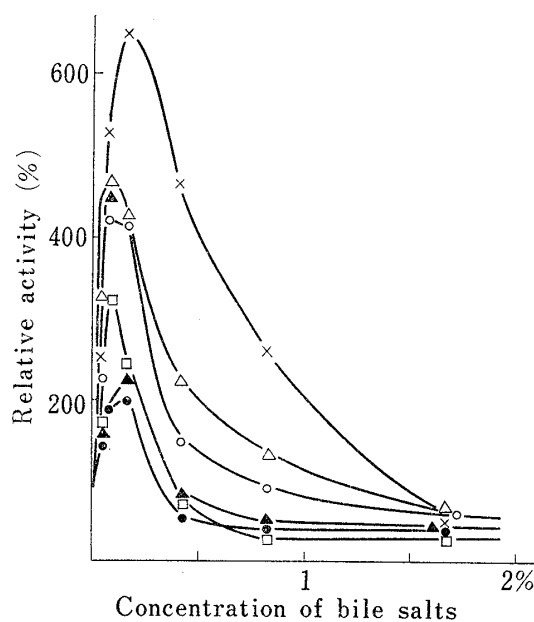

Fig. 3. Effect of Various Bile Salts on DEAE-cellulose-lipase Activity

—●—: sod. ursodeoxycholate
—○—: sod. deoxycholate
—△—: sod. cholate
—▲—: sod. taurocholate
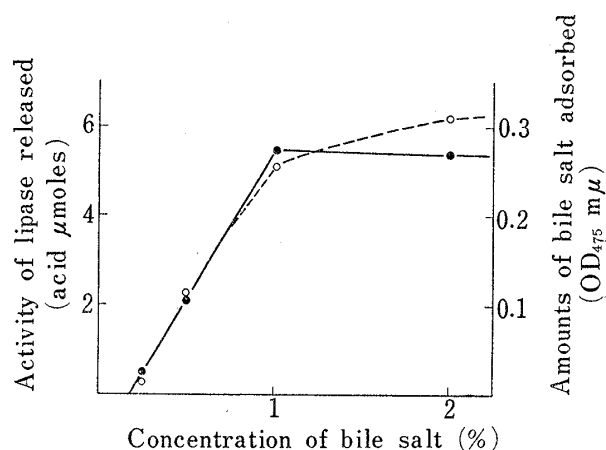—×—: sod. glycocholate
—□—: sod. glycodeoxycholate

Fig. 4. Correlation between Activity of Enzyme Released from DEAE-cellulose-lipase by Addition of V Bile Salt and Amounts of the Salt Adsorbed at the Cellulose

A mixture consisted of 1 ml of McIlvaine buffer (pH 7.0), 0.5 ml of sod. taurocholate solution, 0.5 ml of water and 20 mg of DEAE-cellulose-lipase was incubated at 37° for 10 min and filtered. Activity of the filtrate was assayed in the presence of 1.33 % taurocholate. To a quantity of the residue lyophylized 2 ml of 0.2 n NaOH was added and filtered. The amounts of bile salt in the filtrate were determined.

—●—: activity of lipase released from DEAE-cellulose-lipase
---○---: sod. taurocholate adsorbed at DEAE-cellulose

activity was enhanced twice in the concentration of the salt,[10] about 20% of the lipase bound to DEAE-cellulose appears to be active enzyme. Therefore, it was suggested that in lower concentration of bile salts the activity of the active bound lipase was enhanced 20—30 times higher than the free form.

Action of Protease Towards Bound Lipase——In order to know the action of protease to DEAE-cellulose-lipase, the bound lipase was incubated separately with trypsin or Bacillus Neutral Protease (BNP). At various time intervals the reaction mixture was diluted with water and assayed. As shown in Fig. 5, the bound lipase was more resistant than free enzyme towards action of proteases, especially BNP. The bound lipase remained 63% of the initial activity after incubation with BNP for 60 min, while free lipase was only 26%. A distinct difference was found in remaining activity of both forms of the enzyme, although the susceptibility towards trypsin was less than towards BNP. Therefore, it is supposed that the immobilized molecules where the conformation will be relatively fixed may become unsusceptible towards the action of proteases.
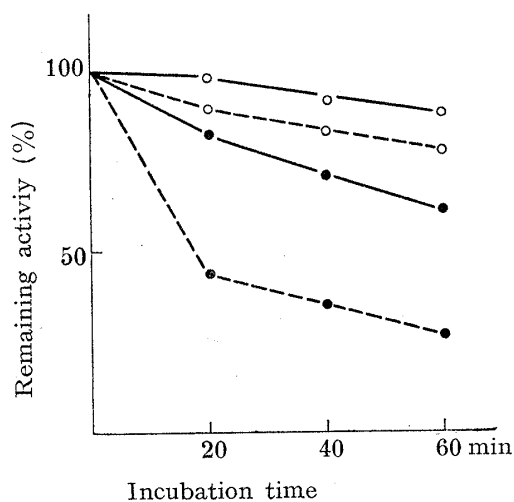
Fig. 5. Action of Proteases towards DEAE-cellulose-lipase

A mixture contained each 1 ml of enzyme solution (or suspension, pH 6.0) and 1 ml of protease solution (0.093 unit of enzyme solution (or suspension, pH 6.0) and 1 ml of protease solution (0.093 unit of trypsin or 0.103 unit of BNP/ml) was incubated at 37° for various time, rapidly cooled, diluted to 4 ml with water and assayed. Solid lines and broken lines represent the action of trypsin and BNP to lipase activity, respectively.
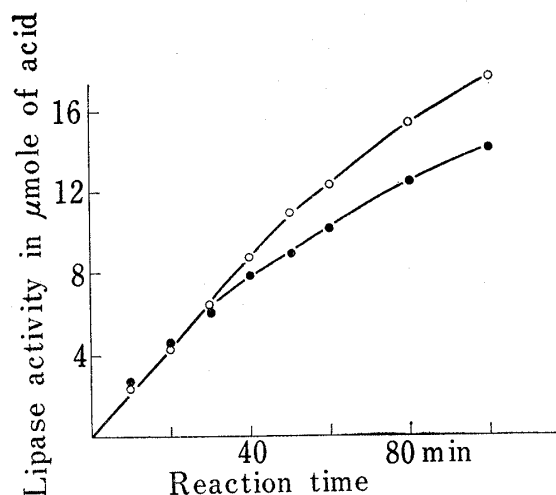●: free   ○: DEAE-cellulose-lipase

Fig. 6. Continuous Enzyme Reaction by Free and DEAE-cellulose-lipase on Olive Oil Emulsion

—●—: free   —○—: DEAE-cellulose-lipase
0.22 unit of lipase was used, respectively.

Lipolysis Curve Michaelis Constant and Activation Energy——Lipolysis by both forms of the enzyme was measured, using 0.22 unit of each lipase. The rate of lipolysis by free lipase decreased more rapidly than by the bound enzyme during incubation time from 40 to 100 min as shown in Fig. 6. Lineweaver-Burk plots of both the lipase activities are shown in Fig. 7. Michaelis constant (K_m) for olive oil was found to be 24.2 and 26.6 (mg/ml) for the free and bound lipase, respectively. Hence, K_m values characterizing the dissociation constants of the enzyme-substrate complex were the same for both forms. Arrhenius plots of both forms of the lipase gave straight lines of almost the same slope, as shown in Fig. 8. The activation energies, calculated from the slope of these lines, were approximately 6360 and 5630 cal/mole for the free and DEAE-cellulose-lipase, respectively. Although the immobilization of the lipase resulted in 11.5% decrease of the activation energy, a remarkable difference in kinetic behavior between the two forms of the lipase was not recognized.
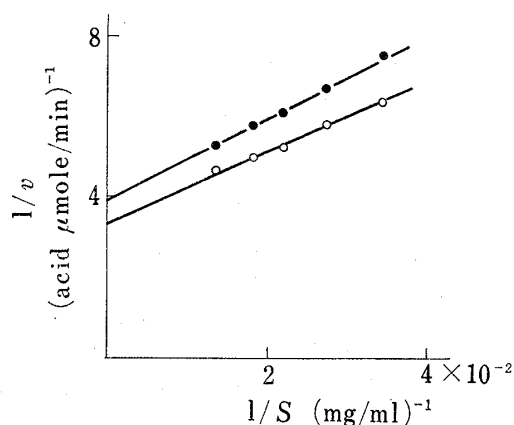
Fig. 7. Lineweaver-Burk Plots of Free and DEAE-cellulose-lipase Activity

0.24 unit of lipase was used, respectively. Experimental details are given in the text.
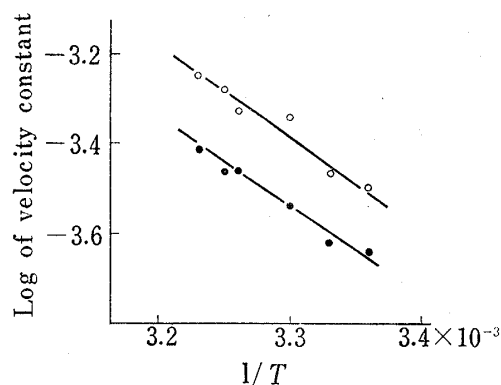—●—: free —○—: DEAE-cellulose-lipase

Fig. 8. Temperature Dependence of Velocity Constants

—●—: free —○—: DEAE-cellulose-lipase

Liberation of Bound Lipase——To study leakage of the lipase from DEAE-cellulose-lipase and inactivation of the enzyme during the operation, 1 liter of 0.1M phosphate buffer (pH 4.6, 7, or 8) was constantly fed into a DEAE-cellulose-lipase column (0.6×3 cm). The rate of elution was maintained at 60 ml/hr, the column was washed with cold water, lyophilized and the lipolytic activity was assayed. The results obtained are shown in Table III. The remaining activity of the bound lipase treated with an acidic buffer was higher than that with an alkaline buffer (pH 8). To clarify the reason for the decrease of lipolytic activity, enzyme content and activity of the insoluble derivative treated with 200—1000 ml of 0.1M phosphate buffer (pH 7.0) by the method as mentioned above were determined. As the result, the activity of the bound lipase decreased with increase of volume of the buffer passed, however, the enzyme contents were approximately 2.5—2.6%, which were the same values as the bound lipase untreated. This indicates no leakage of the enzyme from the carrier but inactivation on it.

Table III. Effect of Washing DEAE-cellulose-lipase Column with Buffers at Several pH

pH of buffer	Residual activity (%)
control	100.0
4.6	80.5
7	71.4
8	62.3

One liter of 0.1M phosphate buffer at each pH was passed through the DEAE-cellulose-lipase column (0.6 × 3 cm) at 20°, washed with water, lyophilized and assayed for the residual activity.

Table IV. Enzyme Content and Remaining Activity of DEAE-cellulose-lipase After Lipolytic Action

Reaction time (hr)	Enzyme content (%)	Remaining activity (%)
0	2.5	100
12	2.4	18
24	2.5	6
36	2.3	0
48	2.2	0

To a mixture consisted of each 2 ml of olive oil emulsion and McIlvaine buffer (pH 7.0) 30 mg (7.5 units) of DEAE-cellulose-lipase was added and incubated for indicated time at 37°. The reaction was stopped by the addition of 5 ml of a mixture of EtOH and acetone (1:1) and filtrated. The residue obtained was successively washed with H_2O, EtOH: acetone (1:1) and ether, and lyophilized. The enyme content in the residue was determined by the method of Lowry, *et al.*

On the other hand, leakage of the lipase from the cellulose during lipolysis was measured using the experimental conditions described in the legend of Table IV. The result also showed

a little leakage of the enzyme from the insoluble complex, although 80% and almost perfect inactivation of the lipase on the carrier was found after 12 and 24 hr, respectively.

On incubating with various buffer solution in the pH range from 2.3 to 12.9 (pH 2.3—8: McIlvaine buffer, pH 9—11: Menzel buffer, pH 12—12.9: glycine-NaOH buffer) at 37°, no leakage of the enzyme was on pH over a wide range from 2.3 to 9, providing that release of the bound lipase from the carrier does not occur at optimum pH, 7.0.

Discussion

Water-insoluble enzyme preparations have been studied extensively by many investigators,[4-8] however, little is known about insoluble lipase preparations.[4b,8] Therefore, it was unclear whether the enzymatic properties of lipase are changed or not, and whether great technical advantages are provided by the immobilization of lipase. To clarify the question, the insoluble derivatives of lipase were prepared by means of covalent bond and ionic bond, using *Mucor* lipase purified. DEAE-cellulose-lipase obtained by linking the enzyme to the cellulose was found to possess the highest activity. In addition to ionic bond, the other factor is considered to be responsible for the immobilization of the lipase. A possible interpretation is that the lipase molecules themselves became insoluble on the carrier, because the lipase was retained on it even after passing with voluminous and high concentration of NaCl solution and was not liberated from the carrier in the pH range from 2.3 to 9. The view may be supported by the fact that the enzyme lyophilized was almost insoluble in water or buffer solutions (pH 2—9), whereas it was soluble in NaOH solution. This property of the lipase may be favorable in immobilization of the enzyme. The low specific activity was probably due to partial denaturation of the lipase bound to carriers and oil particles as substrate being out of contact with the lipase molecules lie in indentations within the folds of the carriers. It was reported that the lipase embedded in ethylcellulose microcapsules did not act the ester containing longer chain, such as olive oil and tween 20, at all,[8] and rabbit muscle aldolase immobilized in a highly cross-linked synthetic polymer exhibited the activity in the vicinity of the surface of the carrier.[16] The reason why DEAE-cellulose-lipase exhibited higher activity than QAE-Sephadex-lipase is considered as follows. A cellulose swells by adsorption of water and even large molecules such as *Mucor* lipase, of which molecular weight is above 200000,[17] may be able to enter into the ionic exchanger,[18] while the lipase may not get into Sephadex A-25 which is a highly closs-linked carrier.[18] Accordingly, the lipase molecules might be bound to DEAE-cellulose more than to QAE-Sephadex (Table I).

As DEAE-cellulose-lipase exhibited the highest activity of four insoluble derivatives, the properties of the lipase preparation were studied. There was very little difference between the free and bound forms of the lipase with respect to optimum pH, optimum temperature, substrate specificity, action pattern, K_m value and the energy of activation (Fig. 7 and 8). This fact suggests that the immobilization did not alter the substantial properties of the enzyme. A significant difference between both forms, however, was detected on pH stability, heat stability and the effect of inhibitors, such as NBS and iodine, and some proteases (Fig. 1, 2, 5, and Table II). The increase in pH and heat stability by immobilization of the lipase may result in preventing the enzyme from some conformational changes in the protein structure, which result from the variation of environment, e.g. pH and temperature, owing to the fixation of the enzyme on a insoluble carrier. The decrease of the susceptibility of the insoluble derivative towards the action of some proteases and of inhibitory effect of NBS and iodine

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17) T. Ogiso and M. Sugiura, "89th Meeting of the Pharmaceutical Society of Japan," Hokkaido, 1969.
18) G. Funazu, *Kagaku To Seibutsu*, **7**, 292 (1970).

is also readily explained by the same argument as mentioned above. The other difference in the two forms of this enzyme was that the activity of the bound lipase was remarkably enhanced in lower concentration of bile salts (Fig. 3). The reason why the lipolytic activity was accelerated is considered as follows; 1) the lipase molecules diminished their negative charge by binding to DEAE-cellulose are adsorbed at oil-water interface more than free lipase, 2) the electrostatic binding of the carrier to oil particles having weak negative charge may cause adsorption of the enzyme to the particles, the adsorption is the first step of lipolysis.[19] On the other hand, the inhibition of lipolytic action in the presence of high concentration of bile salts is found to depend on spliting the complex between the enzyme and DEAE-cellulose, binding the salts as a substitute for the enzyme to the cellulose and releasing partially inactivated lipase (Fig. 4). The slow decrease in initial velocity of lipolysis by the bound lipase as compared with that of free lipase may be due to protection of the enzyme from the inhibition by long chain fatty acids formed during lipolysis,[20] owing to binding of the acids to DEAE-cellulose.

The study on liberation of bound lipase by continuous passing of buffer (Table III) and during lipolysis (Table IV) indicated that the bound enzyme little released from the carrier. This, with an increase in pH and heat stability and protective effect by the immobilization as described above, may be of great advantage to applying the preparation for the continuous enzyme reaction, since in many insoluble derivatives of enzyme the liberation of enzyme from the carrier is known to be one of the weak points; ribonuclease bound to Dowex-2 was liberated from the carrier by water[5a] and amylase bound to acid clay was liberated from it during the hydrolytic reaction of starch.[6b] However, one of the defects of the bound lipase is to become inactive on the carrier during lipolysis for relatively long time at pH 7, 37° (Table IV), because of the enzyme was unstable at the temperature or above.[9]

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20) T. Ogiso and M. Sugiura, *Chem. Pharm. Bull.* (Tokyo), **18**, 663 (1970).