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The Lipase-Catalyzed Reaction of *n*-Alkyl *n*-Butyrates with Methanol[1]

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In order to reveal a difference between the enzyme mechanisms of lipase and esterase, lipase-catalyzed methanolysis was compared with esterase-catalyzed methanolysis.

1) Purified pancreatic lipase and a mold, *Mucor* lipase were found to catalyze methanolysis of *n*-alkyl *n*-butyrates and tri-*n*-butyrin in heterogeneous assay system.

2) The same partitioning ratio (A/H), irrespective of the substrate leaving group, was obtained by our experiments. This indicates formation of a common intermediate during solvolysis. It is reasonable to postulate that this intermediate is an acyl enzyme.

3) Kinetic parameters for lipase and esterase were estimated and compared with each other. Acyl-esterase was more susceptible to nucleophilic attack by methanol than acyl-lipase.

As a rule, hydrolytic enzymes catalyze not only hydrolysis but also solvolysis. For example, the enzyme-catalyzed solvolysis by aliphatic alcohols and amines had been reported in the cases of liver esterase,[3,4] β-galactosidase[5] and 5-hydroxy-N-methylpyroglutamate synthetase.[6] Each solvolysis had been examined in order to confirm the presence of a common intermediate involved in the enzyme reaction, respectively. The efficiency of pancreatic lipase [EC 3.1.1.3] and probably of other lipolytic enzymes are greatly increased by a limited conformational change resulting from adsorption at a hydrophobic surfaces.[7] Therefore, the substrate in an emulsified or micellar form has been employed in the quantitative evaluation of each lipase. Recently, crude enzyme preparations of pancreatic lipase and a mold, *Mucor* lipase were found to catalyze alcoholysis in such a heterogeneous assay system.[8] In this report, lipase-catalyzed alcoholysis was compared with esterase-catalyzed alcoholysis in order to reveal a difference between the enzyme mechanisms of lipase and esterase.

Materials and Methods

Preparation of Enzymes——The crude lipase preparation from *Mucor javanicus* IAM-6108, which had been prepared by ethanol precipitation of the cultural broth filtrate, was kindly supplied from Amano Pharmaceutical Co., Ltd. (Nagoya). *Mucor* lipase was purified according to the method described in the previous report.[9] Porcine pancreatic lipase was purified by the procedures of Garner and Smith[10] with a minor modification. This lipase was pure as judged by disc electrophoresis (two bands, lipase A and B). *Rhizopus delemar* lipase (pure grade) was obtained from Seikagaku Kogyo Co., Ltd. The enzyme preparation of pig liver esterase was identical with that employed for the previous study.[8a]

Other Materials——Methyl, ethyl, *n*-propyl, *n*-butyl, *n*-amyl esters of *n*-butyric acid and tri-*n*-butyrin in the reagent grade were obtained from Tokyo Kasei Kogyo Co., Ltd. Diisopropyl fluorophosphate (DFP)

1) Presented at the 95th Annual Meeting of Pharmaceutical Society of Japan, Nishinomiya, April 1975.
2) Location: *3-1 Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
3) P. Greenzaid and W.P. Jencks. *Biochemistry*, **10**, 1210 (1971).
4) D. Wynne and Y. Shalitin, *Eur. J. Biochem.*, **31**, 554 (1972).
5) T.M. Stokes and I.B. Wilson, *Biochemistry*, **11**, 1061 (1972).
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7) P. Desnuelle, *Adv. Enzymol.*, **23**, 129 (1961).
8) *a*) H. Ishihara, H. Ikezawa, and S. Tejima, *Chem. Pharm. Bull.* (Tokyo), **22**, 413 (1974); *b*) Presented partly at the Annual Meeting of the Japanese Biochemical Society, Tokyo, November 1972.
9) H. Ishihara, H. Ikezawa, and S. Tejima, *Biochim. Biophys. Acta*, **388**, 413 (1975).
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and sodium deoxycholate (SDC) were obtained from Katayama Chemical Industries Co., Ltd. N-Bromosuccinimide (NBS) was obtained from Wako Pure Chemical Industries, Ltd. Sephadex G-75, G-100, G-200 and CM-Sephadex C-50 were the products of Pharmacia Fine Chemicals. Diethylaminoethyl (DEAE)-Cellulose was the product of Brown Co., Ltd.

Assay Procedure——Unless otherwise stated, the enzyme assays were carried out at 30°.

Method 1) Assay Method using pH-Stat——Throughout the purification procedures, lipase activity was determined by the method of Garner and Smith.

Method 2) Assay Method using Gas-Liquid Chromatography (GLC)——When *n*-alkyl *n*-butyrates were used as substrate, enzyme activities were determined by the method reported previously.[8a] The reaction mixture consisted of 1.8 ml of 0.12M phosphate buffer (pH 7.4)-2.1 mM SDC, 0.1 ml of enzyme solution and 0.5 ml of 69% methanolic solution of *n*-alkyl *n*-butyrate (100 μmoles). After reaction with enzyme for 10 min, the rate of transformation of substrate (termed as esterase activity) and that of formation of methyl *n*-butyrate were determined by GLC. For determination of methyl *n*-butyrate, sensitivity was raised (sensitivity=10^3, range=0.4). In the present work, 0.02% *o*-xylene in *n*-hexane was used as the internal standard.

Method 3) Assay Method using pH-Stat and GLC——In the studies on the properties of the purified lipase and esterase, the hydrolytic activity was determined titrimetrically with a pH-stat (Radiometer, Copenhagen) with magnetic stirring at pH 7.4. Standard 0.02N NaOH was used to titrate the released protons. The reaction mixture consisted of 1.8 ml of *n*-propyl *n*-butyrate or tri-*n*-butyrin emulsion, 0.5 ml of 0—100% methanol and 0.1 ml of enzyme solution. Unless otherwise stated, *n*-propyl *n*-butyrate or tri-*n*-butyrin emulsion was prepared by sonication of 1.4 ml of each ester in 34.6 ml of 0.15M NaCl-0.1% SDC, with a Tomy sonifier (100 W, Tomy Seiko Co., Ltd. UR-200P) for 3 min. After a definite time of monitoring the continuous consumption of 0.02N NaOH, the reaction was stopped by addition of 0.5 ml of 0.1% NBS. The contents of the glass titration vessel was transferred immediately to a glass-stoppered test tube containing 2 ml of 0.02% *o*-xylene–*n*-hexane. The tube was then well shaken for 3 min and allowed to stand for a short period. Five μl of the resulting *n*-hexane layer was injected into the GLC (conditions were identical with that employed for Method 2)). Thus, the alcoholytic activity, *i.e.*, the rate for methyl *n*-butyrate formation was determined. Calibration curve for methyl *n*-butyrate was linear from 0.5 μmole up to 5 μmoles per the reaction mixture. The actual recovery of methyl *n*-butyrate was more than 76%. The standard deviation was less than 9.3%.

Unit and Specific Activity——Under the conditions tested, one unit of hydrolytic activity (lipase or esterase) was defined as the amount of enzyme which liberated 1 μequiv. of H^+ from the substrate, while one unit of the esteratic or methanolytic activity was expressed as the amount of enzyme which transformed 1 μmole of *n*-propyl *n*-butyrate or formed 1 μmole of methyl *n*-butyrate. The specific activities were expressed in terms of units per mg protein. Protein concentration was determined by measuring the absorbance at 280 nm.

Results and Discussion

Parallelism of Lipolytic and Methanolytic Activities during the Purification Procedures

Tables I and II show the activities of lipase, esterase and formation of methyl *n*-butyrate in each step of the purification procedures of pancreatic lipase and *Mucor* lipase, respectively. Throughout the overall purification steps, the activity ratio of lipase to esterase was about 500—600: 1 (pancreatic lipase) or about 70—100: 1 (*Mucor* lipase), while that of esterase to formation of methyl *n*-butyrate was 100: 9—11 (pancreatic lipase) or 100: 6 (*Mucor* lipase). When *n*-butyric acid instead of *n*-propyl *n*-butyrate was added as the substrate to the reaction mixture, no appreciable amount of methyl *n*-butyrate was formed. From these data, it is concluded that formation of methyl *n*-butyrate was due to lipase-catalyzed methanolysis, not to the synthetic action (methanol+*n*-butyric acid→methyl *n*-butyrate) of each lipase.

Inactivation by Heating and DFP

In order to obtain exact information on the relationship between lipolytic and methanolytic activities of purified lipase preparation, the changes in two activities were compared after inactivation of the enzymes by heat treatment. As shown in Fig. 1, parallel loss of lipolytic and methanolytic activities was observed. When the purified lipase preparations were treated with DFP according to Maylié, *et al.*,[11] parallel loss of both activities was also observed.

11) M.F. Maylié, M. Charles, and P. Desnuelle, *Biochim. Biophys. Acta*, **178**, 196 (1969).

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Table I. Lipolytic and Methanolytic Activities of Pancreatic Lipase in 3.5M Methanol throughout the Overall Purification Steps

Step	Specific activity			$\frac{(F)}{(E)} \times 100$
	Lipase (L)[a]	Esterase (E)[b]	Formation (F)[c]	
Aqueous extract	30	0.050	0.0055	11
$(NH_4)_2SO_4$ fraction	66	0.11	0.0095	9
DEAE-Cellulose	1000	2.1	0.23	11
Sephadex G-100	2900	5.6	0.62	11
CM-Sephadex C-50	4300	8.2	0.74	9

a) determined by Method 1)
b) determined by Method 2), substrate: *n*-propyl *n*-butyrate
c) methyl *n*-butyrate, determined by Method 2)

Table II. Lipolytic and Methanolytic Activities of *Mucor* Lipase in 3.5M Methanol throughout the Overall Purification Steps

Step	Specific activity			$\frac{(F)}{(E)} \times 100$
	Lipase (L)[a]	Esterase (E)[b]	Formation (F)[c]	
Original powder	21	0.29	0.017	6
Aqueous extract	30	0.41	0.024	6
pH 4.5 fraction	110	1.2	0.071	6
Sephadex G-200	440	4.3	0.26	6
Sephadex G-75	3800	41	2.4	6

a), *b*) and *c*) are the same as shown in Table I.

In 3.5M methanol, about 50% inactivation was attained by a 60 min-incubation of the pancreatic lipase with 50 mM DFP at pH 8.4, and by a 15 min-incubation of the *Mucor* lipase with 8 mM DFP at pH 7.4. On the other hand, 1 mM of DFP completely and immediately inactivated liver esterase activity in 3.5M methanol, pH 7.4. Recently, from the view of sterical hindrance, Brockerhoff[12] has explained the fact that pancreatic lipase is insensitive to concentrated DFP solution. Therefore, it is not possible to know whether the inactivation by DFP results from binding DFP to reactive amino acid in the molecule of lipases or from non-specific inactivation caused by an impurity[13] in DFP reagent. In all cases, these data indicate that methanolysis is an intrinsic property of the lipase itself.

Activities of Lipases on *n*-Alkyl *n*-Butyrates and Formation of Methyl *n*-Butyrate

The purpose of this study was to determine whether the ratio of transformed substrate to methyl *n*-butyrate formed by methanolysis is independent of the nature of the leaving group of substrate in lipase-catalyzed reactions. With *n*-alkyl *n*-butyrates having 1 to 5 carbon atoms in alcohol moieties as substrate, the activities of lipases (from pancreatic, *Mucor* and *Rhizopus*) and liver esterase were measured in the presence of 3.5M methanol. Even though newly formed methyl *n*-butyrate can also become a substrate, the rates of transformation of this ester must be extremely low in these reactions because enzymes employed in this experiment have very poor reactivity for this substrate as shown in Table III. The ratios presented in parentheses in Table III are independent of leaving group. These results are consistent with the concept that water or methanol attacks an intermediate common to all substrates. From our results, however, it is not possible to know the nature of the common intermediate. Recently, Sémériva, *et al.*[14] reported that the hydrolysis of monomeric *p*-nitrophenyl acetate by lipase in the presence of 4% acetonitrile involves the steps of acylation

12) H. Brockerhoff, *Chem. Phys. Lipids*, **10**, 215 (1973).
13) T. Murachi and M. Yasui, *Biochemistry*, **4**, 2275 (1965).
14) M. Sémériva, C. Chapus, C. Bovier-Lapierre, and P. Desnuelle, *Biochem. Biophys. Res. Commun.*, **58**, 808 (1974).

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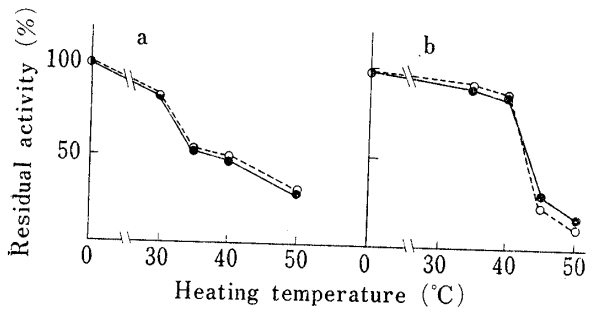

Fig. 1. Inactivation of Purified Lipases by Heating

After heating for 10 min at each temperature, hydrolytic and methanolytic activities in 3.5M methanol were determined by Method 3).
(a) pancreatic lipase, (b) *Mucor* lipase
—●— : hydrolysis, ···○··· : methanolysis

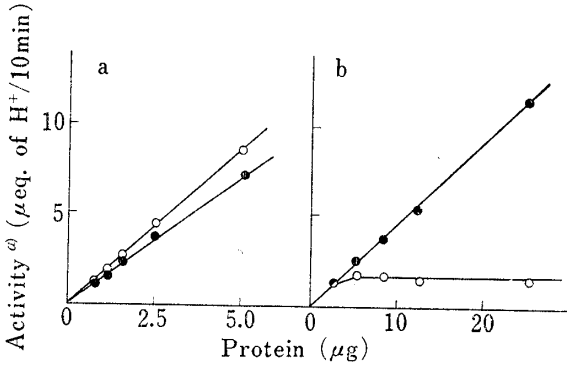

Fig. 2. Enzyme-Catalyzed Hydrolysis of *n*-Propyl *n*-Butyrate

(a) liver esterase, (b) pancreatic lipase
a) determined by Method 3), [MeOH]=0
—○— : -SDC, —●— : +1.8 mM SDC

TABLE III. Relative Activity[a] on *n*-Alkyl *n*-Butyrates in 3.5M Methanol

n-Butyrate ester	Lipase			Esterase liver
	Pancreatic	*Mucor*	*Rhizopus*	
Methyl	0.6	1.0	7.2	9.2
Ethyl	7.3(9)	3.4(6)	9.2(13)	94(73)
Propyl	100(9)	100(6)	100(14)	100(78)
Butyl	100(9)	98(7)	104(14)	89(77)
Amyl	97(8)	71(6)	50(14)	57(76)

The relative activity with each ester was calculated by taking the total activity with *n*-propyl *n*-butyrate as 100.
Numbers in parentheses represent formation of methyl *n*-butyrate, calculated as (methyl *n*-butyrate formed/transformed substrate)×100.
a) determined by Method 2)

and deacylation and that a transient acetyl-lipase intermediate was isolated. Consequently, it is reasonable to postulate that the nature of common intermediate is an acyl enzyme.

In general, *n*-alkyl *n*-butyrates employed in this experiment are good substrates for carboxylesterase [EC 3.1.1.1] and are poor substrates for lipase. Therefore, it was necessary to determine whether or not the activity obtained by lipase preparation in Method 3) was specific for lipase. As shown in Fig. 2 esterase activity in the presence of SDC was slightly lower than that of activity in the absence of SDC. On the other hand, lipase activity in the presence of SDC (1.8 mM) was considerably accelerated and proportional to the enzyme concentration. This phenomenon was in accordance with the intrinsic property of lipase, because the activity of this enzyme is considerably enhanced by interface. Thus, it is concluded that the use of Method 3) is valid in the assay of lipase or esterase activity. Therefore, Method 3) is advantageous for comparison of mechanism of lipase with that of carboxylesterase.

Effect of Methanol on Hydrolysis/Alcoholysis Ratio

Fig. 3 and 4 show the hydrolysis/alcoholysis (H/A) ratio obtained for *n*-propyl *n*-butyrate solvolysis in the presence of various concentrations of methanol. In the presence of methanol, the rate of methanolysis of the ester *via* liver esterase-catalyzed acyl transfer was much higher than that *via* pancreatic lipase-catalyzed acyl transfer. In Fig. 3 and 4, a plot of H/A *vs* $[MeOH]^{-1}$ gives a straight line which has an intercept on the ordinate. As methanol concentration increased, the H/A ratio approached a limiting value. This ratio depended on the concentration of the substrate ester, not on the enzyme concentration. When the substrate concentration was doubled (100→200 mM), lipase-catalyzed methanolysis was increased (Fig. 4) whereas esterase-catalyzed methanolysis was decreased (Fig. 3). As regards liver esterase,

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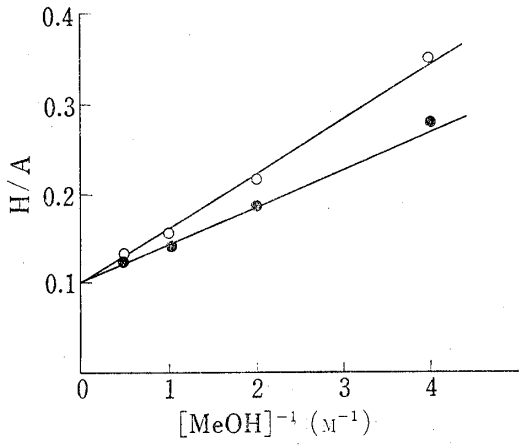

Fig. 3. Effect of Methanol Concentration on H/A Ratio[a] of Liver Esterase-Catalyzed Solvolysis

a) determined by Method 3)
—●— : [*n*-propyl *n*-butyrate] 100 mM
—○— : [*n*-propyl *n*-butyrate] 200 mM

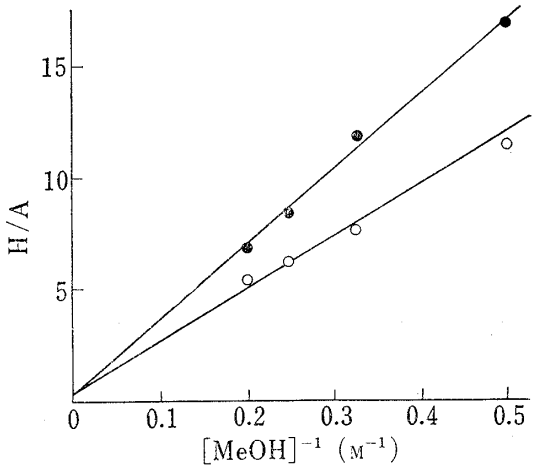

Fig. 4. Effect of Methanol Concentration on H/A Ratio[a] of Pancreatic Lipase-Catalyzed Solvolysis

a) determined by Method 3)
—●— : [*n*-propyl *n*-butyrate] 100 mM
—○— : [*n*-propyl *n*-butyrate] 200 mM

these behaviors agrees with the results reported by Wynne and Shalitin.[4] However, in the case of lipase, the dependence of methanolysis on the substrate concentration contradicts the results obtained in the case of esterase as shown in Fig. 3. Hydrolysis catalyzed by lipase or esterase was less influenced by the substrate concentration than methanolysis.

Kinetic Parameter

Considering that a plot of H/A *vs* $[MeOH]^{-1}$ gave a straight line with an intercept on the ordinate, Wynne and Shalitin[4] proposed Chart 1 and derived the Equation (1). By substituting the data obtained from Fig. 3 and 4 in Equation (1), kinetic parameters for esterase and lipase were obtained and summarized in Table IV.

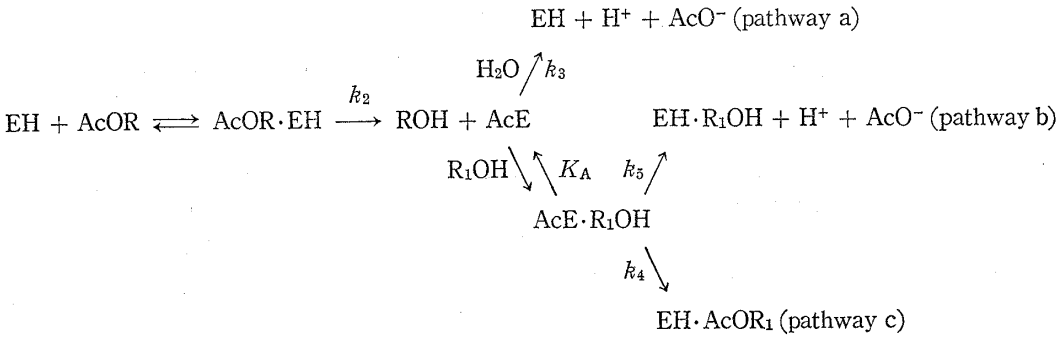

Chart 1[4]

$$\frac{H}{A} = \frac{k_3'}{k_4 K_A} \cdot \frac{1}{[R_1OH]} + \frac{k_5'}{k_4}$$ Equation (1)[4]

where $k'_3 = k_3[H_2O]$, $k'_5 = k_5[H_2O]$

It was revealed from comparison with K_A values, that methanol had a higher affinity for acyl-esterase than for acyl-lipase. Therefore, methanol (2M) is 200-fold superior to water in the solvolysis of acyl-esterase and on the contrast, only 1.7-fold superior to water in the solvolysis of acyl-lipase under the conditions tested. The k_4/k_3' ratio of esterase (=6) was 37.5-fold greater than the ratio of lipase (=0.16). From these data, it can be seen that overall reaction of solvolysis by lipase is liable to proceed through Pathway a in Chart 1, *i.e.*, deacylation

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via hydrolysis (k_3'), and by esterase through Pathway c, *i.e.*, deacylation *via* alcoholysis (k_4). Consequently, the A/H ratio of esterase was greater than the ratio of lipase.

TABLE IV. Kinetic Parameters[a] for Liver Esterase and Pancreatic Lipase at 30°, pH 7.4

	Liver esterase			Pancreatic lipase		
A/H	7.7			0.065		
(A/H).([H_2O]/[MeOH])	200			1.7		
K_A	3M^{-1}			0.2M^{-1}		
	k_3'	k_4	k_5'	k_3'	k_4	k_5'
	1.3	7.8	1	60	10	1

A/H=determined at *n*-propyl *n*-butyrate 100 mM, [MeOH]=2M (A/H).([H_2O]/[MeOH])=the formal reactivity ratio between methanol nucleophile and water.

K_A=the association constant for acyl-enzyme·methanol complex. From the plot of 1/A *vs* [MeOH]$^{-1}$, K_A was obtained as the negative intercept on the abscissa, where A is an initial velocity of formation of methyl *n*-butyrate.

a) determined by Equation (1)

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