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Studies on the Lipase of *Chromobacterium viscosum*. V.[1] Physical and Chemical Properties of the Lipases

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Physical and chemical properties of the two kinds of lipases (lipase A and lipase B) from *Chromobacterium viscosum* were investigated. The results were as follows: Sedimentation constants were 5.35×10^{-13} and 3.82×10^{-13} cm·g/sec·dyne; Molecular weights were 1.2×10^5 and 2.7×10^4; Intrinsic viscosities were 0.060 and 0.051 dl/g; Partial specific volumes were 0.816 and 0.663 cm^3/g; Isoelectric points were 4.7 and 6.9 for the lipase A and B respectively. From the study of ORD, the α-helix content of the lipase A was calculated to be less than 10% and of the lipase B was to be about 20%. The amino acid compositions of the lipases were different from each other and the lipase B did not contain the half-cystine. Lipid was not detected in the both enzymes and carbohydrate was contained only in the lipase A (14%). By the modification with diazonium-1-*H*-tetrazole, the enzymic activities were decreased with increase of the modification of histidine residue in the both lipases, and it was assumed that one mole of the histidine residue in the lipase B was related with the catalytic action of the enzyme. Some properties of the lipases which will be concerned with the affinity of the enzymes on their hydrophobic substrates were also discussed.

Keywords——lipase; *Chromobacterium viscosum*; physical property; amino acid composition; chemical modification

Lipases (glycerol ester hydrolase, EC 3.1.1.3) have a distinctive property that they hydrolyze rapidly the water-insoluble fatty acid esters but very slowly the water-soluble esters. On the other hand, esterases (carboxylic ester hydrolase, EC 3.1.1.1) act preferentially on the water-soluble esters. In their substrate specificities, lipases had been distinguished from esterases.[3] The catalytic action caused by lipase proceeds on the surface of the substrate, and it will be attributable to the adsorption of the enzyme on the surface.[4] It is one of the most interesting problems what the structures or components in the lipase molecule produces the affinity for hydrophobic substrates. And in fact, several hypotheses have so far been formulated in this connection. Tomizuka, *et al.*[5] found that the lipase from *Candida cylindracea* had a high content of hydrophobic amino acid residues and considered that the hydrophobic amino acids would be related to the affinity of the enzyme to lipids. Similar amino acid composition was also observed in the lipases from *Mucor*[6] and *Rhizopus*.[7] Brockerhoff has postulated that hydrophobic region mainly constituting of hydrophobic amino acids and hydrophilic region consisting of hydrophilic amino acids and carbohydrate chain exists in the pancreatic lipase, and these regions are related to the orientation of the enzyme molecule on the substrate interface.[8] Okuda and Fujii has reported that animal adipose tissue

1) This paper forms Part CXXVIII of "Studies on Enzymes" by M. Sugiura. Preceding paper part IV; M. Sugiura and M. Isobe, *Chem. Pharm. Bull.* (Tokyo), **23**, 1226 (1975).
2) Location: *1432–1 Horinouchi, Hachioji-shi, Tokyo, 192–03, Japan.*
3) L. Sarda and P. Desnuelle, *Biochim. Biophys. Acta*, **30**, 513 (1958).
4) G. Benzonana and P. Desnuelle, *Biochim. Biophys. Acta*, **105**, 121 (1965).
5) N. Tomizuka, Y. Ota, and K. Yamada, *Agric. Biol. Chem.* (Tokyo), **30**, 1090 (1966).
6) K. Nagaoka and Y. Yamada, *Agric. Biol. Chem.* (Tokyo), **37**, 2791 (1973).
7) H. Chiba, M. Hisatake, M. Hirose, and E. Sugimoto, *Biochim. Biophys. Acta*, **327**, 380 (1973).
8) H. Brockerhoff, *Chem. Phys. Lipids.*, **10**, 215 (1973).

lipase is composed of esterase and lipid, and that the lipid component induced the affinity of the enzyme to the insoluble substrates.[9] Other lipases that contain carbohydrate[5,6,10] and lipid[11] were reported. On the other hand, lipases which did not contain carbohydrate or lipid were also found.[12]

Multiple forms of lipases have frequently been observed. There are three molecular species of lipases in pancreas[13] and one of the lipase with a high molecular weight is an aggregate of the low molecular weight lipases with lipids. It was also reported[14] that *Rhizopus* sp. produced several species of lipases and there was a relationship of interconversion between the two enzymes. Other multiple forms of lipase from an origin were reported.[15]

In previous papers of this series,[16] two lipases were isolated and purified from *Chromobacterium viscosum*. Furthermore we found that the lipases, as well as the lipases of the other origins, had a characteristic of adsorption not only on the substrate-water interface but also on various interfaces, *i. e.* air/water,[17] water-insoluble organic solvent/water,[18] hydrophobic glass/water,[19] and that these properties were similar to those of detergents.

The present paper deals with the physical and chemical properties of the two lipases from *Chromobacterium viscosum* to elucidate the multiplicity and the molecular aspects of the lipases for the affinity on lipids.

Material and Methods

Enzymes and Assay of Lipolytic Activity——The lipases with a high molecular weight (lipase A) and with a low molecular weight (lipase B) of *Chromobacterium viscosum* were purified from the culture filtrate according to the methods described in previous paper[16] and they were confirmed to be homogeneous with disc electrophoresis. Assay of lipolytic activity was carried out in the same manner as described previously[16b] where olive oil emulsion was used.

Ultracentrifugal Sedimentation——Ultracentrifugal analysis was carried out by a Hitachi Analytical Ultracentrifuge Model UCA-1 with a schlieren optical system, and the sedimentation constant was calculated according to Schachman's method.[20]

Determination of Molecular Weight——Molecular weight of the enzyme was determined according to Whitaker's method[21] with Sephadex G-100 and G-200. Also sodium dodecylsulfatepolyacrylamide gel electrophoresis was carried out according to Weber and Osborn's method[22] with 7.5% gel, and the molecular weight was calculated.

Determination of Isoelectric Point, Viscosity and Specific Volume——Isoelectric point was determined by isoelectric focusing as described by Vesterberg and Svensson[23] with the use of 1% carrier ampholite (pH

9) H. Okuda and S. Fujii, *J. Biochem.* (Tokyo), **73**, 1195 (1973).
10) *a*) Y. Tsujisaka, M. Iwai, and Y. Tominaga, *Agric. Biol. Chem.* (Tokyo), **37**, 1457 (1973); *b*) T. Egelrud and T. Olivecrona, *J. Biol. Chem.*, **247**, 6212 (1972).
11) *a*) M. Funatsu, Y. Aizono, K. Hayashi, M. Watanabe, and M. Eto, *Agric. Biol. Chem.* (Tokyo), **35**, 734 (1971); *b*) P.E. Fielding, V.G. Shore, and C.J. Fielding, *Biochemistry*, **13**, 4318 (1974).
12) W-H. Liu, T. Beppu, and K. Arima, *Agric. Biol. Chem.* (Tokyo), **37**, 2493 (1973).
13) *a*) S. Ramachandran, Y.K. Yip, and S.R. Wagle, *Eur. J. Biochem.*, **12**, 201 (1970); *b*) H. Kimura, T. Kitamura, and M. Tsuji, *Biochim. Biophys. Acta*, **270**, 307 (1972); *c*) R. Verger, L. Sarda, G.H.de Haas, and P. Desnuelle, *Biochim. Biophys. Acta*, **188**, 272 (1969).
14) *a*) M. Semeriva, G. Benzonana, and P. Desnuelle, *Biochim. Biophys. Acta*, **191**, 598 (1969); *b*) M. Iwai and Y. Tsujisaka, *Agric. Biol. Chem.* (Tokyo), **38**, 1249 (1974).
15) *a*) J.D. Schnatz and J. Cortner, *J. Biol. Chem.*, **242**, 3850 (1967); *b*) B. Chorvath and M. Fried, *J. Bacteriol.*, **102**, 879 (1970); *c*) K. Nagaoka and Y. Yamada, *Agric. Biol. Chem.* (Tokyo), **37**, 2791 (1973).
16) *a*) M. Sugiura, M. Isobe, N. Muroya, and T. Yamaguchi, *Agric. Biol. Chem.* (Tokyo), **38**, 947 (1974); *b*) M. Sugiura and M. Isobe, *Biochim. Biophys. Acta*, **341**, 195 (1974).
17) M. Sugiura and M. Isobe, *Biochim. Biophys. Acta*, **397**, 412 (1975).
18) M. Sugiura and M. Isobe, *Chem. Pharm. Bull.* (Tokyo), **23**, 1221 (1975).
19) M. Sugiura and M. Isobe, *Chem. Pharm. Bull.* (Tokyo), **24**, 72 (1976).
20) H.K. Schachman, "Method in Enzymology," Vol. 4, C.P. Clowick and N.O. Koplan(ed), Academic Press, New York, 1975, p. 32.
21) J.R. Whitaker, *Anal. Chem.*, **35**, 1950 (1963).
22) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
23) O. Vesterberg and H. Svensson, *Acta Chem. Scand.*, **20**, 820 (1966).

3—10). Viscosity of the protein solution was measured with a Ostwald's viscometer at 20° and the specific volume was also measured with a pycnometer.

Measurement of Ultraviolet (UV) Absorption Spectrum and Optical Rotatory Dispersion (ORD)——The UV absorption spectrum was taken with a Hitachi Recording Spectrophotometer Model 323. ORD measurement was performed with a Japan Spectroscopic Recording Spectropolarimeter Model J-20 at room temperature near 20°. The values of a_0 and b_0 were calculated by Moffit–Yang's plot.[24]

Analysis of Amino Acid Composition——The hydrolysis of protein was carried out in 3 N *p*-toluenesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole in an evacuated airtight test tube at 110° according to Liu and Chang's method.[25] After neutralization, the hydrolyzate was analyzed with a JEOL Amino Acid Autoanalyzer Model JLC-6AH by the Na^+ two column method. On the other hand, the tryptophan content in the protein was determined spectrophotometrically by Goodwin–Morton's method.[26] The cysteine and cystine contents were assayed colorimetrically with Ellman's reagent.[27]

Assay of Carbohydrate and Lipid——The hexose and hexosamine contents in the enzyme were determined by the phenol–H_2SO_4 method[28] and Elson–Morgan's method,[29] respectively. Assay of lipid was carried out as follows. The protein (10 mg) containing 50 μg of pentadecanoic acid as the internal standard were treated with 5% HCl-methanol solution[30] and extracted with hexane. The fatty acid methyl ester in the extract was assayed by gas chromatography with a column of 2% Silicone OV-1.

Chemical Modification of Histidine Residue——Histidine residue in the lipase was modified with diazonium-1-*H*-tetrazole (DHT) according to the method reported by Takenaka, *et al.*[31]

Results

Sedimentation Analysis

The purified enzymes were dissolved in 10 mM phosphate buffer (pH 7.0) and the solution was passed through a column of Sephadex G–25 which was previously equilibrated with 10 mM phosphate buffer (pH 7.0). The protein solution was concentrated to 2% using a membrane filter. The solution of lipase A was centrifuged at 60000 rpm and 5.3°. On the other

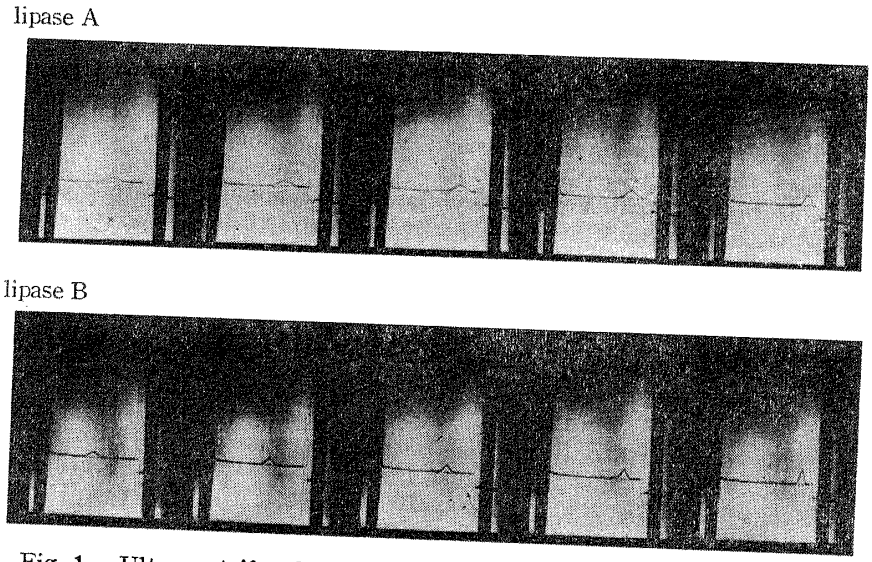

Fig. 1. Ultracentrifugal Analysis of the Lipases from *Chromobacterium*

Lipases were dissolved about 2% in 10 mM phoshate buffer (pH 7.0). After reaching full speed of 60000 rpm, the photographs were taken at a constant intervals of 18 min for lipase A or 24 min for lipase B from right to left.

24) W. Moffit and J.T. Yang, *Proc. Nat. Acad. Sci. U.S.A.*, **42**, 596 (1956).
25) T.Y. Liu and Y.H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).
26) T.W. Goodwin and R.A. Morton, *Biochem. J.*, **40**, 628 (1946).
27) J.F. Robyt, R.J. Ackerman, and C.G. Chittenden, *Arch. Biochem. Biophys.*, **147**, 262 (1971).
28) M. Dubosis, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, **28**, 350 (1956).
29) L.A. Elson and W.T.L. Morgan, *Biochem. J.*, **27**, 1824 (1933).
30) M.E. Mason and G.P. Waller, *Anal. Chem.*, **36**, 583 (1964).
31) A. Takenaka, H. Horinishi, and K. Shibata, *Biochim. Biophys. Acta*, **194**, 293 (1969).

hand, lipase B solution was centrifuged at 60000 rpm and 14.5°. The sedimentation patterns were shown in Fig. 1 and the sedimentation constant ($S_{20,w}$) of lipase A and B was calculated to be 5.35 S and 3.82 S respectively.

Molecular Weight

The molecular weight of the lipase A and B was determined by gel filtration on a column of Sephadex G–200 and G–100 respectively, which was previously equilibrated with 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl. The results were showm in Fig. 2 and the molecular weight of lipase A and B was calculated to be 1.2×10^5 and 2.7×10^4 respectively. Also sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out to estimate the molecular weight. As the results, lipase A dissociated into two subunits and the molecular weights were calculated to be 8.0×10^4 and 5.0×10^4, on the other hand, lipase B showed only a protein band with a molecular weight of 2.6×10^4. These results well agreed with the results from the gel filtration.

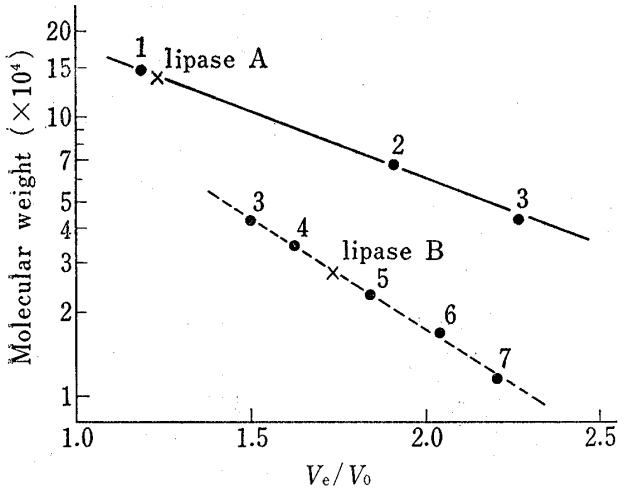

Fig. 2. Determination of the Molecular Weight of the Lipases from *Chromobacterium* by Gel Filtration

The column of Sephadex G-100 (----) and G-200 (——) were equilibrated with 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl. Proteins used as standard; 1, γ-globulin; 2, bovine serum albumin; 3, ovalbumin; 4, pepsin; 5, trypsin; 6, myoglobin; 7, cytochrome c.

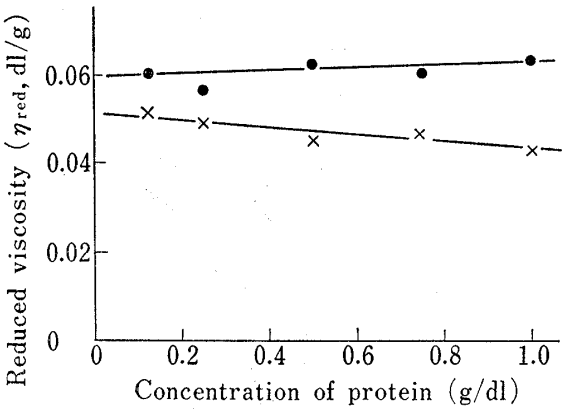

Fig. 3. Viscosity of the Lipases from *Chromobacterium*

The enzymes were dissolved in 10 mM phosphate buffer (pH 7.0) and the viscosity was measured by the Ostwald viscometer in a thermostat bath at 20.0°.
—●—, lipase A; —×—, lipase B.

Intrinsic Viscosity and Partial Specific Volume

The viscosity of the enzymes dissolved in 10 mM phosphate buffer (pH 7.0) was measured with an Ostwald viscometer. From relative viscosity, the reduced viscosity was calculated and shown in Fig. 3 with relation to the protein concentration. The reduced viscosity of lipase A was approximately constant throughout any concentrations of the protein and the intrinsic viscosity was found to be 0.060 dl/g. On the other hand, reduced viscosity of lipase B became smaller with the protein concentration became higher, and the intrinsic viscosity was calculated to be 0.051 dl/g. The partial specific volumes of the lipase A and B were estimated to be 0.816 and 0.663 respectively by weighing the 0.2% protein solution in a pycnometer.

UV Absorption and ORD

The lipases were dissolved in 10 mM phosphate buffer (pH 7.0) and the absorption spectrum of the solution was taken by a spectrophotometer. The absorption spectrum of lipase A showed a maximum at 280 nm and a minimum at 250 nm. The extinction coefficient at 280 nm ($E^{1\%}_{1cm}$) was calculated to be 5.22 and the ratio of absorbance at 280 nm to 250 nm

was 1.7. Althongh lipase B showed a maximum and minimum absorption at the wave length similar to those of lipase A. But the extinction coefficient of lipase B was considerably differed from that of lipase A and the value was estimated to be 7.02. The ratio of absorbancy at 280 nm to 250 nm was 3.8.

ORD spectra of the lipase A and B at UV region were shown in Fig. 4. Both of the proteins showed a minimum optical rotation at 233 nm and the reduced mean residue rotations of lipase A and B at the minimum were calculated to be −1620 and −5010 respectively. On the other hand, the magnitude of the rotation at various wavelength from 700 nm to 300 nm were plotted according to the Moffitt-Yang's equation. From the plot, the parameters, a_0 and b_0, were calculated to be −138 and −54 respectively for the lipase A, and to be −362 and −137 for the lipase B. The α-helix contents of the lipase A and B were calculated to be less than 10% and about 20% respectively from the values of the reduced mean residue rotation at 233 nm and from the b_0.

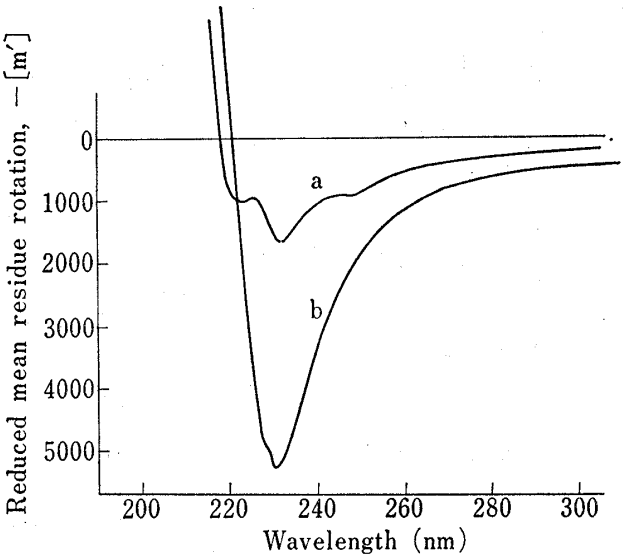

Fig. 4. Optical Rotatory Dispersion of the Lipases from *Chromobacterium*

The enzyme was dissolved in 10 mM phosphate buffer (pH 7.0) and the optical rotatory dispersion was measured.
a, lipase A; b, lipase B.

Table I. Amino Acid Composition of the Lipases from *Chromobacterium* after Acid Hydrolysis

Amino acid	Nearest integered value (mol/mol protein)	
	lipase A	lipase B
Lysine	51	4
Histidine	27	7
Arginine	34	9
Aspartic acid	110	26
Glutamic acid	89	19
Threonine	105	28
Serine	80	22
Glycine	112	18
Proline	34	8
Alanine	120	31
Half-cystine[a)]	2	0
Valine	87	23
Methionine	17	1
Leucine	96	26
Isoleucine	33	8
Tyrosine	29	8
Phenylalanine	21	6
Tryptophan[b)]	3	3

a) Assayed colorimetrically with Ellman's reagent.
b) Assayed spectrophotometrically by Goodwin-Morton's method.

Isoelectric Point

The purified enzymes were subjected to the isoelectric focusing with 1% carrier ampholite (pH 3—10). As the results, single peak of protein and lipolytic activity was obtained at pH 4.7 for the lipase A and at pH 6.9 for the lipase B. Also the results indicated that the enzymes were homogeneous.

Amino Acid Composition

The results of amino acid analysis were shown in Table I. It was found that contents of the sulfur-containing amino acids, such as cysteine and methionine, in the both lipases were very low and that the lipase B was characterized as an enzyme without S–S bond and SH residue in the molecule similarly to the lipase from *Geotrichum*.[10a)] As for the polarity

proposed by Capaldi and Vanderkooi,[32] the values of lipase A and B were calculated to be 44% and 40% respectively and were within or close to the range of many soluble proteins (47±6%). Furthemore, the amino acids in the lipases were classified according to the report of Hatch,[33] where Asp, Asn, Glu, Gln, Lys, Arg, Ser and Thr were polar group, and Val, Lau, Leu, Ileu, Met, Pro, and Phe were apolar group. The ratio of polar to apolar amino acid residues of lipase A and B were 1.6 and 1.5 respectively, and the results showed that the lipases were different from the proteins having a large proportion of apolar residues.

Carbohydrate and Lipid Content

The results of the analysis of carbohydrate and lipid were shown in Table II. The neutral sugar and amino sugar contents of the lipase A were estimated to be 14% as glucose (correspond to 94 mol/mol enzyme) and 0.16% as glucosamine (correspond to 1.1 mol/mol enzyme) respectively. Also, after precipitation with trichloroacetic acid, the sugar contents in the protein did not change significantly. On the contrary, the contents of neutral and amino sugar of lipase B were so low that the enzyme was judged to be not a glycoprotein. The quantitative analysis of lipid components was carried out by means of gas chromatography to determine the long chain fatty acids. The contents of fatty acids in both enzymes were so low that the enzymes were judged to have no lipid components.

Table II. Carbohydrate and Lipid Content in the Lipases from *Chromobacterium*

	Neutral sugar (mol/mol protein as glucose)	Amino sugar (mol/mol protein as glucosamine)	Long chain fatty acid (mol/mol protein as palmitic acid)
Lipase A	14% (94 mol)	0.16% (1.1 mol)	0.05% (0.2 mol)
Lipase B	0.30% (0.4 mol)	0.12% (0.2 mol)	0.01% (0.0 mol)

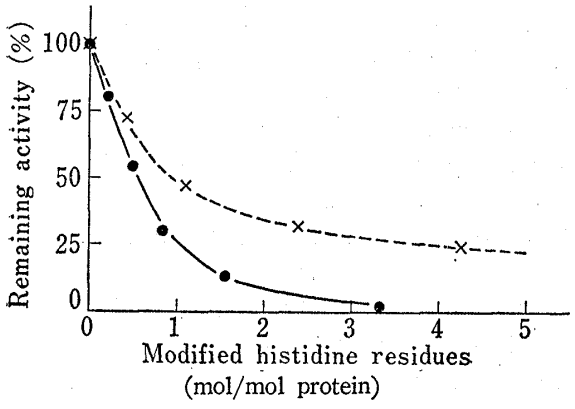

Fig. 5. Chemical Modification of the Lipases from *Chromobacterium* with Diazonium-1-*H*-tetrazole

The enzymes dissolved in 0.7 M borate buffer (pH 8.8) and various concentration of diazonium-1-*H*-tetrazole were mixed and kept the pH at 8.6—9.2 for 1 hr. After the addition of NaN_3, the remaining lipolytic activity and modified histidine residues were assayed.
---×---, lipase A; —●—, lipase B.

Chemical Modification of Histidine

From the preliminary experiment in which the lipases were photoxydized in the presence of methylene blue, it was presumed that histidine residue in the enzyme should have some relationships to the enzymic activity. So an examination was made with diazonium-1-*H*-tetrazole aiming at modifying histidine residues. The results were shown in Fig. 5. The lipolytic activity of lipase A was decreased with the modification of histidine residue but the stoichiometric relationship between the modified residues and enzymic activity was not observed. Furthermore, the modification of the tyrosine residues also proceeded at the same time. Therefore, the role of histidine residues in the activity of lipase A was not able to elucidate. On the other hand,

32) R.A. Capaldi and G. Vanderkooi, *Proc. Nat. Acad. Sci. U.S.A.*, **69**, 930 (1972).
33) F.T. Hatch, *Nature* (London), **206**, 777 (1965).

lipase B lost its enzymic activity with the modification of histidine residues. Under the condition that one mole of histidine residue was modified but tyrosine residues were not, the enzymic activity was decreased to 25%. Also amounts of the modified amino acid residues were assayed by amino acid analysis and the same results were obtained. From the results, it was assumed that one mole of histidine residue in the lipase B was closely related to the enzymic activity.

Discussion

Physical and chemical properties of two kinds of lipases (lipase A and B) purified from *Chromobacterium viscosum* were studied to clarify whether the two lipases are different from each other originally or not. As the results, it became evident that the lipase A and B significantly differed from each other in such properties as amino acid composition, carbohydrate content, isoelectric point and ORD spectrum. Also it was found at sodium dodecylsulfate-polyacrylamide gel electrophoresis that the lipase A dissociated into two subunits with molecular weight of about 80000 and 50000. From these results, it was assumed that the lipase A and B would be originally different protein each other. Many reports[9,13a,b,15] were presented with respect to multiple forms of lipases which are interchangeable each other, and it was elucidated that lipid components participate in these interconversion. The lipases from *Chromobacterium* did not show interconversion and it was agreeable with the absence of lipid in the lipases.

It is one of the other problem what structure of the protein bring the affinity into lipases on the surface of their substrates. And it was considered that hydrophobic structure might be concerned with the affinity of lipase on the surface. Therefore the amino acid composition of the lipases was analyzed and high content of hydrophobic amino acid residues was found.[5,7,12] Lipid contained in the lipase molecule may constitute a hydrophobic region and play an important role in the enzymic action. Many lipases which contain lipids were found.[11] Also it was reported that the carbohydrate in the lipases from pancreas should form a hydrophilic tail and support the orientation of the enzyme molecule into the hydrophobic substrates with its hydrophobic region.[8] On the other hand, the lipases from *Chromobacterium* had neither a high content of hydrophobic amino acid residues nor lipid components in its molecule. And the lipase B was judged to have no carbohydrates. From these results, it was presumed that the principle of the affinity of the lipases on the hydrophobic substrates arised from the higher structure of the protein but not from the other cofactors, and that the hydrophilic and hydrophobic regions in the lipases should show a similar behavior on the hydrophobic interface as those of detergents.

Chemical modifications were carried out and the effect on the enzymic activity was examined. Both of the lipases were deprived of their enzymic activity by the modification with DHT, and it was concluded that one mole of the histidine in the lipase B was closely related to the enzymic action. But the other modification procedures with carbodiimide, acetylimidazole, glutaldehyde, monoiodoacetic acid and diisopropylphosphofluoridate did not give any effects on the enzymic activity of both lipases.