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Studies on the Mechanism of Lipase Reaction. III.[1] Adsorption of *Chromobacterium* Lipase on Hydrophobic Glass Beads

Mamoru Sugiura and Masakazu Isobe

Tokyo College of Pharmacy[2]

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The lipase from *Chromobacterium* was adsorbed on glass beads which was coated with olive oil, liquid paraffin or silicone oil. These adsorption was treated in the Lineweaver-Burk's plot and characters of the adsorption were similar each other regardless of their chemical structure of hydrophobic materials. On the other hand, esterase from porcine liver was not adsorbed on hydrophobic glass beads.

The interaction between the lipase and hydrophobic surface conformed to the Langmuir's adsorption isotherm with a dissociation constant $K=1.4\times10^{-7}$ M. At saturation of the surface with the lipase each protein molecule occupies an average area of 4500 Å^2 per molecule. The lipase adsorbed on hydrophobic surface did not inactivated but activated about 3-fold. It was elucidated that the hydrophobic bond play a major role in the adsorption of the lipase on substrate or hydrophobic surface.

While lipase (glycerol ester hydrolase, EC 3.1.1.3) is fully active on the substrate in the form of aggregates, *i.e.*, emulsion,[3] micelle[4] or monomolecular film[5] but rather inactive upon the dispersed substrate, the reverse is the case with esterase (carboxylic ester hydrolase, EC 3.1.1.1). Several hypotheses have been proposed to explain the characteristic property of lipase. Brockman, *et al.*[6] reported that the reaction of porcine pancreatic lipase with tripropionin showed substantial stimulation in the presence of hydrophobic surface and the enhancement can be ascribed to an increased local concentration of the substrate at the surface. Desnuelle, *et al.*[7] found that the hydrolysis of the monomeric tripropionin or *p*-nitrophenyl acetate by pancreatic lipase were accelerated considerably in the presence of hydrophobic surface or by the addition of water-miscible organic compounds. It is assumed that the hydrophobic interfaces and organic solvents modify the structure of liquid water and causes the acceleration through a transconformation of the enzyme molecule or any other process. The other models for the enzyme action at interface have also been formulated by Desnuelle,[8] Brockerhoff,[9] Wells[10] and Verger, *et al.*[11]

In the previous paper of this series,[12] we found that lipases from various origins have a property of adsorption on oil-water or air-water interface, while esterase and various enzymes which react on water soluble substrates have no such property. The present report will show

1) The work forms part CX of "Studies on Enzymes," proceding paper Part II; M. Sugiura and M. Isobe, *Biochim. Biophys. Acta*, **397**, 412 (1975).
2) Location: *Ueno-sakuragi 1-chome, Taito-ku, Tokyo, 110, Japan.*
3) L. Sarada and P. Desnuelle, *Biochim. Biophys. Acta*, **30**, 513 (1958).
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the interaction between the lipase from *Chromobacterium* and hydrophobic interfaces. The mechanism of lipase reaction was also discussed.

Material and Method

Enzymes——The lipase of *Chromobacterium* was purified as reported previously.[13] The purified enzyme was homogeneous as judged by polyacrylamide gel electrophoresis at pH 9.4. The esterase of porcine liver was obtained from Boehringer Mannheim GmbH.

Materials——Carrier free sodium [^{125}I]iodide was obtained from New England Nuclear. Bovine serum albumin, triglycerides and *p*-nitrophenyl acetate were products of Tokyo Kasei Co., Ltd. Silicone oil DC QF-1 was product of Applied Science Lab. Inc.

Coating of Hydrophobic Materials on Glass Beads——Glass beads (size: 100—200 mesh) were rinsed with detergent solution, rinsed with water, soaked in chromic acid cleaning solution for a day, rinsed to neutrality in distilled water, and dried under vacuum. The surface area of the glass beads was determined with air permeability method[14] as 1700 cm^2/g. One ml of 2% acetone solution of hydrophobic materials and 1 g of glass beads were mixed and the solvent was removed with evaporation. The coated beads were stored under vacuum.

Assay of Enzyme Activities and Protein——The activity of lipase toward olive oil emulsion was assayed according to the method of previous report.[13] The activity of *p*-nitrophenyl acetate hydrolysis by the lipase and esterase was followed at 30° and pH 7.0 by spectrophotometry (400 nm: with the Hitachi Recording Spectrophotometer, Type 323).

The protein content was determined according to the method of Lowry, *et al.*[15]

Disc Electrophoresis——Disc electrophoresis was carried out with the use of 7.5% polyacrylamide gel at pH 9.4, glycine-Tris buffer[16] and electrophoresed at a constant current of 3 mA/tube for 90 min. Staining was carried out using Amido Black 10B (Merck).

Preparation of ^{125}I-Labelled Lipase and Esterase——The method of Greenwood[17] was applied to the preparation of ^{125}I-labelled lipase and esterase. The lipase from *Chromobacterium* (32 μg) or esterase of porcine liver (20 μg) was dissolved in 0.1 ml of 0.1 M phosphate buffer (pH 7.0) and was mixed with 0.1 ml of 10 μM sodium iodide in which 160 uC of sodium ^{125}I iodide was contained. The iodination of protein was carried out by adding 0.1 ml of chloramine-T solution (4 mg/ml) and the reaction was terminated by mixing 0.5 ml of sodium metabisulphite solution (2.4 mg/ml in 0.1 mM phosphate buffer, pH 7.0). To the reaction mixture 0.5 ml of carrier KI solution (10 mg/ml in the 0.1 M phosphate buffer, pH 7.0) and 0.5 ml of bovine serum albumin solution (25 mg/ml in the 0.1 M phosphate buffer, pH 7.0) were added. Separation of ^{125}I-labelled enzyme from the reaction mixture was carried out by gel-filtration with a column (20 × 1 cm) of Sephadex G-50 which was previously equilibrated with 0.1 M phosphate buffer (pH 7.0) containing 7 mg/ml of bovine serum albumin. The radioactivity and enzyme activity of the eluate were assayed and the enzyme was collected.

The radioactivity of ^{125}I in various samples was counted by using a well-type scintillation counter (Aloka Autoganmma Scintillation Spectrometer).

The ^{125}I-labelled *Chromobacterium* lipase, of specific radioactivity 2.5 μC/μg, contains 0.35 atom of total iodine per molecule and retained 89% of enzyme activity through the procedure of labelling. The specific radioactivity of ^{124}I-labelled porcine liver esterase was 0.46 μC/μg and total labelled iodine was 0.56 atom/molecule. The esterase retained 84% of the enzymic activity of the original.

Results

Properties of ^{125}I-Labelled Enzymes

The interaction between hydrophobic surface and ^{125}I-labelled enzymes was studied. Various amount of hydrophobic glass beads and 2 ml of the enzyme solution, in which about 0.01 μg of the ^{125}I-labelled lipase or esterase was contained, were mixed well in a glass tube. After centrifugation, the radioactivity that was not adsorbed on the glass beads but remaining in the aqueous phase was assayed. The adsorbed enzymes were shown as percent of the total radioactivity added (Fig. 1). The ^{125}I-labelled lipase was adsorbed on every hydrophobic glass beads in a similar rate, but the esterase showed only a weak adsorption. These results

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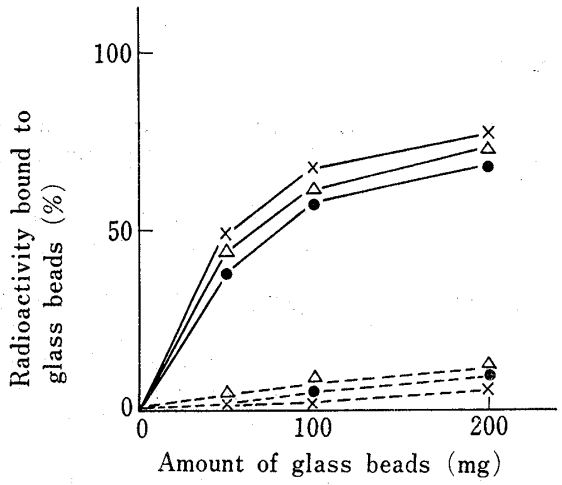

Fig. 1. Adsorption of *Chromobacterium* Lipase and Liver Esterase on Hydrophobic Glass Beads

Various amounts of glass beads which were coated with olive oil (×), liquid paraffin (●) or silicone oil (△) were added to the solution of ^{125}I-labelled *Chromobacterium* lipase (——) and liver esterase (------). After mixing well, the mixture was centrifuged and the glass beads were taken to assay the adsorbed radioactivity.

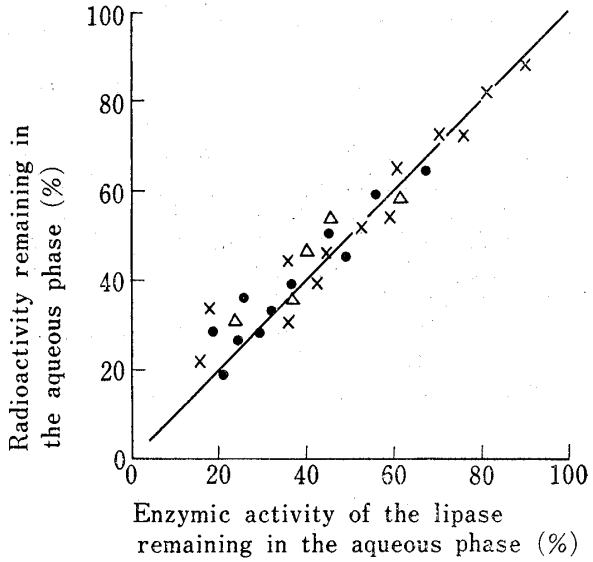

Fig. 2. Adsorption of the Enzymic Activity and Radioactivity on Hydrophobic Glass Beads

Glass beads which were coated with olive oil (×), liquid paraffin (●) or silicone oil (△)were added to the solution of *Chromobacterium* lipase. After the separation of glass beads, enzymic activity and radioactivity remaining in the aqueous phase were assayed.

were in conformity with that of the previous papers[12] and revealed the difference of the surface property between lipase and esterase. If the adsorption of lipase is due to the denaturation of the enzyme resulting from ^{125}I-labeling processes, an erroneous result will be led. Therefore, to confirm the identity of the behavior between ^{125}I-labelled lipase and non-labelled lipase, the same experiment was carried by varying the amount of hydrophobic glass beads and enzyme. The enzymic activity and radioactivity remaining in the aqueous phase were assayed and shown as percent of the added enzyme (Fig. 2). The results indicated that the enzymic activity and radioactivity were directly proportional each other and it was elucidated that the ^{125}I-labelled and non-labelled lipases had the same property with respect to the adsorption on hydrophobic surfaces. The identity between both enzymes were also confirmed in the disc electrophoresis. The ^{125}I-labelled enzyme solution, in which bovine serum albumin was contained, was electrophoresed and the radioactivity of the sliced gel was assayed (Fig. 3). The most of the radioactivity was found at a protein band which was electrophoresed in the same position of that of the native *Chromobacterium* lipase, and the other protein bands which belong to bovine serum albumin or its contaminants contained only a minute amount of radioactivity. From the above results, it was proved that the lipase was labelled specifically by [^{125}I] iodide and the labelled enzyme had same properties as those of native enzyme.

Adsorption of the Lipase on Hydrophobic Glass Beads

The adsorption of the lipase on the various hydrophobic glass beads was treated, as a function of the variation amount of surface area, in the Lineweaver-Burk's plot (Fig. 4). The intercept points corresponding to the $1/V_{max}$ and $1/K_m$ of an enzymic reaction were 2.2—2.7×10^{-5} cpm^{-1} and 1.5—2.0×10^{-2} cm^{-2} respectively. The former value conformed with the reciprocal of added radioactivity and the latter should be assigned the reciprocal of dissociation constant of adsorbed enzyme at the surface. The results from substrate and hydrophobic surface were very similar each other, and it has become apparent that the interaction between the lipase and hydrophobic surface would be closely related to that between the lipase and its substrate. And the other experiment that the adsorption of the ^{125}I-labelled lipase

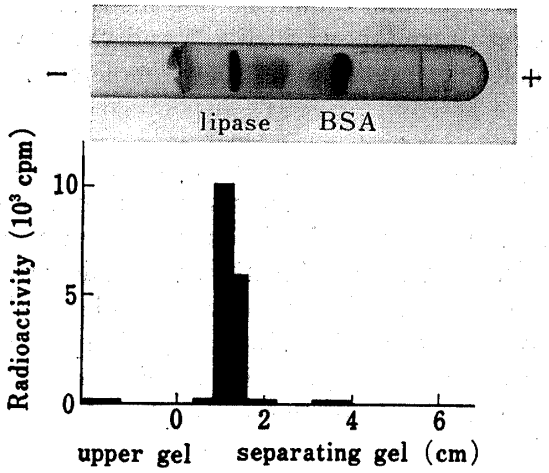

Fig. 3. Disc Electrophoresis of ^{125}I-Labelled *Chromobacterium* Lipase and Radioactivity in the Gel

The solution of ^{125}I-labelled *Chromobacterium* lipase containing 0.007% of bovine serum albumin (BSA) was electrophoresed by the polyacrylamide gel (pH 9.4) at a constant current of 3 mA/tube for 90 min. The gel was sliced and the radioactivity was assayed.

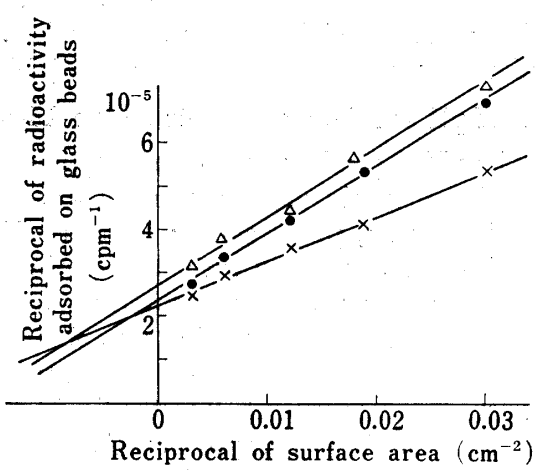

Fig. 4. Lineweaver Burk Plot of the Adsorption of *Chromobacterium* Lipase on Hydrophobic Glass Beads

Various glass beads which were coated with olive oil (×), liquid paraffin (●) or silicone oil (△) were added to the enzyme solution and mixed well. After centrifugation, the supernatant was separated out and the radioactivity was assayed.

on the surface was inhibited competitively by the addition of the native lipase (data was not shown) indicated the identity of the ^{125}I-labelled and native lipase.

The adsorption of the lipase on the surface was also treated by the Langmuir adsorption isotherm. The molar area of binding sites of the lipase on the surface is designated by a and the dissociation constant is K. In this experiment, the surface area of the glass beads (A) was 170 cm^2 and volume of the solution (v) was 2.0 ml. The enzyme adsorbed on the surface (E_{ad}) can be expressed as a function of the enzyme in solution (E_{sol}) by the following equation:

$$E_{sol}/E_{ad} = K \cdot V \cdot a/A + E_{sol} \cdot a/A$$

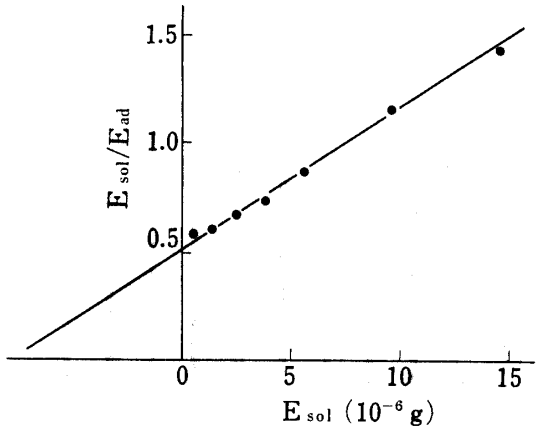

Fig. 5. Langmuir's Plot of the Adsorption of *Chromobacterium* Lipase on Glass Beads Coated with Liquid Paraffin

^{125}I-Labelled *Chromobacterium* lipase was dissolved in 10 mM phosphate buffer (pH 7.0) at various concentrations and 100 mg of glass beads coated with liquid paraffin were added to the enzyme solution. After centrifugation, the amount of the lipase in solution (E_{sol}) and lipase which adsorbed on glass beads (E_{ad}) were assayed.

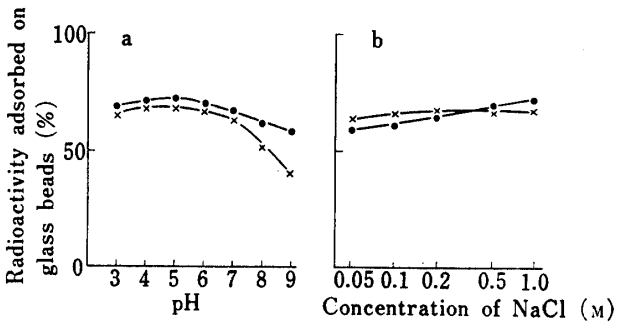

Fig. 6. Effect of pH (a) and NaCl (b) on the Adsorption of *Chromobacterium* Lipase

The ^{125}I-labelled lipase was dissolved in various buffer solution (pH 3—7; McIlvaine, pH 8 and 9; Tris-HCl buffer) or 10 mM phosphate buffer (pH 7.0) containing NaCl. The enzyme solution and 100 mg of glass beads coated with olive oil (×) or liquid paraffin (●) were mixed, and the radioactivity in the aqueous phase was counted.

Thus, by plotting E_{sol}/E_{ad} as a function of E_{sol}, a straight line should be obtained, the slope and intercept of which should yield a and K. The result was shown in Fig. 5 and values were calculated to be $a=2.7\times10^{11}$ cm^2/mol (4500 Å^2/molecule) and $K=1.4\times10^{-10}$ mole/cm^3.

Effect of pH and Inorganic Salts on the Adsorption

The pH dependence of the adsorption of the lipase on glass beads which was coated with olive oil and liquid paraffin were observed. Two ml of various buffer solution (pH 3—7; McIlvaine buffer, pH 8 and 9; 0.1M Tris–HCl buffer) which contained about 10^4 cpm of ^{125}I-labelled lipase was mixed with 100 mg of glass beads. After centrifugation, the radioactivity in the supernatant was assayed and the amount of the adsorbed enzyme was calculated (Fig. 6a). The maximum adsorption was observed at pH 5 and considarable adsorption was also found at every range of pH 4—9. But the adsorption on olive oil at mild alkaline conditions was somewhat less than that on the liquid paraffin and it should be the reason that the surface of olive oil had been charged negatively by free fatty acid which contained in the substrate or released by enzymic reaction. Effect of NaCl on the adsorption of the lipase was also observed. With the concentration of NaCl dissolved in 10 mM phosphate buffer (pH 7.0) increased from 0.05M to 1.0M, the radioactivity adsorbed on glass beads which was coated with olive oil or liquid paraffin was not decreased but increased about 5—10% (Fig. 6b). And effects of the other inorganic salts such as KCl and Na_2SO_4 were similar to that of NaCl.

Effect of Surface Active Agents and Water-miscible Organic Solvents

Effect of sodium dodecylsulfate which is an anionic detergent on the adsorption of the lipase on glass beads was observed. The results were shown in Fig. 7a and it was elucidated that the adsorption of the lipase was strongly inhibited by sodium dodecylsulfate at the concentration of 10^{-3}M. Therefore 10^{-3}M sodium dodecylsulfate did not inactivate the *Chromobacterium* lipase, it is considered that the inhibition of the lipase activity for the hydrolysis of olive oil emulsion resulted from the depression of the interaction between the native enzyme and hydrophobic surface. Similar inhibition of adsorption of the lipase on hydrophobic glass beads was also observed by polyethylene glycol lauryl ether which is a nonionic detergent. Water-miscible organic solvents such as dioxane (Fig. 7b) and acetonitrile inhibited the adsorption and the concentration of organic solvents became 20% the lipase adsorbed on

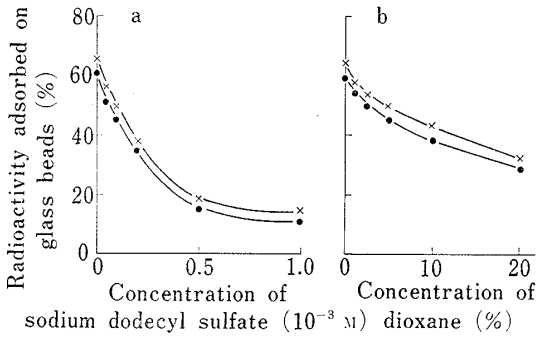

Fig. 7. Effects of Sodium Dodecyl Sulfate (a) and Dioxane (b) on the Adsorption of *Chromobacterium* Lipase

The ^{125}I-labelled lipase was dissolved in 10 mM phosphate buffer (pH 7.0) containing various concentration of sodium dodecyl sulfate or dioxane. After the enzyme solution and 100 mg of glass beads coated with olive oil (×) or liquid paraffin (●) were mixed, the radioactivity of the supernatant was counted.

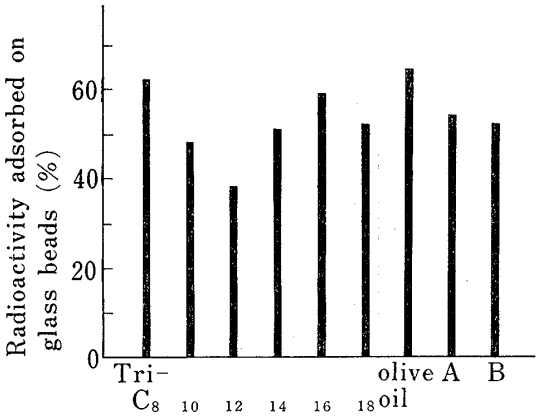

Fig. 8. Adsorption of *Chromobacterium* Lipase on Glass Beads Coated with Various Triglyceride and Hydrophobic Materials

^{125}I-Labelled *Chromobacterium* lipase dissolved in 10 mM phosphate buffer (pH 7.0) and 100 mg of glass beads coated with various triglycerides or hydrophobic materials (A: silicone oil, B: liquid paraffin) were mixed. After centrifugation, the supernatant was taken to assay the radioactivity.

glass beads were reduced to about one-half at that in the buffer solution. The lipase was also stable in the presence of these organic solvents.

Adsorption on Triglycerides

Triglycerides were coated on glass beads with the same method of that for hydrophobic materials. The lipase solution dissolved in 10 mM phosphate buffer (pH 7.0) and 100 mg of coated glass beads were mixed well at 37° and was immediately cooled to 4°. After centrifugation under cooling, the radioactivity in supernatant was assayed and the adsorbed enzyme was calculated (Fig. 8). The amount of adsorbed lipase on the various triglycerides were varied within about 40—60%. There is no relationship between the pattern of the adsorption and the substrate specificity[18] of the lipase for each triglycerides.

Table I. Adsorption of *Chromobacterium* Lipase on Glass Beads and the Activity for the Hydrolysis of *p*-Nitrophenyl Acetate

Reaction system	Esterase activity (10^{-3} U)	Radio-activity (cpm)	Specific activity (10^{-6} U/cpm)
Lipase+substrate	63.4	17560	3.6
Lipase+glass beads +substrate	111.4	17560	6.3
Lipase+glass beads after separation			
glass beads+substrate	90.0	8141	11.1
supernatant+substrate	10.2	3587	2.8

The hydrolysis of *p*-nitrophenyl acetate by *Chromobacterium* lipase were assayed spectrophotometrically in a cuvette at 30° in the presence or absence of 100 mg of glass beads which coated with liquid paraffin. The radioactivity in the reaction mixture was also assayed.

Hydrolysis of *p*-Nitrophenyl Acetate

The hydrolysis of olive oil emulsion by the lipase from *Chromobacterium* was inhibited by the addition of the emulsion of liquid paraffin.[11] The lipase also has an activity for the hydrolysis of *p*-nitrophenyl acetate and it was studied whether the hydrolysis is inhibited or not by the added glass beads which was coated with liquid paraffin. The result was shown in Table I. The reaction was carried in a cuvette at 30° under following up the release of *p*-nitrophenol, and the rate of hydrolysis was not inhibited but accelerated 2-times by adding the glass beads. Therefore all of the added enzymes was not always adsorbed on glass beads in the reaction mixture and the real activity of the adsorbed enzyme was not clear. The following experiment was accordingly carried out. The lipase solution and glass beads was mixed and the mixture was centrifuged. The glass beads on which a part of added enzyme was adsorbed, and supernatant was separated. After the glass beads was once washed with buffer solution, the enzymic activity and radioactivity was assayed. As the result, it was found that the lipase adsorbed on glass beads was activated about 3-fold.

Discussion

In a previous paper of this series,[12a] we had shown that the activity of lipases from various origins was inhibited competitively by water-insoluble organic solvents independent of its chemical structures and presumed that lipases had a property of adsorption on hydrophobic interfaces but esterase had no affinity on interfaces. Comparative studies with the adsorption on air-water interface[12b] were also carried out. From the result, it was confirmed that lipases were adsorbed specifically on surface and had a property similar to those of surface active

18) M. Sugiura and M. Isobe, *Chem. Pharm. Bull.* (Tokyo), 23, 1226 (1975).

agents. In the present paper, it was also confirmed that the lipase from *Chromobacterium* had a property of adsorption on hydrophobic interface but esterase had no such property. The surface properties of the lipase were studied and it was elucidated that the adsorption of the lipase follows to the Langmuir adsorption isotherm which is applicable to the monomolecular adsorption and the lipase adsorbed on liquid paraffin occupies 4500 $Å^2$ per molecule and has a dissociation constant 1.4×10^{-7}M. These properties were similar to those from the kinetical studies on pancreatic lipase.[6] The adsorption of the lipase was not affected largely by various pH of buffer solution or high concentration of inorganic salts that should influences on the interaction with ionic bonds or hydrogen bonds. On the other hand, surface active agents and water-miscible organic solvents that decrease the hydrophobic interaction inhibited the adsorption significantly. From these results, it was elucidated that the hydrophobic bond play a major role in the adsorption of the lipase on substrate and hydrophobic surfaces. The hydrophilic bond should be only adjunctive, but the results that the adsorption on olive oil was inhibited at alkaline conditions shows the possibility of the negative charge in the enzyme protein may play a role in the process of the release of free fatty acid. The optimum pH of adsorption was different from the optimum pH of enzyme action or from the isoelectric point of the enzyme. The lipase adsorbed on the surface of liquid paraffin was not inhibited but activated about 3-fold. Desnuelle, *et al.*[7] reported that the reaction by pancreatic lipase was activated 500-fold in the presence of siliconized glass beads and made an assumption for the characteristic action of lipase on aggregated substrate. Therefore the activation observed in this experiment was very small, it was elucidated that the reaction model of pancreatic lipase could not applicable for the other lipases generally and the more common property of the lipase is the specific adsorption on surfaces. By the property of adsorption, lipases will be concentrate on the surface of the aggregated substrate and situate on a position near to the substrate molecule sterically and the catalytic action will accelerate. But the lipase did not adsorb specifically on the molecule of substrate in the form of molecular dispersion, and esters of water soluble were not or very slowly hydrolyzed by lipase. As esterase has not the property of adsorption on surface, the enzyme will hydrolyze the water soluble substrates and not the water insoluble substrates.