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Studies on the Mechanism of Lipase Reaction. I.[1] Inhibition of Lipase Activity by Emulsion of Organic Solvents

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The hydrolysis of olive oil by the lipase from *Chromobacterium* was inhibited by the addition emulsion of organic solvents. This inhibition was essentially competitive and was observed with emulsion of any organic solvent used in this study, independently of their chemical structure. The competitive inhibition was also observed on the emulsion prepared with various emulsifiers and on the emulsion in which an organic solvent was dissolved. Lipases from porcine pancreas, *Candida* sp. and *Pseudomonas* sp. showed the same behavior. From these results, it was presumed that lipases had a characteristics of adsorption at the interface. This assumption was also supported by the experiment of adsorption to emulsions. On the other hand, the esterase of chiken liver was not inhibited by the emulsion of any organic solvents tested in this study and was not adsorbed to the emulsion.

A possible hypothesis on the mechanism of lipase reaction was discussed in relation to these properties.

Lipase (glycerol ester hydrolase, EC 3.1.1.3) hydrolyzes esters, which are insoluble in water, at the interface between water and lipid.[3] On the other hand, esterase (carboxylic ester hydrolase, EC 3.1.1.1) reactions take place in a homogeneous system and only soluble esters are hydrolyzed.[4] The usual kinetic methods are applicable to esterase reactions[5] but not to lipase reaction. Benzonana and Desnuelle[6] have pointed out the importance of interface area rather than the bulk concentration of insoluble esters, and have treated the lipase reaction by the Michaelis-Menten equation. The lipase reaction is affected by various chemicals, which act not only on the enzyme but also on the substrate. For example, bile salts are an activator of pancreatic lipase[7] and *Mucor* lipase,[8] and is an inhibitor to many other lipases.[9] Free fatty acids[10] and *n*-alcohols[11] inhibit the lipase reaction.

In the previous paper,[12] we reported the enzymic properties and substrate specificity of lipase from *Chromobacterium*. As the enzyme is now pure, stable, and have a unique substrate specificity, it is a good material for studies on the lipase reaction.

In order to clarify the mechanism of lipase reaction, the inhibitory effect of various emulsions on the activity of lipase from *Chromobacterium* and other lipases, was investigated.

1) This forms Part CI of "Studies on Enzyme," proceding paper Part C: M. Sugiura and A. Ito, *Chem. Pharm. Bull.* (Tokyo), "submitted."
2) Location: *Ueno-sakuragi 1-chome, Taito-ku, Tokyo, 110, Japan.*
3) B. Entressangles and P. Desnuelle, *Biochim. Biophys. Acta*, **159**, 285 (1968).
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Material and Method

Enzymes——The lipase B of *Chromobacterium* was prepared as described in the previous repotr.[12] Pancreatic lipase (fraction L_B) was prepared as described by Verger, *et al.*[13] The lipases from *Candida* and *Pseudomonas* were purified by the method of Tomizuka, *et al.*[14] and Sugiura, *et al.*[15] respectively. Liver esterase from chicken was prepared by the procedure as described for pig liver esterase.[16]

Chemicals——Poly vinyl alcohol (PVA)-117 and PVA-210 were kindly supplied by Kurashiki Rayon Co., Ltd. Organic solvents and other reagents were commercial products and obtained either from Tokyo Kasei Co., Ltd. or Wako Chemicals Co., Ltd.

Assay of Lipase and Esterase Activities——The free fatty acid was determined by the method of Dole and Meinertz.[17] As the substrate of lipase, olive oil emulsion was prepared as follows: Olive oil (25 ml) and 75 ml of aqueous PVA solution (2% solution of a 9:1 mixture of PVA-117 and PVA-210) were sonicated for 6 min. Aqueous solution of Tween 20 was used as the substrate for the esterase. The assay of the activity of esterase and lipase was as follows. The reaction mixture contained 1 ml of substrate, 0.5 ml of McIlvaine buffer (pH 7.0), 0.5 ml of organic solvent emulsion or water, and 0.5 ml of enzyme solution. After incubation at 37° for 20 min, 5 ml of a mixture of isopropanol, heptane, and 2N H_2SO_4 (40:10:1, by vol.) was added to terminate the reaction, followed by the addition of 2 ml of water and 3 ml of heptane. After vigorous stirring, the mixture was allowed to stand for 20 min. Three ml of the upper layer was taken and then titrated with 0.01M ethanolic KOH solution, using Thymol Blue as an indicator.

Results

Inhibition of *Chromobacterium* Lipase by the Emulsion of Organic Solvents

Liquid paraffin emulsion in 2% PVA solution was prepared by sonication as described above. Effect of this emulsion on the rate of hydrolysis of olive oil was assayed (Fig. 1).

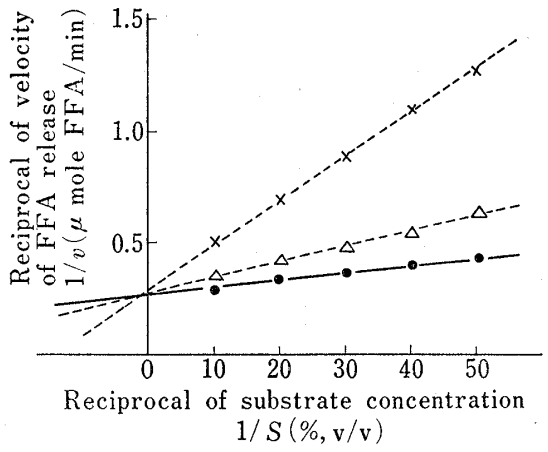

Fig. 1. Inhibition of Activity of the Lipase from *Chromobacterium* by the Emulsion of Liquid Paraffin

Lipase activity was assayed using the emulsion of olive oil as a substrate in the presence 4% (△), 10% (×), or absence (●) of the emulsion of liquid paraffin. Results are expressed as reciprocal of substrate concentration (%, v/v) and velocity of free fatty acid (FFA) release.

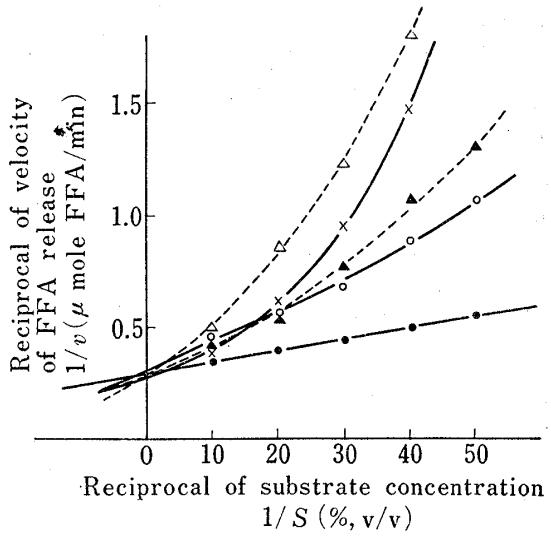

Fig. 2. Inhibition of Activity of the Lipase from *Chromobacterium* by the Emulsion of Various Organic Solvents

Lipase activity was assayed using the emulsion of olive oil as a substrate in the presence of 4% of emulsion of benzene (△), toluene (▲), chloroform (○), carbon tetrachloride (×), or in their absence (●). Results are expressed as reciprocal of substrate concentration (%. v/v) and velocity of free fatty acid (FFA) release.

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The reaction of *Chromobacterium* lipase was inhibited competitively by the emulsion of liquid paraffin. In order to elucidate the mechanism of this inhibition, similar experiments were carried with emulsions of benzene, toluene, chloroform, and carbon tetrachloride (Fig. 2).

The activity of the lipase was inhibited by emulsions of any of the organic solvents tested, particularly in the presence of a low concentration of the substrate. The Lineweaver-Burk plot showed a hyperbolic curve. However, the inhibition was essentially competitive in the range of higher substrate concentration.

The effect of emulsifier was also investigated. Acacia (1%), gelatin (0.2%), and sodium oleate (2 mM) solutions were used as the emulsifier. After addition of Acacia, Lineweaver-Burk plot of the hydrolysis of olive oil in the presence of liquid paraffin became a straight line and the inhibition was found to be competitive (Fig. 3). Similar results were obtained with gelatin (data not shown). However, the rate of hydrolysis of olive oil in the presence of sodium oleate revealed nonlinearity in the Lineweaver-Burk plot, and at a high concentration of the substrate, velocity of hydrolysis became slow. The reason for this was considered to be the unstability of the emulsion of substrate and it was observed that some oil phase separated out during the incubation at a high concentration of the substrate, but extrapolated lines from low concentration of substrate showed that the inhibition was competitive.

Finally, the effect of organic solvents which are dissolved in the substrate was investigated. The results varied widely with solvents tested (Fig. 4). As emulsions of each concentration were prepared individually, the surface concentration and state of the emulsion were different but the average value showed that the inhibition by the organic solvents tested was competitive.

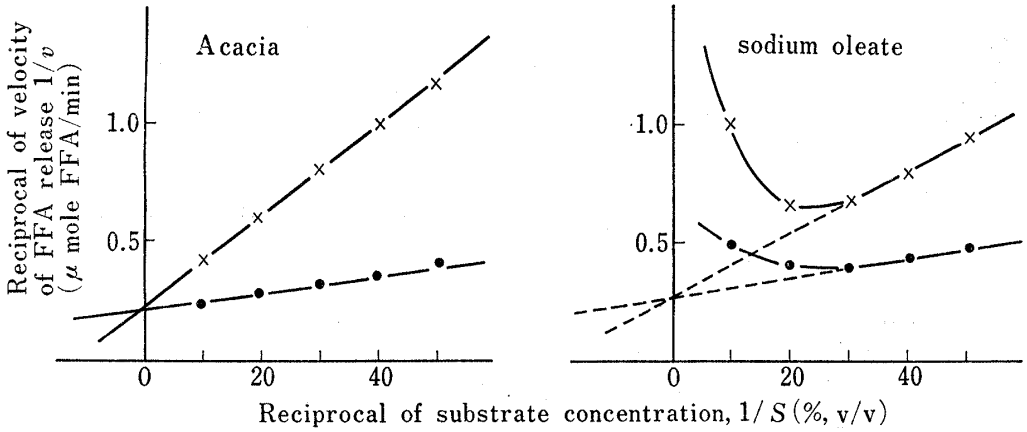

Fig. 3. Effect of Emulsifier on the Inhibition of Activity of the Lipase from *Chromobacterium* by the Emulsion of Liquid Paraffin

Olive oil and liquid paraffin emulsions were prepared by sonication using 1% Acacia solution and 2 mM sodium oleate as an emulsifier. Lipase activity was assayed in the presence of 1% liquid paraffin (×) or in its absence (●). Results are expressed as reciprocal of substrate concentration (%, v/v) and velocity of free fatty acid (FFA) release.

Effect of Emulsions on Other Lipases and Esterase

Effects of emulsions of organic solvents on pancreatic lipase, *Candida* lipase, and *Pseudomonas* lipase were tested to see whether the results shown above is specific for the lipase of *Chromobacterium*. Pancreatic lipase and *Candida* lipase were competitively inhibited by the emulsion of liquid paraffin confirming the results of lipase from *Chromobacterium* (Fig. 5). Similar results were obtained with *Pseudomonas* lipase (data not shown).

The effect of emulsion on the esterase of chicken liver was also examined (Fig. 6). The hydrolysis of Tween 20 by the esterase was not inhibited by emulsions in any condition. The hydrolysis of *p*-nitrophenyl acetate by the esterase was also not inhibited by emulsions of organic solvents (data not shown).

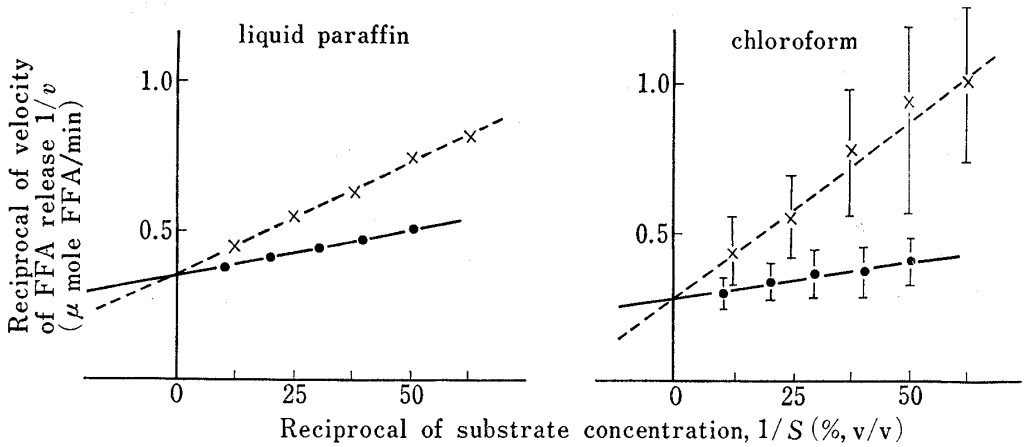

Fig. 4. Inhibitory Effect of the Emulsion of Organic Solvents Dissolved in Substrate on the Activity of Lipase from *Chromobacterium*

Olive oil and organic solvent were mixed and emulsified with 2% PVA solution. Lipase activity was assayed in the presence of 0.8% organic solvent (×) or in its absence (●). Results are expressed as reciprocal of substrate concentration (%, v/v) and velocity of free fatty acid (FFA) release.

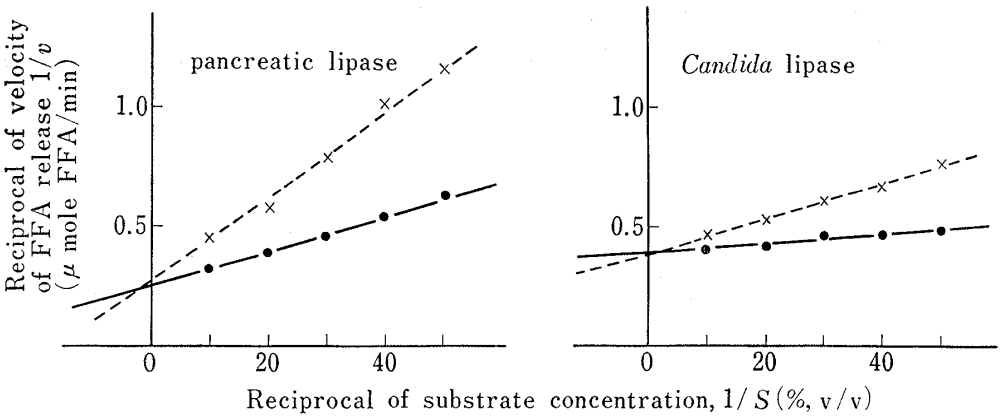

Fig. 5. Inhibition of the Activity of Lipases from Porcine Pancreas and *Candida* by the Emulsion of Liquid Paraffin

Lipase activities were assayed using the emulsion of olive oil as a substrate in the presence of 4% of liquid paraffin (×) or in its absence (●). Results are expressed as reciprocal of substrate concentration (%, v/v) and velocity of free fatty acid (FFA) release.

Adsorption of Enzymes on Emulsions

Possible adsorption of enzymes on emulsions was investigated. After incubation of the enzyme with the emulsion for 20 min at 37° in McIlvaine buffer (pH 7.0), the mixture was centrifuged at 12500×***g*** for 20 min. The activity remaining in the aqueous phase was assayed (Fig. 7).

The activity of the lipase that disappeared from the aqueous phase increased with the concentration of emulsion added. In the emulsion phase of olive oil separated from the aqueous phase, almost all activity that disappeared from the aqueous phase was found. On the other hand, in emulsions of organic solvents, only 20% of the activity was observed. Other lipases used in this study showed a behavior similar to that of lipase of *Chromobacterium*. The adsorption of lipases to emulsions was also supported by the experiment that lipases were not denaturated by saturated solution of the organic solvent and that the disappearance of the activity of lipases from the aqueous phase did not depend on incubation time. On the other hand, the activity of chicken liver esterase was not inhibited nor disappeared by the emulsion of organic solvent.

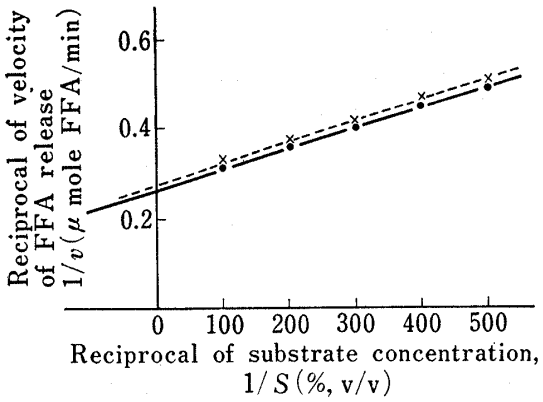

Fig. 6. Effect of the Emulsion of Liquid Paraffin on the Activity of Esterase from Chicken Liver

Esterase activity was assayed using Tween 20 as a substrate in the presence of 4% (×) or absence (●) of the emulsion of liquid paraffin. Results are expressed as reciprocal of substrate concentration (%,v/v) and velocity of free fatty acid (FFA) release.

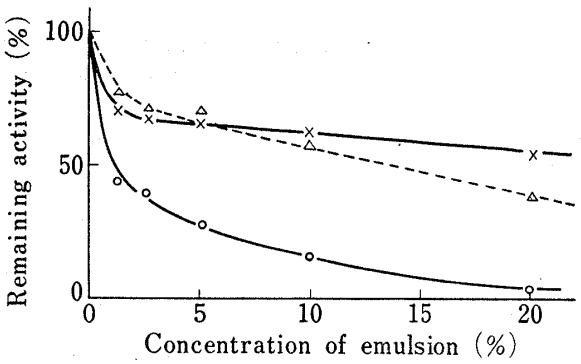

Fig. 7. Adsorption of the Lipase from *Chromobacterium* on the Emulsion

The lipase from *Chromobacterium* was dissolved in McIlvaine buffer (pH 7.0) and the emulsion of olive oil (○), liquid paraffin (△), or benzene (×) was mixed. After centrifugation at 12500×*g* for 20 min, the activity remaining in the aqueous phase was assayed.

Discussion

The result in the present experiments have shown that the activity of lipase was inhibited by emulsions of various organic solvents. The Lineweaver-Burk plot of hydrolysis of olive oil in the presence of liquid paraffin showed a straight line suggesting a competitive inhibition. In the presence of other organic solvents, such as chloroform or benzene, the Lineweaver-Burk plots showed hyperbolic curves but the inhibition was considered to be competitive. Some changes in interfacial properties of emulsion by dissolving of organic solvents might change kinetic properties of the enzyme. The competitive inhibition was also observed on the emulsion prepared with other emulsifier and on the substrate in which organic solvent was dissolved. From these results, it is presumed that lipases was adsorbed on the oil-water interface and that this property is non-specific for chemical structure of the oils tested. Further, adsorption of lipase to the interface was suggested by the results of Fig. 7. Similar experiments had been reported[11,18] on the inhibition of lipase by alcohols, concluding that their inhibitory effect is due to the adsorption of alcohols to the substrate.

On the other hand, esterase was not inhibited by the emulsion of any organic solvent tested in this study and was not adsorbed to the emulsion.

A hypothesis of the mechanism of lipase reaction is presented. Lipases have the nature of being adsorbed on the interface similar to surface active agents, and the enzyme molecule accumulates at a position near the substrate. By this process, a probability for the formation of enzyme-substrate complex may be accelerated, as well as the relationship between intermolecular reaction and intramolecular reaction.[19] The property of adsorption to the interface may differentiate the action of a lipase which hydrolyzes water-insoluble esters from that of an esterase which hydrolyzes soluble esters.

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