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Purification of Microbial Lipases by Glass Beads coated with Hydrophobic Materials[1]

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The lipase of *Chromobacterium viscosum* in the crude enzyme preparation was specifically adsorbed on glass beads coated with various hydrophobic materials. The enzyme adsorbed on siliconized glass beads was not denatured and eluted with 0.1% Triton X-100 and other detergents.

The adsorptive nature of lipase on hydrophobic glass beads was applied to its purification by a one-step process. As the result, lipases of *Chromobacterium*, *Pseudomonas*, *Candida*, and *Rhizopus* were purified 300-, 670, 160-, and 110-fold, respectively. The lipases purified from *Chromobacterium* and *Candida* were examined by disc electrophoresis, and they were judged to be of high purity. Purification of the lipase from *Chromobacterium* by a column method with siliconized glass beads confirmed that this method allowed about 1000-fold purification.

Keywords——lipase; purification; adsorption on surface; hydrophobic affinity; *Chromobacterium viscosum*; affinity chromatography

There have been many reports on the purification of lipase (glycerol ester hydrolase, EC 3.1.1.3) from animal tissues, plants, and microorganisms but most of the purification procedures used in these studies were nonspecific technique such as ammonium sulfate precipitation, gel filtration, ion-exchange chromatography, and so on. Recently affinity chromatography is becoming applicable to the purification of lipases. Since lipases hydrolyze water-insoluble esters preferentially and have a high affinity to adsorb on the interface of water-insoluble esters, Fielding[3] used the plasma lipoprotein in the purification of lipoprotein lipase and obtained a highly pure enzyme by solubilization from the enzyme-substrate complex. Varine, *et al.*[4] found that agarose-linked dioleoylglycerol was an appropriate substance for the affinity chromatography of adipose tissue triacylglycerol lipase. Kosugi and Suzuki[5] tried to use the aliphatic amine-Sepharose, considering that it might serve as an analog of the substrate, for the purification of lipases from *Pseudomonas* sp., *Candida* sp. and hog pancreas. It was observed that heparin-Sepharose[6] and concanavalin A-Sepharase,[6b,c] having a specific interaction with lipoprotein lipase, were useful for the affinity chromatography.

We have found that lipases are adsorbed not only on the interface of substrate but also on various interfaces independent of the molecular structure of the material such as oil-water,[7] air-water,[8] and siliconized glass beads-water.[9] The present paper describes the application of glass beads coated with hydrophobic materials for the purification of microbial lipases.

1) This paper forms Part CXXVII of "Studies on Enzymes" by M. Sugiura.
2) Location: *1432-1 Horinouchi, Hachioji, Tokyo, 192-03, Japan.*
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Experimental

Enzymes——The crude enzyme preparation of *Chromobacterium viscosum*, *Pseudomonas fluorescens*, *Candida cylindracea*, and *Rhizopus* sp. were obtained from Toyo Jozo, Tokyo, Amano Pharmaceutical, Nagoya, Meito Sangyo, Nagoya, and Osaka Saiken Co., Osaka, respectively.

Materials——Silicone DC QF-1 and Silicone OV-1 produced by Applied Science Lab. Inc., U.S.A. and silicone grease produced by Dow Conring Co., U.S.A. were used in the experiment. Triton X-100 and Adekatol SO-120 were obtained from Wako Pure Chemical Ind., Tokyo, and Asahi Denka Co., Tokyo, respectively. Other reagents were the products of Tokyo Kasei Co., Tokyo.

Coating of Hydrophobic Materials on Glass Beads——Glass beads, size of which was defined through filters (80—100 mesh), were soaked in chromic acid cleaning solution for a day and, after rinsing with distilled water, were dried in vacuum. One gram of the glass beads and 1 ml of 2% acetone solution of hydrophobic materials were mixed and the solvent was removed by evaporation.

Assay of Lipolytic Activity and Protein——The activity of lipase was assayed as reported in a previous paper[10] using olive oil emulsion as the substrate. One unit of lipase was defined as the amount of the enzyme which liberates 1 μmole of free fatty acid per minute under the assay condition. The amount of protein was determined according to the method of Lowry, *et al.*[11]

Disc Electrophoresis——Disc electrophoresis was carried out with 7.5% polyacrylamide gel at pH 9.4 and glycine–Tris buffer according to the method of Davis,[12] at a constant current of 4 mA/tube for 90 min. Protein was stained with Amido Black 10B. Lipolytic activity in the gel was assayed as follows; After slice in 1 mm thickness, the gel was minced and extracted with 1 ml of McIlvaine buffer (pH 7.0). The solution was kept at 4° over night and enzymic activity was assayed by the standard method.

Results

Adsorption of Lipase on Hydrophobic Glass Beads

Effect of coating materials on glass beads was studied with respect to the adsorption of lipase from *Chromobacterium viscosum*. One gram of coated glass beads and 10 ml of 0.2% crude enzyme solution were mixed well, the mixture was filtered and the enzymic activity remaining in the aqueous phase was assayed. The results are shown in Table I. The lipase

Table I. Effect of Coating Material of Glass Beads on the Adsorption of *Chromobacterium* Lipase

Coating material	Remaining activity in the aqueous phase (%)
None	85.8
γ-Aminopropyltriethoxysilane	38.1
Stearic acid	31.9
Silicone DC QF-1	10.4
Silicone OV-1	14.4
Silicone grease	9.7

from *Chromobacterium* was adsorbed on all glass beads coated with hydrophobic materials, especially on siliconized glass beads. In the following experiments, Silicone DC QF-1 was used as the coating material.

Effect of pH and ionic strength of the enzyme solution on the adsorption of lipase were also examined. Its results showed that pH between 3 to 9 (pH 3—7, McIlvaine buffer; pH 8 and 9, Tris-HCl buffer) and high concentration (1 M) of NaCl did not significantly affect the adsorption but the addition of $BaCl_2$ or $CaCl_2$ increased the adsorption of the lipase slightly. Relationship between the amount of glass beads and the adsorbed enzyme is shown in Fig. 1. From these results, it is evident that adsorption depends on the surface area of the siliconized glass beads.

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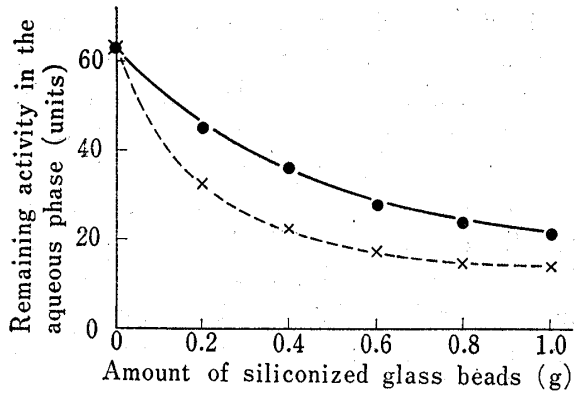

Fig. 1. Adsorption of *Chromobacterium* Lipase on Siliconized Glass Beads

Various amount of glass beads coated with Silicone DC QF-1 and 3.0 ml of 0.5% crude enzyme solution were mixed well, filtered, and the lipase activity remaining in the aqueous phase was assayed. Size of glass beads; —●—: 80—100 mesh, --- x ---: 100—200 mesh.

Table II. Effect of Concentration of Detergents on the Desorption of *Chromobacterium* Lipase from Siliconized Glass Beads

Concentration of detergent (%)	Desorbed enzyme (%)	
	Triton X-100	Adekatol SO-120
0.2	87.7	70.3
0.1	99.5	84.1
0.05	98.2	67.8
0.02	72.4	56.2
0.01	14.8	18.1
0.005	1.5	1.3
None	0.0	0.0

Desorption of Lipase on Siliconized Glass Beads

Desorption of the lipase adsorbed on the siliconized glass beads was examined with various detergents, and among them Triton X-100 and Adekatol SO-120, which are nonionic detergents, gave a good yield. The lipase activity eluted from the glass beads at various concentrations of the detergent is shown in Table II. The most suitable concentrations of both detergent for elution were found to be around 0.1%, and higher concentrations of the detergent were considered to cause inactivation of the enzyme, decreasing the yield of the eluted lipase activity.

Purification of Lipase by Siliconized Glass Beads

Purification of microbial lipases was attempted with siliconized glass beads. Two grams of crude enzyme preparation was dissolved in 200 ml of 10 mM phosphate buffer (pH 7.0) and the solution was centrifuged at 10000 rpm for 15 min. The supernatant and 80 g of siliconized glass beads were mixed well for 30 min, the mixture was filtered, and the glass beads on which lipase was adsorbed were collected. After washing with distilled water, the glass beads on the filter paper were treated with 100 ml of 0.1% Triton X-100 solution and the adsorbed lipase was eluted. The eluate was concentrated to about 5 ml by ultramembrane filtration and the solution was lyophilized. The detergent contained in the lyophilized preparation was removed by extraction with ether. Its results are summarized in Table III. The lipase of *Chromobacterium* was purified from the crude enzyme about 300-fold with a recovery of 34.8% of the enzymic activity. Also lipases of *Candida*, *Pseudomonas*, and *Rhizopus* were purified about 160-, 670-, and 110-fold, respectively.

Table III. Purification of Microbial Lipases by the Siliconized Glass Beads

Origin of lipase		Total activity (units)	Specific activity (units/mg)	Yield (%)
Chromobacterium	Crude	9.65×10^3	4.83	
	Purified	3.36×10^3	1.54×10^3	34.8
Candida	Crude	2.30×10^4	1.15×10	
	Purified	7.27×10^3	1.82×10^3	31.6
Pseudomonas	Crude	4.34×10^3	2.17	
	Purified	1.13×10^3	1.45×10^3	26.0
Rhizopus	Crude	1.42×10^4	7.10	
	Purified	2.98×10^3	8.03×10^2	21.0

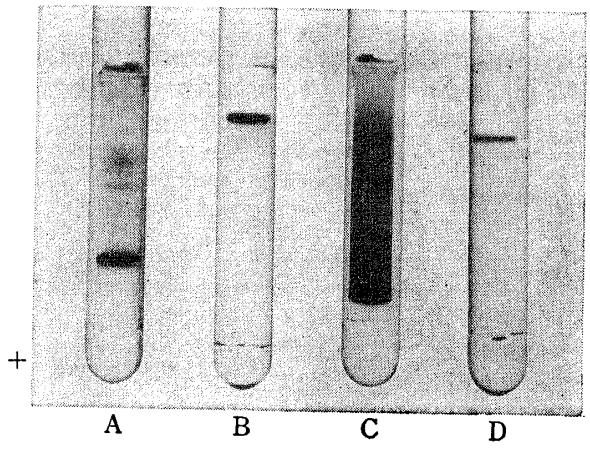

Fig. 2. Disc Electrophoresis of Lipases from *Chromobacterium* and *Candida*

Disc electrophoresis was carried out at a constant current of 4 mA/tube for 90 min with 7.5 % polyacrylamide gel (pH 9.4). Protein was stained with Amido Black 10-B. Crude (A) and purified (B) lipase preparation of *Chromobacterium viscosum*, crude (C) and purified (D) lipase preparation of *Candida cylindracea*.

Further purification of *Chromobacterium* lipase was attempted by the column method. Siliconized glass beads (20 g) suspended in 50% of methanol were packed in a column (3×10 cm), the enzyme solution was charged into the column, and the column was washed with distilled water. The lipase was eluted with 0.1% Triton X-100 solution, and the eluate was collected in 10 ml fractions. By this means, recovery of the activity was increased to 58.3%. On the other hand, the specific activity of the most active fraction reached about 1000-fold higher than that of the crude enzyme.

The lipases purified from *Chromobacterium* and *Candida* by the batch method were examined by disc electrophoresis. From the results shown in Fig. 2, it is evident that both of purified enzyme were electrophoresed in a major protein band and a minor band. Also it was confirmed that both protein bands have a lipolytic activity.

Discussion

It is well known that lipases have a specific adsorptive tendency on the surface of water-insoluble substrates. We found that lipases had a high affinity not only for the substrates but also for various interfaces such as air-water,[8] oil-water,[7] and siliconized glass beads-water,[9] regardless of the molecular structure of the interfacial materials. This adsorption nature of lipases on siliconized glass beads was applied to the one-step purification of microbial lipases.

The lipase from *Chromobacterium* was adsorbed on the glass beads coated with Silicone DC QF-1, Silicone OV-1, or silicone grease, but not on the hydrophilic glass beads treated with γ-aminopropyltriethoxysilane. Amount of the lipase adsorbed on siliconized glass beads increased with the addition of glass beads and may have increased in proportion to the surface area. Adsorption of the enzyme was not affected significantly by pH or ionic strength of the enzyme solution.

The adsorbed enzyme was replaced and desorbed with various detergents. Nonionic detergents such as Triton X-100 and Adekatol SO-120 gave good yields, and there were several detergents among anionic and amphoteric detergents which gave good results. The enzyme activity eluted from the glass beads increased with the concentration of Triton X-100 and Adekatol SO-120, but above 0.2% of the detergents, the yield of the enzyme decreased. The reason for it may be due to the irreversible denaturation of the enzyme by detergents. Amount of the enzyme adsorbed and then eluted from the glass beads increased with the concentration of the crude enzyme solution added to glass beads.

It was found that this purification procedure with siliconized glass beads was effective and lipases in the crude enzyme preparations were highly purified. However, all lipases have a similar property of adsorption on glass beads and it was difficult to separate the lipases from each other in the same solution. Therefore, lipases purified by this method from *Chromobacterium*[10] and *Candida*,[13] in which lipases in multiple forms had been found, show-

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ed two protein bands in disc electrophoresis, and both protein bands had a lipolytic activity. The column method for the purification of lipases with siliconized glass beads was better than the batch method in terms of yield of enzymic activity and maximum specific activity.

In the previous work,[7-9] we had found that lipases were adsorbed on various interfaces and that the adsorption proceeded by the same mechanism as the formation of the enzyme-substrate complex. Therefore, various interfaces, independent of the molecular structure, were able to consider as an analog of the substrate, and the purification method with a column of siliconized glass beads was comparable to the affinity chromatography. There are many reports on the purification of lipase by affinity chromatography, but only a few of them had succeeded in high purification.[6,14] The purification method used in this study was more effective and simple than that of affinity chromatography, which was previously reported.[5] In addition to that, the preparation of siliconized glass beads is easy and the glass beads can be used repeatedly. Therefore, this method can be applied to a large-scale production of microbial lipases with a high purity.

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