# IMMOBILIZATION OF LIPASE FOR FAT SPLITING

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**Abstract**— Lipase (EC 3.1.1.3) from *Candida rugosa (Syn.C.cylindracea)* was immobilized on the polyacrylamide-gel (Bio-Gel P-10) by covalent binding. Since the substrates, triglycerides with long chained fatty acids, are insoluble in water, emulsification of the substrates is necessary for lipase to react. Emulsions containing gum arabic were prepared by homogenization followed by sonication. Tributyrin and triolein were chosen as the substrates in order to compare the reaction properties in terms of the fatty acids chain-length. Kinetics of the enzymatic hydrolysis by soluble and immobilized lipases of tributyrin and triolein have been investigated in a batch-reactor. Soluble lipase was inhibited severely by the substrate at higher concentration of substrate emulsion, whereas immobilization of lipase reduced the substrate inhibition. This was especially true for triolein. Effects of pH and temperature on the lipase were studied. Thermal stability of the lipase was increased considerably when the lipase was immobilized.

## INTRODUCTION

Fat splitting has been exclusively practiced for over a century for the production of fatty acids [1]. Industrial fat splitting was carried out by a chemical process at such drastic conditions as high temperature and high pressure. Due to their drastic conditions, chemical processes cannot be used for heat-sensitive fats and oils, and these processes require costly high-pressure equipment and higher power-input for steam generation. For these reasons, enzymatic fat hydrolysis was applied for the heat-sensitive fats and oils [2].

In contrast to the ordinary enzymes, the substrates of lipase, triglycerides with long-chained fatty acids, are insoluble in water. Instead, they remain as a distinct phase separated from water by an interface. Therefore, it behooves us to review as to how lipase is able to induce such a fast hydrolysis reaction between two immiscible phases, i.e., lipid phase and aqueous phase. Lipase at this interface reacts as follows:

$$\begin{array}{lcl} CH_2\text{–}COOR' & & CH_2OH \quad R'COOH \\ | & & | \\ CH\text{–}COOR'' + 3H_2O & \xrightarrow{\text{Lipase}} & CHOH + R''COOH \\ | & & | \\ CH_2\text{–}COOR''' & & CH_2OH \quad R'''COOH \\ \text{Triglyceride} & & \text{Glycerol} \quad \text{Fatty acids} \end{array}$$

Tributyrin: $R' = R'' = R''' = C_3H_7(C_{4:0})$

Triolein : $R' = R'' = R''' = C_{17}H_{33}(C_{18:1})$

Many papers reviewed the lipase reaction mechanism and many hypotheses were suggested and discussed on the lipase reaction mechanism [3-9]. According to Sarda and Desnuelle [10], the first step of catalytic process is the adsorption of the enzyme to the interface. This adsorption is controlled by several interfacial parameters such as emulsion particle size and surface tension. After adsorption, interfacial activation is carried out. It may result from a conformational change of lipase resulting from adsorption, but this modification is not entirely brought about by conformational change. Finally catalytic reaction occurs at active site of lipase. This hypothesis was confirmed by Chapus and Semeriva [11]. By chemical modification techniques they showed that interfacial activation site and classical active site are located in topologically and functionally distinct regions on the lipase surface.

Typical lipases were classified according to their various specificity [12-13]. The most recommendable lipase for fat splitting is the *Candida rugosa* lipase due to its random specificity. Generally the matrix is important in immobilization process, since its interaction with the enzyme may have an influence on stability and kinetics. Coupling capacity, flow characteristics, stability, ease of activation and cost are also important factors for the selection of the matrix [14-15]. Polyacrylamide gel was selected as a matrix for the enzymatic fat splitting in the substrate emulsion system as a heterocatalytic reaction system.

In this study, kinetics of the immobilized lipases were compared with those of soluble lipases for the

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study of enzymatic fat splitting of triglycerides.

## MATERIALS AND METHODS

### Materials

Lipase from *Candida rugosa*(Syn. *C.cylindracea*) and p-nitrobenzoylazide were purchased from Sigma Chemical Co. (U.S.A.). Polyacrylamide gel (Bio-Gel P-10, 50-100 mesh) was purchased from Bio-Rad Laboratory (U.S.A.). 2,4,6-Trinitrobenzenesulfonate, triolein and tributyrin were purchased from Tokyo Kasei Kogyo Co. (Japan). Gum arabic was obtained from Kishida Chemical Co. (Japan). All other reagents and chemicals used were of analytical grade.

### Methods

#### Preparation of emulsion substrate

Emulsions of substrate were prepared with gum arabic as a main component of the emulsifier which contained sodium chloride and calcium chloride at concentration of 0.137 M and 0.0075 M, respectively [16]. Concentration of gum arabic for tributyrin and triolein were 5% (w/v) and 10% (w/v), respectively. Emulsification of the substrates was carried out by homogenization for 3 min with homogenizer (Oscar Fisher Waring Blendor) followed by sonication for a period of 30 min with macrotip at 70% of power output by sonic dismembrator (Artek Sonic 300). pH of the substrate emulsion was addjusted to the desired value by the addition of 0.1 N sodium hydroxide solution.

#### Immobilization of lipase to polyacrylamide gel

Lipase was immobilized according to the modified method of Inman [17-18]. Diazonium form was prepared just prior to use since it has limited stability, and was cooled to near 0°C.

Aminoethylation time..... Ethylenediamine which was coupled to Bio-Gel P-10 with increasing the coupling time was estimated quantitatively. Functional group density of ethylenediamine to the gel was investigated by the titration methods [17-18] based on dry weight of the gel. The course of the substitution of aminoethyl group in the gel can be observed by TNBS-test qualitatively.

Conditions of immobilization..... Enzyme loading concentration and immobilization time were optimized by determining the bound protein content with respect to enzyme loading concentration and immobilization time. Protein content was estimated by a Lowry's method [19] with bovine serum albumin as a standard at 540 nm. The coarse or non-bound protein which was washed was determined and the immobilized protein content was then computed by the difference.

#### Enzyme kinetics studies

Lipase activity was determined by titrating the free fatty acids which were liberated from triglycerides with sodium hydroxide automatically by a pH stat method (Metrohm Herisau pH stat) [20-21]. Lipase activity was defined as the number of microequivalents of alkali required per unit time. Kientic paramters (apparent Km, apparent Vmax) were obtained by measuring the activity of free or immobilized lipases using substrate emulsions containing various amounts of tributyrin or triolein. pH effects were determined at 20°C. Temperature effects on the lipase activity were determined at the optimum pH. Thermal stability was estimated by preincubating the enzyme at the given temperature for 10 hrs and determining the residual activity of the enzyme.

## RESULTS AND DISCUSSION

### Preparation of immobilized enzyme

Since particle size of emulsion substrates is relatively large, substrate emulsions cannot penetrate the pores of the gels available at present time and lipase which was coupled at the inside of the pores could not react with emulsion substrates. Therefore, pore size of gel must be small enough to block the lipase against the inside coupling. Bead size of gel must be also small, since the enzyme reaction is proportional to the surface area of gel per unit mass. For these reasons, lipase was immobilized to the Bio-Gel P-10 (50-100 mesh).

Substrate emulsion had a buffering capacity, pKa value of which was 5.0-5.5. Initial pH of emulsion was 4.9 and 4.0 for tributyrin and triolein, respectively. Emulsion particle size decreased with the sonication time up to 30 min and leveled off thereafter. Hence, sonication time was fixed at 30 min. Particle size of tributyrin and triolein determined by microscopic methods were 1$\mu$m and 2$\mu$m, respectively.

Time course of the reaction of Bio-Gel P-10 with anhydrous ethylenediamine at 90°C was observed. Aminoethylation time was fixed at 30 min and at this aminoethylation time, the gel possessed the sufficient ethylenediamine for enzyme coupling.

Effects of enzyme loading concentration on the immobilization of lipase were studied. At the low enzyme loading concentration (up to 8 g protein per liter), enzyme was bound in proportion to the enzyme loading concentration with efficiency of about 50%. Therefore, the enzyme loading concentration was optimized to 8 g protein per liter (0.8%; w/v). At this point, immobilized protein capacity was 90 mg protein per 1 g-gel. Effects of the immobilization time, pH and temperature on the protein binding to the gel were estimated. Protein was

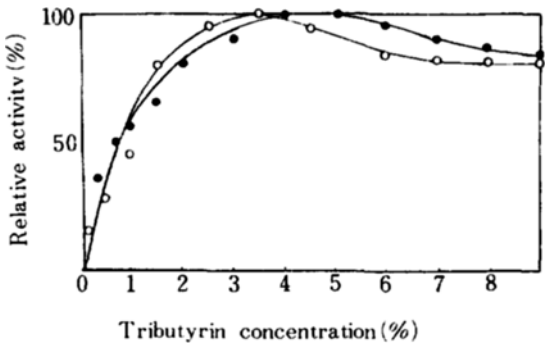


**Fig. 1. Initial reaction rate of lipase to tributyrin.**
○ — ○, soluble enzyme (pH 6.0)
● — ●, immobilized enzyme (pH 7.5)
Temperature, 20℃; paricle size, 1 $\mu$m

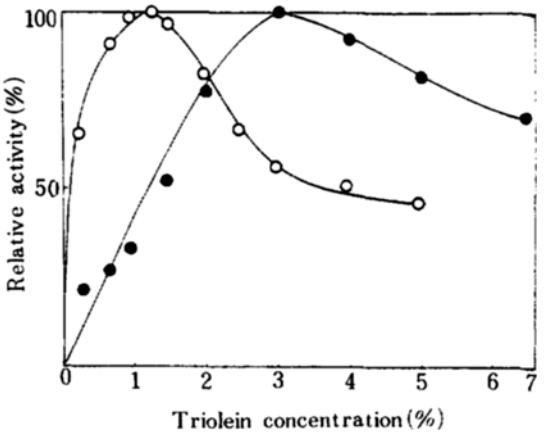


**Fig. 2. Initial reaction rate of lipase to triolein.**
○ — ○, soluble enzyme (pH 7.0)
● — ●, immobilized enzyme (pH 7.5)
Temperature, 20℃; particle size, 2 $\mu$m

bound to maximum point at 10 hrs of immobilization time. Optimum pH was 8.5 and optimum temperature was near 0°C.

**Enzyme kinetics**

Effects of substrate concentration on the initial reaction rate of soluble and immobilized lipases were observed at the specified emulsion particle size as shown in Fig. 1 and Fig. 2. Maximum velocity for free and immobilized lipases for tributyrin were obtained at concentration of 3% (v/v) and 4% (v/v), respectively. For triolein, maximum velocity for free and immobilized lipases were obtained at 1.5% (v/v) and 3% (v/v), respectively.

When substrate concentrations were greater than those as indicated above for tributyrin and triolein, the initial rate was decreased as the substrate concentration was increased. However, drop of the initial rate of the reaction with the increase of substrate concentration for immobilized enzyme is less pronounced than that for soluble lipase. This fact indicates that immobilization reduces the inhibitory effect of high substrate concentration. This is true especially for triolein. Celebi et al. [22] reported that initial reaction rate was decreased steeply with the increase of the concentration of tributyrin (bell shape). But from the data shown in Fig. 1 and Fig. 2, the initial rate was gradually decreased and levelled off for both tributyrin and triolein. Since hydrophobicity of the emulsion is increased in proportion to the substrate concentration, the substrate inhibition at higher concentration is considered to be due to the increasing hydrophobicity. That is to say, the more hydrophobic conditions around the lipase was, the less the lipase activity. However, the initial rate curves were not of bell shape [22], indicating that lipase shows relatively high activity at hydrophobic conditions than any other ordinary enzymes. From these results, it can be concluded that immobilization of the lipase minimized the substrate inhibition, especially for triolein.

Apparent Km value of the immobilized and soluble enzyme for tributyrin were 2.0% (v/v) and 1.7% (v/v), respectively. And for the triolein, apparent Km value was 1.5% (v/v) for immobilized lipase and 0.8% (v/v) for soluble lipase. However, the Michaelis-Menten constants of this enzyme do not have such significant meaning as those of the other enzymes, because Km and Vmax in such a heterocatalytic reaction system as lipase are altered by several environmental factors such as particle size of substrate emulsions, diffusional, and electrostatic effects, and because the concept of substrate concentration in the interfacial reaction is different from that of substrate concentration in aqueous phase.

Effects of pH on the reaction rate of enzyme for tributyrin and triolein are shown in Fig. 3 and Fig. 4, respectively. The reaction rate of soluble enzyme was maximum at pH of 6 and pH of 7 for tributyrin and triolein, respectively. However, in the case of immobilized enzyme reaction rate was maximum around at pH 7.5 in both substrates. Optimum pH of soluble lipase for tributyrin was more acidic than that of triolein. But in immobilized lipases pH profiles were almost identical.

Microenvironment in the interfacial region of immobilized lipase differs from soluble lipase. In the case of polycationic polyacrylamide gel, positive-charged ions (such as hydrogen ions) will be more easily diffused from the carrier on account of electrical repulsion between hydrogen ion and carriers. Hence optimum pH of the immobilized lipase was expected to be shifted toward an acidic region [23-24]. However, the optimum pH was actually shifted from the acidic to the alkaline

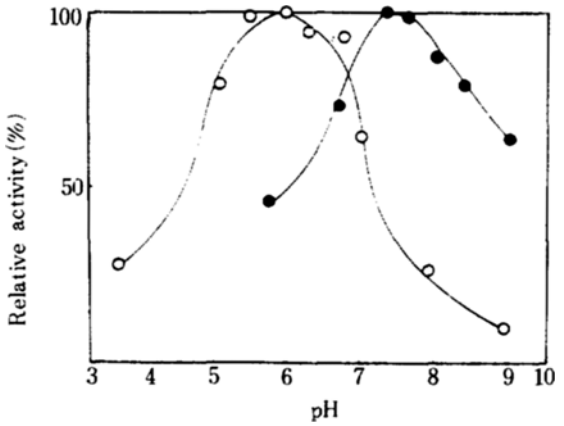


**Fig. 3. Effects of pH on enzyme to tributyrin.**
○ — ○, soluble enzyme
● — ●, immobilized enzyme
Temparature 20℃, tributyrin concentration; 3 %

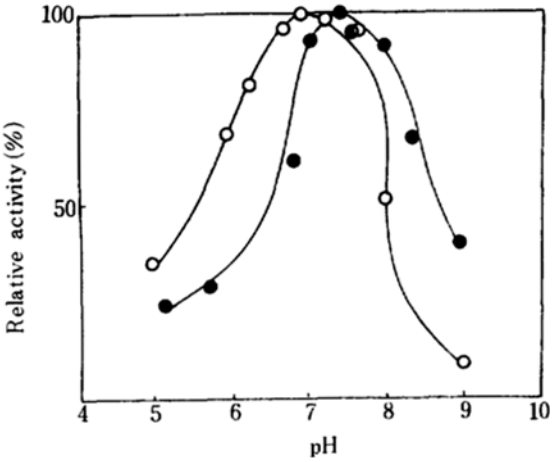


**Fig. 4. Effects of pH on enzyme to triolein.**
○ — ○, soluble enzyme
● — ●, immobilized enzyme
Temperature 20℃, triolein concentration; 2%

region. The reason for this shift may be due to the difference of diffusivity of products between the soluble and immobilized lipases in the interfacial region. In other words, supposing that the shift of pH has no relation to the kinds of substrate and electrostatic potential of carrier, the thickness of diffusion barrier in the interfacial microenvironmental region of the immobilized enzyme is greater than that of soluble lipase. Hence the diffusion rate of fatty acids produced by enzymatic lipase is greater than that of soluble lipase. Hence the the soluble lipase is greater than that in immobilized lipase. Therefore, pH in the microenvironmental region of immobilized lipase is lower than that of soluble lipase due to the remarkable increase of diffusion barrier by immobilization. For this reason, it is believed that the optimum pH of the immobilized enzyme was shifted to alkaline. The reason for the difference of optimum pH shift for tributyrin and triolein is not clear at present time However, the substrate specific conformational change of lipase or the difference in diffusions of the final products or other microenvironmental factors are believed to cause the change of the pH profile for the tributyrin and triolein.

Initial reaction rates of the enzyme at various temperatures for tributyrin and triolein are shown in Fig. 5 and 6, respectively. Temperature profiles were

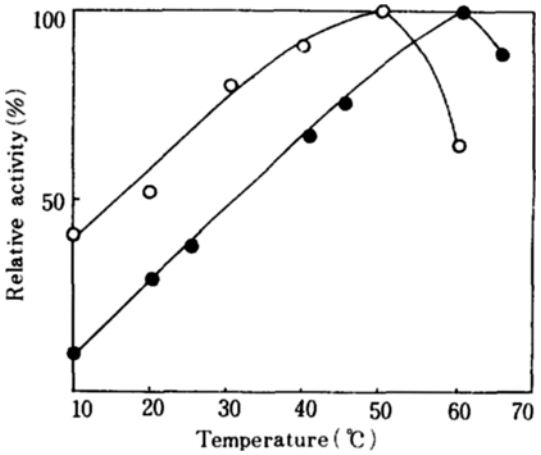


**Fig. 5. Effects on enzyme to tributyrin.**
○ - ○, soluble enzyme
● — ●, immobilized enzyme
Tributrin concentration; 3%, pH 7.0

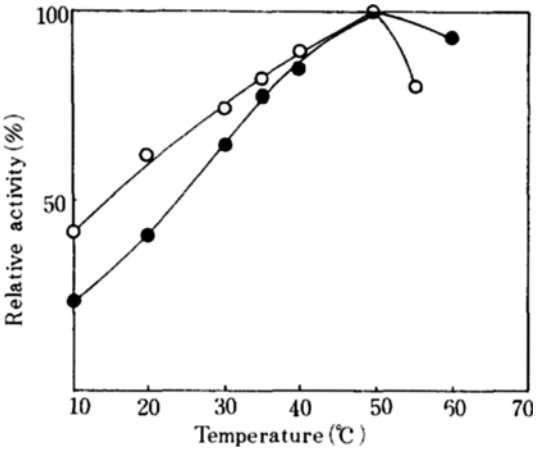


**Fig. 6. Effect of temperature on enzyme to triolein.**
○ — ○, soluble enzyme
● — ●, immobilized enzyme
Triolein concentration; 2%, pH 7.0

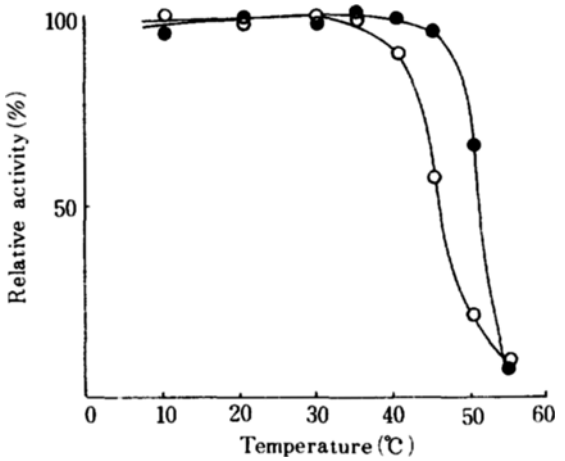


**Fig. 7. Effects of temperature on enzyme stability to tributyrin.**
○ — ○, soluble enzyme
● — ●, immobilized enzyme
Preincubation: time, 10hrs; pH, 7.0 (0.05 M phosphate buffer)
Reaction: pH, 7.0; Temperrature, 20℃; trib-tributyrin concentration, 3%

similar for both tributyrin and triolein. The initial reaction rates for soluble and immobilized lipases for both substrates were highest at 50°C and 60°C, respectively. Activation energy (Ea) expressed by the Arrhenius plot for soluble and immobilized lipases for tributyrin were 9.0 kcal/mol and 12.5 kcal/mol respectively. For triolein, activation energy for soluble and immobilized lipases were 8.5 kcal/mol and 13.5 kcal/mol, respectively. For both substrates, activation energies of immobilized lipases are greater than those of soluble lipases. Thermal stability of the enzyme is shown in Fig. 7. Immobilization of the lipase increased the thermal stability considerably.

In conclusion, immobilization of the lipase reduced the degree of substrate inhibition and, at the same time, increased the stability as shown in Table 1.

**Table 1.** Kinetic properties of lipase.

| Characteristics | Tributyrin | | Triolein | |
|---|---|---|---|---|
| | Soluble lipase | Immobilized lipase | Soluble lipase | Immobilized lipase |
| Optimum pH | 6.0 | 7.5 | 7.0 | 7.7 |
| Optimum temperature (°C) | 35 | 40 | 35 | 40 |
| $K_m$(%)* | 1.5 | - | 0.7 | - |
| $K_m$(%)* | - | 2 | - | 1.5 |
| Maximum activity** ($\mu$mol/hr/mg) | 9,800 | - | 5,000 | - |
| Maximum activity** ($\mu$mol/hr/g-gel) | - | 2,000 | - | 1,400 |
| $E_a$(kcal/mol) | 9.0 | 12.5 | 8.5 | 13.5 |

* $K_m$ are apparent values.
**Maximum activities are the activities of lipase at optimized conditions.

## CONCLUSION

Immobilization of lipase reduced the substrate inhibition and increased the thermal stablity. Fat splitting rate was controlled by such physicochemical parameters as chain length of fatty acids in triglycerides particle size of emulsion, interfacial tension and microenvironmental factors of interface. Diffusivity of substrates and fatty acids between interface and bulk region was the rate limiting step.

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