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Hydrolysis of Triglyceride in Two Phase System Using Immobilized Lipase

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이상계내에서 고정화리파제에 의한 트리글리세리드의 가수분해

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Lipases from *Candida rogosa* and *Rhizopus arrhizus* were immobilized by entrapment with photo-crosslinkable resin prepolymer for the study of fat splitting and interesterification in isooctane-two phase system. Dioctylsulfosuccinate was selected as the most suitable surfactant during the immobilization. Lipase entrapped with hydrophobic photo-crosslinkable resin prepolymer (ENTP-3000) exhibited the highest activity, whereas lipase entrapped with hydrophilic gel (ENT-4000) was more stable in organic solvent. As the degree of hydrophobicity of the immobilization matrix was increased, Vm(app) of the lipase entrapped was increased, but Km(app) was approximately constant. While the optimum pH of the lipases entrapped on hydrophilic gel (ENT-4000) were around pH 7.0 for *Candida* lipase and *Rhizopus* lipase, the reaction rate of the lipases entrapped on hydrophobic gel were less dependent on pH variations for short reaction time. However, for longer reaction time, the lipases from *C. rugosa* and *R. arrhizus* entrapped on hydrophobic gel yielded maximum rate at pH 6.0 and 6.5, respectively. Entrapment method endowed the lipase with thermal stability.

Production of fatty acids from triglyceride or interesterification of fats and oils are of much importance from an industrial point of view. Use of the lipases for the reactions has been attempted as one of the methods to attain specified products and high quality of lipids, and to save the energy. Due to the water-insolubility of lipids, bioconversion of fats and oils in two phase system (or solvent system) by using the microbial lipases has been studied intensively (1-3).

With two phase system it is not necessary to immobilize the enzyme, when the recovery is the sole reason (4). However, immobilization may still be worthwhile because the immobilization protects the enzyme from conformational change upon introduction of an organic solvent into solutions (5).

Bioconversions of various water-insoluble compounds by biocatalysts immobilized with photo-crosslinkable resin prepolymer have been studied (6-8). Taking such advantages of photo-crosslinkable resin prepolymer (9), we performed the triglyceride hydrolysis by entrapping lipases on this matrix in two phase system. The lipases from *Candida rugosa* and *Rhizopus arrhizus* were used for comparative purposes. The two lipases mentioned above were recommended as the suitable enzymes for the fat splitting and interesterification of lipid, respectively (10).

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In this paper, characterisitics of immobilized lipases from *C. rugosa* and *R. arrhizus* for the hydrolysis of olive oil in the two phase system were investigated, and the hydrolysis of olive oil was performed in batch system.

Materials and Methods

Materials

Lipases from *R. arrhizus* and *C. rugosa,* olive oil, and dioctylsulfosuccinate were purchased from Sigma Chemical Co. (St. Louis, MD. USA). Isooctane and benzoinethylether were purchased from Tokyo Kasei Chemical Co., Ltd. (Tokyo, Japan). Dioctylsulfosuccinate (Bis(2-ethylhexyl) sodiumsulfosuccinate, abbreviated as AOT) from Sigma was purified according to the method of Tamamushi and Watanabe (11) and Wong *et al.* (12). All other reagents and chemicals used were of analytical grade.

Three kinds of hydrophilic photo-crosslinkable resin prepolymer, ENT-1000, ENT-2000, and ENT-4000(the number indicates the aproximate molecular weight of poly(ethylene glycol) skeleton) (7) and three kinds of hydrophobic photo-crosslinkable resin prepolymer, ENTP-1000, ENTP-2000, and ENTP-3000(each number indicates the approximate molecular weight of poly(propylene glycol) skeleton) (13) were suplied from Kansai Paints Co., Ltd. (Japan).

Immobilization of lipases

Immobilization of lipases on the hydrophilic and hydrophobic photo-crosslinkable resin prepolymer using the AOT as a surfactant was performed according to the methods reported by other workers (14,15) with modification (16). Immobilization of lipases with copolymer matrix exhibiting the intermediate polarity between extreme hydrophilicity (ENT) and hydrophobicity (ENTP) was performed according to the following procedures; molten mixtures of ENTP-3000 and ENT-4000 thus prepared was mixed with each other according to predetermined ratio, and then stirred vigorously followed by sonication for 5 min. Cooled to room temperature, the lipase solution (20,000 units/ml) was added dropwise to the mixed molten mixture with agitation until the final concentration reached to 20% (v/w). And the immobilization was carried out as described in hydrophilic gel. The gel formed (thickness, *ca.* 1mm) was cut into small pieces (*ca.* 3×3 mm).

Enzyme reaction

Reaction was carried out at 35 °C with agitation (800 rpm) in a 50 ml of reactor. In aqueous medium the lipases entrapped with photo-crosslinkable resin prepolymer were dispersed. The ratio of solvent phase to water phase in the reaction mixture is 8:2 (v/w). Olive oil in isooctane (10% by volume) was used as the solvent phase. Other conditions for the reactions were the same as described in our previous report (1).

Procedures

The methods to determine the lipase activity, degree of hydrolysis, substrate effect, pH effect, temperature effect, and other kinetics were the same as described in the previous paper (1).

One unit of lipase activity was defined as one micromole of fatty acids produced per 1 hr under the analytical conditions.

Results and Discussion

Using the lipase immobilized on the ENTP-3000, the size of the gel and agitation speed of reaction on the lipase activity were investigated as a preliminary step. The lipase from *C. rugosa* was used for this purpose. The result showed that the production rate of fatty acids was increased proportionally with the increase of surface area of the gel, resulting from cutting gel into smaller pieces.

Selection of surfactant for immobilization of lipase

To select the most suitable surfactant, five kinds of surfactant were investigated for immobilization of lipases on ENTP-3000. (Table 1) Fukui *et al.*(14) used Span as a surfactant in immobilization of lipase on the hydrophobic photo-crosslinkable resin prepolymers. However, they neglected the fact that Tween or Span reacts as substrates of lipase (17). Among surfactants, therefore, Tween and Span were excluded in the immobilization of lipase on the

Table 1. Effects of various surfactants on activity of lipase entrapped on ENTP-gel in two phase system.[a]

Surfactant[b]	Relative activity, %
Dioctylsulfosuccinate (AOT)	100
Brij 35	47
Polyethylene glycol 4000	37
Polyethylene glycol 6000	40
Triton X-100	0.51

a ENTP-3000 was used as a hydrophobic gel and isooctane-water for the two phase system.
b Surfactants of Span and Tween were excluded because these surfactants were the substrates of lipase.

photo-crosslinkable resin prepolymer. Table 1 shows the AOT yields maximum activity, and thus we used the AOT throughout the experiment as a surfactant. AOT is an interesting surfactant, because AOT was reported to be the best surfactant in forming reverse micelles for the lipase (18) and alcohol dehydrogenase (19).

Effects of physicochemical properties of matrix on lipase activity

Lipase from *C. rugosa* was immobilized by entrapment on the various types of photo-crosslinkable resin prepolymer. The activity of the immobilized lipase is shown in Table 2. A high activity of the entrapped lipase was obtained when the lipase was immobilized with a hydrophobic photo-crosslinkable resin prepolymer, especially ENTP-3000. Because the hydrophobicity of the entrapping the enzyme may considerably affect the diffusion of hydrophobic substrate such as triglyceride (9), ENTP-3000 yielded highest activity for hydrolysis of triglyceride in two phase system. Table 2 also shows the activity of lipase entrapped with the photo-crosslinkable resin prepolymers of different chain length. The enzyme entrapped with ENTP-3000 among hydrophobic prepolymer and ENT-4000 among hydrophilic prepolymer were found to be most active. The results indicates that the loose net work of gels prepared from longer chain prepolymers facilitates migration of the substrate and product (7), and subsequently, is favorable to the enzyme activity. This results for the hydrolysis of triglyceride using entrapped lipase in two phase system is consistent with the standard reaction system without organic solvent (6).

Table 2. Effects of chain length of prepolymer on lipase activity in two phase system.[a]

Prepolymer	M.W. of main chain	Activity (μmole/g-gel)
Hydrophilic gel		
ENT-1000	ca. 1,000	140.8
ENT-2000	2,000	175.2
ENT-4000	4,000	198.4
Hydrophobic gel		
ENTP-1000	1,000	252.8
ENTP-2000	2,000	289.6
ENPT-3000	3,000	302.4

a Lipase from *C. rugosa* and 10% olive oil were used.

We also investigated the effects of the hydrophobicity or hydrophilicity of the gel on the lipase stability in two phase system (Fig. 1). ENT-4000 and ENTP-3000 were chosen as model of the hydrophilic and hydrophobic gel, respectively. The lipase entrapped with hydrophilic photo-crosslinkable

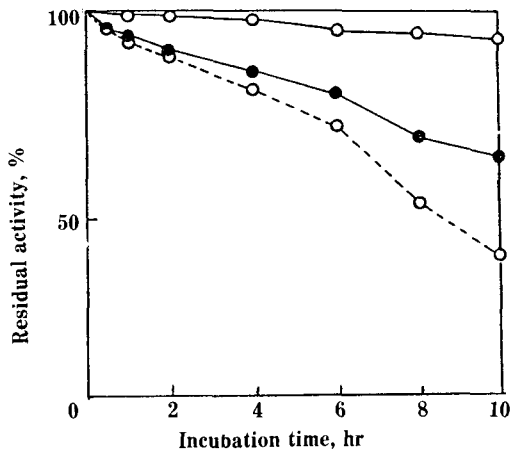

Fig. 1. Effects of gel properties on lipase stability in two phase system.

Reaction conditions: temperature, 35°C; pH of enzyme solution, 6.0; agitation speed, 800 rpm; reactor volume, 50 ml; olive oil concentration, 10 %; reaction time, 30 min; gel thickness, 1 mm; gel size, 3 × 3 mm. These conditions are the same throughout the following figures, unless otherwise specified. The results are mean values of three determinations. Symbols: o---o , lipase of ENTP-3000; ● - ● , lipase of ENTP-3000; ENT-4000 = 9:1; o - o , lipase of ENT-4000.

resin prepolymer was found to be more stable than that with hydrophobic prepolymer. Both photocrosslinkable resin prepolymer stabilized the lipase in two phase system, because immobilization of lipases protected the enzyme from the conformation change of the protein brought about by the organic medium (5). In two phase system, immobilization of the lipase on the matrix excluded organic solvent from the surface of the gel (20). Especially the hydrophilic gel of prepolymer, excluded rapidly nonpolar organic solvents which affected denaturation of protein and as a consequence, hydrophilic gel was observed to be more stable than the hydrophobic gel in organic solvent. In standard reaction system (6), however, hydrophobic gel was reported to be more stable than hydrophilic gel.

Effect of pH on activity of lipase entrapped on the prepolymer

The effect of pH of water phase in which the gel with lipase was dispersed on the lipase activity was examined. ENT-4000 and ENTP-3000 were used as hydrophilic and hydrophobic gels, respectively.

As shown in Fig. 2, the lipase from *C. rugosa* shows that the pH-activity profile of hydrophilic gel-entrapped lipase was shifted to alkaline region by one and half unit of pH in contrast to that of free

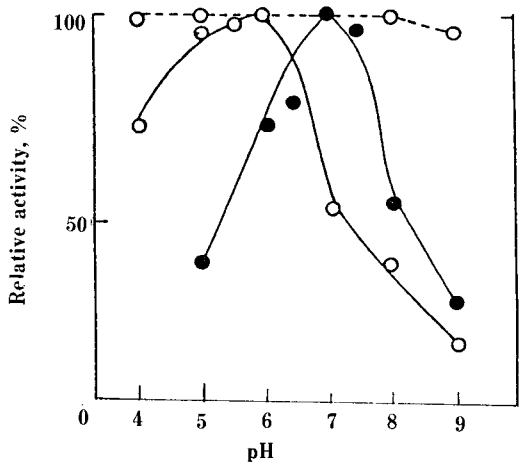

Fig. 2. Effects of pH on activity of entrapped lipase from *C. rugosa*. Symbols:
○－○, free lipase; ○---○, lipase of ENTP-3000; ●－●, lipase of ENT-4000.

lipase in two phase system (1). The same result was obtained in the case of the lipase from *R. arrhizus*. Generally the environment in the vicinity of an immobilized enzyme often differs from that in the bulk phase. The akaline shift of pH optimum by immobilization of lipase with ENT-4000 in two phase system was explained by two possible reasons.

Firstly, the possibility is that hydrophilic gel (ENT-4000) is a polyanionic carrier, and due to the electrostatic interaction, the positively charged ions in the bulk would accumulate around the prepolymer causing the concentration of positive charged molecules to be higher in the surrounding media. Therefore, pH-activity profile of the immobilized lipase with polyanionic carrier will be shifted to akaline region (21).

Secondly, the immobilization of the lipase makes the diffusion barrier between bulk region and surface region of matrix. The products produced by lipase within the vicinity of matrix do not diffuse to the bulk region rapidly. The pH of the surface region of matrix is lower than that of the bulk region, due to the accumulation of the free fatty acids within the surface of immobilized lipase. Therefore, the optimum pH of the immobilized lipase was shifted toward a higher value (1,22).

On the other hand, pH profile of the lipase entrapped on ENTP-3000 was almost plateauristic in the pH range of 2-12 for the first 30 min. This effect can be explained from existence of hydrophobic barrier around the enzyme.

Wingard (23) suggested that electrostatic interactions between the hydrophobic and hydrophilic carrier and substrate often produced a unequal distribution of these species between the micro-and macro-environment, which is called the "partition effect". Therefore, highly hydrophilic hydrogen or hydroxyl ion is largely excluded from the hydrophobic gel (ENTP-3000), and the lipase entrapped on the hydrophobic gel becomes insensitive to the pH-variation of bulk phase. Similar patterns have been reported by many workers; Goldman *et al.* (24) observed the effect mentioned above with papain immobilized on a collodion membrane, and also Lavayre and Baratti (25) observed the same pattern with the lipase im-

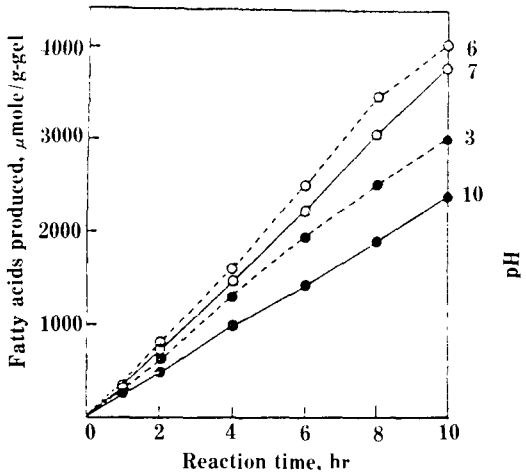

Fig. 3. Time course of fatty acids production at the various pH for entrapped lipase from *C. rugosa* with ENTP-3000.

Each number on the right side of figure represents the pH of enzyme solution.

mobilized on iodopropyl spherosil.

However, for the longer reaction time, the rate of fatty acid production was affected by the pH of bulk phase for the hydrophobic gel (ENTP-3000). Pattern of fatty acid production in two phase system by the lipase from *C. rugosa* entrapped on the hydrophobic gel at different pHs is shown in Fig. 3. The maximum production rate of fatty acids was obtained at pH 6.0. This value was the optimum pH of the free lipase in two phase system. This pattern was also the same for *Rhizopus* lipase.

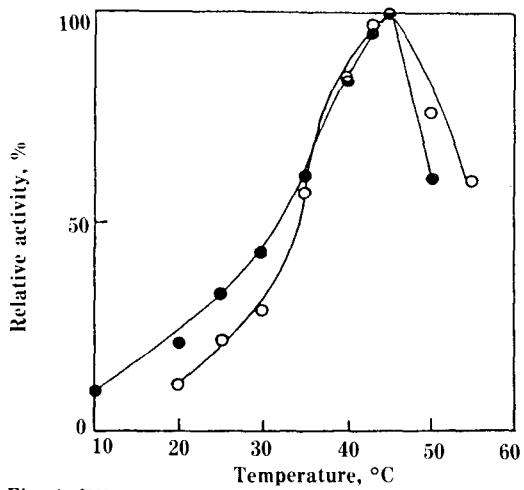

Fig. 4. Effects of tmeperature on activity of entrapped lipase from *C. rugosa* (○) and *R. arrhizus* (●).

Ultimately for the long reaction time, optimum pH of the lipase entrapped on hydrophobic gel in two phase system was the same as those of free lipase.

Effect of reaction temperature on lipase

Temperature dependency of lipase activity is shown in Fig. 4. The optimum temperature was around 45 °C for the both lipases from *C. rugosa* and *R. arrhizus* entrapped on the prepolymer (ENTP-3000). Thus the optimum temperature of ENTP-3000 entrapped lipases were about 5-10 °C higher than those of free lipases. Immobilization sometimes shifts the optimal reaction temperature of enzymes to a higher range. Diffusion of substrate and product in the gels may be facilitated at high reaction temperature. However, reaction temperature of 35 °C was better than 45 °C with regard to the stability of the lipase during repeated reaction.

Values of activation energy (Ea) for the entrapped lipase are 12.4 Kcal/mole and 17.9 Kcal/mole for the lipases from *R. arrhizus* and *C. rugosa*, respectively. For both lipases activation energies of entrapped lipases are greater than those of free lipases (1).

Effects of substrate concentration on activity of entrapped lipase

The activity of entrapped lipase was based on wet gel weight, and one gram of gel contained

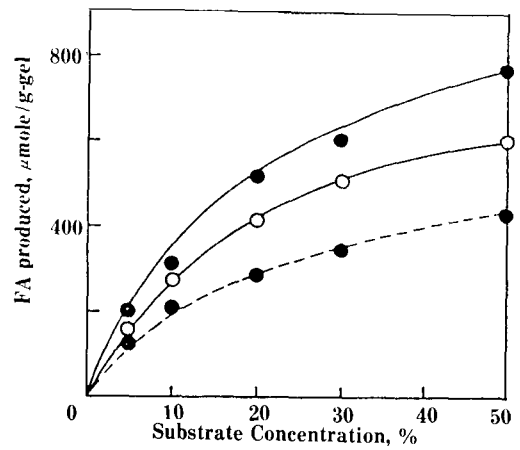

Fig. 5. Effect of substrate concentration on production of fatty acids by entrapped lipase from *C. rugosa*.

Reaction volume is 20 ml. Symbols; ● – ● , lipase of ENTP-3000; ○ – ○ , lipase of ENTP-3000:ENT-4000 = 9:1 ●---● , lipase of ENT-4000.

about 4700 units of lipase from *C. rugosa*. The concentration of substrate was changed in the range of 3-50% (v/v). ENTP-3000 and ENT-4000 were used as the hydrophobic and hydrophilic gel, respectively. The typical ratio of copolymer used in this study was ENT-4000:ENTP-3000=10:90 (w/w).

Fig. 5 shows the fatty acid production by the lipase entrapped on ENT-4000, ENTP-3000, and ENTP-3000:ENT-4000(=10:90) at various olive oil concentration. With the increasing hydrophobicity of gel, the lipase activity was increased, because the diffusion of the olive oil was more rapid in the case of the hydrophobic gel than hydrophilic gel (9).

Since surface area of the entrapped lipase was not changed by the olive oil concentration, a Lineweaver-Burk double reciprocal plot for the entrapped lipase in two phase system could be made in contrast to the free lipase (26). Fig. 6 shows that Vm (app) increased gradually as the polarity of microenvironment around the lipase decreased, whereas Km (app) was approximately same in all matrices. Thus Lineweaver-Burk plot of immobilized lipase is similar to the general irreversible inhibition pattern.

Batch hydrolysis of lipids by entrapped lipase

Under optimized conditions, the production rate

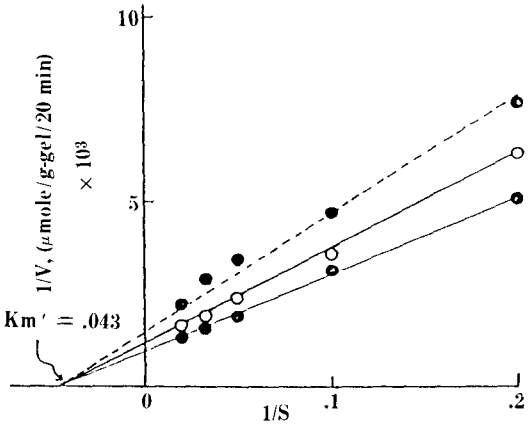

Fig. 6. Lineweaver-Burk plot of entrapped lipase in two phase system.
Symbols: ● – ●, lipase of ENTP-3000; ○ – ○, lipase of ENTP-3000:ENT-4000 = 9:1; ●---●, lipase of ENT-4000.

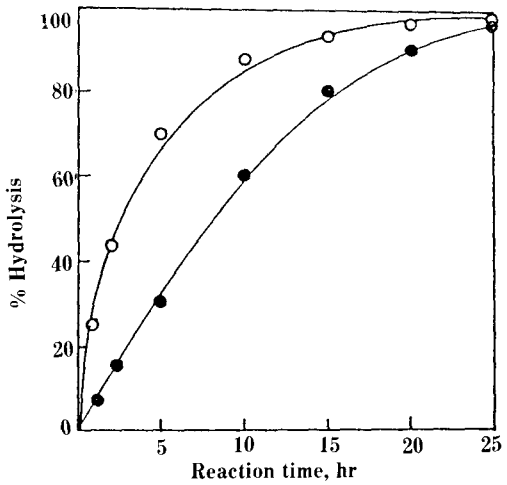

Fig. 7. Batch hydrolysis of olive oil by ENTP-3000 entrapped lipase from *C. rugosa* with 2% olive oil.
Symbols: ○, free lipase, ●, entrapped lipase.

of fatty acids was investigated for the immobilized lipase with 2 % olive oil using the *Candida* lipase. The time course of olive oil hydrolysis with ENTP-3000 entrapped lipase is illustrated in Fig. 7. Although both free and entrapped lipases showed almost the same degree of hydrolysis after 25 hr of reaction, the initial hydrolysis rate with free lipase was higher than that with entrapped lipase.

요 약

*Candida rugosa*와 *Rhizopus arrhizus* 리파제를 photocrosslinkable resin prepolymer에 고정화시켜서 이소옥탄을 유기용매로 사용한 이상계(二相界)를 이용해서 유지분해 및 에스테르교환 반응을 보고자 했다. Dioctylsulfosuccinate가 가장 좋은 surfactant였다. 소수성 젤인 ENTP-3000에 고정된 리파제가 좋은 활성을 나타냈고 친수성 젤인 ENT-4000에 고정된 리파제가 유기용매에 대해 안정했다. 고정화 matrix의 소수성이 증가될수록 Vm(app)는 증가되었으나 Km(app)는 거의 일정했다. 리파제의 최적 pH는 소수성 젤인 ENTP-3000에 고정된 경우 *C. rugosa*와 *R. arrhizus* 리파제에 대해서 각각 6.0과 6.5였으나, 친수성 젤에 고정된 리파제는 짧은 시간 반응에는 pH에 크게 영향을 받지 않았으나 긴 시간 동안 반응시킬 때는 역시 pH 6.0과 6.5에서 각각 *C. rugosa*와 *R. arrhizus* 리파제가 높은

양의 지방산을 분해시켰다. 리파제를 entrapment 시키면 열안정성이 증가됨을 알 수 있었다.

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