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Enzymatic Hydrolysis of Hydrophobic Triolein by Lipase in a Mono-phase Reaction System Containing Cyclodextrin; Reaction Characteristics

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A hydrophobic substrate triolein was hydrolyzed by lipase in a mono-phase reaction system containing cyclodextrin(CD) as emulsifier. The triolein was transformed to an emulsion-like state in the CD containing reaction system in contrast to the oil-droplet like state without CD due to the formation of an inclusion complex between the lipids and CDs. The hydrolysis reaction increased substantially in the CD containing reaction system, and the optimum reaction conditions including the amount of lipase, β-CD concentration, and mixing ratio of triolein and β-CD, were determined. The performance of the enzyme reaction in a mono-phase reaction system was compared with that of a two-phase reaction system which used water immiscible hexane as the organic solvent. The role of a CD in the mono-phase reaction system was elucidated by comparing the degree of the inclusion complex formation with triolein and oleic acid, K_m and V_{max} values, and product inhibition by oleic acid in aqueous and CD containing reaction systems. The resulting enhanced reaction seems to be caused by two phenomena; the increased accessibility of lipase to triolein and reduced product inhibition by oleic acid through the formation of an inclusion complex.

Key words : mono-phase reaction system, cyclodextrin, hydrophobic substrate, triolein, lipase, inclusion complex formation, reaction characteristics

INTRODUCTION

Lipase (triacylglycerol acylhydrolase, E.C. 3.1.1.3) is a widely distributed enzyme that catalyzes both the hydrolysis of lipids and the synthesis of glycerol esters, and also catalyzes transesterification reactions under a certain specific circumstance [1]. Lipase has recently been extensively applied as a tool for the enzymatic synthesis of new materials, for example, useful free fatty acids, monoglyceride, aroma and flavor compounds, biosurfactants, steroid hormones, and biomodified fats *etc.* [2-7].

The lipase reaction is occured in the interfacial layer of hydrophobic containing lipid substrate and hydrophilic including lipase [8]. Therefore, the reaction proceeds relatively slowly because of the limitation of the interfacial layer for reaction, and the product yield is also low. Many attempts have been made to solve above difficulties, for example, a two-phase reaction system consisting of water and a water immiscible organic solvent, mono-phase systems composed of a water immiscible solvent and a minimum amount of water or water and a water miscible solvent, and a reversed micelle system using surfactants as a continuous phase [9-11].

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However, enzyme reaction systems using an organic solvent have several shortcomings including inactivation of the lipase by the organic solvent, and limited practical applicability due to the price, volatility, toxicity, and inflammability of the organic solvent. To overcome this instability of the lipase, Lin [12] screened microbes producing a solvent-tolerant alkaline lipase, and Herniz *et al.* [13] attempted the chemical modification of lipase to maintain enzyme activity in an organic media. Yet these studies are limited to specific microbes, and still require the use of an organic solvent for an enzyme reaction. Consequently, the development of a solvent free lipase reaction system is important to achieve an industrial application of the lipase reaction for various purposes.

Cyclodextrins (CDs) are doughnut-shaped cyclic oligosaccharides of six to eight glucose units linked by α-1,4-glucosydic bond. Outside of CDs has hydrophilic properties due to hydroxyl groups; on the other hand, inside of CDs has hydrophobic properties due to hydrogen bonds. Due to such an unusual property, CDs can form an inclusion complex with many hydrophobic guest molecules including lipids [15-16].

The inclusion complex formation between hydrophobic lipids and CDs can be dispersed more readily in aqueous phases compared to native hydrophobic lipid molecules because of the hydrophilic properties of outside of CD molecules. In our previous work,

the capabilities of the inclusion complex formation of CDs with various molecules were compared and the CD-fatty acid complex was characterized [17], and applied to the fractionation of α-, β-, and γ-CD [18].

The application of a mono-phase reaction system containing CD for the enzymatic hydrolysis of hydrophobic lipid can provide several advantages; an enhanced reaction rate and product yield, and a reduced instability of the enzyme caused by the organic solvent. Potential applications for the above reaction system have been suggested recently [19-21], however, this system has remains at a developmental stage.

In this work, a mono-phase reaction system containing CD was applied to the hydrolysis of lipid using triolein as the standard hydrophobic substrate. The effects of α-, β-, and γ- CD on the hydrolysis of triolein by lipase were analyzed, and the optimum conditions for triolein hydrolysis by lipase in a CD containing reaction system were determined. The role of CD in a mono-phase reaction system was elucidated by comparing the degrees of the inclusion complex formation of triolein and oleic acid, K_m and V_{max} values, and product inhibition by oleic acid both in aqueous and CD containing reaction systems. These results can be utilized for evaluating potential applications of the above reaction system not only for the hydrolysis of lipids but also for other reactions including esterification, transesterification, and other biotransformations using lipase.

MATERIALS AND METHODS

Lipase and Substrate

Crude lipase VII from *Candida rugosa* (E.C. 3.1. 1.3. Sigma Chemical Co., U.S.A) was used as the enzyme, and triolein (Sigma Chemical Co., U.S.A) was used as the hydrophobic standard substrate for the hydrolysis reaction of lipase.

Cyclodextrins(CDs)

Extra pure α-, β-, and γ-CD (CycloLab, Ltd., Hungary) were used as the emulsifier of the hydrophobic triolein.

Measurement of Lipase Activity

The lipase activity was determined by measuring the oleic acid produced from the hydrolysis reaction after 1 hr using 100 mM of triolein in 50 mM of a phosphoric acid-NaCl buffer(pH 7.0) as the substrate. One unit of enzyme was defined as the amount of enzyme producing 1 μmol of oleic acid per hour, and the specific lipase activity used was determined to be 732 units/mg protein.

Measurement of Hydrolysis Yield

The hydrolysis yield of triolein was determined by measuring the oleic acid produced from the triolein, considering a converted triolein as equivalent to three oleic acid molecules. The oleic acid was measured using the Lowry and Tinsley method [24], in which 5 mL of isooctane is added to 1mL of the reaction mixture for the extraction of the lipids, and then 1 mL of cupric acetate-pyridine reagent is added as a coloring reagent. The absorbance of the upper layer of the mixture solution was measured at 715 nm.

Hydrolysis Reaction in a Mono-phase Reaction System Containing CD

100 mM of triolein was added to 50 mM of a phosphoric acid-NaCl buffer (pH 7.0) containing 30 mM of β-CD. The hydrolysis reaction was carried out at 37℃, pH 7.0, and 200 rpm, while changing the amount of lipase and the mixing ratio of β-CD and triolein accordingly.

Hydrolysis Reaction in a Two-phase Reaction System Containing Hexane

20% hexane (Matsunoen Chemicals Ltd. Japan) was added to 100 mM of triolein in 50 mM of a phosphoric acid-NaCl buffer (pH 7.0). The other reaction conditions were the same as for the mono-phase reaction system containing β-CD.

Analysis of Triolein Hydrolysates

Triolein hydrolysates were analyzed using thin layer chromatography (TLC), carried out on Kieselguhr 60 F_{254} plate (Merck, Germany) using hexane: diethyl ether:acetic acid (70:30:3) as the solvent system. 10% cooper sulfate dissolved in a 8% phosphoric acid solution was used as the oxidizing reagent. After heating TLC plate at 150℃ for 1 hr, black spots were detected.

Measurement of Inclusion Complex Formation of β-CD with Triolein and Oleic acid

The degree of inclusion complex formation of β-CD with triolein and oleic acid was estimated by measuring the amount of residual β-CD after inclusion complex formation using the phenolphthalein method [25].

RESULTS AND DISCUSSION

Enzymatic Hydrolysis of Hydrophobic Triolein by Lipase in a Mono-phase Reaction System Containing CD

To examine the effect of CD on the enzymatic hydrolysis of triolein, triolein was hydrolyzed in a mono-phase reaction system containing 30 mM of α-, β-, and γ-CD, and in a reaction system without any CD. Table 1 compares the hydrolysis yield of triolein by lipase in a mono-phase reaction system containing α-, β-, and γ-CD after 12 hrs. The hydrolysis yields increased substantially in the reaction systems containing CD compared to the aqueous reaction system without CD. γ-CD, which has the largest cavity in its molecular structure, exhibited the highest hydrolysis yield compared to α- and β-CD. Since, the differences were not particularly significant, therefore, β-CD was selected as the most effective emulsion state inducing reagent by considering its cost and wide usage.

Photographs of the reaction mixtures of mono-phase reaction systems both with and without CD after 12 hrs are compared in Fig. 1. The reaction mixtures containing CD were fully emulsified throughout the enzyme reaction (A), where as the oil-droplet like state was observed in the aqueous reaction system without CD (B). The major role of CD in a mono-phase reaction system is as an emulsifier to raise the solubility of hydrophobic substrates and products, and its detailed specific role needs to be further analyzed.

Table 1. Comparison of α-, β-, and γ-cyclodextrin for enzymatic hydrolysis of triolein by lipase in the mono-phase reaction system containing CD

	Hydrolysis Yield (%)
None	51.2
α-CD	61.3
β-CD	62.8
γ-CD	63.7

Hydrolysis reaction was carried out at 30 mM of each CDs, 100 mM triolein, 500 units lipase/mmol of triolein, 12 hr, and 37℃

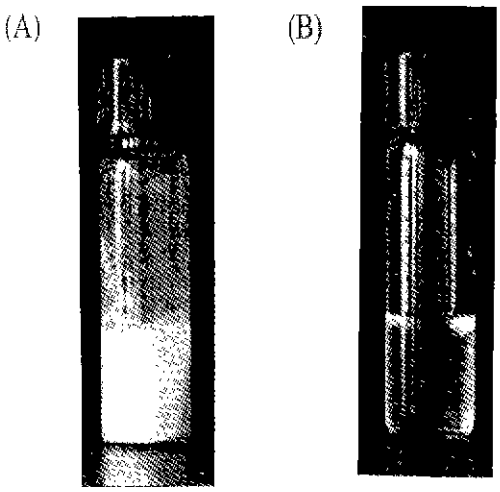

Fig. 1. Photographs of reaction mixtures of the mono-phase reaction system containing CD and without CD after 12 hrs. The reaction was carried out at 100 mM triolein, 500 units lipase/mmol of triolein, pH 7.0, and 37℃. Reaction systems with 30 mM of β-CD(A) and without β-CD (B).

Optimal Reaction Conditions for the Enzymatic Hydrolysis of Triolein by Lipase in a Mono-phase Reaction System Containing CD

Fig. 2(A) compares the effect of the amount of lipase on the hydrolysis yield of triolein in a mono-phase reaction system containing 30 mM of β-CD and 100 mM of triolein. The amount of the enzyme was changed from 100 to 3,000 units of lipase/mmol of triolein, and the reaction was carried out at 37℃ for 12 hr. The hydrolysis yield was increased proportionally as the amount of lipase increased up to 1,000 units of lipase/mmol of triolein, and thereafter, it remained at a similar level without any further increment. This may be due to either the limited reaction surface area of the emulsified triolein or a reverse reaction induced by an excess amount of lipase. The optimal amount of lipase was determined to be 1,000 units of lipase/mmol of triolein. Fig. 2(B) shows the effect of the β-CD concentration on the hydrolysis yield of 100 mM triolein, with changing the β-CD concentration from 10 to 30 mM. The highest hydrolysis yield was achieved at a β-CD concentration of 30 mM, which is the maximum level of β-CD when be dissolved in water at room temperature.

Fig. 2(C) illustrates the effect of the ratio of triolein to β-CD on the conversion. In this experiment, the triolein concentration was varied from 10 to 300 mM, while β-CD was fixed at 30 mM. The maximum hydrolysis yield was achieved at a triolein concentration of 100 mM, the mixing ratio of 10:3

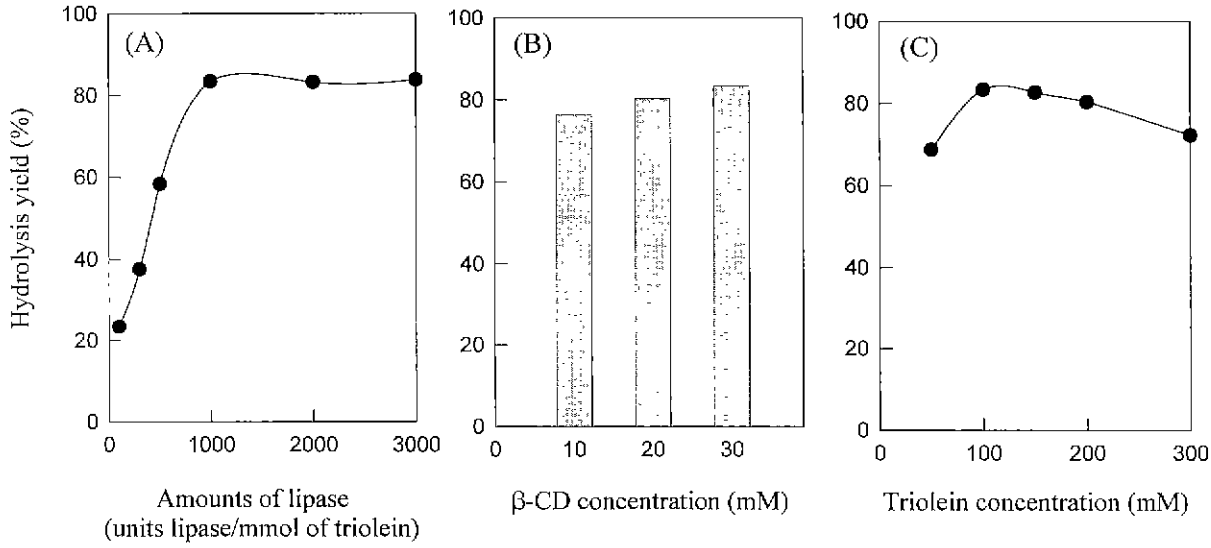

Fig. 2. Reaction conditions for hydrolysis of triolein by lipase in the mono-phase reaction system containing CD. (A) Effect of the amount of lipase, the reaction was carried out at 100 mM triolein, 30 mM β-CD, pH 7.0, 37℃, and 12 hrs. (B) Effect of the concentration of β-CD, the reaction was carried out at 100 mM triolein, 1,000 units lipase/ mmol of triolein, pH 7.0, 37℃, and 12 hrs. (C) Effect of the concentration of triolein, the reaction was carried out at 1,000 units lipase/ mmol of triolein, 30 mM β-CD, pH 7.0, 37℃, and 12 hrs.

(mM of triolein : mM of β-CD), and it decreased gradually as the triolein concentration increased. However, the hydrolysis yield decreased at a triolein concentration of lower than 100 mM. Since the decrement of the hydrolysis yield with a limited amount of triolein is a very unusual compared to other hydrolysis reactions of hydrophobic substrates by lipase [9], further study is needed to clearly understand the above observation.

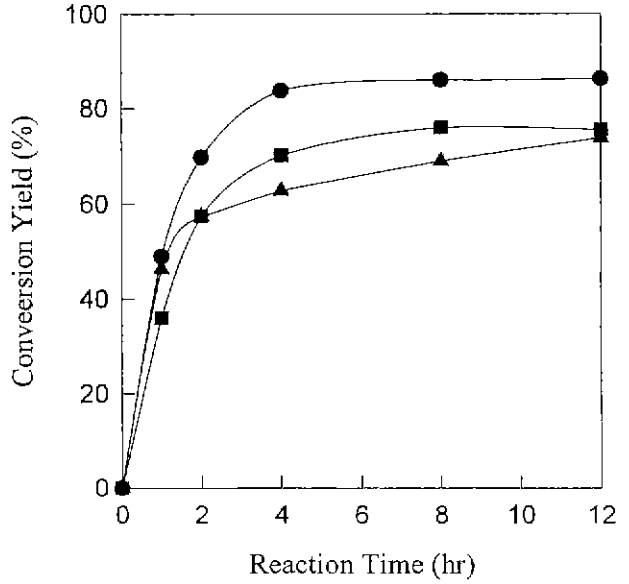

Fig. 3. Progresses of hydrolysis of triolein by lipase in different enzyme reaction systems. Reaction was carried out at 100 mM triolein, 1,000 units lipase/mmol of triolein, pH 7.0, 37℃, 200 rpm, and for 12 hrs, additions of 30 mM β-CD, and 20% hexane, respectively. Reaction systems with 30 mM of β-CD(●) and without β-CD(▲), and 20% hexane(■).

Comparison of a Mono-phase Reaction System with a Two-phase Reaction System

Fig. 3 compares the progress of triolein hydrolysis reaction at the two different enzyme reaction systems; the mono-phase reaction systems with and without β-CD, and the two-phase reaction system using hexane as the organic solvent. The mono-phase reaction system containing β-CD showed better results compared to the water-hexane two-phase-reaction system, and the hydrolysis yields after 12 hrs of incubation reached up to 86.3% and 73.8%, respectively. The β-CD containing reaction system seems to be advantageous over the two-phase reaction system, because the inactivation of the lipase and the reduction of water activity caused by an organic solvent can be prevented. In the absence of β-CD, however, the yield of aqueous enzyme reaction system was reduced to 70.3%, the lowest value compare to above two reactions.

Comparison of the Composition of Triolein Hydrolysates

The profiles of triolein hydrolysate obtained from the reaction systems with and without CD were analyzed by TLC, and compared in Fig. 4. The profiles of the triolein hydrolysates in both reaction systems were not significantly different, and they were composed of triolein, oleic acid, 1,2- and 1,3-diolein, and monoolein. This result is identical with the profiles reported by Sugihara *et al.* [26] who studied the reaction characteristics of lipase from *Penicillium abeanum*. It appears that the catalytic mode of action of the lipase was not modified by the presence of CD molecule.

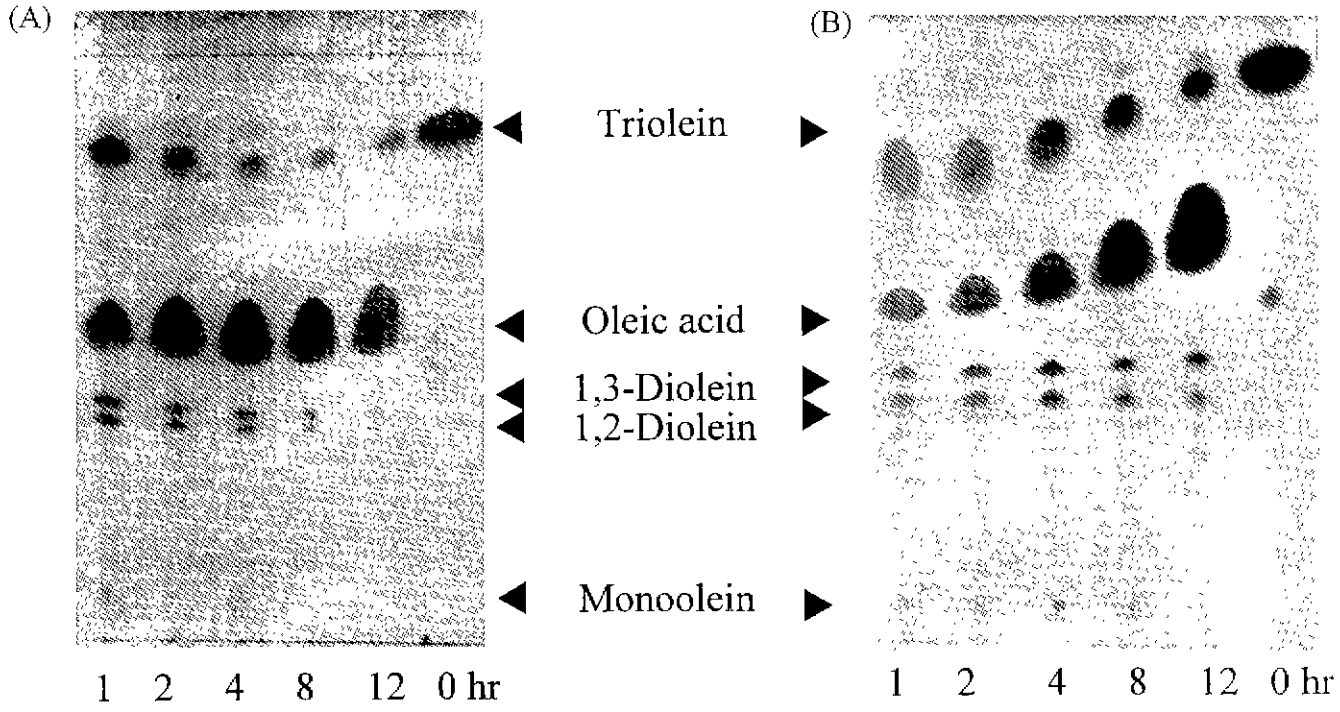

Fig. 4. Compositional profiles of triolein hydrolysates during reaction in the mono-phase reaction system containing CD and without CD. Reaction systems with β-CD(A) and without β-CD(B).

Inclusion Complex Formation of β-CD with Triolein and Oleic acid

The capacities of the inclusion complex formation of β-CD with the hydrophobic substrate triolein and the product oleic acid were compared, after changing the molar mixing ratio of triolein or oleic acid with β-CD. Both of them formed inclusion complex well with β-CD, however, the oleic acid was around 3 times higher than the triolein. Schrenk *et al.* [27] reported that all types of lipids can enter into the cavities of CDs, however, free fatty acid can form a more stable inclusion complex compared to triglyceride.

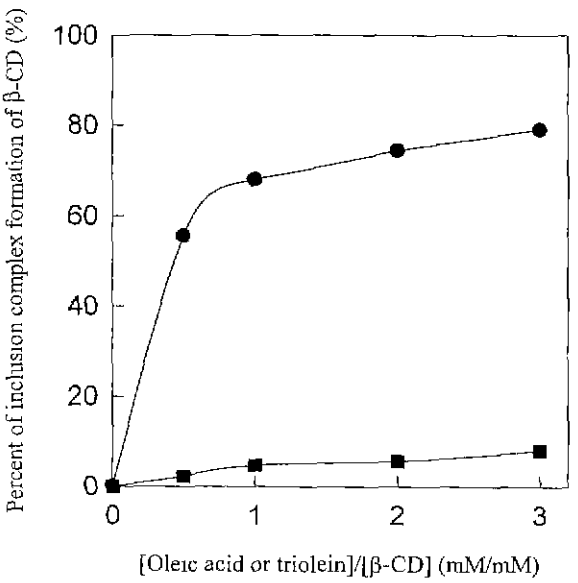

Fig. 5. Capabilities of the inclusion complex formation of oleic acid and triolein with β-CD. The inclusion complex was formed for 30 min at 0-9 mM of oleic acid(●) or triolein(■) at 3 mM β-CD.

Comparison of Kinetic Constants of Lipase Reaction in Mono-phase Reaction Systems With or without CD

Fig. 6 depicts Lineweaver-Burk plots of data obtained from the enzymatic hydrolysis of triolein in the mono-phase systems with and without CD. The K_m values in the reaction system containing β-CD was 4.3 mM compared to 13.6 mM in the aqueous reaction system without β-CD, indicating that the binding affinity between lipase and triolein is increased substantially by the addition of β-CD. Whereas the V_{max} values was not much different, 5.1×10^{-6} mmol/min for reaction system containing β-CD and 8.7×10^{-6} mmol/min of the aqueous reaction system. The enhanced reaction in the presence of β-CD seems to be due to the increased affinity between hydrophobic triolein and lipase.

Comparison of the Product Inhibition of Oleic Acid in Reaction Systems with and without CD

The role of β-CD in a mono-phase reaction system was further elucidated by comparing the product inhibition of additionally supplied oleic acid at the beginning of the reaction. The supplemented

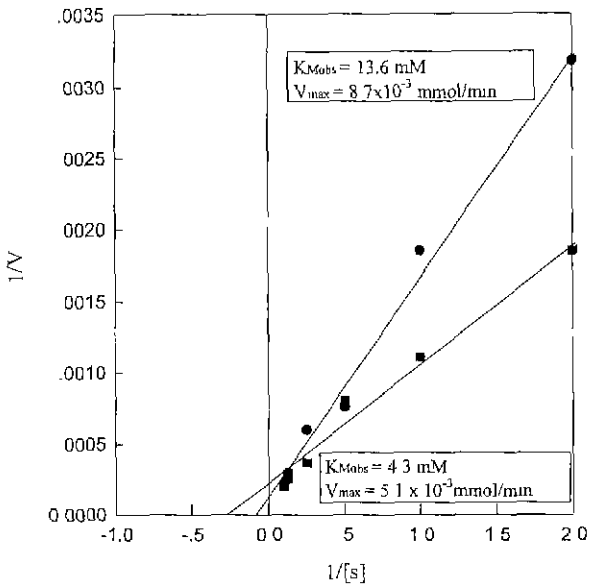

Fig. 6. Lineweaver-Burk plots of hydrolysis reaction of triolein by lipase in the mono-phase reaction systems with or without CD. The reaction was carried out at 0 05-10 mM triolein, 0.015-3 mM β-CD, 10 units/ml lipase, pH 7.0, 37℃, and 30 min. Reaction systems with β-CD(■) and without β-CD(●).

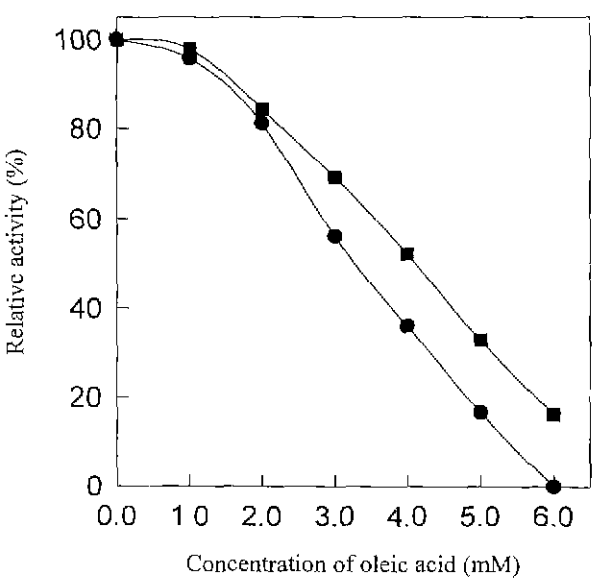

Fig. 7. Effect of CD on product inhibition of oleic acid. The reaction was carried out at 10 mM triolein, 0-6mM oleic acid, 100 units lipase/mmol of triolein, pH 7.0, and 37℃. Reaction systems with β-CD(■) and without β-CD (●).

oleic acid concentration was changed from 1.0 to 6.0 mM, while fixing the triolein concentration at 10 mM. The relative activity, the ratio of hydrolysis yield between added and not added oleic acid, obtained at the different amount of oleic acid was compared in Fig. 7. The relative activity decreased as the oleic acid concentration increased with both reaction systems indicating that inhibition by the oleic acid produced during the reaction. However, the inhibitory effect was reduced in the reaction system containing CD. β-CD may extract oleic acid from the active site of lipase by the formation of

inclusion complex, and therefore by reduce the degree of product inhibition.

Conclusively, the enhanced reaction of the enzymatic hydrolysis of hydrophobic substrate triolein by lipase in a mono-phase reaction system containing CD is caused by two phenomena; firstly, the increment of accessibility of the surface area of the hydrophobic substrate by lipase through the emulsification of the triolein by an inclusion complex formation, and secondly, the reduction of product inhibition caused by oleic acid through the removal of product from the active site of lipase. The detailed function of CD in the above reaction systems and the applicability of the CD-containing reaction system to other enzyme reactions such as esterification, transesterification, and other biotransformations employing hydrophobic compounds as substrates need to be further studied.

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