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学位論文題目	Kinetic Analysis for Triglyceride Hydrolysis Using Lipase in Biphasic Oil-Water System (油水二相系におけるリパーゼを用いたトリグリセリドの加水分解速度論)
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## 論文内容要旨

Triglyceride hydrolysis using lipase has been proposed as a novel method to produce raw materials in food and cosmetic industries such as diacylglycerol, monoacylglycerol, glycerol and fatty acid. In order to design a reactor for utilizing this reaction on industrial scale, constructing a kinetic model is important. Since the substrates are oil and water, the hydrolysis takes place at oil-water interface. The triglyceride has three ester bonds, so that the hydrolysis stepwise proceeds. Thus, the reaction mechanism is very complicated but simplified models were applied up to now. Those models were still not enough to describe the complicated mechanism of the enzymatic triglyceride hydrolysis under wide range of operating conditions. This is because the differences between the interfacial and bulk concentrations of the enzyme, substrates and products and the interfacial enzymatic reaction mechanism were not rigorously considered in the models.

The purpose of this research is to construct a rigorous mathematical model which can describe the stepwise hydrolysis of triglyceride using lipase under wide range of operating conditions. As a model system, the experiments of triolein hydrolysis using a nonspecific lipase are performed in a biphasic oil-water system having a definite interfacial area. A rigorous mathematical model considering the differences between the interfacial and bulk concentrations of the enzyme, substrates and products, and stepwise enzymatic reaction mechanism with two substrates and two products at the interface is proposed. The model is applied to the experimental results obtained under various conditions of the initial enzyme concentration, the interfacial area and the initial concentrations of triolein and a fatty acid. The experiments of the triolein hydrolysis using a lipase with a positional specificity are also performed in the same system and the proposed model is modified by considering the difference of rates for cleaving the respective ester bonds of each substrate to describe the hydrolysis behavior using the specific lipase. Triolein hydrolysis behavior under various conditions either in biphasic or emulsion system is simulated by the models for predicting the appropriate conditions to efficiently produce the desired product such a diacylglycerol.

### Chapter 1 Introduction

The background of this research, present status of the kinetic model for the triglyceride hydrolysis using lipase,

the purpose and outline of this research have been described.

## **Chapter 2 Triolein Hydrolysis Experiments**

The hydrolysis experiments using a nonspecific lipase (CRL) were conducted in a biphasic oil-water system. The oil phase was isooctane containing triolein, and the water phase was phosphate buffer containing lipase. The effects of the initial enzyme concentration, the interfacial area and the initial concentrations of triolein and oleic acid on the triolein hydrolysis behavior were experimentally investigated. The consumption or formation rate of the respective components increased with the initial enzyme concentration and then tended to become constant at saturated enzyme concentration. The reaction rates also increased with the interfacial area. This is because the amounts of lipase and substrates existing at the interface increased. On the other hand, the reaction rates decreased with increasing the initial concentrations of triolein and oleic acid. At the same initial triolein concentration, lower reaction rates and the resulting time delay for giving the maximum concentration of the intermediates were observed when the oleic acid was initially added in the oil phase. Thus, the slower reaction rates were considered to be due to the competitive inhibition by oleic acid.

## **Chapter 3 Construction of a Kinetic Model for Hydrolysis Using Nonspecific Lipase**

In the triolein hydrolysis using nonspecific lipase, the ester bonds of the triolein were stepwise hydrolyzed to produce diolein, monoolein and glycerol while an oleic acid was released at each reaction step. In the biphasic system, triolein, diolein, monoolein and oleic acid were dissolved into the oil phase and glycerol was dissolved into the water phase. The model describing the stepwise hydrolysis of triglyceride by nonspecific lipase in the biphasic oil-water system was formulated on the basis of the following assumptions:

- 1) Each hydrolysis reaction proceeds via a Ping Pong Bi Bi mechanism;
- 2) The inhibition by oleic acid follows the competitive inhibition mechanism;
- 3) The total enzyme concentration at the interface is expressed by Langmuir adsorption isotherm;
- 4) The linear adsorption isotherm provides the relationship for the interfacial and bulk concentrations of the substrates and products;
- 5) The nonspecific lipase evenly cleaves the ester bonds I and II (at the edge and the center of the glycerol backbone, respectively) of each substrate.

Model equations for hydrolysis using nonspecific lipase were derived. There are 13 model constants including the kinetic constants (6), the inhibition constant (1), and the adsorption constants (6). These constants were estimated by fitting the model equations with the 12 sets of experimental data using CRL obtained under various conditions.

## **Chapter 4 Application of the Present Model and Discussion**

The model constructed in chapter 3 was applied to experimental results discussed in chapter 2 to estimate the model constants. The model well described the effects of various operating factors, such as the initial enzyme concentration, the interfacial area and the initial concentrations of triolein and the fatty acid, on the stepwise triolein hydrolysis by nonspecific enzyme. The validity of the estimated values was discussed. The kinetic constants were newly introduced in the present model. By comparing the constant values with the literature values, the inhibition constant and the adsorption constants were considered to be reasonable. The sensitivity

analysis for the model constants having no literature values showed that the reliability of the model constants except for one kinetic constant was considered to be high.

In order to discuss the present model's advantages, the model considering the Ping Pong Bi Bi mechanism without the inhibition by oleic acid, called the PP model and the model considering the conventional Michaelis-Menten mechanism instead of the Ping Pong Bi Bi mechanism, called the MM model, were also applied to the experimental results. The fitted results by the present model were in better agreement with the experimental results under any condition. Thus, in order to rigorously describe the entire process of the triolein hydrolysis, it is important to consider the stepwise reactions based on the Ping Pong Bi Bi mechanism as well as the inhibition by oleic acid.

Triolein hydrolysis behavior under various conditions not only in the biphasic but also in the emulsion system was simulated by the model for predicting the appropriate conditions to efficiently produce the desired product such a diacylglycerol. The maximum diolein yield was almost constant under various conditions. The reaction time giving the maximum value became faster with increasing the initial enzyme concentration and the interfacial area, but became slower with increasing the initial triolein concentration. In the emulsion system, at the same initial enzyme concentration, the total interfacial enzyme concentration decreased with the droplet diameter because of increase in the interfacial area. Therefore, increasing the interfacial area should be accompanied with increasing the initial enzyme concentration to keep interfacial enzyme concentration at saturated condition.

#### **Chapter 5 Modification of the Present Model to Hydrolysis Using Specific Lipase**

The experimental results using the positional specific lipase (PPL) were compared with those using the nonspecific lipase. In the case of PPL, the consumption or formation rates were much slower because of the catalytic activity of PPL was much lower than that of CRL. The maximum concentrations of diolein and monoolein using PPL were much higher than those using CRL, respectively. The positional specificity of PPL seemed to contribute to the difference in the maximum values. The model for the hydrolysis using nonspecific lipase was applied to the experimental results using positional specific lipase. However, poor fitted results were obtained under any conditions. Thus the model was modified to describe the triglyceride hydrolysis by positional specific lipase, and was formulated on the basis of the assumptions 1)-4) of the model for nonspecific lipase, and a following modified assumption:

5') The positional specific lipase does not evenly cleave the ester bonds I and II of each substrate.

Model equations for hydrolysis using specific lipase were derived. There are 21 model constants including the kinetic constants (12), the inhibition constant (1) and the adsorption constants (8). These constants were estimated by fitting the model equations with 8 sets of the experimental data using PPL obtained under various conditions.

#### **Chapter 6 Application of the Modified Present Model and Discussion**

The modified model for positional specific lipase constructed in chapter 5 was applied to the experimental results for PPL under various conditions. The modified model well described the effects of various operating factors, such as the initial enzyme concentration, the interfacial area and the initial triolein concentrations, on the stepwise triolein hydrolysis by positional specific lipase. Therefore, in order to describe the hydrolysis using positional specific lipase, it is important to consider the difference of rates for cleaving the respective ester bonds

of each substrate. Since no literature values were found for the model constants, it is difficult to quantitatively discuss the validity of the estimated values. Then, the model constants related to the rates for cleaving the respective ester bonds of each substrate were qualitatively discussed. The estimated values of these constants showed that the rate for cleaving the ester bond I was faster than the ester bond II. Thus, the estimated values of seven model constants related to the rates for cleaving the respective ester bonds of each substrate were considered to be reasonable.

Triolein hydrolysis behavior using PPL under various conditions not only in the biphasic but also in the emulsion system was simulated by the modified model for predicting the appropriate conditions to efficiently produce diacylglycerol similar to Chapter 4. PPL gave better diolein yields under any condition.

### **Chapter 7 Summary**

The works done in this research were summarized.

# 論文審査結果の要旨

リパーゼ酵素による油脂の加水分解は、高級脂肪酸やグリセリン、高い生理活性を有するモノ、ジアシルグリセロールなどの食品や化粧品原料の新たな生産手法として注目されており、この反応を工業規模で利用する反応装置設計においては、反応速度論モデルの構築が重要となる。この反応は、油水界面で生じること、逐次的に進行すること、二基質二生成物反応であることなどから非常に複雑であり、これまで極度に簡略化された速度論モデルが適用されてきた。本研究では、酵素や基質の界面への吸着・脱離機構、界面での逐次的な二基質二生成物型反応機構を考慮することにより、厳密な数学モデルを構築し、位置選択性のない酵素およびある酵素を用いた場合の広範な条件下での加水分解挙動を解析した。本論文はこれらの結果をまとめ、リパーゼ酵素による油脂の加水分解挙動を解明したもので全編7章よりなる。

第1章は緒論であり、本研究の背景、従来の知見、目的、および意義について述べている。

第2章においては、界面積既知の単純な油水二相系において、位置選択性のないリパーゼ酵素を用いてトリグリセリドの加水分解実験を行い、加水分解挙動に及ぼす諸因子の影響を実験的に明らかにしている。

第3章においては、酵素や基質の油水界面への吸着・脱離、界面での逐次的な反応、二基質二生成物型の酵素基質複合体生成機構、生成物阻害を考慮することにより、新規な数学モデルを導出している。

第4章においては、導出したモデルを種々の条件下での実験結果に適用することにより、モデル定数の推算を行っている。また、他の単純な仮定を用いたモデルとの比較により、本モデルの優位性を検討すると共に、本モデルを用いて、高い生理活性を有する中間生成物であるジアシルグリセロールを効率的に生産できる条件、さらには実用系であるエマルション系での分解挙動をシミュレートしている。

第5章においては、新たに位置選択性のあるリパーゼを用いた加水分解実験を行い、選択性のない酵素との分解挙動の違いを明らかにしている。そして、酵素の位置選択性を考慮することにより、数学モデルの拡張を行っている。

第6章においては、拡張モデルを種々の条件下での実験結果に適用することにより、モデル定数の推算を行うと共に、高付加価値生成物ジアシルグリセロールの生成挙動をシミュレートしている。

第7章は結論であり、研究全体の総括を行っている。

以上、要するに本論文は、数学モデルによるシミュレーションと実験の両面から、種々の選択性を有するリパーゼ酵素による油脂の加水分解反応挙動を定量的に解明したもので、酵素反応を工業的な生産手法として用いるための有効な知見を提供しており、生物反応工学の発展に寄与するところが多い。

よって、本論文は博士（工学）の学位論文として合格と認める。