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Enrichment in γ-Linolenic Acid of Acylglycerols by the Selective Hydrolysis of Borage Oil

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ABSTRACT

Selective hydrolysis of borage oil by *Candida rugosa* lipase immobilized on microporous polypropylene was carried out in an iso-octane-water two-phase system in order to obtain glycerides rich in γ-linolenic acid (GLA). Lipase was immobilized on hydrophobic microporous polypropylene supports by physical adsorption. γ-linolenic acid content in the unhydrolyzed acylglycerols could be raised to 51.7 mol% from an initial content of 23.6 mol% in borage oil with a yield of 59%. A simplified kinetic model was proposed for this selective hydrolysis. The Michaelis constant K_M and the maximal-rate constant V_{max} are 0.107 *M* and 393.9 U/mg-protein, respectively. Product inhibition with a dissociation constant of the enzyme-product complex K_I = 25 m*M* was confirmed. Some properties of the immobilized lipase were also examined.

Index Entries: γ-linolenic acid; selective hydrolysis; immobilized lipase; borage oil; product inhibition.

Abbreviations: ES*, complex of enzyme and substrate molecules; E_1 total mass of enzyme in the reactor (mg); k_{cat}, rate constant in the Michaelis-Menten equation (mmol/min mg protein); K_I, dissociation constant for the complex EP* (M); K_M, apparent Michaelis constant (M);

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P, oleic acid concentration (M); P_o, initial concentration of oleic acid (m*M*); S, substrate concentration (M); S_o, initial value of S (M); t, reaction time (min); V_i, initial velocity of reaction (μmol/min); V_{max}, apparent maximal velocity (μmol/min).

INTRODUCTION

γ-linolenic acid (GLA; all-cis 6, 9, 12-octadecatrienoic acid) is an important intermediate in the normal bioconversion of the essential fatty acid, linoleic acid (18:2n-6), to the eicosanoid precursor arachidonate *(1)*. GLA-rich acylglycerides have been applied in curing certain skin-related and other diseases, such as atopic eczema *(2)*, multiple sclerosis *(3)*, rheumatoid arthritis *(4)*. Recently, many investigators have actively participated in the research for the concentration of GLA from borage oil, evening primrose oil, and fungal oils for pharmaceutical and dietetic purposes. Borage oil has the highest level of GLA among various available sources of GLA, and is suitable as a substrate for the enrichment of GLA. Methods for the enrichment of GLA include urea adduct formation *(5)*, separation on Y-zeolite *(6)*, solvent winterization *(7)*, and enzymatic selective hydrolysis or esterification *(8–10)*. Rahmatullah et al. *(10)* reported that *Candida rugosa* lipase possesses good efficiency in the concentration of GLA from selective hydrolysis of borage oil and evening primrose oil.

Enzymatic selective hydrolysis of borage oil and evening primrose oil were performed in aqueous systems *(10,11)*. Because oils are not soluble in water, and lipase is known to be active at oil–water interface, hydrolysis of oils in organic two-phase system has received extensive attention. Brink et al. *(12)* and Dordick *(13)* gave various reasons to justify the use of organic media instead of aqueous solutions, e.g., increased solubility of nonpolar substrates, enhanced thermostability of enzyme, elimination of microbial contamination, and so on. However, the activity of enzyme at aqueous and organic interface was demonstrated to decrease with reaction time *(14,15)*. Immobilization can be employed to reduce the rate of enzyme deactivation. Many investigators reported that lipase immobilized on hydrophobic supports yields higher lipolytic activity than on hydrophilic supports *(16–18)*. Although lipase is generally immobilized on hydrophobic microporous supports by physical adsorption, leakage of lipase may be made negligible *(19)*.

The purpose of this work is to investigate the hydrolysis of borage oil in iso-octane by *C. rugosa* lipase immobilized by physical adsorption on hydrophobic microporous polypropylene. The immobilized lipase catalyzes the hydrolysis of borage oil at the organic–aqueous interfaces. Some properties of the immobilized lipase and the optimal hydrolysis conditions were described. Michaelis constant K_M, maximal velocity V_{max}

and dissociation constant K_I of the enzyme-product complex for the hydrolysis of borage oil were also determined.

MATERIALS AND METHODS

Materials

Type VII *C. rugosa* lipase (EC 3.1.1.3; triacylglycerol ester hydrolase) and borage oil were purchased from Sigma (St. Louis, MO). Microporous PP (Accurel EP 100, particle size: 200–1000 μm) was a kind gift of Akzo (Obernburg, Germany). Particle size in the range of 300–500 μm was selected as a carrier for enzyme immobilization by using a Tyler sieve. Isooctane was obtained from J. T. Baker (Phillipsburg, NJ) and employed as a solvent. The standards (heptadecanoic acid, 16:0, 18:0, 18:1, 18:2, and 18:3n-3) for GLC analysis were obtained from Sigma, and other standards (20:1, 22:1, and 24:1) were purchased from Nu Check Prep (Elysian, MN). The 18:2 fatty acid standard was also used to construct the calibration curves for fatty acids, as well as in the study of product inhibition. Olive oil emulsion was purchased from Sigma and utilized as a substrate for lipase assay.

Methods

Lipase Immobilization (20)

Ten milligrams of *C. rugosa* lipase powder was dissolved in 250 μL of 0.1 *M* phosphate-buffer solution (pH 7.0). Following centrifugation, the upper clear enzyme solution was removed. An appropriate amount of this clear enzyme solution was pipetted onto a glass sheet. Then, 100 mg of polypropylene prewetted with 0.5 mL of ethanol were added to the enzyme solution and flattened by using a small wood bar. This was then immediately placed into a vacuum oven at 25°C to strip off excess water.

Lipase Assay

The hydrolysis activity of the immobilized lipase was assayed using olive-oil emulsion. Ten milliliters of 10% v/v olive-oil emulsion were incubated at 37°C for 20 min. Immobilized lipase was then added, and the reaction proceeded with stirring by magnetic stirrer at 600 rpm for 20 min. The reaction was terminated by adding 10 mL of acetone:ethanol (1:1 v/v). The liberated free fatty acid was titrated with 0.1 *N* NaOH using a Metrohm 686 Titroprocessor (Herisau, Switzerland). A control test was performed by the same procedure as described above, except that no immobilized lipase was added to the emulsion. One unit of activity is equivalent to 1 μmol of free fatty acid liberated/min at 37°C.

Selective Hydrolysis Reaction

Two milliliters of iso-octane containing borage oil (average MW approx. 873.4) with a concentration of 0.05–0.3 *M* were placed into a screw-cap tube (15 mm ID), and 1 mL of phosphate buffer were added. This mixture was incubated in water bath at 30°C for 20 min, and immobilized lipase was added into the test tube. The reaction mixture was shaken for 1 min by vortex, then returned to the water bath, and stirred by magnetic stirrer (600 rpm) as the reaction proceeded.

The initial rate of lipase-catalyzed selective hydrolysis of borage oil was determined by measuring the amount of liberated free fatty acid. In determining the initial rate, the degrees of conversion for the substrate were maintained below 4%; 0.2 mL of solution in the supernatant of the reaction mixture was removed and added to a test tube containing a mixture of 4.8 mL benzene and 1 mL cupric acetate-pyridine. The reaction was stopped immediately by vigorous shaking of the mixture. Following centrifugation for 5 min, the upper layer was removed and recorded by a spectrophotometer (Metertek SP-810, Taipei, Taiwan) at 715 nm *(21)*.

GLC Analysis of Fatty Acid Components in Acylglycerols (22)

The reaction products resulting from lipase-catalyzed hydrolysis of borage oil were fractionated into water phase containing fatty acid and organic phase containing acylglycerols (tri-, di-, and monoglycerols) by the addition of 1 *N* NaOH of 50% ethanol solution. Acylglycerols were collected by evaporating the organic under nitrogen atmosphere. To convert acylglycerols into fatty acid methyl esters (FAME), 5 mg of acylglycerols, a known amount of internal standard (heptadecanoic acid dissolved in 50 μL of 1,2-dichloroethane) and 50 μL of 0.2 *M* TMSH (trimethylsulfonium hydroxide) methanol solution were added in a test tube. The tube was shaking by vortex for approx 20 s and then placed in air for 15 min. The FAME mixture was then analyzed by a China Chromatography model 8700F (Taipei, Taiwan) gas-liquid chromatography fitted with a flame-ionization detector. The column used was a DB-23 (30 m × 0.53 mm, J and W Scientific, Folsom, CA).

RESULTS AND DISCUSSION

Effects of Temperature on Initial Rate and Stability of the Immobilized Lipase

The effects of temperature on lipase activity and stability were examined and results are shown in Fig. 1. The immobilized lipase shows maximal activity at 45°C, and denaturing occurred rapidly at temperatures higher than 50°C. In the stability study, 4.5 U of immobilized lipase

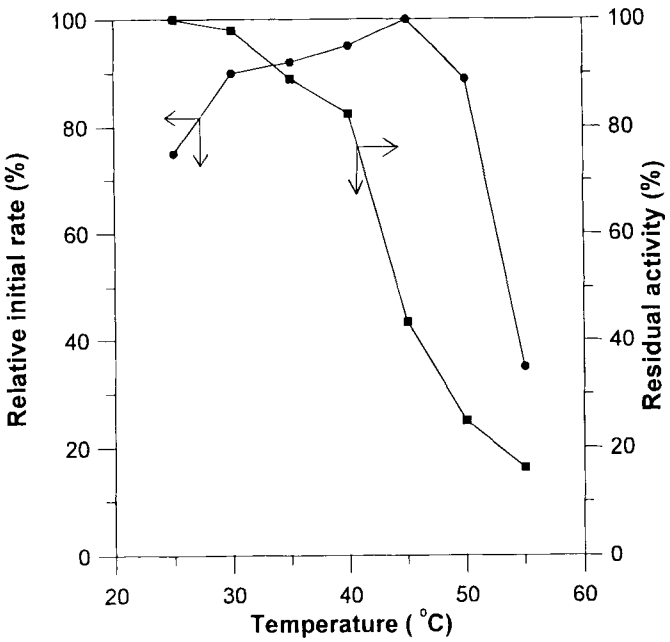

Fig. 1. Effects of temperature on activity (●) and stability (■) of the immobilized lipase. Reaction conditions: enzyme loading 4.5 U, substrate concentration 0.057 *M*, magnetic-stirrer speed 600 rpm. In the thermostability studies, the immobilized lipase was incubated in 0.1 *M* phosphate buffer for 1 h at the indicated temperature; 100% applied to temperatures of 25°C and 45°C for the activity and stability studies, respectively.

was incubated in 0.1 *M* phosphate buffer (pH 7.0) for 1 h at the desired temperature. An optimal operating temperature of 30°C was chosen by an examination of Fig. 1. A lower-reaction temperature is preferred to minimize thermal degradation of GLA.

Storage and Solution Stability

As shown in Fig. 2, immobilized lipase dried under 5 mm Hg-vacuum for 20 min retains approx 87% activity after 30 d of storage at 4°C. When incubated in 0.1 *M* phosphate buffer, the initial rate of immobilized lipase declines with increasing incubation time with a half-life of approx 10 h.

Effect of Reaction Time on the GLA Enrichment

Figure 3 shows the effect of reaction time on the enrichment of GLA content in acylglycerols by the selective hydrolysis of borage oil with immobilized *C. rugosa* lipase in iso-octane at 30°C. Maximal enrichment of GLA was obtained after a reaction time of 4 h, which resulted in a fraction of unhydrolyzed acyglycerols (mono, di, and tri-acylglycerols) of 27% and with 51.7% GLA in the residual acylglycerols. The yield of GLA in acyl-glycerols is 59% at a reaction time of 4 h. Further increase in reaction time

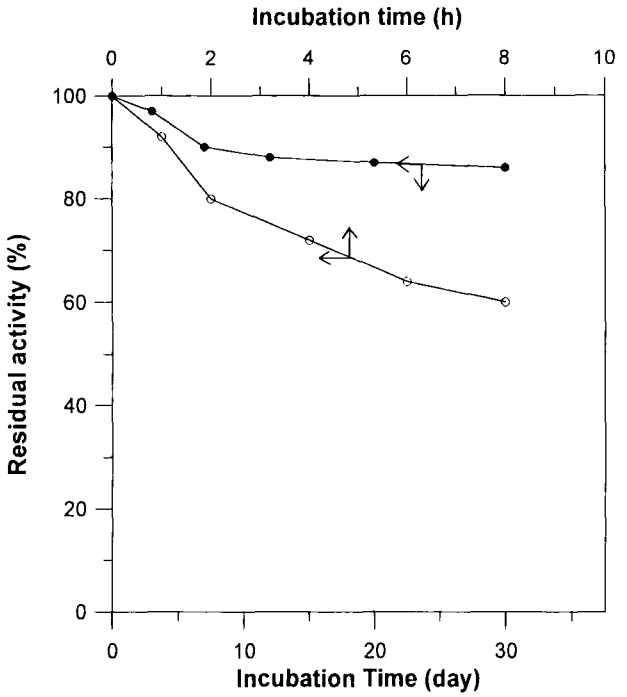

Fig. 2. Storage stability (●) and solution stability (○) of immobilized lipase. Reaction conditions are the same as those in Fig. 1, except that the reaction temperature is 30°C. Initial activity of lipase is taken as 100%.

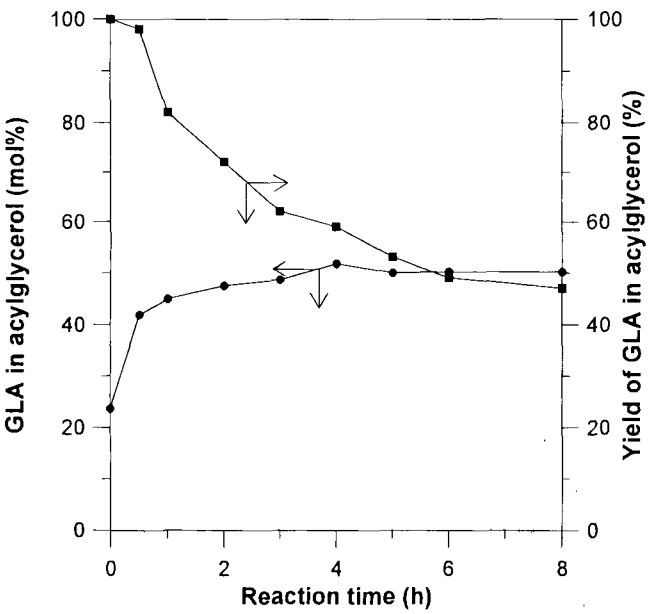

Fig. 3. Effect of reaction time on the GLA enrichment. Reaction conditions: substrate concentration 0.114 *M*, enzyme loading 4.5 U, magnetic-stirrer speed 600 rpm, reaction temperature 30°C.

Table 1
Relationship Between the Hydrolysis Product Concentrations and Reaction Time[a]

	0(h)	0.5	1	2	3	4	5	6	8
TG(mol%)	100	17	8	5	4	3	2.5	2	1
1,2DG	0	14.3	14.8	14.1	12.2	11.3	10.9	10.7	10.7
1,3DG	0	2.2	2.4	2	1.6	1.5	1.4	1.2	1.2
MG	0	0.5	0.8	0.9	1.2	1.2	1.2	1.3	1.3
FFA	0	66	74	78	81	83	84	85	86

[a] Reaction conditions: substrate concentration 0.114 *M*, enzyme content 4.5 U, magnetic stirrer speed 600 rpm, reaction temperature 30°C

leads to a decrease in the yield of GLA. This decreasing tendency was confirmed in a similar study by Rahmatullah et al. *(10)*. Table 1 gives compositions of the product of hydrolysis of borage oil in mol% as the reaction proceeded. At a reaction time of 4 h, 73% of FFA in acylglycerols of borage oil has been liberated by the hydrolysis reaction.

Kinetic Studies

The selective hydrolysis of borage oil is a complicate reaction because substrate is a mixture of various forms of glyceride. Michaelis-Menten kinetics can be employed to described the hydrolysis reaction by assuming that the reaction involves only single substrate (borage oil) and single product (linoleic acid). Linoleic acid was chosen because it is most abundant in the hydrolysis product (approx 50%, data not shown). Results of the initial rate as a function of substrate concentration are shown in Fig. 4. The reaction follows Michaelis-Menten kinetics for substrate concentrations up to 0.3 *M*. The apparent K_M and V_{max} are determined from the Lineweaver-Burk plot with P_o = 0 m*M* in Fig. 5, which are 0.107 *M* and 393.9 U/mg-protein, respectively.

Linoleic acid was employed as an inhibitor for investigating product inhibition. Figure 5 shows initial rate vs reciprocal of substrate concentration with linoleic acid concentration as a parameter. The results indicate that the selective hydrolysis of borage oil catalyzed by immobilized *C. rugosa* lipase is competitively inhibited by linoleic acid. The mechanism of the hydrolysis reaction can be expressed as:

$$\begin{array}{ccccc} & & k_1 & & \\ E + S & & \rightleftharpoons & ES^* & \xrightarrow{k_{cat}} E + P \\ & & k_{-1} & & \\ \Big\uparrow\Big\downarrow K_I & & & & \\ EP^* & & & & \end{array} \qquad (1)$$

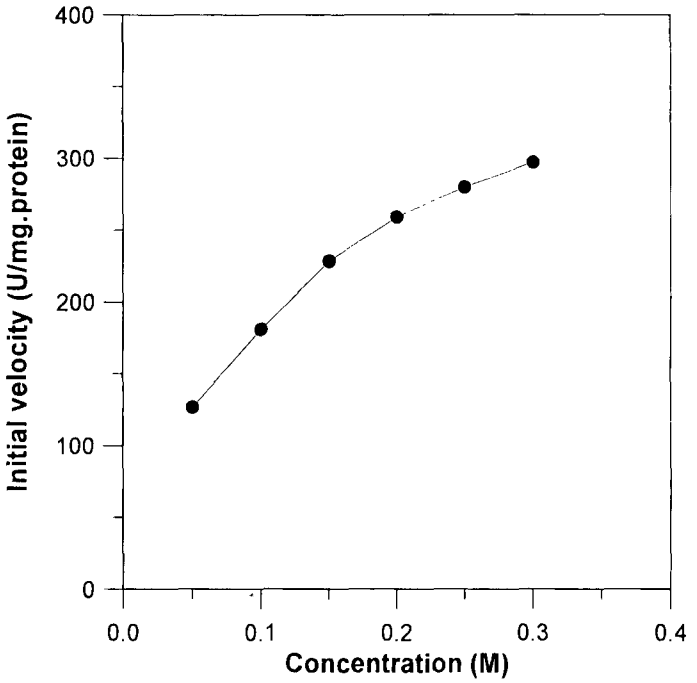

Fig. 4. Relationship between initial rate and substrate concentration. Reaction conditions: enzyme loading 4.5 U, reaction temperature 30°C, magnetic stirrer speed 600 rpm.

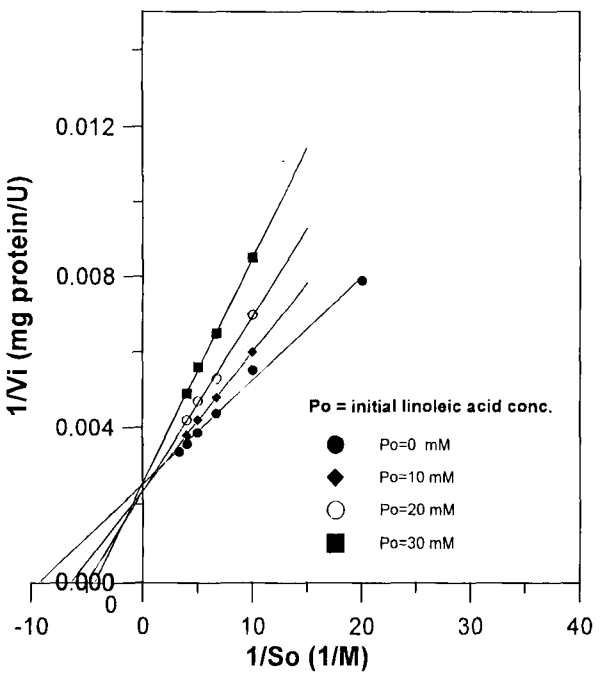

Fig. 5. Lineweaver-Burk plot for the selective hydrolysis of borage oil with linolenic acid concentration as a parameter. Reaction conditions are the same as those in Fig. 4.

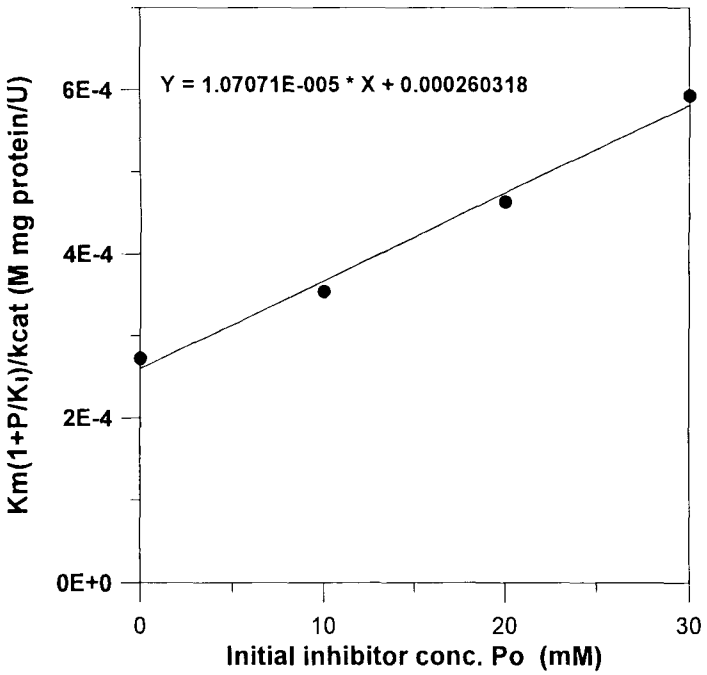

Fig. 6. Relationship between $K_M(1 + P/K_I)k_{cat}$ and initial linoleic-acid concentration. Reaction conditions are the same as those in Fig. 4.

where K_I is the dissociation constant of enzyme-product complex EP*. The initial rate V_i can be described as:

$$Vi = \frac{k_{cat}SE_t}{K_M\left(1+\frac{P}{K_I}\right)+S} \quad (2)$$

where Et is the total amount of enzyme, and S and P are concentrations of substrate (borage oil) and product (linoleic acid), respectively. It is clear from Eq. 2 that the slope of straight line in Fig. 5 is $K_M(1 + P/K_I)(1/k_{cat}E_t)$. A plot of this slope vs inhibitor concentration P should yield a straight line with a slope equal to $K_M/(K_Ik_{cat}E_t)$ as is indicated in Fig. 6. A K_I of 25 m*M* was determined. This small value of K_I reveals that product inhibition plays an important role in the selective hydrolysis of borage oil by immobilized *C. rugosa* lipase.

CONCLUSION

The selective hydrolysis of borage oil catalyzed by immobilized *C. rugosa* lipase was carried out in organic-water system at 30°C. Maximal

enrichment of GLA can be achieved with a reaction time of 4 h, in which GLA content in acylglycerols is raised from 23.6 to 51.7 mol%. Rahmatullah et al. *(10)* reported a GLA content of 47% in acylglycerols from selective hydrolysis of borage oil in aqueous solution. The results indicate that selective hydrolysis of borage oil in organic-water system is a feasible method for the enrichment of GLA. A Michaelis-Menten mechanism with competitive inhibition was proposed to describe the kinetics of this selective hydrolysis. A small K_I of 25 m*M* indicates significant product inhibition.

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