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Effect of Organic Solvents on Lipase for Interesterification of Fats and Oils

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유지의 에스테르교환에 있어서 유기용매가 리파제에 미치는 영향

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Abstract

The effect of organic solvents on the stability and catalytic activity of the microbial lipase from *Rhizopus arrhizus* for interesterification of fats and oils has been examined. The reaction system used was nonaqueous solvent system (two phase system). The solvents examined were 5 hydrocarbons (n-hexane, n-heptane, n-octane, isooctane, and cyclohexane) and 3 ethers (diethylether, diisopropylether, di-n-butylether). The results revealed that diisopropylether and isooctane are superior to the other solvents examined for interesterification of fats and oils in two phase systems.

Introduction

Interesterification is a process which is used in the edible fats and oils industry to alter the composition and therefore the physical properties of triglycerides mixtures. In this process, chemical catalysis by sodium metal or sodium alkoxide is used to promote the migration of fatty acyl groups between glycerol molecules so that the product consist of acylglycerol mixtures in which the fatty acyl groups are randomly distributed among the glycerol molecules.[1-2] By exploiting the benefits of the enzymes such as specificity of lipases and mild reaction conditions, etc.,[3-4] it is possible to produce useful glyceride mixtures which cannot be obtained by conventional chemical interesterification methods.[5-6] There are, in fact, many patents and publications on the enzymatic interesterification of lipids.[7-9]

In recent years, the bioconversion or biotransformation of lipophilic compounds (e.g., steroids and lipids) in two phase systems has been studied intensively, because two phase systems are advantageous when steroids and lipids which are poorly soluble in water are used.[10-11] In contrast to the bioconversion of steroids, however, there are limited number of research papers on the bioconversion of lipids in two phase system. According to Celebi *et al.*, the rate of lipid hydrolysis by lipase from *Candida rugosa* (Syn. *C. cylindracea)* increased appreciably when such solid lipids as tripalmitin and tristerain were dissolved in n-heptane.[12]

Yokozeki *et al.* reported regiospecific interesterification of triglycerides in n-hexane using immobilized lipase from *Rhizopus delema.*[13] However they did not elaborate any rationale as to why they used those specific solvents. From our previous study, issoctane was found to be the most suitable solvent in two phase system for fat splitting by lipase from *C. rugosa.*[14]

The objective of this report is to select the solvent most suitable for interesterification by lipase in terms of lipase stability and cataltic activity of lipase in two phase system. In this study lipase from *Rhizopus arrhizus,* which has 1,3-positional specificity,[3] was used to catalyzed the interesterification of a mixture of triglycerides or triglycerides plus fatty acids.

Materials and Methods

Materials

The highly refined lipase from *Rhizopus arrhizus* (718,000 units per 0.6 *ml* solution, according to the supplier) was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA).

The solvents used were 5 hydrocarbons (h-hexane, n-heptane, n-octane, issoctane and cyclohexane) and 3 ethers (diethylether, disopropylether and di-n-butylether). These solvents were purchased from Tokyo Kasei Chemical Co., Ltd. (Tokyo, Japan), and highly refined olive oil used was specially manufactured by Sigma as lipase substrate. All other reagents and chemicals used were of analytical grade. To maintain the constant water-solvent ratio of each solvent, all of the solvent used were saturated with water before treatments.

Methods

Effects of the solvent on the lipase for interesterification in two phase system were studied by measuring the lipase stability and catalytic activity. The stability and activity of lipase in organic solvents were determined by observing the lipid hydrolysis rate using olive oil as a substrate in a well-stirred batch reactor.

One unit of lipase activity was defined as one micromole of fatty acids produced per 10 min under the analytical conditions.

The stability of lipase in solvent was estimated by determining the residual activity of the lipase after thorough mixing with each solvent for 0.5, 1, 2, 4, 6, 8, and 10 hrs at 30°C. Lipase solution was prepared by adding 0.05 M phosphate buffer (pH 7.0) for dilution up to 3,300 units per 1 *ml* solution at 4°C; 5 *ml* of the lipase solution was added to each solvent (50 *ml*), thermally equilibrated at 30°C, and agitated at 800 *rpm* in the stirred batch reactor. After stopping the agitation at the predetermined time intervals, 200 $\mu\ell$ of the lipase solution was taken off from the underlayer of the mixture of solvent and enzyme solution by microsyringe. This enzyme solution was added to 5 *ml* of 10% (v/v) olive oil-isooctane solution for reaction. The residual activity of lipase was determined by estimating the free fatty acids produced as described below.

Lipase activity in each solvent was determined by assaying the free fatty acids produced. 5 *ml* of lipase solution prepared was added to 50 *ml* of olive oil-solvent (10%, v/v) reaction mixture at 30°C. After incubation for 10 min in the stirred batch reactor, 10 *ml* of 6N-HCl was added at the very end of the reaction and the mixture was agitated for about 30 sec. After stopping the reaction, the supernatant composed of fatty acids and solvent was taken, the solvent was evaporated from this supernatent with N_2 flushing, and the resulting fatty acids were redissolved in issoctane.

Free fatty acid dissolved in issoctane was determined by rapid colorimetric method.[15]

Results and Discussion

There are several possible advantages of biotransformation in two phase system such as maintenance of the high concentration of lipophillic substrate, minimization of the inhibitory effects of substrate and/or product on the enzyme, and easy separation of product and enzyme.[11] However, inevitably there are also potential disadvantages such as enzyme denaturation at the solvent-water interface and enzyme denaturation or inhibition by organic solvents dissolved in the aqueous phase. One objective of using water-immiscible organic solvent is to minimize the exposure of the enzyme to organic solvent. Thus the solubility of the organic solvent in water may be important, since it causes either inhibition of the reaction or denaturation of the enzyme.[10,16] Based on this property, we excluded carbonyls and halogenated hydrocarbons, because they have higher solubility in water. Besides water solubility, toxicity of the solvents and other physicochemical properties of the solvents must be considered in selecting a solvent for biotransformation in two phase system: density, dielectric constant, boiling point, freezing point, interfacial tension, hydrophille-lipophille-balance, etc.,[17] Aromatic hydrocarbons also were excluded, because they are highly toxic to human beings and also because they yielded lower proportions of fatty acids according to our preliminary tests. We included only aliphatic and cyclic hydrocarbons and ethers as the possible solvent. Among the aliphatic hydrocarbons, long chain aliphatic hydrocarbons ($n \geqslant 9$) were also excluded due to their lower yield of fatty acids (data not shown). However, we could not pinpoint any criterion for selecting the solvent for interesterification of lipids on the basis of these physical properties.

The lipases were fairly unstable in all hydrocarbons as shown in Fig. 1. The lipases in all hydrocarbons except n-octane were rapidly deactivated to halves within 2 hr and remained constant thereafter at about 30-50% level. The residual activity of lipase in n-octane was much

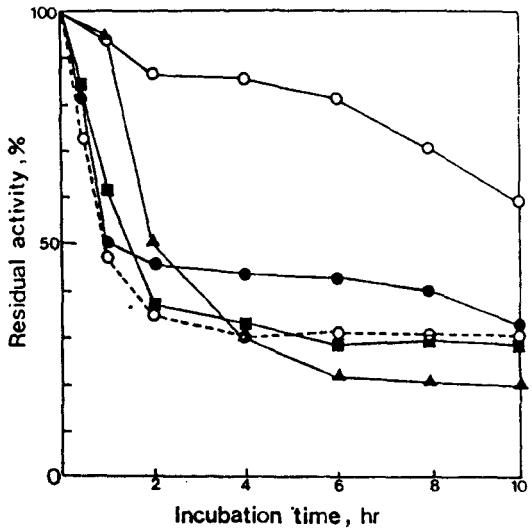

Fig. 1. Stability of lipase in hydrocarbons.
After incubation of lipase with each of the Solvents for 0.5, 1, 2, 4, 6, 8, and 10 hrs at 30°C, residual activity was determined by estimating the fatty acids produced with rapid colorimetric method. Each point represents the mean values of 3 determinations. Symbols: ○–○, n-octane; ●–●, n-hexane; ○- -○, n-heptane; ■—■ isooctane; ▲–▲, cyclohexane

higher than those of other hydrocarbons. Fig. 2 shows the residual activities of lipase in either group decreased rapidly in 2 hr exposition to the solvent and remained constant or slightly decreased thereafter to about 50-70% level. The data shows that the lipase, from *Rhizopus arrhizus*, in organic solvent was substantially unstable compared to that from *Candida rugosa*.[14] However, the patterns of solvent stability between the lipase from *R. arrhizus* and *C. rugosa* were almost the same.[14] The lipase used was highly purified from *R. arrhizus* (ca. 718,000 units per 0.6 *ml*) compared to the *C. rugosa* used in previous report (ca. 78 units per 1 *mg* solid, according to the supplier). From this result, we conclude that the enzyme which was highly purified must be more unstable in organic solvents than less refined from.

Table 1 shows the lipase activity in organic solvents with olive oil at concentration of 10% (v/v). The data indicate that the lipase in isooctane has good lipid hydrolysis rate with poor stability and the lipase in diisopropylether

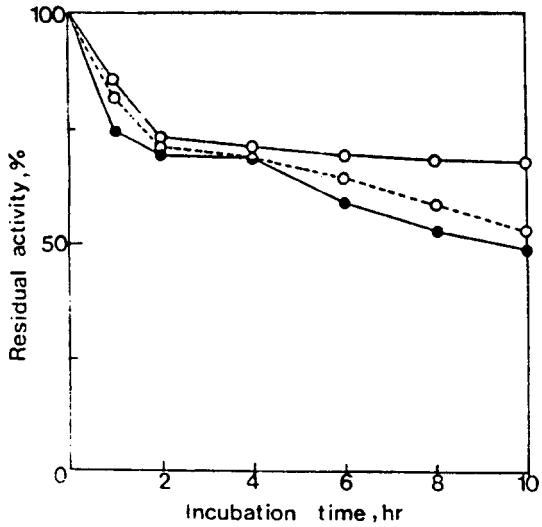

Fig. 2. Stability of lipase in 3 ethers. The procedure is in accordance with the process described in Fig. 1. Symbols: ○-○ diisopropylether; ○--○ dibutylether, ●—● diethylether

Table 1. Activity of *Rhizopus arrhizus* lipase in various organic solvent[a]

Solvent	Lipase activity[b]
Hydrocarbons	
n-Hexane	645 ±7.7
n-Heptane	519 ±31.8
n-Octane	500 ±11.0
Isooctane	1063 ±109.0
Cyclohexane	555 ±7.7
Ethers	
Diethylether	125 ±20.9
Di-n-butylether	592 ±5.4
Diisopropylether	940 ±31.0

a. 10% (v/v) olive oil is dissolved in each solvent.
b. One unit is defined as 1 μmole of fatty acids/10 min/5 *ml* of enzyme solution.
c. Mean value ± SD based on 3 determinations.

has good activity with higher stability. In the case of lipase from *C. rugosa*, isooctane has also good lipase activity.[14] To find out the better solvent between diisopropylether and isooctane, time course of lipid hydrolysis by the lipase from *R. arrhizus* in two phase system was detected (Fig. 3). Diisopropylether yielded lower lipase activity than isooctane within 1 hr reaction, however diisopropylether yielded much higher lipase activity than isooctane after 1 hr reaction time.

In contrast to the lipase from *C. rugosa*, the lipase from *R. arrhizus* in diisopropylether yielded good activity, whereas in diethylether and bi-n-butylether it showed poor activities. We reported previously that the lipase from *C. rugosa* showed poor activities in the ether group although their stability was excellent,[14] because of the probable competitive inhibition of ether moecules as suggested by Brockerhoff.[18] In fact, Bell *et al.* used the diisopropylether in the hydrolysis of triglycerides by mycelial lipase from *R. arrhizus* without elaborating any reason.[19] On the other band, as in the case of *C. rugosa* lipase, the lipase from *R. arrhizus* also was not inhibited by substrate or product in two phase system (data not shown), whereas lipase was inhibited by substrate in emulsion system.[20]

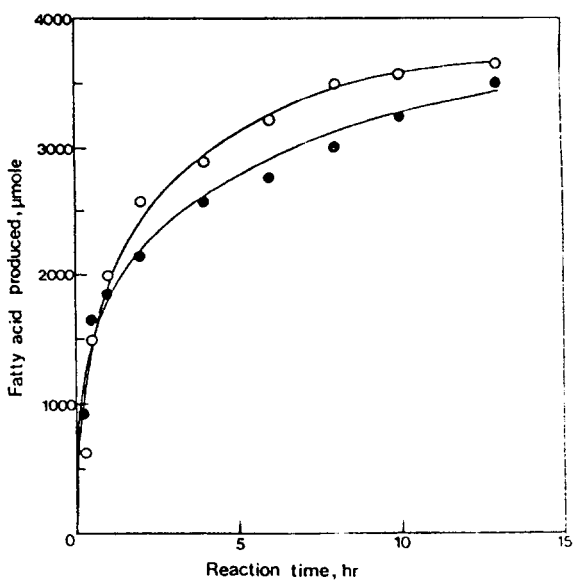

Fig. 3. Time course of lipid hydrolysis between diisopropylether and issoctane with 10% (v/v) oilve oil concentration. Fatty acids produced during the reaction for time intervals; 0.25, 0.5, 1, 2, 4, 6, 8, 10, and 13 hr were represented. Tests were run in duplicate. Symbols: ○-○, diisopropylether: ●-●, issoctane

From the above results, it can be concluded that diisopropylether is the most suitable for enzymatic interesterification in two phase system, and isooctane is also recommendable.

요 약

물에 녹지않는 기질을 사용할 수 있고, 다시 효소를 회수하여 다음에 사용하고자 할때 효소와 기질등 과의 분리등 여러가지 장점이 있는 2상계(二相界)를 사용하여 *Rhizopus arrhizus*의 리파제에 의한 에스테르 교환반응을 위한 기본 실험으로써 물에 녹지 않는 각종 유기용매 중에서 이 반응을 위한 가장 좋은 유기용매를 고르고자 유기용매가 리파제의 안정도와 활성도에 미치는 영향을 보았다. 이 실험에서 사용된 유기용매는 n-hexane, n-heptane, n-octane, isooctane, cyclohexane등 5개의 탄화수소와 diethylether, diiso-propylether, di-n-butylether등 3개의 에테르이다. 실험 결과 diiso-propylether와 isooctane이 다른 유기용매에 비해 월등히 2상계를 이용한 에스테르 교환 반응에 좋은 용매임을 알았다.

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