COMMUNICATIONS

Effects of Organic Solvents on Lipase for Fat Splitting

KEE H. KIM, DAE Y. KWON and **JOON S. RHEE**, Department of Biological Science and Engineering, Korea Advanced Institute of Science and Technology, P.O. Box 150, Chongyangni, Seoul, Korea

ABSTRACT

The effect of organic solvents on the stability and catalytic activity of the microbial lipase from *Candida rugosa* for hydrolysis of triglyceride (fat splitting) has been examined. The solvents examined were 5 hydrocarbons (n-hexane, n-heptane, n-octane, iso-octane and cyclohexane) and 3 ethers (diethylether, diisopropylether and di-n-butylether). The results revealed that iso-octane and cyclohexane are superior to the other solvents examined for enzymatic fat splitting in organic solvent systems.
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INTRODUCTION

In recent years, the bioconversion or biotransformation of steroids and lipids in water-immiscible organic solvents has been studied intensively, because these organic systems are advantageous when steroids and lipids which are poorly soluble in water are used (1,2). In contrast to the bioconversion of steroids, however, there are a limited number of research papers on the bioconversion of lipids in organic solvents. According to Çelibi et al., the rate of lipid hydrolysis by lipase from *Candida cylindracea* (syn. *C. rugosa*) was increased appreciably when such solid lipids as tripalmitin and tristearin were dissolved in n-heptane (2). Bell et al. used the solvent diisopropylether in the hydrolysis of the trglycerides by mycelial lipase from *Rhizopus arrhizus* (3). However, they did not elaborate any rationale as to why they used those specific solvents for hydrolysis of triglycerides (fat splitting).

The objective of this report is to select the solvent most suitable for fat splitting by lipase in terms of lipase stability and catalytic activity of lipase. In this report lipase from *Candida rugosa*, which has random specificity of reaction relative to the positions and fatty acids of triglycerides (4), was used to study the hydrolysis of olive oil.

MATERIALS AND METHODS

The lipase from *Candida rugosa* was purchased from Sigma Chemical Co. (St. Louis, Missouri, USA).

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

Effects of the solvent on the lipase were studied by measuring the lipase stability and activity in a well-stirred batch reactor. One unit of lipase activity was defined as one micromole of fatty acids produced per 20 min under the analytical conditions. The stability of the lipase in solvents was estimated by determining the residual activity of the lipase after thorough mixing with each solvent for 1, 2, 4, 6 and 8 hrs at 35 C. Lipase solution was prepared by dissolving 50 mg of lipase powder (155 units per 1 mg lipase powder, according to the supplier) in 5 ml of 0.05 M phosphate buffer (pH 6.5) at 4 C; 5 ml of the lipase solution was added to each solvent (50 ml), thermally equilibrated at 35 C, and agitated at 1,000 rpm in the stirred batch reactor. After stopping the agitation at the predetermined time intervals, 100 μl of the lipase solution were taken off from the underlayer of the mixture of solvent and enzyme by microsyringe. This enzyme solution was added to a 5% (v/v) emulsion solution of the tributyrin which contained 5% (w/v) gum arabic. The residual activity of lipase was determined by titrating the butyric acid produced with 0.05 N sodium hydroxide (5).

Lipase activity in each solvent was determined by assaying the fatty acids produced by a rapid colorimetric method (6). Phosphate buffer (5 ml, 0.05 M, pH 6.5) including 10 mg lipase powder was added to 50 ml of olive oil-solvent reaction mixture at 35 C. After incuba-

tion for 20 min in the stirred batch reactor, 10 ml of 6N-HCl was added and agitation was continued for about 30 sec. After stopping the agitation, the supernatant composed of fatty acids and solvent was taken, the solvent was evaporated from this supernatant with a rotary vacuum evaporator, and the resulting fatty acids were redissolved in n-hexane. Finally lipase activity in n-hexane was determined by observing the absorbance at 715 nm followed by computing the fatty acids produced from the calibration curve of fatty acids vs. absorbancy.

RESULTS AND DISCUSSION

One objective of using a water-immiscible solvent is to minimize the exposure of the enzyme to organic solvents. Thus the solubility of the organic solvent in water may be important, since it causes either inhibition of the reaction or inactivation of the enzyme as reviewed by Lilly (7). Based on this property, we excluded carbonyls (aldehydes and ketones) and halogenated hydrocarbons, since they have higher solubility in water. Aromatic hydrocarbons also were excluded, because they are highly toxic to human beings and 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 system. Besides the water solubility and toxicity of the solvents, other physicochemical properties of the solvent must be considered in selecting a solvent for enzymatic fat splitting: density, dielectric constant, boiling point, freezing point, protonic activity towards buffers, interfacial tensions, hydrophille-lipophille-balance and propensity for hydrogen bond formation (8). However, we could not pinpoint any criterion for selecting the solvent for fat splitting on the basis of these physicochemical properties.

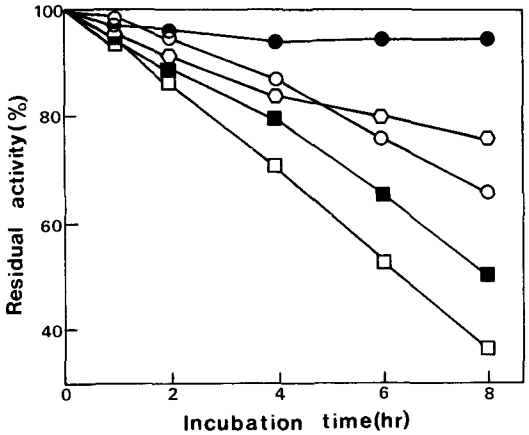

FIG. 1. Stability of lipase in hydrocarbons. After incubation of lipase with each of the solvents for 1, 2, 4, 6 and 8 hrs at 35 C, residual activity was determined by titrating the fatty acids produced in tributyrin emulsion with 0.05 N NaOH. Each point represents the mean value of 3 determinations. Symbols: ○ = n-hexane; □ = n-heptane; ● = n-octane; ■ = iso-octane, and ○= cyclohexane.

The lipases were relatively unstable in all hydrocarbons except n-octane (Fig. 1), whereas the lipases were all stable in the ether group. The residual activities in the ether group slightly decreased up to 2 hr and remained constant thereafter at about the 90-95% level. The lipases in n-octane and the ether group were, in fact, almost as stable as in the aqueous solution at

TABLE 1

Lipase Activity in Various Organic Solvents[a]

Solvent	Lipase activity for olive oil		
	3%[b]	10%	30%
Hydrocarbons			
n-Hexane	15.5 ± 0.2[c]	63.3 ± 0.3	213.3 ± 12.0
n-Heptane	14.1 ± 0.3	42.3 ± 1.3	158.7 ± 4.6
n-Octane	25.2 ± 2.2	42.3 ± 1.2	172.8 ± 6.6
Iso-octane	103.2 ± 1.1	326.2 ± 2.1	710.0 ± 31.0
Cyclohexane	64.1 ± 1.3	180.0 ± 2.5	414.0 ± 16.1
Ether			
Diethyl ether	<5.0	<5.0	15.9 ± 1.5
Diisopropyl ether	<5.0	25.2 ± 0.9	99.0 ± 1.8
Di-n-butyl ether	<5.0	<5.0	<5.0

[a]One unit is defined as one μmole of fatty acids/20 min/5 ml of enzyme solution.
[b]Olive oil concentration, % (v/v).
[c]Mean value ± SD based on 3 samples.

35 C (9). The lipase activity must be measured within the time range in which the activities of lipase do not appreciably change with the types of the solvent. The above results show that the residual activity of the lipase remained at about the 98% level when the enzyme was incubated for 20 min, regardless of the solvents used.

Table 1 shows the lipase activity in the organic solvents used at the substrate (olive oil) concentration of 3, 10 and 30% (v/v). The data indicate that the rate of the enzymatic fat hydrolysis increased with the increase of the substrate concentration up to 30% olive oil, whereas in the emulsion system the rate of fat hydrolysis was increased only up to 5% of olive oil concentration and decreased substantially thereafter with the increase of substrate concentration as shown by Kwon and Rhee (10). According to Linfield et al., the impurities in the olive oil inhibited the lipase activity (11). However, we did not find any inhibition in our study, as we used highly refined olive oil. Furthermore, branched (iso-octane) and cyclic (cyclohexane) hydrocarbons yielded much higher lipase activity than the group of straight-chain aliphatic hydrocarbons. On the other hand, the ether group yielded very poor activities despite their excellent stability. This poor activity probably is due to the competitive inhibition of ether molecules. Brockerhoff suggested, in fact, that the diethylether molecule reacts as a substrate inhibitor, thus inhibiting the activity of the lipase competitively (12).

From the above results, it can be concluded that iso-octane and cyclohexane are better solvents than any of the other solvents examined for enzymatic fat splitting in organic solvent systems.

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