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Beef Tallow Hydrolysis by Immobilized Lipase

KIM, DONG-JOON, DONG-HOON SHIN[1], BYUNG-KI HUR, AND EUN-KI KIM*

Department of Biological Engineering, Inha University, Inchon 402-751, Korea
[1]Graduate School of Biotechnology, Korea University, Seoul 136-701, Korea

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Abstract Beef tallow, which is an industrial lipid substrate, was hydrolyzed by lipase immobilized on a high-density polyethylene (HDPE) powder. Ethanol pre-washing process affected the immobilization efficiency. Half-life of storage of the HDPE at 4°C was 150 days. And after 10 times of repeated use, more than 50% of initial activity remained. An apparent Michaelis constant (K_m) and maximum velocity (V_{max}) were 2.7 M, and 1.4 mmol/min/l for immobilized lipase, and 0.5 M, and 1.9 mmol/min/l for soluble lipase, respectively.

Key words: Beef tallow, lipase, hydrolysis, immobilization

Current process for industrial lipid hydrolysis employs high-temperature and high-pressure reactor such as the ones used in Colgate-Emery method (250°C, 50 atm) [7]. This process has certain disadvantages such as cost of high-energy and formation of byproduct. Enzymatic lipid hydrolysis by lipase, on the other hand, not only produces few byproducts, but also it requires less energy and operates at room temperature under atmospheric pressure as well [4]. Lipase (Triacylglycerol ester hydrolase EC 3.1.1.3) hydrolyzes the lipid into glycerol and fatty acids and is currently produced from yeast, fungi and bacteria [6, 8]. One of the disadvantages of enzymatic lipid hydrolysis is the high cost of enzyme. In fact, many researchers focused on the repeated use of lipase. One approach is to immobilize lipase on the inert hydrophobic support [1, 2, 5]. Kinetic studies of the lipid hydrolysis by immobilized or soluble lipase has been mostly performed using lipid oil such as olive oil [3, 9, 11]. Beef tallow, an animal fat, is widely used as an industrial lipid substrate for soap making that is solid at room temperature (melting point 41°C). Although several thermostable lipases have been reported, very few kinetic data are available for beef tallow hydrolysis by lipase, especially by the immobilized lipase [3]. Characteristics of immobilized lipase, such as the kinetics of lipid hydrolysis, storage stability, activity loss after repeated use, would be important factors for achieving a successful process development of enzymatic beef tallow hydrolysis. In this report, by using *Candida rugosa* lipase, which is stable above the melting point of the beef tallow, optimum conditions of immobilization was determined and the kinetic constants of beef tallow hydrolysis by immobilized lipase was obtained.

*Corresponding author
Phone: 82-32-860-7514; Fax: 82-32-875-0827;
E-mail: ekkim@inha.ac.kr

MATERIALS AND METHODS

Enzymes and Hydrolysis Reaction

Beef tallow was obtained from Moogoongwha Co. (Seoul, Korea). The average molecular weight was determined by analyzing the fatty acids composition. Lipase-OF, a nonspecific lipase produced from *Candida rugosa*, was purchased from Meito Sangyo (Tokyo, Japan). As immobilization supports, high-density polyethylene (HDPE) granule, low-density polyethylene (LDPE) and polyacrylamide-6 were all purchased from Azko Chemicals (Wurzen, Germany). HDPE powder was obtained from Hanwha Company (Taejon, Korea). Average diameter of HDPE powder was 1 mm, whereas the size of granule type support was 1×2×4 mm. Two grams of support in 20 ml of ethanol were stirred for 3 h, followed by filtering through the No. 1 filter paper. Depending on the drying time at 30°C, the ethanol content that remained on the support varied and it was measured by the weight difference after a complete drying. Two grams of support containing ethanol was stirred in 100 ml of distilled water containing lipase (1,080 unit/ml) for 2 h at 150 rpm. Lipase-immobilized support was filtered through the No. 1 filter paper, followed by drying at 37°C for 24 h. Lipid hydrolysis was carried out in the 200 ml solution (500 ml flask) containing beef tallow and 2 g of immobilized HDPE powder for 24 h at 45°C. Flat type impeller at 250 rpm stirred the solution containing the beef tallow and immobilized lipase. For repeated usage,

immobilized lipase was filtered and new medium was added. For kinetic study, beef tallow concentration varied from 0.2 to 3 M.

Analysis

The amount of lipase immobilized was determined by measuring the soluble lipase remaining in the solution after immobilization. One unit of lipase was defined as the amount of enzyme producing 1 μmol of free fatty acid. Lipase activity was determined by measuring the free acid produced from olive oil (5%) solution containing arabic gum (5%) in 0.1 M of potassium phosphate buffer solution (2 ml, pH 7.0) [9]. Fatty acid concentration was determined by titration with 0.1 M of ethanolic KOH using phenolphthaleine as the pH indicator. Monoglyceride or diglyceride was measured by TLC/FID (Iatroscan) (Tokyo, Japan) using benzene: chloroform:acetic acid (70:30:2 v/v) as the solvent. Surface of the support before and after ethanol treatment was examined by scanning electron microscope (SEM).

RESULTS AND DISCUSSION

Ethanol Pre-Washing

Optimum concentration level of ethanol for the pre-washing of HDPE powder was determined by measuring the immobilized lipase at various ethanol concentrations. As shown in Fig. 1, few lipase was immobilized without going through ethanol pre-washing.

More lipase was immobilized on the support by increasing the ethanol concentration, and reached its maximum level at 1 g ethanol/g support. By using a scanning electron microscopy (SEM), we found that ethanol pre-washing removed the impurities on the surface of HDPE powder (picture not shown). Also ethanol pre-washing probably increased the wettability of hydrophobic HDPE support

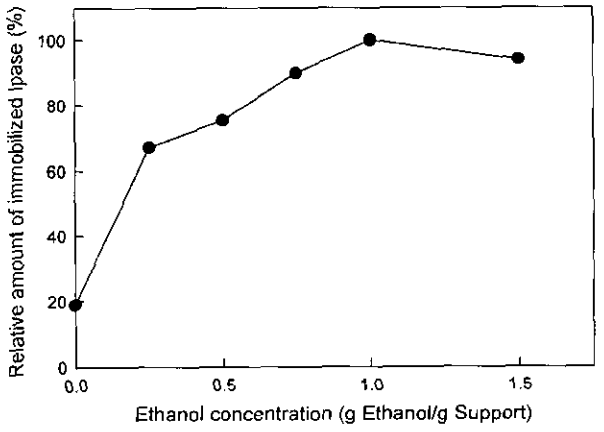

Fig. 1. Effect of ethanol amount in the support on the activity of lipase immobilized on HDPE powder.

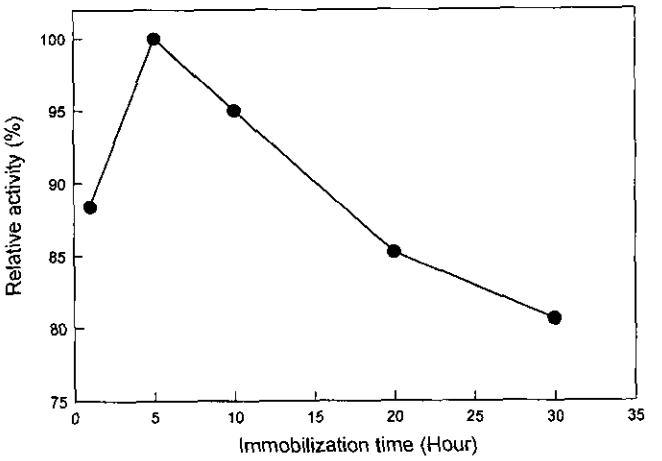

Fig. 2. Effect of contact-time during immobilization on lipase activity.
HDPE powder containing ethanol (1 g ethanol/g support) was stirred in lipase solution without substrate for varying time. After the support was filtered, lipase activity was measured.

with water, since ethanol is less polar than water. As a result, removing the impurities and increasing the wettability both increased the physical attachment of lipase on the hydrophobic support.

Immobilization time affected the activity of immobilized lipase. As shown in Fig. 2, the optimum contact time was for 5 h. Decreased activity after 5 h was probably due to the deactivation of lipase by being in contact with ethanol. Among the various hydrophobic supports, HDPE powder was the most effective support (Table 1). Large surface of HDPE powder probably increased the lipase immobilization.

Storage Stability

Immobilized lipase was stored at 4°C and its activity was determined for 30 days. As shown in Fig. 3, 90% of the initial activity was maintained after 30 days of storage.

Half-life of storage in this storage condition was 150 days. Stability of immobilized lipase during a long-term storage shows that this immobilization method by physical adsorption is very simple, convenient and efficient for practical applications.

Table 1. Immobilization efficiency of various supports.

	Immobilized contents (U/g carrier)	Relative activity (%)	Activity after 4 repeated use (%)
Soluble lipase	-	100	-
HDPE powder	270	95	71
HDPE granule	182	88	58
LDPE granule	138	72	43
PA-6 granule	26	37	12

*Ethanol content of each sample was maintained at 1 g ethanol/g support.

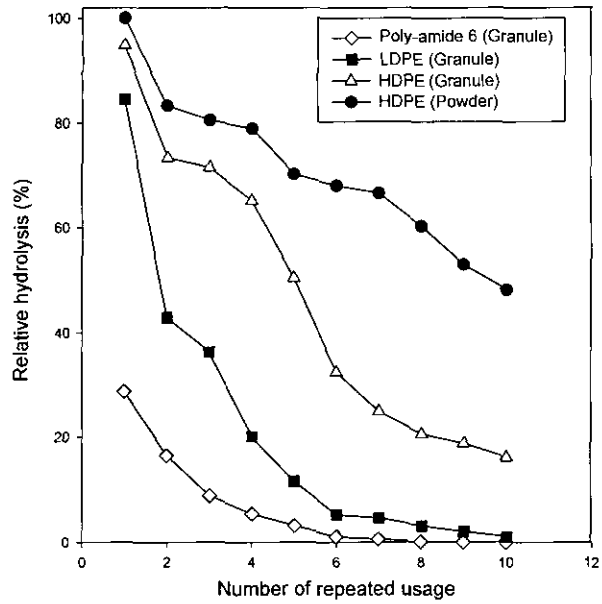

Fig. 3. Storage stability of immobilized lipase.
HDPE powder containing 1g ethanol/ g support was used for immobilization.

Repeated Usage

Hydrolysis of beef tallow was repeated 10 times. After 10 times of repeated usage, more than 50% of the initial activity was retained (Fig. 4). Initial decrease in hydrolysis rate after the first use could be attributed to the insufficient washing of immobilized matrix after immobilization. Among the tested supports, HDPE powder retained more lipase activity after repeated use.

Attempt to recover the soluble lipase after batch hydrolysis resulted in less than 50% yield (data not shown). Although hydrolysis condition using soluble lipase or immobilized lipase was not identical, this comparison shows that the use of immobilized lipase on HDPE powder can be a positive alternative for industrial application. After 5 h of hydrolysis reaction, granular-type supports were easily filtered from the reaction solution. However, in a case of HDPE powder, 20 ml of isooctane were added to 200 ml of reaction solution for an efficient filtration process, since aggregation of the HDPE powder with beef tallow hindered the filtration. More study needs be conducted to provide an efficient filtration and recovery of HDPE powder after the hydrolysis reaction take place.

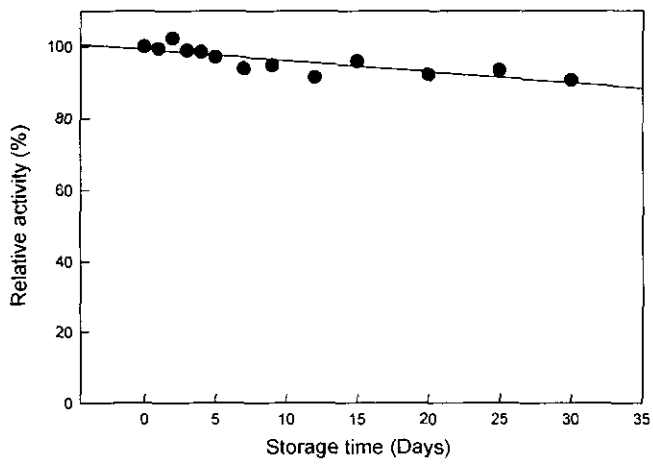

Fig. 4. Repeated use of immobilized lipase.
For immobilization, 2 g of each support (1 g ethanol/g support) was stirred in the lipase solution (1,080 U/l). Hydrolysis rate was measured by titrating the fatty acid after 24 h of reaction time with 0.5 M beef tallow.

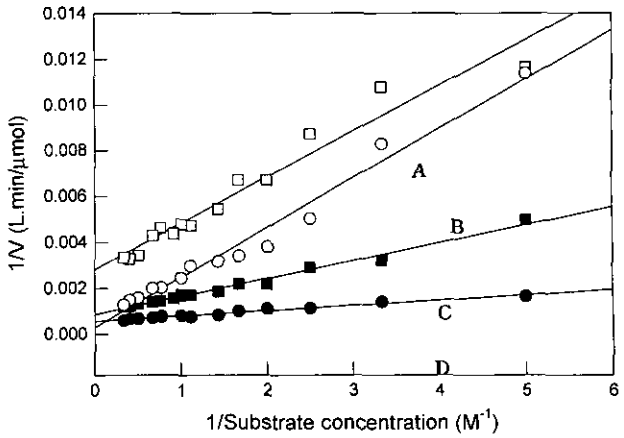

Fig. 5. Lineweaver-Burke plot of soluble and immobilized lipase activity.
A: Immobilized lipase (Water+5% isooctane), B: Soluble lipase (Water only), C: Soluble lipase (Water+5% isooctane), D: Immobilized lipase (Water only).

Hydrolysis Kinetics

Kinetic constants of immobilized lipase on beef tallow were obtained to compare those with soluble lipase hydrolysis. Influence of substrate on the initial hydrolysis rate was investigated at various substrate concentrations. Lineweaver-Burk plot was performed for the kinetics (Fig. 5). Kinetic constants of beef tallow hydrolysis were compared between the soluble and the immobilized lipase (Table 2).

Maximum hydrolysis rate and K_m value of immobilized lipase was 0.7 and 5 times more of the soluble lipase,

Table 2. Kinetic constants of beef tallow hydrolysis by lipase.

Lipase	Kinetic constants	
	$Vmax_{app}$ (μmol/min/l)	Km_{app} (M)
Soluble[a]	1928.3	0.5
Immobilized[a]	1430.5	2.7
Soluble[b]	1055.2	0.8
Immobilized[b]	407.5	1.0

a: Reaction solution contained water, beef tallow and enzyme.
b: Reaction solution (200 ml) contained water, beef tallow, enzyme and isooctane (10 ml).

respectively. In this study, affinity of immobilized lipase to beef tallow was decreased as indicated by the increased Km values. Lipase, during the hydrolysis reaction, was reported to be located between the oil/water interface that was in an active state in the same area [6]. However, when the lipase was immobilized, specific activity decreased as the wettability of support surface decreased, due to the conformational change of the enzyme [10]. Decreased affinity of immobilized lipase, as observed in this study, could be attributed to the same phenomena. Also the support could prevent the direct, physical contact between lipase and substrate, reducing the activity. Addition of isooctane decreased the V_{max} as well as K_m of immobilized lipase (Table 2). Although isooctane increased the affinity of immobilized lipase and beef tallow, the rate of hydrolysis without isooctane was higher than that with isooctane.

Acknowledgments

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