Esterification Kinetics of Long-Chain Fatty Acids and Fatty Alcohols with a Surfactant-Coated Lipase in *n*-Hexane

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ABSTRACT: The esterification reaction kinetics of long-chain fatty acids and fatty alcohols catalyzed with a surfactant-coated lipase in a microaqueous *n*-hexane system were studied. The biocatalytic complex, surfactant-lipase adduct, showed 40 times the activity after a reaction time of 5 h compared to the unmodified lipase in the same reaction system. Various factors that may affect the activity of the modified lipase were studied, such as the influence of substrate fatty acid chainlength, water content, and temperature. By varying the concentration of each of the two substrates while keeping that of the other substrate constant, it was found that the esterification reaction follows Michaelis-Menten kinetics. The surfactant-enzyme complex kinetic parameters were determined with respect to both substrates. It was suggested that the kinetics of the lipase-catalyzed esterification reaction model follow a Ping-Pong Bi Bi mechanism with no substrate or product inhibition.
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KEY WORDS: Esterification, fatty alcohols, lipase, long-chain fatty acids, microaqueous organic media, surfactant-coated lipase, kinetics.

The lipase-catalyzed esterification of carboxylic acids and alcohols in microaqueous organic systems is currently of great industrial interest because of the wide application of this enzymatic reaction to synthesis of useful ingredients in the food, detergent, pharmaceuticals, and cosmetic industries (1–4). For example, compounds resulting from the esterification of long-chain fatty acids with long-chain alcohols offer inexpensive substitutes for other expensive natural oils, such as sperm whale oil, jojoba oil, and other materials used as plasticizers and lubricants (1). These esters can be produced conventionally by simple esterification reactions aided with mineral acids, or with metal catalysts based on cobalt chloride (5). Both conventional technologies lead to the formation of undesirable by-products.

The use of lipases to catalyze esterification of fatty acids and alcohols in microaqueous organic solvents to synthesize "natural"-like esters offers an attractive alternative. In this reaction, lipases that promote the hydrolysis of esters in aqueous systems catalyze the reverse reaction in microaqueous hydrophobic organic solvents. Different lipase preparations have been used for this purpose (6,7). Powdered lipases have been used to synthesize esters in organic solvents. However, because of mass-transfer restrictions, low product yields were obtained (3,8,9). In order to activate lipases and to minimize mass-transfer restrictions in organic solvents, lipases have been solubilized in reverse micelles (10) or covalently attached to polyethyleneglycol (11) or alkyl amines (12–14). Lipases have also been confined in membrane reactors resistant to hydrophobic organic solvents, or adsorbed onto inorganic matrices in an attempt to increase their activity and to regenerate them for further use (4,9). Bioreactors have also been developed to use lipases in supercritical CO_2 (15).

Following the authors' previous studies, it has been shown that lipase complexes coated with fatty acid sugar ester type surfactants gave high esterification activity (16,17). Furthermore, application of the reported lipase modification technique has enabled the conversion of inactive or slightly active crude lipases into active dispersible biocatalysts in organic systems. Recent studies have shown that surfactant-coated lipases can be widely applied as active biocatalysts to catalyze oil and fat interesterification and in esterification/interesterification reactions to separate optically active compounds (18–20).

In all techniques that use lipases as biocatalysts to perform esterification reactions in organic media, the reaction system water content is of crucial importance to determine enzyme activity. It was concluded that lipases vary their activity depending on the water content in the reaction system. However, there is no common explanation describing different lipase activities as a function of the amount of water in the reaction system (21,22). Several research studies dealing with the kinetics of lipase-catalyzed esterification in organic media have recently appeared in the literature. In most cases, lipase preparations were found to follow the Michaelis Menten kinetics with the Ping-Pong Bi Bi mechanism with dead-end inhibition primarily by the alcohol used as a second substrate (19,23–25) .

The aim of the present work was to study surfactant-coated lipases with respect to their catalytic activity and their kinetics. The esterification of lauric acid with dodecyl alcohol in *n*-hexane served as the reaction model.

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MATERIALS AND METHODS

Materials. Lipase Saiken 100, produced from *Rhizopus japonicus*, was supplied by Nagase Biochemicals, Ltd. (Osaka, Japan). All fatty acids and fatty alcohols with better than 99% purity were from Fluka (Buchs, Switzerland). *n*-Hexane and other solvents, all of analytical grade, were obtained from Bio Lab (Israel). Sorbitan monostearate ester was purchased from Kao Pure Chemicals Ind. (Tokyo, Japan). Tris(hydroxymethyl)aminomethane (Tris) was obtained from Sigma (St. Louis, MO).

Lipase modification. The crude lipase preparation, Saiken 100, contained 11% protein; it was coated with sorbitan monostearate according to the authors' previously reported procedure (16,17). A typical surfactant-coated lipase preparation was prepared as follows: Crude lipase (3 g) was dissolved in 1 L Tris buffer at pH 5.5 and magnetically stirred at 10°C. Sorbitan monostearate (0.75 g) dissolved in 20 mL ethanol was added dropwise to the stirred enzyme solution. The resulting colloidal enzyme solution was sonicated for 10 min and then stirred for 3 h at 10°C. The precipitate was collected by centrifugation at 23,500 × *g* and 4°C, followed by freeze-drying. Based on elemental analysis, the protein content of the enzyme–surfactant complex was 11%.

Esterification reaction. The esterification reactions of lauric acid and dodecyl alcohol were initiated by adding modified lipase to 10 mL *n*-hexane that typically contained 100 mmol/L lauric acid and 100 mmol/L dodecyl alcohol. The reaction solution was magnetically stirred and the temperature was maintained at 40°C. Samples of 50 µL were taken periodically, and filtered through 0.45 µm Millipore filters before being mixed with a similar volume of *n*-hexane solution containing *n*-hexadecane as an internal standard. Initial reaction rates were estimated from the slope of plots of the concentrations of fatty acids, dodecyl alcohol, or their corresponding ester at conversions less than 7% vs. time and reported as µmol/(min·mg biocat.). Unless stated otherwise, all experiments were carried out under these conditions. Each esterification reaction was carried out in duplicate. In all experiments, *n*-hexane was dried over 5 Å molecular sieve to reduce its water content to 6 mg/L. The water content in all reaction systems, except those in which the effect of water was investigated, was initially less than 30 mg/L.

Analytical methods. The changes in concentration of fatty acids, fatty alcohols, and their corresponding esters with time were determined in a Hewlett-Packard gas chromatograph (HP 5890; Palo Alto, CA), equipped with a flame-ionization detector. A capillary column, RTX-65TG, 0.53 mm i.d. × 30 m, 0.1 µm film thickness (RESTEK Corp., Bellefonte, PA), was used under these separation conditions: Injector and detector temperatures were maintained at 300°C; initial column temperature was 80°C, for 1 min, thereafter, the oven temperature was raised at a rate of 20°C/min to 270°C; water content in the reaction system was measured with a Karl Fischer titrator 684 KF (Metrohm, Herisau, Switzerland), and the protein contents of the crude and modified lipases were measured by a nitrogen analyzer (model FP-428; Leco, Tokyo, Japan).

RESULTS AND DISCUSSION

Figure 1 shows the time course of the esterification reaction between lauric acid and dodecyl alcohol catalyzed with the crude lipase, Saiken 100, and the sorbitan monostearate-coated lipase without removal of the water produced in the reaction system. Clearly, while the crude lipase showed a very low esterification activity the modified lipase under the same reaction conditions exhibited a 40-fold increase in activity, reaching initial activity values of 2 µmol/min·mg biocat. The conversion of the fatty acid to its corresponding ester following 40 h incubation was barely 5% when crude lipase was used, compared to more than 97% when the lipase modified with sorbitan monostearate was employed. These results show that coating the lipase with the surfactant led to marked enhancement of the enzyme catalytic activity in the esterification reaction of fatty acid and fatty alcohol. The mode of interaction between the enzyme and the surfactant is still not understood. Okahata and Ijino *et al.*(26) proposed that hydrogen bridges between the hydroxyl groups of the surfactant and free amino groups of the enzyme are the main interaction force that leads to the formation of surfactant–lipase complex. Nevertheless, adopting the lipase modification technique described in this study has led to the preparation of a highly active surfactant–enzyme complex to catalyze the esterification reaction between fatty acids and fatty alcohols. The nature of the interaction between the enzyme and the surfactant awaits further elucidation.

Effect of the modified lipase concentration on the reaction rate. The effect of biocatalyst concentration on the esterification reaction rate was studied by varying the amount of the

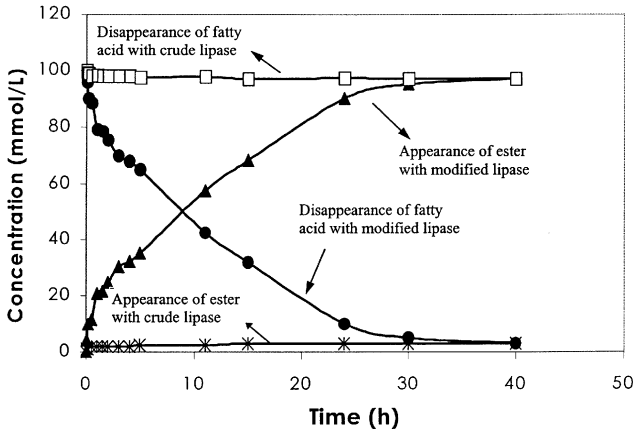

FIG. 1. Time course of the esterification of lauric acid with dodecyl alcohol by the surfactant-coated lipase Saiken 100 and by crude Saiken 100 (control). Reaction conditions: *n*-Hexane (10 mL) with 100 mmol/L lauric acid, 100 mmol/L dodecyl alcohol, and 5 mg of either surfactant-coated or crude lipase (protein content of both was 11%). The reaction system was stirred magnetically and thermostated at 40°C. Each data point represents the mean of two analyses of same reaction.

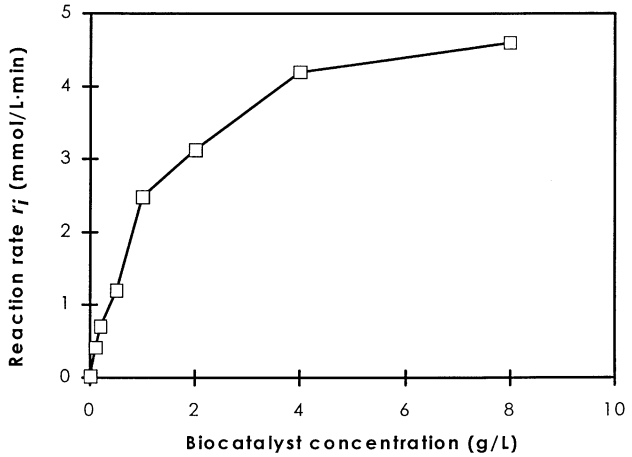

FIG. 2. Effect of the biocatalyst concentration on the esterification reaction rate. Reaction conditions: *n*-Hexane (10 mL) with 20 mmol/L lauric acid, 20 mmol/L dodecyl alcohol and varying concentrations of surfactant-coated lipase. The reaction solution was stirred magnetically and thermostated at 40°C.

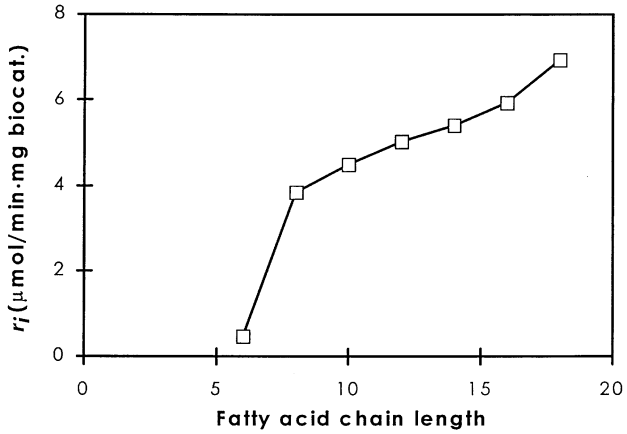

FIG. 3. Effect of the substrate fatty acid chain length on the initial reaction rate, r_i, catalyzed by the modified lipase. Reaction conditions: *n*-Hexane (10 mL) with 100 mmol/L fatty acid, 100 mmol/L dodecyl alcohol, and 5 mg surfactant-coated lipase. The reaction solution was stirred magnetically and thermostated at 40°C.

biocatalyst added to 10 mL *n*-hexane containing 20 mmol/L lauric acid and dodecyl alcohol. Figure 2 shows that increasing the biocatalyst concentration to approximately 2 mg/mL led to a linear increase in the esterification reaction rate. This behavior is an indication of a kinetically controlled enzymatic reaction, in which the reaction system behaves as a homogeneous system. Biocatalyst concentrations above 4 mg/mL did not significantly increase the reaction rate. Therefore, in subsequent experiments the biocatalyst concentration was below 2 mg/mL.

The effect of fatty acid chainlength on the activity of the modified lipase. In the authors' previous study (16) it was found that the fatty acid chainlength of the surfactant that coated the lipase had a significant role in determining the activity of the surfactant–enzyme complex. It was reported that lipase coated with monostearate or monopalmitate sugar esters showed higher activity than that coated with a short-chain sorbitan ester. Similarly, Figure 3 shows that the esterification activity of the modified lipase was also affected by the fatty acid chainlength of the substrate. The results show that fatty acids with a longer alkyl chain, such as palmitic and stearic acids, are better substrates for the modified lipase than those having a shorter alkyl chain. A sharp drop in the reaction rate was observed when the fatty acid chainlength was reduced from 8 to 6 carbons. Similar data were obtained in other research studies employing different lipase sources (19). The chainlength specificity of the crude lipase compared with that of the same modified lipase was not verified in this study because the crude lipase showed a very low esterification activity under the studied conditions (Fig. 1).

The effect of water content on the surfactant-coated lipase activity. The influence of water content on the esterification activity of the modified lipase was investigated by adding various amounts of water to the reaction system (6,22). Other investigators studying this aspect have used water activity as a more indicative measure for the effect of water on lipase activity (8). The authors' previous report showed that water molecules react with the surfactant–lipase complex and lead to desorption of the surfactant from the complex (16,17). It was decided to use water content as an indicator in this study. The lowest water concentration in the reaction system achieved after drying *n*-hexane with molecular sieves and adding the substrates and the enzyme was approximately 20 mg/L. The modified lipase activity was found to be highest when no external water was added to the reaction system (Fig. 4). At this low water content (around 20 mg/L) the reaction medium was cloudy because of dispersion produced after adding the modified lipase. When 30–400 mg/L water was added to the reaction system a sharp decrease in the biocatalyst activity was obtained. It was also noticed that addition of water to the reaction system caused biocatalyst solubilization; at water concentrations over 200 mg/L the reaction medium became transparent. These observations suggest that addition of water to the reaction system results in desorption of the surfactant molecules from the enzyme complex, leading to reduced enzyme activities. The surfactant detached from the complex would be released into the reaction solution; in the presence of water it would produce transparent reverse micelles. Addition of water beyond 400 mg/L did not alter the esterification activity of the modified lipase (Fig. 4). At this point the number of surfactant molecules attached to the enzyme probably remains constant. Under the experimental conditions water could not desorp additional surfactant molecules from the complex. This system response suggests the existence of heterogeneity in the binding affinity of the surfactant molecules to the enzyme. The same effect was observed in other studies using the same lipase modification technique (16,17).

Effect of reaction temperature. The influence of the reaction temperature on the esterification activity of the modified lipase was investigated over the range of 25–69°C. The ini-

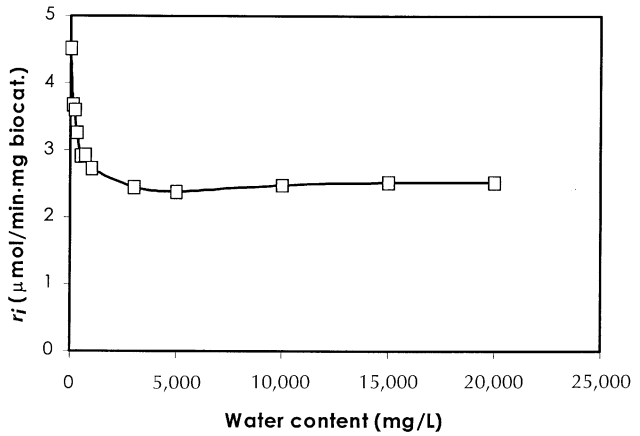

FIG. 4. Effect of water content on the esterification reaction rate with the modified lipase. Reaction conditions: *n*-Hexane (10 mL) with 100 mmol/L lauric acid, 100 mmol/L dodecyl alcohol, and 5 mg surfactant-coated lipase. The reaction solution was stirred magnetically and thermostated at 40°C. For abbreviation see Figure 3.

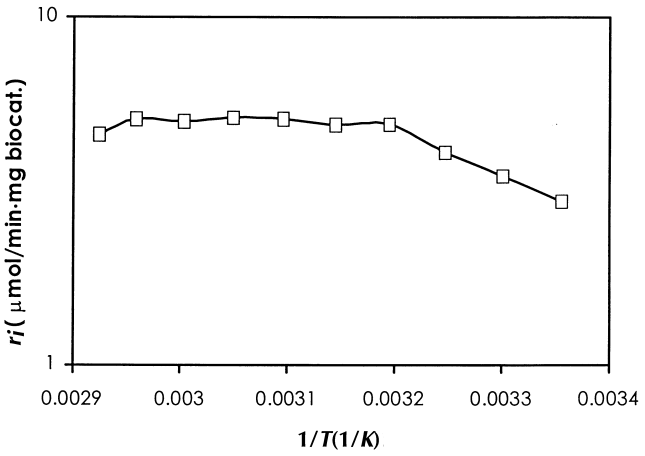

FIG. 5. Arrhenius plot of the esterification reaction with the modified lipase. Reaction conditions were as described in Figure 4 while the temperature of the reaction medium was changed in the range of 25–69°C. For abbreviation see Figure 3.

tial esterification reaction rates were determined and used to construct an Arrhenius plot (Fig. 5). Increasing the reaction temperature up to 45°C led to an increase in the reaction rates. There was no significant further increase in the reaction rates over 45°C. Considering the low stability of the crude enzyme in aqueous systems, in which the optimal temperature is around 35°C, the results indicate that the stability of the surfactant-coated enzyme at relatively high temperatures was due to surfactant protection of the enzyme. The activation energy, E_a, of the esterification reaction was determined from the initial rates in the temperature range of 25–45°C, assuming that changes in K_m are negligible in this small temperature range, and was found to be 27.7 kJ/mol. This value of E_a is in the range of other E_a values determined for other lipase-catalyzed esterification reactions (7). The essentially horizontal plot above 45°C may suggest the desorption of surfactant

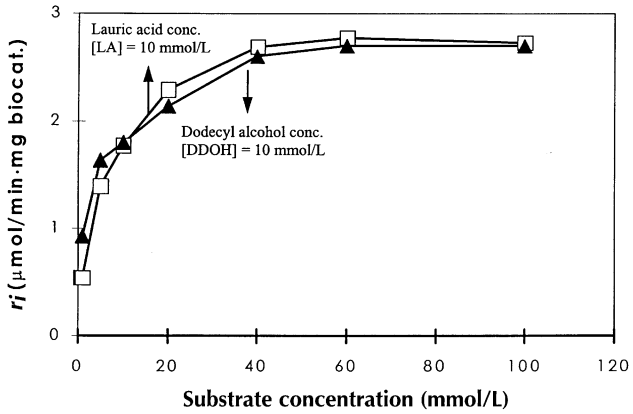

FIG. 6. The effect of each substrate concentration on the esterification reaction rate while the other substrate concentration was kept constant at 10 mmol/L. Reaction conditions were as in Figure 2. For abbreviation see Figure 3.

molecules from the complex as the temperature increased. Such desorption would negatively affect the reaction rate thus counteracting the temperature effect.

Kinetic study of esterification with the surfactant-lipase complex. Since esterification of fatty acids and alcohols is a two-substrate dependent reaction, the effect of each substrate concentration on the reaction rate was separately investigated. Figure 6 shows the effect of each substrate concentration on the esterification reaction rate while the other substrate concentration was kept constant. When lauric acid was the variable substrate, the reaction rates increased with lauric acid concentration and reached a maximum at concentrations over 40 mmol/L. When dodecyl alcohol was the variable substrate, the reaction rates increased proportionally as the concentration of dodecyl alcohol increased and reached a maximum at approximately 40 mmol/L. In both sets of experiments, concentrations over 40 mmol/L did not lead to any increase in reaction rate. These substrate–saturation plots are characteristic of enzymes that follow Michaelis-Menten kinetics with the absence of apparent inhibition by both substrates and their ester at the previously mentioned concentrations.

The Michaelis-Menten kinetic parameters for the modified lipase, V_{max}, $k_{m(LA)}$, and $k_{m(DDOH)}$, were obtained by graphical methods involving primary and secondary plots. Initial reaction rates, r_i, were measured at various lauric acid concentrations while the concentration of dodecyl alcohol remained constant; this set of experiments was then repeated at other fixed dodecyl alcohol concentrations. Plotting $1/r_i$ against the reciprocal of the lauric acid concentrations at the various fixed dodecyl alcohol concentrations gave parallel lines (Fig. 7). This kind of primary plot is typical of a Ping-Pong Bi Bi mechanism in which the modified lipase reacts with lauric acid to form an enzyme–lauric acid complex. Then, a molecule of water is released from the complex to produce the carboxyl-lipase intermediate. This intermediate reacts further with dodecyl alcohol to produce the corresponding ester. The kinetic parameters of the modified lipase can be obtained ac-

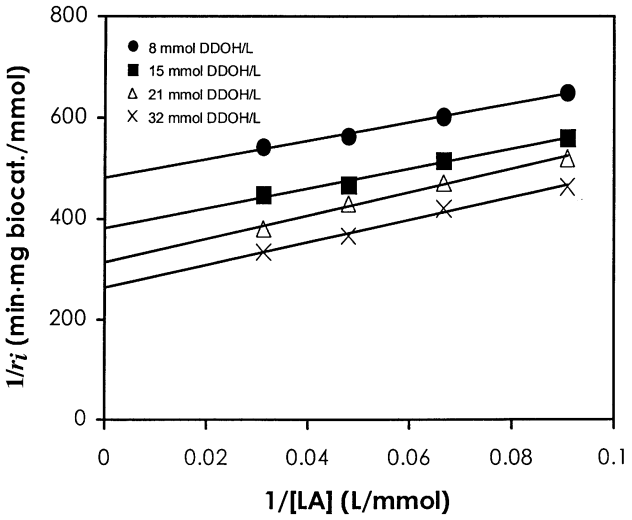

FIG. 7. Double-reciprocal plot of the initial esterification rate (r_L) at varying lauric acid concentrations. The reactions were performed at 40°C, 5 mg modified lipase and with different concentrations of dodecyl alcohol.

cording to the following equation (27):

$$1/r_i = [1 + k_{m(LA)}/[LA] + k_{m(DDOH)}/[DDOH]\ ]\ 1/V_{max} \quad [1]$$

where $k_{m(LA)}$ and $k_{m(DDOH)}$ are the Michaelis-Menten constants with respect to lauric acid and to dodecyl alcohol, respectively, V_{max} is the maximum esterification reaction rate, and [LA] and [DDOH] represent the initial concentrations of lauric acid and dodecyl alcohol, respectively. The slopes of the four parallel lines of Figure 7 are independent of the dodecyl alcohol concentrations, yielding an average value of $k_{m(LA)}/V_{max}$ of 2110 min·mg biocat./L. By plotting the *y*-axis intercepts of the primary plot presented in Figure 7 against the reciprocal of dodecyl alcohol concentrations a secondary plot results that has a slope of $k_{m(DDOH)}/V_{max}$ of 2350 min·mg biocat./L and a *y*-axis intercept of $1/V_{max}$ of 199 min·mg biocat./mmol (Fig. 8). The values for the model parameters that were determined from the graph were: $k_{m(LA)}$ = 10.6 mmol/L, $k_{m(DDOH)}$ = 11.9 mmol/L, and V_{max} = 5.3×10^{-3} mmol/min·mg biocatalyst. The values of the kinetic parameters obtained for the surfactant-coated lipase are of the same order of magnitude as those obtained in another study for a lipase coated with synthetic surfactants that was used as a biocatalyst for esterification reactions (18).

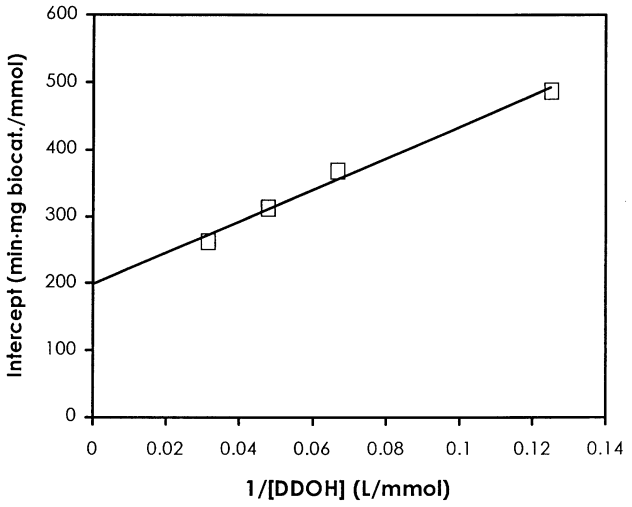

FIG. 8. Intercepts of *y*-axis of Figure 7 vs. the reciprocal of dodecyl alcohol concentrations.

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