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Effects of Methanol on the Catalytic Properties of Porcine Pancreatic Lipase

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Abstract The effect of aqueous methanol on the catalytic properties of porcine pancreatic lipase has been investigated. The k_{cat} values for the hydrolysis of N^{α}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester at 0°C increased in a linear manner with increasing methanol concentration. However, the K_M values were not influenced at methanol concentrations lower than 30% and then began to increase at higher concentrations in an exponential fashion. Based on product analysis, the increase in k_{cat} with increasing methanol concentration can be accounted for by nucleophilic competition of methanol for the acyl enzyme intermediate, indicating that the rate-limiting step of the porcine pancreatic lipase-catalyzed reaction is deacylation under current experimental conditions. The exponential increase in K_M at methanol concentrations higher than 30% is attributed to the hydrophobic partitioning effect on substrate binding. There was no loss of lipase activity over a 4 h period in 60% methanol concentration at pH* 5.5 and 0°C. By monitoring the intrinsic fluorescence and absorbance, no evidence for structural changes by methanol was observed.

Key words: Lipase, rate-limiting step, hydrolysis, methanolysis, methanol solvent

A study of the effect of low temperature on the kinetic constants of enzyme reactions, called cryoenzymology, can provide information concerning the reaction mechanism and transient intermediates of enzyme-substrate complexes, because reactions carried out at low temperature are relatively slow [18]. When cryoenzymological experiments are performed, the decrease in the freezing point of the medium is indispensable, and that can be achieved by adding an organic solvent. Previous cryoenzymological investigations, including proteases such as papain, pepsin, α-chymotrypsin, and β-lactamase, indicated that cryosolvents composed of dimethyl sulfoxide and dioxan would be suitable media for experiments at subzero temperature [4]. However, this solvent system suffers from the drawback of high viscosity [6]. Thus, methanol-based cryosolvents have recently been used, due to their low freezing point, low viscosity, and high solubility of enzyme-substrate complex [12].

Since the mechanism of catalysis by lipases was assumed to be analogous to that of serine proteases [2], many investigations to elucidate the mechanism have been performed [13, 15, 21]. It has been well established that, when the lipase-catalyzed reaction was carried out with an ester substrate in alcohol containing solvents, the mechanism involves the formation of two tetrahedral intermediates [8]. The first tetrahedral intermediate leads to an acyl enzyme and an alcoholic portion of an ester substrate (acylation). The second tetrahedral intermediate, when reacted with water, leads to an acid, and then to a transesterified ester (deacylation), when reacted with alcohol of the solvents. However, it is still controversial whether acylation or deacylation is the rate-limiting step.

In the present study, the kinetics of porcine pancreatic lipase-catalyzed hydrolysis and methanolysis of N^{α}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester in methanol-based cryosolvents have been studied. When the lipase-catalyzed reaction was carried out in water-methanol mixtures, methanol acts as a nucleophile that competes with water in attacking the acyl enzyme intermediate [5]. At the same time, methanol acts as a cosolvent, which brings about change in the hydrophobicity of the medium [1, 14]. From this point of view, our study focused on the effects of these two factors on the kinetic constants of the lipase-catalyzed reaction. The results showed that deacylation is the rate-limiting step in the lipase-catalyzed reaction of N^{α}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester.

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MATERIALS AND METHODS

Reagents

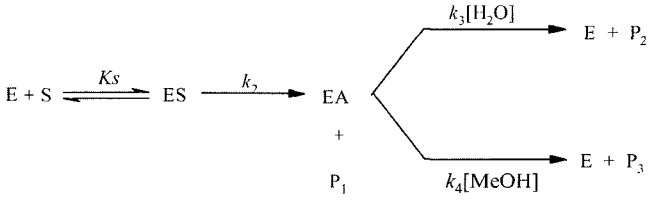
Porcine pancreatic lipase was obtained from Sigma (St. Louis, U.S.A.) and used without further purification. N^{α}-Cbz-L-lysine *p*-nitrophenyl ester, *p*-nitrophenyl acetate, and diethyl *p*-nitrophenyl phosphate were purchased from Sigma (St. Louis, U.S.A.). N^{α}-Cbz-L-lysine *p*-nitrophenyl ester was made into a stock solution of 50 mM in 90% acetonitrile, and this solution was used as the substrate. Spectral-grade methanol was from J. T. Baker (Phillipsburg, U.S.A.), and reagent-grade acetonitrile was from Mallinckrodt (Kentucky, U.S.A.). All other reagents were of analytical reagent grade.

Active Enzyme Determination

The active enzyme was assayed by active-site titration, using diethyl *p*-nitrophenyl phosphate at pH 7.8, 25°C. Under this assay condition, diethyl *p*-nitrophenyl phosphate rapidly acylates lipase with the release of a stoichiometric amount of *p*-nitrophenol in the form of a rapid burst [10, 22]. The amount of released *p*-nitrophenol was measured at 400 nm, using a HP8453 spectrophotometer (Hewlett-Packard, U.S.A.) and, from these results, the molarity of the active enzyme was determined.

Enzyme Reaction

N^{α}-Cbz-L-lysine *p*-nitrophenyl ester was chosen as a substrate for this study, because of the large difference between the rates of acylation and deacylation [6]. The reactions were run with $[S]_o$=0.025-0.5 mM, $[E]_o$=0.03 μM at 0°C and pH* 5.5. The reaction medium was composed of 50 mM acetate buffer containing varying proportions of methanol, and the reaction temperature was controlled with circulating water-methanol mixtures. The sample aliquots were quenched with 10% phosphoric acid to bring the pH to 2. The kinetic parameters of k_{cat} and K_M were determined from the Lineweaver-Burk plot and Hanes-Woolf plot, based on the initial rate according to substrate concentration. All reaction kinetics in triplicate were obtained. The accepted reaction pathway for lipase-catalyzed hydrolysis and methanolysis is shown in Scheme 1, where P_1 is an alcoholic portion of an ester substrate S, P_2 is the carboxylic acid, P_3 is the transesterified ester, and EA is the acyl-enzyme [3].



Scheme 1. Reaction pathway for lipase-catalyzed hydrolysis and methanolysis.

Product Analysis

The reaction products were analyzed, using the Thermo Separation Products gradient HPLC system (TSP Inc., U.S.A.) with a C_{18} reverse-phase column (YMC-H80, U.S.A.). Solvent A was aqueous phosphoric acid, pH 2.5; and solvent B was 100% acetonitrile. A complex gradient beginning at 20% B and leveling off at 90% B was used. Standard samples were used to obtain the following retention times: N^{α}-Cbz-L-lysine, 9.1 min; N^{α}-Cbz-L-lysine methyl ester, 12.3 min; N^{α}-Cbz-L-lysine *p*-nitrophenyl ester, 17.2 min. The peak areas were determined using a Datajet integrator (TSP Inc., U.S.A.).

RESULTS AND DISCUSSION

Stability of Lipase in Methanol Cryosolvent

When an enzyme kinetic study is done in a water-methanol mixture, it must be considered how the stability limitation of the enzyme might affect the reaction. The stability of lipase in aqueous methanol mixture (pH* 5.5, 0°C) was measured by incubating the enzyme for various time periods, prior to assaying for enzyme activity. As shown in Fig. 1, no loss of enzyme activity took place over a period of at least 4 h, even in 60% methanol (Fig. 1). Similar results were also reported by Compton *et al.* [5] who observed no loss of trypsin activity in 70% methanol solvents over a 4.5-h period at 25°C.

Other means to ascertain cosolvent effects on the enzyme structure is to examine the effect of cosolvent on the intrinsic spectral properties of the protein. The intrinsic fluorescence spectrum of lipase was determined at 25°C and pH* 5.5 as a function of methanol concentration. As shown in Fig. 2, the excitation maximum remained

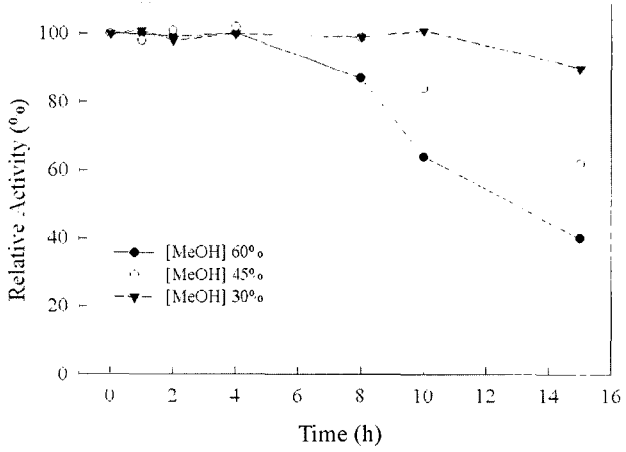


Fig. 1. Stability of lipase at 0°C and pH* 5.5 in aqueous methanol mixed solvent.
Concentration of methanol in aqueous-methanol solvent: ▼, 30% (v/v); ○, 45% (v/v); ●, 60% (v/v).

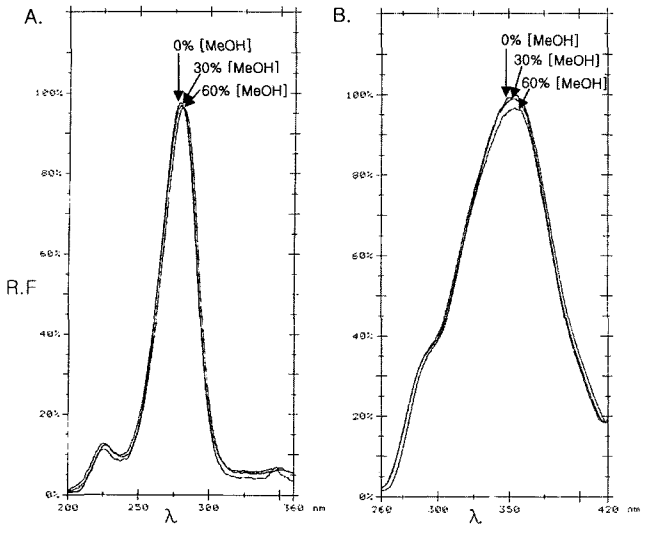


Fig. 2. Relative fluorescence excitation and emission spectra of lipase in aqueous methanol mixed solvent.
(A) The emission spectra were excited at 278 nm, and (B) the excitation spectra were gained at 348 nm.

virtually constant from 0 to 60% methanol (270 nm), and the emission maximum was also constant (350 nm). No evidence for any methanol-induced structural change was observed. Therefore, we could rule out the stability limitations of lipase, when the reaction is carried out in methanol-based cryosolvent.

Effect of Methanol on k_{cat} and K_M for the Hydrolysis of N^{α}-Cbz-L-Lysine *p*-Nitrophenyl Ester

Lipases (acylglycerol acylhydrolase, EC 3.1.1.3) are very diverse in their enzymatic properties and substrate specificities, which make a wide range of industrial applications possible [19]. Therefore, a number of investigations have been made on the catalytic properties of lipase, especially on the rate-limiting step. However, conclusion at the molecular level remains still controversial. Reyes and Hill [16] showed that the acylation step of the lipase-catalyzed reaction was rate-limiting, whereas Paiva *et al.* [11] reported that the deacylation step was rate-limiting, when the lipase reaction was carried out in water-organic solvent system. In order to discriminate the rate-limiting between acylation and deacylation, the effect of increasing methanol concentration on k_{cat} and K_M in the model reaction was analyzed at 0°C by measuring initial velocities, and some of the data are shown in Fig. 3. The k_{cat} values increased with methanol concentration in a linear manner (Fig. 3A), however, the K_M values were not influenced at lower than 30% methanol concentrations and then began to increase at higher concentrations in an exponential fashion (Fig. 3B).

In the lipase-catalyzed reaction of N^{α}-Cbz-L-lysine *p*-nitrophenyl ester, methanol serves as a nucleophile and has

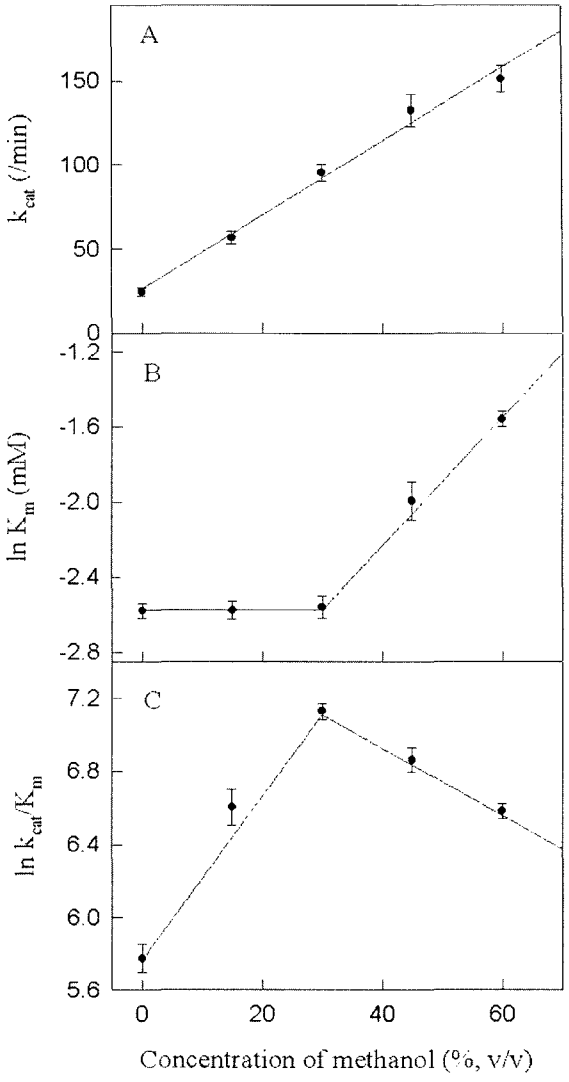


Fig. 3. The effect of methanol on the catalytic parameters for the lipase-catalyzed reaction with N^{α}-Cbz-L-lysine *p*-nitrophenyl ester as substrate.
The reaction was carried out at 0°C and pH* 5.5. $[E]_o$=0.03 μM, $[S]_o$=0.025–0.5 mM. Panel A, k_{cat}; panel B, ln K_m; panel C, ln k_{cat}/K_m.

been known to compete with water in attacking the acyl enzyme intermediate [7, 20]. If this is the case, the k_{cat} values, defined as shown in Eq. (1), will increase with the methanol concentration in a linear manner, implying that the rate-limiting step is deacylation. Figure 3A shows the change of k_{cat} as a function of methanol concentration and, from the results, one can conclude that deacylation is the rate-limiting step. This conclusion is further supported by our other results shown in Fig. 4. If the linear increase in k_{cat} is due to methanol attack on the acyl enzyme, there is a direct quantitative relationship between the observed k_{cat} and the amount of transesterification product, N^{α}-Cbz-L-lysine methyl ester. The product partitioning ratio, R, may be defined as shown in Eq. (2) [5].

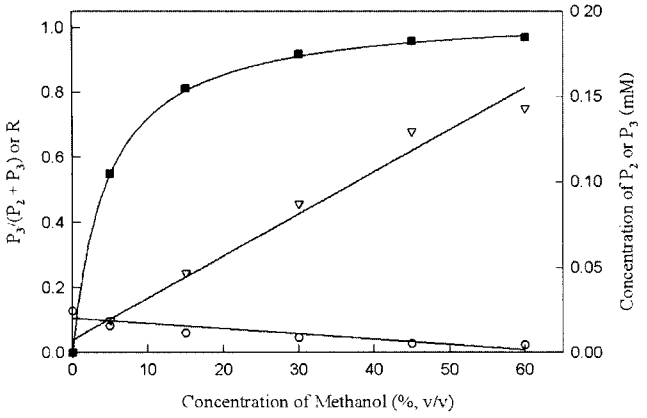


Fig. 4. Comparison of the observed product partitioning ratio, $P_3/(P_2+P_3)$, with that calculated from the observed k_{cat} as a function of methanol concentration.

The reaction was carried out at 0°C and pH 5.5. Symbols are of experimental values, and those were determined by measuring the concentrations of each product (P_2, P_3) that had accumulated over 10-min periods. Data for $P_3/(P_2+P_3)$, ■; for P_2, ○; P_3, ▽. The solid curve for R was calculated from $[k_{cat}(obs)-k_{cat}(0\%)]/k_{cat}(obs)$, as described in the text.

$$k_{cat}=k_3[H_2O]+k_4[MeOH] \quad (1)$$

$$R=\frac{[P_3]}{[P_2+P_3]}=\frac{k_4[MeOH]}{k_3[H_2O]+k_4[MeOH]}=\frac{k_{cat}(obs)-k_{cat}^{\circ}(0\%)}{k_{cat}(obs)} \quad (2)$$

The amount of free acid (P_2) and methyl ester product (P_3) can be determined by HPLC. The hyperbolic solid line in Fig. 4 represents the calculated value of R, based on the observed values of k_{cat} transformed to the corresponding ratio, R, where $k_{cat}(obs)$ is the value for k_{cat} at some methanol concentration and $k_{cat}^{\circ}(0\%)$ is the value in aqueous solution. Excellent agreement was observed between the product partitioning ratios, calculated from the observed values of k_{cat}, and those observed from HPLC analysis. Therefore, it can be concluded that the rate-limiting step is deacylation, and that the increase in k_{cat} with methanol is accounted for by methanolysis.

Figure 3B shows the change of K_M with respect to methanol concentration. Previous studies, using trypsin [5] and amine oxidase [17] which have the reaction pathway (Scheme 1) similar to lipase, have found that an exponential increase in K_M with added methanol is a general tendency in the aqueous organic solvent system, and has been attributed to a hydrophobic partitioning effect on substrate binding: In the model reaction (Scheme 1), $K_M=(k_3+k_4[MeOH])K_s/k_2$, and thus the observed increase in K_M could be due to an increase in K_s. An increase in K_s would reflect a decreased binding affinity of the substrate and would be expected, since the less polar methanol will tend to be more strongly bound in the hydrophobic areas of the substrate binding site than water. As shown in Fig. 3B, however, we observed in the lipase-catalyzed reaction that the K_M values were not influenced by up to about 30% methanol concentrations and then began to increase at higher concentrations in an exponential fashion. These results suggest that substrate binding involves predominantly electrostatic interactions rather than hydrophobic ones [9, 19] in the range of 0–30% methanol concentration. In addition, Petersen *et al.* [15] reported that the relatively larger active site of porcine pancreatic lipase than other enzymes for substrate binding was not affected by methanol up to about 30%. The exponential increase in K_M at higher concentrations is attributed to the above mentioned hydrophobic partitioning effect on K_s.

Figure 3C shows the effect of increasing concentrations of methanol on k_{cat}/K_M. While an increase in k_{cat}/K_M was observed as the methanol concentration increased up to about 30%, the ratio decreased at higher concentrations. The increase in k_{cat}/K_M in the 0–30% methanol concentration range was due to the increase in k_{cat}, suggesting deacylation as the rate-limiting step. The decrease of k_{cat}/K_M at methanol concentration higher than 30% is attributable to the hydrophobic partitioning effect on K_s, as mentioned earlier.

Effect of Methanol on k_{cat} and K_M for the Hydrolysis of *p*-Nitrophenyl Acetate

The experiments were designed to confirm the deacylation as the rate-limiting step and hydrophobic partitioning effect of methanol on the binding of substrate, N^{α}-Cbz-L-lysine *p*-nitrophenyl ester. *p*-Nitrophenyl acetate was chosen as a substrate for this purpose. If the rate-limiting step of the lipase-catalyzed reaction is deacylation, the k_{cat} values will increase with increase of methanol concentration in a linear manner, when the lipase reaction is carried out with *p*-nitrophenyl acetate. And, if methanol hindered the binding of N^{α}-Cbz-L-lysine *p*-nitrophenyl ester to the active site of lipase and the K_M values were consequently increased with the increase of methanol concentration, the K_M values of the lipase-catalyzed reaction with *p*-nitrophenyl acetate would not be influenced by methanol concentration or little effect would be expected, because *p*-nitrophenyl acetate does not have a Cbz-Lys group which binds to the hydrophobic areas of the lipase active site. Figure 5 shows the effect of methanol on k_{cat} and K_M for the hydrolysis of *p*-nitrophenyl acetate. The reactions were run with $[S]_0$=0.025–0.5 mM, $[E]_0$= 0.03 μM at 0°C and pH 5.5. As expected, the linear increase in k_{cat} was observed as methanol concentration increased (Fig. 5A), showing the deacylation as the rate-limiting step. And, the K_M values were not changed with methanol concentration, which indicates that the hydrophobic partitioning effect of methanol was not present, when the lipase-catalyzed reaction was carried out with *p*-nitrophenyl acetate in methanol-based solvents (Fig. 5B). The linear increase in k_{cat}/K_M was observed with

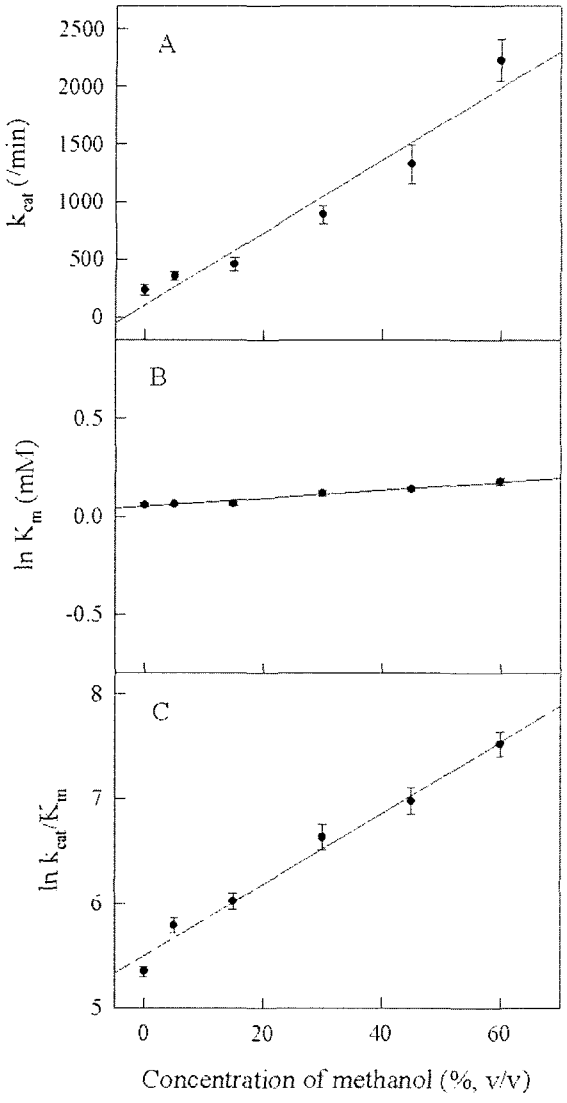


Fig. 5. The effect of methanol on the catalytic parameters for the lipase-catalyzed reaction with *p*-nitrophenyl acetate as substrate. The reaction was carried out at 0°C and pH* 5.5. $[E]_o$=0.03 μM, $[S]_o$=0.025-0.5 mM. Panel A, k_{cat}; panel B, ln K_m; panel C, ln k_{cat}/K_m.

methanol concentration, and this linearity depends on the linear increase of the k_{cat} value (Fig. 5C).

In order to maximize the productivity in enzyme technology, it is necessary to have clear information on the mechanism of catalytic reaction. Lipases are the most frequently used biocatalysts, because they are cheap, available from many sources, and have a broad substrates specificity. However, the mechanism of lipase catalysis at the molecular level is still controversial. Therefore, we examined the effect of methanol on the kinetic parameters of lipase reaction in order to clarify the catalytic mechanism. The results confirmed that the rate-limiting step of the porcine pancreatic lipase-catalyzed reaction is deacylation. Consequently, the information obtained from the present study is expected to be useful, when modeling and simulation of the lipase reaction are performed for the purpose of production of useful materials.

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