

Relationship Between Enhancement of Electrostriction and Decrease of Activation Energy in Porcine Pancreatic Lipase Catalysis

PARK, HYUN¹, KI-SEOG LEE, SEON-MI PARK, KWANG-WON LEE², AUGUSTINE YONGHWI KIM³, AND YOUNG-MIN CHI*

Division of Biotechnology and Genetic Engineering, ¹Korea Polar Research Institute, Korea Ocean Research & Development Institute, Sa-2-Dong 1270, Ansan 426-744, Korea

²Division of Food Science, Korea University, Seoul 136-701, Korea

³Department of Food Science & Technology, Sejong University, Seoul 143-747, Korea

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Abstract The contribution of electrostriction of water molecules to the stabilization of the negatively charged tetrahedral transition state of a lipase-catalyzed reaction was examined by means of kinetic studies involving high-pressure and solvent dielectric constant. A good correlation was observed between the increased catalytic efficiency of lipase and the decreased solvent dielectric constant. When the dielectric constant of solvents was lowered by 5.00 units, the losses of activation energy and free energy of activation were 7.92 kJ/mol and 11.24 kJ/mol, respectively. The activation volume for k_{cat} decreased significantly as the dielectric constant of solvent decreased, indicating that the degree of electrostriction of water molecules around the charged tetrahedral transition state has been enhanced. These observations demonstrate that the increase in the catalytic efficiency of the lipase reaction with decreasing dielectric constant resulted from the stabilization of electrostatic energy for the formation of an oxyanion hole, and that this stabilization was caused by the increase of electrostricted water around the charged tetrahedral transition state. Therefore, we conclude that the control of solvent dielectric constant can stabilize the tetrahedral transition state, thus lowering the activation energy.

Key words: Tetrahedral transition state, electrostriction, high-pressure kinetics, dielectric constant, lipase, activation volume

Electrostatic forces are of great importance in enzyme catalysis, and such forces are thought to play a major part in stabilizing the transition state [1, 11, 25, 26, 29]. In the lipase-catalyzed reaction, a tetrahedral transition state is

formed by hydrogen bonds between the negatively charged carboxyl oxygen atom (called an oxyanion) and two main-chain amide groups of Phe-78 and Leu-154, and the stabilization of this oxyanion in the transition state is essential for catalysis [7, 19]. Despite a plethora of information on the reaction mechanism, not only the dependence of stabilization of the oxyanion hole on the solvent dielectric constant, but also the effect of electrostriction caused by the negatively charged tetrahedral transition state on catalytic efficiency have received relatively little attention in the scientific community. Using molecular dynamics simulations, Warshel and Russel [30] suggested that the stabilization of the oxyanion hole in trypsin, which has a catalytic machinery similar to that present in lipase, includes an interaction between bound water molecules and a negatively charged tetrahedral transition state, and that removal of these water molecules might significantly slow the rate. However, this suggestion has not been experimentally corroborated.

High-pressure kinetics provides a powerful tool for investigating the properties of the reaction transition state, especially the charge distribution [5, 8, 28]. The effect of pressure on an enzyme reaction, as on a chemical reaction, is quantified by the activation volume (ΔV^\ddagger). For example, the activation volume for the catalytic step of an enzyme reaction equals the volume of the system containing the activated complex (V_{ES}^*) minus the volume of the system containing the ground-state complex (V_{ES}) [12]. Changes of the activation volume in enzyme reactions result from two sources [17, 27]: The first is structural contributions caused by changes in the volume of protein itself, while the other is caused by rearrangement of solvent molecules due to change in solvent exposure of the proteins, which modifies the water density. However, according to the results of Low and Somero [12], the latter effect dominates

*Corresponding author

Phone: 82-2-3290-3025; Fax: 82-2-921-3702;

E-mail: ezeg@korea.ac.kr

the former in enzyme reactions. For example, the creation of a charge in the transition state during the reaction increases intermolecular electrostatic forces between charged molecules and solvating molecules, and this electrostricted complex then leads to a reduction in volume, whereas the reverse process has the opposite effect. Moreover, they indicated that the energy barrier (ΔG^\ddagger) and activation volume (ΔV^\ddagger) of an enzyme reaction are lowered by increasing the water density during the formation of the activated enzyme-substrate complex, thereby accelerating the velocity of the reaction [13]. Therefore, effects of solvent polarity on the activation volumes can be used to infer not only the properties of the transition state, but also its degree of hydration in an aqueous solution.

In the present study, in order to elucidate how the solvent dielectric constant affects the degree of electrostriction and whether or not the electrostricted water molecules around the oxyanion hole in the lipase reaction can stabilize the charged tetrahedral transition state, high-pressure kinetic studies combined with the solvent dielectric constant were conducted.

MATERIALS AND METHODS

Reagents

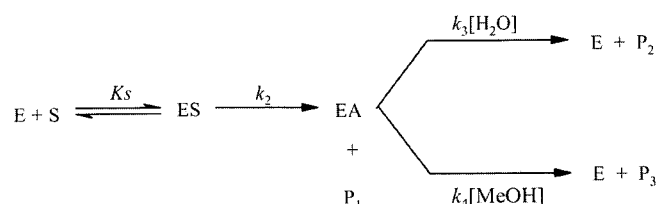
Porcine pancreatic lipase was obtained from Sigma (St. Louis, U.S.A.) and used without further purification. N^α -Cbz-L-lysine *p*-nitrophenyl ester and diethyl *p*-nitrophenyl phosphate were purchased from Sigma (St. Louis, U.S.A.). N^α -Cbz-L-lysine *p*-nitrophenyl ester was prepared as 50 mM stock solution in 90% acetonitrile and used as 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 and 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 [16, 32]. The amount of *p*-nitrophenol released 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.

Kinetic Studies Under High Pressure

Pressurization was performed hyperbarically using N_2 from a high-pressure gas booster (Haskel, U.S.A.), and reaction pressure was regulated using a back-pressure regulator (Tescom, U.S.A.). The high-pressure reactor for enzymatic reaction was made of stainless steel, and



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

reaction temperatures were controlled by cooling the circulator in a water bath. The reaction mixture was added to the high-pressure reactor and full pressure was achieved in less than 1.5 min; a short time compared to the time course linearity of about 25 min. The reaction mixture inside the high-pressure reactor was magnetically stirred and aliquots were removed periodically. In order to conserve the high-pressure required to maintain the reaction, sampling was done through the use of a needle valve and metering valve in series [10].

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 $[S]_0=0.025\text{--}0.5$ mM, $[E]_0=0.03$ μ M at 0°C and pH* 5.5. The preselected dielectric constants of the reaction medium were obtained by adding various concentrations of isopropyl alcohol and by decreasing the dielectric constant of 50 mM acetate buffer, and the reaction temperature was maintained 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 were obtained in triplicate. The accepted reaction pathway for lipase-catalyzed hydrolysis and methanolysis is shown in Scheme 1, where P_1 is the alcoholic portion of an ester substrate, S , P_2 the carboxylic acid, P_3 the transesterified ester, and EA the acyl-enzyme. k_{cat} and K_m were defined, as shown in Eqs. (1) and (2) and described by Burdette and Quinn [2]:

$$k_{\text{cat}} = k_3[\text{H}_2\text{O}] + k_4[\text{MeOH}] \quad (1)$$

$$K_m = (k_3 + k_4[\text{MeOH}])K_s/k_2 \quad (2)$$

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 a phosphoric acid solution, 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^β -Cbz-L-lysine *p*-nitrophenyl ester, 17.2 min.

The peak areas were determined using a Datajet integrator (TSP Inc., U.S.A.).

Measurement of Dielectric Constants

The dielectric constants of water-methanol mixtures and water-methanol-isopropyl alcohol mixtures, in which the ratios of water and methanol concentrations were maintained constant, were measured at a 1 GHz frequency, using an RF Vector Network Analyzer equipped with HP 85070B dielectric probe kit (Hewlett-Packard).

RESULTS AND DISCUSSION

The Effect of Solvent Dielectric Constants on Catalytic Parameters for Lipase-Catalyzed Hydrolysis of N^α-Cbz-L-Lysine *p*-Nitrophenyl Ester

A number of studies have been made on the influence of variation in dielectric strength on the hydrolysis of ester or transesterification by serine protease under conditions that minimize the specific effects of solvents [3, 14, 15, 21]; however, little information is available on lipase. Therefore, in order to understand the effect of solvent dielectric constant on lipase reaction, it would be worthwhile to review the published results on trypsin reaction, because they have a similar catalytic mechanism. By using a variety of solvents, Castaneda-Agullo and Del-Castillo [3] showed that the rate of ester hydrolysis by trypsin increased with diminished dielectric strength and vice versa. However, Maurel [14] observed an opposite effect of dielectric strength on tryptic hydrolysis of benzoyl-L-arginine ethyl ester, when dimethyl sulfoxide was used to control the dielectric strength of the medium. When other solvents were used, the results agreed with those of Castaneda-Agullo and Del-Castillo. This opposite effect of dimethyl sulfoxide can be explained by the results of Compton *et al.* [4] who showed that it has a hydrophobic partitioning effect on substrate binding, but not an electrostatic effect.

Figure 1 shows the effect of solvent dielectric constant on $\ln K_m$, $\ln k_{cat}$, and $\ln k_{cat}/K_m$ for lipase-catalyzed hydrolysis of N^α-Cbz-L-lysine *p*-nitrophenyl ester. The solvent dielectric constant was controlled with isopropyl alcohol, decreasing the dielectric constant of 50 mM acetate buffer, chemically unrelated to the lipase reaction. No deviations from linearity were observed over the range of experimental dielectric constant. In the lipase-catalyzed reaction system, the driving forces involved in the ES complex formation are known to derive from both hydrophobic and electrostatic interactions [22, 24], even though it is still unknown which interactions are predominant. However, no correlation exists between solvent dielectric constant and hydrophobic interaction during the ES complex formation. Therefore, since the hydrophobic partitioning effect of the employed solvents can be ruled out, the change of K_m with varying solvent

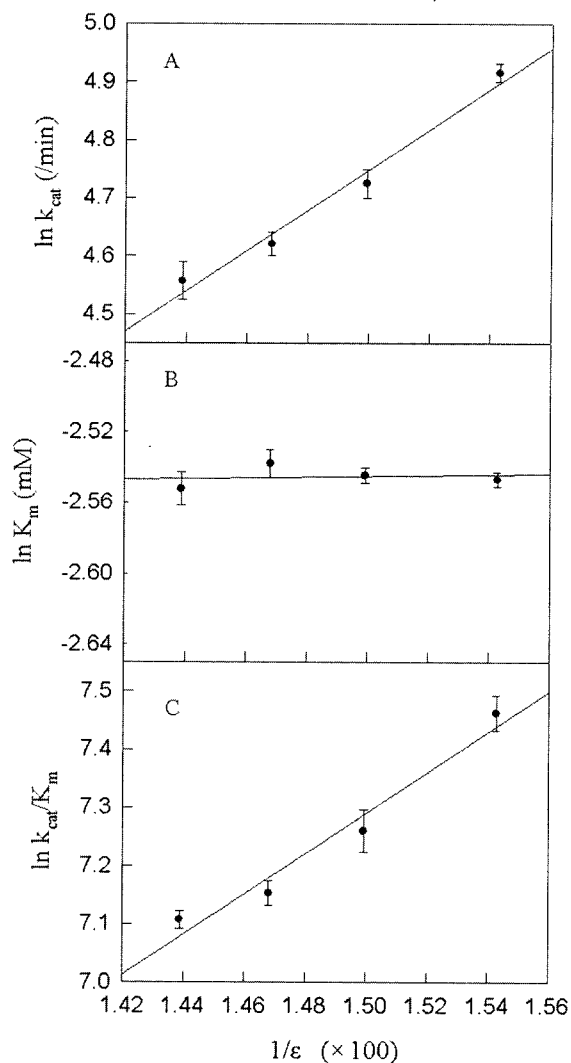


Fig. 1. Effect of the solvent dielectric constant on the catalytic parameters for lipase-catalyzed hydrolysis of N^α-Cbz L-lysine *p*-nitrophenyl ester. The reaction was carried out at 0°C and pH 5.5.

The dielectric constant of the solvents was controlled with isopropyl alcohol which was added up to 7.5% (v/v) maximum of the total volume of the reaction mixture. $[E]_0=0.03$ mM, $[S]_0=0.025-0.5$ mM. Panel A, $\ln k_{cat}$; panel B, $\ln K_m$; panel C, $\ln k_{cat}/K_m$.

dielectric constant must be caused by an electrostatic effect of solvent, suggesting an important role played by electrostatic interaction in the ES complex formation. However, as shown in Fig. 1B, the change of K_m did not occur over the range of experimental dielectric constant. This result suggests that hydrophobic interaction is crucial for the formation of ES complex under present conditions.

The most important catalytic factor in lipase catalysis is electrostabilization of the negatively charged tetrahedral transition state by the oxyanion hole [7]. It is, therefore, logical to assume that electrostatic energy for the formation of the tetrahedral transition state is stabilized by lowering

the solvent dielectric constant. As shown in Fig. 1A, an increase in $\ln k_{\text{cat}}$ with decreasing dielectric constant was observed, indicating stabilization of the oxyanion transition state. Our major aim in the present studies was to understand the relationship between the increased k_{cat} and decreased solvent dielectric constant, which will be presented with the combined effect of pressure and dielectric constant on k_{cat} . Consequently, it was confirmed that the logarithms of the catalytic efficiencies against the reciprocal of the dielectric constant show up in a straight line with a positive slope in lipase, and that these increases are dependent on increased k_{cat} (Fig. 1C).

Combined Effects of Pressure and Dielectric Constant on the Catalytic Parameters for the Lipase-Catalyzed Reaction of N^ε-Cbz-L-Lysine *p*-Nitrophenyl Ester

First of all, in order to appreciate the characteristics of volume change during lipase reaction, the effect of pressure on k_{cat} , K_m , and k_{cat}/K_m was observed. Figure 2 shows the variation of k_{cat} , K_m , and k_{cat}/K_m values of lipase-catalyzed hydrolysis of N^ε-Cbz-L-lysine *p*-nitrophenyl ester as a function of pressure in 30% methanol mixed solvents at 0°C and pH 5.5. When a lipase-catalyzed reaction is carried out in water-methanol mixtures, the methanol used may affect the kinetic constants in three ways; to enhance the nucleophilicity which is reflected in the increase in k_{cat} , to augment the hydrophobic partitioning effect which results in the increase in K_m , and to diminish the dielectric constant of the medium. However, when the lipase-catalyzed reaction was carried out in the range of 0–30% methanol concentrations, no change of K_m was observed [20]. Therefore, 30% (v/v) methanol was used in the present study to modulate the volume change of the lipase reaction in the water-alcohol mixture.

According to the transition state theory, the volume change of an enzyme reaction during a catalytic process is defined by Eq. (3), where k_{cat}/K_m is the catalytic efficiency of an enzyme reaction. The catalytic efficiency term can be split into the activation volume ($\Delta V_{\text{cat}}^\ddagger$) for catalysis and the reaction volume ($\Delta V_{K_m}^{-1}$) for substrate binding, as depicted in Eqs. (4) and (5), respectively. The activation volume (Fig. 2A) and reaction volume (Fig. 2B) were obtained from the slope of the plots, and the linear character of the curves in Fig. 2 indicates that the catalytic mechanism and the active conformation of lipase do not change in the studied pressure range [17].

$$\Delta V^\ddagger = -RT \left(\frac{\partial \ln k_{\text{cat}}/K_m}{\partial P} \right) \quad (3)$$

$$\Delta V_{\text{cat}}^\ddagger = -RT \left(\frac{\partial \ln k_{\text{cat}}}{\partial P} \right)_T \quad (4)$$

$$\Delta V_{K_m}^{-1} = -RT \left(\frac{\partial \ln (K_m^{-1})}{\partial P} \right)_T \quad (5)$$

The positive slope in $\ln k_{\text{cat}}$ (Fig. 2A) means a negative activation volume for the tetrahedral transition state of

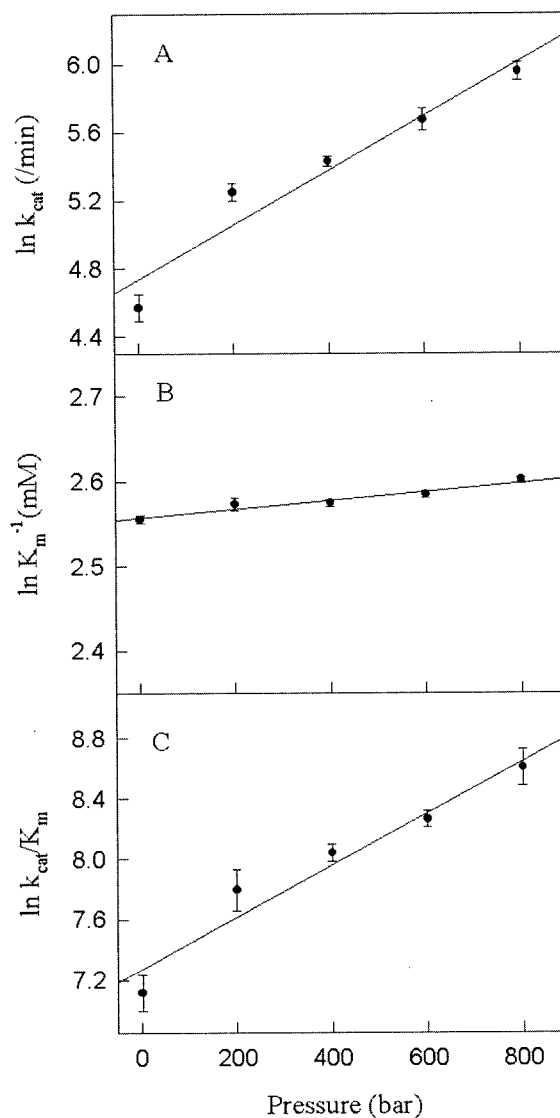


Fig. 2. Pressure dependence of $\ln k_{\text{cat}}$, $\ln (K_m^{-1})$, and $\ln k_{\text{cat}}/K_m$ for the lipase-catalyzed hydrolysis of N^ε-Cbz L-lysine *p*-nitrophenyl ester in 30% methanol mixed solvents at 0°C and pH 5.5.

Activation volume and reaction volume are obtained from the slope of the plots. Panel A, $\ln k_{\text{cat}}$; panel B, $\ln (K_m^{-1})$; panel C, $\ln k_{\text{cat}}/K_m$.

the lipase-catalyzed reaction, and corresponds to activity enhancement induced by pressure. Since the $\Delta V_{\text{cat}}^\ddagger$ reflects the difference in the degree of hydration of the activated complex (ES^\ddagger) and ground-state complex (ES), the negative $\Delta V_{\text{cat}}^\ddagger$ values for k_{cat} of the lipase reaction suggest that the amount of electrostricted water around the ES^\ddagger complex is increased during the formation of the tetrahedral transition state [13].

The combined effects of pressure and dielectric constant on the lipase reaction were studied to elucidate the reason of why the lowered dielectric constant enhances the k_{cat} , as shown in Fig. 1A, and to investigate the relation between dielectric constant and electrostriction around the active site.

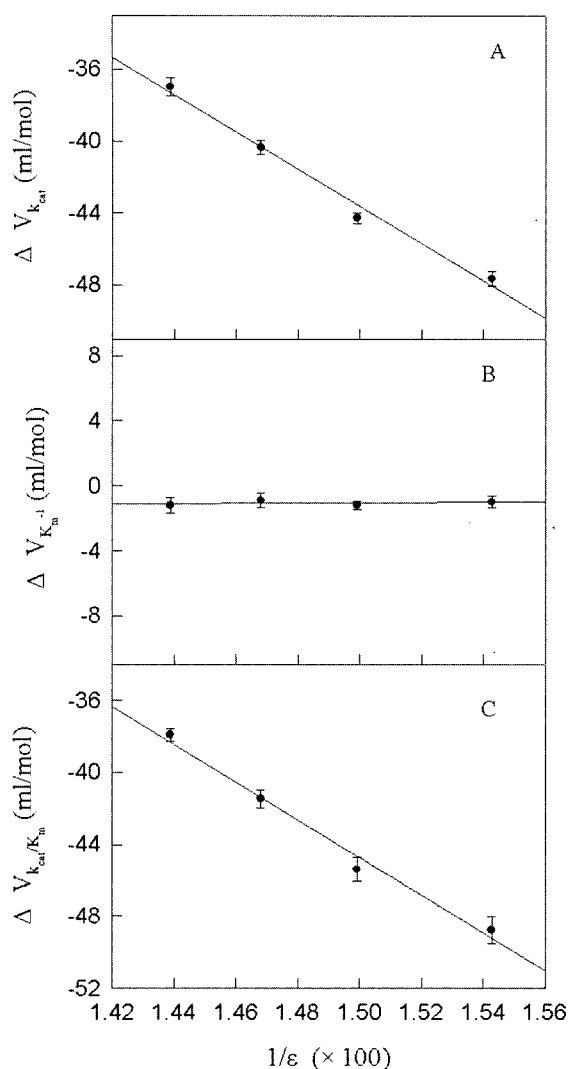


Fig. 3. Effect of solvent dielectric constant on activation volumes for k_{cat} and reaction volumes for K_m^{-1} of the lipase-catalyzed reaction. All experiments were performed at 0°C and pH 5.5 in the pressure range of 1–800 bar.

The dielectric constants of solvents were controlled with isopropyl alcohol, which was added up to 7.5% (v/v) maximum of the total volume of reaction mixture. Panel A, activation volumes for k_{cat} ; panel B, reaction volumes for K_m^{-1} ; Panel C, activation volumes for k_{cat}/K_m^{-1} .

As mentioned earlier, in the lipase-catalyzed hydrolysis of ester, the kinetic constants are affected by the hydrophobicity and dielectric constant of the solvents. However, no correlation exists between activation volumes and hydrophobicity [9]. Therefore, in reactions that proceed through a charged transition state, the change in activation volume with the varied dielectric constants seems to be due to change in the degree of electrostriction on the charged transition state. Figure 3 shows the effects of solvent dielectric constant on activation volumes for k_{cat} and reaction volumes for K_m^{-1} . The lipase-catalyzed reaction was carried out at pH 5.5 and 0°C with N^{α} -Cbz-L-lysine *p*-nitrophenyl ester in a

water-methanol-isopropyl alcohol mixture. While the total volume of the reaction mixtures and the volume ratio of water-methanol (70:30, v/v) were constant, the volume of isopropyl alcohol added was varied to control the dielectric constant of the mixture. The reason of why isopropyl alcohol was selected to control the dielectric constant of the mixtures is that it does not participate in the lipase reaction as a nucleophilic agent. As shown by the plot, the activation volumes for k_{cat} decreased markedly with the lowered dielectric constants, while the reaction volumes for K_m remained unchanged. It is natural that the reaction volumes for K_m of lipase catalysis are not influenced by varying solvent dielectric constants, because substrate binding is primarily governed by hydrophobic interaction between the Cbz-Lys on the substrate and the hydrophobic pocket on the enzyme in 30% methanol concentration (Figs. 1B and 3B). The unchanged values in reaction volume for K_m , as shown in Fig. 3B, indicate that the amount of electrostricted water during the ES complex formation would not vary by lowering the dielectric constant of the solvent.

Based on the widely accepted lipase catalytic mechanism, it can be assumed that the generation of the electrostatic field, which is centered around the protonated active site His-264 and the electrostatic oxyanion hole during the formation of the tetrahedral transition state, may lead to a reduction in activation volume for k_{cat} [7, 8]. Figure 3A shows the negative values of the activation volumes for k_{cat} and strong solvent dielectric dependence of the activation volumes. It is well known that the electric field in the vicinity of an ion is transmitted to greater distances through a medium of low dielectric constant rather than high, and consequently affects a greater volume of the nearby solvent [23]. Moreover, Nakasako *et al.* [18] recently showed through a crystallographic and laser light scattering study that a number of hydration water molecules are densely distributed around the active site of nitrile hydratase. Therefore, the negative $\Delta\Delta V_{cat}^{\ddagger}$ values for k_{cat} as the dielectric constant decreases are attributed to an increase in the degree of hydration around the highly polar transition state.

From these results, it was concluded that the reason of why the lowered dielectric constant of solvent accelerates the rate of the lipase-catalyzed reaction was the enhancement of electrostriction around the charged tetrahedral transition state, and that the greater the number of electrostricted water molecules in the active site, the more the transition state would be stabilized. This conclusion is supported by Xu *et al.* [31] who found that a mutated enzyme having a smaller number of water molecules around the active-site center exhibits a drastic decrease of enzyme activity.

Estimation of Thermodynamic Values

In order to investigate the role of electrostricted water around the tetrahedral transition state, the thermodynamic

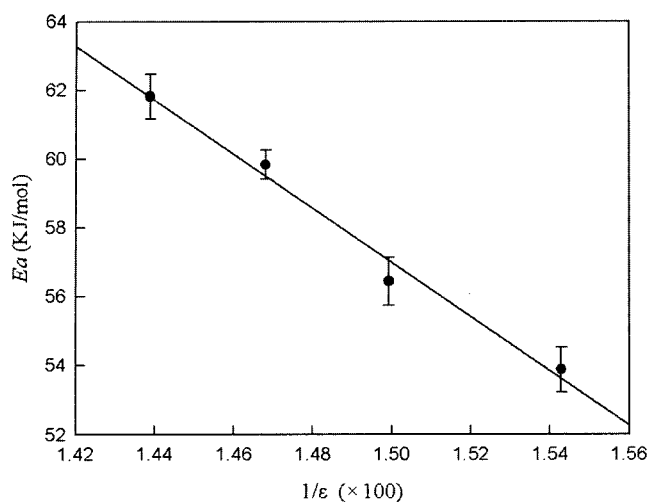


Fig. 4. Activation energy for the lipase-catalyzed reaction as a function of solvent dielectric constant. All experiments were performed at 273–303 K and pH 5.5.

Activation energy was determined from an Arrhenius plot of k_{cat} versus $1/T$, where T is the absolute temperature. From the slope, E_a values were calculated (slope = $-E_a/2.303R$, where R is the gas constant). No deviation from linearity was observed over the experimental temperature range.

functions for the lipase-catalyzed hydrolysis of N^α -Cbz-L-lysine *p*-nitrophenyl ester were determined in medium of variable dielectric constants in the temperature range of 0–30°C. The thermodynamic values observed depended on the dielectric constant of the solvent. As shown in Fig. 4, the activation energy (E_a) determined with the Arrhenius plot for k_{cat} showed decrease with decreasing dielectric constant of solvent: The activation energy of 7.92 kJ/mol was reduced by lowering the solvent dielectric constant by 5.00 units. This result confirmed that the electrostatic interaction of the oxyanion hole with water molecules is important in lowering the activation energy of the reaction, indicating that the increased amount of electrostricted water around the charged tetrahedral transition state caused a reduction in the solvent dielectric constant participating in the stabilization of electrostatic energy for the formation of an activated complex. This consideration was supported by the results of Fig. 5, which showed decrease in the free energy of activation (ΔG^\ddagger) with decreasing dielectric constant of solvents.

The mechanism underlying the effect of solvent polarity on ΔV^\ddagger is that less polar solvents have higher compressibility, therefore, are more constricted by newly generated ion or dipole than the more polar solvent during catalysis [23]. Thus, it can be assumed that a negative $\Delta\Delta V^\ddagger_{\text{cat}}$ value with decreasing dielectric constant of solvent should correspond to a negative $\Delta\Delta S^\ddagger$ value, because an increase in electrostriction corresponds to a decrease in both volume and entropy owing to the loss of freedom of motion within the solvent complex. This consideration is consistent with the results

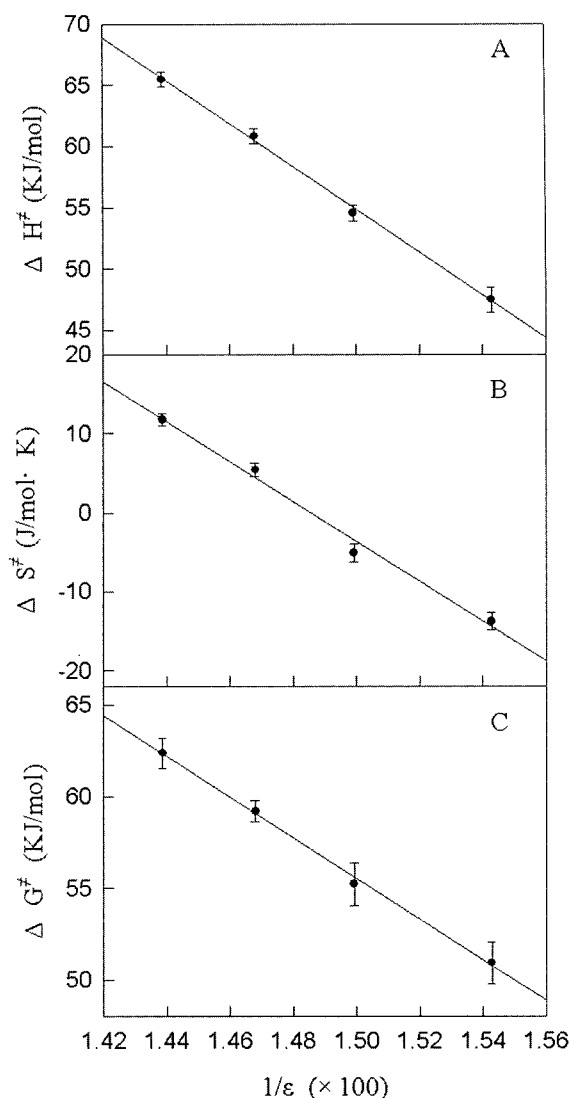


Fig. 5. Activation enthalpy (A), activation entropy (B), and free energy of activation (C) as a function of solvent dielectric constant in 0°C and pH 5.5.

The free energy of activation was estimated from $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$. Activation enthalpy (ΔH^\ddagger) and activation entropy (ΔS^\ddagger) were calculated, using the van't Hoff equation, by applying it to Eyring's transition state theory: $\text{Log}(k_{\text{cat}}) = -[(\Delta H^\ddagger/2.303R)(1/T)] + \text{log}(\kappa_b T/h) + (\Delta S^\ddagger/2.303R)$, where κ_b is the Boltzmann constant and h is the Planck constant.

of Fig. 5, which shows decreases of activation entropy (ΔS^\ddagger) with decreasing dielectric constant of solvents. The value of $\Delta\Delta G^\ddagger$ (i.e. $\Delta\Delta G^\ddagger$ is a negative value) indicates that the transition state is more stable in less polar solvents, because of increased electrostriction in the vicinity of the tetrahedral transition state of the active site, as shown in Figs. 3A, 4, and 5. From these results, we conclude that the control of solvent electrostriction caused by the control of dielectric constant accounts for the stabilization of electrostatic energy for the tetrahedral transition state in the lipase-catalyzed reaction. The present study demonstrated

that investigation of combined effects of high pressure and dielectric constant on kinetic and thermodynamic parameters provides further insight into the relation between solvent electrostriction and electrostatic interaction in lipase catalysis. The negative values of $\Delta\Delta V_{\text{cat}}^{\ddagger}$ and $\Delta\Delta G^{\ddagger}$ with decreasing dielectric constant confirm that the increased degree of electrostriction results in the stabilization of electrostatic interaction in the transition state. Thus, it is concluded that a study of the combined effects of dielectric constant and high pressure can provide useful information on enzymatic reaction mechanisms, especially when the reaction proceeds through a charged transition state.

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