J. Microbiol. Biotechnol. (1998), **8**(6), 656–662

JOURNAL OF
MICROBIOLOGY AND
BIOTECHNOLOGY

Distinction between the Influence of Dielectric Constant and of Methanol Concentration on Trypsin-Catalyzed Hydrolysis and Methanolysis

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Received: September 5, 1998

Abstract To make a distinction between the influence of the dielectric constant and of methanol concentration on trypsin-catalyzed hydrolysis and methanolysis at 0°C, a model reaction of N^{α}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester with water-methanol mixtures was chosen and a kinetic study done. The k_{cat} values increased with methanol concentration, in a linear manner whereas K_M values increased in a log-linear fashion. However, the k_{cat}/K_M ratio increased at lower methanol concentrations than 30% and then began to decrease at higher concentrations. The decrease in k_{cat}/K_M observed at higher than 30% methanol concentrations is attributed to the hydrophobic partitioning effect on substrate binding. On the other hand, the increase in k_{cat}/K_M in the 0~30% methanol concentration range seems to be due to the effect of nucleophilic cosolvent on k_{cat} and of the dielectric constant on K_M. This explanation was verified by measuring the effect of varying the dielectric constant of the medium on kinetic constants with isopropyl alcohol chemically unrelated to the enzyme reaction as the methanol concentration is maintained at a constant level. Therefore, we conclude that the effect of increasing the methanol concentration in the model reaction on the kinetic parameters k_{cat} and K_M is caused by changes in both the nucleophilicity and the dielectric constant of the medium. Based on product analysis, the increase in k_4'/k_3' by decreasing the temperature can be accounted for by the suppression of hydrolytic reactions. This observation indicates that the nucleophile is favored by low temperatures. There was no loss of trypsin activity over a 10 h period in 60% methanol concentration at pH* 5.5, 0°C.

Key words: Cryoenzymology, dielectric constant, hydrolysis, methanolysis, trypsin.

A study of the effect of low temperature on the kinetic constants of enzyme reactions, called cryoenzymology, can provide information concerning the reaction mechanisms and transient intermediates of enzyme-substrate complexes because reactions carried out at low temperatures are relatively slow [19]. 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 like papain, pepsin, and α-chymotrypsin, and β-lactamase indicated that cryosolvents composed of dimethyl sulfoxide and dioxan would be suitable media for experiments at subzero temperatures [4]. However, this solvent system suffers from the drawback of high viscosity. Thus, methanol-based cryosolvents have been recently used due to their low freezing point, low viscosity, and the high solubility of enzyme-substrate complex [9]. Although the drawback of the physical properties of the medium are circumvented by introducing methanol as cosolvent, an organic solvent effect on the kinetic constants still remains [23].

The rate of the reaction in solutions involving ions or dipolar molecules is generally influenced by the dielectric constant of the medium [15, 20]. Therefore, the kinetic parameters of the enzyme reaction in which the ionizable groups of the active site and surface of the enzyme and that of the substrate react with each other, may be expected to be influenced by the dielectric constant of the medium [16, 18]. From this point of view, rates of several enzyme reactions have been measured in water-organic solvent mixtures, and the results have been discussed in relation to the dielectric constant of the medium [22]. However, since it is supposed that the organic additives used to control the dielectric constant of the medium may possibly influence the reaction rates from various causes such as denaturation of enzyme molecules, inhibition of enzyme-substrate complex formation, etc. other than the electrostatic one [1, 6, 7], it is absolutely

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Abbreviations: Cbz, benzyloxycarbonyl; MeOH, methanol; pH*, apparent pH of aqueous organic solvent.

necessary to appreciate the actual relevance of these parameters to the organic solvent effect [13, 17]. Furthermore, by understanding the variation of the kinetic parameters which are caused by these distinguishable factors, it will be possible to design the optimum reaction medium for the production of target products using enzymes.

A number of studies have been made on the synthesis of peptides using protease in organic media. Among them, in particular, it has been recently shown that the lowering of the temperature of the reaction medium results in the increase of nucleophilicity of amino acids and the decrease of hydrolysis of the peptide product [10]. This indicates that a higher yield of peptide can be obtained at low temperatures [12]. Furthermore, in a recent study, it was observed that the peptide synthesis in high yields is possible when working in a frozen system down to 213 K. The explanation of this phenomenon was that protein-water systems still contain a certain amount of unfrozen water at temperatures far below 273 K, as indicated by NMR at about 203 K [21]. From these results, it can be assumed that cryoenzymology is a powerful tool not only in the study of enzyme reaction mechanisms as mentioned above, but also in enzyme-catalyzed synthesis of bioactive material.

In this paper, we report that the thermodynamics and kinetics of trypsin-catalyzed hydrolysis and methanolysis of N^{α}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester in methanol-based cryosolvents have been studied. When the trypsin-catalyzed reaction is carried out in water-methanol mixtures, methanol acts not only as a nucleophile but also as a cosolvent, which brings about changes in the dielectric constant and hydrophobicity of the medium. While the effect of hydrophobicity and nucleophilicity of methanol-based cryosolvents on the catalytic properties of trypsin has been investigated, the relevance of authentic dielectric constant to the cryosolvent effect has never seriously been taken into account. From this point of view, our study focused on the distinction between the influence of the intrinsic dielectric constant and of methanol concentration as a substrate on the trypsin-catalyzed reaction. In addition to that, although it is well recognized that increased yields in peptide synthesis can be obtained when the reaction temperature is lowered, no attempt has been made to explain it through thermodynamic studies. Thus, the reason why lowering of temperature of the reaction medium results in an increased yield in peptide synthesis will be discussed from the thermodynamic point of view using the model reaction.

MATERIALS AND METHODS

Reagents

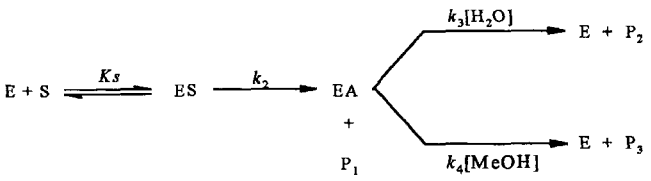
Twice-recrystallized bovine trypsin was obtained from Sigma (St. Louis, U.S.A.) and used without further purification. N^{α}-Benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester, N^{α}-benzyloxycarbonyl-L-lysine, and *p*-nitrophenyl-*p'*-guanidinobenzoate were purchased from Sigma (St. Louis, U.S.A.) and N^{α}-benzyloxycarbonyl-L-lysine methyl ester was obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland). 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. Stock solutions of trypsin were prepared by dissolving it in 1 mM HCl, 0.1 M KCl and were stored at 4°C. 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 *p*-nitrophenyl *p'*-guanidinobenzoate at pH 5.5, 25°C. Under this assay condition, *p*-nitrophenyl *p'*-guanidinobenzoate rapidly acylates trypsin with the release of a stoichiometric amount of *p*-nitrophenol in the form of a rapid burst [20]. The amount of released *p*-nitrophenol was measured at 410 nm using a Beckman DU-64 spectrophotometer 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 its low value of K_M, and because of the large difference rates between acylation and deacylation [8]. The reactions were run with $[S]_o$ = 0.1~0.5 mM, $[E]_o$ = 0.05 μM at 0°C, pH* 5.5. The reaction medium of cryosolvents was composed of 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_3, k_4, k_{cat}, and K_M were determined from the Lineweaver-Burk plot based on the initial rate according to substrate concentration. The accepted reaction pathway for trypsin-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.



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

Product Analysis

The reaction products were analyzed using the Thermo Separation Products gradient HPLC system with a C_{18} reverse-phase column (YMC-H80, U.S.A.). Solvent A was aqueous phosphoric acid, pH 2.5; 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; and N^{α}-Cbz-L-lysine *p*-nitrophenyl ester, 17.2 min. The peak areas were determined using a Datajet integrator.

Measurement of Dielectric Constant

The dielectric constants of water-methanol mixtures and water-methanol-isopropyl alcohol mixtures, in which the ratio of the concentration of water and methanol was maintained constantly, were measured at 1 GHz frequency using an RF Vector Network Analyzer equipped with HP 85070B dielectric probe kit (Hewlett-Packard, U.S.A.).

RESULTS AND DISCUSSION

Stability and Solubility of Trypsin in Methanol Cryosolvent

When an enzyme kinetic study is done in a water-methanol mixture, it must be considered how stability and solubility limitations of the enzyme may affect the reaction. The stability of trypsin in 60% methanol, pH* 5.5, 0°C was measured by incubating the enzyme for various time periods, prior to assaying for enzyme activity. These experiments indicated that no loss in activity took place over a period of at least 10 h (data not shown). Therefore, it was confirmed that we may rule out the stability limitations of trypsin when the reaction is carried out in methanol-based cryosolvent. This conclusion is supported by results of Compton *et al.* [5] who observed no loss of activity of trypsin in 70% methanol solvents over a 4.5 h period even at 25°C. In addition to that, they showed that the maximum solubility of trypsin in 70% methanol was 5 μM by monitoring the absorbance spectrum at 350 nm. However, 0.05 μM of trypsin was used in this study and thus the solubility limitations of trypsin may be excluded.

Effect of Methanol on k_{cat} and K_M for the Hydrolysis of N_{α}-Benzyloxycarbonyl-L-lysine *p*-Nitrophenyl Ester

Although a number of investigations have been made on the catalytic properties of trypsin, especially on the rate-limiting step, the conclusion is still controversial at the molecular level. Antonini *et al.* [2] showed that the acylation step of the catalytic reaction is rate-limiting at pH values above pH 4.8 in aqueous solution. On the other hand, Compton *et al.* [5] reported that the deacylation step is rate-limiting of the trypsin-catalyzed reaction in methanol-based cryosolvent. In order to facilitate discrimination between rate-limiting 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. Some of the data are shown in Fig. 1. The k_{cat} values increased with methanol concentration in a linear manner, whereas K_M values increased in a log-linear fashion. Previous studies, both with trypsin and with other enzymes, have also 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 [2, 18]. That is, in the model reaction (Scheme 1), $K_M = (k_3+k_4[\mathrm{MeOH}])K_S/k_2$, 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.

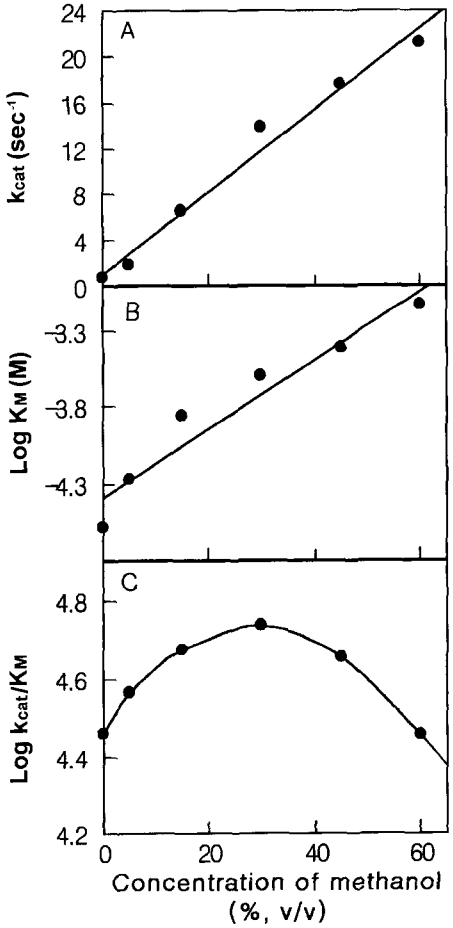


Fig. 1. The effect of methanol on the catalytic parameters for the trypsin-catalyzed reaction of N_{α}-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester.

The reaction was carried out at 0°C and pH* 5.5. $[E]_o = 0.05$ μM, [S] = 0.1~0.5 mM. Panel A, k_{cat}; panel B, *Log* K_M; panel C, *Log* k_{cat}/K_M.

In the trypsin-catalyzed reaction, methanol serves as a nucleophile and has long been known to compete with water in attacking the acyl enzyme intermediate [3]. If this is true, the k_{cat} values, defined as shown in Eq. (1) [5], will increase with the methanol concentration in a linear manner, which means that the rate-limiting step is deacylation. Figure 1A shows the change of k_{cat} with respect to methanol concentration, and from the results one can conclude that deacylation is a rate-limiting step. This conclusion is supported by other results shown in Fig. 2. 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].

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

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

The amount of free acid (P_2) and methyl ester product (P_3) can be determined by HPLC. The solid line in Fig. 2 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} (0%) is the value in aqueous solution. Excellent agreement is 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.

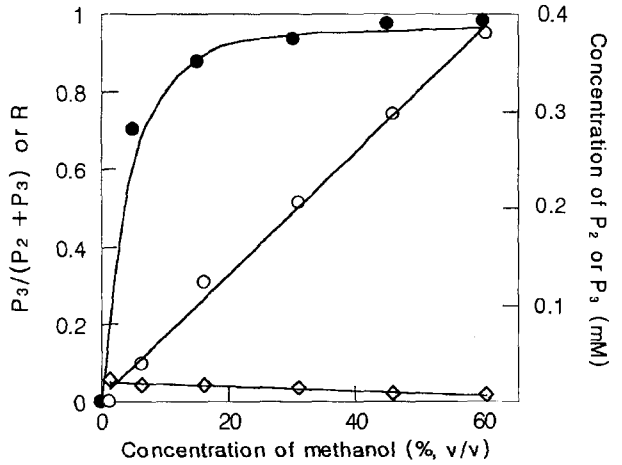


Fig. 2. Comparison of the observed product partitioning ratio, $P_3/(P_2+P_3)$, with that calculated from the observed k_{cat} as a function of methanol concentration.

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 products (P_2, P_3) that had accumulated over a 10 min period. 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.

Figure 1C shows the effect of increasing concentrations of methanol on k_{cat}/K_M. While an increase in k_{cat}/K_M is observed as the methanol concentration increases up to about 30%, at higher concentrations a decrease occurs. The decrease in k_{cat}/K_M observed at higher than 30% methanol concentration is attributed to the hydrophobic partitioning effect on K_M as mentioned earlier. However, the increase in k_{cat}/K_M in the 0~30% methanol concentration range is in contrast to the previous report by Compton *et al.* [5] who found little effect of methanol on the ratio k_{cat}/K_M and suggested, confusingly, the possible perturbation of the catalytic properties of the enzyme. Therefore, we decided to investigate the relevance of the dielectric constant to the methanol-based cryosolvent effect.

Effect of Dielectric Constant on k_{cat} and K_M

When trypsin-catalyzed reaction is carried out in water-methanol mixtures, the increasing methanol concentration may affect the kinetic constants from three points of view. One is to enhance the nucleophilicity which is reflected in the increase in k_{cat} as mentioned above, one is to augment the hydrophobic partitioning effect which results in the increase in K_M, and the remaining one is to diminish the dielectric constant of the medium. It can be noted that, in the trypsin-catalyzed reaction system, the driving forces involved in the ES complex formation are known to derive from approximately equal contributions from both hydrophobic and electrostatic interactions [14]. This means that the K_M values obtained have to be interpreted as being due to the cooperation of two effects, *viz.* an electrostatic one and a hydrophobic one, as previously mentioned. In general, the electrostatic interactions are influenced by the dielectric strength of the medium. Thus, the most effective way to understand the electrostatic characteristics of trypsin reaction is to study the dependence of kinetic constants on the dielectric constant of the medium.

According to the theory of the dielectric constant effect on enzyme reactions which has been developed by Hiromi [11] based on Kirkwood's model, the variation of the K_M values with the dielectric constant, D, of the reaction medium can be represented by the following equation:

$$-\frac{d\ \ln K_M}{d(1/D)} = \frac{\varepsilon^2}{2\kappa T}\left[\frac{L_S}{b_S} + \frac{1}{b_E}(L_E^o - L_{ES}^o)\right] \quad (3)$$

where ε is the protonic charge, κ is the Boltzmann constant, T is the absolute temperature, b_S and b_E are the

radii of the substrate and enzyme, respectively, L_S is the charge configuration functions of substrate, and L_E° and L_{ES}° are the effective charge configuration functions of the enzyme and ES complex. L is a dimensionless quantity which is determined by the radius and the charge state of the molecule, and L° is the charge configuration function constructed with respect only to the small number of charges within the local region of the enzyme or the enzyme-substrate complex in which the catalytic reaction is considered to occur, neglecting all the other charges, which are considered to be unimportant for the reaction and to be fixed during the course of the reaction [11]. Since both the active site of trypsin and the substrate, N_α-Cbz-L-lysine *p*-nitrophenyl ester, are polarized negatively and positively, it can be assumed that the values of L_S and L_E° in Eq. (3) are positive according to the theory of Hiromi [11]. On the other hand, the values of L_{ES}° can be ignored, due to the neutral characteristics of ES complex. Thus, the right side term of Eq. (3) would have a positive value. The fact that the slope $d \ln(1/K_M)/d(1/D)$ is positive implies that the K_M values decrease with decreasing dielectric constant.

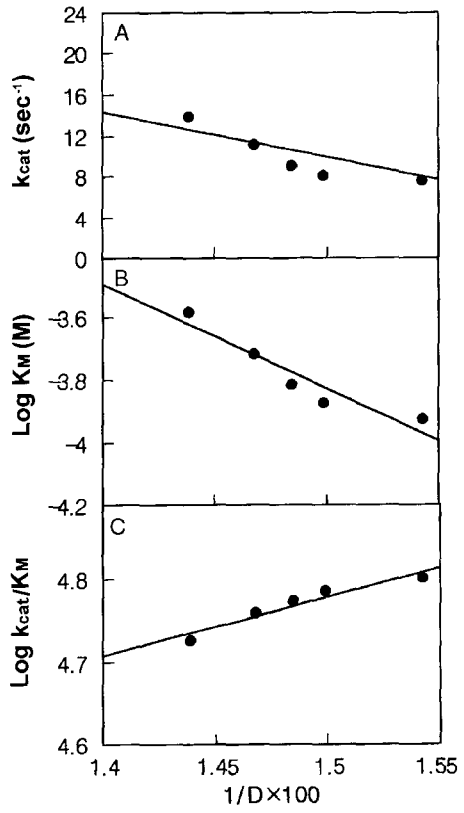


Fig. 3. The effect of the dielectric constant of the medium on the catalytic parameters for the trypsin-catalyzed reaction of N_α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester in water-methanol-isopropyl alcohol mixture.

The reaction was carried out at 0°C and pH* 5.5. The dielectric constant of the medium was controlled with isopropyl alcohol which is added at a maximum up to 7.5%(v/v) of the total volume of reaction mixtures. $[E]_o = 0.05$ μM, $[S] = 0.1$~0.5 mM. Panel A, k_{cat}; panel B, *Log* K_M; panel C, *Log* k_{cat}/K_M.

In order to clarify the dependence of kinetic constants on real dielectric constants and to explain the exponential increase in k_{cat}/K_M in the 0~30% methanol concentration as shown in Fig. 1C, a trypsin-catalyzed reaction was carried out at pH* 5.5, 0°C, with N_α-Cbz-L-lysine *p*-nitrophenyl ester as substrate, in water-methanol-isopropyl alcohol mixtures. While the total volume of reaction mixtures and the volume ratio of water-methanol (70:30, v/v) were maintained constantly, the volume of isopropyl alcohol added was varied to control the dielectric constant of the mixtures. The reason why isopropyl alcohol was selected to lower the dielectric constant of the mixtures is that it does not participate as a nucleophilic agent in the trypsin reaction. Figure 3 shows the linear dependence of k_{cat}, log K_M, and log k_{cat}/K_M on 1/D. Little effect of the dielectric constant on k_{cat} is observed if the decrease in methanol concentration is taken into consideration, as shown in Fig. 3A. However, as shown in Fig. 3B, an exponential decrease in K_M occurs as the dielectric constant of the medium decreases, as predicted in Eq. (3). Thus, it is clear that the increase in k_{cat}/K_M with the decreasing dielectric constant in Fig. 3C is caused by the decrease in K_M. At the same time, it is important to note in Fig. 1B that part of the increase in K_M, which results from the enhancement of the hydrophobic partitioning effect, is offset by the decrease in K_M which is caused by the decreasing dielectric constant. Consequently, we conclude that the dielectric effect on the kinetic constant cannot be eliminated at low methanol concentrations, while it is ignored at higher concentrations.

Estimation of Thermodynamic Values

The effect of temperature on the kinetic parameters was determined for N_α-Cbz-L-lysine *p*-nitrophenyl ester in a 30% methanol solution at pH* 5.5 in the temperature range of 0 to 30°C to calculate the thermodynamic values. The Arrhenius plots for hydrolysis (k_3'), methanolysis (k_4'), and k_{cat} [Eq. (1)] were linear with an activation energy of 72.76 KJ/mol, 58.82 KJ/mol, and 59.45 KJ/mol, respectively, as shown in Table 1. From these values, it is acknowledged that methanol has more nucleophilic reactivity than water and the activation energy for the overall reaction can be lowered by adding more nucleophilic agent. These observations support the previous results that deacylation is rate-limiting.

From the values of activation enthalpy ($\Delta H^\ddagger$) and activation entropy ($\Delta S^\ddagger$) in Table 1, it is supposed that hydrolysis would be accelerated with increasing temperature, since the $\Delta H^\ddagger$ and the $\Delta S^\ddagger$ have positive value and thus the value of activation free energy ($\Delta G^\ddagger$), based on the equation of $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$, will decrease with increasing

Table 1. Thermodynamic values for trypsin-catalyzed hydrolysis and methanolysis of N_α-benzyloxycarbonyl-L-lysine p-nitrophenyl ester in 30% methanol mixed solvent.

Kinetic parameter	ΔH° [a] (KJ/mol)	ΔS° [a] (J/mol · K)	ΔG° [b] (KJ/mol)	E_a [c] (KJ/mol)	$\Delta H^{\ddagger}$ [d] (KJ/mol)	$\Delta S^{\ddagger}$ [d] (J/mol · K)	$\Delta G^{\ddagger}$ [b] (KJ/mol)
k_3' [e] (Hydrolysis)	-	-	-	72.76	70.38	9.81	67.70
k_4' [e] (Methanolysis)	-	-	-	58.82	56.44	-15.61	60.70
k_{cat} (Overall reaction)	-	-	-	59.45	57.07	-12.91	60.59
K_M	-20.09	-4.41	-18.89	-	-	-	-

All the experiments were performed at 273~303 K and pH* 5.5.
[a]Standard enthalpy change (ΔH°) and standard entropy change (ΔS°) were calculated using the integrated van't Hoff equation [$\ln (1/K_M) = -\Delta H^{\circ}/RT + \Delta S^{\circ}/R$]. [b]The values of standard free energy change (ΔG°) and activation free energy ($\Delta G^{\ddagger}$) at 273 K were calculated using the equation of $\Delta G^{\circ} = \Delta H^{\circ} - T\Delta S^{\circ}$ and $\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$. [c]The Arrhenius equation [$k = A \exp(-E_a/RT)$] was used to calculate the activation energy (E_a). No deviations from linearity were observed over the range of 0 to 30°C. [d]Activation enthalpy ($\Delta H^{\ddagger}$) and activation entropy ($\Delta S^{\ddagger}$) were calculated using the van't Hoff equation by applying it to Erying's transition state theory [$\ln (k \cdot h/\kappa T) = -\Delta H^{\ddagger}/RT + \Delta S^{\ddagger}/R$]. [e]$k_3' = k_3$ [H_2O], $k_4' = k_4$ [MeOH]. In the present case, the concentration of H_2O and methanol used were 38.86 M and 9.36 M, respectively.

temperature. On the other hand, methanolysis will increase with decreasing temperature, since the value of $\Delta G^{\ddagger}$ will decrease due to the positive value of $\Delta H^{\ddagger}$ and negative value of $\Delta S^{\ddagger}$. Figure 4 shows the effect of temperature on k_4'/k_3', which is the ratio of the apparent reaction rate of methanolysis over that of hydrolysis. It is clear that hydrolysis is suppressed by lowering the reaction temperature, but nucleophilic attack other than water is accelerated.

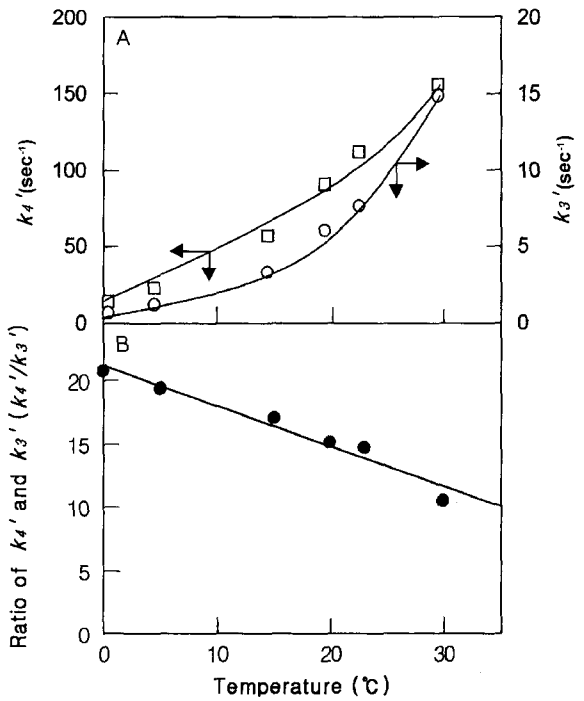


Fig. 4. The effect of temperature on the variation of the catalytic constants of methanolysis (k_4') and hydrolysis (k_3') of N_α-benzyloxycarbonyl-L-lysine *p*-nitrophenyl ester (panel A), and on the ratio of k_4' and k_3' (panel B).
The reaction was carried out in the temperature range of 0°C~30°C at pH* 5.5.

In the present study, we examined the effect of the authentic dielectric constant of the medium on the kinetic parameters of enzyme reaction and the temperature dependence of hydrolysis and transesterification (methanolysis) in the model reaction. The results confirmed that kinetic parameters of the enzyme reaction can be changed by altering the dielectric constant of the medium. Thus, the control of the dielectric constant of the medium is another tool, along with the control of pH and temperature, for the production of useful material in enzyme technology. At the same time, it was ascertained thermodynamically that the suppression of unwanted hydrolytic reaction can be achieved by lowering the reaction temperature. Consequently, the information obtained from the present study will be useful from an industrial point of view.

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