ISSN 0023-1584, Kinetics and Catalysis, 2010, Vol. 51, No. 1, pp. 31–37. © Pleiades Publishing, Ltd., 2010.
Published in Russian in Kinetika i Kataliz, 2010, Vol. 51, No. 1, pp. 35–42.

Studies on Temperature Dependent Kinetics of *Aspergillus awamori* Feruloyl Esterase in Water Solutions[1]

A. E. Fazary, S. Ismadji, and Y. H. Ju

Department of Chemical Engineering, National Taiwan University of Science and Technology, Taipei, Taiwan
e-mail: yhju@mail.ntust.edu.tw

Received November 5, 2008

Abstract—The initial reaction rate (V_0) for the esterification reaction of feruloyl esterase (FAE-II) at different temperatures (288, 298, 308, 318, 328, 338, 348, and 358 K) and various ethyl ferulate concentrations [(2, 4, 6, 8, 10, 12, 14, and 16) × 10^{-4} mol l^{-1} of ethyl ferulate in water] were determined. The Lineweaver–Burk double reciprocal plot yielded the kinetic parameters (maximal velocity V_{max}, Michaelis constant K_m, and second order rate constant V/K). The effects of temperature on those 3 kinetic parameters were presented and discussed. The thermodynamic parameters ΔH^* (enthalpy of activation), ΔG^* (free energy of activation), ΔS^* (entropy of activation), $\Delta G_{E\text{-}S}$ (free energy change of substrate binding), $\Delta G_{E\text{-}T}$ (free energy change of transition state formation), related to that biochemical process were determined and discussed from van't Hoff plot, Arrhenius plot, and Eyring plot.

DOI: 10.1134/S0023158410010064

INTRODUCTION

Structural studies of enzymes have influenced our view of catalysis enormously by providing three-dimensional representations of active sites. However, whereas these time-averaged structures are extremely useful for evaluating and modeling catalysis, they lack the ability to incorporate the dynamic motion that can occur in proteins and their effect on activity. Thus, investigations into whether protein dynamics have an effect on the catalysis of bond formation are receiving considerable attention [1]. From the earliest study on reaction rates, it has been evident that catalysis is strongly influenced by temperature. Thus, temperature must always be controlled if useful results are to be obtained from kinetic experiments. By carrying out measurements at several temperatures, one can deduce important information about reaction mechanisms [2].

Enzymes undergo a variety of denaturation reactions during production, storage and application in industry. Denaturation is the unfolding of the enzyme tertiary structure to a disordered polypeptide in which key residues are no longer aligned closely enough for continued participation in functional or structure stabilizing interactions. It can be reversed if the denaturing influence is removed. A protein is also subject to chemical changes leading to an irreversible loss of activity or inactivation particularly following unfolding [3]. These molecular phenomena give rise to two distinct definitions of in vitro protein stability, viz. thermodynamic (or conformational) stability and long-term (or kinetic) stability. Thermodynamic stability concerns the resistance of the folded protein conformation to denature while long-term stability measures the resistance to irreversible inactivation [4]. Understanding the mechanism of enzyme inactivation and the reversibility or irreversibility of the reactions involved helps in enzyme stability characterization thereby enabling better control over the deactivation process, stabilization approaches and catalytic properties [5]. Enzyme inactivation studies can be of a biochemical/structural nature focusing on the effect of temperature and other agents on the secondary and tertiary structures of enzymes. Another approach is the mathematical simulation of the effect of these agents on the activity of enzymes: the modeling of the activity of enzyme versus the time of inactivation and the quantity of inactivating agent. Stability of an enzyme, as with any kind of catalyst, is important for its application in industry [5].

Kinetic analysis of enzyme deactivation mechanisms is of prime importance allowing for better control over biocatalyst use. Enzyme inactivation is usually fit into a first-order kinetic model. However if one takes into account the various complex networks of reactions taking place during the inactivation process such as dissociation (for multimeric enzymes), denaturation, aggregation, coagulation and chemical decomposition; a need for nonlinear kinetic models arises. More complex kinetic models that consider inactivation partners known as biphasic and grace-period behaviors [6] explain inactivation as a series of many steps all of which may lead to enzyme denaturation. Therefore, it is necessary to separate the differ-

[1] The article is published in the original.

ent steps through which the inactivation of an enzyme takes place.

FAEs (EC 3.1.1.73), including cinnamoyl esterases and cinnamic acid hydrolases, are a subclass of the carboxylic acid esterases (EC 3.1.1) that play a key physiological role in the degradation of the intricate structure of plant cell wall by hydrolyzing the ferulate ester groups involved in the cross-linking between hemicelluloses and between hemicellulose and lignin [7, 8]. In enzymology, feruloyl esterase is an enzyme that catalyzes the chemical reaction: feruloyl-polysaccharide + H_2O ⟷ ferulate + polysaccharide. This enzyme belongs to the family of hydrolases, specifically those acting on carboxylic ester bonds. The systematic name of this enzyme class is other names in common use such as ferulic acid esterase, hydroxycinnamoyl esterase, hemicellulase, accessory enzymes, and FAE-III, cinnamoyl ester hydrolase, FAEA, cinnAE, FAE-I, FAE-II [7, 8].

As the biotechnical industries grow, it is expected that kinetics and thermodynamics will make a significant contribution toward improving the development of new products and toward their economic production. In this paper, we report the experimental results and the discussion for temperature dependent kinetics of native *Aspergillus awamori* FAE-II produced in our laboratory [9].

EXPERIMENTAL

Enzyme

Native *Aspergillus awamori* FAE-II was purified from the fermentation broth of *Aspergillus awamori* strain IFO4033 grew in a culture medium of crude wheat straw as described previously in our work [9].

Enzyme Activity Assay

The activity of *Aspergillus awamori* feruloyl esterase FAE was assayed by the analysis of free ferulic acid (FA) released from ethyl derivative of hydroxycinnamic ester (ethyl ferulate). One unit of FAE activity was defined as the amount of enzyme that liberates 1 μmol ferulic acid per minute, and specific activity was given in units per mg of protein. The assay to measure free ferulic acid released from ethyl ferulate consisted of (2, 4, 6, 8, 10, 12, 14, and 16) × 10^{-4} mol/l solution of ethyl ferulate in water as the substrate in the presence and absence of a suspected chemical denaturant, guanidine hydrochloride (GdnHCl) (1.5 mol/l phosphate buffer (pH ~ 5.5) containing 0.15 M NaCl), 20 μl of enzyme solution and citrate buffer (0.05 M) of pH 6. The reaction mixture was incubated for 10 min at different temperatures (288, 298, 308, 318, 328, 338, 348, and 358 K) and the reaction mixture was assayed using HPLC analytical technique and confirmed by UV-visible spectrophotometry [9]. Data taken from HPLC measurements were used in the calculations. Each experiment was performed in triplicate and the standard deviation was less than 5%.

CALCULATIONS

The activity of FAE-II enzyme was determined in the presence and absence of a suspected chemical denaturant, guanidine hydrochloride (GdnHCl) (1.5 mol dm^{-3} in 50 mmol dm^{-3} phosphate buffer (pH ~ 5.5) containing 0.15 M NaCl), at different temperatures (288, 298, 308, 318, 328, 338, 348, and 358 K) with varying concentrations of substrate and the data are represented graphically in Fig. 1, as rate of reaction (V) against concentration of substrate ([S]), and yield curves with saturation. The relationship is defined by the Michaelis–Menten equation [10], the basic equation of enzyme kinetics:

$$V = \frac{V_{\max}}{1 + \left(\frac{K_m}{[S]}\right)}. \quad (1)$$

To estimate the values of K_m and V_{max} the experimental results were plotted according to the Lineweaver–Burk double reciprocal plot (2), which rearranges the Michaelis–Menten equation into [11]:

$$\frac{1}{V} = \frac{1}{V_{\max}} + \frac{K_m}{V_{\max}}\left(\frac{1}{[S]}\right). \quad (2)$$

Plotting $1/V$ against $1/[S]$ gives a straight line as shown in Fig. 2 where Y intercept = $1/V_{max}$, slope = K_m/V_{max} and X intercept = $-1/K_m$. Using this plot, it can be determined that whether the suspected chemical denaturant acts noncompetitively, competitively, or uncompetitively. This is the most widely used method of linearizing the data, and generally gives the best precision for estimating K_m and V_{max}. The effects of temperature on V_{max}, K_m, and V/K are shown in Figs. 3–5, respectively. The turnover number is defined as $K_{cat} = V_{max}/[E_0]$, where $[E_0]$ is the FAE-II enzyme concentration.

K_m is related to $1/T$ by the Arrhenius equation: $K_m = A\exp(-E_a/RT)$; where the frequency factor A, may be considered as the frequency of collisions with the proper orientation to produce a chemical reaction. This factor can be as large as 10^{13} s^{-1}, which is about the frequency of collisions of molecules in liquids. This form of the Arrhenius equation is the most convenient for graphical purposes, as it shows that a plot of $\ln K_m$ against $1/T$ is a straight line with a slope of $-E_a/R$. This Arrhenius plot provides a simple method of evaluating E_a [12].

We can relate the parameters E_a and A of the Arrhenius theory to the ΔH^* parameter of the Eyring theory by examining the temperature dependence of the logarithm of the rate constants. The thermodynamic parameters for substrate (ethyl ferulate) hydrolysis were calculated by rearranging the Eyring's absolute

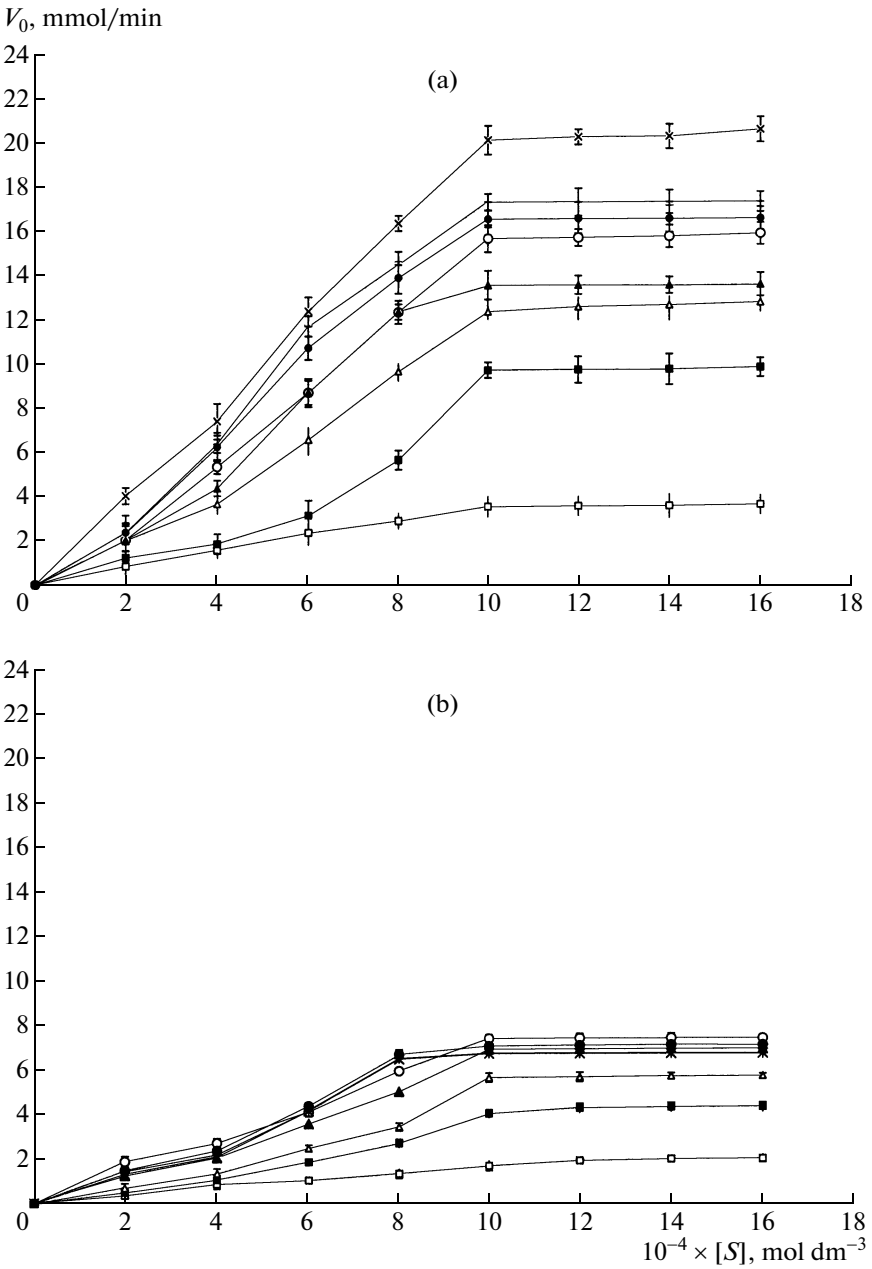


Fig. 1. Initial reaction rate, V_0 versus substrate concentration [S] for the esterification reaction of feruloyl esterase (FAE-II) at different temperatures; (□) 288 K; (■) 298 K; (△) 308 K; (▲) 318 K; (○) 328 K; (●) 338 K; (+) 348 K; (×) 358 K. Data presented are average values ± S.D. of $n = 3$ experiments. (a) Without GdnHCl (b) with GdnHCl (1.5 M in 50 mM phosphate buffer (pH ~5.5) containing 0.15 mol dm^{-3} NaCl).

rate equation derived from the transition state theory [13, 14].

The enthalpy and entropy changes of activation of a chemical reaction provide valuable information about the nature of the transition state, and hence about the reaction mechanism. A large enthalpy change of activation indicates that a large amount of stretching or breaking of chemical bonds is necessary for the formation of the transition state [13, 14].

The entropy of activation gives a measure of the inherent probability of the transition state, apart from energetic considerations. If ΔS^* is large and negative, the formation of the transition state requires the reacting molecules to orient into conformations and approach each other at a precise angle. As molecules vary widely in their conformational stability (i.e. their rigidity and complexity), one might expect that the values of ΔS^* would vary widely between different reactions; which does, in fact, occur [15].

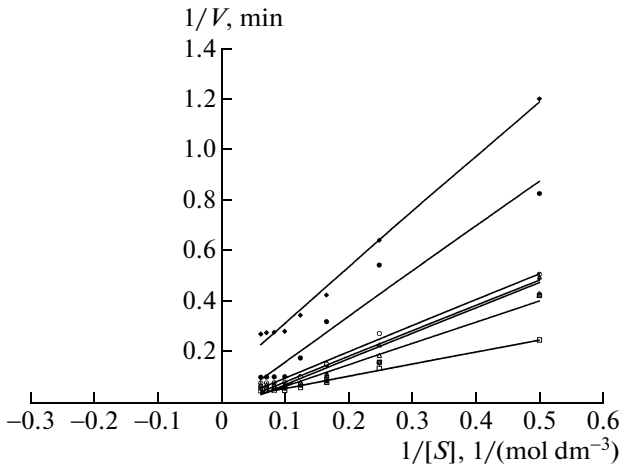


Fig. 2. The Lineweaver–Burk double reciprocal plots showing the reaction rate, $1/V$ versus substrate concentration, $1/[S]$ (mol dm^{-3}), for the esterification reaction of feruloyl esterase (FAE-II) at different temperatures; (□) 288 K; (■) 298 K; (△) 308 K; (▲) 318 K; (○) 328 K; (●) 338 K; (+) 348 K; (×) 358 K. Data presented are average values ± S.D. of $n = 3$ experiments.

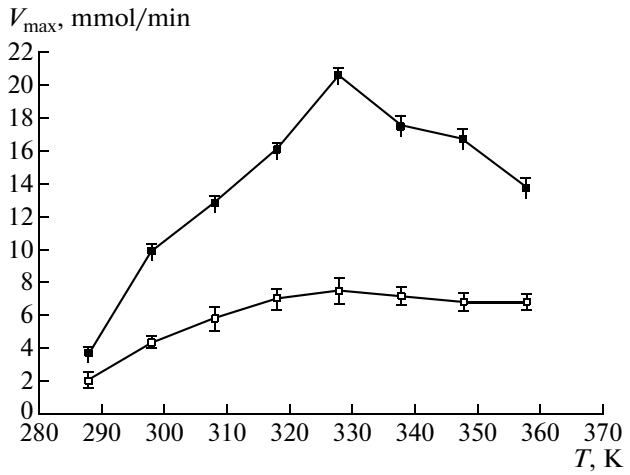


Fig. 3. Effects of temperature on V_{max} for the esterification reaction of feruloyl esterase (FAE-II); (□) with GdnHCl (1.5 mol dm^{-3} GdnHCl in 50 mmol dm^{-3} phosphate buffer (pH ~ 5.5) containing 0.15 mol dm^{-3} NaCl); (■) without GdnHCl. Data presented are average values ± S.D. of $n = 3$ experiments.

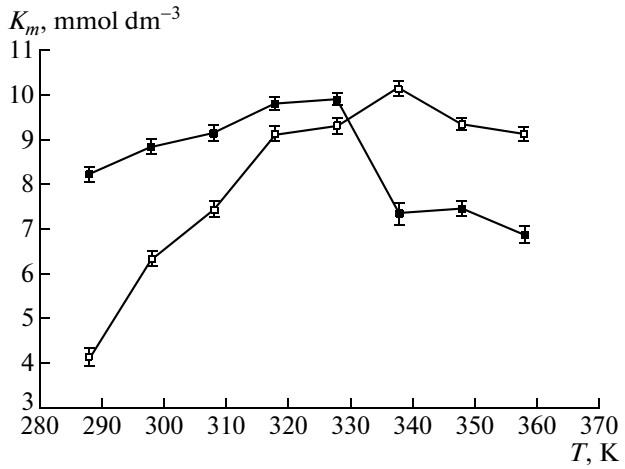


Fig. 4. Effect of temperature on K_m for the esterification reaction of feruloyl esterase (FAE-II); (□) without GdnHCl; (■) with GdnHCl (1.5 mol dm^{-3} in 50 mmol dm^{-3} phosphate buffer (pH ~ 5.5) containing 0.15 mol dm^{-3} NaCl). Data presented are average values ± S.D. of $n = 3$ experiments.

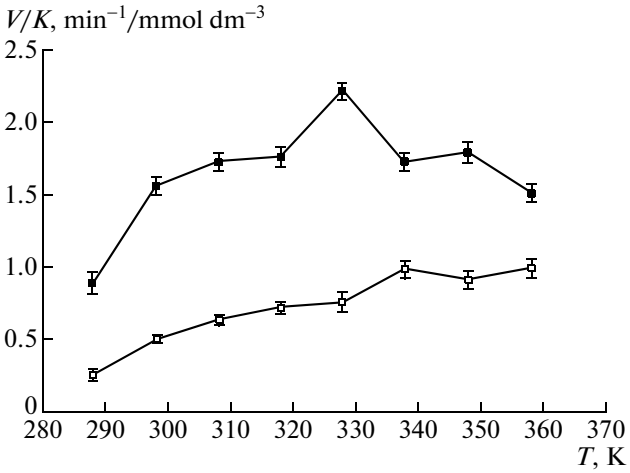


Fig. 5. Effect of temperature on V/K for the esterification reaction of feruloyl esterase (FAE-II); (□) with GdnHCl (1.5 mol dm^{-3} in 50 mmol dm^{-3} phosphate buffer (pH ~ 5.5) containing 0.15 mol dm^{-3} NaCl); (■) without GdnHCl. Data presented are average values ± S.D. of $n = 3$ experiments.

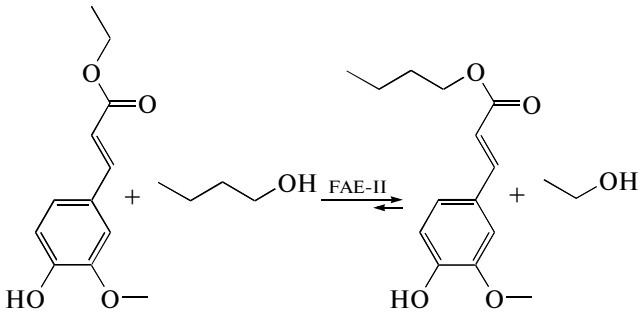
RESULTS AND DISCUSSIONS

Experimental data on the initial reaction rate (V_0) for the transesterification reaction (Scheme 1) of feruloyl esterase (FAE-II) at different temperatures and various ethyl ferulate concentrations in water solutions were plotted as shown in Fig. 1. From the Lineweaver–Burk double reciprocal plot (Fig. 2), kinetic parameters which include maximal velocity V_{max} (μmol/min), Michaelis constant K_m and V/K constant were determined. The effects of temperature on those kinetic parameters were represented graphically in Figs. 3–5, respectively.

The thermodynamic parameters related to the biochemical process which include ΔH^* (enthalpy change of activation), ΔG^* (free energy of activation, ΔS^* (entropy change of activation), $\Delta G_{E\text{-}S}$ (free energy change of substrate binding), $\Delta G_{E\text{-}T}$ (free energy change of transition state formation), were determined from van't Hoff (Fig. 6), Arrhenius (Fig. 7), and Eyring (Fig. 8) plots.

Several types of feruloyl and *p*-coumaroyl esterases can be identified based on their physical properties as well as by their substrate specificity. All enzymes (except *A. awamori p*-coumaroyl esterase) are active on methylferulate and on ethylferulate, which are synthetic substrates commonly used for feruloyl esterase assays [16, 17]. Studies of the activities of feruloyl esterases against natural substrates have focused mainly on xylan and xylan-derived oligosaccharides, from which most enzymes were able to release ferulic acid. Only two of these enzymes, FAEA [18, 19] and CinnAE [20, 21], have been shown to release ferulic acid from pectin. A comparative study using *A. niger* FAEA and CinnAE [18–21] demonstrated a preference of FAEA for substrates with a methoxy group at position 3 of the aromatic ring, and an increase in activity was observed when the number of methoxy groups on the aromatic ring increase.

Some agents such as guanidine hydrochloride (GdnHCl) and urea bring about the disruption of the three-dimensional structure of protein and are known to be used in the studies of protein unfolding and refolding. The mechanism of GdnHCl denaturation is very important in the analysis of results such as those shown in Fig. 1. The kinetics of GdnHCl denaturation of feruloyl esterase (FAE-II) suggests partially unfolding of the protein structure. It has been observed that structural changes produced by denaturants runs in parallel to decrease in enzyme activity. One can concluded that the action of denaturants involves the solublization of amino acid residues that are poorly soluble in water, and bind to protein groups thereby weakening the forces that maintain the native structure of proteins.



Scheme 1. Transesterification reaction of ethyl ferulate with 1-butanol catalyzed by feruloyl esterase (FAE-II).

From our experimental findings shown in Fig. 1, we can conclude that the initial reaction rate (V_0) for the transesterification reaction of feruloyl esterase (FAE-II) increases with increasing temperature from 288 to 328 K and then decreases a little when the temperature is increased from 338 to 358 K. However, the enzyme is still active even at 368 K. As substrate (ethyl ferulate) concentration increases, the initial reaction rate (V_0) for the esterification reaction of feruloyl esterase (FAE-II) increases up to a certain concentration, and then remains constant. The temperature dependence of V_{max} is shown in Fig. 3. V_{max} increases 7-fold from the minimal temperature (288 K) to the

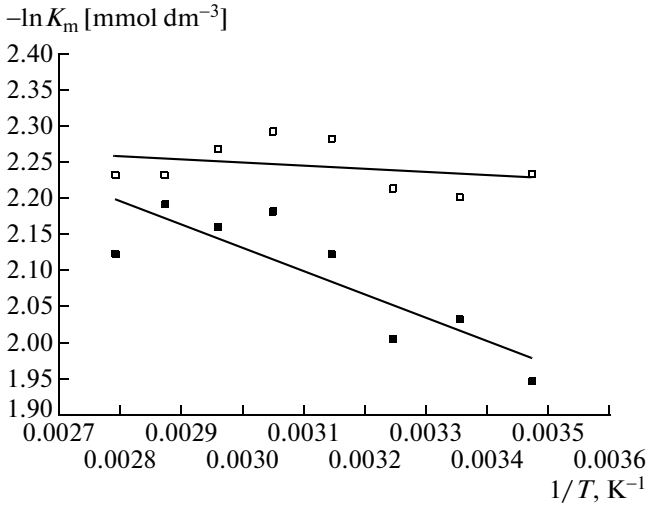


Fig. 6. Van't Hoff plot showing the relationship between K_m and temperature for the esterification reaction of feruloyl esterase (FAE-II); (□) without (GdnHCl); (■) with GdnHCl (1.5 mol dm^{-3} in 50 mmol dm^{-3} phosphate buffer (pH ~ 5.5) containing 0.15 mol dm^{-3} NaCl); the points are experimental values and the lines are the best-fit. Data presented are average values ± S.D. of n = 3 experiments.

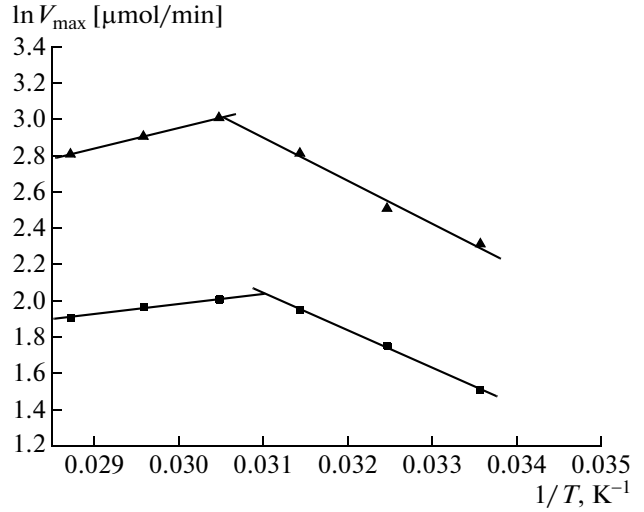


Fig. 7. Arrhenius plot showing the relationship between V_{max} and temperature for the esterification reaction of feruloyl esterase (FAE-II); (▲) without GdnHCl; (■) with GdnHCl (1.5 mol dm^{-3} in 50 mmol dm^{-3} phosphate buffer (pH ~ 5.5) containing 0.15 mol dm^{-3} NaCl); the points are experimental values and the lines are the best-fit. Data presented are average values ± S.D. of n = 3 experiments.

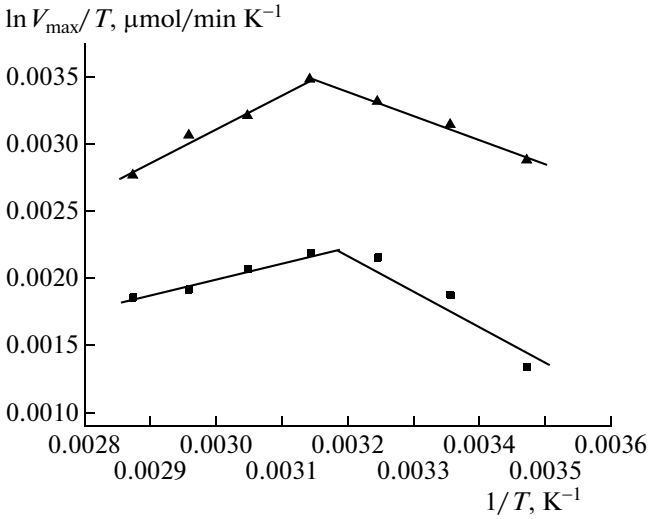


Fig. 8. Eyring plot showing the relationship between V_{max}/T and $1/T$ for the esterification reaction of feruloyl esterase (FAE-II); (▲) without GdnHCl; (■) with GdnHCl (1.5 mol dm^{-3} in 50 mmol dm^{-3} phosphate buffer (pH ~ 5.5) containing 0.15 mol dm^{-3} NaCl); the points are experimental values and the lines are the best-fit. Data presented are average values ± S.D. of $n = 3$ experiments.

maximal temperature (328 K) and almost 2-fold from physiological temperature (310 K) to 338 K. The Michaelis constant, K_m, also shows a similar trend but with only a 2-fold increase from the minimal to maximal temperature (Fig. 4) with no significant increase at any given temperature as seen with V_{max} values. As with the first order rate constant, the second order rate constant, V/K, shows a similar rate of increase; with the values increase 5-fold from the minimal to the maximal temperature (Fig. 5).

The temperature dependence of the equilibrium constant for the binding of feruloyl esterase (FAE-II) was analyzed with van't Hoff plots, and the results are shown in Fig. 6. The data were fitted to van't Hoff equation to determine the change in enthalpy, ΔH^* (−23.55 ± 1.21 kJ/mol), of the binding of the enzyme. The K_m for FAE-II enzyme can be used as an apparent binding constant. The negative value of ΔH^* indicates an exothermic reaction for the binding of feruloyl esterase to ethyl ferulate substrate. The ΔS^* (64.32 J/mol) further suggests that thermal fluctuations cause the K_m to increase as a function of temperature. As temperature increases, the change in entropy is greater in enzyme-substrate complex than for free substrate due to increasing thermal fluctuations. A negative value of ΔG^* (−3.1 kJ/mol) suggests that the enzymatic hydrolysis of ethyl ferulate is spontaneous or thermodynamically favorable. The van't Hoff plot (Fig. 6) is convex, with an apparent break in the data at about 318 K. This suggests that a conformational change is occurring in the feruloyl esterase enzyme (FAE-II) at about 318 K. There are examples for temperature induced conformational changes in enzymes. Among these are: D-amino acid oxidase [23], glutamine synthetase [24], penicillopepsin [25], and tryptophan synthase [26].

Temperature variations of the maximal velocity were analyzed with Arrhenius plots, and the results are shown in Fig. 7. The data were fitted to Arrhenius equation to determine the activation energy, E_a, of the reaction. E_a is the difference in energy between the transition state and the reactants. The slope determines the energy of activation (E_a). The slope is linear between 288–308 K, indicating that the E_a is a constant independent of temperature. The frequency of collisions (A) for each temperature was determined from E_a. Interestingly; A has the greatest value for reaction at 298 K and decreases at higher and lower temperatures (49.86 s^{-1}). According to the Arrhenius theory, the maximal velocity is determined by the ratio of activation energy to the temperature and by the frequency of collisions that produce a reaction. However, after analysis of the residuals, the data showed a systematic error. Therefore, the data were divided into two regions (Fig. 7). The first region, corresponding to values from 288–318 K, gives an E_a of 18.0 ± 1.0 kJ/mol, and a second region from 318–358 K, which gives the value of E_a close to zero. The individual values of the two apparent activation energy obtaining over each corresponding period of Arrhenius linearity can usefully be employed to suggest that: it is possible that convex Arrhenius plots will be more common in biological systems because an enzyme may have specifically evolved such that the lowest-energy states are the reactive ones, thereby leading to low activation energy even as temperature is increased [26]. The values of $\Delta G^*_{E\text{-}S}$ (free energy of substrate binding ≈−0.87 kJ/mol) and $\Delta G^*_{E\text{-}T}$ (free energy of transition state formation ≈−1.36 kJ/mol) of FAE-II enzyme with or without GdnHCl chemical denaturant indicates that the FAE-II enzyme stably works even in the presence of the chemical denaturant.

The temperature dependence of the maximal velocity was analyzed using Eyring equation to determine the enthalpy of activation, ΔH^*. The ΔH^* calculated from the linear fit of all the data in the Eyring plot (Fig. 8) was 3.0 ± 1.2 kJ/mol. However, after examination of the residuals, the data again showed a systematic error. Therefore, the data were divided into two regions as shown in Fig. 8. The first region consisted of values from 288–318 K, which gave a ΔH^* of −17.0 ± 1.0 kJ/mol. A second region, 318–358 K, gave a ΔH^* of 2.4 ± 1.2 kJ/mol. A positive ΔH^* was expected because the transition state involves the breaking of bonds. The calculated ΔS^* is negative (−22.4 kJ/mol), indicating that the formation of the transition state requires the reacting molecules to adopt precise conformations and approach one another at a precise angle and that the molecules are more constrained in the activated complex. Bruice and Benkovic [27] proposed that the preorganization of the activated complex is manifested in the activation enthalpy (ΔH^*)

and not in the activation entropy (ΔS^*). This proposal is based on numerous examples of enthalpies of activation for enzyme-catalyzed and nonenzymatic catalyzed reactions, in which the activation enthalpy varied widely between enzyme-catalyzed and nonenzymatic catalyzed reactions. In contrast, the activation entropy showed very little change between enzyme-catalyzed and nonenzymatic catalyzed reactions. Thus, it will be interesting to test this hypothesis by measuring the temperature dependence for the variety of mutant forms of feruloyl esterase enzyme (FAE-II) that do not exhibit this 1100-fold increase in activity.

ACKNOWLEDGMENT

This work was supported by a grant (NSC94-2214-E011-004) provided by the National Science Council of Taiwan.

REFERENCES

1. Groves, J.T., *Nature*, 1997, vol. 389, p. 329.
2. Radzicka, A. and Wolfenden, R., *Science*, 1995, vol. 267, p. 90.
3. Jencks, W.P., *Catalysis in Chemistry and Enzymology*, New York: Dover, 1987.
4. Gutteridge, A. and Thornton, J., *J. Mol. Biol.,* 2005, vol. 346, p. 21.
5. Hammes, G.G., *Biochemistry*, 2002, vol. 41, p. 8221.
6. Fersht, A., *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, San Francisco: Freeman, 1998.
7. Fazary, A.E. and Ju, Y.H., *Acta Biochim. Biophys. Sin.*, 2007, vol. 39, p. 811.
8. Fazary, A.E. and Ju, Y.H., *Biotechnol. Mol. Biol. Rev.*, 2008, vol. 3, p. 95.
9. Fazary, A.E. and Ju, Y.H., *Biotechnol. J.*, 2008, vol. 3, p. 1264.
10. Savageau, M.A., *J. Theor. Biol.*, 1995, vol. 176, p. 115.
11. Baker, B.M. and Murphy, K.P., *Biophys. J.*, 1996, vol. 71, p. 2049.
12. Laidler, K.J., *Chemical Kinetics*, New York: Harper & Row, 1987, 3rd ed.
13. Laidler, K.J. and King, M.C., *J. Phys. Chem*, 1983, vol. 87, p. 2657.
14. Polanyi, J.C., *Science,* 1987, vol. 236, p. 680.
15. Fersht, A., *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, San Francisco: Freeman, 1998.
16. Vafiadi, C., Topakas, E., Wong, K.K., Suckling, I.D., and Christakopoulos, P., *Tetrahedron: Asymmetry,* 2005, vol. 16, p. 373.
17. Benoit, I., Navarro, D., Marnet, N., Rakotomanomana, N., Lesage-Meessen, L., Sigoillot, J.C., and Asther, M., *Carbohydr. Res.,* 2006, vol. 341, p. 1820.
18. De Vries, R.P., Kester, H.C.M., Poulsen, C.H., Benen, J.A.E., and Visser, J., *Carbohydr. Res.,* 2000, vol. 327, p. 401.
19. De Vries, R.P., Michelsen, B., Poulsen, C.H., Kroon, P.A., Heuvel, R.H.H., Faulds, C.B., Williamson, G., van der Hombergh, J.P.T.W., and Visser, J., *Appl. Environ. Microbiol.*, 1997, vol. 63, p. 4638.
20. Kroon, P.A., Faulds, C.B., and Williamson, G., *Biotechnol. Appl. Biochem.*, 1996, vol. 23, p. 255.
21. Kroon, P.A., Faulds, C.B., Brezillon, C., and Williamson, G., *Eur. J. Biochem.*, 1997, vol. 248, p. 245.
22. Iiyama, K., Lam, T.B.L., and Stone, B.A., *Plant Physiol.,* 1994, vol. 104, p. 15.
23. Shrake, A., Fisher, M.T., McFarland, P.J., and Ginsburg, A., *Biochemistry,* 1989, vol. 28, p. 6281.
24. Allen, B., Blum, M., Cunningham, A., Tu, G.-C., and Hofmann, T., *J. Biol. Chem.*, 1990, vol. 265, p. 5060.
25. Fan, Y-X., McPhie, P., and Miles, E.W., *Biochemistry,* 2000, vol. 39, p. 4692.
26. Truhlar, D.G. and Kohen, A., *Proc. Natl. Acad. Sci. U. S. A.*, 2001, vol. 30, p. 848.
27. Bruice, T.C. and Benkovic, S.J., *Biochemistry,* 2000, vol. 39, p. 6267.