Kinetics of a Thermolysin-Catalyzed Peptide Formation Reaction in Acetonitrile–Water

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Thermolysin-catalyzed peptide condensation reactions from acylamino acids and amino acid amides were studied in an acetonitrile (MeCN)–water mixed solvent in a homogeneous system. A kinetic analysis of the condensation reaction was performed by taking into account the simultaneous, solvent-induced, gradual inactivation of the enzyme. Although the dependence on the concentration of the carboxyl component gave linear double-reciprocal plots, that on the amine component at 70% MeCN showed an apparent substrate inhibition profile. An analysis of the apparent K_m and k_{cat} parameters for both components at 40% MeCN indicated that the condensation reaction proceeded by a random Bi–Bi mechanism. The K_m values were estimated to be 5.5 mM for Cbz–Phe and 110 mM for $LeuNH_2$, and k_{cat} was 4—5 s^{-1}. The K_m values for Cbz–Phe and $LeuNH_2$ were also evaluated at 70% MeCN to be 5 and 100—150 mM, respectively. This indicates that the catalytic property of this enzyme is not significantly influenced by the existence of a higher concentration of such an organic solvent, except for the second nonproductive binding of the amine component.

Thermolysin (TLN: E.C. 3.4.24.4)[1] catalyzes the condensation of an *N*-acylamino acid (C-component) and an amino acid amide (A-component) (Eq. 1: AC, acyl-group; Xxx and Yyy, amino acid residue).[2] Most of the TLN-catalyzed condensation reactions are thermodynamic-controlled by the equilibrium constant of the following equation:

$$\begin{aligned}&\text{AC–NH–Xxx–COOH} + \text{H}_2\text{N–Yyy–CONH}_2 \rightharpoonup \\ &\qquad \text{AC–NH–Xxx–CONH–Yyy–CONH}_2 + \text{H}_2\text{O}\end{aligned} \quad (1)$$

Several other equilibrium processes are also involved, and ionizations of the amino and carboxylic groups in

$$\begin{aligned}&\text{AC–Xxx–COOH} \rightleftharpoons \text{AC–Xxx–COO}^- + \text{H}^+ \\ &\text{H}_2\text{N–Yyy–CONH}_2 + \text{H}^+ \rightleftharpoons {}^+\text{H}_3\text{N–Yyy–CONH}_2\end{aligned} \quad (2)$$

significantly influence the yield of the product peptide. Therefore, controlling these equilibrium processes is important for improving the reaction yield before trying to activate the enzyme in various ways.[3] For this purpose, as well as in order to increase the solubility of the reactants (or some time the products), organic solvents are frequently introduced, either in heterogeneous system comprising aqueous and immiscible organic phases, a homogeneous reacting phase generating product precipitation, or a totally homogeneous system.

Although in recent years the genetic production of solvent-resistant mutant enzymes has started for proteases,[4] fundamental investigations concerning the kinetic and thermodynamic aspects of condensation or synthetic reactions have not been sufficiently performed, even with natural enzymes,[5,6] compared with those efforts to elucidate the mechanisms of hydrolytic catalysis.[7—9] For that (the third in the above) a totally homogeneous system is preferable. Regarding this, we have reported on the effects of dimethyl sulfoxide (DMSO) on TLN in terms of the kinetics of a synthetic reaction at relatively high concentrations of the organic solvent.[10] We found that the catalytic characteristics of TLN are basically maintained, even in 70% DMSO, if the parallel, solvent-induced inactivation of the enzyme is taken into consideration.

For some enzymes, DMSO and/or dimethylformamide (DMFA) have been reported to show milder effects on the structure and activity. However, more volatile solvents are preferable from practical aspects, even with aqueous mixed-solvent systems. Acetone, acetonitrile (MeCN), tetrahydrofuran, and 2-propanol were often taken as a water-miscible solvent (their vapor pressure reaches, e.g., 50 mmHg (1 mmHg=133.322 Pa) at −6.5 °C, 14.0 °C, −3.2 °C, and 26.9 °C in a pure solvent, respectively, while DMFA or DMSO does so at 74 °C or well above 100 °C, respectively). Our preliminary test, however, showed that only MeCN can afford meaningful enzymatic activity among these solvents at concentrations higher than 40%. We thus studied the kinetics of the TLN-catalyzed dipeptide condensation reaction in an aqueous-MeCN solvent at a high content of the latter.

Experimental

Materials: Thermolysin was purchased from Daiwa Kasei Co., Ltd. (Osaka, Japan; Lot TIDC391). Its concentration was determined spectrophotometrically.[1,8] *N*-[3-(2-Furyl)acryloyl]leucineamide (Fua–Gly–$LeuNH_2$) from the Peptide Institute (Minoo, Japan) was used as a hydrolytic substrate.[8] *N*-Benzyloxycarbonyl-L-amino acids (Cbz–Xxx) and amino acid amides ($XxxNH_2$) were purchased from Sigma (St. Louis, Mo. USA) or the Peptide Institute. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)

was purchased from Dojindo Laboratories (Kumamoto, Japan). Acetonitrile (MeCN) was an HPLC-grade reagent, obtained from Nacalai Tesque (Kyoto, Japan). The other chemical reagents were commercially available.

Methods: Peptide condensation reactions were carried out with A-component in excess of the C-component. In most cases, the buffering action was taken by the A-component; after an aqueous solution of the C- and A-components had been adjusted at the desired pH with dilute acid (HCl), an organic solvent was mixed. The reaction mixture in a small test tube was incubated in a thermostated water bath (37 °C). Aliquots were removed and analyzed by HPLC (Shimadzu LC10A-Cosmosil 5C18-P) after quenching by an excess amount of dioxane/5% phosphoric acid mixture. The eluent contained MeCN (30—40%(v/v)), phosphoric acid (pH 3), and triethylamine (50 mM, M=mol dm^{-3}).

Inactivation of the enzyme was followed by adding an aliquot (of appropriate volume) from an incubated enzyme mixture to a standard medium (0.1 M Hepes pH 6.5, 0.01 M $CaCl_2$, 2% (v/v) DMSO) containing the substrate (Fua–Gly–$LeuNH_2$). The absorbance change at 320—340 nm, due to a cleavage of this substrate at the Gly–Leu bond, was followed by a spectrophotometer (Union-SM401; Union Giken, Hirakata, Japan).

The pH value in the mixed solvent (pH^*) with a varying composition was not measured or adjusted, since it had no physicochemical significance. Therefore, the following description of "pH" only concerns the value of the aqueous solution to which the organic solvent was added.

Results and Discussion

(1) Apparent Equilibrium Yield. (a) Substrate Dependence: Since our previous study[10] showed that the TLN-catalyzed condensation reaction proceeded in a homogeneous system with 30—80% (v/v) MeCN, and that the highest yield was obtained after a 5 h reaction at 70 or 80% MeCN, the substrate specificity for the apparent equilibrium yield of the condensation was studied at 70% MeCN with high concentrations of TLN (15 μM). Figure 1 shows the result in a bar graph. With the same A-component, a more hydrophobic C-component gave better yields and, with the same C-component, a more hydrophobic A-component gave higher yields. Cbz–Arg is an exception; with this cationic C-component, $LeuNH_2$ somehow gave a better yield than did $PheNH_2$. Very high yields were obtained with a combination of $PheNH_2$ or $LeuNH_2$ and Cbz–Phe or Cbz–Trp.

(b) pH Dependence: The pH dependence of the apparent equilibrium yield (after 5 h) was studied for Cbz–Phe/$LeuNH_2$ between pH 4.5 and 9.0 (Fig. 2, ○) in 70% MeCN. Higher yields were obtained between pH 6 and 7.5. The pH dependence of the equilibrium yield was practically determined based on the dissociation constants of the carboxylic groups of the C-component (pK_{a1}), since the perturbation of the co-existing organic solvent is more effective upon pK_{a1} than on pK_{a2}.[11] For these dissociations, MeCN has a weaker effect than does DMSO, and therefore the yield in MeCN–water became pH-independent at a lower pH value than in DMSO–water. The hydrolytic activity of TLN (—) is optimal at around pH 6.5 in an aqueous medium.[8]

(2) Apparent Rate of Condensation. As was the case for the reaction in DMSO, TLN shows a time-dependent inactivation when exposed in an MeCN–water medium. Thus, a kinetic analysis of the condensation reaction, which requires lower enzyme concentrations, can be performed only with a full consideration of the co-occurring inactivation during the reaction period.

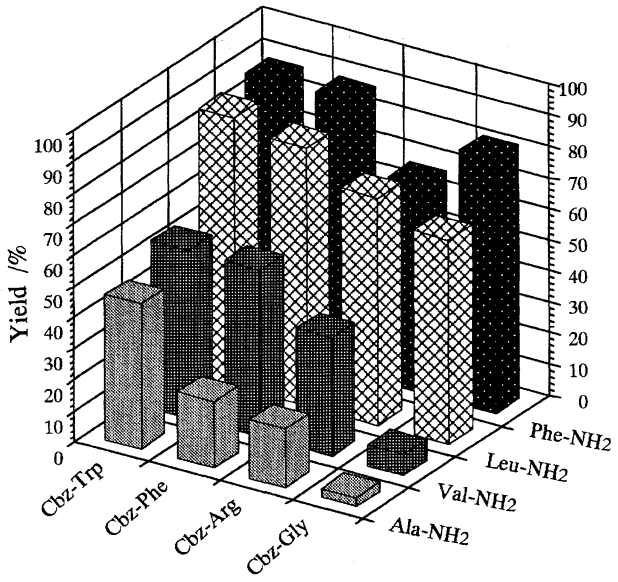


Fig. 1. Peptide condensation yield in 70% (v/v) MeCN with various C- and A-components after 5 h reaction. [TLN]=15 μM, [Cbz–Xxx]=5 mM, [YyyNH$_2$]=100 mM. pH 6.5, 37 °C.

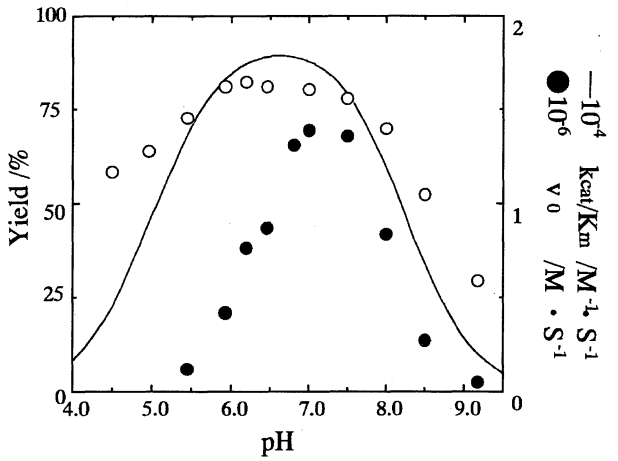


Fig. 2. pH dependence of the peptide condensation yield by TLN in 70% MeCN after 5 h reaction (○), compared with the apparent initial rate of condensation (●) and the reported second-order rate of the hydrolytic reaction (—).[8] For both, [Cbz–Phe]=5 mM, [$LeuNH_2$]=100 mM, 37 °C. [TLN]=15 μM (○) or 1 μM (●).

Figure 3 shows a time profile of the activity loss at pH 7 in 70% MeCN. In contrast to the case of DMSO-water, the loss in activity did not show a simple exponential decay. Instead, a sudden inactivation after a gradual decrease in the activity for a certain period was reproducibly observed. This might have been related to the aggregation of solvent-denatured proteins. The apparent (first-order) rate constant

of the inactivation process could therefore be evaluated only for the first phase, within which our kinetic measurements of the condensation reaction were well completed.

Taking into consideration such an inactivation, the time profile of the peptide condensation was analyzed. Practically linear time-dependences were obtained at [TLN] < 2 μM (Fig. 4), and the kinetic data were collected under such conditions. At [Cbz–Phe]=5 mM and [$LeuNH_2$]=100 mM, the apparent rate of the peptide condensation changed with the pH, as shown in Fig. 2 (●). We thus know that the left half of the pH profile of the apparent "equilibrium" yield is actually determined by the equilibrium, while the right half is, at least partly, determined kinetically.

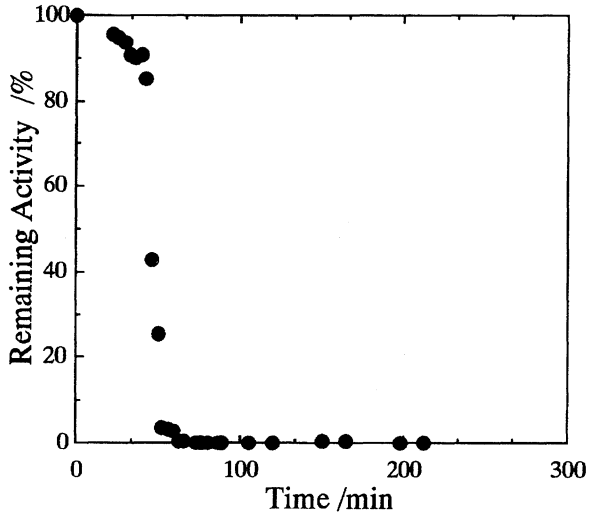


Fig. 3. Time profiles of the inactivation of TLN in 70% MeCN–aqueous solution. [TLN]=30 μM, $CaCl_2$=0.01 M, 0.1 M Hepes (pH 7), 37 °C. Activity was measured in aqueous buffer (Hepes; pH 6.5) with FuaGlyLeuNH_2.

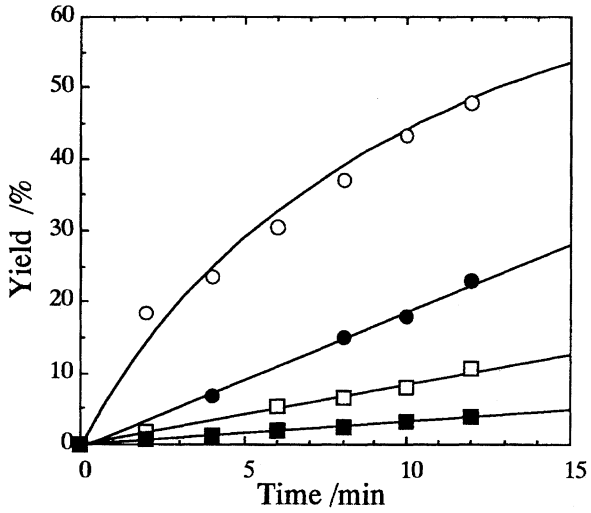


Fig. 4. Time course of the peptide condensation reaction from Cbz–Phe and $LeuNH_2$ at various enzyme concentrations in 70% MeCN. ○, [TLN]=5 μM; ●, 2 μM; □, 1 μM; ■, 0.5 μM. [Cbz–Phe]=5 mM, [$LeuNH_2$]=100 mM. pH 6.5, 37 °C.

(3) Substrate Concentration Dependence of the Rate. (a) Carboxyl Component Concentration: When the concentration of the A-component ($LeuNH_2$) was fixed, sufficiently linear L–B plots were obtained with various concentrations of Cbz–Phe in 40 and 70% MeCN (Fig. 5-a,b). The condensation reaction is a two-substrate, two-product reaction, and a proper Bi–Bi mechanism should be applied for a kinetic analysis. Since TLN is believed to have no acyl- (or other covalent) enzyme intermediate, the possibility of the so-called Ping-Pong Bi–Bi mechanism, a standard mechanism for serine proteases, is very low, and either the ordered Bi–Bi[5a] or the random Bi–Bi[5b,6] mechanism is to be applied.

According to the Cleland kinetics,[12] a discrimination of these two mechanisms can be made by comparing the position of the intersection point of L–B plots at different fixed

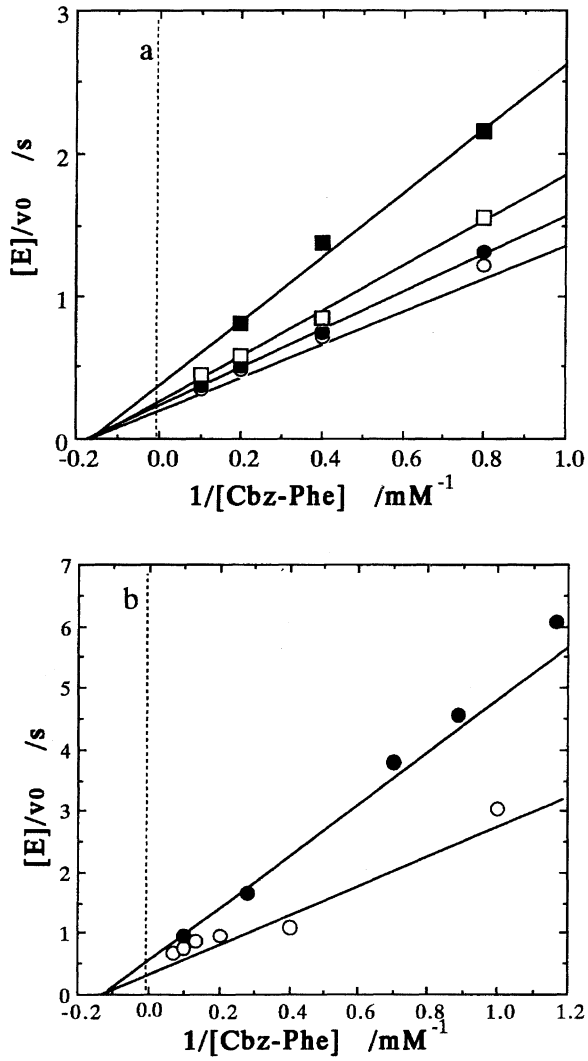
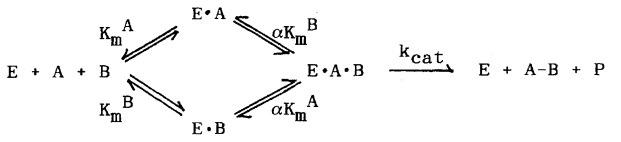


Fig. 5. Lineweaver–Burk plot of the initial rate of the condensation reaction in 40% (a) and 70% (b) MeCN at various Cbz–Phe concentrations with fixed concentrations of $LeuNH_2$. pH 6.5, 37 °C. a, 40% MeCN. [$LeuNH_2$]=294 mM (○), 220 mM (●), 147 mM (□), and 73 mM (■). [TLN]=0.5 μM. b, 70% MeCN. [$LeuNH_2$]=100 mM (○) and 60mM (●). [TLN]=1 μM.

concentrations of the other component. In both cases in Fig. 5, the intersections seem to be in the second quadrant or on the X-axis. This indicates, when the rapid equilibrium hypothesis can be applied, that Cbz–Phe is not the second-binding substrate in the ordered Bi–Bi mechanism. Either the random Bi–Bi with a cooperativity factor (α, see below) ≤ 1, or an ordered one with Cbz–Phe binding first to the enzyme can be applied.

(b) Amine Component Concentration: For further analysis the dependence on the concentration of the A-component with various fixed concentrations of the C-component must be examined. The result at 40 or 70% MeCN is shown in Fig. 6. In 70% MeCN (b), although the dependence on the A-component concentration was not simple, an apparent substrate inhibition was observed, which is fundamentally similar to the case of 70% DMSO.[10] This apparent substrate inhibition phenomenon was not observed in 40% MeCN, which affords an evaluation of $K_{m(app)}$ and $k_{cat(app)}$ by an ordinary procedure.

(4) Possible Mechanism of the Condensation. The intersection in Fig. 6-a seems to be in the second quadrant, and very close to the X-axis. This and Fig. 5-a indicate that the reaction proceeds by the random Bi–Bi mechanism (Eq. 3), with the cooperativity factor being close to unity.



(3)

This mechanism has been postulated for the TLN-catalyzed condensation reaction in lower concentrations of DMFA or DMSO by Reichmann & Kasche[5b] as well as Wayne & Fruton.[6] This conclusion is also compatible with the result in 70% MeCN (Fig. 5-b); we understand that the TLN-catalyzed condensation reaction can be explained by the random Bi–Bi mechanism even at a higher concentration of a less-mild organic solvent, such as MeCN. Thus, the apparent K_m and k_{cat} parameters in the initial rate analysis with fixed or varying concentrations of the C-component (A) or A-component (B) are described as follows:

$$K^A_{m(app)} = \alpha K_m{}^A(1 + K^B_m/[B])/(1 + \alpha K^B_m/[B]),$$
$$k^A_{cat(app)} = k_{cat}/(1 + \alpha K^B_m/[B]), \quad (4)$$
$$K^B_{m(app)} = \alpha K_m{}^B(1 + K_m{}^A/[A])/(1 + \alpha K_m{}^A/[A]),$$

and

$$k_{cat(app)}{}^B = k_{cat}/(1 + \alpha K_m{}^A/[A]).$$

When α is very close to unity, these are simplified as

$$K_{m(app)}{}^A = K_m{}^A,$$
$$k_{cat(app)}{}^A = k_{cat}/(1 + K_m{}^B/[B]), \quad (5)$$
$$K_{m(app)}{}^B = K_m{}^B,$$

and

$$k_{cat(app)}{}^B = k_{cat}/(1 + K_m{}^A/[A]).$$

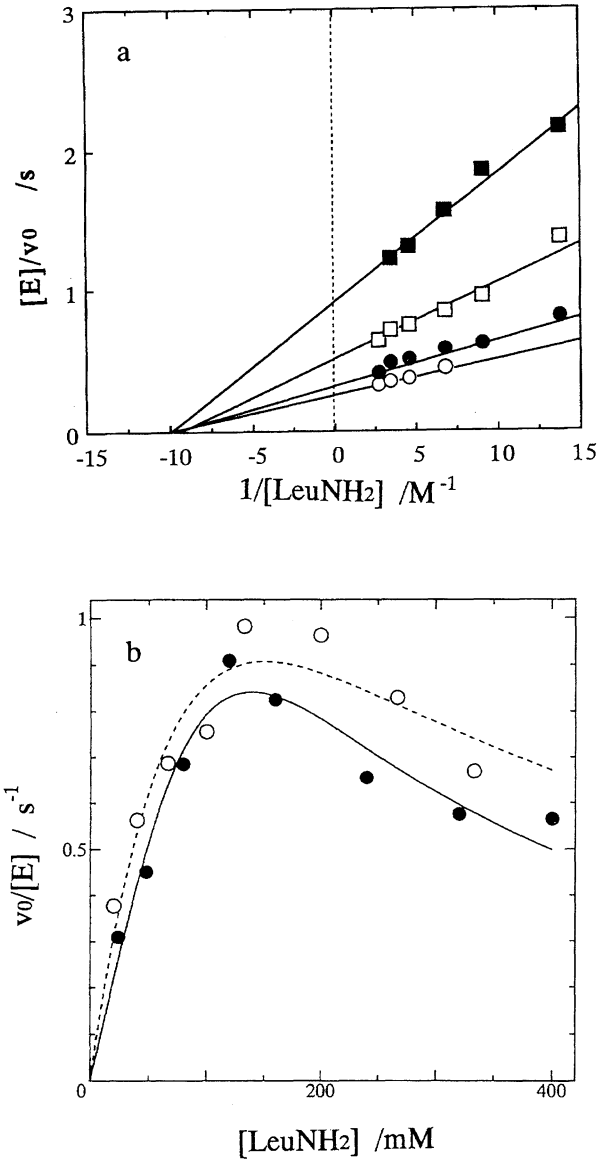


Fig. 6. Initial rates of the condensation reaction in 40% (a) and 70% (b) MeCN at various $LeuNH_2$ concentrations with fixed concentrations of Cbz–Phe. a: 40% MeCN. [Cbz–Phe]=10 mM (○), 5.0 mM (●), 2.5 mM (□), and 1.3 mM (■). [TLN]=0.5 μM, pH 6.5, 37 °C. b: 70% MeCN. [Cbz–Phe]=5 mM. [TLN]=1.2 μM, pH 6.5 (●) or 7.0 (○), 37 °C. Curves in the figure are arbitrary.

Thus, the "apparent" Michaelis constant can be considered to be the actual K_m, and the apparent k_{cat} values are influenced by the term $1/(1+K_m/[A \text{ or } B])$. The results given in Figs. 5 and 6 indicate that $K_m{}^A$, $K_m{}^B$, and k_{cat} are 5.5 mM, 110 mM, and around 4—5 s^{-1}, respectively, at 40% MeCN and pH 6.5. At 70% MeCN, although the data were not simply analyzed, $K_m{}^A$ can be estimated to be about 5 mM (pH 6.5), which is similar to the value at 40% MeCN. At pH 7.0, $K_m{}^A$ (70% MeCN) increased to 24.2 mM and $K_m{}^B$ (40% MeCN) decreased to 65 mM (detailed data not shown). These pH dependences are related to the ionization of the carboxylate (pK_{a1}) and the amino (pK_{a2}) groups. If pK_{a1} of Cbz–Phe in

70% MeCN and pK_{a2} of $LeuNH_2$ in 40% MeCN are estimated to be 4.8 and 8.1,[11] respectively, the K_m values for the non-ionized forms of each substrate (left-side forms in Eq. 2) are calculated to be about 0.1 mM (for Cbz–Phe–COOH at 70% MeCN) and 5 mM (for H_2N–$LeuNH_2$ at 40% MeCN).

An apparent substrate-inhibition phenomenon was seen at 70% MeCN, but not at 40%. Though there are some possibilities that such a dependence was brought about through an effect of the organic solvent on the proton activity in the solution, or by some hydrodynamic effect of the concentrated solution of hydrophobic amine in aqueous organic solvent, this can be explained by saying that the solvent composition affected the affinity of the second molecule of the A-component to inhibit the catalytic reaction. In the case of the two-phase reaction system of water/ester, it was shown[13] that two molecules of the C-component can be bound on the TLN-active site, but, in homogeneous system as the present one, two molecules of the A-component can be bound at a high concentration of water-miscible organic solvents, one of them being nonproductive and acting as an inhibitor of further productive binding of the substrate. From the lower concentration range of Fig. 6-b, $K_{m(app)}{}^B$ (equals to $K_m{}^B$ for the random mechanism with $\alpha=1$) was roughly estimated to be 100—150 mM, which is not significantly different from the $K_m{}^B$ at 40% MeCN.

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