Kinetics of the Peptide Formation Catalyzed by Thermolysin in a Homogeneous Aqueous–Organic System

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The effect of an organic solvent on the thermolysin-catalyzed peptide condensation reaction from acylamino acid and amino acid amide was investigated. Profiles of the apparent equilibrium yield were roughly divided into three regions: precipitation-driven (mostly low to medium organic-solvent concentration), clear and homogeneous (middle to rather high concentration), and inactivated conditions (very high (>80%) concentration).

In the second, homogeneous region (70%(v/v) DMSO), the kinetics of the condensation reaction were followed by taking into account the simultaneous, solvent-induced gradual inactivation of the enzyme. The dependence on the carboxyl component concentration gave apparent K'_m and k'_{cat} parameters for Cbz–Phe and Cbz–Trp; we found that even at 70% DMSO, thermolysin had similar rate parameters and activity to those in 10% DMSO [Wayne and Fruton, *Proc. Natl. Acad. Sci. U.S.A.*, **80**, 3241 (1983)]. The amine-component concentration dependence showed distinct substrate-inhibition profiles for both $LeuNH_2$ and $PheNH_2$.

Thermolysin, a thermostable metal neutral protease from *B. thermoproteoliticus*,[1] can catalyze the condensation of *N*-acylamino acid and an amino acid amide (Eq. 1: AC, acyl-group; AA and AA′, amino acid residue), and some industrial applications have been performed.[2]

$$AC\text{–}AA + AA'\text{–}NH_2 \longrightarrow AC\text{–}AA\text{–}AA'\text{–}NH_2 \qquad (1)$$

Though we have investigated several kinetic aspects of free and immobilized thermolysin,[3,4] our understanding of the synthetic reactions has remained insufficient. During peptide condensation by thermolysin, most of the reactions are controlled thermodynamically, and several equilibrium processes are involved. The ionization of amino and carboxylic groups, as well as the reaction equilibrium of Eq. 1, determines the yield of the peptide product. Therefore, the influences upon the equilibrium processes affect the reactions before activation of the enzymes in some way, for example, through chemical modifications or physical optimization.[5,6] To realize these and also to increase the solubility of the reactants (or the products), organic solvents are often introduced into the reaction system. From an engineering perspective, a heterogeneous system comprising aqueous and immiscible organic phases might be preferred; or a solvent system that allows high solubility for the substrates and a lower level for the product could promote a precipitation of the product and shift the equilibrium towards production. Occasionally, nearly organic systems containing nonaqueous "water mimics" and a small amount of water have been proposed.[7]

The effect of organic solvents on biocatalysis has generally had increasing importance in bioconversions, since the applications have broadened. There have been few reports concerning the effects of organic solvents on thermolysin[8—10] in terms of the kinetics of a synthetic reaction in a homogeneous system. Furthermore, most of them were for a rather low concentration of the organic solvent.

Here, we performed a kinetic study of the condensation reaction in a homogeneous system. We first studied the solvent concentration dependence of the peptide yield and sought a clear and homogeneous system. Thereafter, we performed some kinetic analyses.

Experimental

Materials: Thermolysin was purchased from Daiwa Kasei Co., Ltd. (Osaka, Japan; Lot TIDC391); its concentration ([TLN]) was determined spectrophotometrically.[1,3] *N*-[3-(2-Furyl)acryloyl]leucine amide ($FuaGlyLeuNH_2$), used in a hydrolytic study, was purchased from the Peptide Institute (Minoo, Japan) or prepared in our laboratory.[3] *N*-Benzyloxycarbonyl-*L*-amino acids and amino acid amides 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). Dimethyl sulfoxide (DMSO), *N*,*N*-dimethylformamide (DMF), dioxane, and acetonitrile (MeCN) were HPLC-grade reagents, and were obtained from Nacalai Tesque (Kyoto, Japan). The other chemical reagents were commercially available.

Methods: A condensation reaction was carried out

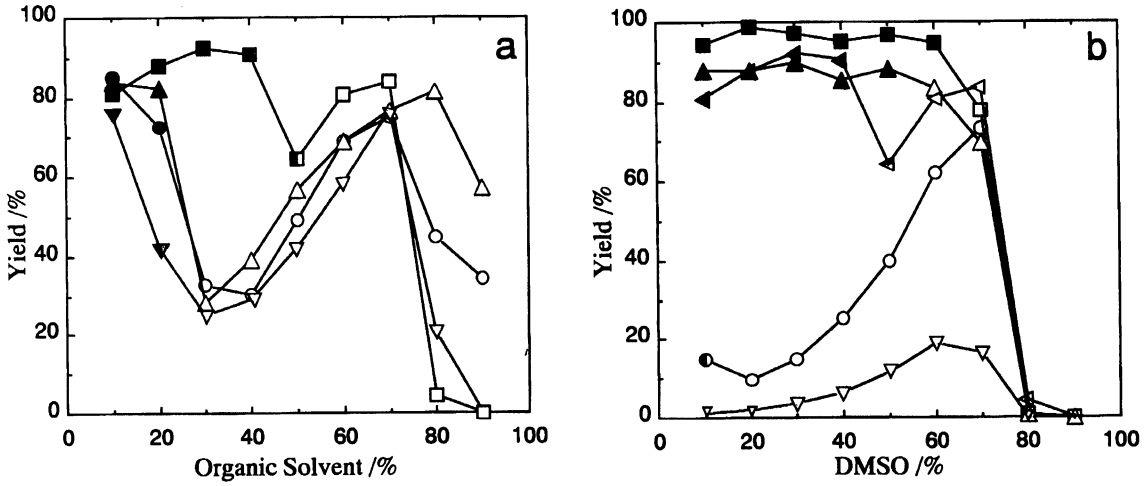

Fig. 1. Peptide yield after a 5 h reaction in various concentrations of organic solvent. a. In different solvents with Cbz–Phe+$LeuNH_2$. ○, Dioxane; □, DMSO; △, MeCN; ▽, DMF. b. In DMSO with various substrates. □, Cbz–Phe+$PheNH_2$; △, +$ValNH_2$; ▽, +$AlaNH_2$; ◁, +$LeuNH_2$, ○, Cbz–Trp+$LeuNH_2$. [TLN]=15 μM, [Cbz–Xxx]=10 mM, [$YyyNH_2$]=50 mM. pH 7.0 (0.2 M Hepes), 37 °C. Closed and open symbols represent heterogeneous and homogeneous, respectively.

in the indicated solvent composition with amino acid amide in excess of the carboxyl component. The reaction mixture in a small test tube was incubated in a thermostated water bath (normally at 37 °C). The reaction process was followed by analyzing an aliquot of the reaction mixture by HPLC (Shimadzu LC6A-Cosmosil 5C18-P) after quenching by mixing with excess dioxane and 5% phosphoric acid. The eluent contained MeCN (30—40%(v/v)) and phosphoric acid (pH 3) containing triethylamine (50 mM, 1 M=1 mol dm^{-3}).

The inactivation of the enzyme was followed by removing an aliquot (of appropriate volume) from the incubated enzyme mixture, which was added to the substrate (FuaGlyLeuNH_2) in the standard medium (0.1 M Hepes pH 6.5, 0.01 M $CaCl_2$, 2%(v/v) DMSO).

The pH value in the mixed solvent (pH*) with a varying composition was not measured or adjusted, since it has no physicochemical significance. Therefore, the following description of "pH" only concerns the value of the buffer with which the solution was prepared.

Results and Discussion

(1) Apparent Equilibrium Yield. (a) Co-Solvent Dependence: Figure 1 contains the peptide yield obtained after 5 h of reactions under various solvent conditions (a) and with different substrate combinations (b). The resulting "pseudo-equilibrium" yields can be classified into three categories. The first shows those obtained in systems that precipitate (shown as solid symbols in the figures); rather high yields were brought about due to the inclination of the condensation equilibrium caused by the creation of a second solid phase. The second category showed a low or moderate yield, of clear and homogeneous solutions. The third was for a much higher concentration of the organic solvent (80 and 90%) and gave only a few % yield. Although some of them were clear solutions, others contained precipitates of the enzyme. Since the boundaries of these categories were fundamentally determined by the solubility of the produced peptide and they critically depend on the nature of the solvent as well as the substrate combination.

At 70% solvent, most of the substrates gave a homogeneous system. Under these conditions we measured the substrate-dependence of the pseudo-equilibrium yield. The results are given in Fig. 2. $LeuNH_2$ showed a better yield; the highest was obtained with the combination of this amide and Cbz–Phe in DMSO/water.

(b) pH Dependence: The pH dependence of the equilibrium yield (Y_{eq}) was determined between pH 5 and 8.5; the results are shown in Fig. 3. With some minor exceptions, the full processes approaching the equilibrium were followed and the equilibrium yield was obtained after several hours even at a lower pH. The pH dependence of Y_{eq} can be seen at the right end of the graph; a higher yield was obtained when the pH was above 7. As discussed by Homanberg et al.,[11] the pH dependence of the equilibrium yield is fundamentally determined by the dissociation constants of the amino and carboxylic groups of each component (pK_{a2} and pK_{a1}, respectively). The perturbation of these by the co-existing organic solvent is more effective upon pK_{a1} than on pK_{a2}. Most of the solvent effect on the total yield (in a homogeneous system) is due to the effect on pK_{a1}, for which DMSO has a very marked effect.[11] In 80% DMSO/water, the pK_{a1} of *N*-acetylglycine is reportedly as high as 6.93, more than 3 units higher than that in aqueous solution. The effect on pK_{a2} was only 0.1 unit. A shift in pK_{a1} of this magnitude results in a large increase in the apparent equilibrium constant (K_{app}), since K_{app} is proportional to $1/(1+10^{(pH-pK_{a1})})$. Although the (hydrolytic) activity of thermolysin itself is optimal around pH 6.5 in an aqueous medium,[3] the rate necessary to approach the

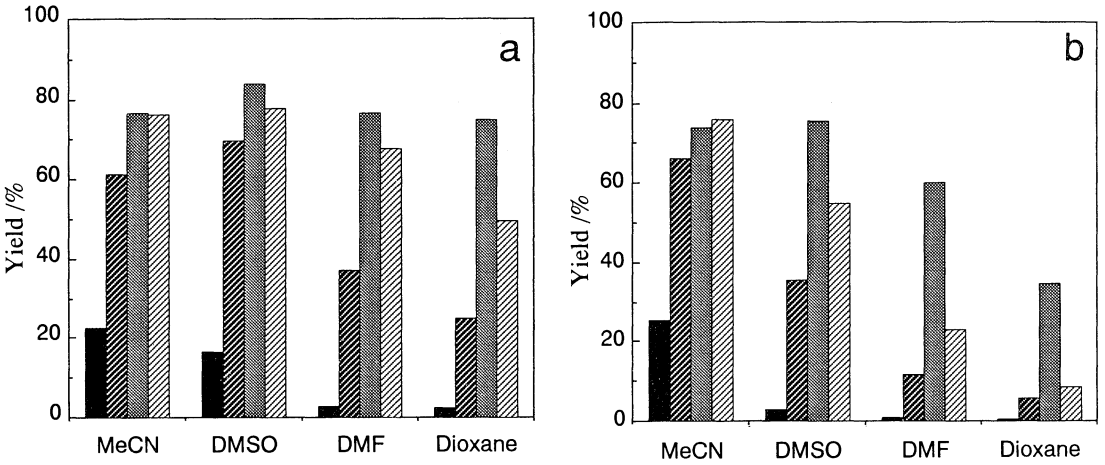

Fig. 2. Peptide condensation yield in a 70% (v/v) organic-aqueous solution with various amino acid components. Reaction conditions were as described in the legend to Fig. 1. a. With Cbz–Phe as the carboxyl component. ■, +$AlaNH_2$; ▨, +$ValNH_2$; ■, +$LeuNH_2$; ▨, +$PheNH_2$. b. With Cbz–Trp as the carboxyl component. Patterns are the same as in a.

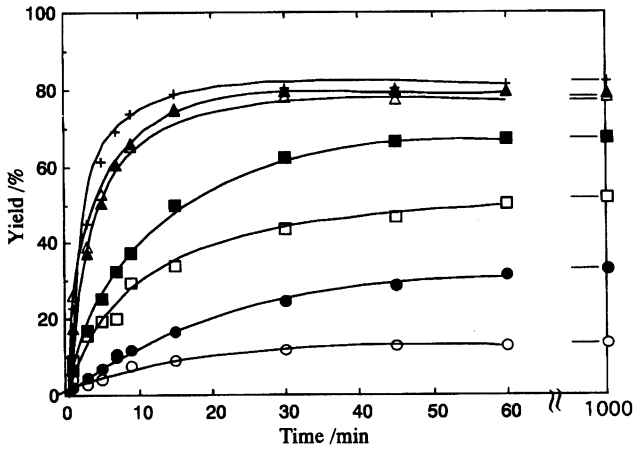

Fig. 3. pH dependence of the peptide condensation by thermolysin in 70% DMSO. ○, pH 5.0; ●, 5.5; □, 6.0; ■, 6.5; △, 7.0; ▲, 7.5; +8.0. [TLN]=15 μM, [Cbz–Phe]=10 mM, [$LeuNH_2$]=50 mM, 37 °C. Hepes (0.2 M) or Mes (0.2 M) were used.

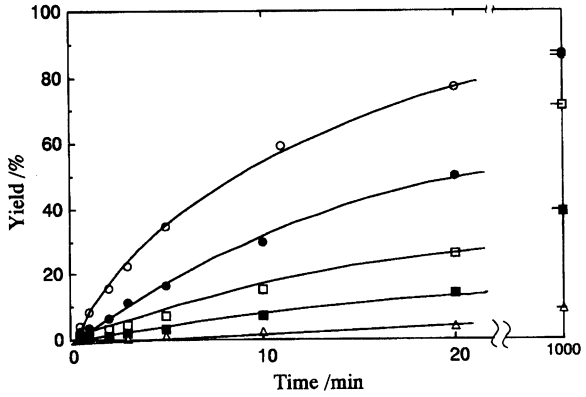

Fig. 4. Time course of the peptide condensation reaction from Cbz–Phe and $LeuNH_2$ at lower enzyme concentrations. ○, [TLN]=5 μM; ●, 2 μM; □, 1 μM; ■, 0.5 μM; △, 0.2 μM. [Cbz–Phe]=5 mM, [$LeuNH_2$]=100 mM. pH 7.0, DMSO 70%, 37 °C.

apparent condensation equilibrium became faster along with an increase in the pH up to 8, as can be seen in Fig. 3.

(2) Kinetics of the Condensation. (a) Enzyme Concentration Dependence: To follow the kinetic processes of the condensation reaction, a lower enzyme concentration was necessary. We studied the enzyme-concentration dependence of the formation (Fig. 4). At the same time, the yield after prolonged periods in the presence of various amounts of enzyme was checked. The figure shows that the initial phase of the reaction curves obeyed normal kinetic profiles, and the initial rate analysis gave an almost linear dependence on the enzyme concentration; however the "equilibrium" yield was dependent upon the enzyme concentration. This apparent dependence indicated that the enzyme, especially at lower concentrations, was inactivated by the solvent during the reaction.

(b) Inactivation of the Enzyme: Figure 5 contains the time profile of the activity loss at pH 7 in various DMSO concentrations (a) and at various pH in 70% DMSO (b). In 10% to 50% DMSO, the apparent first-order rate constant of the inactivation process (k_{dis}) was around 2 to 3×10^{-5} s^{-1}. This became faster in 70% DMSO, being 6×10^{-5} s^{-1} at pH 6, 7, and 8 and 1.3×10^{-4} s^{-1} at pH 5 or 9. At 80% DMSO or more, k_{dis} was very large and not measurable by this batch-wise method.

The value of k_{dis} was dependent upon the concentration of the enzyme and Ca^{2+}. At pH 7 and 70% DMSO the apparent k_{dis} was as large as 2.0×10^{-4} s^{-1} for [TLN]=60 μM and [Ca^{2+}]=200 μM; it further increased to 3.5×10^{-4} s^{-1} for [TLN]=2 μM and [Ca^{2+}]=20 μM. The [TLN] dependence was extrapolated to zero concentration and the k^0_{dis} value was calculated to be 3.7×10^{-4}

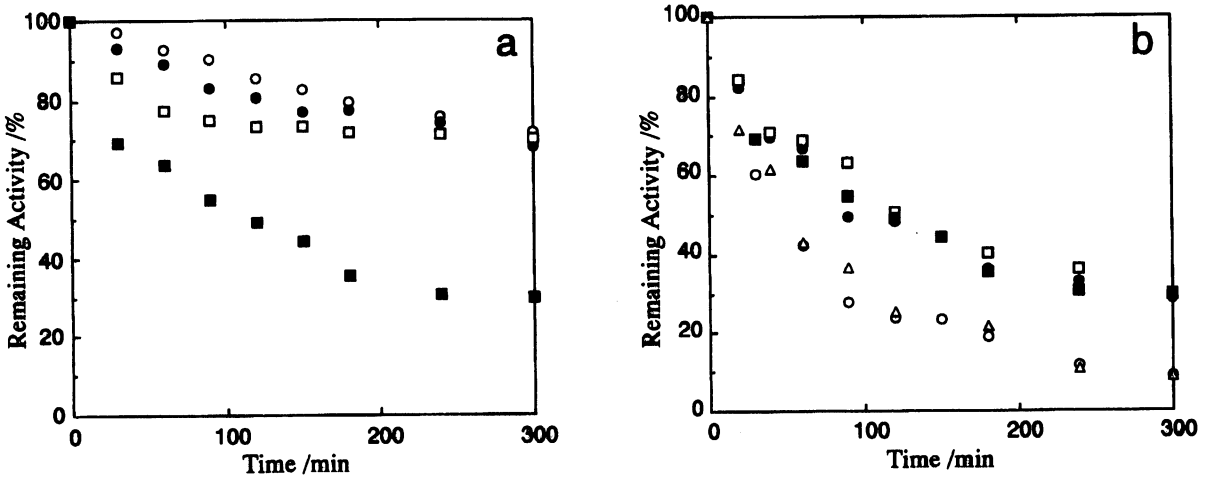

Fig. 5. Time profiles of the inactivation of thermolysin in DMSO-aqueous solution. a. With various concentration of DMSO at pH 7. ○, DMSO 10%; ●, 30%; □, 50%; ■, 70%. b. At various pH in 70% DMSO. ○, pH 5.0; ●, pH 5.8; □, pH 6.9; ■, pH 7.7; △, pH 9.0. [TLN]=30 μM, $CaCl_2$=0.01 M, 0.2 M Hepes or Mes, 37 °C.

s^{-1}. A PAGE analysis of the incubated enzyme solution after kinetic measurements showed no sign of facilitated autolysis.

(c) Revaluation of the Time Profile: Thus, the inactivation process was taken into consideration when analyzing the time profile of the peptide condensation. For example, Fig. 6 was drawn to show the effect of the revaluated time profile after considering the inactivated enzyme during the process. A very linear rate profile was obtained. By doing similar revaluations, kinetic data were collected. When the amine component ($LeuNH_2$) was fixed at 100 mM, a sufficiently linear Lineweaver–Burk plot was obtained for both Cbz–Phe and Cbz–Trp (data not shown). The apparent calculated kinetic parameters are summarized in Table 1.

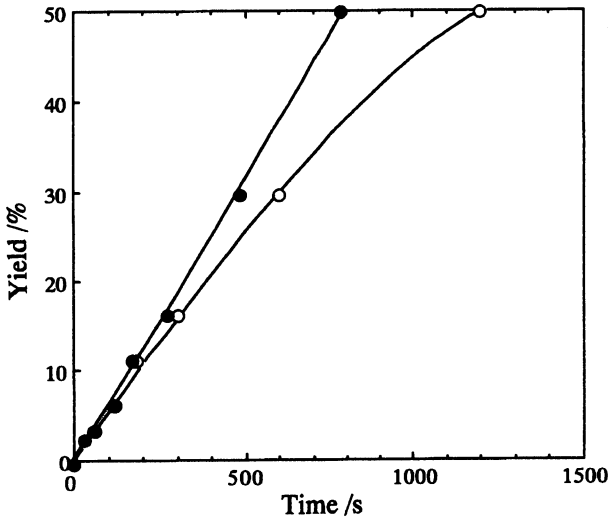

Fig. 6. Revaluation of the time profile of the condensation reaction accounting for inactivation of the enzyme. ○, Experimental yield; ●, revised one. [TLN]=2 μM. Other conditions are as in Fig. 4.

Table 1. Apparent Kinetic Parameters for the Carboxyl Component[a)]

DMSO concn	Carboxyl component	$K^{A}_{m(app)}$ mM	$k^{A}_{cat(app)}$ s^{-1}	$k^{A}_{cat(app)}/K^{A}_{m(app)}$ $M^{-1}s^{-1}$
70	Cbz–Phe	29+/−1	12+/−0.5	410
	Cbz–Trp	30+/−10	7+/−3	230
10[b)]	Cbz–Phe	42+/−1	18+/−2	430

a) [TLN]=0.2 μM, [$LeuNH_2$]=100 mM, pH 7, 37 °C.
b) Taken from Ref. 9. The k_{cat} value in the original paper (105 min^{-1}) was very small and contradicts the value calculated by $(k'_{cat}/K'_m)/K'_m$ (1050 min^{-1}). Thus the latter value is cited here.

Since the protease-catalyzed condensation reaction is a two-substrate and two-product reaction, a kinetic analysis of the proper Bi–Bi mechanism should be applied. Usually, however, a rather simplified apparent kinetic analysis has been performed for these systems; here, also, we could calculate only the apparent parameters. Since thermolysin is believed to have no acyl-enzyme intermediate, the peptide-condensation reaction catalyzed by this enzyme can be described by a random Bi–Bi mechanism;[12)] the apparent K_m and k_{cat} parameters in the initial rate analysis with a fixed concentration of B (amine component) and varying concentration of A (carboxyl component) would be

$$K^{A}_{m(app)} = K^{A}_{m} \quad (2)$$

and

$$k^{A}_{cat(app)} = k_{cat}/(1 + K^{B}_{m}/[B]). \quad (3)$$

Here, the affinity of the free enzyme (E) towards B is assumed to be similar to that of an enzyme-acid complex (E·A).

The values obtained here can be compared with the values reported by Wayne and Fruton, obtained using 10% DMSO. The $K_{m(app)}$ and $k_{cat(app)}$ values did

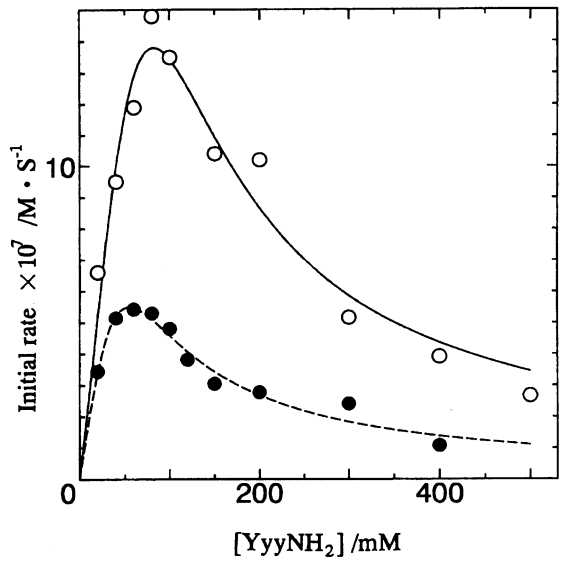

Fig. 7. Initial rate of the condensation reaction at various concentrations of the amine component with Cbz–Phe as the carboxyl component. ○, With $LeuNH_2$; ●, with $PheNH_2$. [TLN]=1 μM; [Cbz–Phe]=5 mM; DMSO 70%, pH 7, 37 °C.

not differ significantly in two concentrations of DMSO. Though the Ca^{2+} concentration and pH are somehow different, they were evaluated with the same [$LeuNH_2$]. The difference in the carboxyl component is mostly reflected in the $k_{cat(app)}$ parameter. In a rapid equilibrium random Bi–Bi mechanism, this difference can be deduced to an actual k_{cat} parameter with a common amine component.

(d) Effect of Amine Component Concentration: Figure 7 shows the initial rate obtained with various concentrations of the amine component ($LeuNH_2$ or $PheNH_2$) in 70% DMSO. This was not a simple saturation (Michaelis–Menten type) dependence. Instead, the apparent rate was maximal for either amine component. The maximal values differed and the rate for $LeuNH_2$ was over two-times higher than that for $PheNH_2$. These profiles indicate substrate inhibition. A simple phenomenological equation for such inhibition

$$E + S \overset{K_S}{\rightleftharpoons} E{\cdot}S \overset{k_2}{\rightarrow} E + P \quad (4)$$

and

$$E{\cdot}S + S \overset{K_{SS}}{\rightleftharpoons} S{\cdot}E{\cdot}S, \quad (5)$$

could be applied. Then, the rate would be expressed as

$$\text{rate} = (k_2[S]/K_S)/\{1 + [S]/K_S + [S]^2/(K_S{\cdot}K_{SS})\}. \quad (6)$$

However, the increase at a lower concentration is almost linear, and a numerical analysis only gave K_S as being very large (>50 mM), with a K_{SS} around 100 mM for both. The ratio of the k_2 value between $LeuNH_2$ and $PheNH_2$ was around 2.5. Though Nakanishi and Matsuno have described[13] that for the two-phase reaction system thermolysin can bind two carboxyl components in the active site, the present result in a homogeneous aqueous–organic system implies that two amine components can be bound and that one of them is nonproductive, acting as an inhibitor of further productive binding of the substrate. Though these curves were obtained at a lower concentration of the carboxyl component (A), Eqs. 2 and 3 show that the K_m behavior was not interfered with by the non-saturating conditions of A. The difference in the highest initial rate between the two amine components could be ascribed to the difference in $k^B_{cat(app)}$ ($=k_{cat}/(1+K^A_m/[A])$ with common A), or to their different balance of K_S and K_{SS}.

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