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Kinetic Study of Thermolysin-Catalyzed Synthesis of N-(Benzyloxycarbonyl)-L-Phenylalanyl-L-Leucine Ethyl Ester in an Ethyl Acetate Saturated Aqueous System

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Abstract The kinetics of the thermolysin-catalyzed synthesis of N-(benzyloxycarbonyl)-L-phenylalanyl-L-leucine ethyl ester (Z-Phe-LeuOEt) from N-(benzyloxycarbonyl)-L-phenylalanine (Z-Phe) and L-leucine ethyl ester (LeuOEt) in an ethyl acetate saturated aqueous system in a batch operation were studied. The kinetics for the synthesis of Z-Phe-LeuOEt were expressed using a rate equation for the rapid equilibrium random bireactant mechanism. The four kinetic constants involved in the rate equation were determined numerically by the quasi-Newton method so as to fit the calculated results with the experimental data. Within the pH and temperature range examined, the k_{cat} value for the synthesis of Z-Phe-LeuOEt reached a maximum at pH 7.0 and 45°C, whereas the affinity between Z-Phe and thermolysin reached a maximum at pH 6.0 and 40°C. The inhibitory effect of Z-Phe on the condensation reaction decreased as the pH and temperature decreased. In contrast, the affinity between LeuOEt and thermolysin remained unchanged within the pH and temperature range examined. Therefore, it was concluded that the protonation state of the carboxyl groups of Z-Phe was more important than that of the amino groups of LeuOEt for the synthesis of Z-Phe-LeuOEt in the present solvent system. The equilibrium yield at pH 6.0 and 30°C was 8% higher than that at pH 7.0 and 40°C, although the rate was much slower. This result suggested that the affinity between the enzyme and the substrate rather than the overall rate was a more important factor affecting the equilibrium yield, when the peptide synthesis was carried out in a product-precipitation system.

Key words: Enzymatic peptide synthesis, ethyl acetate saturated aqueous system, thermolysin

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Oligopeptides are becoming increasingly important in view of their biological activities, including antibiotic [20, 26], hormonal [13], enzyme inhibitory [21], and immunomodulating characteristics [2]. Therefore, the enzymatic synthesis of peptides using the reversal of a hydrolysis reaction has attracted much attention in recent years [8, 9, 10, 16, 23]. Enzymatic peptide synthesis has certain merits over chemical methods. For example, the reaction can be carried out stereospecifically under mild conditions with or without the protection of side chains. Yet one of the defects with the enzymatic method is the low equilibrium yield of the condensation product, resulting from the suppression of the condensation reaction by a thermodynamically unfavorable hydrolysis. However, this problem can be circumvented by introducing a water-miscible or immiscible organic cosolvent [8, 9, 10]. Moreover, if the condensation product is precipitated in the reaction mixture due to its solubility, a high equilibrium yield of the product can be obtained.

Thermolysin, a thermostable and typical metallo-neutral protease from *Bacillus thermoproteolyticus*, has a specificity against hydrophobic or bulky amino acid residues at the amino side of the splitting point in a peptide or protein substrate. Isowa *et al.* [6] first showed that thermolysin can catalyze the reaction for peptide bond formation. Since then, a number of studies have been focused on the thermolysin-catalyzed synthesis of peptides in an aqueous-organic biphasic system to overcome the solubility limitation of hydrophobic amino acid in an aqueous system and to shift the equilibrium of the reaction toward condensation [9, 16, 25]. However, when the reaction is carried out in an aqueous-organic biphasic system, although the synthetic yield is high, the rate of synthesis is quite low, because the enzyme and hydrophobic substrate exist separately in the aqueous and organic phase, respectively. In order to circumvent this problem, Reslow *et al.* studied thermolysin-catalyzed peptide synthesis in water/acetonitrile mixtures

[19], however, only 80% of the equilibrium yield of dipeptide was obtained, which is unsatisfactory.

Accordingly, to enhance the equilibrium yield of dipeptide in an aqueous organic one-phase system, the possibility of utilizing ethyl acetate as a cosolvent was examined, and the thermolysin-catalyzed rate, equilibrium yield, and various factors affecting the synthesis of N-(benzyloxycarbonyl)-L-phenylalanine-L-leucine ethyl ester (Z-Phe-LeuOEt), which is indispensable for the production of Leu-enkephalin (L-Tyr-Gly-Gly-L-Phe-L-Leu), an opioid peptide, were investigated in a 0.25 M Tris/HCl buffer saturated with ethyl acetate containing 5 mM $CaCl_2$. The overall reaction mechanism can be expressed by equation (1).

$$\text{Z-Phe+LeuOEt} \rightarrow \text{Z-Phe-LeuOEt+H}_2\text{O} \quad (1)$$

The condensation product, Z-Phe-LeuOEt, was a highly insoluble substance with no dissociating side groups in the present homogeneous aqueous-organic solution. Therefore, the reaction described by equation (1) was identified as a product-precipitation system and treated as an irreversible reaction.

MATERIALS AND METHODS

Reagents

The crystalline thermolysin (1× crystallized) was obtained from Sigma (St. Louis, U.S.A.) and used without further purification. The N-(Benzyloxycarbonyl)-L-phenylalanine (Z-Phe), L-leucine ethyl ester (LeuOEt) and N-[3-(2-furyl)acryloyl]-glycyl-L-leucinamide were purchased from Sigma (St. Louis, U.S.A.), and the N-(Benzyloxycarbonyl)-L-phenylalanyl-L-leucine ethyl ester, an objective product in the present study, was obtained from the Protein Research Foundation (Osaka, Japan) and used for calibration. All other reagents were of analytical reagent grade.

Determination of Enzyme Activity

The activity of the enzyme purchased was measured using N-[3-(2-furyl)acryloyl]-glycyl-L-leucinamide as the substrate under pseudo-first-order conditions ($[S] \ll K_m$), as described by Feder *et al.* [3]. The reaction was initiated at 25°C by adding 4.8 μg/ml of thermolysin into 0.1 M Tris buffer, pH 7.2, in which 0.83 mM of the substrate was dissolved, and the thermolysin-catalyzed hydrolysis of furylacryloyl dipeptides was monitored by spectrophotometrically measuring the decrease of absorbance at 345 nm using a Beckman DU-64 spectrophotometer. Using the initial linear time course, the first-order rate constant (k) was obtained, and the molarity of the active enzyme was routinely estimated from the relation of equation (2), derived under pseudo-first-order conditions.

$$k=k_{cat}[E]/K_m \quad (2)$$

The representative value of k_{cat}/K_m, $9.46\times10^3\ M^{-1}sec^{-1}$, was calculated by Feder *et al.* [3] under the same conditions as in the present and this value was then used throughout the experiment.

Enzyme Reaction

The rate of the thermolysin-catalyzed Z-Phe-LeuOEt synthesis was measured at various temperatures and pHs in a 0.25 M Tris/HCl buffer saturated with ethyl acetate containing 5 mM $CaCl_2$. The enzyme concentration in the reaction mixture was 5.0 μM, and the acid component of the substrate, Z-Phe, was used within a range of 1–80 mM, while the amine component, LeuOEt, was maintained at 40 mM. After dissolving the two substrates in 7.5 ml of a 0.25 M Tris/HCl buffer saturated with ethyl acetate, the pH was adjusted to the desired value with 6 M HCl or 5 M NaOH solution. The enzyme was also separately dissolved in 2.5 ml of the same buffer at the prescribed concentration. After mixing these two solutions in a stoppered glass bottle placed on a magnetic stirrer, the reaction was started with vigorous magnetic stirring and the reaction temperature was controlled using a circulating water bath. The initial velocity of the synthetic reaction was determined from the linear part of the time course. In most cases, 0.2 ml of sample aliquots were taken at the appropriate intervals and immediately diluted with 0.8 ml of acetonitrile to stop the reaction. The resulting solution was then analyzed by an HPLC (Dionex 500, U.S.A.) with a C18 column (4.6×300 mm, YMC-H80, U.S.A.). A mixture of acetonitrile/water (70/30 v/v), adjusted to pH 2.5 with phosphoric acid, was used as the elution buffer for the HPLC with a flow rate of 0.5 ml/min.

RESULTS AND DISCUSSION

Effects of Z-Phe and LeuOEt Concentrations on the Initial Rate for Synthesis of Z-Phe-LeuOEt

A number of studies have been performed on the thermolysin-catalyzed synthesis of peptides containing phenylalanine in an aqueous-organic solvent system [1, 6, 9, 12, 15, 25]. Among them, Nakanishi *et al.* [15] noted the inhibitory effect of Z-Phe on the initial rate of Z-Phe-PheOMe synthesis at higher concentrations, however, all other researchers have disregarded this effect. Accordingly, the current study investigated the dependence of the synthetic rate of Z-Phe-LeuOEt on the concentrations of Z-Phe and LeuOEt. Figure 1 shows the effects of Z-Phe and LeuOEt concentrations on the initial rate of synthesis at a fixed LeuOEt (40 mM) or Z-Phe (5 mM) concentration. At lower Z-Phe concentrations, the dependence of the initial rate followed typical Michaelis-Menten behavior. However, at higher Z-Phe concentrations, the inhibitory effect of Z-Phe could not be ignored. On the other hand, the

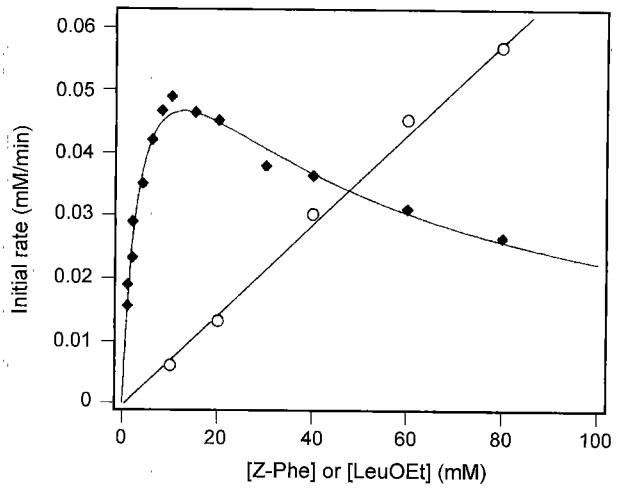

Fig. 1. Effects of Z-Phe and LeuOEt concentrations on initial rate for synthesis of Z-Phe-LeuOEt in 0.25 M Tris/HCl buffer saturated with ethyl acetate, containing 5 mM $CaCl_2$ at pH 7.0, 40°C.

The concentrations of LeuOEt (◆) and Z-Phe (○) were fixed at 40 mM and 5 mM respectively. The solid lines are calculated curves based on equation (3).

dependence of the initial rate on the LeuOEt concentrations seemed to be linear within the concentration range studied, indicating a high K_m value for LeuOEt and no inhibitory effect of LeuOEt. Therefore, if the inhibitory effect of Z-Phe was taken into consideration, the reaction mechanism for the thermolysin-catalyzed synthesis of Z-Phe-LeuOEt from Z-Phe and LeuOEt can be proposed, as shown in Fig. 2. This proposed mechanism is a rapid equilibrium random bireactant system that coincides with Michaelis-Menten kinetics, assuming that Z-Phe has an affinity for both sides of the active site [5, 14, 17]. From the proposed reaction mechanism, the initial rate for the synthesis of Z-Phe-LeuOEt can be expressed by equation (3), when the synergistic factors, β_1 and β_2, are assumed to be 1 for simplicity.

$$V=\frac{d[\text{Z-Phe-LeuOEt}]}{dt}$$

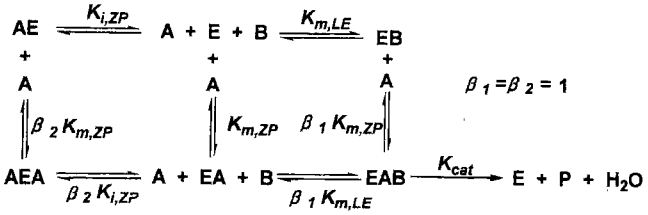

Fig. 2. Proposed reaction mechanism for thermolysin-catalyzed synthesis of Z-Phe-LeuOEt from Z-Phe and LeuOEt.

A: Z-Phe; B: LeuOEt; P: Z-Phe-LeuOEt; E: thermolysin; $K_{m,ZP}$, $K_{m,LE}$: dissociation constant for enzyme-Z-Phe and enzyme-LeuOEt complexes, respectively; $K_{i,ZP}$: inhibitor constant of Z-Phe; β_1, β_2: synergistic factor.

$$=\frac{k_{cat}[E]_0[A][B]}{(K_{m,ZP}+[A])(K_{m,LE}+[B]+K_{m,LE}[A]/K_{i,ZP})} \quad (3)$$

where $K_{m,ZP}$ and $K_{m,LE}$ are the dissociation constants for enzyme-Z-Phe and enzyme-LeuOEt, respectively, A and B signify Z-Phe and LeuOEt, respectively, and $K_{i,ZP}$ is the inhibitor constant of Z-Phe.

The kinetic constants involved in the proposed model were determined numerically by the variable metric method, which is sometimes called the quasi-Newton method [18], and is an optimization algorithm that can identify the minimum of the function of several variables, so as to fit the results calculated by equation (3) with the experimental data. The $K_{m,ZP}$, $K_{m,LE}$, and $K_{i,ZP}$ values were determined to be 4.1 mM, 229 mM, and 39.3 mM, respectively. The k_{cat} value was 105.2 min^{-1}. The $K_{m,ZP}$ and k_{cat} values obtained were similar in magnitude to those reported by Nakanishi *et al.* [15] who observed 6.5 mM and 125 min^{-1} for the $K_{m,ZP}$ and k_{cat} values with a synergistic factor of 1 when the thermolysin-catalyzed synthesis of Z-Phe-PheOMe from Z-Phe and PheOMe was carried out under the same conditions as in the current study. However, the $K_{i,ZP}$ value (39.3 mM) indentified here was smaller than that of Nakanishi *et al.* [15] (300 mM), thereby indicating a greater inhibitory effect of Z-Phe in the present study. From these results, it can be concluded that the inhibitory effect of Z-Phe on the thermolysin-catalyzed synthesis of peptides is influenced by the nature of the amine component. This explanation is also supported by the results of Kunugi *et al.* [12] who demonstrated the effect of the amine component concentration on the peptide condensation reaction.

Combined Effects of Temperature and Z-Phe Concentration on the Initial Rate and Kinetic Constants for Synthesis of Z-Phe-LeuOEt

In order to elucidate the temperature dependence of the kinetic constants, $K_{m,ZP}$, $K_{m,LE}$, $K_{i,ZP}$, and k_{cat}, the combined effects of temperature and the Z-Phe concentration on the initial rates for the synthesis of Z-Phe-LeuOEt in a 0.25 M Tris/HCl buffer saturated with ethyl acetate, pH 7.0, containing 5 mM $CaCl_2$ were investigated at a fixed LeuOEt concentration (40 mM), and the kinetic constants were calculated as mentioned earlier. The results are shown in Figs. 3 and 4. As shown in Fig. 3, the initial rate of Z-Phe-LeuOEt synthesis reached a maximum when the reaction was carried out at 40°C with a ratio of 10 mM Z-Phe to 40 mM LeuOEt. However, although the effect of temperature on the reaction velocity was clear in this result, the temperature dependence of the kinetic constants could not be determined. For example, information on the temperature dependence of the inhibitory effect of Z-Phe on the thermolysin-catalyzed synthesis of Z-Phe-LeuOEt cannot be gained by the result shown in Fig. 3. Therefore, an attempt was made to calculate four kinds of kinetic

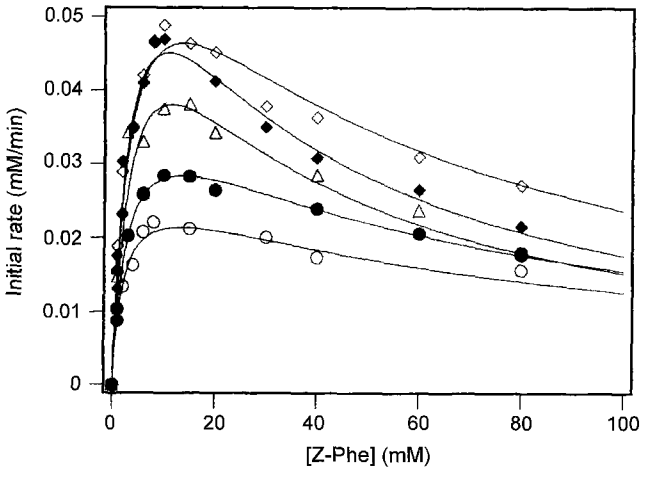

Fig. 3. Combined effects of temperature and Z-Phe concentrations on the initial rate for synthesis of Z-Phe-LeuOEt in 0.25 M Tris/HCl buffer saturated with ethyl acetate, pH 7.0, containing 5 mM $CaCl_2$.

The reaction was carried out within a temperature range of 30–50°C with 40 mM of LeuOEt. ○: 30°C; ●: 35°C; ◇: 40°C; ◆: 45°C; △: 50°C. Symbols are experimental values, and all lines are calculated curves based on equation (3).

constants based on the results of Fig. 3 and equation (3). As shown in Fig. 4, it was observed that each of the kinetic constants was influenced differently by temperature.

The dependence of the k_{cat} values on temperature was a bell-shaped curve within a temperature range of 30°C–55°C, and the temperature optimum was at 45°C (Fig. 4A). In contrast, the dissociation constant, $K_{m,ZP}$, for the thermolysin-Z-Phe complex increased in a linear manner as the temperature increased, indicating that the affinity between them increased with decreasing temperature (Fig. 4B). At the same time, the inhibition effect, $K_{i,ZP}$, of Z-Phe was minimal as the temperature decreased (Fig. 4C), while the dissociation constant for the thermolysin-LeuOEt complex remained apparently unaffected within the temperature range examined (Fig. 4D). From these results, it can be assumed that the optimum temperature of 40°C for the synthetic rate of Z-Phe-LeuOEt is a compromise temperature between k_{cat}, $K_{m,ZP}$ and $K_{i,ZP}$, therefore, there is a possibility that the optimum temperature for the equilibrium yield of Z-Phe-LeuOEt formation is not 40°C, as will be discussed later.

Combined Effects of pH and Z-Phe Concentration on the Initial Rate and Kinetic Constants for Synthesis of Z-Phe-LeuOEt

The pH dependence of the initial rate and kinetic constants of the synthesis of Z-Phe-LeuOEt at 40°C were determined between pH 6.0 and 8.5, and the results are shown in Fig. 5 and Table 1. The optimum pH for the initial rate was around 7 in 0.25 M Tris/HCl buffer saturated with ethyl acetate containing 5 mM $CaCl_2$, the same as that determined for N-(benzyloxycarbonyl)-L-phenylalanyl-L-phenylalanine methyl ester synthesis by Nakanishi *et al.* [15], and N-furylacryloylglycyl-L-leucinamide hydrolysis by Kunugi *et al.* [11], who showed that the k_{cat}/K_m profile could be explained by a simple bell-shaped curve with pk_1=5.0 and pk_2=8.25. In general, when enzyme-catalyzed peptide bond formation and hydrolysis are carried out in a water-organic

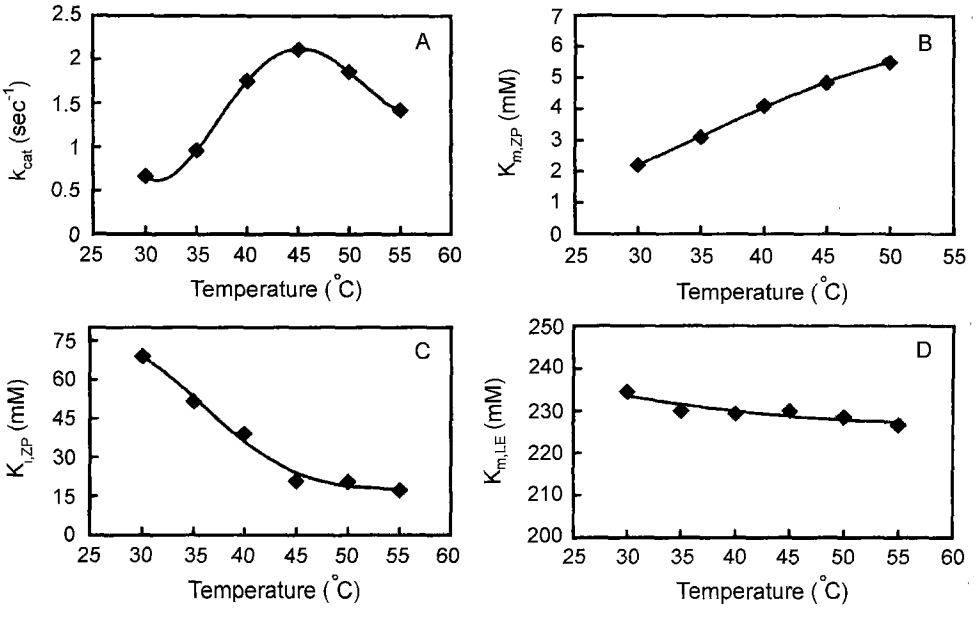

Fig. 4. Effects of temperature on the kinetic constants of thermolysin-catalyzed synthesis of Z-Phe-LeuOEt.

The values of each kinetic constant are calculated from the results of Fig. 3 and equation (3), as described in the text. Panel A, k_{cat}; Panel B, $K_{m,ZP}$; Panel C, $K_{i,ZP}$; Panel D, $K_{m,LE}$.

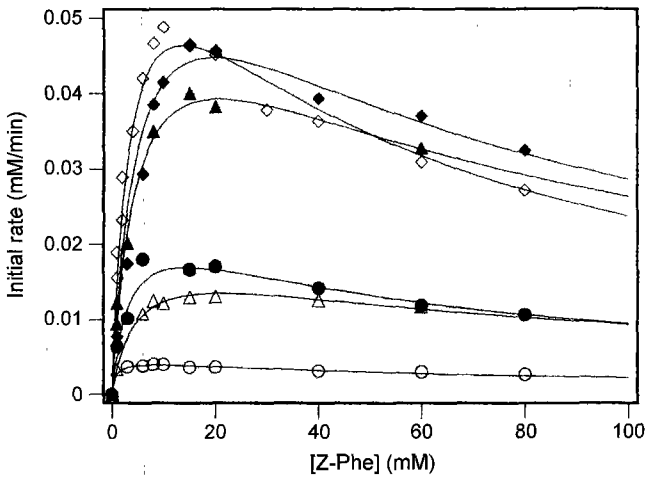

Fig. 5. Combined effects of pH and Z-Phe concentrations on the initial rate for synthesis of Z-Phe-LeuOEt in 0.25 M Tris/HCl buffer saturated with ethyl acetate containing 5 mM $CaCl_2$.
The reaction was carried out within a pH range of 6.0–8.5. ○: pH 6.0; △: pH 6.5; ◇: pH 7.0; ◆: pH 7.5; ▲: pH 8.0; ●: pH 8.5. The symbols are experimental values, and all lines are calculated curves based on equation (3).

solvent system, the added organic cosolvent affects the dissociation constant, not only of the amino acid residues of the active site but also of the amino and carboxyl groups of the two substrates. Moreover, the catalytic process must pass through the same reaction mechanism in both the hydrolysis and synthesis of a peptide bond by a given enzyme. Therefore, from the results of Fig. 5 and the results by two other groups [11, 15], it can be assumed that the present solvent system did not affect the dissociation constants of the reactants.

Table 1 shows the pH dependence of four kinds of kinetic constants calculated from the results of Fig. 5 and equation (3). The optimum pH for k_{cat}, $k_{cat}/K_{m,ZP}$ and $k_{cat}/K_{i,ZP}$ was achieved at 7. In contrast, as the pH decreased, the affinity between thermolysin and Z-Phe increased markedly, and the inhibition effect of Z-Phe decreased. Meanwhile, the affinity between thermolysin and LeuOEt remained unaffected within the pH range examined. From these results, it can be concluded that the protonation state of the carboxyl groups of Z-Phe was more important than that of the amino groups of LeuOEt for the synthesis of Z-Phe-LeuOEt in the present solvent system, and that the pH optimum of 7 was a compromise pH between k_{cat}, $K_{m,ZP}$ and $K_{i,ZP}$.

Courses of Synthesis of Z-Phe-LeuOEt in the Ethyl Acetate Saturated Aqueous System

As noted in Fig. 4 and Table 1, although the inhibitory effect of Z-Phe decreased as the pH and temperature decreased and the affinity between Z-Phe and thermolysin reached a maximum at pH 6.0 and 40°C, the optimum rate for the synthesis of Z-Phe-LeuOEt was obtained at pH 7.0 and 40°C. Moreover, according to recent reports [4, 7, 22, 24], it has been shown that lowering the temperature of the reaction medium results in a decrease in the hydrolysis of the peptide product. Therefore, in order to compare the equilibrium yield of Z-Phe-LeuOEt at pH 7.0 and 40°C and pH 6.0 and 30°C, a catalyzed reaction of 10 μM thermolysin was carried out in 0.25 M Tris/HCl buffer saturated with ethyl acetate containing 5 mM $CaCl_2$, with 20 mM Z-Phe and 40 mM LeuOEt as the substrates. In addition, the equilibrium yield and rate in the ethyl acetate saturated aqueous system and aqueous/organic biphasic system were compared to confirm the difference in each case. The aqueous/organic biphasic system was composed of 0.25 M Tris/HCl buffer, pH 7.0, saturated with ethyl acetate as the aqueous phase, and ethyl acetate saturated with 0.25 M Tris/HCl buffer, pH 7.0, as the organic phase. In this biphasic system, the reaction was started by mixing 5 ml portions of the aqueous and organic phases with the same substrate concentrations and continued with vigorous mixing by magnetic stirring at pH 7.0 and 40°C. At appropriate times, 0.2 ml samples of the organic phase were taken for analysis. The equilibrium state was confirmed when the yield did not change while the reaction continued, and the results are shown in Fig. 6.

As expected, the equilibrium yield in the aqueous/organic biphasic system was higher than that in the ethyl

Table 1. Dependence of the kinetic constants of thermolysin-catalyzed synthesis of Z-Phe-LeuOEt on pH in an ethyl acetate saturated aqueous system.

Kinetic parameters	pH					
	6.0	6.5	7.0	7.5	8.0	8.5
k_{cat} (sec^{-1})	0.11	0.60	1.75	1.65	1.39	0.46
$K_{m,ZP}$ (mM)	0.74	3.60	4.10	5.50	5.19	4.89
$K_{m,LE}$ (mM)	232.0	230.4	229.3	230.0	231.1	229.9
$K_{i,ZP}$ (mM)	76.73	49.19	43.53	59.50	69.38	76.21
$k_{cat}/K_{m,ZP}$ ($sec^{-1} \cdot mM^{-1}$)	0.15	0.17	0.43	0.3	0.27	0.10
$k_{cat}/K_{m,zp}$ ($sec^{-1} \cdot mM^{-1}$)	0.001	0.012	0.045	0.028	0.020	0.006

The reaction was carried out under the same conditions as described in the text, except pH. For pH 6.0 and 6.5, a 0.25 M MES/NaOH buffer was used, and for pH 7.0–8.5, a 0.25 M Tris/HCl buffer was used.

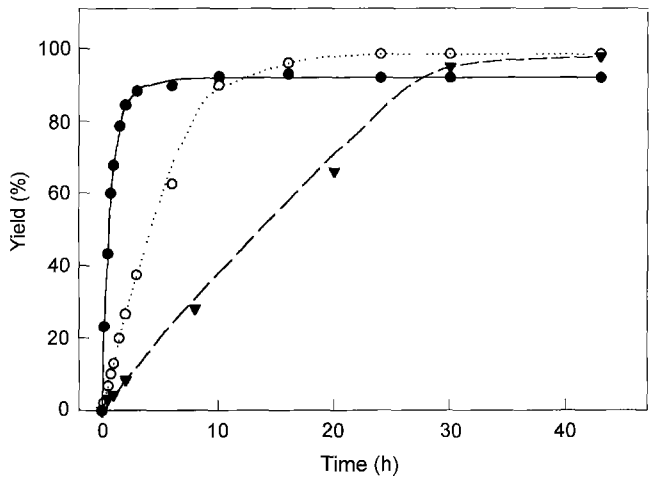

Fig. 6. Courses of synthesis of Z-Phe-LeuOEt at pH 7.0, 40°C (●) and at pH 6.0, 30°C (▲) in an ethyl acetate saturated aqueous system and at pH 7.0, 40°C (○) in an aqueous/organic biphasic system.
The reactions in each case were carried out with 20 mM Z-Phe, 40 mM LeuOEt, and 10 μM thermolysin containing 5 mM $CaCl_2$.

acetate saturated aqueous system, although the rate was much slower. However, it was unexpected to observe that the equilibrium yield of Z-Phe-LeuOEt at pH 6.0 and 30°C was higher than that at pH 7.0 and 40°C, when the synthesis was performed in the ethyl acetate saturated aqueous system. The yield was almost the same as that in the aqueous/organic biphasic system. From these results, it was concluded that the affinity between the enzyme and the substrate, rather than the overall rate, was a more important factor affecting the equilibrium yield when the peptide synthesis was carried out in a product-precipitation system, even though the overall rate was much slower.

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