
Takeshi Inagaki,* Koji Tadasa,† and Hiroshi Kayahara

Department of Bioscience and Biotechnology, Shinshu University, 8304 Minamiminowa, Kamin, Nagano 399-45, Japan

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Thermolysin-catalyzed peptide synthesis using N-(benzylxycarbonyl)-L-phenylalanine (Z-Phe) and L-phenylalanine methyl ester (Phe-OMe) as substrates was done mainly in a water-organic one phase solvent system. The organic solvent content used was less than the saturation concentration in buffer. With organic solvents with high log P values, the enzymatic activity increased as the organic solvent content increased; but further increases in the organic solvent content decreased the enzymatic activity, showing an “organic activity” profile. On the other hand, with organic solvents of low log P values, the enzymatic reaction was inhibited even by the initial addition of organic solvents. When a correlation between maximum activities and log P values or Hildebrand solubility parameters was investigated, a linear correlation was obtained among the same category of organic solvents, but not between categories. This suggests that the direct effect of organic solvents on the microenvironment of the enzyme largely depends on the molecular structure of the solvents.

Enzymatic syntheses in organic solvents have been of great interest. In these syntheses, the choice of an organic solvent as a reaction medium is a key for succeeding in the enzymatic syntheses. At present, it is mostly trial and error to search for this reaction system. Many workers have attempted to correlated enzymatic activities to characteristic parameters of organic solvents.1–4) One of these parameters, log P, is the logarithm of the partition coefficient as a measure for solvent hydrophobicity. The other, the Hildebrand solubility parameter, δ, is described in Materials and Methods as a scale for solvent polarity. The results obtained by many investigators showed that the correlation between enzymatic activities and organic solvents did not depend upon a single parameter. The effects of organic solvents on the enzymatic reaction system have been noted to be two kinds: one is a phenomenon called the indirect effect, that the partitioning of substrates and products, mass transfer, and shifts of chemical equilibria influence the reaction rate in the two-phase system5–7); another is a direct effect, that organic solvent molecules in reaction media directly affect microenvironment of the enzyme, in particular, at the active site.8) These dual effects are different in origin, therefore the effects should be discussed separately. The direct effect represents the genuine relation between the enzyme molecule and the organic solvents, except for problems of the distribution, mass transfer, and chemical equilibria. Water-immiscible solvents were demonstrated to influence the activity of x-chymotrypsin directly with relation to phosphate buffer concentration.9) The binding of organic solvent molecules to the active site was also demonstrated to affect the stereo- and sequence-specificity of x-chymotrypsin.10)

Although it has been suggested that the effects of organic solvents on an active site depended on the molecular structures of the organic solvents instead of their physicochemical constants,9–11) nothing has been reported about the correlation between their molecular structures and enzymatic activities. This study was done to examine the direct effect between the organic solvents including their structures and the enzymatic activities in the thermolysin-catalyzed peptide synthesis of N-(benzylxycarbonyl)-L-phenylalanine-L-phenylalanine methyl ester (Z-Phe-Phe-OMe).

Materials and Methods

Chemicals. Crystalline thermolysin from Bacillus thermoproteolyticus (EC 3.4.24.4) with a specific activity of 8960 PU/mg protein was obtained from Daiwa Kasei K.K. (Osaka, Japan) and was used without further purification. N-(benzylxycarbonyl)-l-phenylalanine (Z-Phe) and l-phenylalanine methyl ester hydrochloride (Phe-OMe·HCl) were prepared in our laboratory by the usual methods using the reagents of benzylxycarbonyl chloride (Z-Cl) (Peptide Institute, Inc., Osaka, Japan) and thionyl chloride (SOCl₂) (Nacalai Tesque, Inc., Kyoto, Japan), respectively. Their identification and purity were checked by melting point measurements and HPLC; they were recrystallized to be homogeneous. Organic solvents were used without further purification. Organic solvents and all other chemicals were of analytical grade and were purchased either from Wako Pure Industries, Ltd. (Osaka, Japan) or from Nacalai Tesque, Inc.

Solubility of organic solvents in buffer solution. For the water-immiscible organic solvents, five ml of the dehydrated solvent was mixed with 20 ml of buffer solution by vigorous magnetic stirring for 10 min. After the two phases were clearly separated at 40°C, a portion of the aqueous phase was withdrawn and analyzed to estimate an exact saturating concentration of organic solvents by gas chromatography (Shimadzu GC-6A, Kyoto, Japan) using a Porapak Type Q (Waters) column (3.0 mm × 1.0 m) and a thermal conductivity detector. Helium was used as the carrier gas at a flow rate of 40 ml/min.

Measurement of enzyme activity. The initial rate of the thermolysin-catalyzed peptide synthesis of Z-Phe-Phe-OMe was measured at 40°C in a 0.25 M tri(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 7.4, containing 5 mM calcium chloride (CaCl₂) with or without

* A student of United Graduate School of Agricultural Sciences, Gifu University.
† To whom all correspondence should be addressed.
addition of solvents and used to calculate the enzyme activity. The reaction was done in a 5-ml bottle with a screw cap. Z-Phe and Phe-OMe·HCl were dissolved in a reaction medium at 10 mM and 40 mM as final concentrations, respectively.

**Enzyme reaction.** After addition of the substrates, the pH of the solution was readjusted to the prescribed value with 4 N NaOH at 40°C. The enzyme, thermolysin, was also separately dissolved in the equivalent medium at 0.033 mM as a final concentration. The reaction was started by adding the enzyme solution. The reaction mixture (1 ml as a total volume) was vigorously stirred with a magnetic stirrer in a recirculating water bath at 40°C. The initial rate was calculated from a linear part of the course of the reaction. After the reaction was stopped by adding 0.04 ml of 5 N hydrochloric acid (HCl), the reaction mixture was appropriately diluted with the HPLC eluent. A portion of this solution (0.002 ml) was used for a high performance liquid chromatography (HPLC) analysis.

**HPLC analysis.** The product was measured by HPLC (Shimadzu LC-9A) using a LiChroPrep 100 RP-18 column (250 x 4 mm, Merck) eluted with water-methanol-perchloric acid (20:80:0.1, v/v). The peak of product was detected with a UV detector (Shimadzu SPD-6A) at 254 nm by comparing with the synthesized standard, and was calibrated with an integrator (Shimadzu C-R6A) in comparison with known concentrations.

**Estimation of Hildebrand solubility parameter.** The values of the solubility parameter, \(\delta\), were estimated from the Hildebrand expression:

\[
\delta = \left[ \rho (\Delta H^v - RT) / M \right]^{0.5}
\]

wherein \(\rho\), specific gravity (g/cm\(^3\)); \(\Delta H^v\), molar heat of vaporization (J/mol); \(R\), gas constant (J/mol·K); \(T\), absolute temperature (K); \(M\), molecular weight (g/mol).

**Results and Discussion**

**Effects of \(n\)-alcohols**

It is known that a small amount of water called “water activity” exerts great influence on the enzymatic activity in organic solvents.\(^{17-19}\) Reslow et al.\(^{20}\) showed that a small amount of water in organic solvent systems influenced the microenvironment of enzyme and that water-miscible solvent molecules (low log \(P\) values) adsorbed in this area, which should be an active site, in place of water, caused the enzymatic activation. Some polar organic solvents (ethylene glycol, glycerol, or formamide) activated the enzyme in organic solvents containing a small amount of water.\(^{21}\) Therefore, these results should support the idea of the substitution of the organic solvents for water at the site of the active center. We have demonstrated a similar phenomenon in the thermolysin-catalyzed peptide synthesis of Z-Phe-Phe-OMe, that a small volume of water-immiscible organic solvents in buffer solutions influenced the enzymatic activity —we tentatively call this phenomenon “organic activity”—. To evaluate the implication of the “organic activity” in the water activity, the relation between the enzyme and organic solvents was investigated. The enzymatic activities in varied organic solvent contents have been measured with various organic solvents having different physical properties such as log \(P\), or the Hildebrand solubility parameter, \(\delta\).

Figure 1 shows the effects of \(n\)-alcohols as organic solvents. With the effects of organic solvents in a buffer, it is usually said that an organic solvent exerts only a direct effect on an enzyme in an organic solvent-saturating buffer system.\(^{10}\) With the alcoholic solvents used, the maximum activities of the enzyme came before the concentration of organic solvents was reached saturation, pH changes in the reaction solutions through the reactions were only ±0.01, when the enzyme activities were evaluated. It is, therefore, supposed that an external parameter such as pH does not affect the enzyme activity in this reaction. All \(n\)-alcohols higher than \(n\)-pentanol had similar tendencies. On the other hand, with lower \(n\)-alcohols than \(n\)-pentanol (\(n\)-butanol and \(n\)-propanol) the enzyme was inhibited even at the initial addition of organic solvents. This result suggests that \(n\)-alcohols inherently have small differences of the effects on the enzymatic activity between the four and five-carbon chains.

**Effects of other organic solvents**

Figures 2–4 show the effects of several structural types of organic solvents having different log \(P\) values. We found two differences in these results in comparison with that of \(n\)-alcohols. First, with methyl \(n\)-alkyl ketones, maximum activities are given over the saturated concentrations (Fig. 3). As mentioned above (Fig. 1), this may be explained by the fact that the maximum activities are not necessarily obtained at the saturated concentration of organic solvents. Organic solvents of lower log \(P\) values (methyl ethyl ketone and acetone) also inhibited the enzymatic activities. Second, with \(n\)-alkyl acetates, methyl acetate (water-miscible), with the lowest log \(P\), activates the enzyme and moreover does not inactivate it to a certain extent at higher contents (Fig.
Fig. 3. Effects of Methyl n-Alkyl Ketones on Activity of Thermolysin.
The concentrations of organic solvents are presented as 100% saturation when the buffers are saturated with each organic solvent: \(\square\), methyl ethyl ketone; \(\odot\), methyl n-propyl ketone; \(\blacklozenge\), methyl n-butyl ketone; \(\varnothing\), methyl n-pentyl ketone; \(\blacktriangle\), methyl n-hexyl ketone; \(\cdots\cdots\), acetone [the dotted line is drawn supposing that the saturating concentration is the same as that of methyl ethyl ketone, 18.6% (v/v)].

Fig. 4. Effects of n-Alkyl Acetates on Activity of Thermolysin.
The concentrations of organic solvents are presented as 100% saturation when the buffers are saturated with each organic solvent: \(\bullet\), ethyl acetate; \(\odot\), n-propyl acetate; \(\varnothing\), n-butyl acetate; \(\square\), n-pentyl acetate; \(\blacklozenge\), n-hexyl acetate; \(\cdots\cdots\), methyl acetate [the dotted line is drawn supposing that the saturating concentration is the same as that of ethyl acetate, 6.6% (v/v)].

4). This obviously differs from the results in other kinds of organic solvents; this result is different from that observed by Piura et al.22)

The results from Figs. 1-4 do not seem to give the linear correlation between the log \(P\) values of the water-immiscible organic solvents and the activation of the enzyme. Then, the relation of the enzymatic activity with log \(P\) values and with Hildebrand solubility parameters, \(\delta\), are illustrated in Fig. 5. Figure 5-a shows that there is no correlation between the log \(P\) values and the maximum activities obtained from Figs. 1-4. Furthermore, the maximum activities was not correlated to the Hildebrand solubility parameters as shown in Fig. 5-b. A linear correlation between the maximum activities and the Hildebrand solubility parameters, however, was demonstrated in the equivalent line of structure of the organic solvents. The straight lines obtained are directed separately, and it seems that they do not correlate each other.

Figure 5 suggests that the molecular structure of organic solvents may be associated with the direct effect on the enzyme in the case of thermolysin-catalyzed peptide synthesis used. In the buffer dissolving organic solvents at lower than saturated concentrations, organic solvents, getting rid of the indirect effect, would exert the effect (direct effect) on the microenvironment of the enzyme, particularly on the active site.

**Equilibrium of organic solvent between in and around enzyme**

Considering the general conception that an active site forms a hydrophobic pocket, the organic solvent molecules
Table Kinetic Parameters for Solvent-free and Solvent-containing (n-pentanol) System in Thermolysin-catalyzed Reaction

<table>
<thead>
<tr>
<th>Organic solvent</th>
<th>$k_m$ (mm)</th>
<th>$k_{cat}$ (min⁻¹)</th>
<th>$k_{cat}/k_m$ (min⁻¹/mm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>39.2</td>
<td>101.3</td>
<td>2.6</td>
</tr>
<tr>
<td>n-Pentanol</td>
<td>6.0</td>
<td>38.5</td>
<td>6.4</td>
</tr>
</tbody>
</table>

Initial concentration of Z-Phe varied from 3.2 to 14.0 mm (six runs); that of Phe-OMe was kept at 40 mm. The concentration of n-pentanol was 0.23 m (saturating concentration). Other experimental conditions see Materials and Methods. The $K_m$ and $k_{cat}$ values were estimated from Lineweaver-Burk plots. Wayne and Fruton gave the kinetic parameters for the thermolysin-catalyzed synthesis from Z-Phe and Phe-OMe. They are: $K_m$, 20 mm; $k_{cat}$, 125 min⁻¹; $k_{cat}/K_m$, 6.25 min⁻¹ mm⁻¹ for Z-Phe. The difference between their and our finding should be caused by the different conditions used.

distributed into the active site could be equilibrated with the bulk phase at the saturating concentration of organic solvents, or the active site could become a microenvironment with organic solvent excess. Because the maximum activities were obtained before reaching saturating concentrations of n-alcohols (Fig. 1) or n-alkanes (Fig. 2), the latter may be right. To inquire into this possibility, we checked the change of activity per enzyme unit (Fig. 6).

The specific activity per enzyme concentration ($v/[E]_o$) converged at lower or higher enzyme concentrations, and kept different fixed values for every organic solvent content between 0.032 mm and 0.052 mm of the enzyme. This seems to indicate that the results of the constant $v/[E]_o$ values in the range of 0.032 mm to 0.052 mm of the enzyme tell that the organic solvent densities at the active site of the enzyme may be similar to those in the bulk water-phase surrounding the enzyme. That is, the excess intrusion of the organic solvent molecules over the equilibrium into the active site would not occur. The convergence of the $v/[E]_o$-values at 0.016 mm and 0.066 mm of the enzyme to the limiting values is caused by the lower initial rates (like an induction phase) at around 0.016 mm and accelerating initial rates (like a logarithmic phase) at around 0.066 mm, respectively. The reason for this is not clear.

Table shows $k_{cat}$ and $K_m$ values measured in the water-organic one phase solvent system (n-pentanol) or in the non-organic solvent system. When the n-pentanol was added to the reaction system, the $k_{cat}/K_m$ increased and the $K_m$ decreased. The organic solvents improve the binding of substrates and accelerate the rate of the reaction. Our conclusion from these results is that the direct effect of organic solvents on the active site of the enzyme would be mainly dependent on the structure of the organic solvent itself.

References