

## Thermolysin Activation by Water-miscible Organic Solvents in Aqueous-organic One-phase Reaction System is not Dominated by Their Physical or Physicochemical Parameters

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**Summary.** Thirteen water-miscible organic solvents, except for alcohols, with infinite solubility in water; and two physical and eighteen physicochemical parameters were adopted for enzymatic peptide synthesis in water-organic one-phase reaction system. Results showed that the activation of thermolysin by water-miscible organic solvents has striking similarity with that of water-immiscible solvents. They exhibited common activation profile and kinetic mechanism. It was demonstrated that activation is not controlled by any of the solvent parameters-suggesting the involvement of specific atoms and/or groups.

**Keywords :** water-miscible solvent ; thermolysin ; physicochemical parameter ; direct effect ; indirect effect

Biocatalysis in organic milieus has been of great interest.<sup>1~4)</sup> Several enzymes have been found to work in nearly anhydrous organic solvents<sup>5,6)</sup>. Until recently, enzyme reactions have been demonstrated in many different solvent systems. It seems a general consensus that high biocatalytic activity in organic milieus is favored in relatively hydrophobic solvents and none or low activity is observed in relatively hydrophilic solvents<sup>7)</sup>. In a more recent report, it has been stated that neither solvent polarity nor water immiscibility by itself is essential for better or optimal enzymic activity<sup>8)</sup>. Therefore, the optimization of biocatalytic process remains a critical problem. Previously, we have reported the *direct effect*<sup>9~11)</sup> of organic solvents in thermolysin-catalyzed peptide synthesis<sup>12,13)</sup>. We mostly used water-immiscible solvents and also found that hydro-

phobic solvents greatly activate thermolysin-catalyzed peptide synthesis and hydrolysis. Recently, we have found that all polar alcohols inhibit thermolysin<sup>14)</sup>. Quantitative correlation between solvent parameters and enzymatic activity in the presence of organic solvents has seen greater attention<sup>15~19)</sup>. Although higher  $\log P^{20)}$  and  $\delta^{21)}$  seem to be correlated with high enzyme activity, a clear-cut correlation between enzymatic activity and solvent parameter has not been attended yet. Recently, we have shown that inhibition of thermolysin by water-miscible organic solvents linearly correlates with most of the solvent parameters<sup>14)</sup>. It was demonstrated that structurally similar monoalcohols and diols show very linear relationship with most physicochemical parameters. A natural question then arises. Does activation by polar solvents also correlates with the solvent parameters? In the present article, we will address this question by utilizing the *direct effect* of water miscible organic solvents in the thermolysin-catalyzed peptide synthesis of Z-Phe-Phe-OMe.

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Received 15 April

Accepted 7 July

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Abbreviations used : Z-Phe-Phe-OMe, N-(benzyloxycarbonyl)-L-Phenylalanyl-L-Phenylalanine Methyl ester.

## Materials and Methods

### Chemicals

Crystalline thermolysin from *Bacillus thermo- proteolyticus* (EC 3.4.24.2) was obtained from Daiwa Kasei K.K. (Osaka, Japan) and was used without further purification. Substrates (Z-Phe and Phe-OMe) were synthesized chemically by conventional method using the reagents of benzyloxycarbonyl chloride (Z-Cl) (Peptide Institute, Inc., Osaka, Japan) and thionyl chloride (SOCl<sub>2</sub>) (Nakalai Tesque, Inc., Kyoto, Japan). Their identification and purity were checked by TLC and HPLC; they were recrystallized to be homogeneous. Organic solvents and all other chemicals were of analytical grade and were purchased either from Wako Pure Industries, Ltd. (Osaka, Japan) or from Nakalai Tesque, Inc. (Kyoto, Japan).

### Organic Solvents

Thirteen water-miscible organic solvents were selected for investigation; acetonitrile; formamide; acetone; tetrahydrofuran; dimethylformamide; pyridine; dioxane; triethylamine; ethylamine; allylamine; 1,3-dioxolane; ethyleneglycoldimethylether and ethyleneglycolmonoacetate.

### Physicochemical Parameters

In total, eighteen physicochemical parameters were chosen. Among them, ten parameters were primarily considered for correlation because they are commonly used for various water-miscible organic solvents. These are;  $\epsilon$  (dielectric constant),  $\delta$  (Hildebrand Solubility parameter), log P (the partition coefficient of substances between water and octanol), D (dipole moment),  $E_T^N$  (empirical parameter of solvent polarity),  $D_N$  (donor number),  $A_N$  (acceptor number),  $\alpha$  (a scale of ability of solvent to donate a proton in a solvent-to-solute hydrogen bond),  $\beta$  (a scale of ability of solvent to accept a proton in a solvent-to-solute hydrogen bond) and  $\pi^*$  (an index of solvent dipolarity) and their values for each solvent are shown in the Table I. Other parameters such as ET, Y, Z, log $k_{ion}$ , F, W, XB, and AN were

not directly correlated with activation because they have properties similar to  $A_N$ . Two physical parameters including molecular weight and size of solvents were also considered. Values of Physicochemical parameters, except for  $D_N$  and  $A_N$ , were taken from literature or text<sup>15,22,23</sup>. Most of the values of  $D_N$  and  $A_N$  were determined in our laboratory following the methods described elsewhere<sup>24</sup>.

### 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 0.25M Tris-HCl buffer, pH 7.4, containing 5 mM calcium chloride (CaCl<sub>2</sub>) with or without addition of solvents and used to calculate the enzyme activity. 0.25M Tris-maleate buffer, pH 7.4 at 40°C, was used for estimation of enzymatic activity in the presence of amines (triethylamine, ethylamine and pyridine) to overcome the pH adjustment problems. The reaction was done in a 5-ml bottle with a screw cap. Z-Phe and Phe-OMe were dissolved in reaction medium at 20 mM and 40 mM as final concentrations, respectively.

### Enzyme reaction

The reaction was carried out following the method described previously<sup>12,13</sup>. After addition of the substrates, the pH of the solution was readjusted to prescribed value with 4 M NaOH at 40°C. The enzyme, thermolysin, was also separately suspended in the equivalent medium at 30  $\mu$ M 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 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 40  $\mu$ l of 5 M HCl the reaction mixture was appropriately diluted with the HPLC eluent. A portion of this solution (2  $\mu$ l) was used for HPLC analysis.

### HPLC analysis

The resulting dipeptide (Z-Phe-Phe-OMe) was analyzed by HPLC using the method described elsewhere<sup>12,15</sup> except for the product obtained in the medium containing pyridine (in that case, the

product was extracted with ethyl acetate from reaction mixture and the solution was dried up in oil bath, then the extract was dissolved in methanol and measured). The peak of product was detected with a UV detector (Shimadzu SPD-6A) at 254 nm by comparing with the synthesized standard.

## Results and Discussion

Solvent effect on enzyme catalysis has been noted to be of two types: the *direct effect*<sup>9-13</sup>) and the *indirect effect*<sup>25-28</sup>). Our interest is focused on the direct effect because the effect is considered to be the direct or genuine relationship between the enzyme and organic solvent molecule in aqueous-organic one-phase reaction system.

Figure 1 depicts the activation profiles of thermolysin by water-miscible organic solvents. It is evident that the activation intensities vary greatly with the types of solvent. The maximal activities in the presence of 1-2 % (v/v) DMF (a), acetone (b), and 1,3-dioxolane (c) in buffer were around 2.5 times, 1.5 times and 1.13 times, respectively, comparing with that in buffer. All other organic solvents showed activation profiles that coincided with any one of the profiles shown in the Fig. 1. The present article is mainly focused on the *direct*

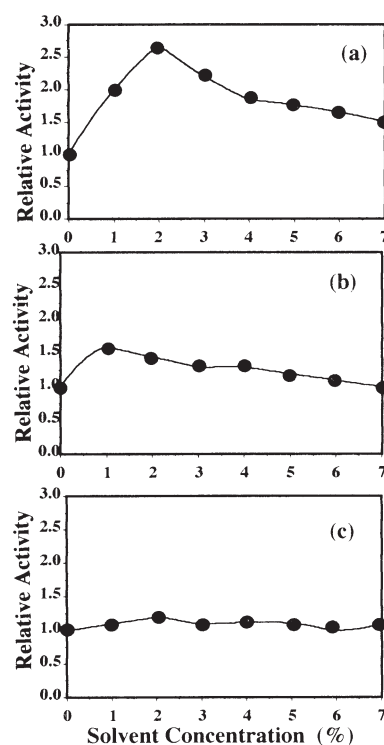


Fig. 1. Effect of Water-miscible Organic Solvents on Activation of Thermolysin.

(a), DMF (b), acetone, (c), 1,3-dioxolane. Concentrations of Z-Phe and Phe-OMe were 10 mM and 20 mM, respectively. Reaction was carried out in Tris-HCl or Tris-maleate buffer pH 7.4 at 40°C. Relative activity 1.0 denotes initial reaction rate in buffer. Each point was repeated five times and then average was made. Further details in the text.

Table I. Physicochemical Parameters of Activating Water-miscible Organic Solvents<sup>a</sup>

Solvent	M.W.	Size <sup>b</sup>	log P	$\epsilon$	$E_T^N$	$\delta$	D	$\alpha$	$\beta$	$\pi^*$	$D_N$	$A_N$
1. Acetonitrile	41.05	1.273	-0.33	36.02	0.460	12.11	3.92	0.19	0.31	0.75	14.1	19.3
2. Formamide	45.04	0.750	-1.61	111.0	0.799	19.40	3.71	0.71	-	0.97	24.0	39.8
3. Ethylamine	45.08	1.464	-0.20	6.94	-	-	1.22	-	-	-	156.8	20.3
4. Allylamine	57.10	1.063	-0.05	-	-	-	-	-	-	-	159.0	20.3
5. Acetone	58.08	1.266	-0.23	20.49	0.355	9.62	2.90	0.08	0.48	0.71	17.0	12.5
6. THF	72.11	1.051	0.49	7.58	0.207	9.52	1.70	0.00	0.55	0.58	20.00	8.00
7. DMF	73.09	1.059	-1.00	36.71	0.404	11.79	3.82	0.00	0.69	0.88	26.6	19.3
8. Dioxolane	74.04	0.943	0.68	-	-	-	-	-	-	-	22.8	16.8
9. Pyridine	79.10	1.018	0.71	12.30	0.302	10.62	2.15	0.00	0.64	0.87	33.1	14.2
10. Dioxane	88.10	0.967	-1.10	2.20	0.164	9.67	-	0.00	0.37	0.55	14.8	10.8
11. EG.Dimet.Ether	90.12	1.151	-0.75	7.20	-	-	-	0.00	0.41	0.53	20.0	12.5
12. Triethylamine	101.1	1.374	1.58	2.42	0.043	-	0.9	0.00	0.71	0.14	61.0	2.30
13. EG.Monoacetate	104.1	0.902	-2.97	-	-	-	-	-	-	-	8.97	33.4
14. Water <sup>c</sup>	18.02	1.00	-1.29	78.54	1.000	23.53	6.47	1.17	0.18	1.09	42.0	54.8

<sup>a</sup>Arranged in the order of molecular weight. When different values were available for a single parameter, the value proposed in the most recent literature was taken. <sup>b</sup>Size/diameter was calculated from the reciprocal values of density ( $den/g\ cm^{-3}$ ). <sup>c</sup>Values for water was mentioned for reference. Further details in text.

effect of organic solvents, which may occur at the lower content of organic solvents in water. It is, therefore, reasonable that the activation exerted upon addition of small volume of miscible solvents in water. The same was the case in water-immiscible organic solvents<sup>12,13</sup>). We, however, observed variation of a published report<sup>29</sup>). Here, we found that thermolysin is activated by classical protein denaturant like formamide.

Previously, we have demonstrated that C<sub>5</sub> or longer alcohols have linear relationships between maximum activity and solvent parameters including log P and  $\delta$  within equivalent solvent structural categories of solvents<sup>12,13</sup>). Recently, we have reported a very linear relationship exists between inhibition by alcohols and most of their physicochemical parameters<sup>14</sup>). It was demonstrated that miscible monoalcohols of C<sub>4</sub> or smaller and diols show direct linearity with most solvent parameters. The data clearly demonstrated that enzymic activity by organic solvents is governed by the structure of solvents. In the present study, we

attempted to correlate 'extent of activation' ( $\mu\text{mol. product/min. mg enzyme at maximal activities exerted by solvents and is denoted as 'activity'}$ ) and solvent parameters. Figure 2 depicts the correlation plots for log P and A<sub>N</sub>. Evidently, no significant correlation exists between activity and these physicochemical parameters. Other physicochemical parameters showed much less correlation. Correlation between enzymic activity and physical parameters of organic solvents has seen very less attention. In this study, we attempted to correlate physical parameters of solvents including molecular weight and solvent size. Size/diameter was calculated from the reciprocal density values ( $\text{den/gm cm}^{-3}$ ; mass per unit volume; as described in the recent Chemical Handbook) of solvents. We focused on the solvent size/diameter because the accessibility of solvents to the microenvironment of active site was of particular interest. Figure 3 shows their relationship with activity. As seen, there exist no significant rela-

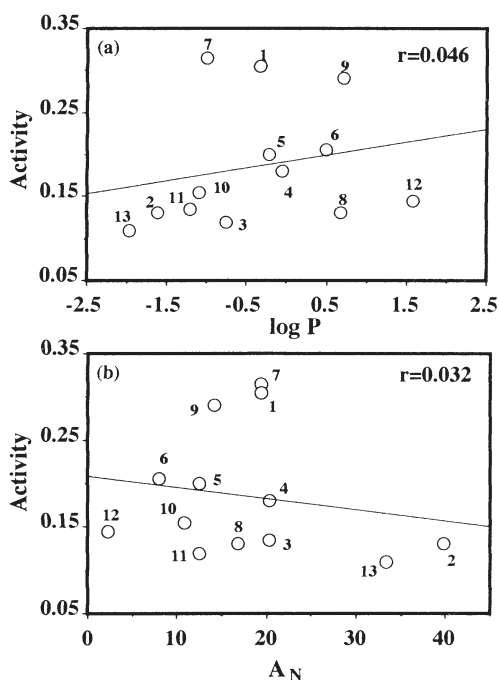


Fig. 2. Correlation Between Activity and Physicochemical Parameters of Water-miscible Organic Solvents.

(a), log P and (b), A<sub>N</sub>. Activity denotes maximum activity exerted by each solvent. Table I depicts the names of organic solvents that correspond to the numbers in this Fig. Further details in text.

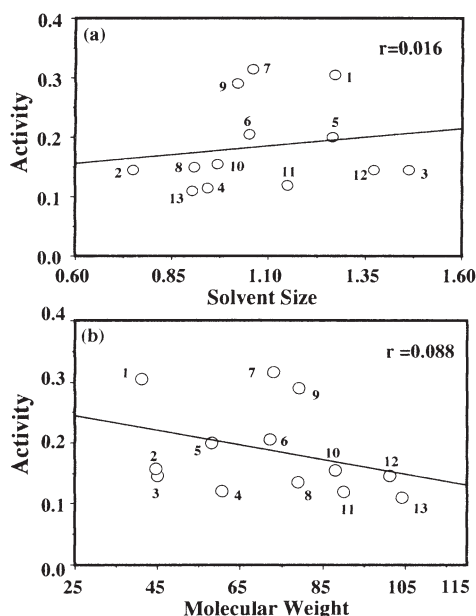


Fig. 3. Correlation Between Activity and Physical Parameters of Water-miscible Organic Solvents.

(a), Solvent size and (b), molecular weight. Activity denotes maximum activity exerted by each solvent. Table I depicts the names of organic solvents that correspond to the numbers in this Fig. Further details in text.

tionship between activity and the physical parameters as well. When the value of water was incorporated in the plots, the correlation coefficients become much less. As demonstrated, the activation of thermolysin by miscible solvents does not seem to be governed by any of their physical or physicochemical parameters. The values of correlation coefficients are depicted in the Table II. The values are very small when all solvents are considered. The reason of such phenomenon is very clear. Water-miscible solvents that were found to activate the thermolysin catalysis in the present study are quite dissimilar in their structures. It deserves to mention that the miscible solvents selected were the maximum suitable ones for the reaction conducted. Conversely, inhibiting miscible alcohols were structurally similar to each other ( $C_4$  or shorter normal alcohols and diols) and resulted very linear correlations<sup>14</sup>. However, we observed an interesting phenomenon in the present investigation. Better correlation coefficients were observed when solvents containing nitrogen were excluded from the plots (Table

II, last entry). One can see that the correlation coefficients become significantly larger upon exclusion of nitrogen containing solvents. It could be predicted that nitrogen containing solvents might have some differences in governing activity than those are not. Therefore, functional groups or atoms in the organic solvents may play an important role in this case.

From the data presented, it was concluded that the general belief of 'hydrophilic solvents-low activity; hydrophobic solvents-high activity' is not always the case. We have demonstrated that acetonitrile, THF and DMF with log P values -0.33, 0.49 and -1.00, respectively, greatly activate thermolysin. And the evidence of no significant correlation between activity and any of the physical or physicochemical parameters, and increase in correlation coefficients upon exclusion of nitrogen containing solvents may indicate the implications of specific atoms and/or groups in this particular case. This needs to be elucidated and awaits further study.

Table II. Correlation Coefficients<sup>a</sup> for Activation vs. Physicochemical Parameters of Different Groups of Activating Organic Solvents

Parameters	Correlation Coefficient (All Solvents)	Correlation Coefficient (All Except Containing 'N') <sup>b</sup>
log P	0.046	0.270
$\epsilon$	0.000	0.267
$E_T^N$	0.004	0.365
$\delta$	0.112	0.660
D	0.254	0.354
$\alpha$	0.144	—
$\beta$	0.149	0.439
$\pi^*$	0.232	0.070
$D_N$	0.027	0.423
$A_N$	0.032	0.439
MW	0.088	0.639
Size	0.016	0.297

<sup>a</sup>Values were obtained from the plots of maximum activities exerted by the organic solvents versus their physical and physicochemical values as shown in Fig. 4 and Fig. 5. <sup>b</sup>Values after solvents containing nitrogen in their structure were excluded. Further details in text.

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## 水溶性有機溶媒による水-有機溶媒単相反応系におけるサーモリシンの活性化はその物理的、物理化学的パラメーターと相関をもたない

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### 要 約

水可溶性有機溶媒によるサーモリシンの活性化は、水不溶性有機溶媒の場合の活性化プロファイルと反応機構の点で非常に類似していた。この実験ではアルコール類を除く13種類の有機溶媒を、水-有機溶媒単相系に適用し、その活性と18種類の物理化学的パラメーター、2種類の物理的パラメーターとの相関を求めた。その結果、活性はどのパラメーターとも相関をもたず、有機溶媒中に含まれる特異的原子または原子団と関係するらしいことが分かった。

**キーワード：**サーモリシン, 物理的パラメーター, 物理化学的パラメーター, 直接効果, 間接効果