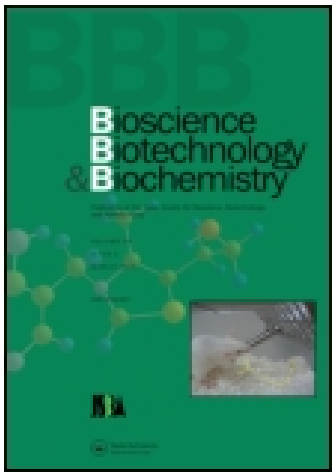

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Effects of Normal and Their Branched Alcohols with Structurally Minimal Variation on Kinetic Parameters in Thermolysin-catalyzed Peptide Hydrolysis and Synthesis of N-(Benzyloxycarbonyl)-L-phenylalanyl-L-phenylalanine and Its Methyl Ester

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Note

Effects of Normal and Their Branched Alcohols with Structurally Minimal Variation on Kinetic Parameters in Thermolysin-catalyzed Peptide Hydrolysis and Synthesis of *N*-(Benzyloxycarbonyl)-L-phenylalanyl-L-phenylalanine and Its Methyl Ester

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When higher alcoholic solvents were added to the reaction medium, and the enhancement of the enzyme activity, followed by its reduction then inactivation, were observed in thermolysin-catalyzed peptide hydrolysis and synthesis. The organic solvent content used was less than the saturating concentration in the buffer (*i.e.*, water-organic one-phase system). The kinetic parameters, K_m and k_{cat}, at the alcoholic concentration giving maximal enzyme activity in these reactions changed linearly with increasing log *P* values of the alcohols and consequently k_{cat}/K_m as well. When the branched isomers of alcohols with structurally minimal variation of which log *P* was equivalent theoretically, were used as annexments, the kinetic parameters were also changed. The results, especially the changes of K_m for each organic solvent, suggested that each alcohol should act at the active site of the enzyme in its own effective mode.

The effects of organic solvents on enzymes has been investigated.[1–5] Chemically there are water-miscible and water-immiscible organic solvents. These two types of organic solvents have been thought to have different modes of effects on enzymes.[6] One of the distinguishable points for the water-immiscible organic solvents is that they generate effects on the distribution, the mass transfer, and the chemical equilibria of the substrates and products.[7–9] These phenomena in the aqueous-organic two-phase systems are termed the indirect effects, in a lump.[6] The direct effects, on the contrary, represent the correlation of the organic solvent molecule with the enzyme molecule (in its active site).[10] The direct effects may be small and neglected in the two-phase systems. These effects, however, will be the genuine relation between the enzyme molecule and the organic solvent molecule during the enzyme reactions, and so are very interesting.[1]

We have reported that in an aqueous-organic one-phase system some water-immiscible organic solvents enhanced the enzyme activity in the thermolysin-catalyzed peptide synthesis of Z-Phe-Phe-OMe from Z-Phe and Phe-OMe.[1] And we concluded that the maximal acceleration of the enzyme activity by the organic solvents depended on the structure of the organic solvents, by use of the physicochemical parameters (log *P*: the logarithm of the partition coefficient defined by the octanol–water system as a measure for solvent hydrophobicity,[11] and δ: Hildebrand solubility parameter as a scale for solvent polarity[12,13]). Accordingly the acceleration did not depend on both log *P* and δ of the organic solvents. Within the equivalent structural category of the organic solvents, however, the activity change can be related with such physicochemical parameters.

In the case of higher alcohols such as *n*-pentanol, *n*-hexanol, *n*-heptanol, and *n*-octanol, the results suggested that the maximal activities of the enzyme were given at the equilibrated concentration of the organic solvents between the active site of the enzyme and the surrounding phase in a one-phase medium. This evidence would manifest the fact that the organic solvent molecules directly interact with the active site of the enzyme molecule. It would be very interesting to understand the relevancy of the active site of the enzyme to the structure of the organic solvent molecule, but it is very difficult to do so now.

This article deals with the kinetical parameters for understanding the relationship of the active site of thermolysin with organic solvent molecules, using higher alcohols as annexments, in the cases of the synthesis and hydrolysis of Z-Phe-Phe-OMe and Z-Phe-Phe-OH.

Crystalline thermolysin from *Bacillus thermoproteolyticus* (EC 3.4.24.4) with a specific activity of 8960 PU/mg protein was used without further purification. Organic solvents and all other chemicals were of analytical grade and were available from Nacalai Tesque, Inc. (Kyoto, Japan) or Wako Pure Industries, Ltd. (Osaka, Japan), and the Peptide Institute, Inc. (Osaka, Japan).

Amino acid and dipeptide derivatives were prepared in our laboratory as shown in ref. 1. Z-Phe-Phe-OH were prepared by the alkali saponification of its methyl ester using 1.2 eq. of 1 N NaOH. The identification and purity of these synthetic substrates were checked by melting point measurements and HPLC; they were recrystallized to be homogeneous. The reason for the difference in substrates that were used for the peptide synthesis and hydrolysis was due to their solubility in the reaction media. Z-Phe-Phe-OMe as a substrate has very low solubility or is insoluble; the solubility was undetectable (<0.02 mM), in the reaction media, so it was impossible to get the measurable activities of the enzyme. Z-Phe-Phe-OH, on the contrary, has a reasonable solubility (<13 mM) in the buffer solution and <45 mM in the buffer saturated with *n*-pentanol, giving appreciable activities of the enzyme.

The initial rates of the thermolysin-catalyzed peptide synthesis and hydrolysis were measured in the aqueous-water-immiscible one-phase system and used to calculate their respective enzyme activities.[1] Z-Phe-Phe-OH was used at 3 mM to obtain the enzyme activities. The enzyme reactions and HPLC analyses were achieved as shown in ref. 1.

The maximal activation of thermolysin-catalyzed synthesis by the C4 to C8 alcohols came before the buffer was saturated with each alcohol (forming a one-phase solution), as described earlier.[1] The kinetic parameters were investigated at each alcohol concentration giving the maximal activation of the enzyme and the data were shown in Fig. 1a. The maximal activities, which are proportional to k_{cat}/K_m, shown in earlier report, have a linear correlation with log *P* of the alcohols. The individual values of k_{kat} and K_m are also proportional to log *P*.

In the hydrolysis, the profiles of the activation and inactivation of thermolysin by the alcohols were almost equivalent, though the activities were smaller on the whole than in the case of the synthesis,

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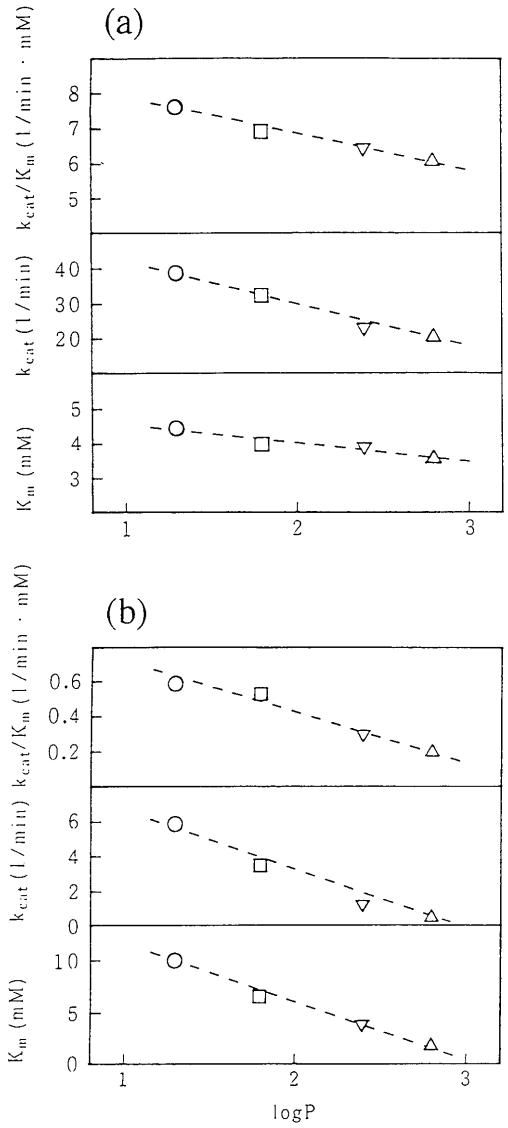

Fig. 1. Relationship of Log *P* for *n*-Alcohols with Kinetic Parameters in Thermolysin-catalyzed Reactions.

The kinetic parameters in thermolysin-catalyzed synthesis (a) and hydrolysis (b) were obtained at each *n*-alcohol concentration when the activities were maximal. The relation between *n*-alcohol concentrations and the activities is shown in ref. 1: ○, *n*-pentanol; □, *n*-hexanol; ▽, *n*-heptanol; △, *n*-octanol.

due to the low solubility. The resulting kinetic parameters at the alcoholic concentrations giving the maximal activities are shown in Fig. 1b. The inclination pattern of each kinetic parameter to log *P* proved to be almost the same as those in the synthesis. These data suggest that the higher alcohols should act in their own interaction modes with the enzyme molecule at the active site, as well as the case of the synthesis.

We showed in our earlier report[1] that the organic solvents that did not belong to the equivalent categories in structure had random activities in regard to the separate log *P*. Here, we tried to learn the relation of the structure with log *P* by investigating the changes of the activities in thermolysin-catalyzed peptide synthesis using the isomers of methyl-butanol as annexments, which have structurally minimal variation. Though these isomers have the same log *P*-values theoretically, the activities of three isomers, 3-methyl-1-butanol, 2-methyl-1-butanol, and 1-methyl-1-butanol (2-pentanol) are lower than those of 4-methyl-1-butanol (*n*-pentanol), and are between *n*-pentanol's and *n*-butanol's activities. The results obtained show that *n*-alcohols are different in structure from non-*n*-isomeric alcohols, accordingly endowing

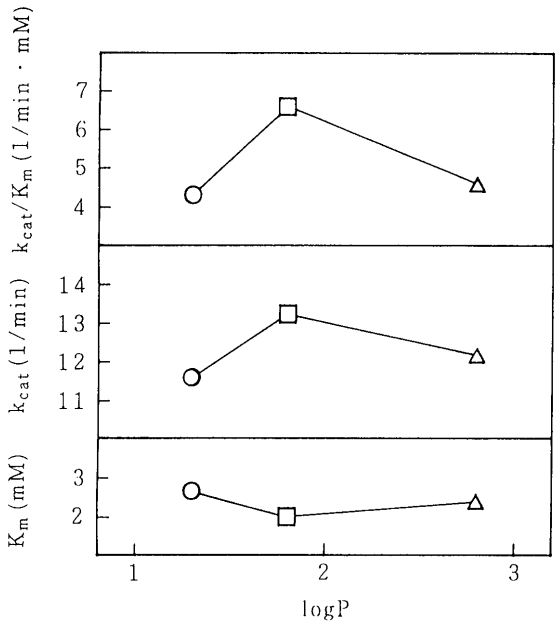

Fig. 2. Relationship of Log *P* for 2-Alcohols with Kinetic Parameters in Thermolysin-catalyzed Synthesis.

The values were measured at each alcoholic concentration when the activities were maximal: ○, 2-pentanol; □, 2-hexanol; △, 2-octanol.

Table Kinetic Parameters for Isomers of methyl-butanol in Thermolysin-catalyzed Peptide Synthesis and Hydrolysis

Organic solvent	Synthesis			Hydrolysis		
	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1} \cdot mM^{-1}$)
n-Pentanol	4.5	34.0	7.6	10.0	5.9	0.59
3-Methyl-1-butanol	3.6	20.2	5.6	9.2	5.1	0.55
2-Methyl-1-butanol	3.1	14.7	4.7	7.5	3.0	0.43
2-Pentanol	2.7	11.6	4.3	5.6	2.7	0.34
n-Butanol	9.0	14.3	1.6	15.4	2.0	0.13
No alcohol[a]	39.2	101.2	2.6	—	—	—

Initial concentration of Z-Phe was varied from 3.2 to 14.0 mM (six data points); that of Phe-OMe was kept at 40 mM. That of Z-Phe-Phe-OH was varied from 1.0 to 10.0 mM. The concentration of isomers of methyl-butanol applied was when the maximal activity was obtained; *n*-butanol concentration was 75% saturation for the synthesis and 50% saturation for the hydrolysis. The K_m and k_{cat} values were estimated with the Lineweaver–Burk plot method. The data of *n*-butanol are for comparison.

[a] The data were quoted from ref. 1.

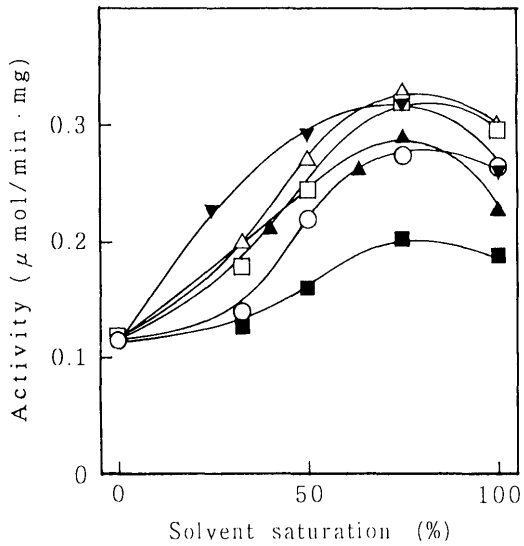

Fig. 3. Effects of Isomers of Methyl-pentanol on Activity in Thermolysin-catalyzed Synthesis.

The concentrations of organic solvents are presented as 100% saturation where the buffers are saturated with each organic solvent: ▲, *n*-hexanol; ○, 4-methyl-1-pentanol; △, 3-methyl-1-pentanol; □, 2-methyl-1-pentanol; ■, 2-hexanol; ▼, *n*-pentanol.

different activities, even though their structures are almost in the equivalent category. Kinetic parameters obtained at the maximal activities for each isomers are shown in the Table. The results shown in Fig. 2 will support the above finding. The values of K_m, k_{kat}, and k_{cat}/K_m for secondary alcohols (2-pentanol, 2-hexanol, and 2-octanol), but not belonging to *n*-alcohols, are given separately and not combined with a linear relation against log *P*. The changes of the activities in the thermolysin-catalyzed peptide hydrolysis using the equivalent isomers were also investigated in the case of the synthesis. A similar pattern of change was also obtained in this case. Kinetic parameters obtained at the maximal activities for each isomer are shown in the Table. Each kinetic parameter is differently derived and had a similar pattern as to the change in the values.

These data support again that each organic solvent would act at the active site of the enzyme in its own effective mode, showing the effect depended on the difference in structure of the organic solvents. To demonstrate these experimental facts, another set of isomers, methyl-pentanol, was accepted as annexments. The results for the synthesis were depicted in Fig. 3. The activities obtained are not in good order, but the separate mode of behavior of the isomers in the active site of the enzyme are suggestive.

The log *P*-values for organic solvents were calculated theoretically from hydrophobic fragmental constants as measured by Rekker.[11] The organic solvents with complicated structures, however, often have with the deviated log *P*-values. We tried to calculate those of the isomers of methyl-butanol experimentally. The values obtained demonstrated that the deviation within log *P*-values for the isomers was small and negligible.

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