Variety of Effect of n-Alcohols on Microenvironment of Some Proteases in Peptide Synthesis or Z (N-Benzyloxycarbonyl) -Amino Acid Ester Hydrolysis.

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Summary

To investigate the interaction of proteases (thermolysin, α-chymotrypsin, papain and pepsin) with organic solvent molecules, buffer systems in which n-alcohols were dissolved as to make a one-phase medium were used in the case of peptide synthesis or Z-amino acid hydrolysis as model reactions. The chain length of n-alcohols gave different characteristics of the effective patterns to the enzymes, and also each protease was affected in different modes of effective profiles by n-alcohols. Activity of α-chymotrypsin was increased by n-alcohols higher than n-hexanol grdually with increase of their concentration just over the saturation to the buffer. The activation was proposed to be non-essential by kinetics. Meanwhile, n-pentanol to α-chymotrypsin showed to be an uncompetitive inhibitor.

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Key words : protease, microenvironment, thermolysin, α-chymotrypsin, papain, pepsin

There have been published a lot of articles concerning effects of organic solvent on enzymes.[1-4] They were aimed at investigating the behavior of organic solvents in buffer, especially to promote enzyme reaction resulting in high yield of reaction products. To achieve those studies, many investigators have tried to correlate potent behavior of organic solvents to their own physicochemical characteristics. The physicochemical parameters taken up were dielectric constant, dipole moment, logP (a measure of hydrophobicity),[5] and Hildebrand solubility parameter (δ ; a scale of polarity).[6,7] They, however, failed to correlate organic solvent behavior to the physicochemical parameters. The failure was ascribed to usage of aqueous-organic two phase media, because such medium-systems brought about two different modes of effect.[8] One of them is an indirect effect, which is dominated by the distribution, the mass transfer, and the chemical equilibirea of substrates and products. The other is a direct effect, which stands for the essential correlation of enzyme molecule, may be at its active site, with organic solvent

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molecule. Judging from the above, we easily understand which is better to elucidate the genuine correlation of enzyme molecule with organic solvent molecule.

Recently we published the articles[9~11] to make clear the correlation of thermolysin and glucosidase with several organic solvents. We achieved the experiments which were designed to know the essential correlation of enzyme molecule to organic solvent molecules, using aquaous-organic one phase media. The results obtained showed positive effects (enzyme acceleration) as follows: (1) the effect depended on the structure of organic solvents and (2) in spite of the above, organic solvents of equivalent line of structure accelerated the enzyme with linear relation against the physicochemical parameters, logP and δ.

This article deals with the correlation of enzyme molecule to organic solvents (n-alcohols) in extended protease -reactions, in which thermolysin, α-chymotrypsin, papain, and pepsin are included.

Materials and Methods

Materials Proteases used and their suppliers were as follows: thermolysin (EC 3. 4. 24. 4) from Daiwa Kasei K.K.; α-chymotrypsin (EC 3. 4. 21. 1) and pepsin (EC 3. 4. 23. 1) from Sigma Chemical Co., Ltd.; papain (EC 3. 4. 22. 1) from Merck Co., Ltd. N-Alcohols were from Nakarai Tesque Inc. or Wako Pure Chemical Industries. Amino acid and other reagents for N- or C- terminal protecting were from Peptide Institute Inc. All these enzymes and solvents were used without further purification.

Preparation of amino acid derivatives Z(benzyloxycarbonyl)-Phe was prepared by a normal chemical method with Z-Cl. Z-Phe $-NH_2$ was synthesized using EEDQ (N-ethoxycarbony1-2-Ethoxy-1, 2-dihydroquinoline) and NH_4HCO_3 as a modification reagent. Removal of the Z-group was curried out with CTH (catalytic transfer hydrogenation) method, then obtained Phe-NH_2. Z-Gly-OMe was synthesized from Z-Gly, methanol and $SOCl_2$ by heating under refluxing.

Enzyme reactions The reactions were carried out at 40°C. The enzyme was dissolved in 0.5ml Tris-malate-NaOH buffer (0.25M), pH7.0, and mixed with the substrate solution of the equivalent buffer in a 5.0ml screw cap container. The intial concentrations were as follows: Z-Phe. 10mM; Phe-NH_2 40mM and Z-Gly-OMe, 10mM. In the case of thermolysin reaction 5.0mM $CaCl_2$ was added and in the papain reaction 1.25μML-Cys and 0.5μM EDTA were added. The final concentrations of enzymes were as follows: thermolysin, 8.0μM; α-chymotrypsin, 200μM; papain, 2.5μM and pepsin, 200μM. The reaction was started by the addition of the enzyme stock solution. The reaction mixture was vigorously stirred with a magnetic stirrer. The initial reaction rates were calculated

for different experiments from a linear part of the progress curve. The reaction was stopped by adding 0.005ml of 6N HCl.

HPLC analysis After the reaction was stopped, 1.5ml of methanol was added to the mixture and supernatant obtained by centrifugation (10,000rpm for 3min.) was used as an analysing sample. HPLC analysis was achieved with LiChrospher RP-18 coulumn (ϕ4.0×69.0mm, Merck) by the use of 65 % (v/v) methanol containing 0.1 % perchloric acid as an eluent at a flow rate of 1.0ml/min. Absorption at 254nm or 210nm was monitored with Shimadzu LC-6A (Shimadzu Co.) and UVIDEC-100V(Japan spectroscopic Co. Ltd.). The products were evaluated by identification of its elution time with those of corresponding compounds chemically synthesized as standards and was calibrated with an integrator (Shimadzu C-R5A) in comparison with known concentrations.

Measurement of apparent kinetic parameters The substrate concentration of Z-Phe was

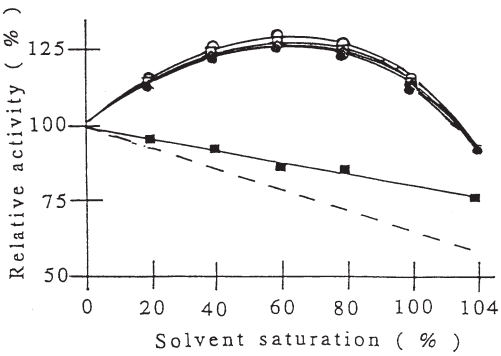


Fig. 1. Effects of n-Alcohols on Activity of Thermolysin.

The concentration of organic solvents are presented as 100% saturation when the buffers are saturated with each organic solvent: ■, n-butanol; ●, n-pentanol; ◇, n-hexanol; △, n-heptanol; □, n-octanol; ○, n-nonanol, ⋯, n-propanol [the dotted line is drawn supposing that the saturating concentration is same as that of n-butanol]. The relative activities were estimated on the basis of the activity without organic solvents under the same conditions including the enzyme concentration, the substrate concentration, the reaction temperature, and the reaction time.

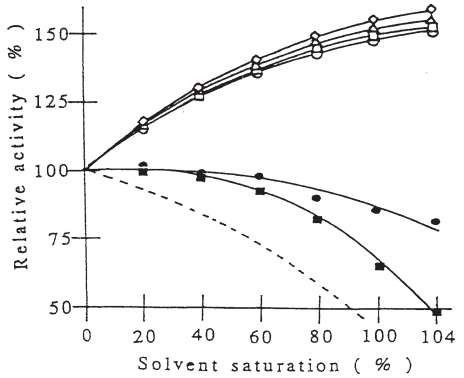


Fig. 2. Effects of n-Alcohols on Activity of α-chymotrypsin.

The concentration of organic solvents are presented as 100% saturation when the buffers are saturated with each organic solvent: ■, n-butanol; ●, n-pentanol; ◇, n-hexanol; △, n-heptanol; □, n-octanol; ○, n-nonanol; ⋯, n-propanol [the dotted line is drawn supposing that the saturating concentration is same as that of n-butanol]. The relative activities were estimated on the basis of the activity without organic solvents under the same conditions including the enzyme concentration, the substrate concentration, the reaction temperature, and the reaction time.

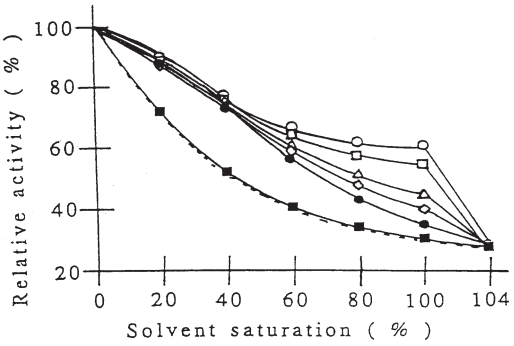


Fig. 3. Effects of n-Alcohols on Hydrolysis Activity of Papain.

The concentration of organic solvents are presented as 100% saturation when the buffers are saturated with each organic solvent : ■, n-butanol ; ●, n-pentanol ; ◇, n-hexanol ; △, n-heptanol ; □, n-octanol ; ○, n-nonanol ; ⋯, n-propanol〔the dotted line is drawn supposing that the saturating concentration is same as that of n-butanol〕. The relative activities were estimated on the basis of the activity without organic solvents under the same conditions including the enzyme concentration, the substrate concentration, the reaction temperature, and the reaction time.

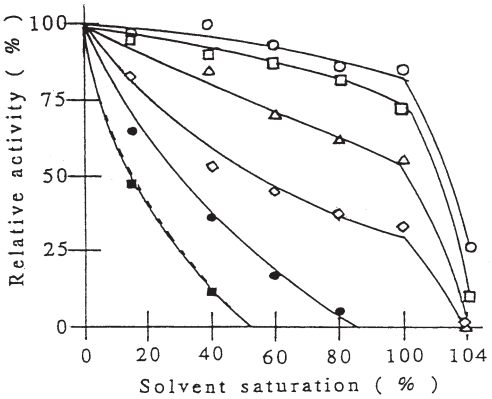


Fig. 4. Effects of n-Alcohols on Activity of Pepsin.

The concentration of organic solvents are presented as 100% saturation when the buffers are saturated with each organic solvent : ■, n-butanol ; ●, n-pentanol ; ◇, n-hexanol ; △, n-heptanol ; □, n-octanol ; ○, n-nonanol ; ⋯, n-propanol〔the dotted line is drawn supposing that the saturating concentration is same as that of n-butanol〕. The relative activities were estimated on the basis of the activity without organic solvents under the same conditions including the enzyme concentration, the substrate concentration, the reaction temperature, and the reaction time.

varied ranging from 5.0mM to 40mM (5～7runs) ; that of Phe-NH_2 was kept at 40mM. The apperent K_m and V_{max} values were estimated from Lineweaver-Burk plot.

Results and Disucussion

Selection of n-alcohols

In the previous paper,[4] we used n-alcohols, n-alkanes, methyl-n-alkyl ketones, and n-alkyl acetates as annexments. Among them, n-alcohols showed clear effective modes for thermolysin. One of effective modes was presented by n-alcohols higher than n-pentanol. They showed an increasing acceleration before the saturation concentration of each alcohol, followed by inhibition to the saturation concentration of alcohols. The lower n-alcohols than n-butanol, however, showed increasing inhibition at a small amount of the annexment concentration. From the results, we selected n-alcohols as annexments.

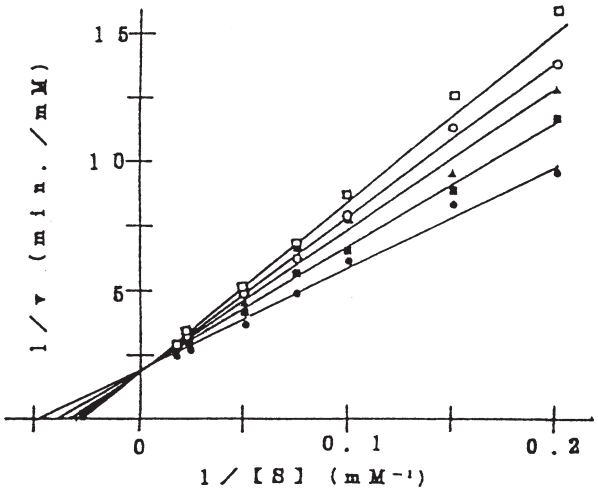


Fig. 5. Lineweaver-Burk Plots for Various n-Hexanol Containing Medium in α-Chymotrypsin-Catalyzed Reaction.

The concentration of n-hexanol which reach the saturated concentration was defined as 100%. The concentrations of added organic solvent were : ●, 0% (buffer only) ; ■, 25% ; ▲, 50% ; □, 75% ; and ○, 100% respectively. Other experimental conditions were described under Material and Method.

Comparison of effective patterns

To compare the effects of n-alcohols on proteases, we selected four enzymes as follows: thermolysin, which is a presentatitive belonging to metal proteases; α-chymotrypsin, which is a presentative belonging to serine proteases; papain, which is a presentative belonging to cysteine proteases; pepsin, which is a presentative belonging to

Table Kinetic Parameters for Solvent-free and Solvent-containing (n-hexanol) Systems in α-Chymotrypsin-Catalyzed Reaction.

Solvent saturation (%)	K_m (mM)	V_{max} (min. xmM)	k_{cat} (min^{-1})	k_{cat}/K_m ($mM^{-1}\times min^{-1}$)
0	32.2	0.56	2.77	8.6×10^{-2}
25	30.7	0.56	2.77	9.2×10^{-2}
50	26.6	0.54	2.77	10.4×10^{-2}
75	22.2	0.54	2.70	12.2×10^{-2}
100	20.0	0.53	2.63	13.2×10^{-2}

The kinetic parameters were calculated from Lineweaver-Burk plots (Fig. 5). Other experimental conditions were contained in the text.

aspartic proteases.

The results for four enzymes are shown in Figs. 1～4. Each protease gave different pattern of effects. Thermolysin has two types of effective modes, which are resemble to the case of the result[8] using Phe-OMe as a basic component, excluding the inhibition by n-pentanol, which acclerated the rate in the case of Phe-OMe. This difference should be caused by the substitution of -OMe for $-NH_2$. This phenomenon must be explained by more additional data.

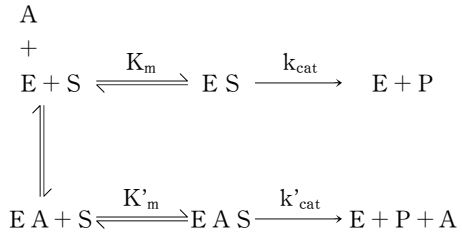
α-Chymotrypsin, on the contrary, accelerated the rate with increasing concentration of n-alcohols till just over the their saturation, exhibiting the same types of effective modes as themolysin. This reason is speculated in the later part.

Papain and pepsin, however, caused no activation both by any n-alcohols and at any concentration of n-alcohols. These results are unexpected, and are not made clear at this point of time.

Activation by α-chymotrypsin

As shown in Fig.2, α-chymotrypsin was activated by higher alcohols than n-hexanol. We tried to demonstrate how the activation occurred in the case of n-hexanol. Fig.5 shows Lineweaver-Burk plot for n-hexanol. This figure shows that n-hexanol activates α-chymotrypsin nonessentially. The reaction proceeds under the conditions of no n-hexanol, and therefore it will be rational that the type of activation is nonessential.

The scheme is shown as fllows:



Enzymatic scheme

Kinetic estimation for activation

The kinetic parameters for the activation by α-chymotrypsin were obtained in Table. From the data, it is understandable that the increasing k_{cat}/K_m with raising n-hexanol concentration is controlled mainly by Km-values. Decreasing Km with rasing n-hexanol concentration stands for the increasing affinity of the enzyme with n-hexanol molecules. This affinity may be caused by appropriately fitted n-hexanol molecules into appropriately hydrophobic pockets in the enzyme active site.

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ペプチド合成またはZ(ベンジルオキシカルボニル)—アミノ酸エステル加水分解における２，３のプロテアーゼの微環境へのn-アルコール効果の多様性

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要　　約

サーモリシン，α—キモトリプシン，パパインおよびペプシンなどの数種のプロテアーゼの微環境と，有機溶媒分子との相互作用を，単相系を形成するような，n-アルコール添加緩衝液系で検討した。反応モデルとしては，ペプチドの合成およびアミノ酸エステルを用いて行った。その結果，有機溶媒の酵素への影響の形式は，n-アルコールの鎖長によって様々であった。さらに各プロテアーゼは独自の影響形式を示した。n-ヘキサノールより大きなアルコールでは，α—キモトリプシンの場合，各アルコールの緩衝液への飽和濃度以上でも，その活性は減少することなく，徐々に上昇した。その場合の活性化形式は，動力的学的にnonessential type とみなすことが出来た。一方，α—キモトリプシンに対してn-ペンタノールは阻害剤として働き，その阻害形式は uncompetitive type であることが分かった。

キーワード：n-アルコール，プロテアーゼ，酵素の微環境，サーモリシン，α—キモトリプシン，パパイン，ペプシン