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Enzyme Reactions in Microstructured Media. Subtilisin Catalysis in Alkyl Glucopyranosides Aggregates

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In order to understand the effect of the structures of the surrounding medium on biocatalytic reactions, hydrolytic catalysis by subtilisin Carlsberg in a ternary system containing octyl β-D-glucopyranoside (β-OG), water, and octanol (OcOH) was studied, with special reference to the enzyme reactivity and the morphology of the surfactant aggregates.

First, the effect of β-OG and other alkyl D-glucopyranosides (AG) on the subtilisin reaction was studied in the surfactant/water binary system; all four surfactants tested were found to inhibit the subtilisin catalysis.

Then, a detailed phase diagram of β-OG/water/octanol was determined and several liquid crystalline phases were found.

When the subtilisin reactions were measured in several compositions in the phase diagram, the maximum of the apparent rate was observed in reversed micells as well as in the reversed hexagonal phase at a certain [water]/[surfactant] ratio (w_o). The apparent optimal w_o was calculated to be around 5—6. These results were compared with the results in another ternary system of Aerosol OT/water/isooctane.

Enzymes are catalytic biomolecules which are generally considered to exert their functions in an isotropic and continuous environment like ordinary aqueous solutions. However, in many cases they are forced to work in a more or less anisotropic and micro-heterogeneous environment, as in biological membranes, which have considerable structural asymmetry or anisotropy. An organelle can be considered to be a highly fabricated microorganization which contains a number of ordered systems of biological factors.

Therefore, in order to construct models of them, we must prepare asymmetric or organized structures in artificial systems.[1] Recently, as one type of microstructured system, the characteristic textures organized by low- or high-molecular surfactants and their relation with the activity of an enzyme, which is placed in these structured environments, has attracted keen attention.[2,3] The study of enzyme reactions in the presence of surfactants has been developed in relation to detergent enzymes, and also as a specific area of enzymatic or biological conversions in organic solvent systems, reverse micells.[2,4,5] Reversed micells are considered to be ternary systems comprising a surfactant, water, and an organic solvent. In some cases they show highly organized structures, such as liquid crystals.[6—8]

Enzymatic reactions occurring in such a structured medium are interesting as model reactions of membrane enzymes or enzymes working in various organelles. In application, it is also interesting how we can control enzymatic reactions through a reorganization of the surrounding medium or structure.

This newer field involving enzyme studies was initiated by a Russian group,[2] followed by French researchers.[3] They are closely related to structural studies on surfactant systems.

We take alkyl D-glucopyranosides (AG; mostly octyl (OG)) as the surfactant. This kind of nonionic surfactant shows a milder effect on proteins, and has been used as solubilization reagents of membrane proteins.[9—12] A study concerning the structure in a binary system containing AG has also been carried out.[13,14]

Since proteolytic reactions in the presence of various surfactants have been intensively studied in relation to the activity and structural stability of enzymes, we started to test the effect of AG on protease in a surfactant/water binary system, and then proceeded to a ternary one. Since in most of the previous studies carried out in this field Aerosol OT was used as the surfactant,[1,4] we compared the result of AG with some results obtained in Aerosol OT/water/isooctane ternary systems.

Experimental

Materials. Alkyl D-glucopyranosides were donated by Nippon Fine Chemical Co. (Osaka, Japan) or purchased from Nacalai Tesque Co. (Kyoto, Japan). Subtilisin Carlsberg was purchased from Sigma (Mo, USA; Lot 53H0085 and 90H0171). Suc–Ala–Ala–Pro–Phe–pNA (SucAAPFpNA) and Cbz–Gly–Gly–Leu–pNA (CbzGGLp-

NA) were purchased from Sigma and the Peptide Institute (Osaka, Japan), respectively. Octanol (OcOH), Aerosol OT (AOT; bis(2-ethylhexyl) sodium sulfosuccinate), isooctane, *N*-(*trans*-cinnamoyl)imidazole, and several buffering reagents were purchased from Sigma or Nacalai Tesque.

Method. The phase diagrams of the ternary systems were drawn mostly as a result of observations with a polarized microscope (Nikon OPTIPHOT-POL)[15] coupled with a titration method. The concentration of subtilisin was determined by a kinetic titration method using *N*-(*trans*-cinnamoyl)imidazole.[16] The catalytic activity of subtilisin, both in binary and ternary systems, was measured against SucAAPFpNA by observing the absorbance increase due to the liberated *p*-nitroaniline using a Shimadzu UV-2200 spectrophotometer under temperature control by circulating thermostated water through observation-cell jackets. In some cases a more hydrophobic substrate (CbzGGLpNA) was used for a comparison.

Results and Discussion

Effect of AG in a Binary System. In a binary system of AG/water, the presence of the surfactant affected the subtilisin reactions as shown in Fig. 1. All four types of alkyl D-glucosides (octyl α-D-glucopyranoside, octyl β-, decyl α-, and decyl α/β- (α/β=5/2)) showed an inhibitory effect. Though a longer alkyl chain showed a stronger effect, the individual inhibitory effect does not seem to be dependent on anomerism, and the apparent inhibition was mostly determined by the solubility of AG; in this sense β-OG has the highest solubility, and, as a result, it inhibited to the greatest extent. This inhibition seemed to be reversible; after prolonged storage in the presence of the surfactant, the activity of the enzyme showed a higher value than the control, due to the lack of autolytic inactivation of the enzyme (Fig. 2).

The inhibition by β-OG was practically independent on the pH (from 4.8 to 9.2: Fig. 3)), and the Dixon plot shown in Fig. 4 indicates competitive inhibition, having a K_i of 7.5 mM (at pH 8.5, 25 °C, M=mol dm^{-3}). Since the critical micellar concentration of β-OG was reported to be as 20 mM,[12] these observations were well below the cmc. However, this single K_i value can apparently reproduce the inhibitory behavior in a binary system well above the cmc, up to 65 %(w/w) of β-OG, as shown in Fig. 4(b).

Phase Diagram of Ternary System. Fig-

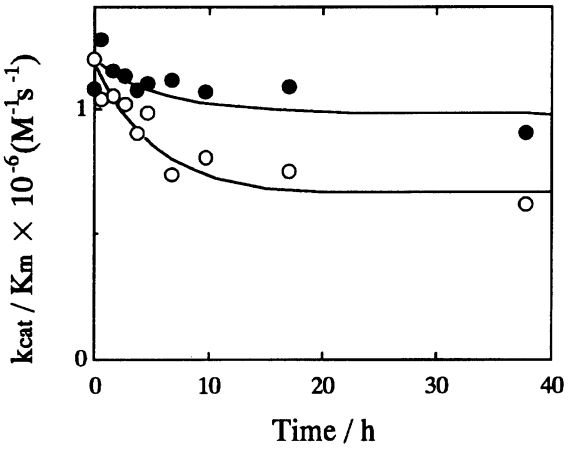

Fig. 2. Effect of β-OG addition on the stability of subtilisin in the stock solution. Incubated at 25 °C, pH 8.5 (0.1 M Taps/NaOH). Activity was measured with SucAAPFpNA and shown in the apparent second-order rate constant. ●, in the presence of 0.17%(w/w) β-OG; ○, control without addition of β-OG.

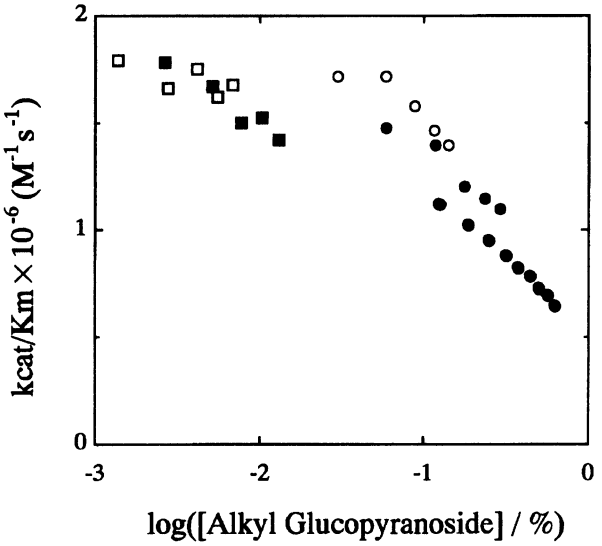

Fig. 1. Effect of AG addition on subtilisin hydrolysis of SucAAPFpNA. Activity is in the apparent second order rate constant. 30 °C, pH 8.5 (0.1 M Taps/NaOH). [S] =21 μM, [E] =39—46 nM. ○, α-OG, ●, β-OG; □, α-DG; ■, α/β-DG.

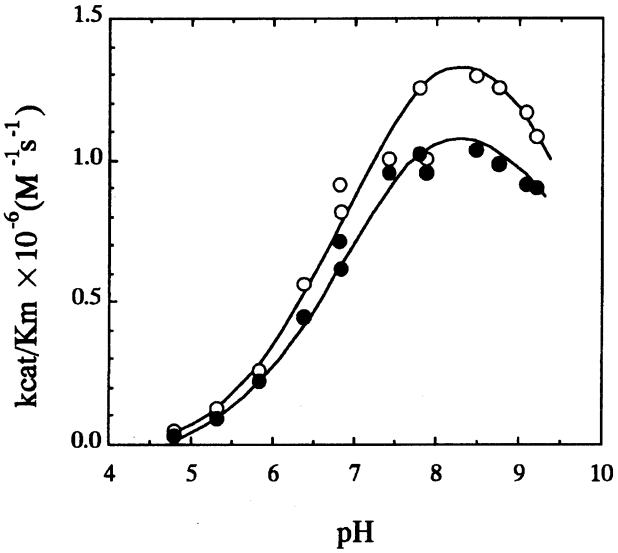

Fig. 3. pH dependence of the inhibitory effect of β-OG on subtilisin. ●, values obtained in the presence of 0.16%(w/w) β-OG; ○, control without addition of β-OG. [E] =44 nM. [S] =23 μM. 25 °C. Mes, Hepes, and Taps were used.

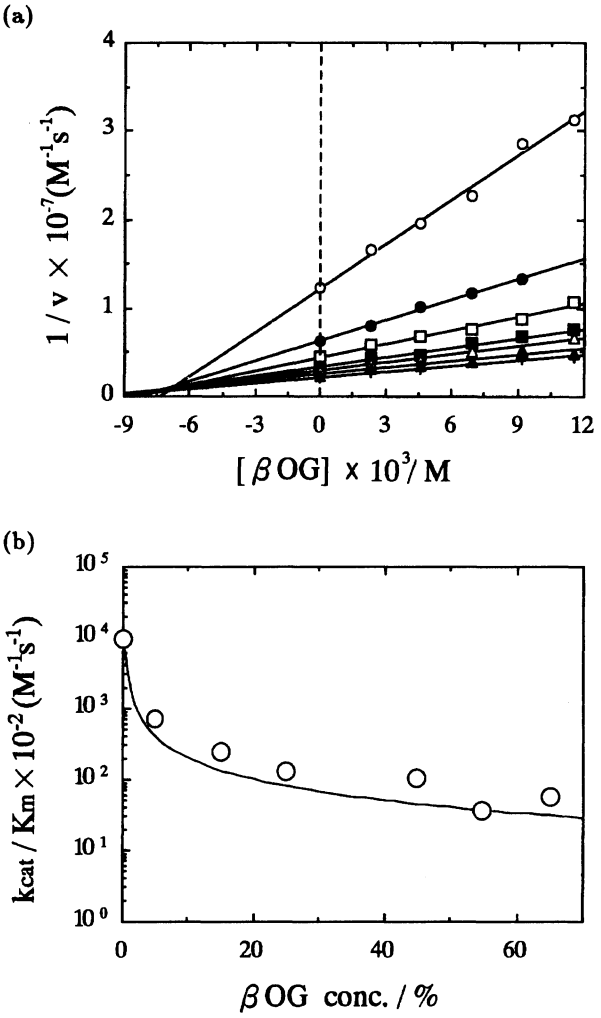

Fig. 4. Dixon plot for the inhibition by β-OG (a) and the inhibitory profile at higher concentrations of β-OG (b). (a) 25 °C, pH 8.5 (0.1 M Taps/NaOH). [S] =9.90 μM (○), 1.98 μM (●), 2.96 μM (□), 3.95 μM (■), 4.94 μM (△), 5.92 μM (▲), and 6.91 μM (+). The intersection in the 2nd quadrant indicates a competitive mechanism and the ordinate shows K_i =7.5 mM. (b) [E] =0.115 μM, [S] =21 μM. Solid curve was calculated with the K_i value of 7.5 mM.

ure 5 is a phase diagram of the β-OG/water/OcOH ternary system. It also shows the case for the β-OG/buffer (0.1 M Tris-HCl)/OcOH. There are some influences of the presence of a buffering reagent; those obtained for an aqueous mixture containing the enzyme or the substrate were practically equivalent to the latter. These diagrams are fundamentally in accordance with a rougher one once reported by Chopineau et al.[3] In our observation, however, the reversed micell (L_2) was more extended, and much more complicated liquid crystalline states were detected. Most of the liquid crystalline states generally described for a micellar ternary system[6—8] were found. Along the line of around 60% of β-OG, the water/β-OG system exhibits a normal hexagonal (E); an increase in the octanol content moves it to lamellar (D) and then further increases to reversed hexagonal (F). At around 70% of β-OG, near or on the water side, the anisotropy disappeared and the phase was assigned to normal cubic, according to the conclusion by Chung and Jeffrey.[14]

Subtilisin Reaction in Ternary System. In this ternary system, the relative activity of the enzyme was highly affected by the size and form of each phase. Along six lines drawn on the phase diagram (α–α to ζ–ζ) in Fig. 5, the reaction rate was measured for the hydrolysis of SucAAPFpNA (Eq. 1),

$$\text{SucAAPFpNA} + \text{H}_2\text{O} \underset{\text{(Subtilisin)}}{\rightarrow} \text{SucAAPF} + \text{pNA} \qquad (1)$$

The obtained apparent second-order rate constants ($M^{-1}\,s^{-1}$) of this reaction are given in Figs. 6 and 7.

In the L_2 phase, a certain maximum of the rate was observed, and the optimum size of the reversed micell or the optimum ratio of $[H_2O]/[\beta\text{-OG}]$ (mol/mol;$=w_o$) was considered. The activity contour in the L_2 phase was drawn using an interpolation method, as shown in Fig. 8. Such a maximum rate was also observed in the reversed cylindrical diameters (F; Fig. 7(b)), though the rate constant is fairly smaller. In all of these cases the optimum $[H_2O]/[\beta\text{-OG}]$ was calculated to be around 5—6. On the contrary, subtilisin hydrolyzed the hydrophobic substrate (CbzGGLpNA) very slowly in the L_2 phase, and no apparent maximum was observed for this substrate (Fig. 9). These facts indicate that CbzGGLpNA is located in either the organic phase or in the depth of the surfactant layer, while SucAAPFpNA is distributed in the aqueous area or around its boundaries. The Michaelis–Menten analysis for SucAAPFpNA at points A and B of Fig. 8 gave the following kinetic parameters (Table 1): A (OG/water/OcOH =33.1/5.5/61.4), k_{cat}= 0.17 s^{-1}, K_m=0.29 mM, k_{cat}/K_m=5.9×10^2 $M^{-1}\,s^{-1}$; B (OG/water/OcOH=31.5/10.1/58.4), k_{cat}=1.7 s^{-1}, K_m=0.21 mM,k_{cat}/K_m =8.4×10^3 $M^{-1}\,s^{-1}$. These are compared with the values in an aqueous medium (0/100/0): 410 s^{-1}, 0.23 mM, and 1.8×10^6 $M^{-1}\,s^{-1}$, respectively (at 30 °C, pH 8.0, 0.1 M Tris-HCl). Certainly, the k_{cat} parameter was found to be highly perturbed. Since the concentration of the substrate is described in terms of the total volume, the possible change in the actual concentration in the water pool is to be counterbalanced by some factors involved in the evaluation of the apparent K_m to give a rather constant value. We did not observe any indications of so-called "super-activation" in either the L_2 or F phase.

In other liquid crystalline states, the enzyme reaction became very slow; in some cases, however, a phase transition gave an apparent discontinuous change in the rate. In the present case of the peptide hydrolytic reaction, both the extent of the reaction and the substrate concentration were so low that they showed no apparent effect on the phase transition during the reaction

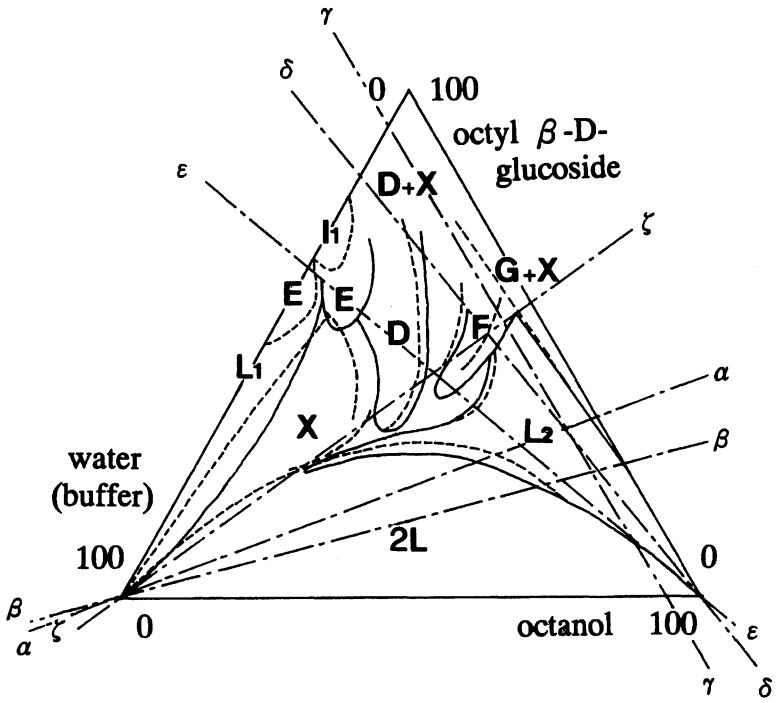

Fig. 5. Phase diagram obtained for β-OG/water or buffer/OcOH. Dotted boundaries are those for water and solid ones for buffer (0.1 M Tris/HCl). At 30 °C. L_1, normal micell; L_2 reversed micell; E, normal hexagonal; F, reversed hexagonal; D, lamellar; I_1, normal cubic; 2L, octanol/water (macro)emulsion; G, surfactant crystal; X, non-determined structure.

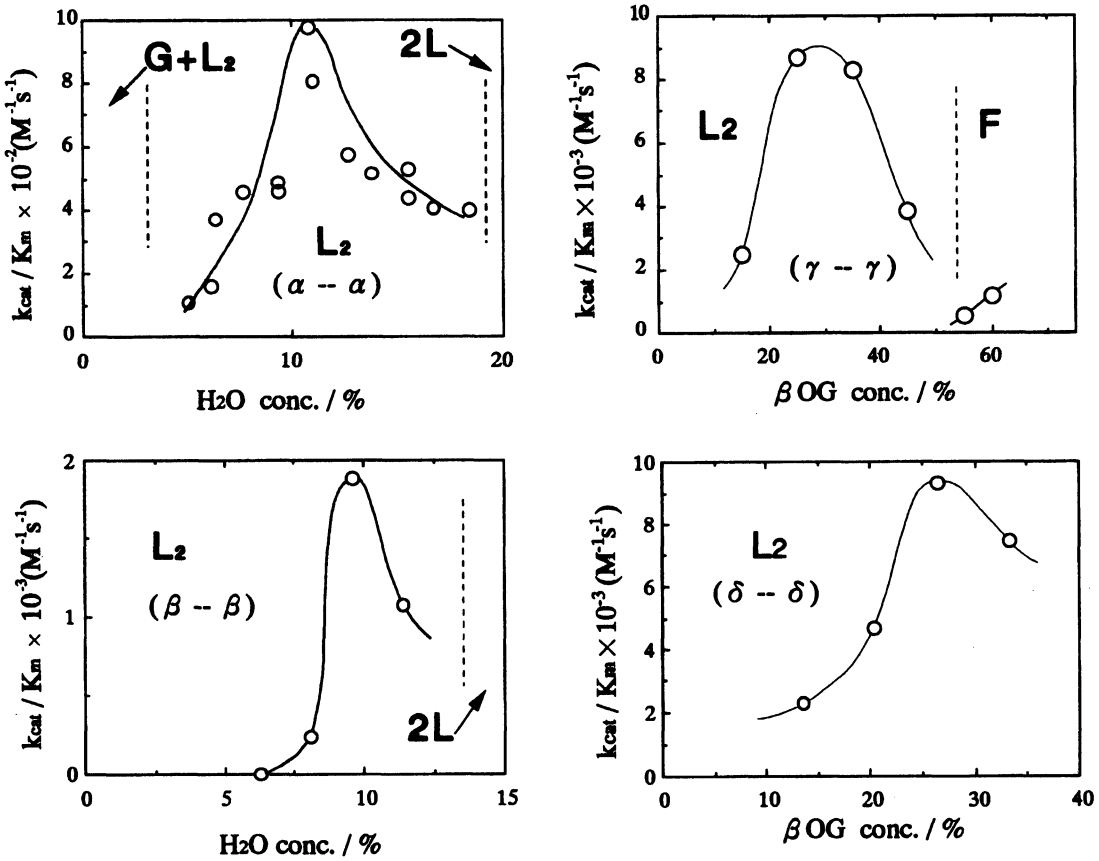

Fig. 6. Apparent subtilisin activity in various compositions of the medium along four lines (α–α, β–β, γ–γ, and δ–δ) in Fig. 5. 30 °C, pH 8.0 (0.1 M Tris/HCl). [E] =0.17—0.31 μM. [S] =20—33 μM. [β-OG]/[OcOH] =0.53 (w/w) for α–α, and 0.33 for β–β. [water] =8.0%(w/w) for γ–γ and [β-OG]/[water] =5.0 (w/w) for δ–δ.

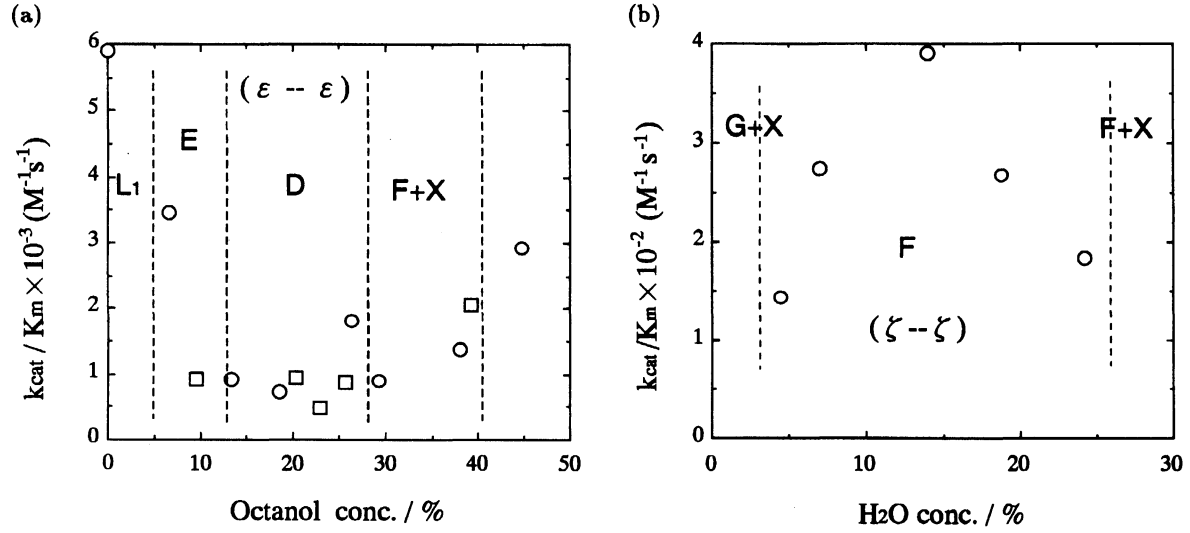

Fig. 7. Apparent subtilisin activity in various compositions of the medium along two lines (ε–ε, and ζ–ζ) in Fig. 5. 30 °C, pH 8.0 (0.1 M Tris/HCl). [E] =0.20—0.33 μM. [S] =22—23 μM. [β-OG]/[water] =1.85 (w/w) for ε–ε and [OcOH]/[β-OG] =1.5 for ζ–ζ.

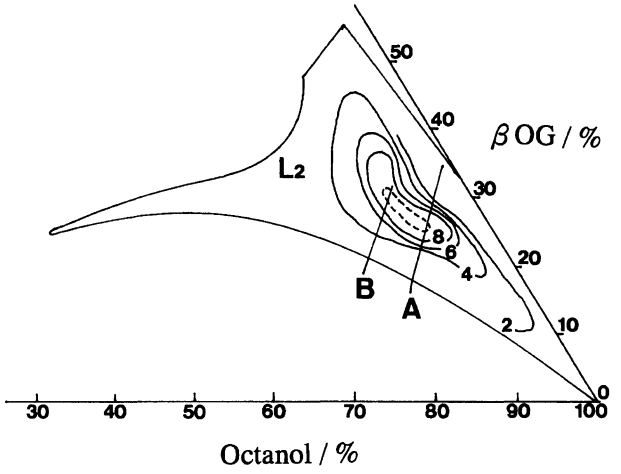

Fig. 8. Activity contour in L_2 phase drawn by interpolation method from the data in Figs. 6 and 7. Numbers are in $\times 10^{-3}$ $M^{-1}s^{-1}$. For A and B. see text.

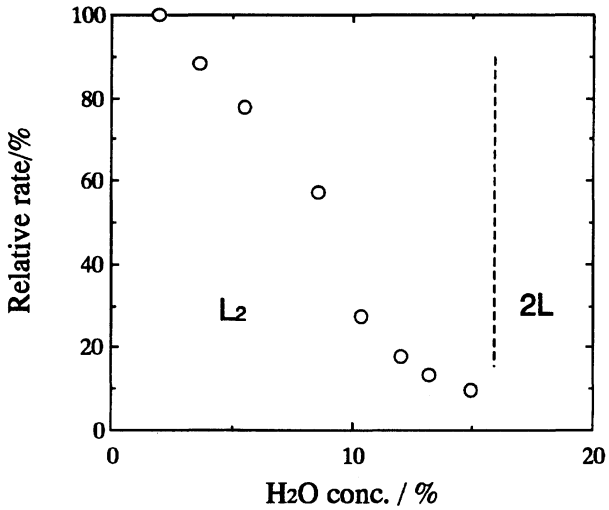

Fig. 9. Relative rate of CbzGGLpNA hydrolysis in L_2 phase catalyzed by subtilisin. 30 °C, pH 8.0 (0.1 M Tris/HCl). [E] =0.37 μM, [S] =56 μM. Composition changed along the line of β–β. The rate is described relative to the value at water concn =2.0% (w/w).

process. However, the consumption of water by a hydrolytic reaction or its production by a condensation reaction with a higher substrate concentration (% level) can cause a phase transition due to progress of the reaction, and could be a self-controlling reaction system. This kind of idea was proposed by Chopineau et al.,[3] in whose case the enzymatic conversion of β-OG to octanol and glucose was the motive force. Furthermore, by providing an external field or force to the system, the enzymatic reaction could be controlled through morphological changes in the liquid crystalline phase. For that purpose, further studies on the phase behavior of various systems are to be performed.

Comparison with the AOT System. So far, certain numbers of studies concerning micellar enzymology have been carried out in systems containing AOT. We thus performed a comparative study in an AOT/water-(buffer)/isooctane system. Figure 10 shows a phase di-

Table 1. Kinetic Parameters at the Medium Composition of A and B in Fig. 8 and in Buffer (O)[a)]

	Composition	k_{cat}	K_m	k_{cat}/K_m
	water/OG/OcOH	s^{-1}	mM	$M^{-1}s^{-1}$
A	5.5/33.1/61.4	0.17	0.29	5.9×10^2
B	10.1/31.5/58.4	1.7	0.21	8.4×10^3
O	100 / 0 / 0	410	0.23	1.8×10^6

a) 30 °C, pH 8.0, 0.1 M Tris-HCl.

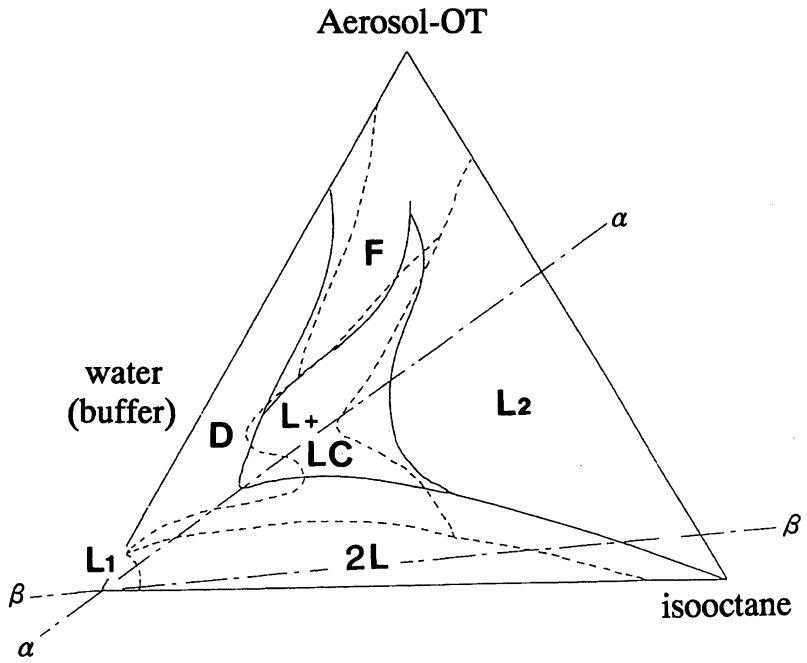

Fig. 10. Phase diagram for AOT/water or buffer/isooctane. Dotted boundaries are for water and taken from Ref. 17. Solid ones are for buffer (0.1 M Tris/HCl) and measured in this study. Symbols are as in Fig. 5 except for L+LC, liquid/liquid crystal mixture, and 2L, isooctane/water emulsion.

agram of this system. Our observation of the buffer system is compared with the reported water system.[17]

In the L_2 phase, the subtilisin reaction against SucAAPFpNA was measured along the two lines in the phase diagram. The result is shown in Fig. 11 in the form of the apparent second-order rate constant against $[H_2O]/[AOT]$ (w_o). In this case an apparent maximum in the rate was also observed, though no super-activation was detected; the optimal w_o was 20 or 30, considerably higher than in the case of β-OG. AOT also showed an inhibitory action towards subtilisin; the measured K_i was 0.37 mM (0.1 M Tris-HCl pH 8, 25 °C, against SucAAPFpNA), 20-times stronger than that of β-OG. In spite of this, the apparent rate constant observed in AOT reversed micell was fairly larger than that in β-OG, especially in a highly organic medium (β–β). This might be related to the lower solubility and CMC of AOT in water (ca. 0.6 and 0.05%, respectively) and with the larger size of the water pool of an AOT reversed micell. The latter results from the anionic nature of the hydrophilic group of AOT and the fact that the ionic solvation requires a greater number of water molecules per surfactan.

Several explanations have been proposed concerning the apparent optimal w_o for enzyme reactions in reversed micells.[18—23] Some of them quote the inter-micellar diffusion or exchange of the substrate being the key process; others postulate the optimal size in the micell or the domain of the enzyme reaction being crucial. Both of the present two surfactants offer further complicated factors of molecular inhibition of the enzyme by the surfactant. The apparent activity is the

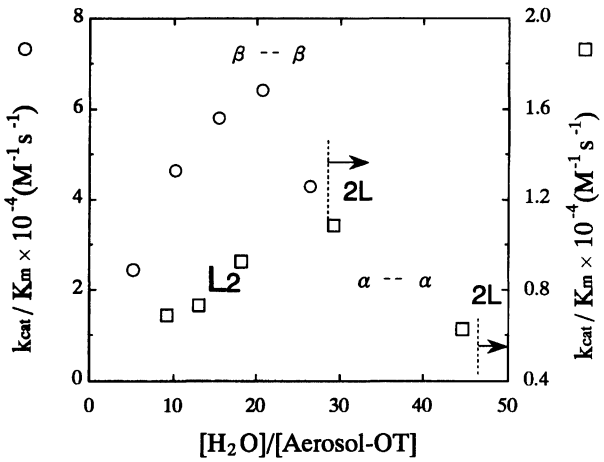

Fig. 11. Apparent subtilisin activity in L_2 phase of AOT/water/isooctane measured with SucAAPFpNA. 25 °C, pH 8.0 (0.1 M Tris/HCl), [E] =0.34 μM, [S] =15 μM. □, for [AOT]/[isooctane]=0.67 (w/w); line α–α. ○, for [AOT]/[isooctane]=0.03 (w/w); line β–β.

total sum or the product of diffusion (exchange), distribution of the enzyme, the substrate, and also the surfactant (in the neighbor of the enzyme). Since we observed an apparent maximum in the F phase of the β-OG system, some diffusion or exchange mechanisms will be favored to explain this. However, the strong effect on k_{cat} and the smaller effect on the K_m parameters might suggest that some changes in the enzyme hydration/solvation or protein structure take place in part. Such an interpretation could occur, especially in

the case of AG, since they form a smaller sized water pool, and the nonionic or hydrogen bonding nature of the head group might facilitate a direct interaction with the enzyme proteins. Our recent study showed that β-OG also exhibits inhibitory action on thermolysin, but not on trypsin, chymotrypsin, or carboxypeptidase Y. By surveying the reaction of these enzymes in ternary systems containing β-OG, further information concerning the mechanisms of enzyme reactions in reversed micells and surfactant aggregates will be obtained.

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