Fluorescence Spectroscopic Study of α-Chymotrypsin as Relevant to Catalytic Activity in Aqueous–Organic Media[1)]

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The effects of the composition of aqueous–organic mixed solvents on steady state fluorescence emission of α-chymotrypsin were investigated. In all the solvent systems, maximum wavelength of fluorescence emission shifted initially to higher wavelength and then to lower wavelength by increasing water content in organic solvents. The results were interpreted in terms of the changes in microenvironment of tryptophan residues in the enzyme due to conformational modification of the enzyme. The maximum emission wavelength of chymotrypsin is well correlated to its catalytic activity for the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester. Activity of chymotrypsin decreased or it was totally inhibited at solvent compositions which give long emission wavelength of the enzyme. The results suggest that the fluorescence spectroscopy may be useful for detection of conformational changes and alteration of catalytic activity of the enzyme.

Enzymes have been widely used as catalysts in organic synthesis and asymmetric transformations of chiral compounds.[2—5)] It has been recognized that enzymes work not only in aqueous solutions but also in many organic solvents.[6—8)] Especially the ability of proteases to catalyze the synthetic reactions of esters and peptides, which are the reverse reactions of the hydrolysis of these compounds, in media composed mainly of organic solvents have been well documented.[9—11)] In these reactions catalytic activities of enzymes are strongly affected by the nature and composition of solvents. Furthermore, substrate specificity and enantiospecificity of the enzymes are often altered by changing the solvents.[12—14)]

These results suggest that enzyme structures are profoundly influenced by the molecular environment. Organic solvents may perturb the native structure of the enzymes and modify their activity and specificities. Also, numerous studies have reported that enzymes are deactivated in organic solvents probably due to unfolding of the peptide chains; typical examples are deactivation of proteases in alcohols and polar aprotic organic solvents.[15—17)]

Most of the studies on enzyme functions in nonaqueous media have been based on the kinetic measurements. Most of enzymes are insoluble in media with high concentrations of organic solvents. Therefore spectroscopic studies such as NMR and CD (circular dichroism), which are often employed for aqueous solutions, are not applicable to the studies of enzymes in these media. In the present study we investigated the changes in fluorescence properties of α-chymotrypsin (CT) in several aqueous–organic mixed solvents in order to detect the structural changes in the enzyme. Since the dissolution of an enzyme in a solvent is not a prerequisite for measuring fluorescence spectra,[18)] the emission spectra of the tryptophan residues in CT would give some information on the structural changes in wide range of the solvent compositions. The results are discussed in relevant to the effects of solvent composition on the catalytic activity of CT.

Experimental

Materials. α-Chymotrypsin (CT) having a specific activity of 46 units (μmol min^{-1} mg^{-1}) at pH 7.8 and 25°C for hydrolysis of *N*-benzoyl-L-tyrosine ethyl ester was a product of Sigma Chemical Co. *N*-Acetyl-L-tyrosine ethyl ester (ATEE) and *N*-acetyl-L-tryptophan ethyl ester were also products of Sigma. Organic solvents were guaranteed grade and purchased from Wako Pure Chemical Co. The solvents were dried on 3A molecular sieves.

Measurement of Fluorescence Spectra. Steady state fluorescence spectra of CT and *N*-acetyl-L-tryptophan ethyl ester in aqueous–organic solvents were taken with Shimadzu RF-5000 instrument with excitation wavelength of 295 nm and band width of 5 nm. The samples contained 2.5 mg CT in 10 ml solvents and were magnetically stirred in a temperature-controlled (30°C) cell. Maximum emission wavelength of a sample was determined as average of more than four measurements.

Kinetic Measurement. A solution of ATEE in a mixture of organic solvent and water was added to an aqueous solution of CT. Pure water was used instead of buffer solutions throughout the experiment in order to avoid the precipitation of the buffer components in solutions of high concentrations of organic solvent. The mixture was incubated at 30°C with reciprocal shaking (about 150 cycles per min). At intervals a part of the mixture was filtered by a poly(tetrafluoroethylene) membrane filter and the filtrate was injected into HPLC (Shimadzu LC-6A). A Shim-pack CLC-ODS column (0.15 m×6.0 mm) was used and eluted with water–acetonitrile (50/50 by volume). Acetanilide was used as an internal standard, and reaction components were detected with a UV detector at 270 nm. The reaction rates were calculated from the initial increase in the amounts of *N*-acetyl-L-tyrosine.

Results and Discussion

It is known that the fluorescence of proteins is due to constituent aromatic amino acids, tryptophan, and tyrosine. In the present work fluorescence of CT was excited by UV light at 295 nm, because at this wavelength only tryptophan residues are excited and also

intertryptophyl energy transfer is negligible.[19—21] Under this condition, observed fluorescence spectra are regarded as the sum of contributions of individual tryptophan residues in the protein.

It is also known that fluorescence of a tryptophan residue is sensitive to its microenvironment. In general, the wavelength of maximum emission should shift to lower values and intensity of emission will increase as the polarity of the environment of the fluorophore decreases.[19,22] A typical example of solvent effect on tryptophyl fluorescence is shown in Fig. 1, in which the maximum wavelength (λ_{em}) and intensity of the fluorescence of *N*-acetyl-L-tryptophan ethyl ester are plotted against water content (%) in acetonitrile–water mixed solvents. In going from pure acetonitrile to water, λ_{em} shifts from 333 to 353 nm. Therefore, it may be assumed that any changes in λ_{em} of tryptophan residues in CT will be an indication of the change in microenvironment of the residues due to structural perturbation of the protein.

CT has eight tryptophan residues. It has been inferred that none of them are highly exposed to solvent, and therefore the λ_{em} of CT (331—333 nm in aqueous solutions) is hardly affected by solvents or ionic quenchers unless the conformational changes of the enzyme occurs. It was reported that the position of the emission spectra of CT is independent of hydration or solubilization.[18] Judging from the λ_{em} of native CT, most of tryptophan residues are considered to be in hydrophobic environment. The maximum emission wavelength of CT in several aqueous–organic media was measured and the results are discussed in terms of microenvironment of CT and as relevant to its catalytic activity.

a) Acetonitrile. In Fig. 2, λ_{em} and emission intensity of CT are plotted against water content in acetonitrile–water mixed solvents. By increasing water content, λ_{em} initially shifts to higher wavelength up to 345 nm at about 70% water and then to lower wavelength. In pure water the emission wavelength is 332 nm which is close to the normal emission of native CT in buffer solutions or in solid state. Interestingly, the profile of emission intensity is almost reversal of that of emission wavelength (Fig. 2). The shifts of λ_{em} to higher wavelength may be the indication of the change in microenvironment of tryptophan residues in CT due to interactions with the solvent. Probably, at water contents between 30 and 80%, where λ_{em} shifts to higher wavelength, some structural changes of CT may occur which increase the polarity of the environment of tryptophan residues; that is the exposure of tryptophan residues to the solvent. The situation is very similar to denaturation by urea. It has been reported that the denaturation of proteins by high urea concentrations which causes practically complete unfolding of a protein globule, gives rise to the shifts of fluorescence spectral maxima toward 350—353 nm.[19,23]

In the present experiment, part of CT is insoluble and suspended in the solvents at water contents below

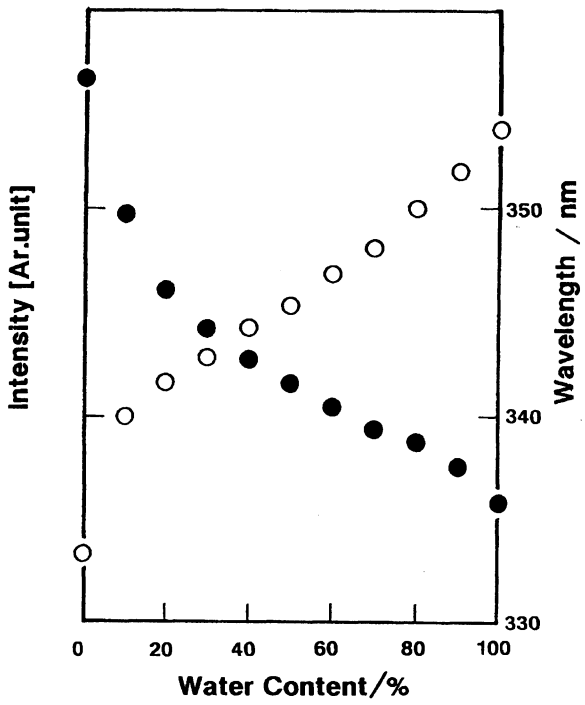

Fig. 1. Wavelength and intensity of maximum fluorescence emission of *N*-acetyl-L-tryptophan ethyl ester in acetonitrile–water. ○: wavelength; ●: intensity.

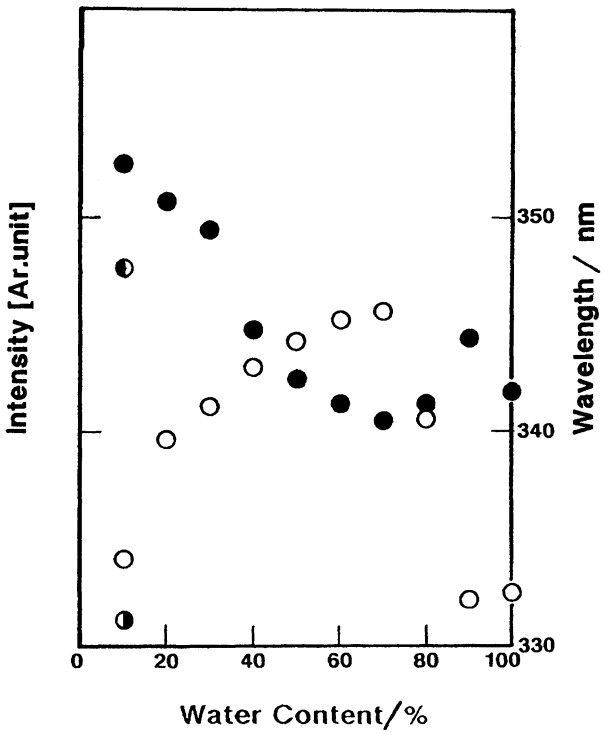

Fig. 2. Wavelength and intensity of maximum fluorescence emission of CT in acetonitrile–water. ○: wavelength; ●: intensity; ◐ and ◑: wavelength and intensity of filtrate, respectively.

40%. In these solvents, both suspended and solubilized CT will contribute to emission. In was found that solubilized CT exhibits much higher λ_{em} than that of suspended CT; for example after filtration of CT suspension in a solvent with 10% water, the filtrate showed λ_{em} at around 347 nm as compared to that of CT suspension at 333 nm (Fig. 2). So far, relatively few studies have appeared on the fluorescence spectroscopy of solid enzymes.[18] However, the above results suggest that this technique may be useful in studying modification of enzyme structure not only in solutions but also in solid (suspended) state.

It may be reasonably assumed that any structural modifications of an enzyme would give rise to changes in its catalytic activity and specificities. Figure 3 shows the reaction rate and product yield of the hydrolysis of *N*-acetyl-L-tyrosine ethyl ester (ATEE) by the catalysis of CT in acetonitrile–water. It can be seen clearly that CT loses activity at water contents between 40 and 70%. This phenomenon seems to be closely related to the red shift of λ_{em} described above and may most probably be ascribed to the structural modification of CT. At water contents below 40%, probably the aggregation of CT stabilizes the enzyme against destructive action of the solvent. As described previously, catalysis of CT at high concentrations of organic solvents have been well recognized and utilized for ester or peptide synthesis.

b) Ethanol. Ethanol has been considered to be a toxic solvent for many enzymes.[15] It has been generally recognized that, by increasing ethanol contents, enzymes lose activity probably due to unfolding of the peptide chains. However, recent studies revealed that this solvent can be used for synthetic reactions by proteases such as ester and peptide synthesis.[10,11] The activity is strongly dependent on the water content, and therefore analysis of fluorescence properties would give some information on structural changes of the enzymes in ethanol–water mixtures.

Figures 4 and 5 show the fluorescence properties and reaction profile of ATEE hydrolysis in ethanol–water. Again in this case, by addition of ethanol to aqueous CT solution, maximum emission shifts to higher wavelength giving a maximum at around 50% water. By further decreasing water contents, λ_{em} exhibits a blue shift. These spectral variations are very similar to those in acetonitrile-water, and furthermore, a close relationship between λ_{em} and hydrolysis rate of ATEE is observed (Fig. 5). At 50% water, catalysis of CT is completely suppressed.

c) 1,4-Dioxane and THF. The dependency of λ_{em} of CT on water content in 1,4-dioxane–water (Fig. 6) has some common feature to that in ethanol–water. Maximum λ_{em} is observed at around 50% water content, and there are lower λ_{em} regions at higher and lower water contents. This implies that at least part of CT is not deactivated at high concentrations of 1,4-dioxane as demonstrated by kinetic study (Fig. 7). Small but not negligible activity of CT is retained for hydrolysis of ATEE in 1,4-dioxane containing 10% water, although 1,4-dioxane is known to be a competitive inhibitor of CT.[23,24]

In the case of THF–water, emission profile of CT is similar to that in 1,4-dioxane–water, except that maximum λ_{em} is obtained at about 70% water (Fig. 8). This

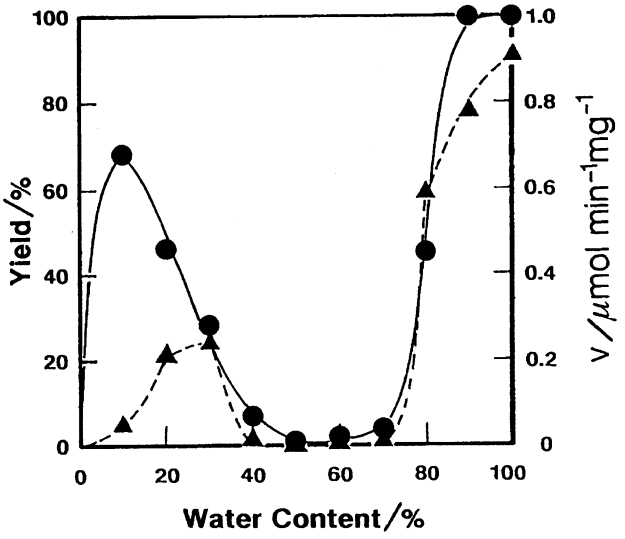

Fig. 3. Reaction rate and product yield of hydrolysis of ATEE in acetonitrile–water. CT 2.5 mg, ATEE 10 mM, solvent 10 ml; ▲: reaction rate; ●: Ac–L-Tyr–OH yield (24 h).

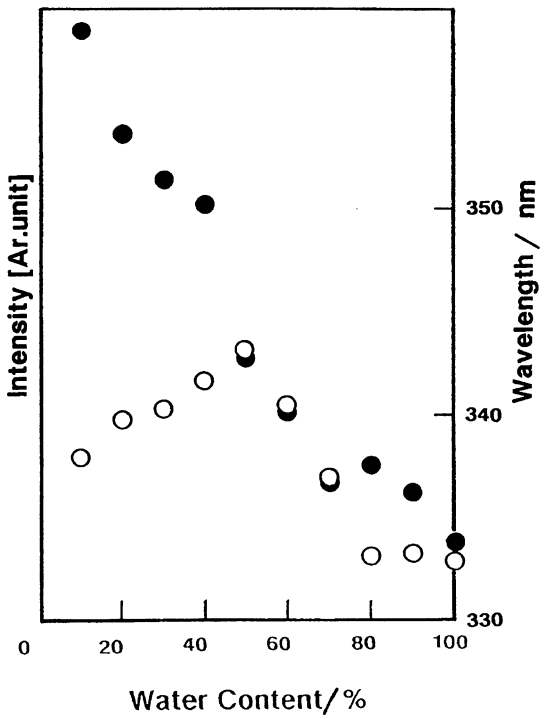

Fig. 4. Wavelength and intensity of maximum fluorescence emission of CT in ethanol–water. ○: wavelength; ●: intensity.

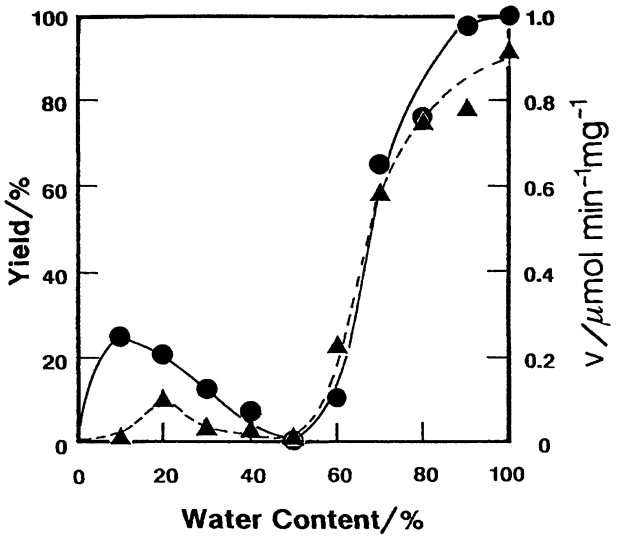

Fig. 5. Reaction rate and product yield of hydrolysis of ATEE in ethanol–water. CT 2.5 mg, ATEE 10 mM, solvent 10 ml; ▲: reaction rate; ●: Ac–L-Tyr–OH yield (24 h).

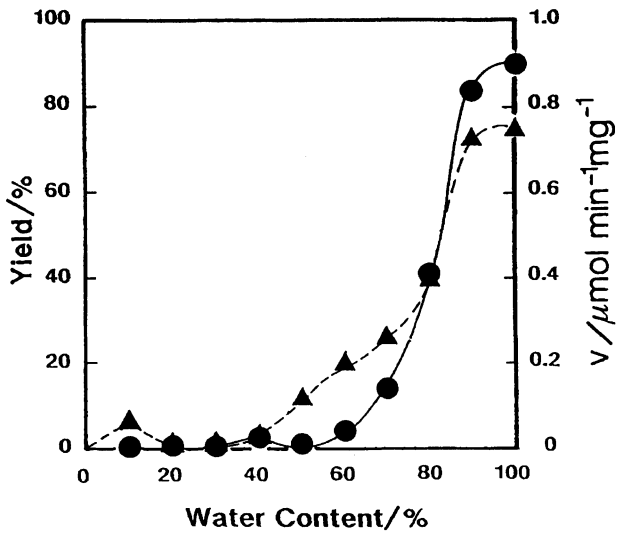

Fig. 7. Reaction rate and product yield of hydrolysis of ATEE in 1,4-dioxane–water. CT 2.5 mg, ATEE 10 mM, solvent 10 ml; ▲: reaction rate; ●: Ac–L-Tyr–OH yield (24 h).

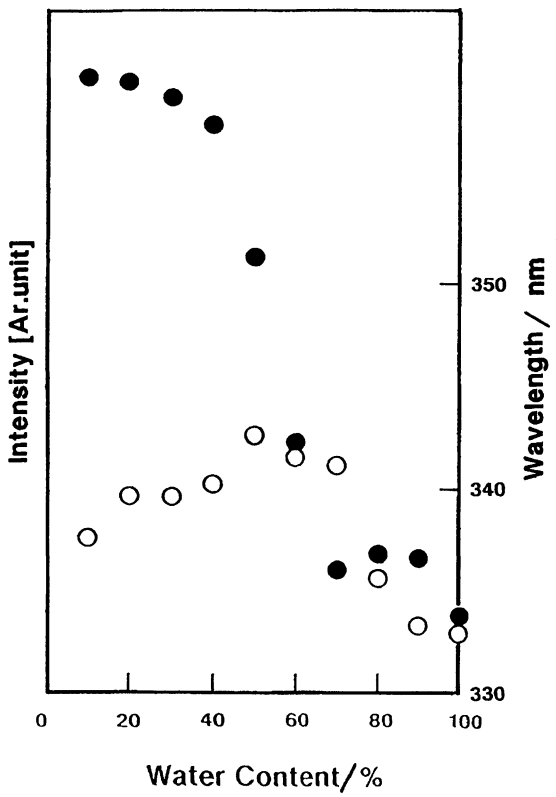

Fig. 6. Wavelength and intensity of maximum fluorescence emission of CT in 1,4-dioxane–water. ○: wavelength; ●: intensity.

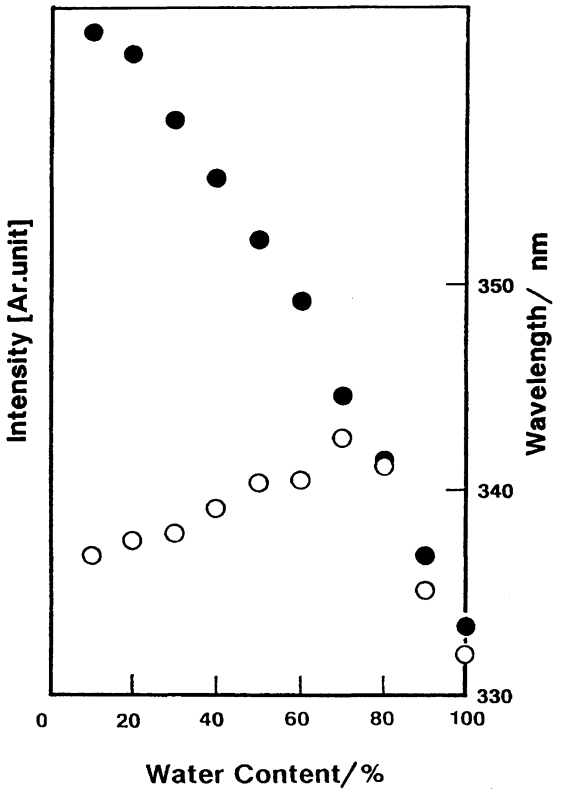

Fig. 8. Wavelength and intensity of maximum fluorescence emission of CT in THF–water. ○: wavelength; ●: intensity.

corresponds to the complete retardation of activity of CT by addition of only 30% THF (Fig. 9). Thus, both in 1,4-dioxane and THF–water systems, significant relationship is observed between λ_{em} and activity of CT.

d) *N*,*N*-Dimethylformamide (DMF). DMF is one of the solvents which have strong solubilizing power for many polar substances. Especially for biocatalytic conversions of amino acids and related compounds in organic solvents, selection of solvent is prerequisite in which the substrates are soluble. DMF has been a candidate for this purpose. However, DMF is known to be a competitive inhibitor of many enzymes and most

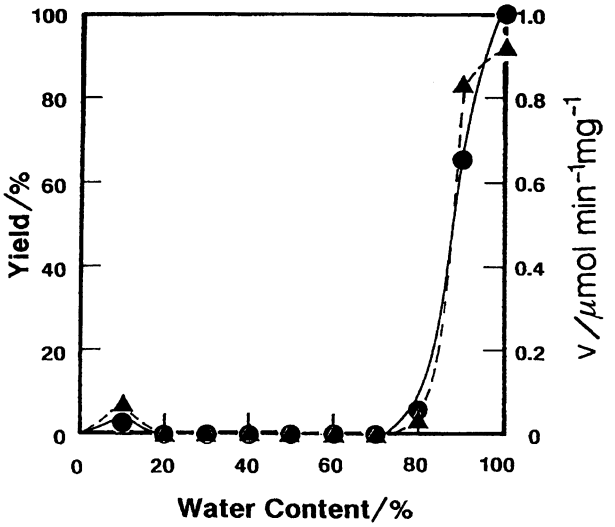

Fig. 9. Reaction rate and product yield of hydrolysis of ATEE in THF–water. CT 2.5 mg, ATEE 10 mM, solvent 10 ml; ▲: reaction rate; ●: Ac–L-Tyr–OH yield (24 h).

of enzymes lose catalytic activity in solvents with high DMF contents. An exception is subtilisin Carlsberg which was reported to retain its activity in anhydrous DMF.[25] In our experiences, catalytic activity of CT for ester or peptide synthesis was completely suppressed in DMF.[26,27] By addition of 50% or more water, activity is recovered and the reaction systems have been utilized for peptide synthesis[28,29] and optical resolution of amino acids.[30]

In Fig. 10 maximum emission wavelength of CT and its intensity are plotted against water content in DMF. With increase in water content, λ_{em} slightly shifts to higher wavelength and then sharply shifts to lower wavelength giving a maximum at about 30% water. Again this is closely correlated to the change in activity for ATEE hydrolysis shown in Fig. 11. The situation is comparable to those described above for several organic solvents, but kinetic study reveals that CT completely loses catalytic activity in DMF with water contents below 30% (Fig. 11). This indicates that DMF denatures CT more effectively than other solvents probably due to its strong interaction with the enzyme molecules.

The present results appear of interest particularly in view of enzyme utilization for synthetic reactions in water-containing organic solvents because many of these reactions are necessarily carried out in these systems. Enzymes often lose catalytic activity at high concentrations of organic solvents or their specificities are altered at specific solvent compositions. The fluorescence spectroscopic measurements may be useful for prediction of changes in enzyme catalysis in these reaction systems.

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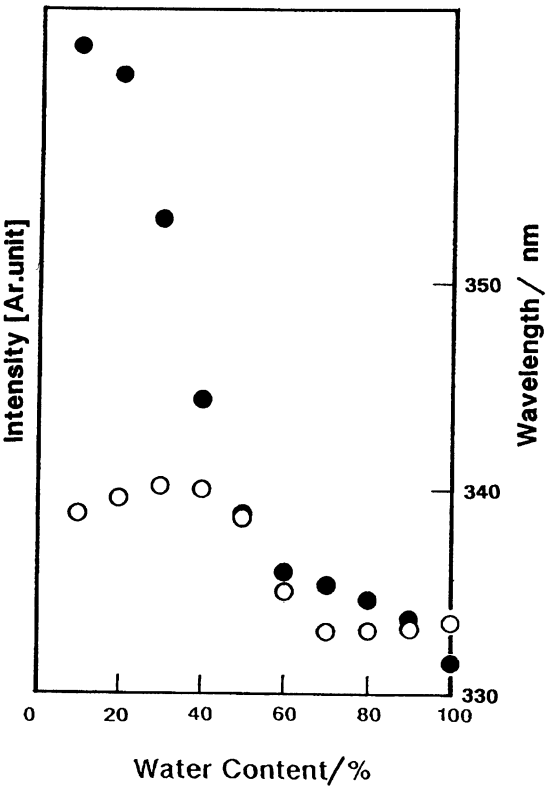

Fig. 10. Wavelength and intensity of maximum fluorescence emission of CT in DMF– water. ○: wavelength; ●: intensity.

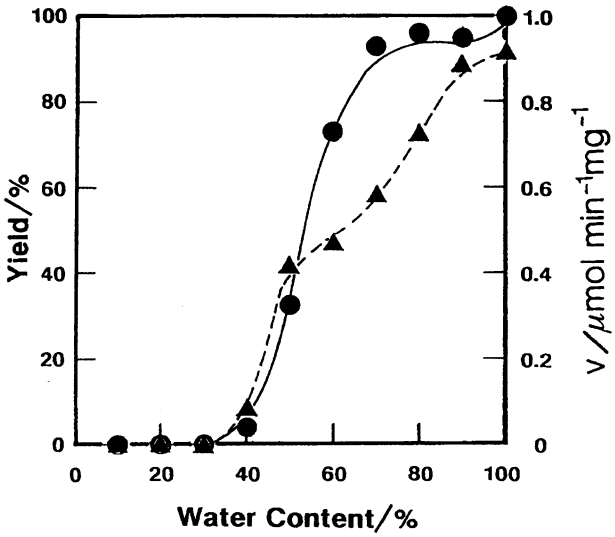

Fig. 11. Reaction rate and product yield of hydrolysis of ATEE in DMF–water. CT 2.5 mg, ATEE 10 mM, solvent 10 ml; ▲: reaction rate; ●: Ac–L-Tyr–OH yield (24 h).

References

1) Enzymatic Reactions in Aqueous–Organic Media. Part 18. Part 17: Y. Tomiuchi, K. Ohshima, and H. Kise,

Bull. Chem. Soc. Jpn., **65**, 2599 (1992).
2) S. Fukui and A. Tanaka, *Yuki Gosei Kagaku Kyokai Shi*, **41**, 384 (1983).
3) J. B. Jones, *Tetrahedron*, **42**, 3351 (1986).
4) C. -S. Chen and C. J. Sih, *Angew. Chem., Int. Ed. Engl.*, **28**, 695 (1989).
5) A. M. Klibanov, *Acc. Chem. Res.*, **23**, 114 (1990).
6) A. Zaks and A. M. Klibanov, *Science*, **224**, 1249 (1984).
7) T. Yamane, *J. Jpn. Oil Chem. Soc.*, **36**, 59 (1987).
8) J. S. Dordick, *Enzyme Microb. Technol.*, **11**, 194 (1989).
9) C. -H. Wong, *Science*, **244**, 1145 (1989).
10) H. Kise, A. Hayakawa, and H. Noritomi, *J. Biotechnol.*, **14**, 239 (1990).
11) H. Kise, *Yuki Gosei Kagaku Kyokai Shi*, **49**, 42 (1991).
12) H. Kitaguchi, P. A. Fitzpatrick, J. E. Huber, and A. M. Klibanov, *J. Am. Chem. Soc.*, **111**, 3094 (1989).
13) P. A. Fitzpatrick and A. M. Klibanov, *J. Am. Chem. Soc.*, **113**, 3166 (1991).
14) T. Nagashima, A. Watanabe, and H. Kise, *Enzyme Microb. Technol.*, **14**, 842 (1992).
15) T. T. Herskovits, B. Gadegbeku, and H. Jaillet, *J. Biol. Chem.*, **245**, 2588 (1970).
16) A. A. Klyosov, N. V. Viet, and V. Berezin, *Eur. J. Biochem.*, **59**, 3 (1975).
17) D. S. Clark and J. E. Bailey, *Biochim. Biophys. Acta*, **788**, 181 (1984).
18) G. Vermunicht, N. Boens, and F. C. De Schryver, *Photochem. Photobiol.*, **53**, 57 (1991).
19) E. A. Burstein, N. S. Vedenkina, and M. N. Ivkova, *Photochem. Photobiol.*, **18**, 263 (1973).
20) G. Desie, N. Boens, and F. C. De Schryver, *Biochemistry*, **25**, 8301 (1986).
21) K. J. Willis and A. G. Szabo, *Biochemistry*, **28**, 4902 (1989).
22) R. M. Guinn, H. W. Blanch, and D. S. Clark, *Enzyme Microb. Technol.*, **13**, 320 (1991).
23) G. E. Clement and M. L. Bender, *Biochemistry*, **2**, 836 (1963).
24) K. Tanizawa and M. L. Bender, *J. Biol. Chem.*, **249**, 2130 (1974).
25) A. Zaks and A. M. Klibanov, *J. Biol. Chem.*, **263**, 3194 (1988).
26) H. Kise and H. Shirato, *Enzyme Microb. Technol.*, **10**, 582 (1988).
27) H. Kise, K. Fujimoto, and H. Noritomi, *J. Biotechnol.*, **8**, 279 (1988).
28) J. B. West and C. -H. Wong, *J. Org. Chem.*, **51**, 2728 (1986).
29) C. F. Barbas, III, J. R. Matos, J. B. West, and C. -H. Wong, *J. Am. Chem. Soc.*, **110**, 5162 (1988).
30) Y. Tomiuchi, K. Ohshima, and H. Kise, *Bull. Chem. Soc. Jpn.*, **65**, 2599 (1992).