Concentration- and Structure-Dependent Effects of Amides on Protease Activity in Organic Solvents

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The catalytic activity of α-chymotrypsin (CT) in the transesterification of *N*-acetyl-L-tyrosine methyl ester to its ethyl ester in aqueous-organic media was markedly enhanced by replacing a part of water with formamide. The activity of CT was strongly dependent on the formamide/water ratio, and excess formamide retarded the activity. Addition of formamide to reaction mixtures at constant water contents exhibited similar activation–deactivation profiles for CT. A kinetic study revealed that the rate acceleration is due to an increase in k_{cat} rather than a change in K_m. At a given concentration of amides (0.5 M, M=mol dm^{-3}), propionamide and DMF were much less effective than formamide for activation of CT. The results suggest that formamide interacts with CT in a different way from water.

There is rapidly growing interest in enzymatic reactions in organic media.[1—4] A variety of enzymes have been utilized in organic solutions for synthetic reactions and optical resolution of chiral compounds. It is well-documented that the activity of an enzyme is a function of the nature of the organic solvent and the water content in the reaction mixture.[5—7] In general, water is required for activity of enzymes in organic solvents. However, in the case of synthetic reactions of esters or peptides by proteases or lipases, water is a competitive reactant producing hydrolysis products. For example, peptide synthesis by proteases from amino acid esters is competitive with hydrolysis of the substrates.[7—9] Also, the product yield may be significantly decreased by secondary hydrolysis of the products.

In 1989 Kitaguchi and Klibanov reported that a part of water can be replaced with "water mimics" such as formamide and ethylene glycol for synthetic reactions of peptides by thermolysin in *t*-pentyl alcohol.[10] It was assumed that these compounds form multiple hydrogen bonds with the enzyme, thus maintaining the active conformation of the enzymes with a significant reduction of hydrolysis. However, the experimental results indicated that complete replacement of water with a water mimic leads to deactivation of the enzyme. Similar results were reported later for ester synthesis by a lipase in heptane,[11] but a systematic investigation of the effects of water mimics has not been reported.

In the course of a study on the transesterification of amino acid esters by proteases in organic solvents, it was found that replacement of a part of water with formamide or its related compounds dramatically enhanced the catalytic activity of α-chymotrypsin (CT). Enhancement of catalytic activity was also observed by addition of these compounds to the aqueous-organic reaction media at a constant water content. This article describes the results of a detailed study on the activation and deactivation of CT by aliphatic amides and *N*-substituted formamides.

Experimental

α-Chymotrypsin (CT) was purchased from Sigma Chem. Co. *N*-Acetyl-L-tyrosine methyl ester (ATME) was prepared by the reaction of *N*-acetyl-L-tyrosine with methanol in the presence of thionyl chloride. Guaranteed grade amides were purchased from Wako Pure Chemical Co. and used without further purification. Ethanol and organic solvents were also of guaranteed grade and dried on molecular sieves 3A.

Transesterification of ATME to ATEE was carried out as follows: An aqueous solution of an enzyme was added to a solution of ATME, ethanol, and an amide in an organic solvent. The total reaction volume was 10 ml. The reaction mixture was incubated at 30 °C with constant reciprocal shaking (about 150 cycles per min). Aliquots of the reaction mixture were taken at intervals, filtered through polytetrafluoroethylene membrane filters, and injected into a Shimadzu LC-6A HPLC. 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. The rate of transesterification was calculated from the initial increase in the amounts of ATEE. All the reactions were carried out at 30 °C. Yields of the products were measured after 24 h reactions.

Results and Discussion

Transesterification of *N*-acetyl-L-tyrosine methyl ester (ATME) to its ethyl ester (ATEE) is effectively catalyzed by α-chymotrypsin (CT) in organic solvents containing small amounts of water. The reaction is competitive with the hydrolysis of ATME to form *N*-acetyl-L-tyrosine (AT), and the yield of ATEE is dependent on the water content for reactions in acetonitrile as shown in Fig. 1. The maximum yield of ATEE was about 65% at water contents of 2—3%. Similar results were obtained in propylene carbonate or *t*-butyl alcohol; the maximum yields of ATEE were obtained at 2—3% water, and the yield decreased at higher water contents due to an increase in the hydrolysis product.

Thus, a part of water was replaced with formamide. We expected an enhancement of ATEE synthesis due to a retardation of hydrolysis. The results of reactions in acetonitrile, propylene carbonate, and *t*-butyl alcohol

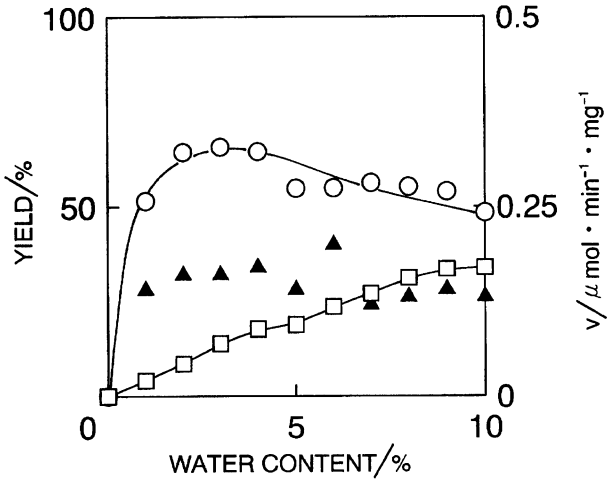

Fig. 1. Transesterification of ATME in acetonitrile–water. ATME 10 mM, CT 2 mg, EtOH 1 ml, (MeCN + H_2O) 9 ml. ▲: transesterification rate; ○: ATEE yield; □: AT yield.

are shown in Fig. 2. Interestingly, both transesterification and hydrolysis were accelerated by replacement of a part of water with formamide, giving maximum reaction rates at 1/5—1/4 replacement depending on the nature of the solvents (total molar amounts of water plus formamide were kept constant at 16.7 mmol for acetonitrile or propylene carbonate and 11.1 mmol for *t*-butyl alcohol, which correspond to 3 and 2% water, respectively, the optimum water contents in the absence of formamide). With further increases in the formamide/water ratio, the rates of both transesterification and hydrolysis decreased, and at complete replacement of water with formamide, the enzyme was deactivated. The ratio of the rate of transesterification to that of hydrolysis was not much affected by the formamide/water ratio. These results suggest that formamide acts as an activator of CT in a different way from water which has been considered to activate enzymes by enhancing the conformational flexibility of the enzymes.[10]

The above results led us to investigate the effects of the addition of formamide at a constant water content. As shown in Fig. 3, addition of formamide markedly increased the rate of ATEE formation. It should be noted that the reaction mixtures contained 3 or 2% water which, as mentioned above, are optimum for reactions without formamide. Also, it should be noted that the rate increase was much larger and the retardation of activity by excess formamide was more relaxed as compared to the cases of water replacement with formamide (Fig. 2). These results suggest again that formamide activates or inhibits the enzyme in a different way from water and to extents that can not be achieved by water. Similar results were obtained for transesterification in ethanol as the reactant and a solvent (Fig. 4).

The transesterification by CT obeys Michaelis–Menten kinetics. The kinetic parameters listed in Table 1 were obtained by Lineweaver–Burk plots for reactions in the absence and presence of formamide. It is obvious that acceleration of the reaction by formamide is due to an increase in k_{cat}. Assuming that deacylation

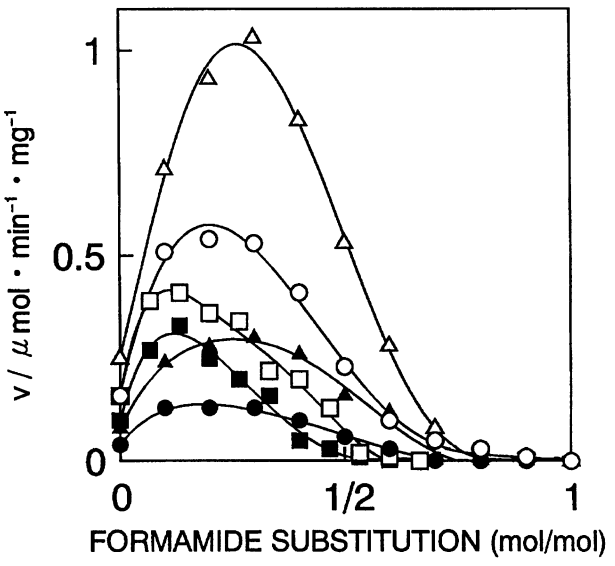

Fig. 2. Substitution of formamide for water in organic solvents. ATME 10 mM, CT 2 mg, EtOH 1 ml, (organic solvent+formamide+H_2O) 9 ml, (formamide+H_2O) 16.7 mmol for acetonitrile and propylene carbonate, and 11.1 mmol for *t*-butyl alcohol. ○ (acetonitrile), △ (propylene carbonate), □ (*t*-butyl alcohol): transesterification rate; ● (acetonitrile), ▲ (propylene carbonate), ■ (*t*-butyl alcohol): hydrolysis rate.

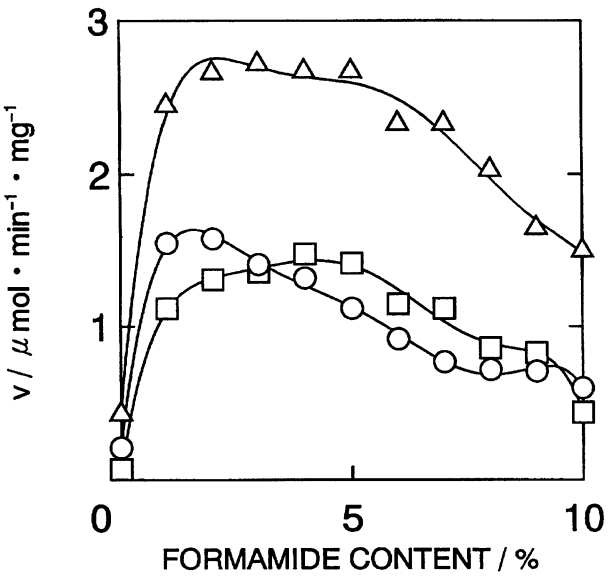

Fig. 3. Effect of addition of formamide on the transesterification rate of ATME in organic solvents. ATME 10 mM, CT 1 mg, EtOH 1 ml, H_2O 0.3 ml for acetonitrile and propylene carbonate, and 0.2 ml for *t*-butyl alcohol, (organic solvent+formamide) 8.7 ml. ○: acetonitrile; △: propylene carbonate; □: *t*-butyl alcohol.

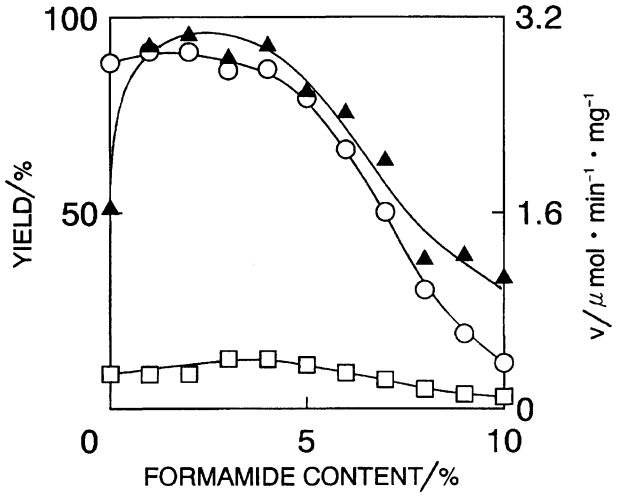

Fig. 4. Effect of addition of formamide on the transesterification of ATME in ethanol. ATME 10 mM, CT 1 mg, H_2O 0.5 ml, (EtOH+formamide) 9.5 ml. ▲: transesterification rate; ○: ATEE yield; □: AT yield.

Table 1. Kinetic Parameters for Transesterification of ATME to ATEE by CT in Acetonitrile[a)]

Additive	K_m/mM	k_{cat}/s^{-1}	$(k_{cat}/K_m)/M^{-1}s^{-1}$
—	20.2	0.2	9.9
Formamide	24.4	1.2	49.2

a) Formamide 5 mmol, CT 1 mg, H_2O 0.3 ml, total volume 10 ml.

of the acyl-enzyme intermediate is rate-determining,[3)] this result seems to suggest that formamide promotes the nucleophilic attack of ethanol on the acyl-enzyme.

As described above, it has been assumed that formamide forms multiple hydrogen bonds to thermolysin in an organic solvent.[10)] Therefore, the effect of substitution of the amide hydrogens with methyl groups was studied. Figure 5 illustrates the effects of addition of *N*-methylformamide (MFA) and *N,N*-dimethylformamide (DMF) on the transesterification of ATME to ATEE in acetonitrile. It can be seen that maximum acceleration of the reaction was larger for MFA and smaller for DMF than formamide. DMF hardly affected the reaction at contents below 20%. This seems to indicate that hydrogen bonding is essential for the interaction of the amides with CT. The reduction of CT activity at higher concentrations of formamide seems to suggest that formamide interacts with CT at or near the binding site of ethanol. Alternatively, there is a possibility that excess formamide causes a conformational change in CT like urea.[12,13)]

Figure 6 summarizes the effects of aliphatic amides, along with *N*-substituted amides, on transesterification. At a given concentration of the unsubstituted amides (0.5 M), formamide is the most effective, followed by acetamide and propionamide. Since each amide may

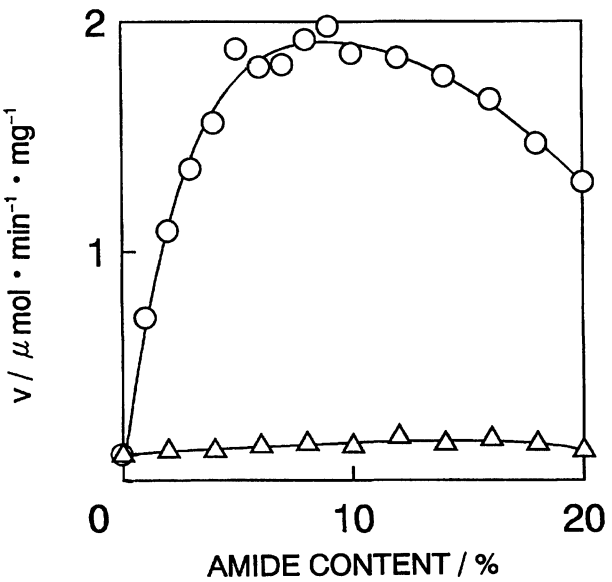

Fig. 5. Effect of addition of MFA (○) or DMF (△) on transesterification of ATME in acetonitrile. ATME 10 mM, CT 1 mg, EtOH 1 ml, H_2O 0.3 ml, (organic solvent+MFA or DMF) 8.7 ml.

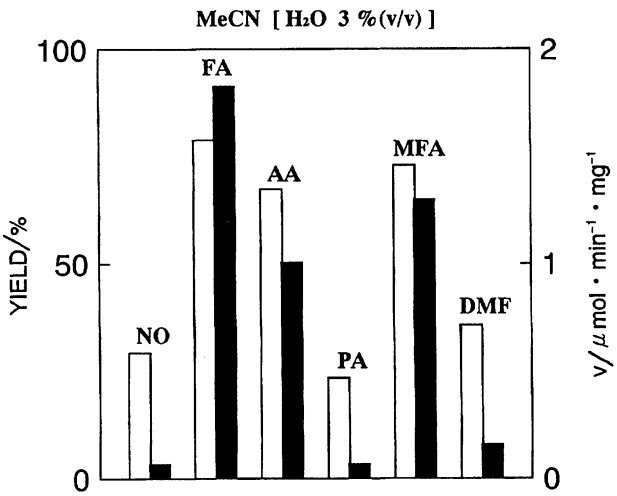

Fig. 6. Transesterification of ATME in acetonitrile with or without amides. ATME 10 mM, CT 1 mg, amide 5 mmol, EtOH 1 ml, H_2O 3%. NO: without amide; FA: formamide; AA: acetamide; PA: propionamide; MFA: *N*-methylformamide; DMF: *N,N*-dimethylformamide. ■: transesterification rate; □: ATEE yield.

have its own optimal concentration for activation of the enzyme, and there may also be an optimal water concentration for each amide, it seem difficult to draw any distinct conclusion on the structural effects of the amides. However, it is obvious that short chain amides are preferable for activation of the enzyme. Also, polar interactions rather than hydrophobic interactions are important between the amides and CT.

In summary, it was found that the catalytic activity of CT is markedly enhanced by formamide or its

related compounds for transesterification of ATME to ATEE in organic solvents. However, excess formamide retards the activity, and complete replacement of water with formamide deactivates the enzyme. The results suggest that formamide works as an activator of CT by a different mechanism from water, although structural effects of amides suggest the importance of amide hydrogens probably for hydrogen bonding to CT. Our attention is now focused on possible structural changes in CT by amides, and a spectroscopic study on CT and other serine proteases in organic solvents is in progress.

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