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Enzymatic Reactions in Aqueous-Organic Media. II.[1] Effects of Reaction Conditions and Selectivity in the Esterification of Aromatic Amino Acids by α-Chymotrypsin in Alcohols

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N-Acetylated aromatic amino acids were esterified by α-chymotrypsin suspended in alcohols. The reaction rate and the yields of the esters depended primarily on the amount of the aqueous buffer solution in which α-chymotrypsin was dissolved. The reaction rate increased but the equilibrium yield of the esters decreased with increasing concentration of water. High substrate selectivity and stereospecificity were observed for L-aromatic amino acids; and therefore, in this system α-chymotrypsin was considered to be an effective catalyst which maintains its native conformation. When the amount of the substrate was increased, the present reactions gave higher yields of the esters than the reactions in chloroform (two-phase method).

Recently, the area of enzymatic reactions in the presence of organic solvents, either miscible or immiscible with water, is rapidly growing and is drawing much attention. These reactions are of great interest in view of both basic studies on the medium effects on enzyme catalysis and the application of enzymatic reactions to organic synthesis. For example, hydrolytic enzymes, such as α-chymotrypsin (CT), have been employed as catalysts for various ester syntheses in aqueous-organic two-phase systems.[2-12] It has been considered that in this system the decreased amount of water and the low solubility of the products in the aqueous phase shift the reaction equilibrium to the synthesis of esters. However, when a hydrophobic (water-immiscible) organic solvent is used, the main drawback of the method is that if one of the reactants RCOOH such as amino acids is hardly soluble in the organic phase, it would be concentrated in the aqueous phase (Fig. 1a). This often causes a substrate inhibition of the enzyme, leading to low yields of the esters.[7,13]

The synthetic reactions by hydrolytic enzymes in water-hydrophilic (water-miscible) organic solvents have also been reported.[9,14-16] By using limited amounts of water in solvents, the equilibrium of the reaction can be shifted to favor the synthesis. However, the catalytic activity of the enzymes is often impaired at high concentrations of organic solvents, and the yields of products have been rather limited. For example, it has been reported that *N*-acetyl-L-tyrosine ethyl ester was obtained in 25—30% yields by CT-catalyzed reactions of *N*-acetyl-L-tyrosine with ethanol in 50—60% water, but that CT was inactivated at lower concentrations of water.[14]

The present study concerns with the esterification of *N*-acetylated aromatic amino acids by CT at very high concentrations of alcohols. The reaction system is heterogeneous containing suspended CT which can be easily separated by filtration like immobilized enzymes. The results for the effects of various reaction conditions on the reaction rate and the yield of the esters, as well as substrate selectivity and stereospecificity of the reaction, are reported.

Experimental

Materials. Bovine pancreatic α-chymotrypsin (CT) (EC 3.4.21.1) having a specific proteinase activity, with *N*-benzoyl-L-tyrosine ethyl ester (BTEE), of 50 units (μmol-min^{-1}) mg^{-1} of protein (pH 7.8 at 25 °C) was purchased from Sigma Chemical Co. *N*-Acetyl-L-tyrosine (ATy), its ethyl ester (ATyE), *N*-acetyl-D-tryptophan, -D-phenylalanine, and -L-alanine were also purchased from Sigma. *N*-Acetyl-L-tryptophan (AT), -L-phenylalanine (AP), their ethyl esters (ATE and APE), and *N*-acetyl-L-leucine were obtained from Nakarai Chemical Co. Alcohols and chloroform of guaranteed grade were also obtained from Nakarai and used without further purification.

Reactions and Measurements. Typically, a solution of CT (0.01 g) in 0.5 ml of phosphate buffer (0.1 M (1M=1 mol dm^{-3}), pH 6.8) was added to a solution of AT (0.05 g, 0.20 mmol) in 20 ml of ethanol. The mixture was incubated with constant reciprocal shaking (140 cycle per min) at 30 °C for 24 h (method A). Unless otherwise stated method A was used throughout this study. Alternatively, AT was first dissolved into the CT solution and then ethanol was added (method B). When chloroform was used as a solvent (two-phase method), the total volume of the organic solution was also 20 ml. In all the methods, after the reaction the solvents were evaporated with a rotary evaporator, and the amounts of AT and ATE in the residue were determined by a HPLC (JASCO Tri Rotar SR-1) using a JASCO Finepak SIL C18 column eluted with water-acetonitrile (50/50 by volume). Acetanilide was used as an internal standard.

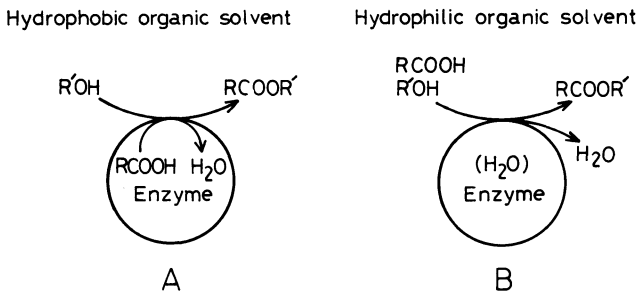

Fig. 1. Schematic representation of enzymatic ester synthesis in two-phase (A) and the present (B) systems.

The initial rate of esterification was measured by a determination of the amounts of AT and ATE in the reaction mixtures at intervals. A 4 μl aliquat of the reaction mixture, from which CT had been removed by filtration with a regenerated cellulose type membrane filter, was injected directly inot HPLC and analyzed under the same conditions as described above.

The CD spectra of CT solutions in ethanol or water were obtained with a JASCO spectropolarimeter model J-20.

Results and Discussion

Ester Synthesis in Chloroform (Two-Phase Method). In order to elucidate the effect of a water-immiscible organic solvent we at first investigated the esterification of AT by CT in chloroform.[1] Figure 2 shows the time courses of esterification with 1 M ethanol in chloroform and with different amounts of buffer solution in which CT was dissolved. It can be seen that the conversion (yield) of ATE at equilibrium increases with the decrease in the amount of the buffer. Although longer reaction time was required to attain the equilibrium with 0.25 ml of the buffer solution, more than 90% conversion was obtained after 24 h. Without water, however, the reaction was totally inhibited, indicating that a minimum amount of water is essential to the catalytic activity of CT. Furthermore, no reaction occurred without CT under the same conditions, ruling out the possibility of catalysis by ionic species in the buffer solutions.

The dependency of the equilibrium conversion on water content may be explained simply by a mass action law which predicts that with decreasing amounts of water the equilibrium shifts to ester synthesis. It is considered that the reaction occurs in the water droplets in which CT is dissolved. With lower water contents the amount of free water available for hydrolysis may be small enough to suppress the hydrolysis.

When the concentration of ethanol in chloroform was increased with a constant total volume of the organic phase (20 ml), the change in ATE yield was found to be rather small (less than 10%); the data are presented in Table 1. It had been considered that high concentration of water-miscible solvents such as ethanol would impair the enzyme activity and for this reason two-phase method has been employed. However, the above result indicates that chloroform can be replaced as a solvent by ethanol without any serious inactivation of CT. In neat ethanol the reaction mixture is a suspension of hydrated CT which can be separated by filtration with a glass or a membrane filter. Since the reactions in alcohols are clean and easy to work up, and, as will be mentioned later, the substrate inhibition is much less than in two-phase method, most of the reactions were carried out in alcohols.

Ester Synthesis in Ethanol. The effects of the reaction conditions on the ester yield were extensively studied in order to obtain optimum conditions. Figures 3 to 5 exhibit plots of the yields of ethyl esters of AT, ATy, and AP after 24 h reactions in ethanol against the volume-% of aqueous buffer solutions in which CT

Table 1. Effect of EtOH Concentration on ATE Yield[a]

EtOH in $CHCl_3$/M	ATE yield/%
1	83
3	86
5	77
8.6 (50 vol-%)	79
17.1 (neat)	78

a) AT 0.05 g (0.20 mmol), CT 0.01 g, organic phase 20 ml, phosphate buffer (0.1 M, pH 6.8) 0.5 ml, 30 °C, 24 h.

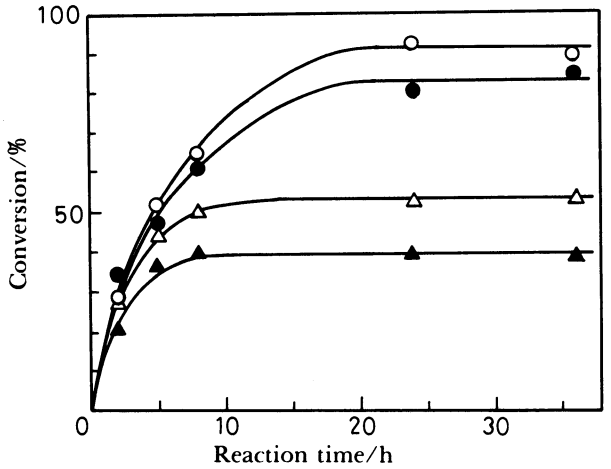

Fig. 2. Time courses of CT-catalyzed esterification of AT in chloroform. AT 0.05 g (0.20 mmol), CT 0.01 g, 1 M EtOH in chloroform (20 ml), 30 °C, phosphate buffer (0.1 M, pH 6.8) ○: 0.25 ml, ●: 0.5 ml, △: 1.0 ml, ▲: 2.0 ml.

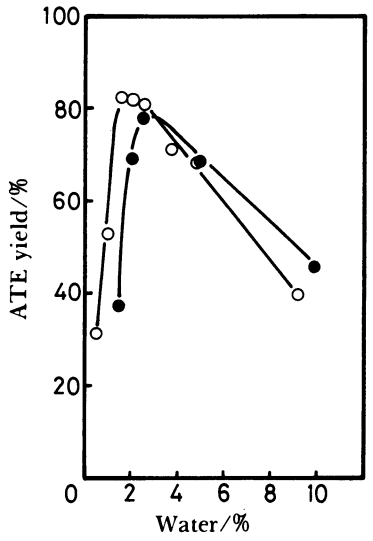

Fig. 3. Effect of water content on ATE yield. AT 0.05 g (0.20 mmol), CT 0.01 g, EtOH 20 ml, phosphate buffer (0.1 M, pH 6.8), 30 °C, 24 h. ○: Method A, ●: method B.

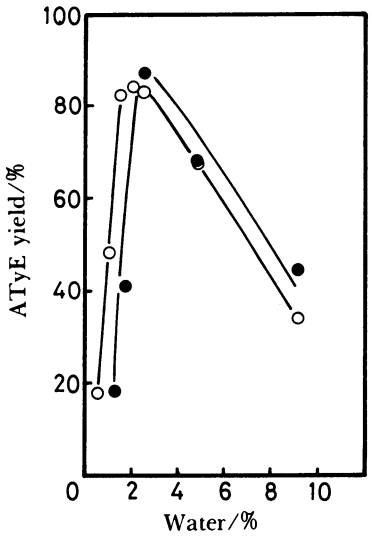

Fig. 4. Effect of water content on ATyE yield. ATy 0.045 g (0.20 mmol). Other reaction conditions and methods are the same as those in Fig. 3.

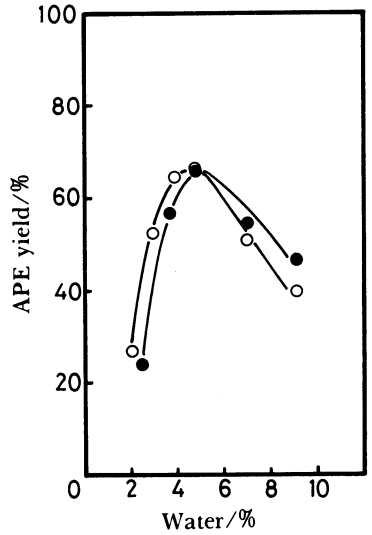

Fig. 5. Effect of water content on APE yield. AP 0.042 g (0.20 mmol). Other reaction conditions and methods are the same as those in Fig. 3.

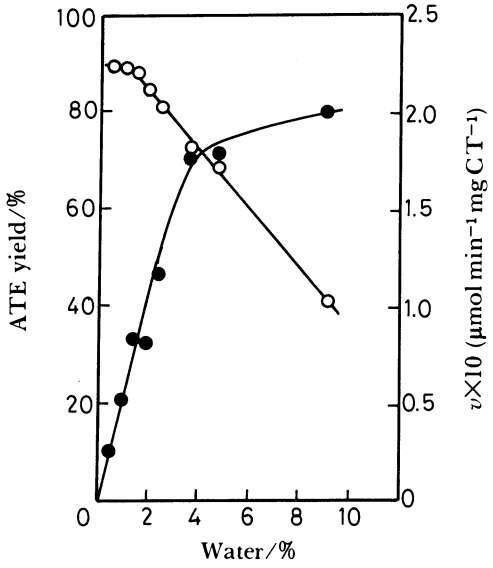

Fig. 6. Effect of water content on ATE yield (○) and the reaction rate (●) of esterification of AT. In the region of low water content, reaction time was increased up to 15 days. AT 0.05 g (0.20 mmol), CT 0.01 g, phosphate buffer (0.1 M, pH 6.8), 30°C.

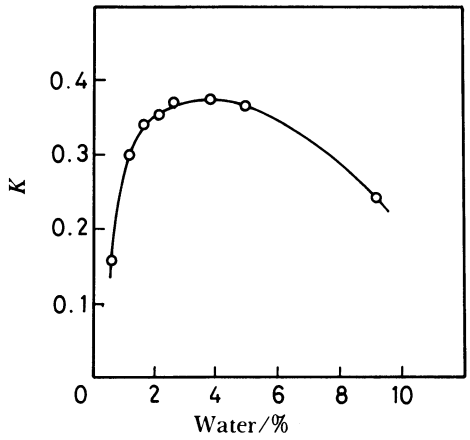

Fig. 7. Change in K with water content. K=[ATE]-[water]/[AT][EtOH].

was dissolved. The results for the two methods (method A and B, see Experimental) are compared in each Figure. The yields of the three esters exhibit a similar dependency on the water content, although the water contents which gave maximum yields are different. Like the reactions in chloroform mentioned above, the decrease in the yield with increasing amount of water may be explained by the shift of the equilibrium to hydrolysis. Alternatively, the increase in the solubility of CT in ethanol-water may be responsible for the decrease in the yield, since, as will be mentioned later, CT loses activity by dissolving in the mixed solvent.

The marked decrease in the yields at lower water concentrations may be the consequence of the slower reaction rate due to the decrease in the degree of hydration of CT which is considered to be essential to the catalytic activity of CT. This consideration may be supported by the fact that, as shown in Fig. 6, the rate of ATE synthesis decreased with a decreasing amount of water. Furthermore, the yield of ATE at prolonged reaction times up to 15 days which were considered to be enough to attain the equilibrium shows quite different dependency on water content from those shown in Fig. 3. In the lower range of water content, a prolonged reaction time gave much higher yields than those for 24 h reactions shown in Fig. 3. This suggests that the amount of active enzyme decreases with the decrease in water content for probably not only ATE but also ATyE and APE synthetic reactions. As in the

Table 2. Effect of Reaction Temperature[a]

Temp/ °C	$v \times 10^2$ μmol min^{-1} mg^{-1}	Yield/% 24 h	48 h	72 h
−3	3.2	73	71	
10	8.3	83	84	
20	12	82	82	
30	12	81	81	
35	12	85		
40	4.9	41	54	60
50	4.7	6.7	7.0	

a) AT 0.05 g, CT 0.01 g, EtOH 20 ml, phosphate buffer (0.1 M, pH 6.8) 0.5 ml.

Table 3. Effect of the Amount of Substrate[a]

AT/g	Reaction time/h	pH	ATE yield/% In EtOH[b]	In $CHCl_3$[c]
0.05	24	6.8	81	78
0.05	48	6.8	81	
0.075	24	6.8	79	
0.075	48	6.8	80	
0.10	24	6.8	86	
0.10	48	6.8	80	
0.15	24	6.8	75	
0.15	48	6.8	81	
0.15	72	6.8	84	
0.20	24	6.8	70	
0.20	48	6.8	85	
0.20	72	6.8	83	
0.20	96	6.8	85	4.4
0.20	96	5.0		2.7
0.20	96	8.6		3.5
0.20	96	9.0		2.0
0.25	24	6.8	26	
0.25	96	6.8	58	3.0

a) CT 0.01 g, phosphate buffer (0.1 M) 0.5 ml, 30 °C. b) EtOH 20 ml. c) 1 M EtOH in $CHCl_3$ 20 ml.

reaction in chloroform, in the absence of water the reaction was totally inhibited.

Figure 7 shows a plot of the calculated equilibrium constant K for ATE synthesis against water content. The K value may be affected by several factors, such as the dielectric constant of the medium; however, much slower reactions at very low concentrations of water are probably responsible for the sharp decrease in K in this region. It was found that CT loses activity at 50 °C, probably due to denaturation; the optimum temperatures for the ester synthesis were between 10 and 35 °C (Table 2).

Effect of the Amount of the Substrate. It was found that an increase in the initial amount of AT caused a decrease in the yield of ATE after 24 h reaction. However, in ethanol the yield of ATE increased upon increasing the reaction time, whereas in chloroform the yield was largely suppressed regardless of the reaction time and pH of the buffer solution. The results are summarized in Table 3.

These results imply that, when the initial amount of AT was increased, for example 0.2 g of AT in 20 ml of

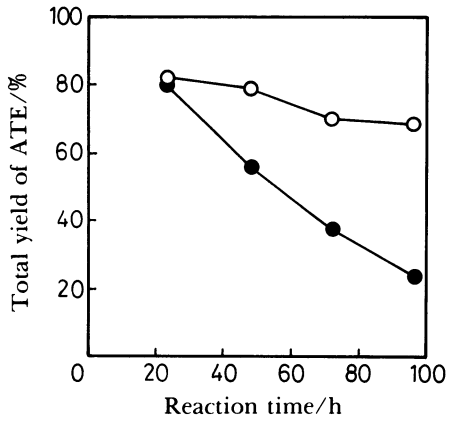

Fig. 8. Change in the yield of ATE in consecutive reactions. 0.05 g each of AT was added every 24 h. CT 0.01 g, EtOH (○) or chloroform (●) 20 ml, phosphate buffer (0.1 M, pH 6.8) 0.5 ml, 30 °C.

solvent, the reaction in ethanol is under kinetic control, but other factors must be taken into consideration for the reactions in chloroform, since the yields were very low even after prolonged reactions (below 5% after 96 h, Table 3). No distinct conclusion can be drawn from the present study, but it is probable that excess AT, which is considered to be concentrated in water phase, hinders CT activity.

Stability of Chymotrypsin. One of the most important aspects of enzymatic reactions is the stability of enzymes in the reaction media. Figure 8 shows the change in the total ATE yield in the course of successive reactions in which an equal amount of AT (0.05 g) was added every 24 h to the reaction mixtures containing constant amounts of CT and water. The total yield in ethanol decreased from 82 to 68% in going from the first reaction with 0.05 g AT for 24 h to the fourth reaction with total 0.20 g AT for 96 h. The yield decreased more rapidly in chloroform than in ethanol. The above result can not be attributed only to the instability of CT in chloroform, since the accumulation of AT may impair CT activity as mentioned above. The result, however, again exhibits the preference of the reaction in ethanol to that in chloroform when larger amounts of AT were employed.

Selectivity in Ester Synthesis. *N*-Acetylated L-alanine and L-leucine could not be esterified under the same conditions as those for aromatic amino acids. Also the yields of ethyl esters of *N*-acetylated D-tryptophan and D-phenylalanine were negligibly low. These results clearly indicate that substrate selectivity, stereospecificity, and therefore the native conformation of CT were strictly maintained in the present reaction system.

Klibanov et al.[17] reported that porcine pancreatic lipase maintained a high stability in various alcohols and that at very low water concentrations the enzyme exhibited a high activity, even at 100 °C. It seems from these results that enzymes require only small amounts

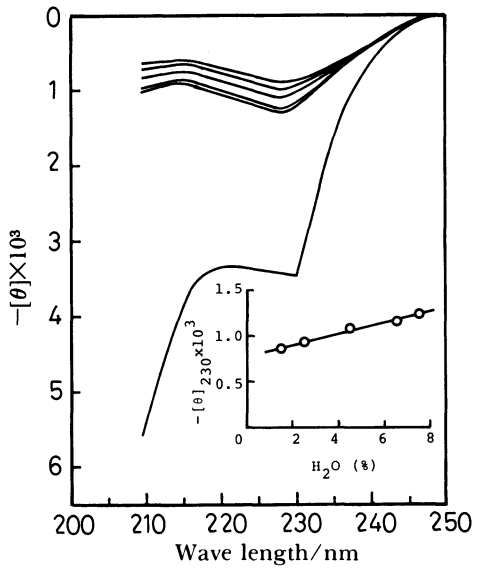

Fig. 9. CD spectra of CT in EtOH. From upper to lower water contents (%) are 1.5, 2.5, 4.5, 6.5, 7.5, and in neat water (pH 6.8). Insertion is the plot of $-[\theta]$ at 230 nm against water content.

Table 4. Effect of Alcohol Structure on AT Ester Synthesis[a)]

Alcohol	$v\times10^2$	Ester yield/%	
	μmol min^{-1} mg^{-1}	24 h	48 h
Methanol	0	0	0
Ethanol	12	81	81
1-Propanol	21	83	82
2-Propanol	17	78	79
1-Butanol	21	83	84
2-Butanol	12	65	78
2-Methyl-1-propanol	17	80	83
2-Methyl-2-propanol	0	0	0
1-Hexanol	0	40	61

a) AT 0.05 g, CT 0.01 g, alcohol 20 ml, phosphate buffer (0.1 M, pH 6.8) 0.5 ml, 30 °C.

of water for the maintenance of their native conformation in alcoholic solutions.

It was found that in the present system about 85% of added CT is suspended in ethanol and the rest of CT which is solubilized in ethanol is inactive. It has been reported that the content of α-helix in CT can be evaluated by the CD band at 230 nm, and the intensity of this band can be correlated with the enzyme activity.[18-20] Our data in ethanol is presented in Fig. 9. The CD spectrum in a phosphate buffer (pH 6.8) is also given in the Figure. It can be clearly seen that the intensity ($-[\theta]$) at 230 nm in ethanol decreases with a decrease in the water content, and is much lower than that in a buffer solution. Thus, the inactivity of solubilized CT in ethanol-water may be interpreted by the change in its conformation, probably due to a hydrophobic interaction between the enzyme and ethanol.

The results for the reactions of AT with different alcohols are summarized in Table 4. It is interesting that methanol and 2-methyl-2-propanol (*t*-butyl alcohol) are specifically inactive, while other alcohols gave esters in fairly good yields. Although no definite conclusion can be derived on the structure-reactivity relationship for the alcohols, it seems that CT exhibits rather a loose selectivity for the structure of alcohols as in the case of peptide synthesis in water where the amine components to some extent affect the reaction rate.[21]

Conclusion

The esterification of *N*-acetylated aromatic amino acids was realized in alcohols containing small amounts of water. The reaction mixture is considered to contain hydrated α-chymotrypsin (CT) suspended in alcohols which catalyzes the reaction, while CT in solution was found to be inactive. The reaction rate and the yield of the esters depended primarily on the water content in the reaction mixtures. The optimum reaction temperatures were between 10 and 35 °C. When the initial amount of AT was increased, CT suffered less of a substrate inhibition in ethanol than in chloroform. From the study of substrate selectivity, CT is considered to maintain its native conformation in this reaction system.

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