Tetrahedron Letters,Vol.26,No.49,pp 6081-6084,1985 0040-4039/85 $3.00 + .00
Printed in Great Britain

SYNTHESIS OF AROMATIC AMINO ACID ETHYL ESTERS BY α-CHYMOTRYPSIN IN SOLUTIONS OF HIGH ETHANOL CONCENTRATIONS†

Hideo Kise* and Hitoshi Shirato
Institute of Materials Science, University of Tsukuba
Sakura, Niihari, Ibaraki 305, Japan

Abstract: N-Acetyl-L-tryptophan and N-acetyl-L-tyrosine were converted to their ethyl esters by α-chymotrypsin in water-ethanol mixed solvents with ethanol concentration higher than 90 %. The effects of the solvent composition and the stability of α-chymotrypsin in these solutions are described.

Extensive studies of the effects of dipolar organic solvents on the kinetics of α-chymotrypsin(CT)-catalyzed hydrolyses were reported by Bender et al.[1,2] Enzymatic reactions below 0 °C (cryoenzymology) were also studied in mixed solvents such as methanol-water.[3] Although in several cases the addition of small amounts of organic solvents enhanced the enzyme activity,[4,5] there has been a general consideration that enzymes lose catalytic activity in solutions of high concentrations of water miscible organic solvents due to unfolding.[6]

The enzymatic reactions in the presence of organic solvents have been studied also with a view to utilize the hydrolytic enzymes as synthetic catalysts.[7-11] However, the lack of stability of enzymes in organic solvents has been the main problem in the organic synthesis by enzymes.

Recently studies have been made on the enzymatic synthesis in water-water immiscible organic solvents (two-phase method),[12-16] but little has been known about the enzymatic synthesis in homogeneous water-organic solvent systems. This paper describes a new approach to the enzymatic reactions in high concentrations of organic solvent; the conversion of aromatic amino acids to ethyl esters by α-chymotrypsin (CT) in ethanol containing small amounts of water.

In a typical reaction, CT (0.01 g) in 0.5 ml phosphate buffer (0.1 M, pH 6.8) was added to a mixture of N-acetyl-L-tryptophan (AT, 0.05 g, 0.203 mmol) and ethanol (20 ml). The mixture was incubated at 30 °C with constant shaking for 24 h. In a two-phase method, the initial reaction mixture

consisted of 1 M ethanol in chloroform (20 ml), 0.05 g of AT, and 0.01 g of CT in 0.5 ml phosphate buffer (0.1 M, pH 6.8). After the reaction, the solvents were evaporated by rotary evaporator, and the amounts of AT and N-acetyl-L-tryptophan ethyl ester (ATE) were determined by HPLC (column packing was JASCO Finepak SIL C18 and eluent was H_2O/acetonitrile 50/50 by volume).

Table 1. Effect of EtOH concentration on ATE yield[a)]

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

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

At first we studied the ATE synthesis by two-phase method, since it was considered that high concentration of ethanol in the homogeneous systems, which was required for the shift of equilibrium toward ATE synthesis, would impair the catalytic activity of CT. Actually however, when the concentration of ethanol was increased in the two-phase reactions with a constant volume of organic phase, the reaction system changed from heterogeneous to homogeneous, but the yield (conversion) of ATE decreased by less than 10 % (Table 1). The result indicates that the activity of CT was not much diminished in solutions of high ethanol concentrations (above 90 %) giving fairly high yield of ATE.

Since the yield of ATE was considered to be thermodynamic control and to depend primarily on the water/ethanol ratio, the effect of water content was examined. The results are collected in Table 2, along with those of N-acetyl-L-tyrosine ethyl ester (ATyE). It can be seen that the decrease in water content largely increased the yield of the esters. Thus one may expect that minimum amounts of water would favor the ester synthesis, but the limited solubility of CT made it difficult to study the reaction with lower contents

Table 2. Effect of water concentration in EtOH on ester yield[a)]

H_2O (vol %)	ATE		ATyE	
	Yield (%)	Kx10[b)]	Yield (%)	Kx10[b)]
2.4	78	4.1	87	4.1
4.9	69	3.8	68	3.7
9.8	46	3.0	45	2.8

a) CT 0.01 g in phosphate buffer (pH 6.8), AT or ATy 0.05 g, 24 h at 30 °C.

b) $K=[ATE \text{ or } ATyE][H_2O]/[AT \text{ or } ATy][EtOH]$.

of water. It was found, however, that without water the reaction was totally inhibited. The result suggests that a small amount of water is essential for the catalytic activity of CT.

The equilibrium constant K increased with the decrease in water content (Table 2) in favor of the ester synthesis. The reason for the change in K is not clear, but it is likely that the change in the nature of the reaction medium such as dielectric constant would cause the shift of the equilibrium.

Figure 1 shows the dependence of ATE yield on the pH value of the buffer solutions in which CT was dissolved. The pH profile is quite different from those of ordinary hydrolyses of peptides or amino acid esters in water where the activity of CT increases rapidly at pH 6-7 and reaches almost constant at pH 8.

From the practical point of view, one of the most important aspects in enzymatic reactions is the stability of the enzymes in the reaction media. Figure 2 shows the change in the total ATE yield in the course of a successive reaction in which an equal amount of AT (0.05 g) was added every 24 h to the reaction mixture containing a constant amount of CT. The total yield of ATE decreased from 82 % to 68 % in going from the first reaction with 0.05 g AT in 24 h to the fourth reaction with total 0.20 g AT in 96 h. The Figure

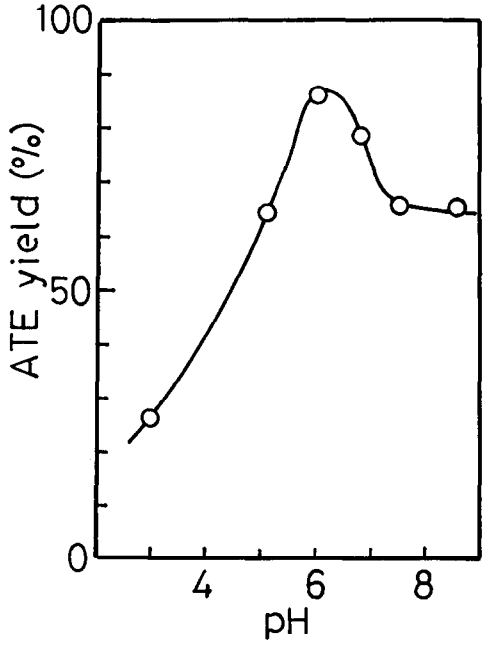

Fig. 1. Effect of pH on ATE synthesis. CT 0.01 g in 0.1 M phosphate buffer (0.5 ml), EtOH 20 ml, AT 0.05 g, 30 °C, 24 h.

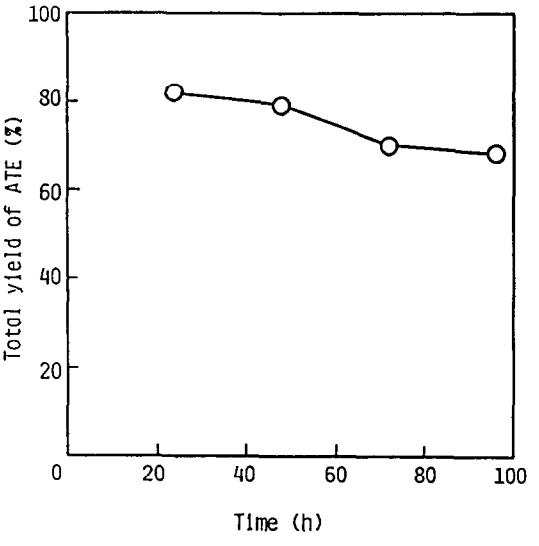

Fig. 2. Change in activity of CT in consecutive ATE synthesis. EtOH 20 ml, CT 0.01 g in 0.1 M phosphate buffer (0.5 ml, pH 6.8), AT 0.05 g each, 30 °C.

exhibits fairly good stability of CT in the present reaction medium. It was found that N-acetyl-D-tryptophan could not be esterified at all under the same conditions as those for the L-isomer. This indicates that CT maintains its native conformation in these reaction mixtures.

It should be noted that Klibanov and a coworker observed high stability of porcine pancreatic lipase in various alcohols.[17] They claimed that with much reduced amounts of water the enzyme exhibited high activity even at 100 °C. This and our results seem to suggest that enzymes require only small amounts of water for the catalytic activity in alcoholic solutions.

The conformation of CT in water-ethanol solutions by spectroscopic method and the change in the substrate selectivity in these systems are currently under investigation.

References

1) G. E. Clement and M. L. Bender, Biochemistry, 2, 836 (1963).
2) K. Tanizawa and M. L. Bender, J. Biol. Chem., 249, 2130 (1974).
3) B. H. J. Bielski and S. Freed, Biochim. Biophys. Acta, 89, 314 (1964).
4) K. H. Tan and R. Lovrien, J. Biol. Chem., 247, 3278 (1972).
5) J. L. Miles, E. Morey, F. Crain, S. Gross, J. S. Julian, and W. J. Canady, J. Biol. Chem., 237, 1319 (1962).
6) T. T. Herskovits, B. Gadegbeku, and H. Jaillet, J. Biol. Chem., 245, 2588 (1970).
7) E. A. Sym, Biochem. J., 30, 609 (1936).
8) M. Yamashita, S. Arai, S. Tanimoto, and M. Fujimaki, Biochim. Biophys. Acta, 358, 105 (1974).
9) K. Morihara and T. Oka, Biochem. J., 163, 531 (1977).
10) G. A. Homandberg, J. A. Mattis, and M. Laskowski, Jr., Biochemistry, 17, 5220 (1978).
11) K. Nilsson and K. Mosbach, Biotech. Bioeng., 26, 1146 (1984).
12) R. G. Ingalls, R. G. Squires, and L. G. Butler, Biotech. Bioeng., 17, 1627 (1975).
13) A. M. Klibanov, G. P. Samokhin, K. Martinek, and I. V. Berezin, Biotech. Bioeng., 19, 1351 (1977).
14) P. Kuhl, A. Könnecke, G. Döring, H. Däumer, and H.-D. Jakubke, Tetrahedron lett., 21, 893 (1980).
15) B. Cambou and A. M. Lkibanov, J. Am. Chem. Soc., 106, 2687 (1984).
16) H. Schutt, G. Schmidt-Kastner, A. Arens, and M. Preiss, Biotech. Bioeng., 27, 420 (1985).
17) A. Zaks and A. M. Klibanov, Science, 224, 1249 (1984).

†Enzymatic Reactions in Aqueous-Organic Media. 1.

(Received in Japan 22 August 1985)