Enzymatic Reactions in Aqueous-Organic Media. XVII. Optical Resolution of Amino Acid Esters by Enzymatic Hydrolysis in Organic Solvents[1)]

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Enantiospecific hydrolysis of DL-tyrosine ethyl ester was carried out using α-chymotrypsin (CT) as a catalyst in organic solvents, and L-tyrosine was obtained with high optical purity. Activity and enantiospecificity of CT were found to be greatly altered by water content in the reaction media, and generally high enantiospecificity was observed at low water contents. Many of natural and unnatural amino acid esters were resolved by hydrolysis in organic solvents using CT, subtilisin Carlsberg, or subtilisin BPN′ as catalysts.

The abilities of enzymes to act as effective catalysts in organic solvents are well documented.[2–5)] There are many advantages in employing organic solvents for enzymatic reactions, such as increase in substrate solubility and shift of reaction equilibrium in favor of synthesis as opposed to hydrolysis. Thus, a variety of enzymes have been utilized in organic solvents for ester or peptide synthesis[6,7)] and conversion of lipophilic substrates.[8,9)]

It has been demonstrated that enzyme properties can be altered by the nature of reaction medium. In particular, substrate specificity[10,11)] and enantiospecificity[12,13)] of enzymes can be different in organic solvents from those in aqueous solutions. When a hydrophilic (water-miscible) organic solvent is used for an enzyme reaction, water content can also be a crucial factor controlling enzyme specificities.[14–16)] We already reported an unusual solvent effect on protease activity for hydrolysis of amino acid esters in acetonitrile,[17)] and change in substrate specificity with composition of water–acetonitrile mixed solvents.[11)] In the present work, we investigated the effects of reaction medium, especially the nature of organic solvent and composition of aqueous-organic cosolvents, on activity and enantiospecificity of serine protease for hydrolysis of racemic amino acid esters. Our attention has been focused on reactions at high concentrations of organic solvents, since hydrolytic reactions under these unusual conditions have rarely been studied. The advantages of using high concentrations of organic solvents for hydrolytic reactions will be demonstrated as a new method of optical resolution of amino acids.

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 purchased from Sigma Chemical Co. Subtilisin Carlsberg (protease type VIII)(STC) and subtilisin BPN′ (protease type XXVII) (STB) having specific proteinase activities for casein of 11.6 and 6.8 units, respectively, and hydrochlorides of esters of DL-phenylalanine, tryptophan, alanine, valine, and threonine were also purchased from Sigma. The hydrochlorides of other DL-amino acid ethyl esters were prepared by the reactions of DL-amino acids with ethanol in the presence of thionyl chloride. In the case of DL-tyrosine and DL-DOPA (3,4-dihydroxyphenylalanine), free esters were obtained by dehydrochlorination with aqueous solutions of sodium carbonate and recrystallized from ethyl acetate or ethyl acetate–ether. Other ester hydrochlorides were recrystallized from ethanol–ether. The purity of the products was confirmed by NMR and elemental analysis. All the solvents used were guaranteed grade and dried on 3A molecular sieves.

Optical Resolution. Typically, an organic solution (18 ml) of DL-amino acid ethyl ester (or its hydrochloride) was added to an aqueous solution (2 ml) of an enzyme (5 mg). Except for esters of DL-tyrosine and DL-DOPA, equimolar amounts of triethylamine to ester salts were added to the organic solution for dehydrochlorination of the esters. The reaction mixture was incubated with constant reciprocal shaking (about 150 cycles per min) at 30 °C for 24 h. After the reaction, precipitates were collected by filtration and dried under vacuum. The product was dissolved in water and the solution was subjected to ultrafiltration with Advantec UP-20 ultrafilter. Then the product was crystallized by evaporation of water and dried under vacuum.

Specific optical rotation of the products was measured in 1 or 5M HCl solution (1 M=1 mol dm^{-3}) with a polarimeter JASCO DIP-140 (optical path length 100 mm). Average of ten measurements was taken as the specific rotation of the sample. In the case of DL-DOPA, crude hydrolysis products were dissolved in 1M HCl and, after ultraflitration, specific optical rotation was measured.

Results and Discussion

Solvent Effect on Resolution of DL-Tyrosine Ethyl Ester. The ability of α-chymotrypsin (CT), either free or immobilized to solid supports, to act as a catalyst for ester or peptide synthesis in organic solvents is well documented.[6,7,18)] As for ester and peptide synthesis, activity of CT is strongly dependent on the nature of organic solvents.[11,19–22)] Therefore, initially we studied on the effect of the nature of organic solvents on the activity and enantiospecificity of CT for hydrolytic reactions. Table 1 summarizes the results of hydrolysis of

Table 1. Resolution of DL-Tyrosine Ethyl Ester by CT[a)]

Solvent	Yield/%	e.e./%	*E*
Methanol	0	—	—
Ethanol	34	96	80
1-Propanol	46	89	39
2-Propanol	41	85	22
1-Butanol	20	95	49
2-Methyl-1-propanol	13	>99	>230
2-Butanol	42	96	102
2-Methyl-2-propanol	39	83	18
1-Pentanol	38	93	49
3-Methyl-1-butanol	41	92	46
2-Methyl-2-butanol	41	82	18
Tetrahydrofuran	49	>99	>752
1,4-Dioxane	49	95	126
Acetonitrile	48	>99	>646
Acetone	50	>99	>1060
DMF[b)]	32	92	37
DMA[b)]	41	>99	>412

a) DL-Tyrosine ethyl ester 50 mM, CT 10 μM, solvent 18 ml, water 2 ml, 30 °C, 24 h. b) Water 50%.

DL-tyrosine ethyl ester by CT in organic solvents containing 10% water. Since the solubilities of tyrosine are very low in all of the solvents used, the hydrolysis products were precipitated and easily separated by filtration. It can be seen that in general aprotic solvents, THF, 1,4-dioxane, acetonitrile, and acetone, gave higher product yields than alcohols. Similarly to ester and peptide synthesis,[23–25)] activity of CT for hydrolysis was markedly retarded in methanol. Only a small amount of tyrosine methyl ester was detected, which was produced by transesterification between tyrosine ethyl ester and methanol. This implies that water does not act as a nucleophile for acyl-enzyme intermediate, probably because the binding site of nucleophile to CT may be occupied exclusively by methanol. Interestingly, in propanols and higher alcohols, hydrolysis of tyrosine ethyl ester predominated over transesterification in spite of large excess of alcohols over water. This may be the consequence of the fact that hydrolysis product is insoluble and isolated from the reaction equilibrium which controls the product distribution in solution.

As will be described later, CT loses catalytic activity in DMF and DMA (*N,N*-dimethylacetamide) at water contents below 30%. It may be assumed that the conformation of CT is significantly altered, or, alternatively, the organic solvents replace water molecules at nucleophile binding sites, since DMF is known to be a competitive inhibitor and binds to the same binding site of CT as water.[26)] At 50% or higher water contents, the reactions gave hydrolysis product in fairly high yields.

The enantiomeric excess (e.e.) of the produced tyrosine is listed in Table 1. Optically pure products were obtained in 2-methyl-1-propanol, THF, acetonitrile, acetone, and DMA. However, since the value of e.e. is generally a function of conversion of the substrate, the ability of an enzyme to discriminate between enantiomers of racemic substrates is normally expressed as the *E* value.[27–29)] The calculated *E* values in Table 1 demonstrate that CT exhibits high ability of enantiomer discrimination in acetone, THF, and acetonitrile, followed by DMA, 2-methyl-1-propanol, and 1,4-dioxane. It should be noticed that, except for 2-methyl-1-propanol, CT gives high *E* values in aprotic solvents. No distinct conclusions can be drawn at present, but the above results seem to suggest that the nature of solvents strongly influences the rigidity of enzymes[13)] thereby changing the enantiospecificity of the enzymes.

Effect of Water Content. As described previously, water content in an organic solvent is a strong factor to control catalytic activity and in some cases substrate specificity of an enzyme. However, few reports have appeared on the effects of water content on enzyme enantiospecificity, especially at high concentrations of organic solvents.[13)] Therefore, the next step was to investigate the dependence of activity and enantiospecificity of CT on solvent composition for hydrolysis of racemic tyrosine ethyl ester.

Figure 1 demonstrates the effect of water content in mixed solvents of acetonitrile–water on the yield and e.e. of the hydrolysis product of racemic tyrosine ethyl ester. Dramatic change in activity of CT is observed by changing water content, especially at about 70% water.[17)] The loss of activity at 70% water may most probably be ascribed to conformational change of peptide chains in the enzyme, since by changing the solvent composition, activity of CT was recovered. When an aqueous phosphate buffer solution was used instead of pure water as a solvent, poor results were obtained with respect to yield and e.e. value of the hydrolysis product (Fig. 2). It was found that, in pure water or buffer

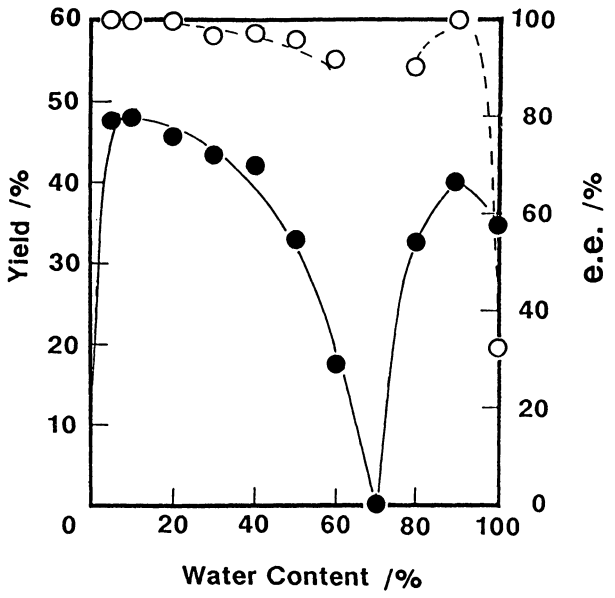

Fig. 1. Resolution of DL-tyrosine ethyl ester in acetonitrile–water. DL-Tyrosine ethyl ester 50 mM, CT 10 μM, 30 °C, 24 h. ○: e.e.; ●: yield.

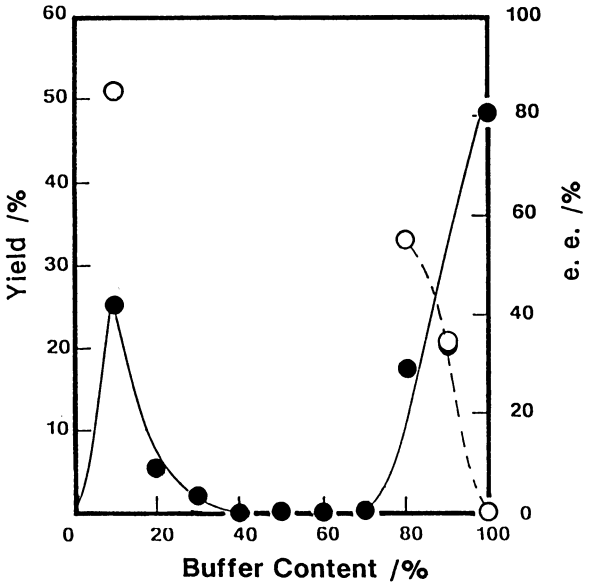

Fig. 2. Resolution of DL-tyrosine ethyl ester in acetonitrile–aqueous phosphate buffer (0.1 M, pH 8.0). DL-Tyrosine ethyl ester 50 mM, CT 10 μM, 30 °C, 24 h. ○: e.e.; ●: yield.

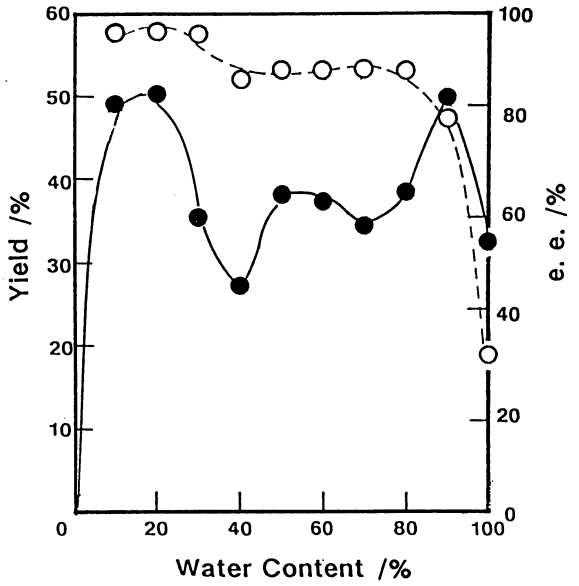

Fig. 4. Resolution of DL-tyrosine ethyl ester in 1,4-dioxane–water. DL-Tyrosine ethyl ester 50 mM, CT 10 μM, 30 °C, 24 h. ○: e.e.; ●: yield.

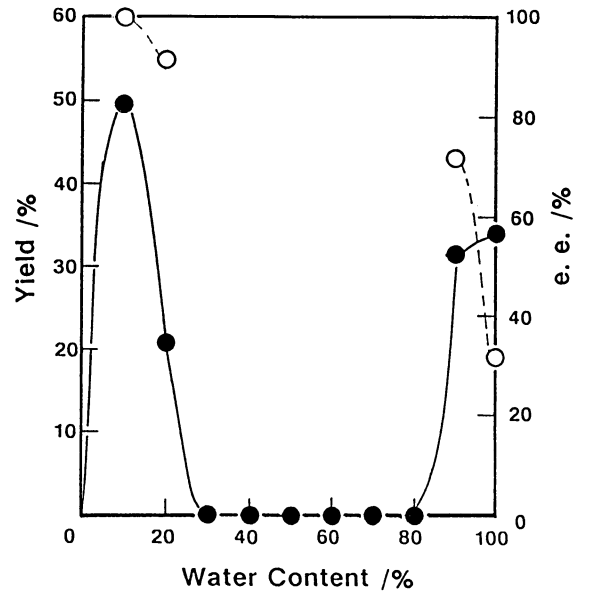

Fig. 3. Resolution of DL-tyrosine ethyl ester in THF–water. DL-Tyrosine ethyl ester 50 mM, CT 10 μM, 30 °C, 24 h. ○: e.e.; ●: yield.

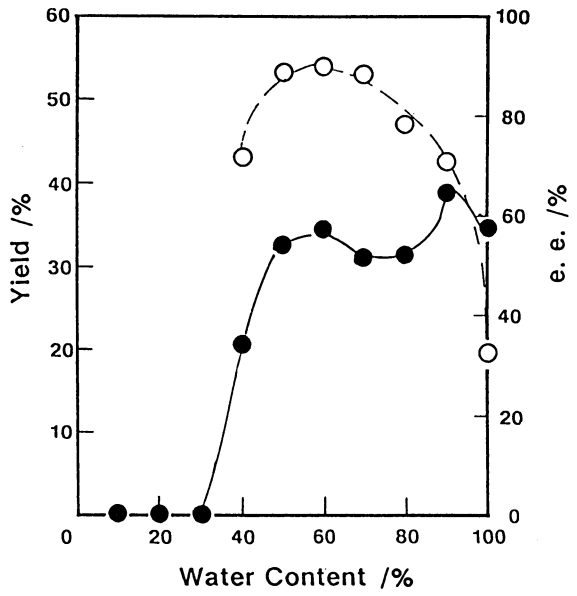

Fig. 5. Resolution of DL-tyrosine ethyl ester in DMF–water. DL-Tyrosine ethyl ester 50 mM, CT 10 μM, 30 °C, 24 h. ○: e.e.; ●: yield.

solutions, hydrolysis of tyrosine ethyl ester occurred without enzyme. This suggests that the reaction is catalyzed by acidic or basic species, and this non-enzymatic hydrolysis may be responsible for the low e.e. values in aqueous solutions as shown in Figs. 1 and 2.

When THF was used as a cosolvent, loss of catalytic activity was observed at water contents between 30 and 80% (Fig. 3), while in the case of 1,4-dioxane, relatively high activity was retained at the whole range of the solvent composition (Fig. 4). Interestingly, high values of e.e. were obtained at low water contents for these organic solvents. Ten to 20% water is optimum for effective optical resolution of DL-tyrosine ethyl ester in these solvents.

It has been known that DMF, which is one of the solvents with strongest solubilizing power for organic compounds including amino acid derivatives, often causes inactivation of enzymes.[30,31] As shown in Fig. 5, CT exhibits activity at water contents higher than 40% affording hydrolysis products in fairly good yields.

Table 2. Resolution of Amino Acid Ethyl Esters in Acetonitrile[a)]

Amino acid	CT			STC			STB		
	Yield	e.e.	*E*	Yield	e.e.	*E*	Yield	e.e.	*E*
Tyrosine	48	>99	>646	45	96	118	40	97	129
Phenylalanine[b)]	46	87	32	37	94	56	37	70	8
Tryptophan	43	92	50	6	—	—	0	—	—
Alanine	9	80	10	44	86	27	39	77	12
Valine[b)]	5	—	—	5	—	—	6	—	—
Leucine	5	86	14	37	94	56	30	97	99
Threonine[b)]	0	—	—	0	—	—	0	—	—
p-Chlorophenylalanine	3	82	10	17	78	9	13	99	230
DOPA	50	>99	>1060	38	88	27	49	81	22
Phenylglycine	9	84	12	4	54	3	3	—	—
2-Aminobutanoic acid	10	—	—	20	92	30	26	95	54
2-Aminopentanoic acid	7	67	5	22	80	11	34	85	18
2-Aminohexanoic acid	7	—	—	24	84	15	32	95	61

a) Amino acid esters 50—100 mM, enzyme 5 mg, acetonitrile 18 ml, water 2 ml, 30 °C, 24h. Yield and e.e. in %. b) Methyl ester.

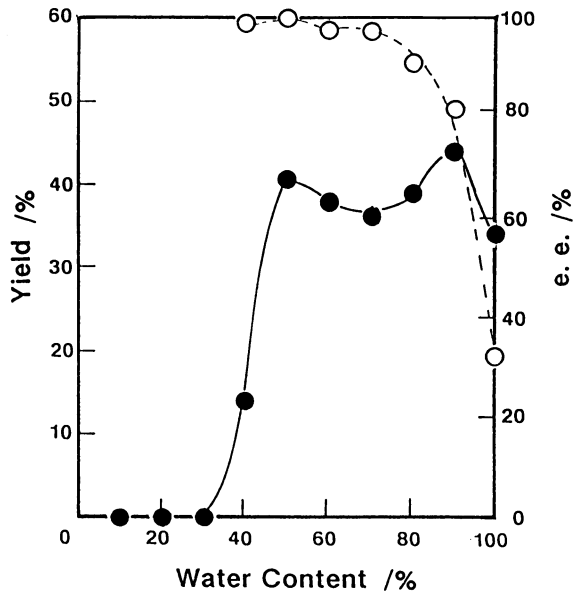

Fig. 6. Resolution of DL-tyrosine ethyl ester in DMA–water. DL-Tyrosine ethyl ester 50 mM, CT 10 μM, 30 °C, 24 h. ○: e.e.; ●: yield.

The highest value of e.e. was obtained at about 60% water. Similarly to DMF, DMA exhibits catalytic activity at water contents above 40%, but gives hydrolysis products with much higher e.e. than DMF (Fig. 6).

Preparative Resolution of Racemic Amino Acid Esters. The medium effects described above on activity and enantiospecificity of CT for racemic tyrosine ethyl ester indicated that cosolvent systems of selected aprotic organic solvents with 10 to 20% water were optimum media for optical resolution of amino acids esters. This led us to study on preparative resolution of structurally different amino acid esters. Table 2 summarizes the results of optical resolution of several amino acid esters including unnatural amino acid esters in acetonitrile containing 10% water. Three serine proteases, CT, subtilisin Carlsberg (STC), and subtilisin BPN' (STB), were used as catalysts, in order to examine substrate specificities of these enzymes under these unusual reaction conditions. It can be seen that CT is highly specific to natural aromatic amino acids and DOPA (3,4-dihydroxyphenylalanine) affording hydrolysis products with high e.e. and *E* values. STC and STB are specific to aromatic amino acids except for tryptophan and also to several aliphatic amino acids such as alanine and leucine. STB is the best catalyst for resolution of linear aliphatic amino acids (the last three compounds in Table 2). For all the enzymes esters of 2-amino-2-phenylacetic acid (phenylglycine) and *p*-chlorophenylalanine were poor substrates.

In conclusion, it was found that serine proteases (CT, STC, and STB) maintain catalytic activity in many organic solvents at low water contents, and they exhibit high abilities of enantiomeric discrimination in hydrolysis of racemic amino acid esters. The method can be applied to effective resolution of many amino acids. The advantages in using organic solvents for resolution by hydrolysis are high enantiospecificity of enzymes, inhibition of nonenzymatic hydrolysis, and easy separation of enantiomers by virtue of low solubilities of the reaction products.

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