Enantiospecificity of α-Chymotrypsin at S'_1 Site for Peptide Synthesis in Aqueous–Organic Media

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The enantiospecificity of α-chymotrypsin (CT) at S'_1 site in aqueous–organic media was investigated for peptide synthesis from *N*-acetyl-L-tyrosine ethyl ester (ATEE) as an acyl donor and alanine or serine derivatives as acyl acceptors. It was found that catalytic activity of CT is a function of nature of organic solvent, and, among the solvents used, CT exhibited high activity in *t*-butyl alcohol, acetone, and acetonitrile. The relative reactivity of L-alaninamide as an acyl acceptor to that of D-isomer was also significantly dependent on nature of solvent. CT exhibited high specificity to L-isomer in these three organic solvents. The enantiospecificity is also a function of water content in reaction media. In acetonitrile, the maximum reaction rates of ATEE with L- and D-alaninamide were obtained at 10 and 6% water, respectively, and relative reactivity of D-isomer increased with decrease in water content. Structural effects of acyl acceptors on their reactivity are striking and discussed in terms of stability of their complexes with the acyl-enzyme intermediate.

Recently extensive studies have been made on enzymatic reactions in organic solvents with restricted amounts of water.[1] In these reaction systems, enzymes form aggregates and are usually suspended as small particles in solutions. It has been recognized that enzyme activities are functions of many factors such as nature of organic solvent and water content in reaction media.[1] Furthermore, substrate specificity of enzymes can be altered by changing organic solvent or water content.[2—4]

Organic solvents may perturb enzyme structure by direct interaction with enzymes or by changing dissociation state of ionic groups of the enzymes. The hydrophobic interactions between a substate and the binding site of an enzyme may also be altered by switching from one solvent to another. These would lead to changes in enantiospecificity of the enzymes. In fact, several enzymatic reactions have been reported in which enantiospecificities of enzymes are dependent on nature of organic solvent; examples are aminolysis,[5] ester synthesis,[6] and transesterification[7—10] by subtilisin or lipases. Furthermore, a complete reversal of enzyme enantiospecificity was reported upon a change in the solvent.[11] Also it was found that water content in organic solvents affects enantiospecificity of enzymes in ester synthesis[12] or transesterification.[8]

It is known that a number of biologically important peptides contain D-amino acid residues.[13—15] Incorporation of D-amino acid derivatives into peptides by enzymatic reactions has been reported by several workers.[16—20] The reactions have been carried out mostly in aqueous or aqueous–organic cosolvent systems. Relatively few studies have included the reactions in organic solvents with restricted amounts of water.[21—23] So far, solvent effect on enantiospecificity of proteases for peptide synthesis has not been studied extensively. In the present work, the enantiospecificity of α-chymotrypsin (CT) was investigated for peptide synthesis from *N*-acetyl-L-tyrosine ethyl ester (ATEE) and amino acid amides or esters in aqueous–organic mixed solvents. CT is one of the enzymes which have been most frequently utilized for peptide synthesis owing to its high catalytic activity in both aqueous and organic solutions. Our attention has been focused on effects of nature of organic solvents and water content on ability of CT to discriminate enantiomeric amino acid derivatives as amine components (acyl acceptors) in peptide synthesis. The binding mode of amine components at S'_1 site of CT is also discussed as relevant to their reactivity for peptide synthesis.

Experimental

Materials. α-Chymotrypsin (EC 3.4.21.1, CT) from bovine pacreas and *N*-acetyl-L-tyrosine ethyl ester (ATEE) were purchased from Sigma Chem. Co. Hydrochlorides of L-alaninamide, L-alanine methyl ester, and L-serine methyl ester were also purchased from Sigma. Hydrochlorides of D-alanine methyl ester and D-serine methyl ester were prepared by the reaction of D-alanine and D-serine with methanol in the presence of thiony chloride and purified by recrystallization from methanol–ether. D-Alanine methyl ester was then converted to D-alaninamide hydrochloride by reaction with ammonia followed by treatment with hydrogen chloride.[24] The product was purified by recrystallization from methanol–ethyl acetate. The purity of the products was confirmed by NMR and elemental analysis. All the solvents were guaranteed grade and dried on molecular sieves 3A.

Peptide Synthesis. A typical synthetic reaction of peptide from ATEE and L- or D-alaninamide hydrochloride was carried out as follows: A solution of alaninamide hydrochloride (0.1 mmol) and triethylamine (0.1 mmol) in water (0.45 ml) was added to a solution of ATEE (0.1 mmol) and acetanilide (25 mg), which was an internal standard for HPLC analysis, in acetonitrile (10 ml). Then, a solution of CT (0.5 mg) in water (0.05 ml) was added to the above solution, and the mixture was incubated at 30 °C with constant reciprocal shaking (about 150 cycles per min). This method guarantees enough hydration of the enzyme, which is in dispersed (solid) state, to be activated even at the initial stage of the reaction. Triethylamine was used for dehydrochlorination of alaninamide hydrochloride to form free

alaninamide. The final concentrations of ATEE and alaninamide were 10 mM. Aliquots of the reaction mixture were taken at intervals and filtered by poly(tetrafluoroethylene) membrane filters and injected into an HPLC (Shimadzu LC-6A). Shim-pack CLC-ODS column (0.15 m×6.0 mm) was used and eluted with water–acetonitrile (50/50 by volume). Reaction components were detected with a UV detector at 270 nm. The initial reaction rate was calculated from the slope of linear relation between reaction time and amounts of the products using a least square method.

Result and Discussion

Time Course of Peptide Synthesis. Figures 1 and 2 illustrate time course of reactions of *N*-acetyl-L-tyrosine ethyl ester (ATEE) with L- and D-alaninamide, respectively, by the catalysis of α-chymotrypsin (CT) in acetonitrile containing 4.8% water. It is known that the reaction proceeds via an acyl-enzyme intermediate, *N*-acetyltyrosyl-CT.[25] The competitive nucleophilic attack of alaninamide and water on the intermediate gives dipeptide (Ac–L-Tyr–Ala–NH_2) and hydrolysis product (Ac–L-Tyr–OH), respectively (Scheme 1). As shown in Fig. 1, when L-alaninamide was used, the amount of hydrolysis product was small throughout the reaction, reflecting much higher reactivity of L-alaninamide than water. In contrast, when D-alaninamide was used as an amine component, considerable amounts of hydrolysis product were formed at early stage of the reaction. However, with increase in reaction time, hydrolysis product diminished while the amount of peptide increased monotonously. After 24 h reaction, the dipeptide (Ac–L-Tyr–D-Ala–NH_2) was obtained in 95% yield which is comparable to that from L-isomer. The result indicates that hydrolysis product again enters into reaction to form acyl-enzyme intermediate and then peptide (Scheme 1). This means that peptide formation is thermodynamically favorable over hydrolysis, and it seems that the present method is useful for peptide systhesis containing D-amino acid residues as well as L-isomers.

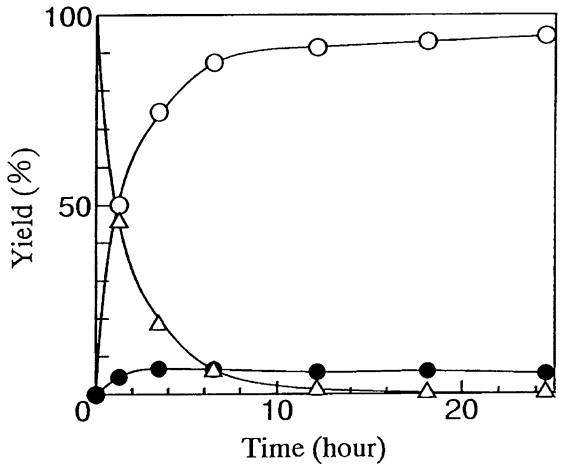


Fig. 1. Time course of reaction of ATEE with L-alaninamide by CT. ATEE 10 mM, L-alaninamide 20 mM, CT 1 mg, acetonitrile 10 ml, water 0.5 ml, 30 °C. ○: Ac–L-Tyr–L-Ala–NH_2; ●: Ac–L-Tyr–OH; △: ATEE.

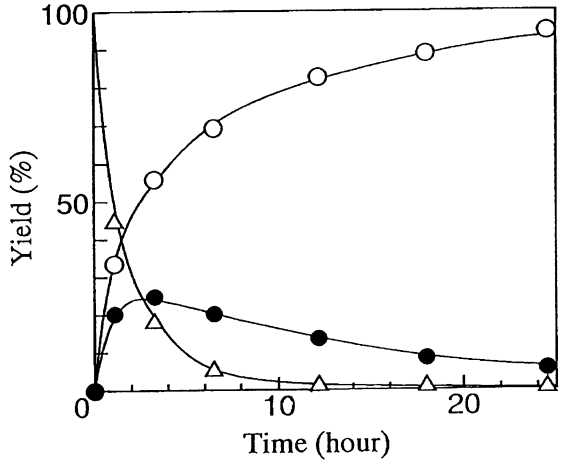
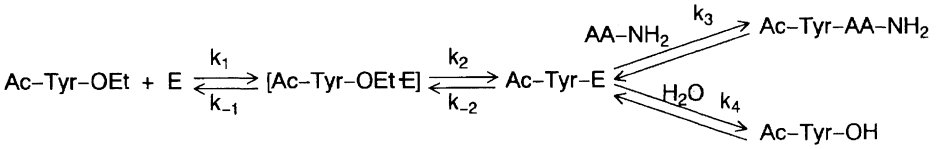


Fig. 2. Time course of reaction of ATEE with D-alaninamide by CT. ATEE 10 mM, D-alaninamide 20 mM, CT 1 mg, acetonitrile 10 ml, water 0.5 ml, 30 °C. ○: Ac–L-Tyr–D-Ala–NH_2; ●: Ac–L-Tyr–OH; △: ATEE.

Effect of Organic Solvent. It has been reported that reaction rate of peptide synthesis by CT in organic solvents is strongly dependent on nature of organic solvent.[4,26,27] Therefore, reactivity of L- and D-alaninamide with ATEE was investigated in several organic solvents and the results are summarized in Table 1. It can be seen that rate of peptide formation is markedly altered on going from a solvent to another. Among the solvents used *t*-butyl alcohol, acetone, and acetonitrile are preferable to others in terms of catalytic activity of CT. *t*-Pentyl alcohol, which was reported to be a competitive inhibitor of CT,[28] is a poor solvent as compared to its close homologue *t*-butyl alcohol.

The reaction selectivity, which is expressed by $v_L/(v_L+v_H)$ or $v_D/(v_D+v'_H)$ in Table 1, is not so much dependent on nature of solvent as total reaction rate (v_L+v_H or $v_D+v'_H$). This implies that relative reactivity and local concentration of alaninamides and water around active site of the enzyme are not much affected by nature of these solvents. No correlations were found between activity of CT and solvent parameters, such as dielectric constant and log P[29] which has been frequently employed for enzymatic reactions in organic solvents. In the present reactions, hydrophobic solvents are not suitable because of low solubilities of substrates, and therefore it is difficult to examine solvent effects over wide range of solvent parameters.

The enantiospecificity of CT expressed by v_L/v_D alters significantly by changing organic solvent as seen in Table 1. There is a correlation between catalytic activity and enantiospecificity of CT in these organic solvents; the higher the activity (v_L or v_D), the higher the enantiospecificity (v_L/v_D). An inspection of Table 1



Scheme 1.

Table 1. Solvent Effect on Initial Reaction Rate of ATEE with L- and D-Alaninamide by α-Chymotrypsin[a)]

Solvent	Initial rate (μmol min^{-1} mg^{-1})[b)]								
	L-Alaninamide				D-Alaninamide				
	v_L	v_H	v_L+v_H	$v_L/(v_L+v_H)$	v_D	v'_H	$v_D+v'_H$	$v_D/(v_D+v'_H)$	v_L/v_D
t-Butyl alcohol	7.5	1.9	9.4	0.80	1.7	6.4	8.1	0.21	4.5
Acetone	5.6	0.56	6.16	0.91	2.1	2.8	4.9	0.43	2.7
Acetonitrile	5.0	0.40	5.40	0.93	1.9	2.1	4.0	0.48	2.7
THF	1.3	0.14	1.44	0.90	0.71	1.3	2.01	0.35	1.8
1,4-Dioxane	0.77	0.057	0.827	0.93	0.51	0.55	1.06	0.48	1.5
Propylene carbonate	0.23	0.062	0.292	0.79	0.16	0.31	0.47	0.34	1.4
t-Pentyl alcohol	0.13	0.065	0.195	0.67	0.08	0.22	0.30	0.26	1.7

a) ATEE 10 mM, alaninamide hydrochloride 10 mM, triethylamine 10 mM, solvent 10 ml, water 0.5 ml, CT 0.5 mg, 30 °C. b) v_L and v_D: peptide synthesis; v_H and v'_H: hydrolysis.

reveals that relaxation of enantiospecificity in solvents from top to bottom of the table comes mainly from decreases in v_L rather than change in v_D. The result may be attributed to modification of enzyme structure which results in decrease in specific binding of the substrate to the enzyme.

Effect of Water Content on Enantiospecificity. It is well-known that activities of proteases for peptide synthesis in organic solvents are strongly influenced by water content.[4,22,26,27,30)] However, the effect of water content on enantiospecificity of proteases for peptide synthesis has been rarely investigated. Therefore, next we studied the effect of water content on enantiospecificity of CT for coupling reactions of ATEE with L- or D-alaninamide in acetonitrile containing different amounts of water. As expected, by increasing water content, reaction selectivity for peptide synthesis (ratio of rate of peptide formation to that of hydrolysis) decreases sharply (Figs. 3 and 4). However, it should be noted that, at 4.5% water (2.5 M, 1 M=1 mol dm^{-3}), peptide formation from D-alaninamide (10 mM) is comparable to hydrolysis in spite of large excess of water. This means that D-alaninamide is still about 250 times more reactive than water. More interestingly, it can be seen clearly that enantiospecificity expressed by v_L/v_D is also a strong function of water content (Fig. 5); the value of v_L/v_D increases more than 20 fold by changing water content from 2 to 40%. Maximum reaction rates with D- and L-alaninamides were obtained at about 6 and 10% water, respectively. At water contents below 6%, the ratio are lower than 2, indicating a significant relaxation of enantiospecificity.

Several reports have appeared on the effects of water content on enantiospecificity of enzymes in organic solvents. For example, enantiospecificity of a lipase for

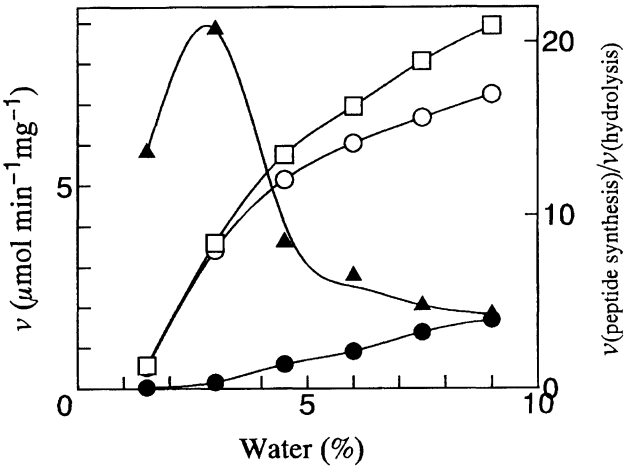


Fig. 3. Effect of water content on reaction rate of ATEE with L-alaninamide by CT. ATEE 10 mM, L-alaninamide 10 mM, CT 0.5 mg, acetonitrile–water 10 ml, 30 °C. ○: Ac-L-Tyr-L-Ala-NH_2; ●: Ac-L-Tyr-OH; □: total rate; ▲: relative rate.

ester synthesis from 1-butanol and racemic 2-bromopropanoic acid in hexane increased with increase in water content.[12)] However, in the case of transesterification between racemic 1-phenylethanol and vinyl butanoate in dioxane, the enantiospecificity of subtilisin Carlsberg decreased with increase in water content.[8)] These results suggest that effect of water content on enzyme specificity is highly specific to the nature of enzyme and solvent.

The result obtained in the present work may be ratinalized as illustrated in Fig. 6. It has been deduced that, in the case of peptide synthesis from ATEE and amino acid amides by CT, the nucleophile is bound

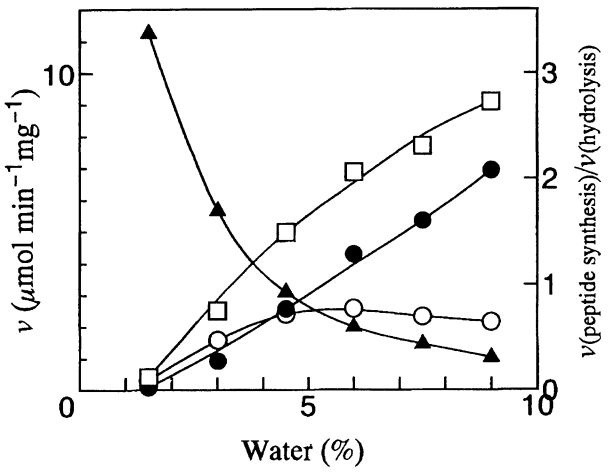


Fig. 4. Effect of water content on reaction rate of ATEE with D-alaninamide by CT. ATEE 10 mM, D-alaninamide 10 mM, CT 0.5 mg, acetonitrile–water 10 ml, 30 °C. ○: Ac–L-Tyr–D-Ala–NH_2; ●: Ac–L-Tyr–OH; □: total rate; ▲: relative rate.

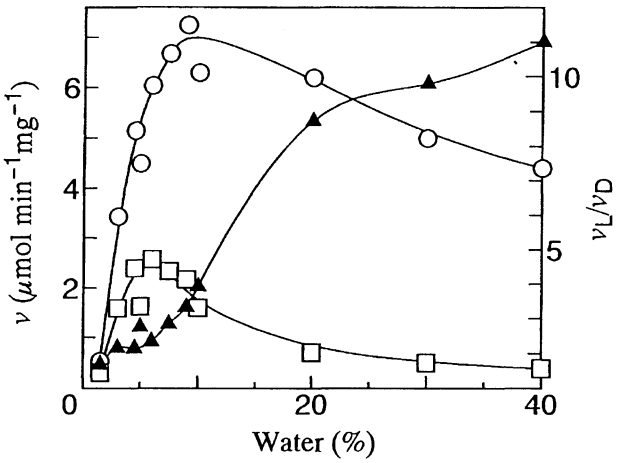


Fig. 5. Effect of water content on reaction rate of ATEE with L- and D-alaninamide by CT. ATEE 10 mM, alaninamide 10 mM, CT 0.5 mg, acetonitrile–water 10 ml, 30 °C. ○: Ac–L-Tyr–L-Ala–NH_2; □: Ac–L-Tyr–D-Ala–NH_2; ▲: relative rate (v_L/v_D).

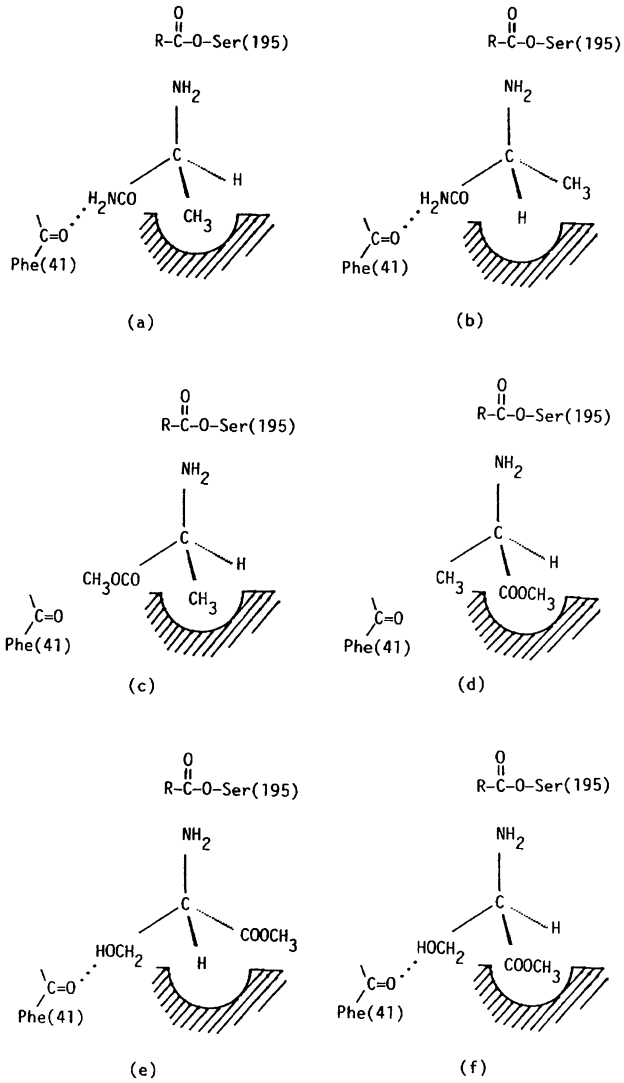


Fig. 6. Schematic representation of binding of amine components to reaction site of CT. (a): L-alaninamide; (b): D-alaninamide; (c): L-alanine methyl ester; (d): D-alanine methyl ester; (e): L-serine methyl ester; (f): D-serine methyl ester.

to the acyl enzyme before deacylation occurs.[25] On the other hand, based on a crystallographic study of a Michaelis complex of CT with a trypsin inhibitor,[31] it has been proposed that the alanine residue (Ala-16) in the inhibitor at P_1' position (carboxyl-terminal side of the sensitive bond) makes at least 15 contacts with CT, including hydrophobic interactions of the Ala-16 methyl group with Cys-42 and His-57 of CT.[32] The methyl group points away from the mass of the enzyme. Glycine derivatives lacking the beta carbon are of lower reactivity and D-amino acids cannot be accommodated. In addition, the amide proton in Arg-17 of the inhibitor (which corresponds to amide proton of alaninamide in peptide synthesis) has hydrogen bond contact with C=O of Phe-41 in CT; estimated bond length is 2.7 Å.[31] Thus, as shown in Fig. 6a, it is likely that, in peptide synthesis, L-alaninamide is bound to acyl-enzyme by two-point interaction before deacylation (peptide formation) occurs; that is hydrophobic interaction of methyl group and hydrogen bonding of amide hydrogen. The binding energy may be utilized for the following deacylation step. Decrease in water content will diminish interaction of the methyl group with hydrophobic pocket of CT, causing lower reactivity of L-alaninamide.

On the contrary, a productive complex of D-alaninamide with CT would be such that as shown in Fig. 6b. In this case binding would be due only to hydrogen bonding with Phe-41, resulting in slower reaction with acyl-enzyme in water than that for L-isomer. However,

decrease in water content may not so much affect the binding as for L-isomer, and this will lead to low enantiospecificity at low water contents.

It has been reported that esters of L-alanine are poor nucleophiles for peptide synthesis with ATEE by the catalysis of CT.[4] Similar results were obtained for esters of leucine and valine.[16,25] These facts also support the consideration that hydrogen bond between amide hydrogen of a nucleophile and CT plays an important role for its binding to the enzyme. As shown in Fig. 7, reaction of L-alanine methyl ester with ATEE is much slower than its amide counterpart, and almost identical to that of D-alanine methyl ester (Fig. 8). The result may be interpreted by very weak binding of L-ester due to lack of hydrogen bonding to the binding pocket (Fig. 6c). In this case, productive binding of D-ester may be such that as shown in Fig. 6d; the difference in binding energies between L- and D-esters may be very small as compared to that for amides.

The above results led us to examination of reactivity of serine methyl esters as amine components. Serine has a hydroxyl group on beta carbon which may be a hydrogen donor at binding pocket of CT just like amide groups of alaninamides. Possible productive binding states are illustrated in Figs. 6e and 6f for L- and D-serine methyl esters, respectively. Figures 9 and 10 show that both esters enter into reaction much more rapidly than alanine esters. Furthermore, difference is small

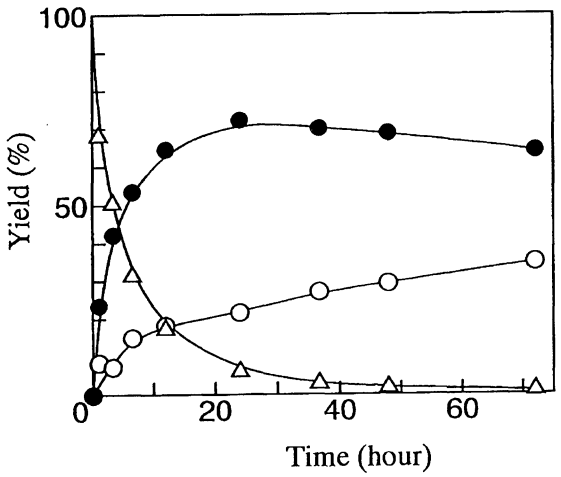


Fig. 7. Time course of reaction of ATEE with L-alanine methyl ester by CT. ATEE 10 mM, L-alanine methyl ester 20 mM, CT 1 mg, acetonitrile 10 ml, water 0.5 ml, 30 °C. ○: Ac–L-Tyr–L-Ala–OMe; ●: Ac–L-Tyr–OH; △: ATEE.

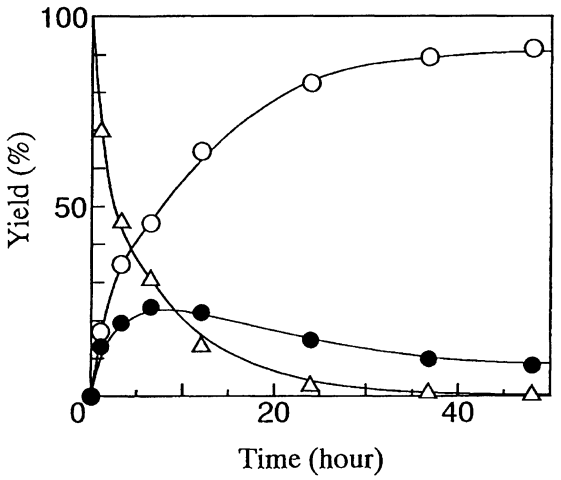


Fig. 9. Time course of reaction of ATEE with L-serine methyl ester by CT. ATEE 10 mM, L-serine methyl ester 20 mM, CT 1 mg, acetonitrile 10 ml, water 0.5 ml, 30 °C. ○: Ac–L-Tyr–L-Ser–OMe; ●: Ac–L-Tyr–OH; △: ATEE.

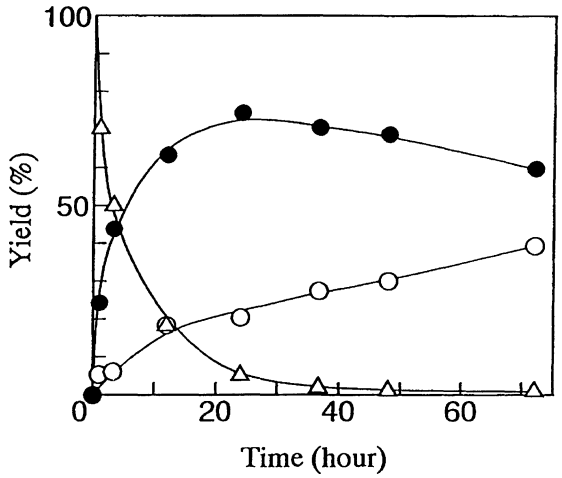


Fig. 8. Time course of reaction of ATEE with D-alanine methyl ester by CT. ATEE 10 mM, D-alanine methyl ester 20 mM, CT 1 mg, acetonitrile 10 ml, water 0.5 ml, 30 °C. ○: Ac–L-Tyr–D-Ala–OMe; ●: Ac–L-Tyr–OH; △: ATEE.

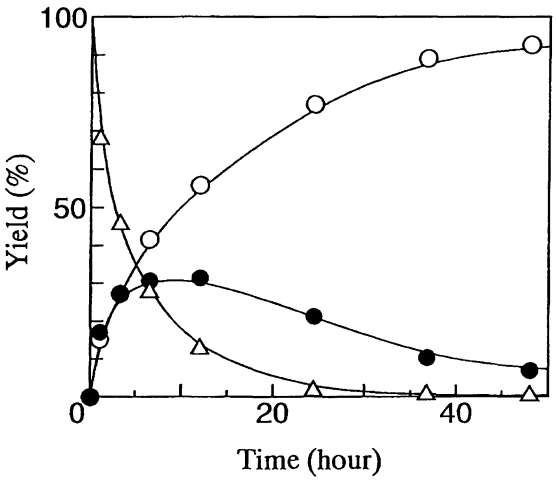


Fig. 10. Time course of reaction of ATEE with D-serine methyl ester by CT. ATEE 10 mM, D-serine methyl ester 20 mM, CT 1 mg, acetonitrile 10 ml, water 0.5 ml, 30 °C. ○: Ac–L-Tyr–D-Ser–OMe; ●: Ac–L-Tyr–OH; △: ATEE.

between reaction rates of L- and D-serine methyl esters, which probably reflects small energy difference between complexes of the esters with acyl-CT. The hydrogen bond between hydroxyl groups of serine methyl esters and CT would strongly contribute to complex formation and accelerate peptide formation.

In summary, enantiospecificity of CT for peptide synthesis is a strong function of the reaction medium. The nature of organic solvent and water content profoundly affect the relative reactivity of enantiomeric amine components. The results may be interpreted by difference in medium effects on stability of complexes between the enantiomeric substrates and CT, which is caused by different binding structures of the enantiomers. The importance of hydrogen bonding between amine components and acyl-CT was demonstrated, which illustrates the structural effects of amine components on enantiospecificity of CT for alanine and serine derivatives. The present results may contribute to the better understanding of the effects of solvent and substrate structure on enantiospecificity of CT in aqueous-organic media.

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References

1) a) Yu. L. Khmelnitsky, A. V. Levashov, N. L. Klyachko, and K. Martinek, *Enzyme Microb. Technol.*, **10**, 710 (1988); b) A. Zaks and A. J. Russell, *J. Biotechnol.*, **8**, 259 (1988); c) J. S. Dordick, *Enzyme Microb. Technol.*, **11**, 194 (1989); d) C. -S. Chen and C. J. Sih, *Angew. Chem., Int. Ed. Engl.*, **28**, 695 (1989); e) A. M. Klibanov, *Acc. Chem. Res.*, **23**, 114 (1990); f) H. Kise, A. Hayakawa, and H. Noritomi, *J. Biotechnol.*, **14**, 239 (1990).

2) A. Zaks and A. M. Klibanov, *J. Am. Chem. Soc.*, **108**, 2767 (1986).

3) A. Ferjancic, A. Puigserver, and H. Gaertner, *Appl. Microbiol. Biotechnol.*, **32**, 651 (1990).

4) T. Nagashima, A. Watanabe, and H. Kise, *Enzyme Microb. Technol.*, **14**, 842 (1992).

5) H. Kitaguchi, P. A. Fitzpatrick, J. E. Huber, and A. M. Klibanov, *J. Am. Chem. Soc.*, **111**, 3094 (1989).

6) S. Parida and J. S. Dordick, *J. Am. Chem. Soc.*, **113**, 2253 (1991).

7) L. T. Kanerva, J. Vihanto, M. H. Halme, J. M. Loponen, and E. K. Euranto, *Acta Chem. Scand.*, **44**, 1032 (1990).

8) P. A. Fitzpatrick and A. M. Klibanov, *J. Am. Chem. Soc.*, **113**, 3166 (1991).

9) R. Bovara, G. Carrea, L. Ferrara, and S. Riva, *Tetrahedron: Asymmetry*, **2**, 931 (1991).

10) K. Nakamura, Y. Takebe, T. Kitayama, and A. Ohno, *Tetrahedron Lett.*, **32**, 4941 (1991).

11) S. Tawaki and A. M. Klibanov, *J. Am. Chem. Soc.*, **114**, 1882 (1992).

12) H. Kitaguchi, I. Itoh, and M. Ono, *Chem. Lett.*, **1990**, 1203.

13) P. Manavalan and F. A. Momany, *Biochem. Biophys. Res. Commun.*, **105**, 847 (1982).

14) S. Wolfe, A. L. Demain, S. E. Jensen, and D. W. S. Westlake, *Sciences*, **226**, 1386 (1984).

15) J. E. Baldwin, R. M. Adlington, N. J. Turner, B. P. Domayne-Hayman, H. -H. Ting, A. E. Derome, and J. A. Murphy, *J. Chem. Soc., Chem. Commun.*, **1984**, 1167.

16) K. Morihara and T. Oka, *Biochem. J.*, **163**, 531 (1977).

17) D. D. Petkov and I. B. Stoineva, *Tetrahedron Lett.*, **25**, 3751 (1984).

18) K. Nilsson and K. Mosbach, *Biotechnol. Bioeng.*, **26**, 1146 (1984).

19) J. B. West and C. -H. Wong, *J. Org. Chem.*, **51**, 2728 (1986).

20) C. F. Barbas, III, J. R. Matos, J. B. West, and C. -H. Wong, *J. Am. Chem. Soc.*, **110**, 5162 (1988).

21) A. L. Margolin, D. -F. Tai, and A. M. Klibanov, *J. Am. Chem. Soc.*, **109**, 7885 (1987).

22) M. Reslow, P. Adlercreutz, and B. Mattiasson, *Eur. J. Biochem.*, **177**, 313 (1988).

23) J. M. Ricca and D. H. G. Crout, *J. Chem. Soc., Perkin Trans. 1*, **1989**, 2126.

24) R. W. Chambers and F. H. Carpenter, *J. Am. Chem. Soc.*, **77**, 1522 (1955).

25) L. Riechmann and V. Kasche, *Biochim. Biophys. Acta*, **830**, 164 (1985).

26) P. Clapes, P. Adlercreutz, and B. Mattiasson, *Biotechnol. Appl. Biochem.*, **12**, 376 (1990).

27) P. Clapes, P. Adlercreutz, and B. Mattiasson, *J. Biotechnol.*, **15**, 323 (1990).

28) J. L. Milles, E. Morey, F. Crain, S. Gross, J. S. Julian, and W. J. Canady, *J. Biol. Chem.*, **237**, 1319 (1962).

29) C. Laane, S. Boeren, K. Vos, and C. Veeger, *Biotechnol. Bioeng.*, **30**, 81 (1987).

30) J. M. Cassells and P. J. Halling, *Enzyme Microb. Technol.*, **10**, 486 (1988).

31) D. M. Blow, C. S. Wright, D. Kukla, A. Ruhlmann, W. Steigmann, and R. Huber, *J. Mol. Biol.*, **69**, 137 (1972).

32) A. R. Fersht, D. M. Blow, and J. Fastrez, *Biochemistry*, **12**, 2035 (1973).