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Lipase-Catalyzed Peptide Synthesis Using Z-Amino Acid Esters as Acyl Donors in Aqueous Water-Miscible Organic Solvents

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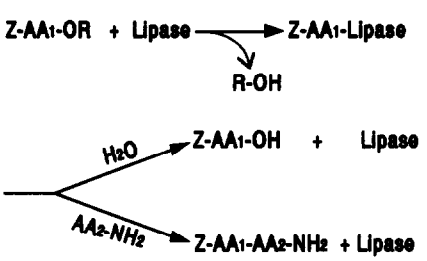
Abstract : The lipase(PPL)-catalyzed peptide synthesis(Z-Phe-Phe-NH2) in aqueous water-miscible organic cosolvents was studied using Z-Phe-OEt as an ester substrate and Phe-NH2 as a nucleophile. It was found that peptide yield increased with an increase of the concentration of DMF. At a higher concentration than 50%(v/v), however, peptide yield decreased rapidly. The optimal pH of an aqueous buffer and concentration of Phe-NH2 were 8 and 10-fold excess over Z-Phe-OEt, respectively. DMF, MeOH and DMSO were found to be effective cosolvents to promote peptide synthesis. The effect of reaction temperature was studied. It was found that both selectivity for peptide synthesis and the stability of PPL increased when the temperature was lowered. The selectivity reached about 90% at -10℃ where the peptide synthesis became rather slow. The rate of peptide synthesis in aqueous-organic solvent mixture was good at room temperature. In a typical experiment (Z-Phe-OEt: 20 mM, Phe-NH2: 200 mM, 10℃, 50% DMF, PPL : 4 $mgmL^{-1}$), Z-Phe-OEt was consumed almost completely within 6 h, yielding about 80% Z-Phe-Phe-NH2. The specificity of ester substrates toward PPL was also studied.

INTRODUCTION

In recent years, enzymatic, namely protease-catalyzed peptide synthesis has been investigated extensively.[1-3] The advantages of enzymatic peptide synthesis are free from racemization, minimal activation and side chain protection, mild reaction conditions, and high regio- and stereoselectivity. Furthermore, the reaction can be carried out in a mixture of water and water-miscible organic solvent, which is capable of dissolving hydrophobic substrates. With these advantages, protease-catalyzed peptide synthesis generally suffers from shortcomings including an undesirable hydrolysis of the growing peptide chain due to an amidase activity, a narrow substrate specificity, and deactivation due to organic solvents. In addition, the enzymatic method is not generally applicable to peptides involving D-amino acids because of the L-specificity of proteases.

Recently, two groups[4-6] have found that lipase-catalyzed peptide formation took place between an *N*-protected amino acid ester and a nucleophile such as an amino acid amide. The peptide synthesis proceeds in a similar manner as with that catalyzed by a protease(kinetically controlled reaction)[2]; both peptide syntheses are due to the esterase activity of the enzyme. The ester substrate(acyl donor) forms an acyl-enzyme intermediate by interaction with a lipase, the aminolysis of which with an amino acid derivative(nucleophile) yields the

desired dipeptide(Scheme 1). On the other hand, the nucleophilic attack of water to the intermediate results merely in the hydrolysis of the ester substrate. Since lipases are nonproteases with no amidase activity, undesirable cleavage of peptide bonds does not take place during peptide synthesis. They used *N*-benzyloxycarbonyl(Z)-amino acid 1-glyceryl(Glc) and methyl(Me)[4,5] or 2-chloroethyl(Mce)[6] esters as acyl donors, and carried out the peptide syntheses in two phase systems[4,5] or in anhydrous organic solvents[6], in order to eliminate the undesirable hydrolysis of the ester substrates. However, at the same time, the rate of peptide synthesis generally became very low. For this reason, the methods in the literature[3-5] are not applicable to practical peptide synthesis at present.



Scheme 1. Lipase-catalyzed peptide synthesis. AA denotes an amino acid residue.

In order to overcome this drawback, we undertook lipase-catalyzed peptide synthesis in a mixture of water and water-miscible organic solvent, *N,N*-dimethylformamide(DMF). The reason why the reaction medium was chosen is that such an aqueous organic solvent was expected not only to dissolve a hydrophobic ester substrate but also to enhance the activity of a lipase. Furthermore, it has been reported that DMF is a good cosolvent for protease-catalyzed peptide syntheses.[7-9] In the presence of water, however, a problem that undesirable hydrolysis of an ester substrate should be eliminated, arises newly. In the present study, as a model reaction we selected the peptide synthesis of Z-Phe-Phe-NH_2 using Z-Phe-OEt as an acyl donor and Phe-NH_2 as an acyl acceptor. We used free porcine pancreas lipase(PPL) as a catalyst for peptide synthesis. The influence of an added organic cosolvent, pH of aqueous buffer, nucleophile concentration, and reaction temperature on peptide yield was investigated. In addition, the effect of structures of ester substrates on the specificity of PPL was also examined.

RESULTS AND DISCUSSION

Fig.1 presents a typical time course of PPL-catalyzed synthesis of Z-Phe-Phe-NH_2 in 50% DMF-aqueous buffer(pH 8). The reaction completed within about 6 h and peptide yield reached about 80%, indicating that the undesirable hydrolysis of the ester substrate was well depressed under the reaction conditions. The reaction time was improved significantly as compared with that required for the reaction in an anhydrous organic solvent[6] or in a two-phase system.[4,5] As expected, the secondary hydrolysis of Z-Phe-Phe-NH_2 was not observed(Fig.1). Furthermore, the isolation of the product from 50% DMF-aqueous buffer was easy; Z-Phe-Phe-NH_2 could be successfully separated from the reaction mixture by precipitation with water and the subsequent filtration. However, the peptide synthesis was much slower than that catalyzed by proteases. For example, Clavet *et al.*[9] have reported that α-chymotrypsin-catalyzed synthesis of Z-Phe-Leu-NH_2 from Z-Phe-OMe and Leu-NH_2 completed within 30 min in *ca.* 50% DMF-aqueous buffer. This difference could be attributed to the fact that a Z-amino acid ester is generally a less reactive substrate for lipases, while proteases have a very high activity toward the substrate. It is interesting to note that the peptide yield or product distribution(peptide/acid) for PPL was comparable to that with α-chymotrypsin.[9]

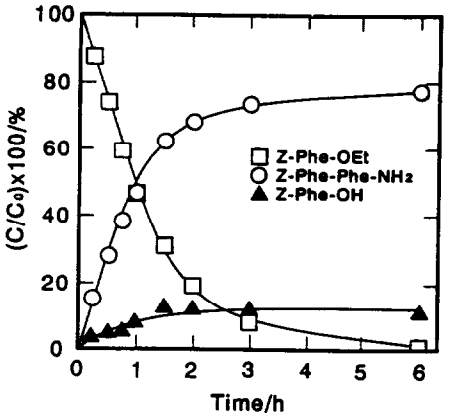


Fig.1. Time course of the synthesis of Z-Phe-Phe-NH_2 using Z-Phe-OEt as an ester substrate and Phe-NH_2 as a nucleophile. Reaction conditions : Z-Phe-OEt, 20mM ; Phe-NH_2, 200mM ; DMF, 50% ; buffer, pH 8 ; 15℃ ; PPL, 4 $mgmL^{-1}$.

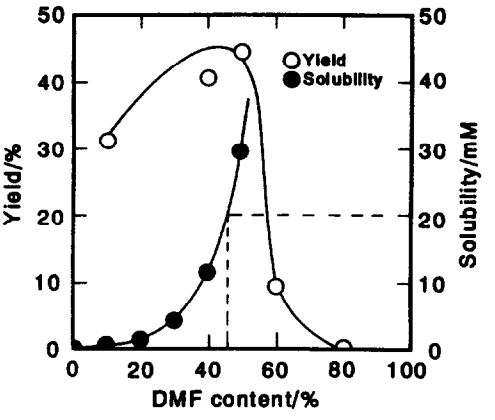


Fig.2. Effect of DMF-content on the synthesis of Z-Phe-Phe-NH_2. Reaction conditions : Z-Phe-OEt, 20mM ; Phe-NH_2, 40mM ; buffer, pH 8 ; 24℃ ; 6 h ;PPL, 4 $mgmL^{-1}$. Solubility of Z-Phe-OEt was measured at 24℃.

Fig.2 shows the influence of DMF-content on peptide yield. It decreased rapidly at a high DMF-content(>50%), which may be explained in terms of inhibition with DMF. For comparison, kinetic parameters of the Michaelis-Menten equation were measured(30℃) for the hydrolysis of Z-Phe-OEt. K_m and k_{cat} were found to be 22 mM and 16 μ mol h^{-1}mg-$powder^{-1}$, respectively, at 50% DMF. At 60% DMF, K_m became 2.5 times as large as that at 50% DMF, and k_{cat} reduced to one-third of that at 50% DMF. Such an effect of water-miscible organic solvent on K_m and k_{cat} is known for papain.[10] In contrast, peptide yield also decreased at a low DMF-content probably because of the incomplete dissolution of Z-Phe-OEt. In order to confirm this, the solubility of Z-Phe-OEt in the reaction medium was measured. The filled circles in Fig.2 show the solubility of Z-Phe-OEt at various DMF-contents. As can be seen in Fig.2, the complete dissolution of the ester substrate is achieved at about 45% of DMF-content(broken line). This is consistent with the finding that a maximum yield of the dipeptide was obtained at around about 50% DMF, indicating that the complete dissolution of the ester substrate is required for PPL to express a high catalytic activity. Therefore, the subsequent peptide synthesis was carried out in the solution containing 50% water-miscible organic cosolvent.

Fig.3 shows the effect of nucleophile concentration on peptide synthesis. Z-Phe-OEt was consumed almost completely under the reaction conditions of Fig.3. The yields of both peptides(Z-Phe-Phe-NH_2 and Z-Phe-Ala-NH_2) can be seen to be nonlinear in nucleophile concentration. A similar phenomenon has been observed in α-chymotrypsin-catalyzed peptide synthesis.[11,12] As can be seen in Fig.3, the yields of both peptides became almost constant at a nucleophile concentration higher than 200 mM(10-fold excess of a nucleophile over the ester substrate). Therefore, the subsequent reaction was carried out at a nucleophile concentration of 200 mM. The effect of nucleophile concentration on peptide yield was almost identical for Phe-NH_2 and Ala-NH_2, suggesting that PPL does not strictly discriminate a nucleophile.

Fig.4 shows a pH profile for the peptide synthesis in 50% DMF. The optimal pH was found to be about 8. The pH values given are of the buffer solution containing the nucleophile before the addition of DMF dissolving

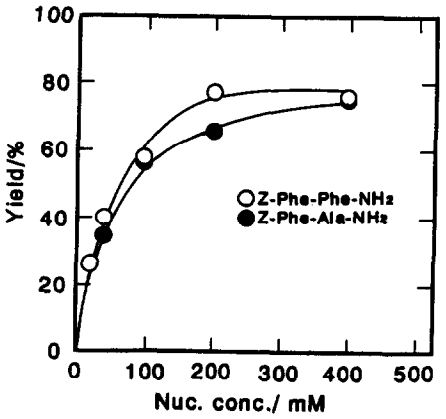


Fig.3. Effect of nucleophile concentration on the syntheses of Z-Phe-Phe-NH2 and Z-Phe-Ala-NH2. *Reaction conditions : DMF, 50% ; buffer, pH 8 ; 15 ℃ ; 6 h* ; PPL, 4 mgmL^{-1}. Open circle : Z-Phe-OEt(20mM) + Phe-NH2. Filled circle : Z-Phe-OEt(20mM) + Ala-NH2.

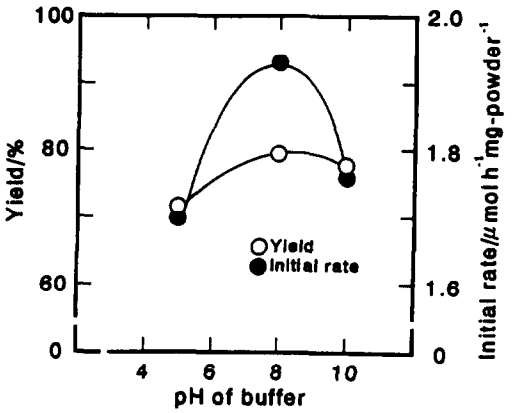


Fig.4. Effect of pH value of aq. buffer on the synthesis of Z-Phe-Phe-NH2. Reaction conditions : Z-Phe-OEt, 20mM ; Phe-NH2, 200mM ; DMF, 50% ;10℃ ; 6 h ; PPL, 4 mgmL^{-1}.

Z-Phe-OEt. Interestingly, lipase-catalyzed peptide synthesis was not affected so strongly with pH values. In contrast, it is well known that α-chymotrypsin-catalyzed peptide synthesis is sensitive to a high pH value, and above pH 9 the peptide yield decreases significantly because of denaturation of the enzyme.[12]

Table 1. Effect of Water-Miscible Organic Cosolvents on Peptide Synthesis a) (Z-Phe-OEt + Phe-NH2 ⟶ Z-Phe-Phe-NH2)

Cosolvent	Dielectric constant b)	Peptide yield/% 6 h	24 h
Dioxane		14	19
Acetone		62	68(12h)
Ethanol		35	48
MeOH	60.3	83(3h) c)	-
Acetonitrile		17	21
DMF	66.5	77 c)	-
Ethylene glycol	64.5	51	62
DMSO	76.0	86 c)	-

a) Z-Phe-OEt, 20mM ; Phe-NH2, 200mM ; cosolvent, 50%(v/v) ; pH of aq. buffer, 8 ; PPL, 4mgmL^{-1} ; 24℃. b) Cited from Refs. 12 and 13. c) Z-Phe-OEt was consumed almost completely.

Table 1 presents the effect of an added organic cosolvent on peptide synthesis. It was reported that peptide yield increased with an increase of the polarity (dielectric constant) of a cosolvent mixture in α-chymotrypsin-catalyzed peptide synthesis.[12] In the present study, however, peptide yield was not well correlated with the values of dielectric constants of the respective cosolvent mixtures, although all of them could not be obtained from the literature. In the instances of dimethyl sulfoxide(DMSO) and DMF, Z-Phe-OEt was consumed completely within 6 h, giving the dipeptide in good yields(77-86%). The addition of MeOH enhanced greatly the peptide synthesis; both Z-Phe-OEt and Z-Phe-OMe(formed by transesterification) disappeared within 3 h, yielding 83% Z-Phe-Phe-NH2. In this instance, the transesterification took place so rapidly that both Z-Phe-OEt and Z-Phe-OMe might act as ester substrates. For example, under similar conitions(2-fold excess of Phe-NH2 over Z-Phe-OEt), Z-Phe-OEt reduced to 4% of the initial concentration after 1 h, yielding Z-Phe-OMe(53%), Z-Phe-Phe-NH2(19%), and Z-Phe-OH(23%). This finding indicates

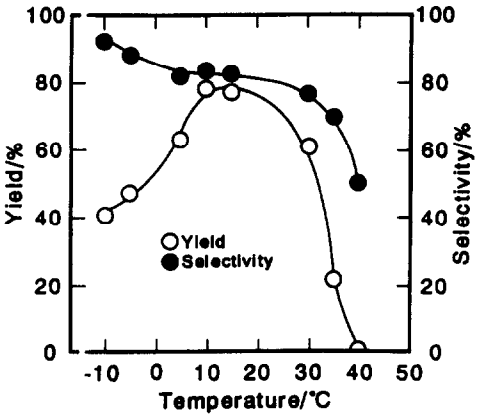


Fig.5. Effect of temperature on the synthesis of Z-Phe-Phe-NH2. Reaction conditions : Z-Phe-OEt, 20mM ; Phe-NH2, 200mM ; DMF, 50% ; buffer, pH 8 ; 6 h ; PPL, 4 mgmL^{-1}.

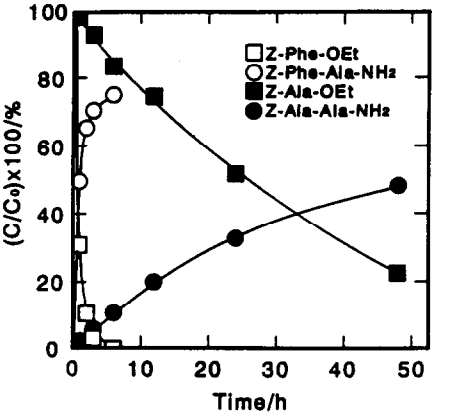


Fig.6. Comparison of reactivity between Z-Phe-OEt and Z-Ala-OEt. Reaction conditions : buffer, pH 8 ; 24℃ ; PPL, 4 mgmL^{-1}. Open symbol : Z-Phe-OEt(20mM) + Ala-NH2(200mM) ; DMF, 50%. Filled symbol : Z-Ala-OEt(20mM) + Ala-NH2(400mM) ; DMSO, 50%.

that the transesterification can take place rapidly in 50% aqueous MeOH. Therefore, it was suspected that the effect of MeOH might be due to the difference in reactivity between the ethyl and methyl esters. In a separate experiment, however, we found that the reactivity of Z-Phe-OMe (under the reaction conditions of Fig.1) was comparable to that of Z-Phe-OEt(Fig.1). Consequently, the solvent effect of MeOH may be attributed to its nature. From Table 1, it is apparent that DMSO, MeOH and DMF are good cosolvents.

Fig.5 shows the influence of reaction temperature on peptide yield. The selectivity for peptide synthesis(filled circle) increased steadily with a decrease of temperature, reaching about 95% at -10℃. This finding indicates that the relative rate of hydrolysis and aminolysis depends on reaction temperature and also that the latter, namely peptide synthesis is favored with a decrease of temperature. A similar temperature effect is known for α-chymotrypsin-catalyzed peptide synthesis,[7,11] and explained in terms of a stronger binding of a nucleophile to the acyl-enzyme intermediate before deacylation at lowered temperature.[7,11b] As can be seen in Fig.5, the temperature-dependency of peptide yield(open circle) has a maximum at 10℃. A rapid decrease in peptide yield at a high temperature is the result of thermal deactivation of PPL. In contrast, a gradual decrease in peptide yield at a low temperature can be explained in terms of the reduced rate of peptide synthesis. This explanation is consistent with the finding that unreacted ester substrate remained in the reaction mixture after 6 h. For example, at -10℃, dipeptide yield increased up to about 81% after 24 h. A similar temperature-dependency was also observed for the initial rate of the peptide synthesis.

Fig.6 shows the comparison of reactivity between Z-Phe-OEt and Z-Ala-OEt as an ester substrate. It can be seen that Z-Ala-OEt is much less reactive than Z-Phe-OEt. The difference in reactivity between Phe and Ala could be attributed to the hydrophobicity of the side chain, suggesting that a lipase such as PPL expresses a substrate specificity toward an amino acid residue like proteases. It is well known that the substrate specificity(log(k_{cat}/K_m)) of α-chymotrypsin can be correlated with physical properties of the ester substrates such as hydrophobicity(log P) and molar refractivity(MR).[14]

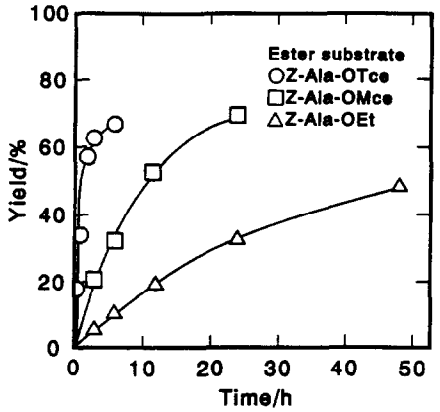


Fig.7. Effect of activation of the ester moiety on peptide synthesis. Reaction conditions : ester substrate, 20mM ; Ala-NH2, 400mM ; DMSO, 50% ; buffer, pH 8 ; 24℃ ; PPL, 4 $mgmL^{-1}$.

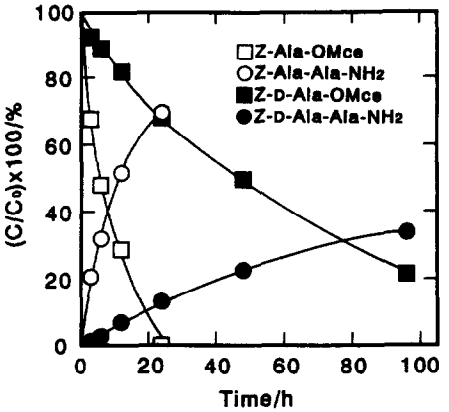


Fig.8. Time course of peptide synthesis using D- and L-Ala-OMce as ester substrates. Reaction conditions : DMSO, 50% ; buffer, pH 8 ; 24℃ ; PPL, 4 $mgmL^{-1}$. Open symbol : Z-Ala-OMce(20mM) + Ala-NH2(400mM). Filled symbol : Z-D-Ala-OMce(20mM) + Ala-NH2(400mM).

Fig.7 shows the effect of the ester moiety of an acyl donor on the rate of dipeptide synthesis(Z-Ala-Ala-NH2). When Z-Ala-OMce and Z-alanine 2,2,2-trichloroethyl ester(Z-Ala-OTce) were used as acyl donors, peptide synthesis was enhanced significantly as compared with that from Z-Ala-OEt and completed within 24 and 6 h, respectively. The yield of Z-Ala-Ala-NH2 reached about 70% in these instances; the remainder was Z-Ala-OH formed from undesirable hydrolysis of the ester substrates. This finding indicates that a less reactive amino acid ester can be converted to a highly reactive one by the introduction of electron-withdrawing groups into the ester moiety, namely by the activation of the ester carbonyl carbon. Barbas *et al.* have also found that Z-phenylalanine cyanomethyl ester(an active ester for chemical peptide synthesis)[15] was about 10 times reactive than the corresponding methyl ester in enzymatic peptide synthesis.[8] It can be seen in Figs. 6 and 7 that the reactivity of Z-Ala-OTce is comparable to that of Z-Phe-OEt. It is worth noting that no dipeptide synthesis was observed in the absence of PPL under the same conditions.

When Z-D-Phe-OEt was used as an acyl donor, no dipeptide formation was observed, indicating that PPL discriminates the L-enantiomer of an amino acid residue under these reaction conditions. The finding that hydrolysis of Z-D-Phe-OEt did not take place under the same conditions, also supports this result. It is interesting to note that PPL expresses an activity toward the L-enantiomers of amino acids like proteases. Contrary to this, in the instance of CCL(a lipase from *Candida Cylindracea*)-catalyzed peptide synthesis, it has been reported that the incorporation of D-Phe into the *N*-terminal residue of a certain dipeptide was achieved in a 45%-yield using Z-D-Phe-Glc as an acyl donor.[5] Miyazawa *et al.* have reported that the enantioselectivity of CCL-catalyzed hydrolysis of Z-amino acid 2-chloroethyl esters was generally poor(i.e.,7%e.e.(L-isomer)[16] for Ala).[17] Therefore, the difference in enantioselectivity between PPL and CCL could be attributed to the origin of a lipase used as a catalyst.

On the other hand, when Z-D-Ala-OMce was reacted with Ala-NH2, the formation of the D-L peptide

amide took place, although the reaction was very slow as compared with that of the L-L peptide amide(Fig.8). This can be attributed to a small side chain of Ala ; the L-specificity of PPL may relax as the side chain of an amino acid becomes small. The finding demonstrates that PPL can incorporate a D-amino acid with a small side chain such as Ala to the *N*-terminal residue of dipeptides. In separate experiments, we carried out PPL-catalyzed hydrolysis of Z-DL-Ala-OMce in 20% DMF-aqueous buffer(pH 8), and measured e.e.(L-isomer) of the hydrolysis product. As expected, PPL hydrolyzed the L-enantiomer preferentially but e.e.(L-isomer) varied depending on conversion of the ester substrate. At a conversion of 44%, e.e.(L-isomer) was found to be 68%, indicating that the D-enantiomer was also hydrolyzed to some extent. This finding is well consistent with the PPL-catalyzed synthesis of Z-D-Ala-Ala-NH_2 described above.

It is well known that a protease such as α-chymotrypsin is deactivated rapidly in the presence of a high concentration of DMF. For example, West and Wong have reported that free α-chymotrypsin(0.2 mM enzyme) lost nearly 50% of the original activity within 5 min in 50% DMF-aqueous buffer (pH 7 or 9.1).[12] Then we investigated deactivation of PPL in 50% DMF-aqueous buffer(pH 8). The deactivation became significant with an increase of the temperature, suggesting that the activity of PPL dissolved in 50% DMF was sensitive to temperature. For example, the remaining activites at 30 and 40℃ after 1 h were 82 and 18% of the original one, respectively. However, the original activity was retained at 10 and 20℃ for 12 h. The results indicate that free PPL is stable in 50% DMF at a temperature lower than 20℃. This tendency for deactivation of PPL with temperature is well consistent with the peptide yield shown in Fig.5.

In conclusion, the major advantage of using water-miscible instead of water-immiscible organic solvents and anhydrous organic solvents alone appears to be both the increased solubility of substrates and the enhanced activity of PPL, making peptide synthesis fast.

EXPERIMENTAL

Porcine pancreas lipase(EC 3.1.1.3, Type II crude) was obtained from Sigma. Amino acids were purchased from Peptide Institute Inc. The other chemicals were of commercial products(reagent grade) and used without further purification. Amino acid amides(Phe-NH_2 and Ala-NH_2)[18] were prepared by ammonolysis of the corresponding amino acid methyl esters.[19] Z-Dipeptide amides used as references for HPLC were synthesized by the method of Anderson *et al.*[20] Amino acids were of L-configuration unless otherwise noted.

Ester substrates. Z-Amino acid ethyl esters were prepared by acylating the corresponding amino acid ethyl esters with benzyloxcarbonyl chloride(Z-Cl).[12] Z-Phe-OEt : oil ; yield, 69%. Z-D-Phe-OEt : oil ; yield, 72%. Z-Ala-OEt : oil ; yield, 78%.

Z-Amino acid 2-chloroethyl esters were synthesized by reacting the corresponding Z-amino acids with 2-chloroethanol using *N,N'*-dicyclohexylcarbodiimide(DCC) as a condensing agent in the presence of 4-(dimethylamino)pyridine. Z-Ala-OMce : m.p. 47-48℃ ; yield, 41% (Found : C, 54.90 ; H, 5.75 ; N, 4.72%. Calcd for $C_{13}H_{16}NO_4Cl$: C, 54.65 ; H, 5.64 ; N, 4.90%). Z-D-Ala-OMce : m.p. 47-48℃ ; yield, 60% (Found : C, 54.80 ; H, 5.77 ; N, 4.97%).

Z-Ala-OTce was synthesized similarly from Z-Ala-OH and 2,2,2-trichloroethanol ; m.p. 39-40℃ ; yield, 53% (Found : C, 44.30 ; H, 4.16 ; N, 4.04%. Calcd for $C_{13}H_{14}NO_4Cl_3$: C, 44.03 ; H, 3.98 ; N, 3.95%). This was reported previously as an oil by Dhaon *et al.*[21]

Enzymatic peptide synthesis. The given amino acid amide was dissolved in phosphate buffer(pH 8) and pH was adjusted to the desired value with aq. NaOH or HCl. The pH given is of the aqueous solution before

addition of the organic solution. The cosolvent containing the ester substrate was added, and the solution was allowed to stand at a prescribed temperature with stirring. Solid enzyme was then added. At appropriate time intervals, aliquots(1 mL) were taken from the reaction mixture. The enzymatic reaction was quenched by adding DMF solution(2 mL) containing an internal standard. Typical reaction conditions were as follows : ester substrate, 20 mM ; nucleophile, 200 mM ; organic cosolvent, 50% ; buffer, pH 8 ; PPL, 4 $mgmL^{-1}$.

Isolation of Z-Phe-Phe-NH2. A phosphate buffer(pH 8)-DMF(1:1, v/v), 50 mL, containing Z-Phe-OEt(20 mM), Phe-NH_2(200 mM), and PPL(2 $mgmL^{-1}$) was stirred at 15℃ for 12 h. The product was precipitated by the addition of an equal volume of cold water, and the mixture was cooled in a freezer for 1 h. After filtration, the precipitate was washed with water and dried in vacuum; m.p. 240-241℃; yield, 78%. Found: C, 69.97; H, 6.09; N, 9.53%. Calcd for $C_{26}H_{27}N_3O_4$: C, 70.10; H, 6.11; N, 9.43%.

Analysis. The composition of the reaction mixtures was determined by HPLC. A JASCO 880 pump, a JASCO 875 UV monitor and a SIC chromatcorder 11 were assembled to carry out HPLC analysis with a JASCO Finepak SIL C_{18} column(250 x 4.6 mm). The eluent was 10 mM H_3PO_4-K_2HPO_4 buffer(pH 2.6) + 50 mM Na_2SO_4 containing 35, 40 or 50%(v/v) acetonitrile. The column effluent was monitored at 254 nm where the Z-protecting group of the ester substrates and products has a strong light absorbance. Peak identification was made by comparing retention times with those of authentic compounds. Quantitative analysis was carried out by an internal standard method.

Assay. The activity was assayed by determining the release of Z-Phe-OH from Z-Phe-OEt(for 10 min) by HPLC. PPL was dissolved in 50% DMF-phosphate buffer(pH 8) and the solution(PPL,4 $mgmL^{-1}$) was allowed to stand at a prescribed temperature(10, 20, 30, and 40℃). The substrate solution was 10 mM Z-Phe-OEt in DMF. Typical assay contained 6 mL of buffer(pH 8), 4 mL of substrate solution, and 2 mL of the PPL solution being assayed.

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