α -Chymotrypsin as the Catalyst for Peptide Synthesis

By KAZUYUKI MORIHARA and TATSUSHI OKA

Shionogi Research Laboratory, Shionogi and Co. Ltd., Fukushima-ku, Osaka 553, Japan

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α -Chymotrypsin (EC 3.4.21.1)-catalysed syntheses of peptides were performed with various *N*-acylated amino acid or peptide esters as donors, and amino acid derivatives, peptides or their derivatives as acceptors. Under optimal conditions the synthesis was almost quantitative. As acceptor nucleophiles, free amino acids or the ester derivatives were inadequate, but amino acid amides or hydrazides, di- or tri-peptides, or the amides, hydrazides and esters of the peptides were useful. The nucleophile specificity for synthesis was markedly similar to the leaving-group specificity in hydrolysis; hydrophobic or bulky amino acid residues were most efficient at both P_1' and P_2' positions [notation of Schechter & Berger (1967) *Biochem. Biophys. Res. Commun.* 27, 157–162], but L-proline as well as D-amino acid residues were the worst choices. The synthesis was further dependent on the solubility of the products synthesized; a higher yield of products was expected with lower solubility. As donor esters, good substrates were all useful. Accordingly, fragment condensation was possible by using *N*-acylated peptide esters and various peptides. The present study suggested that α -chymotrypsin may become a useful tool for peptide synthesis.

Substantial evidence indicates that chymotrypsin (EC 3.4.21.1)-catalysed hydrolyses proceed in two steps (Hess, 1971). The first reaction is acylation of serine-195 residue of the enzyme and the second is a subsequent deacylation. Water is not unique in the latter reaction; for example, replacement by amine or alcohol derivatives (nucleophile N) may result in changes in the rate and products (Scheme 1). Therefore peptides are expected to be synthesized enzymically when amino acid derivatives, peptides or the derivatives are used as nucleophile N in Scheme 1.

In fact, since Bergmann & Fraenkel-Conrat (1937) first demonstrated papain-catalysed amide (anilide)-bond synthesis, many workers have reported enzymic synthesis of peptides, especially with papain (EC

3.4.22.2) (Kimmel & Smith, 1957). Concerning synthesis by chymotrypsin, Bergmann & Fruton (1938) synthesized Bz*-Tyr-Gly-NH₂ from Bz-Tyr and Gly-NH₂, and Blau & Waley (1954) obtained oligomeric peptides from Tyr-NH₂, Phe-NH₂ or Tyr-Tyr. Epand (1969) found that Bz-Tyr-Gly-NH₂ is produced by chymotrypsin hydrolysis of Bz-Tyr-OEt in

* Abbreviations for lesser-known substituents: Ac, acetyl; Bz, benzoyl; Cbz, benzyloxycarbonyl; *t*-Bu, *t*-butyl ester; OEt, ethyl ester. Abbreviated designations of amino acid derivatives, peptides or their derivatives conform to the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature (1972). Except when specified, the constituent amino acids were all of the L-configuration.

Scheme 1.

the presence of Gly-NH₂, although detailed information on the synthesis was not reported.

Fastrez & Fersht (1973) have shown that the following chymotrypsin-catalysed synthesis depends on the concentration of the acceptor nucleophile:

The yields of the product (with [Ac-Phe-OEt] = 2.5mM) were 51.8, 75.5 and 86.0% when the concentrations of Gly-NH₂ were 0.10, 0.25 and 0.50M respectively, and were 24.3, 48.1, 69.0 and 81.8% when those of Ala-NH₂ were 0.010, 0.025, 0.05 and 0.10M respectively. The reason was ascribed to inhibition of the secondary hydrolysis of the synthesized product by the same enzyme in the presence of a high concentration of the corresponding nucleophile. They further found that 1M-Gly-NH₂ or -Ala-NH₂ was 11 or 44 times more reactive (respectively) than 55M-water for deacylation of acyl-chymotrypsin.

The study by Fastrez & Fersht (1973) suggested the possibility of peptide synthesis with chymotrypsin as the catalyst, although the yields from the acceptor nucleophiles used were markedly low. The present study was thus undertaken to investigate whether, if this defect can be overcome, chymotrypsin can become a useful catalyst for peptide synthesis. Various *N*-acylated amino acid or peptide esters were used as donors, and various amino acid derivatives, peptides or their derivatives as acceptors.

Materials

Enzyme

α -Chymotrypsin (three-times recrystallized) obtained from Worthington Biochemical Corp., Free-

hold, NJ, U.S.A., was used in this study without further purification.

Amino acid derivatives, peptides, or their derivatives

Ac-Phe-OEt, Cbz-Phe-OEt, Ac-Tyr-OEt, Ac-Trp-OEt, Cbz-Gly-Leu-NH₂, Cbz-Gly-D-Leu-NH₂, Cbz-Gly-Pro-NH₂, Leu-NH₂·HCl, Leu-OEt·HCl, Leu-Gly, Leu-Gly-Gly, Gly-Gly, Gly-Gly-Gly, Gly-Leu, Gly-Pro and L-leucine were supplied by the Protein Research Foundation, Osaka, Japan. Glu-NH₂ (isoglutamine), His-NH₂·2HCl, Ser-NH₂·HCl, Tyr-NH₂·HCl, Gly-Ala, Val-Ala, Leu-Phe, Phe-Leu, Tyr-Leu-NH₂ and Gly-Gly-Tyr-NH₂ were obtained from Cyclo Chemical Corp., Los Angeles, CA, U.S.A., and Lys-Leu·2HBr, Ala-Ala and Ala-Ala-Ala from Miles-Yeda Ltd., Rehovot, Israel. Cbz-Ala-Phe-OMe (Mori-hara *et al.*, 1974), Cbz-Gly-Pro-Phe-OEt (Mori-hara & Oka, 1977), Ac-Ala-Ala-Phe-OMe (Mori-hara *et al.*, 1974), Ala-Ala-Ala-OBu^t, AcOH (Mori-hara & Oka, 1973), Ala-Ala-OBu^t (Mori-hara & Oka, 1973), Ala-NH₂·HCl (Mori-hara *et al.*, 1969a), Val-NH₂·HCl (Mori-hara *et al.*, 1969a), Ile-NH₂·HCl (Mori-hara *et al.*, 1969a) and Cbz-Phe-Tyr-Ala (Mori-hara *et al.*, 1969a) were prepared by methods described previously.

Gly-Leu-NH₂·HCl, Gly-D-Leu-NH₂·HCl, Gly-Pro-NH₂·HCl and Phe-Tyr-Ala were obtained by catalytic hydrogenolysis (palladium black) of the corresponding Cbz-peptides in the usual manner (Schwyzer & Rittel, 1961). Cbz-Val-OBu^t was synthesized as usual by using 2-methylpropene/H₂SO₄ (Anderson & Callahan, 1960). Various Cbz-amino acid amides were prepared by ammonolysis of the mixed carboxylic acid anhydrides synthesized from Cbz-amino acids and ethylchlorocarbonate. Catalytic hydrogenolysis of these products gave Val-OBu^t and various amino acid amides. Phe-NHNH₂ was prepared by the reaction of Cbz-Phe-OEt with

Table 1. *Identification of various compounds*

All melting points are uncorrected. Elementary analyses were performed in the Shionogi Research Laboratory.

Compounds	M.p. (°C)		Elementary analysis (%)			
			C	H	Cl	N
Val-OBu ^t ·HCl	148–149	Calc.	51.54	9.61	16.90	6.68
		Found	52.00	9.69	16.94	6.77
Gly-NH ₂ ·HCl	208–210	Calc.	21.73	6.38	32.07	25.34
		Found	22.01	6.34	31.98	25.51
Pro-NH ₂	93–95	Calc.	52.61	8.83		24.54
		Found	53.12	8.64		24.07
DL-Leu-NH ₂ ·HCl	227–230	Calc.	43.24	9.07	21.28	16.81
		Found	43.75	9.16	21.18	16.71
D-Leu-NH ₂ ·HCl	241–243	Calc.	43.24	9.07	21.28	16.81
		Found	43.73	9.08	20.90	16.58
Phe-NH ₂ ·HCl	236–238	Calc.	53.87	6.53	17.67	13.96
		Found	53.69	6.68	17.48	13.98
Phe-NHNH ₂ ·2HCl·½H ₂ O	267–270	Calc.	42.27	6.07	27.72	16.43
		Found	42.46	6.22	27.29	16.40

hydrazine hydrate in methanol as solvent, and by the successive catalytic hydrogenolysis of the product. The identification data of these products are presented in Table 1.

Methods

Enzymic synthesis of peptides

Except where specified, the reaction mixture (1 ml) contained 0.1M donor ester, 0.1M acceptor nucleophile, 0.02mM- α -chymotrypsin, 10% (v/v) dimethylformamide, and 0.2M- $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer, pH10. The reaction was performed by stirring vigorously for 2 min at room temperature (25°C) and was stopped by the addition of 1 ml of 1M-HCl; the mixture was then evaporated to dryness under reduced pressure. The dried material was suspended in 1 ml of 1M-HCl and the product was repeatedly extracted with ethyl acetate. The ethyl acetate layer usually consisted of *N*-acylated materials such as the starting donor ester, the hydrolysate of the donor ester (*N*-acylated amino acids or *N*-acylated peptides) and the product. Some of the products were not extracted with ethyl acetate, e.g. Ac-Phe-PheNHNH₂, Ac-Phe-Lys-Leu etc. In such cases, the products were extracted with ethanol from the dried material; the extract should contain the starting acceptor nucleophile together with the above-mentioned *N*-acylated materials.

To determine the product yield directly instead of by the t.l.c. method as described below, the reaction was carried out in the modified conditions as follows: (1) the reaction was scaled up to 10ml; (2) the concentration of the starting materials was increased to 0.2M; (3) the time of agitation was increased to 10min to complete the reaction on a large scale.

Quantitative determination of product yields

There were three components, all of them *N*-acylated materials (starting-donor ester, hydrolysed acid and the product) in the ethyl acetate extract, and four (the three *N*-acylated compounds plus the acceptor nucleophile) in the ethanol extract. These were separated by t.l.c. in various solvents as follows: a mixture of chloroform and acetic acid (I, 17:3; I', 2:1; II, 3:1; III, 4:1; V, 9:1; VI, 39:1; all v/v), and a mixture of chloroform, methanol and acetic acid (IV, 80:20:3; VII, 45:3:2; VIII, 90:10:3; all by vol.). After being sprayed with conc. H_2SO_4 , the plate was heated at 200°C for 10–30 min. The colour intensities of the three or four spots on the plate were determined by using a trial device consisting of a densitometer (manufactured by Dr. H. Hakata of this laboratory) with two-dimensional scanning and two-wavelength differentiation (A_{550} – A_{630}) to compensate for the irregular shape of the spots, after the plate had been soaked, under reduced pressure, in liquid paraffin. The product yield was determined by calcu-

lating the ratio against the total intensities of all the spots of *N*-acylated materials.

In the 10ml-scale experiment, the product was isolated to determine its yield directly.

Identification

The products extracted with ethyl acetate or ethanol as above were used as starting materials. Some were crystallized easily from the extracts by using solvents, and the others were obtained after further washing of the ethyl acetate portions with 5% (w/v) NaHCO_3 or column chromatography on silica gel. When the product yields were too low for crystallization to be possible, they were isolated by preparative t.l.c. After the purity was checked by t.l.c., identification was made in the usual manner.

Solubility test

Various amounts of compounds were suspended in 1 ml of 0.2M- $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH10, containing 10% dimethylformamide, and the mixtures were stirred slowly overnight at room temperature. After filtration on a glass filter, the compounds remaining on the filter were washed with small amounts of water to remove the salt and the adhering dimethylformamide. The weights of the insoluble materials were examined after drying *in vacuo*, from which the solubilities at room temperature (25°C) in the solution were calculated.

Results

Optimal results for peptide synthesis

The synthesis of Ac-Phe-Leu-NH₂ was studied under various experimental conditions by using Ac-Phe-OEt and an equimolar amount of Leu-NH₂ as the starting materials. The results indicated that a considerably alkaline pH is suitable for synthesis (Fig. 1a), and a considerable amount of enzyme is required in comparison with that for hydrolysis (Fig. 1b). Fig. 1(c) indicates that the synthetic reaction is almost instantaneous, being completed within 2 min under these conditions. Secondary hydrolysis of the product was negligible owing to its low solubility (9.2mm at 25°C) in the reaction mixture, even though the reaction was continued for 20h at room temperature. Higher product yield resulted with increased concentration of the starting materials (Fig. 1d). Below a starting-material concentration of 10mM, the product yield markedly decreased owing to preferential hydrolysis of the donor ester rather than synthesis of the product and/or secondary hydrolysis of the synthesized product. At a starting-material concentration above 0.2M, vigorous stirring became difficult within 1 min owing to the abundant formation of insoluble product, and thus there was a possible incomplete use of Ac-Phe-OEt within 2 min of reaction.

Fig. 1. Optimal conditions for peptide synthesis by α -chymotrypsin

Except where specified, the reaction mixture (1 ml) contained 0.1 M-Ac-Phe-OEt, 0.1 M-Leu-NH₂HCl, 0.02 mM- α -chymotrypsin, 0.2 M-Na₂CO₃/NaHCO₃ buffer, pH 10, and 10% (v/v) dimethylformamide. The mixture was stirred vigorously for 2 min at room temperature (25°C). (a) Effect of pH. The pH was controlled with 1 M-NaOH during the reaction with a pH-stat in the presence of 0.1 M-KCl (0.2 M-Na₂CO₃/NaHCO₃ buffer was omitted). (b) Effect of enzyme concentration. (c) Effect of reaction time. (d) Effect of the concentration of the starting materials. In (a)–(d): ●, Ac-Phe-OEt remaining; ○, Ac-Phe-Leu-NH₂ produced. The calculation of the percentages was based on the ratio of the amount produced by the enzymic synthesis to the theoretical amount when synthesized completely.

Acceptor-nucleophile specificity in synthesis

Amino acids or their derivatives. The experiment was carried out with Ac-Phe-OEt as a donor and amino acids or their derivatives as nucleophiles, as shown in Table 2. Both the free amino acid and the ester derivatives were unsuitable for synthesis, whereas the amide or hydrazide derivative were useful. To examine the P₁'-position (notation of Schechter & Berger, 1967) specificity for peptide synthesis, the following experiment was performed with various amino acid amides as nucleophiles. The results are summarized in Table 3, which gives the product identifications and product yields determined by t.l.c. Table 3 indicates that the effects of the various amino acid amides are as follows: Val-NH₂, Leu-NH₂, Ile-NH₂, Phe-NH₂, Tyr-NH₂, Ala-NH₂ > Gly-NH₂ > Ser-NH₂, His-NH₂ > Glu-NH₂ > D-Leu-NH₂ > Pro-NH₂. Hydrophobic or bulky amino acid

residues at position P₁' are suitable for synthesis. Glu-NH₂ is less suitable for synthesis, which might be ascribed to its negative charge. Interestingly, D-Leu-NH₂ can be used for synthesis, although the reaction is much slower than that of the corresponding L-form. Thus the resolution of DL-Leu-NH₂ was possible in the synthesis. Pro-NH₂ is the most unsuitable material for the synthesis. We could not detect any product in the reaction mixture even though the concentration of the starting materials was increased to above 0.25 M.

To check the product yields determined by t.l.c. as described above, 10 ml-scale experiments were performed with a starting-material concentration of 0.2 M (Ac-Phe-OEt and an amino acid amide), and in which the reaction was continued for 10 min. After concentration *in vacuo*, the products were extracted with ethyl acetate, which was successively washed with 1 M-HCl, water, 5% (w/v) NaHCO₃ and water.

Table 2. *Effects of amino acids or their derivatives as nucleophiles*

The reaction mixture (1 ml) contained 0.1 M-Ac-Phe-OEt, 0.1 M acceptor nucleophile, 0.02 mM- α -chymotrypsin, 0.2 M- $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$ buffer (pH 10) and 10% (v/v) dimethylformamide. The mixture was stirred vigorously for 2 min at room temperature (25°C). The R_F values of products were calculated by using the hydrolysates of the donor ester as control ($R_F = 1.0$), and the roman numeral in parentheses shows the solvent used for t.l.c. (see the Methods section; this notation is also used in Tables 3-7).

Acceptor nucleophile	Identification		Product yield (%)
	Product	R_F	
Leu	Ac-Phe-Leu		0
Leu-OEt	Ac-Phe-Leu-OEt		0
Leu-NH ₂	Ac-Phe-Leu-NH ₂ *	1.4 (I)	78
Val-OBu [†]	Ac-Phe-Val-OBu [†]	1.4 (I)	19
Phe-NHNH ₂	Ac-Phe-Phe-NHNH ₂ ‡	0.7 (I)	79

* The identification is described in Table 3.

† Not identified.

‡ The product was identified by comparing its hydrolysate by α -chymotrypsin with Phe-NHNH₂ on t.l.c.

After drying over Na_2SO_4 , the products were crystallized by using ethyl acetate and/or methanol. The products were identified in the usual manner, and the yields of Ac-Phe-Gly-NH₂, Ac-Phe-Ala-NH₂, Ac-Phe-Leu-NH₂ and Ac-Phe-Tyr-NH₂ were 38, 55, 85 and 78% respectively. When DL-Leu-NH₂ was used, the yield of the L-form was almost quantitative (48%). Nevertheless, a 27% yield of Ac-Phe-D-Leu-NH₂ was obtained even with D-Leu-NH₂ as a nucleophile. These results were comparable with those in Table 3.

To determine the relation between product yields and their solubilities, the saturated concentrations of typical products and their starting materials were determined under the same conditions used in the synthesis. The concentrations (in parentheses) were as follows: Ac-Phe-Gly-NH₂ (0.23 M), Ac-Phe-Ala-NH₂ (33 mM), Ac-Phe-Leu-NH₂ (9.2 mM), Ac-Phe-D-Leu-NH₂ (21 mM), Ac-Phe-Tyr-NH₂ (5.6 mM), Ac-Phe-OEt (50 mM), Leu-NH₂ (>2.6 M) and Tyr-NH₂ (0.67 M). Although the solubility of Ac-Phe-OEt is low, α -chymotrypsin can attack it at concentrations above 0.2 M when the ester is suspended in the reaction mixture. With a few exceptions the product

Table 3. *Effects of various amino acid amides as nucleophiles*
For methods used and for further explanation, see the legends to Tables 1 and 2.

Acceptor nucleophile	Identification							Product yield (%)	
	Product	R_F	M.p. (°C)	Elementary analysis (%)			0.1 M*	0.25 M*	
				C	H	N			
Gly-NH ₂	Ac-Phe-Gly-NH ₂	0.3 (I)	186	Calc.	59.30	6.51	15.96	47	72
				Found	59.19	6.50	15.93		
Ala-NH ₂	Ac-Phe-Ala-NH ₂	0.3 (I)	242.5–243.5	Calc.	59.86	6.96	14.96	68	85
				Found	60.17	6.89	14.85		
Ser-NH ₂	Ac-Phe-Ser-NH ₂	0.3 (III)	212–214	Calc.	57.33	6.53	14.32	47	55
				Found	57.69	6.36	14.14		
Val-NH ₂	Ac-Phe-Val-NH ₂	1.3 (II)	284	Calc.	62.93	7.59	13.76	78	—
			(decomp.)	Found	62.85	7.58	13.66		
His-NH ₂	Ac-Phe-His-NH ₂ †	0.15 (IV)						42	54
Pro-NH ₂	Ac-Phe-Pro-NH ₂							0	0
Leu-NH ₂	Ac-Phe-Leu-NH ₂ , $\frac{1}{2}$ H ₂ O‡	1.4 (I)	256.5–258	Calc.	63.21	7.93	13.01	78	—
				Found	63.29	8.07	12.82		
DL-Leu-NH ₂	Ac-Phe-Leu-NH ₂ ,H ₂ O§	1.4 (I)	253–256	Calc.	60.51	8.07	12.45	48	—
				Found	60.78	7.67	12.19		
D-Leu-NH ₂	Ac-Phe-D-Leu-NH ₂ , $\frac{1}{2}$ H ₂ O	0.8 (I)	215–218	Calc.	62.17	7.98	12.79	0	39
				Found	62.34	8.12	12.66		
Ile-NH ₂	Ac-Phe-Ile-NH ₂	1.5 (I)	274–275.5	Calc.	63.93	7.89	13.15	81	—
				Found	63.95	7.88	12.99		
Phe-NH ₂	Ac-Phe-Phe-NH ₂ , $\frac{1}{2}$ H ₂ O	1.5 (I)	266–268	Calc.	67.28	6.61	11.77	75	—
				Found	67.33	6.55	11.74		
Tyr-NH ₂	Ac-Phe-Tyr-NH ₂	0.5 (V)	249–251	Calc.	65.06	6.23	11.38	71	—
				Found	64.78	6.24	11.10		
Glu-NH ₂	Ac-Phe-Glu-NH ₂	0.5 (I)	209–211	Calc.	57.31	6.31	12.53	24	25
				Found	57.43	6.20	12.45		

* Concentration of the starting materials.

† The product was identified by comparing its hydrolysate by α -chymotrypsin with His-NH₂ on t.l.c.

‡ $[\alpha]_D^{25} - 19.8 \pm 4.8^\circ$ (0.126% in methanol).

§ $[\alpha]_D^{25} - 17.8 \pm 2.5^\circ$ (0.232% in methanol).

|| $[\alpha]_D^{25} + 82.7 \pm 1.2^\circ$ (1.028% in methanol).

yields mentioned above seem to be roughly parallel to their solubilities. For example, in spite of the low solubility of Ac-Phe-D-Leu-NH₂, its yield is small.

Dipeptides, tripeptides and their derivatives. We continued our study with dipeptides, tripeptides or their derivatives as acceptor nucleophiles, as shown in Table 4. Among the dipeptides tested, Leu-Phe and Phe-Leu are the best for synthesis; they contain hydrophobic or bulky amino acid residues at both P₁' and P₂' positions. The hydrophobic residues appear to be more efficient at the P₂' rather than the P₁' position (i.e. Gly-Leu > Leu-Gly). Lys-Leu is also useful for synthesis, indicating that the ε-amino group does not usually interfere with the synthesis.

Both Gly-Gly and Gly-Pro are very unsuitable residues.

Blocking the terminal carboxyl groups in dipeptides results usually in increased efficiency (Gly-Leu-NH₂ > Gly-Leu, Ala-Ala-OBu^t > Ala-Ala). The moderate efficiency of Tyr-Leu-NH₂ in comparison with Phe-Leu may be ascribed to its low solubility (95 mM at 25°C). Gly-D-Leu-NH₂ is also efficient for synthesis, although the yield is smaller than that of the corresponding L-form. Gly-Pro-NH₂ is much less suitable than Gly-D-Leu-NH₂, indicating that L-proline is a poor residue at both P₁' and P₂' positions even compared with D-amino acid residues. Tripeptides seem better than dipeptides as nucleophiles

Table 4. *Effects of various peptides or their derivatives as nucleophiles*
For methods used and for further explanation, see the legends to Tables 1 and 2.

Acceptor nucleophile	Product	Identification						Product yield (%)	
		R _F	M.p. (°C)	Elementary analysis (%)				0.1 M*	0.25 M*
					C	H	N		
Gly-Gly	Ac-Phe-Gly-Gly							0	0
Gly-Ala	Ac-Phe-Gly-Ala, $\frac{1}{2}$ H ₂ O	0.4 (II)	187–190	Calc.	56.55	6.38	12.36	44	74
				Found	56.41	6.34	12.59		
Gly-Leu	Ac-Phe-Gly-Leu, EtOH	0.7 (II)	109–110	Calc.	59.76	7.71	10.20	47	82
				Found	60.00	7.58	10.20		
Gly-Pro	Ac-Phe-Gly-Pro							0	0
Ala-Ala	Ac-Phe-Ala-Ala†	0.2 (I)						17	
Val-Ala	Ac-Phe-Val-Ala, $\frac{1}{3}$ H ₂ O	0.4 (I)	228.5–230.5	Calc.	59.52	7.27	10.96	48	65
				Found	59.44	7.20	10.96		
Leu-Gly	Ac-Phe-Leu-Gly, $\frac{1}{2}$ H ₂ O	0.4 (V)	199–201	Calc.	59.53	7.43	10.68	16	39
				Found	59.41	7.21	10.67		
Leu-Phe	Ac-Phe-Leu-Phe†	0.4 (I')						64	
Phe-Leu	Ac-Phe-Phe-Leu†	0.7 (III)						80	
Lys-Leu	Ac-Phe-Lys-Leu†	0.6 (IV)						33	75
Gly-Pro-NH ₂	Ac-Phe-Gly-Pro-NH ₂ ‡	0.5 (III)						<10	<10
Gly-Leu-NH ₂	Ac-Phe-Gly-Leu-NH ₂ , $\frac{1}{3}$ H ₂ O§	0.6 (II)	224–225	Calc.	60.05	7.53	14.74	81	
				Found	60.01	7.52	14.81		
Gly-D-Leu-NH ₂	Ac-Phe-Gly-D-Leu-NH ₂	0.4 (III)	216–218	Calc.	60.62	7.50	14.88	57	
				Found	60.57	7.51	14.84		
Ala-Ala-OBu ^t	Ac-Phe-Ala-Ala-OBu ^t †	0.7 (III)							48
Tyr-Leu-NH ₂	Ac-Phe-Tyr-Leu-NH ₂	0.6 (II)	244–246 (decomp.)	Calc.	64.71	7.10	11.61	47	
				Found	64.58	7.02	11.59		
Gly-Gly-Gly	Ac-Phe-Gly-Gly-Gly							0	0
Leu-Gly-Gly	Ac-Phe-Leu-Gly-Gly	0.3 (I)	148–150	Calc.	58.05	6.96	12.90	52	75
				Found	58.08	7.02	12.80		
Ala-Ala-Ala	Ac-Phe-Ala-Ala-Ala†	0.3 (III)						46	
Phe-Tyr-Ala	Ac-Phe-Phe-Tyr-Ala¶	0.8 (I')						24	
Gly-Gly-Tyr-NH ₂	Ac-Phe-Gly-Gly-Tyr-NH ₂ ¶	0.8 (IV)						81	
Ala-Ala-Ala-OBu ^t	Ac-Phe-Ala-Ala-Ala-OBu ^t	0.7 (I)	232–233	Calc.	60.49	7.61	11.76	67	90
				Found	60.49	7.66	11.70		

* Concentration of the starting materials.

† The product was identified by comparing its hydrolysate by α-chymotrypsin with the corresponding nucleophile on t.l.c.

‡ Not identified.

§ $[\alpha]_D^{25} + 21.0 \pm 0.6^\circ$ (1.050% in methanol).

|| $[\alpha]_D^{25} + 43.7 \pm 0.8^\circ$ (1.020% in methanol).

¶ Amino acid analysis of the hydrolysate (6M-HCl, 120°C, 20h) confirmed its composition.

(Ala-Ala-Ala > Ala-Ala, Leu-Gly-Gly > Leu-Gly), except Phe-Tyr-Ala and Gly-Gly-Gly. The small yield of Phe-Tyr-Ala may be ascribed to its low solubility. Blocking of the carboxyl group in tripeptides seems to result in higher synthesis yield (Ala-Ala-Ala-OBu^t > Ala-Ala-Ala).

Generally speaking, the yields with di- or tripeptides appear to be less than those with the corresponding amides. This might partly be due to the difference in their solubilities. To clarify this, the effect of reaction time was examined with 0.25M-Ac-Phe-OEt as a donor and 0.25M-Leu-Gly, -Gly-Ala or -Leu-Gly-Gly as a nucleophile (the other conditions being those described in Table 2). The product yields from reaction for 2 and 60 min, and for 20 h were as follows (in parentheses): Ac-Phe-Leu-Gly (39, 22 and 3%), Ac-Phe-Gly-Ala (74, 47 and 7%) and Ac-Phe-Leu-Gly-Gly (75, not determined and 35%). The data indicate that a reaction longer than

2 min results in greater decrease in yields; this is in marked contrast with the reaction with Leu-NH₂ (Fig. 1c). The lower yield thus can be ascribed to the secondary hydrolysis of the products by the same enzyme owing to their high solubility in the reaction mixture (saturated concentrations: Ac-Phe-Leu-Gly, 1M; Ac-Phe-Leu-Gly-Gly, 0.4M).

The 10 ml-scale experiment was performed with 0.2M-Ac-Phe-OEt and 0.2M-Gly-Leu or 0.2M-Leu-Gly-Gly as the starting materials (10 min reaction). The reaction was stopped by the addition of 5 ml of 1M-HCl. After concentration *in vacuo* the products were extracted with ethyl acetate in the presence of 1M-HCl. With Ac-Phe-Gly-Leu the extract was applied on the column of silica gel, and the product was eluted with chloroform/methanol (4:1, v/v). The product was crystallized from chloroform/ethanol, and identified in the usual way. The yield was 37%. With Ac-Phe-Leu-Gly-Gly, the above-

Table 5. *Effects of Ac-Tyr-OEt and Ac-Trp-OEt as donor esters*

The methods are as described in Table 2, except that the reaction mixture (1 ml) contained 0.1M donor ester and 0.1M-Leu-NH₂. For further explanation, see the legends to Tables 1 and 2.

Donor ester	Product	<i>R_F</i>	Identification					Product yield (%)
			M.p. (°C)	Elementary analysis (%)				
				C	H	N		
Ac-Tyr-OEt	Ac-Tyr-Leu-NH ₂	1.5 (IV)	249–250	Calc.	60.88	7.51	12.53	76
				Found	61.02	7.51	12.39	
Ac-Trp-OEt	Ac-Trp-Leu-NH ₂	1.7 (III)	249–250.5 (decomp.)	Calc.	63.67	7.31	15.63	41
				Found	63.29	7.37	15.12	

Table 6. *Effect of Cbz-Phe-OEt as a donor ester*

The reaction mixture (1 ml) contained 0.04M of starting materials (Cbz-Phe-OEt and a nucleophile), 0.2mM- α -chymotrypsin and 20% (v/v) dimethylformamide, the other conditions being as described in Table 2. The reaction was continued for 5 min. For further explanation, see the legends to Tables 1 and 2.

Acceptor nucleophile	Product	Identification						Product yield (%)
		<i>R_F</i>	M.p. (°C)	Elementary analysis (%)				
				C	H	N		
Gly-NH ₂	Cbz-Phe-Gly-NH ₂ , $\frac{1}{2}$ MeOH	0.4 (V)	130–132	Calc.	63.06	6.24	11.31	34
				Found	62.90	6.03	11.32	
Ala-NH ₂	Cbz-Phe-Ala-NH ₂	0.6 (V)	211–213	Calc.	65.03	6.28	11.37	58
				Found	65.18	6.28	11.17	
Leu-NH ₂	Cbz-Phe-Leu-NH ₂	0.7 (VI)	192.5–193.5	Calc.	67.13	7.10	10.21	73
				Found	67.09	6.83	10.23	
Tyr-NH ₂	Cbz-Phe-Tyr-NH ₂ , $\frac{1}{3}$ H ₂ O	0.4 (I)	224–226	Calc.	67.65	5.90	9.10	87
				Found	67.14	5.94	9.03	
Leu-Gly	Cbz-Phe-Leu-Gly	0.4 (V)	154–155	Calc.	63.95	6.65	8.95	31
				Found	63.75	6.60	8.81	
Val-Ala	Cbz-Phe-Val-Ala	0.3 (V)	207–209	Calc.	64.21	6.68	8.95	39
				Found	63.97	6.50	8.72	
Leu-Gly-Gly	Cbz-Phe-Leu-Gly-Gly, $\frac{1}{3}$ H ₂ O	0.5 (II)	194–197	Calc.	60.17	6.55	11.02	48
				Found	59.84	6.50	10.96	

Table 7. Effect of *N*-acylated peptide esters as donors

When the donor ester was Cbz-Ala-Phe-OMe, the reaction mixture contained 0.15 mM- α -chymotrypsin and 35% (v/v) dimethylformamide; when it was Ac-Ala-Ala-Phe-OMe or Cbz-Gly-Pro-Phe-OEt, 0.02 mM- α -chymotrypsin and 10% (v/v) dimethylformamide, or 0.02 mM- α -chymotrypsin and 40% (v/v) dimethylformamide were used respectively. Acceptor nucleophile and concentration of the starting materials are shown in the table. The reaction was continued for 5 min. The other conditions are described in the legend of Table 2. For further explanation, see the legends to Tables 1 and 2.

Reaction mixture			Identification			Elementary analysis (%)				Product yield (%)
Donor ester	Acceptor nucleophile	Concn. (M)	Product	R_F	M.p. (°C)		C	H	N	
Cbz-Ala-Phe-OMe	Gly-NH ₂	0.1	Cbz-Ala-Phe-Gly-NH ₂ · $\frac{1}{2}$ H ₂ O	0.2 (III)	192-193	Calc.	61.31	6.20	13.00	43
	Ala-NH ₂	0.1	Cbz-Ala-Phe-Ala-NH ₂	0.3 (III)	244.5-247	Found	61.25	6.19	13.04	
	Leu-NH ₂	0.1	Cbz-Ala-Phe-Leu-NH ₂	0.7 (V)	228-230	Found	62.74	6.36	12.73	53
	Phe-NH ₂	0.1	Cbz-Ala-Phe-Phe-NH ₂	0.6 (V)	255-256	Found	62.46	6.44	12.36	79
Ac-Ala-Ala-Phe-OMe	Phe-NHNH ₂	0.1	Cbz-Ala-Phe-Phe-NH ₂	0.7 (VII)	225-227	Calc.	64.42	6.90	11.55	69
	Phe-Leu	0.1	Cbz-Ala-Phe-Phe-Leu	0.8 (VIII)	225-227	Found	67.46	6.20	10.85	
Cbz-Gly-Pro-Phe-OEt	Leu-NH ₂	0.1	Cbz-Gly-Pro-Phe-Leu-NH ₂	0.4 (II)	290-294 (decomp.)	Calc.	66.68	6.66	8.89	95
	Leu-NH ₂	0.1	Cbz-Gly-Pro-Phe-Leu-NH ₂ · $\frac{2}{3}$ H ₂ O	1.2 (IV)	162-163.5	Found	66.37	6.75	8.81	69
Ala-Ala-Ala-OBu ¹	Gly-Gly	0.14	Cbz-Gly-Pro-Phe-Leu-NH ₂	0.5 (I)	155-157.5	Found	59.59	7.63	15.23	77
	Ala-Ala-Ala-OBu ¹	0.1	Cbz-Gly-Pro-Phe-Ala-Ala-OBu ¹ ·CH ₃ OH	0.8 (III)	192-194	Calc.	60.80	7.14	11.82	48
				0.2 (I)		Found	60.85	7.15	11.84	
				0.4 (I)		Found	59.99	6.52	12.34	49
				0.2 (III)		Found	60.16	6.51	11.85	
						Found	60.46	7.21	11.13	
						Found	60.45	7.17	11.21	

* The product was identified by comparing its hydrolysate by α -chymotrypsin with Phe-NHNH₂ on t.l.c.

mentioned ethyl acetate extract was concentrated *in vacuo* and the residue was washed repeatedly with chloroform. The product was crystallized from ethyl acetate and was identified in the usual manner. The yield was 43%. In comparison with the product yields determined by t.l.c., they were considerably lower. This could partly be ascribed to the longer reaction period (during which secondary hydrolysis would occur). The acceptor nucleophile (Tyr-Leu-NH₂ or Leu-Gly-Gly, for example) is not hydrolysed at all by chymotrypsin in the reaction (10 min).

Effects of the type of donor esters

In place of Ac-Phe-OEt, either Ac-Tyr-OEt or Ac-Trp-OEt can also be used as a donor ester, as is shown in Table 5. The conditions that are required for the use of Cbz-Phe-OEt as a donor are as follows: (1) 20% dimethylformamide must be used to dissolve the donor ester to a concentration of 40 mM; (2) enzyme concentration needs to be increased to 0.2 mM (10 times higher than usual) to compensate for the inactivation by the dimethylformamide; (3) the duration of agitation must be extended to 5 min to complete the reaction. Table 6 shows results for the reaction with various acceptor nucleophiles, which is analogous to that observed in the experiment with Ac-Phe-OEt as a donor ester.

The next experiment was carried out with peptide esters such as Cbz-Ala-Phe-OMe, Ac-Ala-Ala-Phe-OMe and Cbz-Gly-Pro-Phe-OEt as donor esters. Owing to the low solubility of most of them, the reaction was performed in the presence of a high concentration of dimethylformamide ($\leq 40\%$). The results are summarized in Table 7, which indicates a fairly high yield even with peptide esters as donors. When peptides are used as nucleophiles, the synthesis can be regarded as fragment-condensation.

The 10 ml-scale experiment was performed for 10 min with Cbz-Gly-Pro-Phe-OEt and Leu-NH₂ as starting materials, the other conditions being those described in Table 7. To stop further reaction, 5 ml of 1 M-HCl was added to the reaction mixture. After concentration *in vacuo*, the product was extracted with ethyl acetate, which was successively washed with 1 M-HCl, water, 5% NaHCO₃ and water. After drying over Na₂SO₄, the product was concentrated and crystallized with the same solvent. The product was identified as Cbz-Gly-Pro-Phe-Leu-NH₂ and its yield was 58%. These results indicate that *N*-acylated peptide esters are useful as donors for peptide synthesis if they are good substrates for α -chymotrypsin.

Discussion

Our present study indicates that α -chymotrypsin is a potent catalyst in peptide synthesis. For example, Ac-Phe-Leu-NH₂ was synthesized almost quanti-

tatively under optimal conditions when Ac-Phe-OEt and Leu-NH₂ were used as starting materials. This conflicts with the previous view that chymotrypsin may not be very useful for peptide synthesis, unlike papain (Fink & Bender, 1969) or bovine liver esterase (Goldberg & Fruton, 1969). The conditions for synthesis were very similar to those with bovine liver esterase, which has been studied by Goldberg & Fruton (1969). These latter workers showed that considerably more enzyme and a more alkaline pH are necessary for synthesis than hydrolysis. This information was applied to our chymotrypsin-catalysed synthesis of peptides. Another very important condition was the concentration of the starting materials, which will be discussed below.

Also important for peptide synthesis with α -chymotrypsin was selection of the amino acid derivatives, peptides or their derivatives used as nucleophiles. Scheme 1 (see the introduction) indicates that the synthesis of Ac-Phe-N depends on the k_{+4} of the nucleophile (N), as well as its concentration ([N]). This is true in the synthesis of Ac-Phe-Gly-NH₂ and Ac-Phe-Ala-NH₂, with Gly-NH₂ and Ala-NH₂ respectively as nucleophiles; the product yields almost paralleled the k_{+4} values of the corresponding nucleophiles (which were given in the introduction). The k_{+4} values of the other nucleophiles have never been determined. However, this study indicates that the P₁'-position specificity for synthesis, determined with various amino acid amides as nucleophiles (Ac-Phe-OEt as donor), was similar to that for hydrolysis. The latter specificity was determined (Mori-hara *et al.*, 1969b; Mori-hara & Oka, 1977) with Cbz-Tyr-Abc-NH₂ and Cbz-Gly-Pro-Phe-Abc-Abc

$\uparrow \qquad \qquad \qquad \uparrow$
 (where Abc = various amino acid residues; the arrows show the bonds split) as substrates. Hydrophobic or bulky amino acid residues were suitable for both hydrolysis and synthesis. Synthesis may partly be related to the low solubility of the products, which is discussed below. Pro-NH₂ never participated in peptide synthesis with α -chymotrypsin; a similar case was observed in hydrolysis, i.e. Cbz-Gly-Pro-Phe-Pro-Leu was insensitive to α -chymotrypsin.

As to the stereospecificity, resolution of DL-Leu-NH₂ was possible by asymmetric synthesis:

The yield of the L-product was almost quantitative. Synthesis with D-Leu-NH₂ was much slower than that of the L-form. Similar stereospecificity has been observed with papain (Milne & Stevens, 1950). Interestingly, the stereospecificity of papain for anilide or hydrazide synthesis is dependent on the acyl group for blocking the α -amino group in the donor (Bennett & Niemann, 1950; Schuller & Nie-

mann, 1951); stringent stereospecificity was observed with $\text{CH}_3\text{CO}-$ or $\text{C}_6\text{H}_5\text{CO}-$ but less with $\text{CH}_3\text{OCO}-$, $\text{C}_2\text{H}_5\text{OCO}-$ or $\text{C}_6\text{H}_5\text{CH}_2\text{OCO}-$.

The P_2' -position specificity for synthesis was also similar to that for hydrolysis, which has been determined with Cbz-Tyr-Gly-Abc and Cbz-Gly-Pro-Phe-Gly-Abc (Moriyama *et al.*, 1969b; Moriyama & Oka, 1977) as substrates. Hydrophobic or bulky amino acid residues were also efficient in both reactions, with L-proline being the worst choice. Stereospecificity was also observed. These results may indicate that the k_{+4} values of nucleophiles are closely related to the leaving-group specificity of substrates containing the corresponding nucleophiles as leaving groups. Further, the acceptor nucleophiles used for synthesis may occupy the same position (subsite) in the active site of α -chymotrypsin as the leaving groups in hydrolysis. Similar binding modes with nucleophiles have been observed with papain (Fink & Bender, 1969).

Various ester substrates, such as Ac-Tyr-OEt, Ac-Trp-OEt, Cbz-Phe-OEt, Cbz-Ala-Phe-OMe, Cbz-Gly-Pro-Phe-OEt and Ac-Ala-Ala-Phe-OMe, were useful as donor esters for peptide synthesis, together with Ac-Phe-OEt. However, the reaction had to be performed in the presence of a high concentration of dimethylformamide ($\leq 40\%$, v/v) with some esters, such as those of Cbz-Phe or Cbz-peptide, which have low solubility. Ester substrates may be better donors than peptide (e.g. Ac-Phe-Leu-Gly) or amide substrates (e.g. Ac-Phe-NH₂) or their hydrolysates of α -chymotrypsin (e.g. Ac-Phe-OH). Since the rate-determining step with the latter donors is the acylation process, the synthesis appears to be too slow to allow accumulation of products, which undergo secondary hydrolysis. This may not be the case when the products are precipitated in the reaction mixture (K. Moriyama & T. Oka, unpublished work). The products synthesized by α -chymotrypsin seem to be good substrates for the same enzyme, and thus must be hydrolysed as soon as they are produced (secondary hydrolysis) while they are still soluble.

With ester substrates, preferential synthesis may take place rather than secondary hydrolysis for the following reason. The rate-determining step is deacylation in ester hydrolysis of α -chymotrypsin ($k_{+2}/k_{+3} \approx 70$, in Ac-Phe-OEt hydrolysis; Zerner *et al.*, 1964), which may result in abundant accumulation of acyl-enzyme in the reaction mixture. Since the rate-determining step is acylation in peptide- or amide-bond hydrolysis by α -chymotrypsin ($k_{+3}/k_{+2} = 2000$ in Ac-Phe-NH₂ hydrolysis; Zerner *et al.*, 1964), the ratio (2000:1) may roughly correspond to that of the rates of synthesis and secondary hydrolysis of products. The real ratio, k_{+4}/k_{+2} , must be larger than that, k_{+3}/k_{+2} , because $k_{+4} > k_{+3}$ (an essential factor for peptide synthesis). Even if corrections are made in the k_{+3}/k_{+2} ratio for the different leaving groups

(Moriyama *et al.*, 1969b; Fersht *et al.*, 1973; Moriyama & Oka, 1977), the rate of synthesis must be markedly higher than that of the secondary hydrolysis of products. Thus synthetic products accumulate in the reaction mixture until the corresponding ester substrates are consumed, whether the products are soluble or not.

A higher concentration of nucleophile in the reaction mixture disturbs the hydrolysis of substrates containing the corresponding nucleophiles as leaving groups (Fastrez & Fersht, 1973). Therefore the secondary hydrolysis seems negligible at the initial stage of the synthetic reaction owing to the high concentration of nucleophiles, and it gradually increases together with the decrease in nucleophile concentration in the reaction mixture. In this case, too, the concentration of the starting materials, especially the nucleophiles, is very important, as it affects the inhibition of secondary hydrolysis of products.

In relation to the concentration of starting materials, the solubility of the products is very important in enzymic synthesis of peptides. When the products are precipitated in the reaction mixture, secondary hydrolysis does not occur; the reverse may be observed when the products remain soluble. The following examples illustrate this:

Both synthetic reactions are depicted in Fig. 2, assuming that the k_{+4} values of both nucleophiles are identical. Since the rate of hydrolysis of Cbz-Tyr-

Leu-NH₂ by α -chymotrypsin is considerably higher than that of Cbz-Tyr-Ala-NH₂ (Moriyama *et al.*,

1969b), the k_{+4} value of Leu-NH₂ must be higher than that of Ala-NH₂. Ala-NH₂ at 1 M is 44 times more reactive than 55 M-water for deacylation of acyl-chymotrypsin (Fastrez & Fersht, 1973). Accordingly, the nucleophilicity of 0.25 M-Leu-NH₂ may be calculated as 20 times more reactive than 55 M-water (assuming that the ratio of nucleophilicity of Leu-NH₂/Ala-NH₂ = 2). To simplify Fig. 2, the hydrolysis is assumed to be 5% and the synthesis 95% in the deacylation process, with the rate of the synthetic reaction gradually decreasing with consumption of the ester substrate.

The solubilities of the products Ac-Phe-Leu-NH₂ and Ac-Phe-Leu-Gly are 9.2 mm and 1 M respectively in the reaction mixture. Accordingly, most of Ac-Phe-Leu-NH₂ synthesized is precipitated in the reaction mixture, and does not undergo secondary hydrolysis (Fig. 2a). Even with the soluble product (9.2 mm), secondary hydrolysis must be small, owing to inhibition by the high concentration of Leu-NH₂ in the reaction mixture, even in the later stage of the

Fig. 2. Correlation between synthesis and secondary hydrolysis

The reaction was performed under optimal conditions, with 0.25M-Ac-Phe-OEt as common donor and (a) 0.25M Leu-NH₂ and (b) 0.25M Leu-Gly respectively as acceptors: —, synthesis; ----, secondary hydrolysis. The solubility of Ac-Phe-Leu-NH₂ is about 10mM, which is shown by a straight line in (a).

synthetic reaction. The product yield may thus be almost quantitative. On the other hand, Ac-Phe-Leu-Gly must be hydrolysed immediately on synthesis, with the rate of synthesis being faster than that of secondary hydrolysis, and the difference between the two rates being the product yield (Fig. 2b). To obtain a good yield, the end of the reaction must be monitored to prevent excessive secondary hydrolysis.

Amino acid amides or tripeptides are usually better nucleophiles than dipeptides, and blocking the C-terminal group of dipeptides or tripeptides increases the extent of synthesis. This may be related to the decreased solubility of the products. As for the products derived from Ac-Phe-OEt as donor, Ac-Phe-Abc-NH₂ (Abc = L-alanine, L-valine, L-leucine, D-leucine, L-isoleucine and L-phenylalanine), Ac-Phe-Gly-Leu, Ac-Phe-Leu-Phe, Ac-Phe-Phe-Leu, Ac-Phe-Gly-Leu-NH₂, Ac-Phe-Gly-D-Leu-NH₂, Ac-Phe-Ala-Ala-OBu^t, Ac-Phe-Gly-Gly-Tyr-NH₂ and Ac-Phe-Ala-Ala-Ala-OBu^t are precipitated in the reaction mixture; the product yields are usually high except in a few cases (e.g. Ac-Phe-D-Leu-NH₂). Although all the other products such as Ac-Phe-Abc-NH₂ (Abc = glycine, L-serine, L-histidine and L-glutamic acid), Ac-Phe-dipeptide (dipeptide = Gly-Gly, Gly-Ala, Ala-Ala, Val-Ala, Leu-Gly and Lys-Leu), and Ac-Phe-tripeptide (tripeptide = Gly-Gly-Gly, Leu-Gly-Gly and Ala-Ala-Ala) are soluble in the reaction mixture, their product yields also depend on their solubility. For example, the solubility of Ac-Phe-Leu-Gly-Gly is 0.4M; therefore the product must be soluble in the reaction mixture when the concentration of the starting materials is 0.25M, similarly with Ac-Phe-

Leu-Gly. The yield of the former is much better than that of the latter product in relation to the degree of solubility. The secondary hydrolysis seems to require more time, owing to the lower solubility of the products even when they are soluble in the reaction mixture. The negligible yield of Ac-Phe-Gly-Gly and Ac-Phe-Gly-Gly-Gly may be ascribed to their high solubility, which makes them markedly susceptible to secondary hydrolysis.

There are some differences in product yield that depend on the kinds of nucleophiles used; for example, Phe-Leu is a better nucleophile than Phe-Tyr-Ala or Tyr-Leu-NH₂, where the solubility of the tripeptide or dipeptide derivative is low in the reaction mixture (<0.1M). This might be related to the low concentration of nucleophiles in the deacylation process. Therefore the product yield seems to increase when the difference of solubility between the starting materials and the products is more marked, assuming that the k_{+4} values of the nucleophiles are equal.

The synthetic reaction with ester substrates was very fast, as was shown by the present study, and the possibility of side reactions was therefore decreased. For example, either Tyr-Leu-NH₂ or Gly-Gly-Tyr-NH₂ used as nucleophiles could be hydrolysed to some extent at the peptide and/or amide bond. However, the rate of synthesis is much faster than that of secondary hydrolysis as mentioned above. The rate of hydrolysis of nucleophiles must be much slower than that of secondary hydrolysis, because the N-terminal group is not blocked. Thus preferential synthetic reaction may be considered to take place before hydrolysis of the nucleophiles. Similarly, condensation between the hydrolysates of ester substrates by α -chymotrypsin and nucleophiles or between the nucleophiles may be negligible, because the reaction is markedly slow in comparison with that of synthesis with ester substrates. The negligible side reactions may be another merit of the method using ester substrates as donors.

When the solubility of the donor esters is low, the synthetic reaction should be performed in the presence of a high concentration of dimethylformamide ($\leq 40\%$), with other conditions being similar to those with Ac-Phe-OEt as donor. The product yields mainly depend on the kinds of nucleophiles, and correspond to those with Ac-Phe-OEt as donor. Thus good ester substrates are all useful for peptide synthesis, regardless of their solubility. This further suggests that the correlation in solubility between donor esters and products in the reaction mixture containing a high or low concentration of dimethylformamide is dependent on the kinds of nucleophiles used for synthesis, but independent of the solubility of the donor esters.

Fragment condensation was possible when peptide esters and peptides or their derivatives were used as starting materials. For example:

The product yields depended on the kinds of nucleophiles used, and they corresponded to those observed with Ac-Phe-OEt as a donor. Fragment condensation is probably difficult when longer molecules are used as donors and acceptors because of the small difference in solubility between the starting materials and the products.

We investigated whether or not α -chymotrypsin could be used for peptide synthesis in place of the chemical method. In some cases, the synthesis was almost quantitative, with the nucleophilicity of the acceptor nucleophiles and the solubility of the products being the most important conditions. The concentration of the starting materials is also very important in (1) nucleophilic attack of the nucleophiles and (2) inhibition of secondary hydrolysis by the nucleophiles, regardless of product solubility. When the solubility of the product is low, more products may be precipitated with the increased concentration of the starting materials. Trypsin (EC 3.4.21.4) is known to possess much sharper specificity than α -chymotrypsin, although its mechanism of action is very similar. We can thus expect it to undergo reactions analogous to those described in the present paper. The merits of the enzymic method for peptide synthesis are: (1) the reaction is stereospecific; (2) the functional groups of side chains in amino acid residues usually do not need to be blocked for peptide synthesis; (3) fragment condensation is possible in some cases.

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