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Thermolysin-Catalyzed Synthesis of Peptide Amides[1)]

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Enzymatic condensation by thermolysin between various amino acid amides and N^{α}-protected or unprotected peptides was examined. As models, three protected tetrapeptide amides, Boc–Trp–Met–Asp–X, Boc–Ser–Glu–Ala–X and Boc–Ser–Lys–Ala–X, and two unprotected tetrapeptide amides, H–Trp–Met–Asp–X and H–Phe–Met–Arg–X, were prepared [X = Phe–NH_2, Leu–NH_2, Phe–NHEt, Val–NH_2, Ala–NH_2, (*p*-fluoro)Phe–NH_2], and the effects of various experimental conditions (pH, solvent and time) were examined. In addition, the C-terminal of oxidized insulin B-chain was elongated to an amide by addition of various amides mentioned above with the aid of thermolysin.

Keywords——enzymatic synthesis; thermolysin-catalyzed synthesis; peptide amide synthesis; optimal pH conditions; oxidized insulin B–Phe^{31}–NH_2; tetragastrin; FMRF-NH_2

Many biologically active peptides have an α-amide structure at their carboxyl termini and in most cases, such amide groups play an important role in their biological activities. Recent work on prohormone processing has revealed that the C-terminal amide group is generated from the Gly residue adjacent to paired basic amino acid residues by tryptic-like cleavage, followed by the action of tissue-specific α-amidating enzymes.[2-7)] Recently, gene technology has made it possible to produce a number of peptides and proteins, but not C-amidated products, since the bacteria or yeast organisms employed lack the above necessary enzymatic machinery. In order to overcome this limitation of the recombinant deoxyribonucleic acid (DNA) method, α-amidating enzymes are under intensive investigations.[8-11)]

We have examined an alternative way of preparing C-amidated peptides by enzymatic condensation of amino acid amides and peptides. Protease-catalyzed peptide synthesis has some advantages over chemical synthesis.[12-14)] The most attractive feature of the enzymatic method is that synthesis can be performed in principle without side chain protection of constituent amino acid residues, since proteases specifically activate the α-carboxyl or the α-amino group at the reaction site, with retention of the chiral integrity. In the present studies, by using thermolysin as a catalyst, we prepared the following three protected tetrapeptide amides by condensation of H–Phe–NH_2 and the respective tripeptides, *i.e.*, Boc–Trp–Met–Asp–Phe–NH_2 (C-terminal of gastrin),[15)] Boc–Ser–Glu–Ala–Phe–NH_2 (C-terminal of rat calcitonin gene-related peptide, CGRP)[16)] and Boc–Ser–Lys–Ala–Phe–NH_2 (C-terminal of human CGRP).[17)] Next, by using unprotected tripeptides, H–Trp–Met–Asp–Phe–NH_2 and H–Phe–Met–Arg–Phe–NH_2 (molluscan cardioexcitatory neuropeptide, FMRF amide)[18)] were similarly prepared. In these experiments, H–Phe–NH_2 was replaced by other amino acid amides and optimum reaction conditions (pH, solvent effects and reaction time) were examined. In addition, by using thermolysin, condensation reactions between oxidized insulin B-chain and H–Phe–NH_2 or four other amides were examined.

First, the model tripeptide units, Boc–Trp–Met–Asp–OH, Boc–Ser–Glu–Ala–OH and

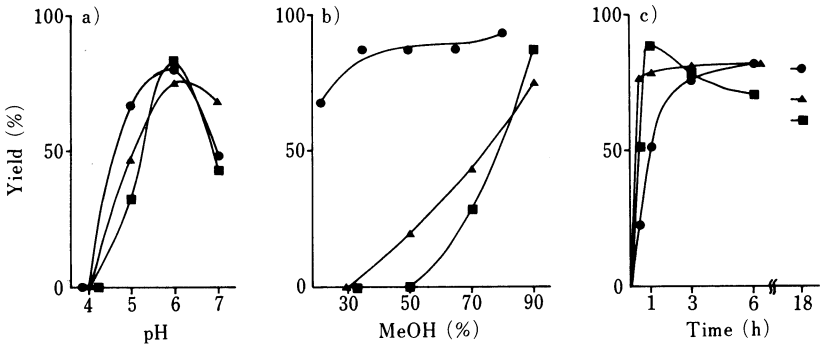

Fig. 1. Various Reaction Conditions for the Thermolysin-Catalyzed Syntheses of Boc–Trp–Met–Asp–Phe–NH_2 (●), Boc–Ser–Glu–Ala–Phe–NH_2 (▲) and Boc–Ser–Lys–Ala–Phe–NH_2 (■)

a) Optimum pH. b) Concentration of MeOH. c) Reaction time.
Conditions: a) ●, (50% MeOH, 18 h), ▲■, (90% MeOH, 3 h); b) ●, (pH 6.0, 18 h), ▲■, (pH 6.0, 3 h); c) ●, (50% MeOH, pH 6.0), ▲■, (90% MeOH, pH 6.0).

Boc–Ser–Lys–Ala–OH, were prepared chemically by known methods. A mixture of thermolysin, H–Phe–NH_2 and each protected tripeptide (0.001 : 5 : 1 mol eq) in aqueous methanol (20%—90%) was stirred at various pH values (4 to 7) at room temperature for 3 h or 18 h. Each product was quantified by high-performance liquid chromatography (HPLC). Under these conditions, the optimum pHs for the syntheses of three peptides, Boc–Trp–Met–Asp–Phe–NH_2, Boc–Ser–Glu–Ala–Phe–NH_2 and Boc–Ser–Lys–Ala–Phe–NH_2, were judged to be around 6.0, as shown in Fig. 1-a, regardless of the presence or absence of the free carboxyl or amino side chain functions in the two reactants. Generally, in a high concentration of organic solvents, dissociation of functional groups, including the α-carboxyl and the amino group, seems to be well suppressed to shift the equilibrium towards α-peptide bond formation.[19] Thus, the effects of methanol concentration were examined, and the results are shown in Fig. 1-b. The yield of Boc–Trp–Met–Asp–Phe–NH_2 was not much affected by the methanol concentration. However, in the cases of Boc–Ser–Glu–Ala–Phe–NH_2 and Boc–Ser–Lys–Ala–Phe–NH_2, the yields were immensely affected by the amount of methanol employed. In the former case, the product, Boc–Trp–Met–Asp–Phe–NH_2, precipitated during the reaction, because of poor solubility in the solvent system employed. Thus, the undesirable hydrolysis of the established peptide bond was avoided. In the latter two cases, Boc–Ser–Glu–Ala–Phe–NH_2 and Boc–Ser–Lys–Ala–Phe–NH_2 are both rather soluble in the reaction solvent and thus, reversible hydrolysis of the peptide bond took place partially, depending on the concentration of methanol.

Since the enzymatic peptide-forming reaction is a reversible reaction, the time required for the optimum yield was next examined. At the optimum pH, a less soluble compound, Boc–Trp–Met–Asp–Phe–NH_2, required 6 h to reach the maximum yield, while the two rather soluble peptides, Boc–Ser–Glu–Ala–Phe–NH_2 and Boc–Ser–Lys–Ala–Phe–NH_2, required only 1 h. In the case of Boc–Ser–Lys–Ala–Phe–NH_2, the yield decreased according to the time of exposure to thermolysin. The results suggest that a longer treatment is necessary for a peptide less soluble in the solvent, but not for peptides soluble in the solvent.

In the above experiments, we examined various reaction conditions, when H–Phe–NH_2 was employed. Next, we replaced H–Phe–NH_2 by five other amide derivatives, H–Leu–NH_2, H–Phe–NHEt, H–Val–NH_2, H–Ala–NH_2 and H–DL-(*p*-fluoro)Phe–NH_2, and the results are shown in Table I.

As expected, amide derivatives with a more hydrophobic nature, such as H–Phe–NHEt,

TABLE I. Thermolysin-Catalyzed Condensation of Various Amides with Boc–Trp–Met–Asp–OH or Boc–Ser–Glu–Ala–OH or Boc–Ser–Lys–Ala–OH

Amino component	Carboxyl component					
	Boc–Trp–Met–Asp–OH (a)		Boc–Ser–Glu–Ala–OH (b)		Boc–Ser–Lys–Ala–OH (c)	
	Yield (%)	t_R[a] (min)	Yield (%)	t_R[a] (min)	Yield (%)	t_R[a] (min)
Phe–NH_2	86	5.8	81	8.7	79	7.6
Leu–NH_2	57	5.3	31	6.5	34	5.6
Phe–NHEt	78	8.1	83	13.5	94	11.6
Val–NH_2	38	4.3	31	3.7	31	3.5
Ala–NH_2	Trace	3.7	9	3.0	0	—
DL-(*p*-Fluoro)Phe–NH_2	53[b]	6.7	56	10.7	54	9.4

The reaction mixture contained 50 mM carboxyl component, 250 mM amino component and 50 μM thermolysin. Reaction conditions: (a) 50% MeOH, pH 6.0, 18 h; (b) and (c), 90% MeOH, pH 6.0, 3 h. *a*) Eluent for HPLC: (a), 45% CH_3CN in 0.2% TFA; (b), 25% CH_3CN in 0.2% TFA; (c), 22% CH_3CN in 0.2% TFA. *b*) L-Configuration.

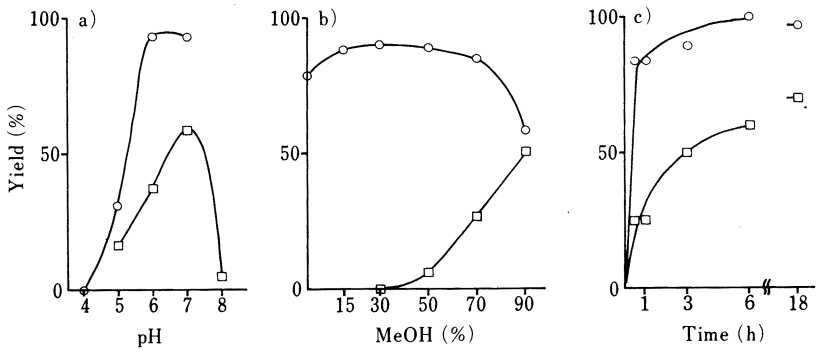

Fig. 2. Various Reaction Conditions for the Thermolysin-Catalyzed Syntheses of H–Trp–Met–Asp–Phe–NH_2 (○) and H–Phe–Met–Arg–Phe–NH_2 (□)

a) Optimum pH. b) Concentration of MeOH. c) Reaction time.
Conditions: a) ○, (50% MeOH, 3 h), □, (90% MeOH, 3 h); b) ○, (pH 6.5, 3 h), □, (pH 7.0, 3 h); c) ○, (50% MeOH, pH 6.5), □, (90% MeOH, pH 7.0).

gave higher yields than the others, since thermolysin, a metallo endopeptidase, has a high substrate specificity to the imino side of hydrophobic amino acids as the reaction site. A fairly good co-relation between the yields and their retention times in HPLC was observed. It is clear that each retention time reflects the hydrophobicity of each condensed amino component. As a modified amino component, we examined H–DL-(*p*-fluoro)Phe–NH_2. Thermolysin gave the product in nearly 50% yield. Its all-L-configuration was ascertained by chromatographic comparison with a standard sample prepared by the established method.

Next, thermolysin-catalyzed condensation reactions between various amides and unprotected tripeptides, H–Trp–Met–Asp–OH and H–Phe–Met–Arg–OH, were examined. A mixture of H–Phe–NH_2 and H–Trp–Met–Asp–OH or H–Phe–Met–Arg–OH (5 : 1 mol eq) was treated with thermolysin (0.001 mol eq) in aqueous methanol (0%—90%) at room temperature for 3 h. The optimum pHs for the syntheses of two peptides, H–Trp–Met–Asp–Phe–NH_2 and H–Phe–Met–Arg–Phe–NH_2, were 6.5 and 7.0, respectively, as shown in Fig. 2-a. These values were higher than pH 6.0, observed in the preparations of the three N^{α}-protected tetrapeptides stated earlier. From theoretical studies, the optimum pH is given by the following equation[20]: $pH_{opt} = 1/2(pK_{ac} + pK_{aa})$, where pK_{ac} and pK_{aa} are the isoelectric

points of the carboxyl and amino components, respectively. In these experiments, we did not measure the isoelectric point of any component involved. However, it is certain that an unprotected peptide has an additional amino function compared with an N^{α}-protected peptide and this difference accounts for the difference of the optimum pH values observed in these two parallel experiments. We also examined the effect of concentration of methanol in the solvent system. As shown in Fig. 2-b, H–Trp–Met–Asp–Phe–NH_2 showed less solvent effects than observed in the case of Boc–Trp–Met–Asp–Phe–NH_2, while H–Phe–Met–Arg–Phe–NH_2 suffered considerable solvent effects, because of its rather good solubility in the reaction solvent.

Next, the relationship between the yield and time course was examined and the results are shown in Fig. 2-c. In the synthesis of H–Trp–Met–Asp–Phe–NH_2, a relatively high yield was obtained within 1 h, while in the case of H–Phe–Met–Arg–Phe–NH_2, the yield remained

TABLE II. Thermolysin-Catalyzed Condensation of Various Amides with H–Trp–Met–Asp–OH or H–Phe–Met–Arg–OH

Amino component	Carboxyl component				
	H–Trp–Met–Asp–OH (a)		H–Phe–Met–Arg–OH (b)		
	Yield (%)	t_R[a] (min)	Yield (%)	Yield[b] (%)	t_R[a] (min)
Phe–NH_2	89	7.5	50	82	8.9
Leu–NH_2	78	5.8	48	56	7.1
Phe–NHEt	48	13.3	38	69	16.2
Val–NH_2	51	4.1	7	28	4.3
Ala–NH_2	Trace	3.1	0	13	4.0
DL-(*p*-Fluoro)Phe–NH_2	76[c]	9.1	23	47	11.4

The reaction mixture contained 50 mM carboxyl component, 250 mM amino component and 50 μM thermolysin. Reaction conditions: (a), 50% MeOH, pH 6.5, 3 h; (b), 90% MeOH, pH 7.0, 3 h. *a*) Eluent for HPLC: (a), 25% CH_3CN in 0.2% TFA; (b), 20% CH_3CN in 0.2% TFA. *b*) 500 mM amino component and 18 h reaction. *c*) L-Configuration.

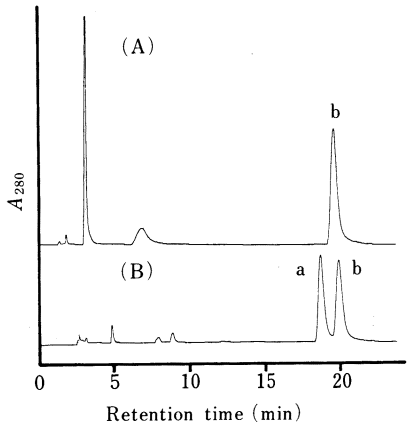

Fig. 3. HPLC Analysis of the Enzymatic (A) and the Chemical (B) Preparations of H–Trp–Met–Asp–(*p*-fluoro)Phe–NH_2

Column: Nucleosil $5C_{18}$ (4.0 × 200 mm). Solvent: 20% CH_3CN in 0.2% TFA. Flow rate: 1.0 ml/min. a. H–Trp–Met–Asp–D-(*p*-fluoro)Phe–NH_2, b. H–Trp–Met–Asp–L-(*p*-fluoro)Phe–NH_2.

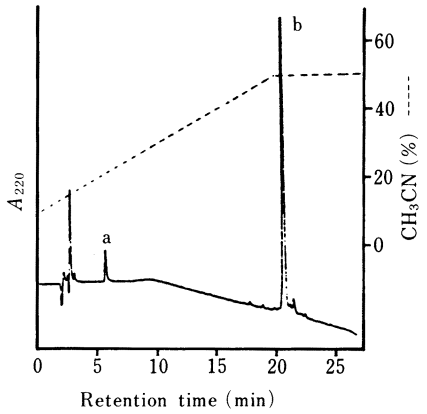

Fig. 4. HPLC Separation of a Lysyl Endopeptidase Digest of Oxidized Insulin B–Phe[31]–NH_2

Column: Nucleosil $5C_{18}$ (4.0 × 200 mm). Solvent: gradient of CH_3CN in 0.2% TFA. Flow rate: 1.0 ml/min. a. H–Asp–Phe–NH_2, b. des-Ala[30]-oxidized insulin B-chain.

at 50% after 3 h, presumably due to reversible hydrolysis of the peptide bond. It seems worthwhile to note that under these conditions, thermolysin did not give any polymerized product, such as H–(Trp–Met–Asp)$_n$–OH or H–(Phe–Met–Arg)$_n$–OH. As in the former experiments, we condensed various amide derivatives with H–Trp–Met–Asp–OH or H–Phe–Met–Arg–OH by the use of thermolysin, and the results are shown in Table II.

As stated earlier, amide derivatives with more hydrophobic nature gave higher yields. In these experiments, the yield of H–Phe–Met–Arg–Phe–NH_2 was somewhat low. However, every yield was increased when the amount of each amino component was increased from 5 to 10 eq and the reaction time was elongated from 3 h to 18 h as shown in Table II. As suggested in enzymatic synthesis,[21] higher concentrations of reactants are particularly effective for peptide bond formation, when the product is in a dissolved state.

Unlike α-chymotrypsin[22] or papain,[23] thermolysin is known to recognize solely the N-terminal amino acid residue with L-configuration.[24,25] In the above experiments, we prepared three Boc-tetrapeptides containing (*p*-fluoro)Phe–NH_2. We examined the L-configuration of the incorporated (*p*-fluoro)Phe–NH_2 residue in H–Trp–Met–Asp–(*p*-fluoro)Phe–NH_2. As shown in Fig. 3, H–Trp–Met–Asp–DL-(*p*-fluoro)Phe–NH_2 was prepared as a standard by a chemical method starting from DL-(*p*-fluoro)Phe–NH_2. This peptide exhibited two peaks in HPLC at the retention times of 18.7 and 20.0 min, which were thought to be due to the D-form and the L-form. The enzymatic preparation showed one peak with the retention time of 20.0 min. Thus, we confirmed that only the amino component with the L-configuration was incorporated into the peptide chain by thermolysin.

After conducting these model experiments, we examined whether this thermolysin-catalyzed reaction could be applied to the syntheses of longer peptides. As an example, thermolysin-catalyzed condensation of oxidized bovine insulin B-chain with various amide components was examined. In contrast to the reactions with small peptides, the present reactions had to be carried out at a low concentration of the acyl component, because of its poor solubility in various solvents. A mixture of H–Phe–NH_2 and oxidized insulin B-chain (10 μmol : 1 μmol) in an organic solvent (0.2 ml) was treated with thermolysin (0.01 μmol) at room temperature for 3 h, and the product was isolated by HPLC. The product was characterized by amino acid analysis, after 6 N HCl hydrolysis, and one residue of Phe was shown to have been incorporated. In addition, digestion of this product by lysyl endopeptidase gave two peaks in HPLC (Fig. 4). One consisted of Ala and Phe after 6 N HCl hydrolysis, and showed the same retention time in HPLC as that of chemically synthesized H–Ala–Phe–NH_2. The amino acid composition of the other peptide was in good agreement with the theoretical values for des-Ala^{30}-oxidized insulin B-chain. From these results, we concluded that the product obtained by thermolysin is oxidized insulin B–Phe^{31}–NH_2.

The reactions performed in 70% aqueous methanol or 70% aqueous 1,4-butanediol were

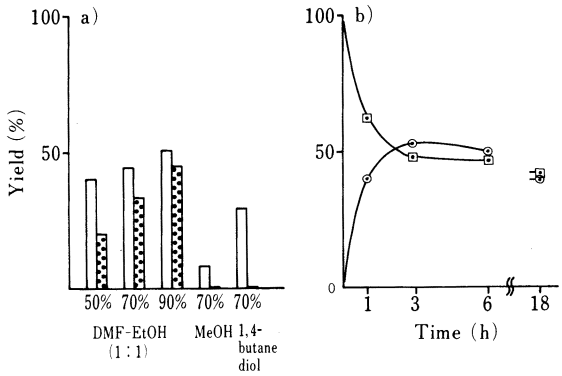

Fig. 5. Reaction Conditions for the Thermolysin-Catalyzed Synthesis of Oxidized Insulin B–Phe^{31}–NH_2

a) Organic solvent. b) Reaction time.
Conditions: a) (pH 6.0, 3 h), open columns indicate remaining oxidized insulin B-chain, dotted columns indicate oxidized insulin B–Phe^{31}–NH_2; b) [90% DMF–EtOH (1 : 1, v/v), pH 6.0], ⊡, (oxidized insulin B-chain), ⊙, (oxidized insulin B–Phe^{31}–NH_2).

TABLE III. Thermolysin-Catalyzed Condensation of Various Amides with Oxidized Insulin B-Chain

Amino component (M)	Oxidized insulin B-chain remaining (%)	Product yield (%)	Retention time of product[a] (min)
Phe–NH_2 (0.05)	51	53	12.6
Phe–NH_2 (0.1)	48	57	12.6
Leu–NH_2 (0.1)	13	84	12.3
Phe–NHEt (0.1)	29	73	14.0
Val–NH_2 (0.1)	21	70	11.2
DL-(*p*-Fluoro)Phe–NH_2 (0.1)	43	45	13.2

The reaction mixture, containing 5 mM oxidized insulin B-chain, 50 μM thermolysin and various concentrations of amino component in the presence of 90% DMF–EtOH (1 : 1, v/v) was stirred at pH 6.0 for 3 h. *a*) HPLC was carried out on a Nucleosil $5C_{18}$ column (4.0 × 200 mm) using gradient elution with CH_3CN (30→50%/20 min) in 0.2% TFA.

unsatisfactory, but much higher yields were obtained when a mixture of aqueous DMF–EtOH (1 : 1) was used as a solvent, and aqueous 90% DMF–EtOH (1 : 1) gave the highest yield (53%) after a 3 h reaction (Fig. 5-a). The yield decreased gradually, when the mixture was kept for 6 h, as shown in Fig. 5-b. Since oxidized insulin B-chain has many peptide bonds susceptible to thermolysin, a higher concentration of organic solvent also played an important role here to improve the yield. Next, we replaced H–Phe–NH_2 with other amide derivatives as in the model experiments. As shown in Table III, fairly good yields were obtained.

As described above, thermolysin catalyzed the condensation reaction of various amide derivatives with not only N^{α}-protected peptides, but also unprotected peptides in relatively high concentrations of organic solvents by suppressing undesired hydrolysis of the peptide bond. This enzymatic thermolysin method may be a useful tool for conversion of synthetic peptides into amides.

Experimental

The *Rf* values in thin layer chromatography (TLC) performed on silica gel (Kieselgel 60, Merck) refer to the following solvent systems: Rf_1 $CHCl_3$–MeOH (10 : 1), Rf_2 $CHCl_3$–MeOH (20 : 1), Rf_3 $CHCl_3$–MeOH–AcOH (5 : 1 : 0.5), Rf_4 $CHCl_3$–MeOH–AcOH (10 : 1 : 0.2), Rf_5 *n*-BuOH–AcOH–pyridine–H_2O (4 : 1 : 1 : 2), Rf_6 *n*-BuOH–AcOH–H_2O–AcOEt (3 : 1 : 1 : 5), Rf_7 $CHCl_3$–MeOH–H_2O (65 : 35 : 10, lower phase). HPLC was conducted with a Shimadzu LC-4A model. Acid hydrolysis (6 N HCl) was carried out at 110 °C for 18 h, and amino acids were determined with a Hitachi 835 analyzer. Trp-containing peptides were hydrolyzed by 4 M methanesulfonic acid (MSA) containing 0.2% 3-(2-aminoethyl)-indole (Pierce) at 110 °C for 18 h. Thermolysin and lysyl endopeptidase were obtained from Wako Pure Chemicals. Bovine insulin was purchased from Sigma. The starting materials and the standard peptides were prepared chemically by the known methods. For brevity, experimental scales for easily preparable dipeptide derivatives are omitted.

N^{α}-Deprotection——The N^{α}-protecting group, Boc, was cleaved by TFA (*ca.* 10 ml per 1 g of a peptide) in the presence of thioanisole (2 eq or more) at room temperature for 30 min, or by 3 N HCl/dioxane (*ca.* 20 ml per 1 g of a peptide) at room temperature for 1 h. After evaporation of the solvent *in vacuo*, the residue was treated with dry ether. The resulting powder was collected by filtration, dried over KOH pellets *in vacuo* for 3 h and then used for the condensation reaction.

Condensation Reactions——The HOBt/DCC and the active ester condensations were performed at room temperature. A mixed anhydride (MA) was prepared using isobutyl chloroformate and allowed to react with an amino component in an ice-bath for 3 h.

Purification——Unless otherwise mentioned, products were purified by one of the following procedures. Procedure A: For purification of a protected peptide soluble in AcOEt, the extract was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O–NaCl, dried over Na_2SO_4 and concentrated. Procedure B: For purification of a peptide less soluble in AcOEt, the crude product was triturated with ether and 5% citric acid. The resulting powder was washed with 5% citric acid, 5% $NaHCO_3$ and H_2O. For purification of peptide containing the free carboxyl group, 5% citric

acid and H_2O were used for washing.

Boc-Trp-Met-Asp-OH——A mixture of a 3 N HCl/dioxane-treated sample of Boc-Met-Asp-OH (3.00 g, 8.24 mmol), Boc-Trp-ONp (3.51 g, 8.24 mmol) and Et_3N (3.46 ml, 24.7 mmol) in DMF-H_2O (30 ml-20 ml) was stirred for 16 h and concentrated. The product was purified by procedure B, followed by column chromatography on silica gel (2.5 × 20 cm) using $CHCl_3$-MeOH (20 : 1) as an eluant. The product was finally precipitated from AcOEt with petroleum ether; yield 3.36 g (61%), mp 126—127 °C, $[\alpha]_D^{25}$ −15.2° (c=0.5, MeOH), Rf_3 0.35. *Anal.* Calcd for $C_{25}H_{34}N_4O_8S$: C, 54.53; H, 6.22; N, 10.18. Found: C, 53.59; H, 6.21; N, 10.06.

H-Trp-Met-Asp-OH——Boc-Trp-Met-Asp-OH (1.00 g, 1.82 mmol) was treated with TFA and the resulting powder was dissolved in 0.05 N NH_4OH (20 ml). The solution was applied to a column of DEAE-Sephadex A-25 (2.0 × 30 cm), which was eluted with a linear gradient of NH_4HCO_3 (pH 8.0, 0.02 M/1.0 M = 500 ml/500 ml). The fractions corresponding to the main peak (tube Nos. 98—118, 10 ml each, monitored by measuring $OD_{280\,nm}$) were combined and lyophilized; yield 615 mg (75%), mp 202—203 °C, $[\alpha]_D^{25}$ +3.2° (c=0.5, 1 N, NH_4OH), Rf_5 0.38. Amino acid ratios in a 4 M MSA hydrolysate: Asp 1.10, Met 1.00, Trp 1.02 (recovery of Asp, 86%).

Boc-Trp-Met-Asp-Phe-NH_2——A mixture of a TFA-treated sample of Boc-Met-Asp-Phe-NH_2 (5.20 g, 10.2 mmol) which was prepared from Boc-Met-OTcp and H-Asp-Phe-NH_2, Boc-Trp-ONp (5.20 g, 12.3 mmol) and Et_3N (2.85 ml, 20.4 mmol) in DMF-DMSO (75 ml-75 ml) was stirred for 16 h and concentrated. The product was purified by procedure B, followed by recrystallization from MeOH and ether; yield 5.44 g (77%), mp 214—215 °C, $[\alpha]_D^{25}$ −28.7° (c=0.3, MeOH), Rf_3 0.56. Amino acid ratios in a 4 M MSA hydrolysate: Asp 1.00, Met 0.93, Phe 0.95, Trp 0.92 (recovery of Asp, 100%). *Anal.* Calcd for $C_{34}H_{44}N_6O_8S$: C, 58.60; H, 6.36; N, 12.06. Found: C, 58.48; H, 6.26; N, 11.79.

H-Trp-Met-Asp-Phe-NH_2——The title compound was synthesized previously by an enzymatic method.[26]

Boc-Ser(Bzl)-Glu(OBzl)-Ala-OH·DCHA——A suspension of Boc-Glu(OBzl)-Ala-OH·DCHA (5.89 g, 10.0 mmol) in AcOEt (150 ml) was washed with 5% citric acid, dried over Na_2SO_4 and concentrated. The residue was treated with TFA. The resulting powder was dissolved in DMF (20 ml) together with *N*-methylmorpholine (2.24 ml, 20.0 mmol). An MA [prepared from 2.95 g (10.0 mmol) of Boc-Ser(Bzl)-OH] in THF (50 ml) was added to the above ice-chilled solution, and the solution was stirred for 3 h and concentrated. The product was purified by procedure A, and dissolved in ether (100 ml) together with DCHA (1.99 ml, 10.0 mmol). The resulting solid was recrystallized from AcOEt and petroleum ether; yield 6.58 g (86%), mp 113—114 °C, $[\alpha]_D^{25}$ −8.0° (c=0.2, MeOH), Rf_4 0.40. *Anal.* Calcd for $C_{42}H_{62}N_4O_9$: C, 65.77; H, 8.14; N, 7.30. Found: C, 65.29; H, 8.08; N, 7.33.

Boc-Ser-Glu-Ala-OH——A suspension of Boc-Ser(Bzl)-Glu(OBzl)-Ala-OH·DCHA (5.90 g, 7.70 mmol) in AcOEt (100 ml) was washed with 5% citric acid, dried over Na_2SO_4, and concentrated. The residue in MeOH (60 ml) was hydrogenated over Pd black as a catalyst. The solution was filtered, the filtrate was concentrated and the residue was precipitated from MeOH with ether; yield 2.86 g (92%), mp 166—167 °C, $[\alpha]_D^{25}$ −24.0° (c=0.2, MeOH), Rf_6 0.45. Amino acid ratios in a 6 N HCl hydrolysate: Ser 0.84, Glu 0.98, Ala 1.00 (recovery of Ala, 85%).

Boc-Ser(Bzl)-Glu(OBzl)-Ala-Phe-NH_2——A suspension of Boc-Ser(Bzl)-Glu(OBzl)-Ala-OH·DCHA (383 mg, 0.50 mmol) in AcOEt (30 ml) was washed with 5% citric acid, dried over Na_2SO_4, and concentrated. The residue was dissolved in DMF (5 ml) together with *N*-methylmorpholine (55 μl, 0.50 mmol), H-Phe-NH_2·HCl (100 mg, 0.50 mmol), HOBt (101 mg, 0.75 mmol) and DCC (129 mg, 0.63 mmol). The solution, after being stirred for 16 h, was filtered, and the filtrate was concentrated. The product was purified by procedure A, followed by precipitation from MeOH with ether; yield 330 mg (90%), mp 196—197 °C, $[\alpha]_D^{25}$ −21.0° (c=0.2, MeOH), Rf_1 0.57. *Anal.* Calcd for $C_{39}H_{49}N_5O_9$: C, 64.00; H, 6.75; N, 9.57. Found: C, 63.76; H, 6.68; N, 9.64.

Boc-Ser-Glu-Ala-Phe-NH_2——Boc-Ser(Bzl)-Glu(OBzl)-Ala-Phe-NH_2 (240 mg, 0.33 mmol) in MeOH (10 ml) was hydrogenated over Pd black as a catalyst. The solution was filtered, the filtrate was concentrated and the residue was precipitated from MeOH with ether; yield 83 mg (46%), mp 197—198 °C, $[\alpha]_D^{25}$ −15.0° (c=0.2, DMF), Rf_3 0.26. Amino acid ratios in a 6 N HCl hydrolysate: Ser 0.87, Glu 0.98, Ala 1.00, Phe 0.94 (recovery of Ala, 91%).

Boc-Ser(Bzl)-Lys(Z)-Ala-OH·DCHA——A suspension of Boc-Lys(Z)-Ala-OH·DCHA (7.00 g, 11.0 mmol) in AcOEt (150 ml) was washed with 5% citric acid, dried over Na_2SO_4 and concentrated. The residue was treated with TFA. The resulting powder was dissolved in DMF (20 ml) together with *N*-methylmorpholine (2.46 ml, 24.0 mmol). An MA [prepared from 3.25 g (11.0 mmol) of Boc-Ser(Bzl)-OH] in THF (50 ml) was added to the above ice-chilled solution, and the solution was stirred for 3 h and concentrated. The product was purified by procedure A, and dissolved in ether (100 ml) together with DCHA (2.19 ml, 11.0 mmol). The resulting solid was recrystallized from MeOH and ether; yield 7.34 g (83%), mp 152—153 °C, $[\alpha]_D^{25}$ −5.6° (c=0.5, MeOH), Rf_4 0.38. *Anal.* Calcd for $C_{44}H_{67}N_5O_9$: C, 65.24; H, 8.34; N, 8.64. Found: C, 64.97; H, 8.38; N, 8.88.

Boc-Ser-Lys-Ala-OH——A suspension of Boc-Ser(Bzl)-Lys(Z)-Ala-OH·DCHA (3.00 g, 3.87 mmol) in AcOEt (100 ml) was washed with 5% citric acid, dried over Na_2SO_4, and concentrated. The residue in MeOH (60 ml) was hydrogenated over Pd black as a catalyst. The solution was filtered, the filtrate was concentrated and the residue was precipitated from MeOH with ether; yield 1.06 g (68%), mp 72—74 °C, $[\alpha]_D^{25}$ −19.0° (c=0.4, MeOH), Rf_5 0.40. Amino acid ratios in a 6 N HCl hydrolysate: Ser 0.93, Ala 1.00, Lys 0.89 (recovery of Ala, 77%).

Boc-Ser(Bzl)-Lys(Z)-Ala-Phe-NH_2——A suspension of Boc-Ser(Bzl)-Lys(Z)-Ala-OH·DCHA (405 mg, 0.50 mmol) in AcOEt (30 ml) was washed with 5% citric acid, dried over Na_2SO_4, and concentrated. The residue was

dissolved in DMF (5 ml) together with *N*-methylmorpholine (55 μl, 0.50 mmol), H-Phe-NH_2·HCl (100 mg, 0.50 mmol), HOBt (101 mg, 0.75 mmol) and DCC (129 mg, 0.63 mmol). The solution, after being stirred for 16 h, was filtered, and the filtrate was concentrated. The product was purified by procedure A, followed by precipitation from MeOH with ether; yield 330 mg (85%), mp 193—194 °C, $[\alpha]_D^{25}$ −18.0° (*c*=0.2, MeOH), Rf_1 0.49. *Anal.* Calcd for $C_{41}H_{54}N_6O_9$: C, 63.55; H, 7.02; N, 10.85. Found: C, 63.33; H, 7.00; N, 11.04.

Boc-Ser-Lys-Ala-Phe-NH_2——Boc-Ser(Bzl)-Lys(Z)-Ala-Phe-NH_2 (232 mg, 0.30 mmol) in MeOH (10 ml) was hydrogenated over Pd black as a catalyst. The solution was filtered, the filtrate was concentrated and the residue was precipitated from MeOH with ether; yield 65 mg (39%), mp 205—207 °C, $[\alpha]_D^{25}$ −26.0° (*c*=0.2, MeOH), Rf_5 0.57. Amino acid ratios in a 6 N HCl hydrolysate: Ser 0.96, Ala 1.00, Phe 0.99, Lys 0.99 (recovery of Ala, 89%).

Boc-Phe-Met-Arg(Mts)-OH——A TFA-treated sample of Boc-Met-Arg(Mts)-OH (2.50 g, 4.25 mmol) which was prepared from Boc-Met-OTcp and H-Arg(Mts)-OH,[27] was dissolved in DMF (15 ml) together with *N*-methylmorpholine (477 μl, 4.25 mmol). An MA [prepared from 1.13 g (4.25 mmol) of Boc-Phe-OH] in THF (40 ml) was added to the above ice-chilled solution. The solution was stirred for 3 h and concentrated. The product was purified by procedure A, followed by column chromatography on silica gel (2.5 × 20 cm) using $CHCl_3$–MeOH–AcOH (15 : 1 : 0.1) as an eluant. The product was finally precipitated from MeOH with ether; yield 1.92 g (61%), mp 115—117 °C, $[\alpha]_D^{25}$ −8.0° (*c*=0.4, MeOH), Rf_4 0.20. *Anal.* Calcd for $C_{34}H_{50}N_6O_8S_2$: C, 55.57; H, 6.86; N, 11.44. Found: C, 55.18; H, 6.89; N, 11.58.

H-Phe-Met-Arg-OH——Boc-Phe-Met-Arg(Mts)-OH (735 mg, 1.00 mmol) was treated with 1 M TFMSA–thioanisole[28] in TFA (30 ml) in the presence of ethanedithiol (1.68 ml, 20.0 mmol) in an ice-bath for 1 h, then dry ether was added. The oily product was dissolved in H_2O (20 ml) and passed through a column (2.0 × 10 cm) of Amberlite IRA-400 (acetate form). The eluate was applied to a column (2.0 × 20 cm) of SP-Sephadex C-25, which was eluted with a linear gradient of $AcONH_4$ [0.01 M (pH 4.5)/0.5 M (pH 7.0) = 600 ml/600 ml]. The fractions corresponding to the main peak (tube Nos. 42—50, 17 ml each, monitored by measuring $OD_{230\,nm}$) were combined and lyophilized; yield 427 mg (83%), mp 80—81 °C, $[\alpha]_D^{25}$ −10.5° (*c*=0.4, MeOH), Rf_5 0.41. Amino acid ratios in a 6 N HCl hydrolysate: Met 1.02, Phe 1.00, Arg 1.00 (recovery of Arg, 82%).

Boc-Phe-Met-Arg(Mts)-Phe-NH_2——A mixture of Boc-Phe-Met-Arg(Mts)-OH (367 mg, 0.50 mmol), *N*-methylmorpholine (55 μl, 0.50 mmol), H-Phe-NH_2·HCl (100 mg, 0.50 mmol), HOBt (101 mg, 0.75 mmol) and DCC (129 mg, 0.63 mmol) in DMF (5 ml) was stirred for 16 h and filtered, and the filtrate was concentrated. The product was purified by procedure A, followed by recrystallization from AcOEt and ether; yield 394 mg (89%), mp 136—137 °C, $[\alpha]_D^{25}$ −19.3° (*c*=0.3, MeOH), Rf_1 0.36. *Anal.* Calcd for $C_{43}H_{60}N_7O_9S_2$: C, 58.48; H, 6.85; N, 11.10. Found: C, 58.43; H, 6.99; N, 11.54.

H-Phe-Met-Arg-Phe-NH_2——Boc-Phe-Met-Arg(Mts)-Phe-NH_2 (220 mg, 0.25 mmol) was treated with 1 M TFMSA–thioanisole in TFA (5 ml) in the presence of ethanedithiol (420 μl, 5.00 mmol) in an ice-bath for 1 h, then dry ether was added. The oily product was dissolved in H_2O (10 ml) and passed through a column (1.0 × 10 cm) of Amberlite IRA-400 (acetate form). The eluate was applied to a column (2.0 × 15 cm) of CM-Cellulofine, which was eluted with a linear gradient of $AcONH_4$ [0.01 M (pH 4.5)/0.2 M (pH 7.0) = 600 ml/600 ml]. The fractions corresponding to the main peak (tube Nos. 44—58, 17 ml each, monitored by measuring $OD_{230\,nm}$) were combined and lyophilized; yield 120 mg (67%), mp 87—88 °C, $[\alpha]_D^{25}$ −8.8° (*c*=0.4, 1 N AcOH). Rf_5 0.56. Amino acid ratios in a 6 N HCl hydrolysate: Met 0.96, Phe 1.88, Arg 1.00 (recovery of Arg, 100%).

Boc-Trp-Met-Asp(OBzl)-DL-(*p*-Fluoro)Phe-NH_2——A mixtur of a TFA-treated sample of Boc-Met-Asp(OBzl)-DL-(*p*-fluoro)Phe-NH_2 (1.24 g, 2.00 mmol), which was synthesized by the HOBt/DCC procedure starting from a TFA-treated sample of Boc-DL-(*p*-fluoro)Phe-NH_2, *N*-methylmorpholine (220 μl, 2.00 mmol), Boc-Trp-OH (608 mg, 2.00 mmol), HOBt (407 mg, 3.00 mmol) and DCC (515 mg, 2.50 mmol) in DMF (15 ml) was stirred for 16 h and filtered, and the filtrate was concentrated. The product was purified by procedure A, followed by recrystallization from MeOH and AcOEt to give the title compound (1.39 g, 86%), which showed two spots on TLC (Rf_1 0.49 and 0.50). *Anal.* Calcd for $C_{41}H_{49}FN_6O_8S$: C, 61.18; H, 6.14; N, 10.44. Found: C, 61.01; H, 6.18; N, 10.27.

H-Trp-Met-Asp-DL-(*p*-Fluoro)Phe-NH_2——A 3 N HCl/dioxane-treated sample of the above peptide amide (200 mg, 250 μmol) was dissolved in MeOH–H_2O (3 ml–1.5 ml) and hydrogenated with HCOOH (1.5 ml) and 10% Pd–C (300 mg) for 2 h. The solution was filtered, and the filtrate was concentrated. The residue was precipitated from MeOH with ether; yield 121 mg (92%). Amino acid ratios in a 4 M MSA hydrolysate: Asp 1.02, Met 0.97, DL-(*p*-fluoro)Phe 1.00, Trp 0.82 [recovery of DL-(*p*-fluoro)Phe, 78%]. Rf_6 0.29 and 0.30.

Oxidized Insulin B-Chain——Bovine insulin (500 mg) was oxidized with performic acid,[29] and fractionated by the method of Sanger[30] to give oxidized insulin B-chain (107 mg).

Enzymatic Syntheses of Protected or Unprotected Model Peptides——Except where otherwise specified, a carboxyl component (10 μmol), an amino component (50 μmol) and thermolysin (0.01 μmol) were dissolved in an appropriate solvent (200 μl). After adjustment of the pH of the reaction mixture by addition of 7% NH_4OH or 2 N HCl, the mixture was stirred at room temperature for various times and diluted to 10 ml with 1 N HCl–MeOH (1 : 9, v/v). An aliquot (5 μl) was subjected to analysis by HPLC using a Nucleosil $5C_{18}$ column (4.0 × 200 mm), which was eluted with an appropriate concentration of CH_3CN in 0.2% TFA at a flow rate of 1.0 ml per min. Trp-containing peptide was monitored by measuring UV absorption at 280 nm and the others at 220 nm. The product yields were

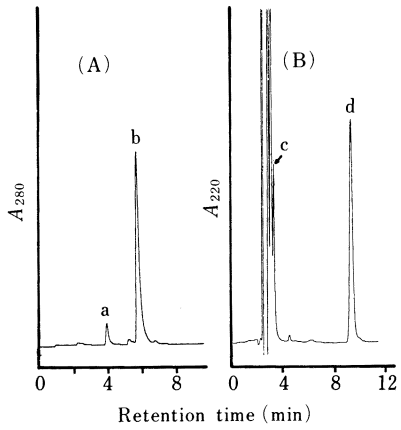

Fig. 6. HPLC Profile of the Coupling Product Formed by Thermolysin: (A), Boc–Trp–Met–Asp–Phe–NH_2; (B), H–Phe–Met–Arg–Phe–NH_2

Column: Nucleosil $5C_{18}$ (4.0 × 200 mm). Solvent: (A), 45% CH_3CN in 0.2% TFA; (B), 20% CH_3CN in 0.2% TFA. Flow rate: 1.0 ml/min. a. Boc–Trp–Met–Asp–OH, b. Boc–Trp–Met–Asp–Phe–NH_2, c. H–Phe–Met–Arg–OH, d. H–Phe–Met–Arg–Phe–NH_2.

TABLE IV. Amino Acid Compositions of Oxidized Insulin B-Chain Derivatives

	Oxidized insulin B-chain	Oxidized insulin B-chain derivatives				
		B–Phe^{31}–NH_2	B–Leu^{31}–NH_2	B–Phe^{31}–NHEt	B–Val^{31}–NH_2	B–(*p*-fluoro)Phe^{31}–NH_2
Ala[a]	(2)	2.00	2.00	2.00	2.00	2.00
Val	(3)	—	—	—	3.61	—
Leu	(4)	—	5.06	—	—	—
Phe	(3)	3.93	—	3.90	—	—
(*p*-Fluoro)Phe[b]						0.95
Ethylamine[c]				0.92		

Acid hydrolyses were carried out in 6 N HCl with phenol at 110 °C for 24 h (numbers in parentheses are theoretical). *a*) Diagnostic amino acid. *b*) (*p*-Fluoro)Phe was eluted behind Phe. *c*) Ethylamine was co-eluted with Arg.

calculated from the ratio of peak areas between the reaction solution and the standard solution of authentic sample (Fig. 6). When various amides were used, the coupling yields were estimated from the ratio of peak areas to that of the corresponding authentic sample with C-terminal –Phe–NH_2 without correction of the molecular extinction coefficient.

Enzymatic Syntheses of Oxidized Insulin B-Chain Amide Derivatives——Oxidized insulin B-chain (1 μmol), an amino component (10 μmol) and thermolysin (0.01 μmol) were dissolved in reaction solvent (200 μl). After adjustment of the pH of the mixture with 7% NH_4OH, the mixture was stirred at room temperature for various times and diluted to 10 ml with 0.1 N HCl–DMF (9 : 1, v/v). An aliquot (5 μl) was subjected to analysis by HPLC using a Nucleosil $5C_{18}$ column (4.0 × 200 mm), which was eluted with a gradient of CH_3CN (30→50%/20 min) in 0.2% TFA at a flow rate of 1.0 ml per min. Synthetic yields of oxidized insulin B-chain amide derivatives were estimated from the ratio of peak areas to that of the standard solution of oxidized insulin B-chain.

Identification of Products——Identification of enzymatically synthesized peptides was performed by amino acid analysis. The eluate expected to contain the desired compound in HPLC was collected and concentrated. The residue was hydrolyzed with 6 N HCl or 4 M MSA. In all cases, the results were close to the anticipated values. Amino acid compositions of oxidized insulin B-chain derivatives are shown in Table IV.

Synthesis of Oxidized Insulin B–Phe^{31}–NH_2——Oxidized insulin B-chain (10.5 mg, 3.00 μmol) and H–Phe–NH_2·HCl (12.0 mg, 60.0 μmol) were dissolved in a mixture of H_2O (60 μl) and DMF–EtOH (1 : 1, v/v, 540 μl), (pH 6.0). After addition of thermolysin (1.04 mg), the mixture was stirred for 3 h. The reaction was terminated by addition of 1 N HCl (1.0 ml), and the precipitate was removed by centrifugation. The supernatant was applied to a column (1.0 × 25 cm) of Nucleosil $5C_{18}$, which was eluted with 34% CH_3CN in 0.2% TFA at a flow rate of 4.0 ml per min. The eluate fractions containing the product were combined and lyophilized; yield 4.0 mg (37%).

Digestion of Oxidized Insulin B–Phe^{31}–NH_2 by Lysyl Endopeptidase——Oxidized insulin B–Phe^{31}–NH_2 (500 μg) was digested with lysyl endopeptidase (10 μg) in 0.2 M $AcONH_4$ (pH 9.0, 100 μl) at room temperature for 1 h. The reaction was terminated by addition of 1 N HCl (80 μl), and the solution (20 μl each) was applied to a Nucleosil $5C_{18}$ column (4.0 × 200 mm). The eluates corresponding to the two peaks (retention times of 5.7 and 20.5 min) obtained by gradient elution with CH_3CN in 0.2% TFA were collected separately and evaporated (Fig. 4). Amino acid

composition of one peptide (retention time of 5.7 min): Ala 1.00, Phe 0.96. Amino acid composition of the other peptide (retention time of 20.5 min) (numbers in parentheses are theoretical): Asp 1.92 (2), Thr 1.00 (1), Ser 0.95 (1), Glu 2.83 (3), Pro 1.02 (1), Gly 3.00 (3), Ala 1.00 (1), cysteic acid 1.92 (2), Val 2.75 (3), Leu 3.92 (4), Tyr 1.90 (2), Phe 2.90 (3), Lys 0.85 (1), His 1.38 (2), Arg 1.02 (1).

References and Notes

1) Except when otherwise specified, amino acid and peptide derivatives are of the L-configuration. The following abbreviations are used: Z = benzyloxycarbonyl, Boc = *tert*-butoxycarbonyl, Bzl = benzyl, OBzl = benzyloxy, Mts = mesitylene-2-sulfonyl, Np = *p*-nitrophenyl, Tcp = trichlorophenyl, DCC = dicyclohexylcarbodiimide, HOBt = *N*-hydroxybenzotriazole, TFA = trifluoroacetic acid, TFMSA = trifluoromethanesulfonic acid, DMF = dimethylformamide, DMSO = dimethylsulfoxide, THF = tetrahydrofuran, DCHA = dicyclohexylamine.
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