Peptide Syntheses with Proteinases. Fragment Condensation of ZLeuGlnGlyOH or ZGlnGlyOH with HLeuValNH$_2$ Using Metalloproteinases

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The crude preparations of microbial metalloproteinase from *Bacillus subtilis var. amyloliquefaciens* (Prolisin) catalyzed the condensation of ZLeuGlnGlyOH or ZGlnGlyOH with HLeuValNH$_2$ in the presence of the serine-proteinase inhibitor from potatoes to give the corresponding Z-penta or tetra-peptide amide in good yields in an aqueous buffer solution. Two other metalloproteinases, from *Bacillus thermoproteoliticus* (Thermolysin or Thermoase) and *Streptomyces caespitosus* (Tacynase N), also catalyzed this reaction; however, they gave poorer results than Prolisin. The yields of the coupling products were affected most by the concentration of the substrates, among the several factors investigated. Addition of a small amount of an organic solvent sometimes promoted the yield for this method.

In previous papers[1] we reported that the peptide linkage is catalytically formed by proteinases from two suitably protected amino acids and peptides and that the catalytic specificities of the enzymes are similar to those in the peptide hydrolysis. Since microbial metalloproteinases hydrolyze specifically the peptide linkage with an amino acid residue carrying a hydrophobic side chain at the amino side of the linkage,[2] they are expected to catalyze the condensation between a C-terminal protected peptide which has this amino acid residue as its N-terminal and an N-terminal protected peptide. Moreover, the proteinases do not usually catalyze the hydrolysis of esters or amides of amino acids or peptides,[2] so that appropriate use of them is very effective for the peptide synthesis.

We investigated their catalytic ability in the peptide bond formation in accordance with their characteristics in hydrolysis. In this article we report that three microbial metalloproteinases catalyze the condensation of ZGlnGlyOH or ZLeuGlnGlyOH with HLeuValNH$_2$ to give ZGlnGlyLeuValNH$_2$ (Secretin 24—27) or ZLeuGlnGlyLeuValNH$_2$ (Secretin 23—27) in good yields. The crude crops from the reaction mixture were almost pure without further purification. Furthermore, it is noticeable that even crude preparations of metalloproteinase were effective in the reaction, under the condition of using serineproteinase inhibitor from potatoes.

Materials and Methods

All amino acids used are of the L configuration. The following abbreviations are used: PU=protease unit; EDTA =ethylenediaminetetraacetic acid; Tris=tris(hydroxymethyl)-methanamine; Z=benzyloxycarbonyl; BOC=*t*-butoxycarbonyl; OSu=*N*-hydroxysuccinimide ester; DCCI=dicyclohexylcarbodiimide; DCU=*N,N'*-dicyclohexylurea; HOBt=1-hydroxybenzotriazole. Thin layer chromatography was performed on precoated TLC plates Silica Gel 60 F254 (from Merck) using the following solvent system: *s*-BuOH-3% aqueous ammonia (8 : 3 by vol). See Ref. 1b for other analytical procedures, execpt that here the UV spectra for the assaying of the protease activity of the proteinases were determined on a Hitachi Recording Spectrophotometer 323.

Serineproteinase Inhibitor from Potatoes. The crude inhibitor (115 g) was obtained by the method of Melville and Ryan[3] from potatoes (60 kg) and used without further purification. This inhibitor will be called potato inhibitor here after in this article.

Proteinases. All proteinases were used without further purification. Protease activity was assayed at pH 7.2 using Hammarsten casein (Merck) by the method of Tsuru *et al.*[4] Thermolysin and Thermoase were dissolved in 0.2 M AcONa–0.2 M Ca(OAc)$_2$ aqueous solution (pH 8.1) and the others in 5×10^{-3} M Ca(OAc)$_2$ aqueous solution. Protease activity was also assayed in the presence of 1/500 M EDTA or of potato inhibitor.

Prolisin:[5] Two lots of Prolisin obtained from *Bacillus subtilis var. amyloliquefaciens* were purchased from Ueda Chemical Co. (Neyagawa, Osaka) and characterized as follows. One of the preparations, which was manufactured May 19, 1976 and stated to have a protease activity of 7.7×10^5 PU/g, was demonstrated to contain metalloproteinase, serine-proteinase, amylase, and other proteins and showed the protease activity of 1.9×10^6 PU/g according to Tsuru's method. This specimen of the Prolisin will be called Prolisin A hereafter in this article. The protease activity was 1.45×10^6 PU/g in the presence of potato inhibitor in 10 times the weight of the enzyme (Table 1) and was 5.1×10^5 PU/g in the presence of 1/500 M EDTA. Hence the rough ratio between metallo- and serineproteinase was 14 : 5 PU/PU. The other preparation, which was manufactured on February 8,

Table 1. Protease activity of proteinases in the presence of potato inhibitor

	P.I.:Proteinase wt/wt	Activity PU/g
Prolisin A	0	1.90×10^6
	1:1	1.73
	5:1	1.60
	10:1	1.49
	50:1	1.49
Prolisin B	0	7.2×10^5
	1:2	5.6
	1:1	5.4
	3:2	5.4
Tacynase N	0	2.60×10^5
	1:1	2.47
	3:1	2.37
	5:1	2.33
	10:1	2.30
Thermoase	0	4.0×10^6
	1:1	3.7
	3:1	3.2
	5:1	3.1

1975 and stated to have the activity of 3.0×10^5 PU/g, was found to contain metalloproteinase, serineproteinase, a small amount of amylase, other proteins, and starch as an excipient in about 65% of the weight and exhibited a protease activity of 7.2×10^5 PU/g according to Tsuru's method. This specimen of the Prolisin will be called Prolisin B hereafter in this article. The protease activity was 5.4×10^5 PU/g in the presence of potato inhibitor in equal weight of the enzyme (Table 1) and was 1.6×10^5 PU/g in the presence of 1/500 M EDTA. Hence the rough ratio between metallo- and serineproteinase was 27 : 8 PU/PU.

Tacynase N[6] *(with a Stated Protease Activity of* $\geqslant 10^5$ *PU/g)*: This enzyme preparation obtained from *Streptomyces caesptitosus* was purchased from Kyowa Hakko Kogyo Co. (Tokyo, Japan) and found to show a protease activity of 2.6×10^5 PU/g according to Tsuru's method. The enzyme was slightly inhibited by potato inhibitor in 10 times the weight of the enzyme (2.3×10^5 PU/g) (Table 1) and almost completely inhibited by 1/500 M EDTA ($<6\times10^3$ PU/g)

Thermolysin[7] *and Thermoase*: Both enzyme preparations obtained from *Bacillus thermoproteoliticus* were purchased from Daiwa Kasei Co. (Osaka, Japan). Thermoase was an unpurified specimen and its protease activity, which was stated to be 1.6×10^6 PU/g, was found to be 4.0×10^6 PU/g according to Tsuru's method and was reduced to 3.1×10^6 PU/g in the presence of potato inhibitor in 5 times the weight of the enzyme (Table 1) and to 8.5×10^5 PU/g in the presence of 1/500 M EDTA. Thermolysin was the enzyme purified from Thermoase; its protease activity, which was stated to be 8.0×10^6 PU/g, was shown to be 1.8×10^7 PU/g according to Tsuru's method.

Detection and Inhibition of Esterase Action of Crude Proteinase Preparations. The esterase action was detected by observing the drop in pH, on addition of proteinase, of a solution of 0.1 M HPheOEt·HCl (10 ml). The pH had been previously adjusted at 7.0 with 1 M NaOH. The esterase action of Prolisin A,B, and Thermoase were repressed almost completely by the potato inhibitor in 3 times, equal, and equal weight of the enzyme (Table 2); however, that of Tacynase N was not completely inhibited by the potato inhibitor even in 5 times the weight of the enzyme (Table 2). Accordingly, the enzymatic syntheses of peptides with Prolisin A, B, and Thermoase were carried out in the presence of the potato inhibitor in 3 times, equal, and equal weight of the enzyme for 3—17 h, while that with Tacynase N in the presence of the inhibitor in equal weight of the enzyme for 3 h. Thermolysin has scarcely any esterase action, so that the condensation with Thermolysin was carried out in the absence of the potato inhibitor.

Table 2. Inhibitory effect of potato inhibitor on the esterase action of proteinases[a]

	P.I.:Enzyme wt/wt	pH		
		0.5 h	3 h	17 h
Prolisin A	0	4.8	4.1	3.9
	3:1	6.9	6.8	6.4
	5:1	7.0	6.9	6.4
		3/4 h	4 h	20 h
Prolisin B	0	4.6	4.1	4.0
	1:2	6.9	6.8	6.8
	1:1	6.9	6.9	6.9
		1 h	2.5 h	8 h
Tacynase N	0	6.6	5.7	4.8
	1:1	6.7	6.2	5.6
	5:1	6.8	6.5	6.0
		1.5 h	3 h	24 h
Thermoase	0	6.2	5.4	4.1
	1:1	6.9	6.7	6.3
	2:1	6.9	6.8	6.5

a) Amounts of each enzyme used: Prolisin A, 0.035 g; Prolisin B, 0.2 g; Tacynase N, 0.2 g; Thermoase, 0.1 g.

The Enzymatic Syntheses of Peptides (General Procedure). Appropriate amounts of an enzyme and, when necessary, of potato inhibitor were dissolved in an adequate volume of buffer (unless otherwise stated 0.2 M Tris–HCl buffer (pH 8, containing 5×10^{-2} M $Ca(OAc)_2$) was used) and the undissolved materials were filtered off if required. The solution was added to a mixture of a carboxyl and an amine component. Then 4 M NaOH of equimolar quantity with the amine component was added. The mixture was stirred at 38—40 °C for a certain period. The product was collected on a filter by suction, washed thoroughly in succession with 1 M aqueous ammonia, 1 M HCl, and water, and dried *in vacuo* over P_2O_5 at room temperature.

Peptide Syntheses According to the Chemical Methods. Ordinary chemical syntheses were used to prepare some peptides for substrates in the enzymatic synthesis and for the purpose of identification of the products by comparison.

HValNH$_2$·HCl (I): ZValNH$_2$ (50.0 g, 0.2 mol) was hydrogenated over 10% Pd–C (2 g) in MeOH (300 ml) containing 1 M HCl (21 ml). After filtering the catalyst, the filtrate was evaporated to give a white powder, which was recrystallized from MeOH and ether: yield 29.3 g (96%); mp 281—282 °C (dec); $[\alpha]_D^{24}$ +34.1° (*c* 1, MeOH).

Found: C, 39.52; H, 8.77; N, 18.38; Cl, 23.08%. Calcd for $C_5H_{13}N_2OCl$: C, 39.35; H, 8.58; N, 18.36; Cl, 23.23%.

ZLeuValNH$_2$ (II): To a cooled solution (−10 °C) of ZLeuOH (103.5 g, 0.39 mol) and HValNH$_2$·HCl (59.3 g, 0.39 mol) in DMF (1500 ml) were added *N*-methylmorpholine (41 g, 0.41 mol), HOBt (58.1 g, 0.43 mol), and DCCI (88.6 g, 0.43 mol) dissolved in DMF; the mixture was stirred at 0 °C for 3 h and at room temperature for 17 h. After filtering DCU, the filtrate was concentrated and water was added. The slightly brownish precipitate obtained was washed successively with 1 M aqueous ammonia, 1 M HCl, and water, air dried, and dried *in vacuo* over P_2O_5 at 60 °C to give a white powder: yield 103.3 g (72.9%); mp 238—239.5 °C; $[\alpha]_D^{20}$ −24.2° (*c* 1, AcOH) (lit,[8] mp 231—232 °C; $[\alpha]_D^{20}$ −24.4±0.5° (*c* 1.8, AcOH)).

HLeuValNH$_2$·HCl (III): ZLeuValNH$_2$ (17 g, 0.0468 mol) was hydrogenated over 10% Pd–C (2 g) in MeOH (200 ml) containing 1 M HCl (50 ml). After the reaction, the catalyst was removed by filtration and the filtrate was evaporated to give a white powder, which was recrystallized from MeOH and ether: yield 10.8 g (86.9%); mp 153—155 °C; $[\alpha]_D^{26}$ +28.2° (*c* 1, AcOH). Found: C, 49.87; H, 9.05; N, 15.89; Cl 13.15%. Calcd for $C_{11}H_{24}N_3O_2Cl$: C, 49.71; H, 9.10; N, 15.81; Cl, 13.34%.

ZGlnGlyOH (IV): This was obtained from ZGlnGlyOEt (18.3 g, 0.05 mol) according to the procedure described by E. Wünsch *et al.*:[8] yield 15.1 g (90%); mp 185—187 °C; $[\alpha]_D^{28}$ −2.8° (*c* 1, DMF) (lit,[8] mp 183 °C; $[\alpha]_D^{20}$ −3.1±0.5° (*c* 2, DMF)).

ZGlnGlyLeuValNH$_2$ (V): This was obtained from ZGlnGlyOH (20.4 g, 0.06 mol) and HLeuValNH$_2$·HCl (15.9 g, 0.06 mol) according to the procedure described by E. Wünsch *et al.*:[8] yield 25.5 g (77.5%); mp 243.5—246 °C; $[\alpha]_D^{30}$ −26.0° (*c* 1, AcOH); R_f 0.75. (lit,[8] mp 239.5—240.5 °C; $[\alpha]_D^{20}$ −24.6±0.5° (*c* 1.8, AcOH)). Found: C, 56.91; H, 7.39; N, 15.17%. Calcd for $C_{26}H_{40}N_6O_7$: C, 56.92; H, 7.35; N, 15.32%.

ZLeuGlnGlyOEt (VI): This was obtained according to the procedure described by E. Wünsch *et al.*[8] A solution of HGlnGlyOEt·HCl (0.05 mol equivalent) was obtained by the hydrogenation of ZGlnGlyOEt (18.2 g, 0.05 mol) over 10% Pd-C (2 g) in DMF–H_2O (250 : 70 ml) with 1 M HCl (50 ml) added portionwise to keep the solution at pH 4. After the reaction the catalyst was filtered off and to the filtrate was added DMF (100 ml). To the cooled solution (−10 °C) were added Et_3N (5 g, 0.05 mol), pyridine (3.9 g, 0.05 mol), and ZLeuOSu (18.1 g, 0.05 mol) dissolved in DMF. The mixture was stirred at −10 °C for 2 h and at room temperature for 40 h. The suspension was concentrated to give a white powder, which was washed with water thoroughly, air dried, and dried *in vacuo* over P_2O_5 at 60 °C: yield 23.0 g (96%); mp 214 °C; $[\alpha]_D^{25}$ −14.5° (*c* 1, DMF).

Found: C, 57.88; H, 7.16; N, 11.58%. Calcd for $C_{23}H_{34}N_4O_7$: C, 57.72; H, 7.16; N, 11.71%.

ZLeuGlnGlyOH (VII): A solution of ZLeuGlnGlyOEt (19.5 g, 0.04 mol) in DMF (300 ml) at 50 °C, after addition of MeOH–H_2O (100 : 50 ml), was allowed to cool to room temperature. 1 M NaOH (52 ml) was then added portionwise over 1 h, and the solution was stirred at room temperature for 2 h. After the reaction, water (100 ml) was added and undissolved materials were filtered off. The filtrate was acidified by adding Amberlite CG120 (H form, 3.5 mmol/g, 15 g) and stirring for 10 min, then the resin was filtered off. The filtrate was concentrated to syrup, which was triturated with water. The precipitate which formed was collected by suction, washed with water, air dried, and dried *in vacuo* over P_2O_5 at 60 °C: yield 16.3 g (84%); mp 185—186.5 °C; $[\alpha]_D^{30}$ −13.8° (*c* 1, DMF).

Found: C, 56.13; H, 6.89; N, 12.55%. Calcd for $C_{21}H_{30}N_4O_7$: C, 55.99; H, 6.71; N, 12.44%.

ZLeuGlnGlyLeuValNH$_2$ (VIII): *a) HGlnGlyLeuValNH$_2$·HCl (IX)*. This was obtained according to the procedure described by E. Wünsch *et al.*[8] except that DMF–H_2O (3 : 1) was used as the solvent of hydrogenation in place of MeOH–H_2O (3 : 1). ZGlnGlyLeuValNH$_2$ (5.5 g, 0.01 mol) was hydrogenated in DMF–H_2O (3 : 1, 200 ml) over 10% Pd-C (0.5 g) with the addition of two 5 ml portions of 1 M HCl. The catalyst was removed and the filtrate was used immediately in the subsequent step.

b). VIII: To the cooled solution (−10 °C) obtained from the above procedure were added Et_3N (1.0 g, 0.01 mol), pyridine (0.8 g, 0.01 mol), and ZLeuOSu (4.3 g, 0.012 mol) dissolved in DMF (30 ml). The mixture was stirred for 4 h at −10 °C and for 36 h at room temperature. The reaction mixture was concentrated to give a gelatinous mass, which was washed with water three times, air dried, then washed with boiling ethyl acetate (100 ml), air dried, and dried *in vacuo* over P_2O_5 at 60 °C: yield 6.2 g (93.7%); mp 273—275 °C; $[\alpha]_D^{28}$ −33.9 ° (*c* 0.5, AcOH); R_f 0.65.

Found: C, 57.99; H, 7.53; N, 14.58%. Calcd for $C_{32}H_{51}N_7O_8$: C, 58.07; H, 7.77; N, 14.82%.

BOCLeuGlnGlyLeuValNH$_2$ (X): It was synthesized by the same procedure as in the case of the compound VIII. The compound IX obtained from the compound V (2.75 g, 0.005 mol) using 10% Pd-C (0.3 g) and 1 M HCl (5 ml) in DMF–H_2O (3 : 1, 100 ml) was allowed to react with BOCLeuOSu (2 g, 0.006 mol) in the presence of Et_3N (0.5 g) and pyridine (0.4 g): yield 2.7 g (87%); mp 237—238 °C (dec); $[\alpha]_D^{25}$ −18.4° (*c* 0.5, DMF) (lit,[8] mp 234—235 °C (dec); $[\alpha]_D^{20}$ −19.4° (*c* 1, DMF)). Found: C, 55.29; H, 8.52; N, 15.84%. Calcd for $C_{29}H_{53}N_7O_8$: C, 55.48; H, 8.51; N, 15.62%.

Results and Discussion

I. The Influence of the Nature of Buffer Solutions upon the Protease Activity of Metalloproteinase. *Bacillus subtilis* metalloproteinase had been reported to be disadvantageously affected by phosphate ions,[9] so the effect of three buffers (**A** McIlvaine, pH 7; **B** Tris–HCl, pH 7; **C** citrate buffer, pH 6.5) on the protease activity of the metalloproteinase in Prolisin was investigated using varying concentrations in the presence of potato inhibitor to suppress serineproteinase action (Table 3). Tris–HCl buffer solution reduced the activity least among the three and kept about 75% of the initial activity even after 23 h. The other two buffers were inadequate as a medium for the enzymatic synthesis of peptides. Moreover, since metalloproteinases are generally most active in the neutral pH region from 6—9.0 and the pH of the medium slightly fell toward the acidic side during the course of the reaction, it is recommended to use a buffer adjusted to the alkaline side, pH 8 being the best, and in a concentration of 0.2—0.4 M to maintain the correct pH.

Table 3. Influence of three buffers on the protease activity of Prolisin B[a]

	Buffer concn (M)		Activity remaining (%)			
			1 h	4 h	6 h	23 h
	Na_2HPO_4	Citric acid				
A	0.4	0.2	71	37	32	0
	0.2	0.1	74	55	48	0
	0.1	0.05	83	66	58	0
B	0.2		94	89	83	72
	0.1		95	91	83	75
	0.05		95	93	84	79
C	0.1		69	38	25	0
	0.05		88	68	59	21

a) A mixture of Prolisin B (0.1 g) and the potato inhibitor (0.1 g) was incubated in each buffer solution containing 2×10^{-3} M $Ca(OAc)_2$ (10 ml) at 40 °C and the protease activity was assayed at intervals.

II. The Effect of Potato Inhibitor on Commercial Crude Metalloproteinases.[10] The esterase action of crude metalloproteinase preparations probably is due to some serineproteinase which was present in the preparations. Since the serineproteinase, in addition to esterase action, shows amidase action, the crude metalloproteinases in such a condition are inadequate for the present study and must be used in the presence of some suitable serineproteinase inhibitor. Among several serineproteinase inhibitors, the potato inhibitor[11] was chosen and used, for it is attractive to use and not toxic. Consequently, the condensation between ZGlnGlyOH and HLeuValNH$_2$ with Prolisin in the presence of potato inhibitor gave the product in 75% or above of the theoretical yield (see below). On the other hand, the above reaction in the absence of the inhibitor gave a product which showed 3 spots on TLC, one of which corresponded to those of the object matter. Furthermore, though the amount of the product gradually

increased with time and reached a maximum after 3 h, it was far less than that from the reaction in the presence of the potato inhibitor.

III. Fragment Condensation of ZGlnGlyOH or ZLeuGlnGlyOH with HLeuValNH$_2$ by the Aid of Prolisin.

The enzymic condensation between ZGlnGlyOH and HLeuValNH$_2$ (Reaction 1) or ZLeuGlnGlyOH and HLeuValNH$_2$ (Reaction 2) was examined in the search for suitable coupling conditions with respect to the substrate concentration, the enzyme concentration, the reaction period, the reaction temperature, the pH of the buffer, the effect of organic solvent, and the nature of amino-protecting groups, Z or BOC.

1) The Influence of the Substrate Concentration on the Yield: When Reaction 1 was carried out at the substrate concentration of 300 mM in the presence of Prolisin A in 0.2 M Tris-HCl buffer (pH 8), the condensation product was obtained in 71% of the theoretical yield; however, the reaction at the substrate concentration of 73 mM gave a surprisingly low yield of the product under otherwise the same conditions (Fig. 1). Reaction 2 exhibited results similar to those of Reaction 1 (Fig. 1). These findings indicate that the substrates in higher concentrations will gave better results in these reactions, but that too high a substrate concentration interrupts the progress of the reaction owing to rapid caking of the reaction mixtures.

2) The Influence of the Enzyme Concentration on the Yield: Figure 2 shows the results of Reaction 1 with enzyme concentrations increasing from 0.3—19 mg/ml at 167 (a), 235 (b), and 300 mM (c) substrate concentrations under conditions similar to those described under

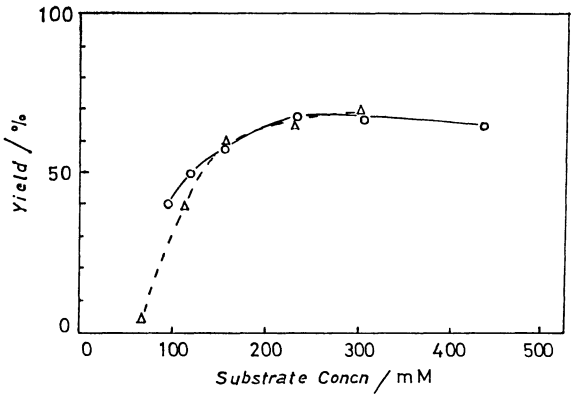

Fig. 1. The influences of substrate concentrations on the syntheses of ZGlnGlyLeuValNH$_2$ with Prolisin A and of ZLeuGlnGlyLeuValNH$_2$ with Prolisin B. Buffer solution used: 0.2 M Tris-HCl (pH 8, containing 5×10^{-2} M Ca(OAc)$_2$). △----△: An equimolar amount of ZGlnGlyOH and HLeuValNH$_2$·HCl (73—300 mM respectively), Prolisin A (4.25 mg/ml), and potato inhibitor (12.5 mg/ml) were used. The reactions were carried out according to the general procedure in the buffer (4 ml) at 40 °C for 3 h. ○——○: An equimolar amount of ZLeuGlnGlyOH and HLeuValNH$_2$·HCl (97.6—444 mM respectively), Prolisin B (24 mg/ml), and potato inhibitor (24 mg/ml) were used. The reactions were carried out according to the general procedure in the buffer at 40 °C for 17 h. Mps and R_f of all products were comparable with those of the products in the investigation III-6 (Table 4 or 5).

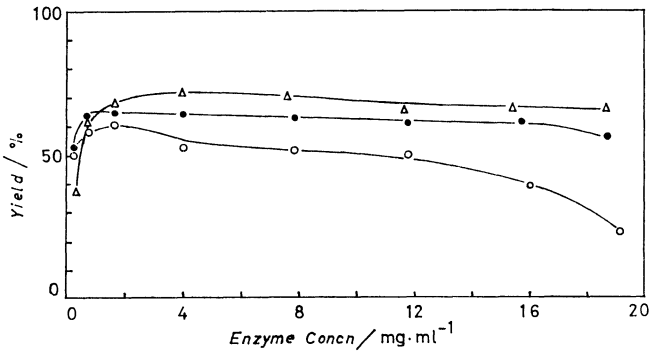

Fig. 2. The effect of enzyme concentration on the yield of ZGlnGlyLeuValNH$_2$. An equimolar amount of ZGlnGlyOH and HLeuValNH$_2$·HCl (167 (○——○), 235 (●——●), and 300 (△——△) mM respectively), Prolisin A (0.3—19 mg/ml), and potato inhibitor(0.9—57 mg/ml, Prolisin A: potato inhibitor=1 : 3 (wt/wt)) were used. The reactions were carried out in 0.2 M Tris-HCl (pH 8, containing 5×10^{-2} M Ca(OAc)$_2$, 4 ml) according to the general procedure at 40 °C for 3 h. Mps and R_f of all products were comparable with those of the products in the investigation III-6 (Table 4).

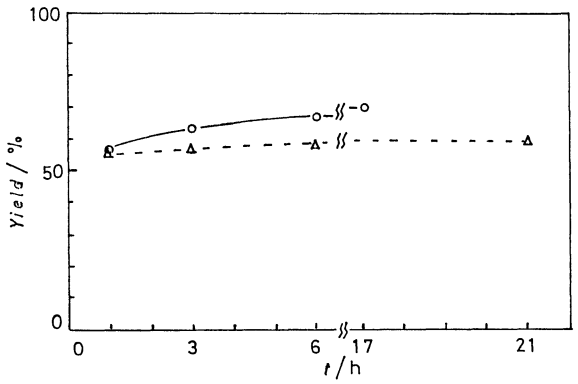

Fig. 3. The influence of the reaction period on the yield of ZGlnGlyLeuValNH$_2$ and ZLeuGlnGlyLeuValNH$_2$ with Prolisin B. The reactions were carried out in 0.2 M Tris-HCl (pH 8, containing 5×10^{-2} M Ca(OAc)$_2$) at 40 °C according to the general procedure. △----△: ZGlnGlyOH and HLeuValNH$_2$·HCl (307 mM respectively), Prolisin B(30.8 mg/ml), potato inhibitor (30.8 mg/ml), and the buffer (3 ml) were used. ○——○: ZLeuGlnGlyOH and HLeuValNH$_2$·HCl (235 mM respectively), Prolisin B (23.5 mg/ml), potato inhibitor (23.5 mg/ml), and the buffer (4 ml) were used. Mps and R_f of all products were comparable with those of the products in the investigation III-6 (Table 4 or 5).

the heading III-1. It can safely be said that the optimal yield is reached at a rather low concentration of enzyme, *i.e.*, 1.6—3.7 mg/ml.

3) The Influence of the Reaction Period on the Yield: Figure 3 illustrates the influence of the reaction period of both condensations (1—21 h) on the yield and the virtual completion of the reactions in 1 or 3 h.

4) The Influence of the Reaction Temperature on the Yield: J. D. McConn *et al.*[9] reported that the activity of *Bacillus subtilis* metalloproteinase has its maximum

at 58 °C in the presence of 5.5×10^{-4} M calcium acetate and was reduced to about 2/3 at 40 °C. However, when Reaction 1 was carried out for 3 h in the presence of Prolisin A under conditions similar to those of III-1 at 20—60 °C, the yield was almost unaffected by variations in temperature.

5) The Influence of the Buffer pH: When Reaction 2 was carried out under conditions similar to those of III-1, the change in the pH of the buffer had no perceptible influence in the range of 6.5—8.5. Therefore, in our examination of other factors influencing the peptide bond formation, the reactions were carried out always in a buffer of pH 8.

6) The Effect of Organic Solvent: Many organic solvents miscible with buffer solutions were examined for their effect on the yield of the product in both condensations under conditions similar to those of III-1 with Prolisin A or B (Tables 4 and 5). The results showed that small amounts (15% of total volume) of dioxane, *n*-BuOH, and MeOH brought about good results in both reactions and acetonitrile in one reaction; however, the addition of more solvent than 32% of the total volume gave unsatisfactory results. It is important to note that these products were generally purer than that from the reaction without organic solvent. It is not understood why better results were brought about by Prolisin A than by Prolisin B in Reaction 2.[12]

7) The Effects of the Amino-protecting Groups: As shown in Table 6, BOC for the amino-protecting group in place of Z brought about unsuccessful results under the same conditions as those of III-1. In this case organic solvents did not give the desired results, in contrast to the former two examples.

IV. Synthesis of ZGlnGlyLeuValNH$_2$ and ZLeuGlnGlyLeuValNH$_2$ with Other Enzymes. Since differences among several microbial metalloproteinases in the rate of hydrolysis of peptide bonds have been reported,[13] the above two reactions were examined further by using Thermolysin (Thermoase) and Tacynase N. The reactions were performed under the conditions most suitable for Prolisin; however, the two enzymes produced poorer results than Prolisin (Table 7). The differences observed with the three enzymes seem to correspond to those in the hydrolysis, though careful examinations will be necessary for each enzyme in view of the fact that the high concentration of Thermolysin brought about good results.

Table 4. The effect of organic solvent on the synthesis of ZGlnGlyLeuValNH$_2$[a]

Substrate (mM)	Prolisin A (mg/ml)	Inhibitor (mg/ml)	Org. solv.	Yield (%)
300	3.9	11.6	None	70.7
254	3.3	9.8	*n*-BuOH	79.4
254	9.8	29.3	*n*-BuOH	78.4
254	3.3	9.8	Dioxane	71.8
254	9.8	29.3	Dioxane	76.7
254	3.3	9.8	CH_3CN	62.4
254	3.3	9.8	THF	68.1
254	3.3	9.8	MeOH	82.9[b]

a) ZGlnGlyOH (0.44 g) and HLeuValNH$_2$·HCl (0.34 g) were allowed to react in a mixed solution of 0.2 M Tris-HCl buffer (pH 8, containing 5×10^{-2} M Ca(OAc)$_2$, 4 ml), organic solvent (0.8 ml), and 4 M NaOH (0.32 ml) at 40 °C for 3 h. b) Mp 245—247 °C; $[\alpha]_D^{30}$ −23.4° (*c* 1, AcOH); R_f 0.75; Found: C, 56.79; H, 7.31; N, 15.42%. Calcd for $C_{26}H_{40}N_6O_7$: C, 56.92; H, 7.35; N, 15.32%. Mps and R_f of all the other products were found comparable with the above data.

Table 5. The effect of organic solvent on the synthesis of ZLeuGlnGlyLeuValNH$_2$ with prolisin A or B[a]

Substrate (mM)	Prolisin (mg/ml)	Inhibitor (mg/ml)	Org. solv. (%)	Total vol. (ml)	Yield (%)
235	B, 23.5	23.5	Done	4.25	68.6
198	B, 19.8	19.8	Dioxane (15.8)	5.05	75.6
160	B, 16.0	16.0	Dioxane (32.0)	6.25	17.5
138	B, 13.8	13.8	Dioxane (41.4)	7.25	0
198	B, 19.8	19.8	THF (15.8)	5.05	70.4
160	B, 16.0	16.0	THF (32.0)	6.25	0
198	B, 19.8	19.8	CH_3CN (15.8)	5.05	74.9
160	B, 16.0	16.0	CH_3CN (32.0)	6.25	0
198	B, 19.8	19.8	CH_3NO_2 (15.8)	5.05	67.4
198	B, 19.8	19.8	$HOCH_2CH_2$-OMe (15.8)	5.05	65.7
198	B, 19.8	19.8	*n*-BuOH (15.8)	5.05	74.9
235	A, 8.0	23.5	None	2.125	70.7
198	A, 6.7	19.8	*n*-BuOH (15.8)	2.525	82.8
198	A, 6.7	19.8	Dioxane (15.8)	2.525	87.3[b]
198	A, 6.7	19.8	MeOH (22.0)	2.725	81.0

a) ZLeuGlnGlyOH and HLeuValNH$_2$·HCl (1 or 0.5 mmol each) were allowed to react in a mixed solution of 0.2 M Tris-HCl (pH 8, containing 5×10^{-2} M Ca(OAc)$_2$), an organic solvent, and 4 M NaOH at 40 °C for 3 h (Prolisin A) or 17 h (Prolisin B). b) Mp 260—262 °C; $[\alpha]_D^{25}$ −30.4° (*c* 0.5, AcOH); R_f 0.65; Found: C, 57.97; H, 7.66; N, 14.86%. Calcd for $C_{32}H_{51}N_7O_8$: C, 58.07; H, 7.77; N, 14.82%. Mps and R_f of all the other products were found comparable with the above data.

Table 6. Synthesis of BOCLeuGlnGlyLeuValNH$_2$ with Prolisin A[a]

Substrate (mM)	Prolisin (mg/ml)	Inhibitor (mg/ml)	Org. solv. (%)	Total vol. (ml)	Yield (%)
444	7.5	22.2	None	2.25	3.0
235	8.0	23.5	None	2.125	19.8[b]
198	6.7	19.8	*n*-BuOH (15.8)	2.525	0
198	6.7	19.8	Dioxane (15.8)	2.525	0

a) BOCLeuGlnGlyOH and HLeuValNH$_2$·HCl (1 or 0.5 mmol each) were allowed to react in a mixed solution of 0.2 M Tris-HCl (pH 8, containing 5×10^{-2} M Ca(OAc)$_2$), organic solvent and 4 M NaOH at 40 °C for 3 h. b) After one recrystallization from MeOH. Mp 230—232 °C; $[\alpha]_D^{25}$ −19.2° (*c* 0.25, DMF); Found: C, 55.54; H, 8.39; N, 15.24%. Calcd for $C_{29}H_{53}N_7O_8$: C, 55.48; H, 8.51; N, 15.62%.

Table 7. Syntheses of ZGlnGlyLeuValNH$_2$ (I) and ZLeuGlnGlyLeuValNH$_2$ (II) with thermolysin (thermoase) or tacynase N[a]

	Subst. (mM)	Protease (mg/ml)	Inhibitor (mg/ml)	Total vol. (ml)	Yield (%)
I	300	Thermoase (2.3)	2.3	4.325	55.5[b]
I	300	Tacynase N (46.2)	46.2	4.325	16.4
II	235	Thermolysin	none	4.25	53.6[c]
II	444	Thermolysin	none	2.25	63.3
II	235	Tacynase N (47.0)	47.0	4.25	36.6

a) A mixture of ZGlnGlyOH and HLeuValNH$_2$·HCl (1.3 mmol each) or ZLeuGlnGlyOH and HLeuValNH$_2$·HCl (1.0 mmol each) was stirred in a combined solution of 0.2 M Tris-HCl (pH 8, containing 5×10^{-2} M Ca(OAc)$_2$) and 4 M NaOH at 40 °C for 17 h (Thermolysin and Thermoase) or 3 h (Tacynase). b) Mp 246—247.5 °C. c) Mp 258.5—259 °C; $[\alpha]_D^{29}$ −33.4° (*c* 0.5 AcOH); Found: C, 57.27; H, 7.74; N, 14.77%. Calcd for $C_{32}H_{51}N_7O_8\cdot1/2H_2O$: C, 57.29; H, 7.81; N, 14.62%.

Conclusions

It was found that ZLeuGlnGlyOH or ZGlnGlyOH is catalytically coupled with HLeuValNH$_2$ by several metalloproteinases to give protected Secretin 23—27 or 24—27 peptide fragments in good yields. Since these reactions are performed in an aqueous solution and the crude products obtained from the reaction mixture are approximately pure in most cases, the after treatments are much easier than with the conventional methods. The lengthening of the peptide chain of a carboxyl component from 2 to 3 seemed to have no influence on the yield. The addition of a small amount of suitable organic solvents usually assisted the enzymatic reaction to give pure products in good yields.

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