Hydrolytic Cleavage of Pyroglutamyl-Peptide Bond. I. The Susceptibility of Pyroglutamyl-Peptide Bond to Dilute Hydrochloric Acid[1,2]

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The susceptibility of the pyroglutamyl-peptide bond in some biologically active peptides, dog neuromedin U-8 fragment (pGlu-Phe-Leu-Phe-Arg-Pro-Arg-OH), human big gastrin fragment (pGlu-Leu-Gly-Pro-OH) and thyrotropin releasing hormone (TRH) fragments (pGlu-His-Pro-OH, pGlu-His-OH), to 1 N HCl under mild conditions and/or at 60 °C was studied. It was found that the N-terminal portion of pGlu-peptides is extremely labile to acid hydrolysis, giving not only the ring-opened product of the pyrrolidone moiety of the pGlu residue, but also the cleavage product of the pGlu-peptide linkage. The ring-opening reaction predominated over the cleavage reaction in hydrolysis of the four peptides in 1 N HCl at 60 °C. The ring-opening reaction and the cleavage reaction of pGlu-peptide linkage proceeded faster than the cleavage of internal peptide bonds. The rate of hydrolysis was affected by the reaction temperature, and the ring-opening reaction was greatly diminished at 4 °C in comparison with the cleavage reaction. Thus, the phenomenon that the pGlu-peptide bond is susceptible to dilute HCl as compared to the other peptide bonds appears to be a general one.

Key words pyroglutamyl-peptide bond; cleavage reaction; hydrolysis; hydrochloric acid; dog neuromedin U-8; thyrotropin releasing hormone

Instability of synthetic peptides is occasionally observed during purification procedures such as RP-HPLC and gel-filtration under acidic conditions, resulting in deamidation and cleavage of peptide bonds.[3] Our preliminary communication[2] revealed that pGlu-peptides are highly sensitive to dilute HCl under mild conditions and are hydrolyzed to give complicated hydrolysates which involve not only the ring-opened product of the pyrrolidone moiety of the pGlu residue, but also the cleavage product of the pGlu-peptide linkage. The present paper describes the identification of the decomposition products, the main pathway of the decomposition, the decomposition rate, the accumulation rate of the hydrolysates, and the influence of temperature on the hydrolysis of model peptides related to biologically active peptides, dog neuromedin U-8[4] (d-NMU-8) (1—7)-OH (pGlu-Phe-Leu-Phe-Arg-Pro-Arg-OH[5]), human big gastrin[6] (1—4)-OH (pGlu-Leu-Gly-Pro-OH) and thyrotropin releasing hormone[7] (TRH) (1—3)-OH and TRH-(1—2)-OH (pGlu-His-Pro-OH and pGlu-His-OH) in dilute HCl.

Results and Discussion

We have often encountered some decomposition of NMU-8 (Fig. 1) and related peptides during purification, especially by gel-filtration on a column of Toyopearl HW-40 using 10—25% MeCN in 5 mM HCl as the eluent. It was also found that d-NMU-8 and its analogs are highly sensitive to dilute HCl, generating complicated hydrolysates which may involve not only the deamidation products of the two carboxamide functions of the C-terminal Asn amide, but also several N-terminal decomposition products, whereas porcine NMU-8[8] (p-NMU-8) was decomposed only at the C-terminal Asn amide. Since d- and p-NMU-8 share a common C-terminal heptapeptide amide structure, that is, d-NMU-8 has a pGlu residue in place of Tyr^1 of p-NMU-8, the complex decomposition of d-NMU-8 could be interpreted by assuming that the susceptibility of the N-terminal portion of d-NMU-8 to dilute acid is due to not only the ring-opening reaction[9] of the pyrrolidone moiety of the pGlu residue, but also unknown reactions at the N-terminal.

porcine	p-NMU-8	H-$\overset{1}{\text{Tyr}}$-Phe-Leu-Phe-Arg-Pro-Arg-$\overset{8}{\text{Asn}}$-NH_2
dog	d-NMU-8	pGlu-Phe-Leu-Phe-Arg-Pro-Arg-Asn-NH_2

Fig. 1. Amino Acid Sequences of Neuromedin U-8 (NMU-8)

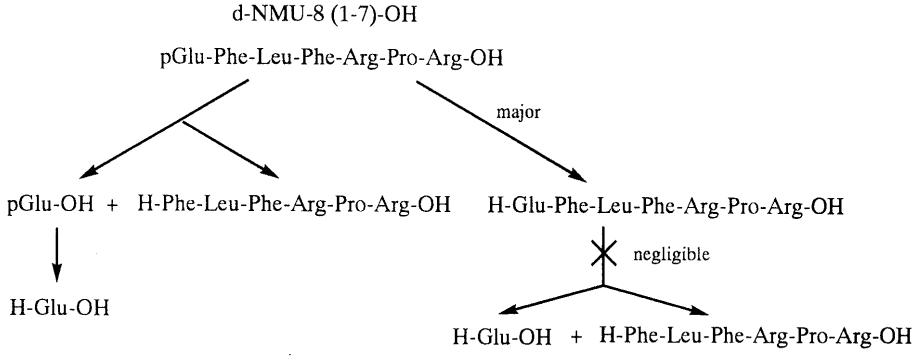

Chart 1

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In order to study the decomposition of the N-terminal portion of d-NMU-8, the hydrolysis of d-NMU-8 (1—7)–OH[5] (pGlu–Phe–Leu–Phe–Arg–Pro–Arg–OH), lacking the Asn amide, in dilute HCl was examined by RP-HPLC.

When a solution of d-NMU-8 (1—7)–OH at a concentration of 10^{-3} mol/l in 10% MeCN in 5 mM HCl was allowed to stand at 25 °C for 50 h, or 1 N HCl at 4 °C for 11 h, or 1 N HCl at 25 °C for 20 min, decomposition occurred, resulting in the formation of two hydrolysates which were detected as small peaks on RP-HPLC. The incubation of d-NMU-8 (1—7)–OH in 1 N HCl at 60 °C resulted in an increase of the two hydrolysates and a decrease of the starting material, which appeared on RP-HPLC as peaks a, b and c, respectively (Fig. 2A). Peak a was assigned to NMU-8 (2—7)–OH (H–Phe–Leu–Phe–Arg–Pro–Arg–OH), peak b to [Glu[1]]–NMU-8 (1—7)–OH (H–Glu–Phe–Leu–Phe–Arg–Pro–Arg–OH) and peak c to d-NMU-8 (1—7)–OH on the basis of amino acid analysis and direct comparison with the authentic peptide on the RP-HPLC. pGlu–OH was similarly assigned to peak d, based on RP-HPLC (Fig. 2C). These peaks were confirmed by the coelution, and H–Glu–OH was analyzed directly by an amino acid analyzer system. The results suggested that the decomposition occurred exclusively at the N-terminal of d-NMU-8 (1—7)–OH, that is, the partial acid hydrolysis of d-NMU-8 (1—7)–OH caused not only ring-opening[9] of the pyrrolidone moiety, but also cleavage of the pGlu–Phe bond without appreciable cleavage of the internal peptide bonds. It is well known that the lactam of the pGlu residue is readily hydrolyzed,[9] but the susceptibility of the pGlu–peptide bond to dilute HCl under mild conditions has not been reported. To elucidate the main pathway of the decomposition, the hydrolysis of [Glu[1]]–NMU-8 (1—7)–OH was examined. Incubation of this peptide under the same conditions as described for d-NMU-8 (1—7)–OH for 6 h produced d-NMU-8 (1—7)–OH (3.2%) and NMU-8 (2—7)–OH (1.3%), and about 87% of the starting material remained intact. d-NMU-8 (1—7)–OH was regenerated by the formation[9] of the pyrrolidone moiety derived from the ring closure of the N-terminal Glu residue of [Glu[1]]–NMU-8 (1—7)–OH. The Glu–Phe linkage of [Glu[1]]–NMU-8 (1—7)–OH was partially cleaved by the acid to give NMU-8 (2—7)–OH. The pGlu residue of d-NMU-8 (1—7)–OH was converted by ring-opening reaction to a Glu residue to give [Glu[1]]–NMU-8 (1—7)–OH, which did not undergo extensive peptide bond cleavage reaction. Therefore, the results indicated that the pGlu–Phe bond of d-NMU-8 (1—7)–OH might be directly hydrolyzed to give pGlu–OH and NMU-8 (2—7)–OH (Chart 1).

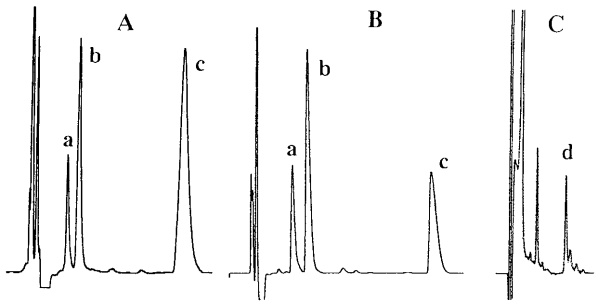

Fig. 2. HPLC Profiles of d-NMU-8 (1—7)–OH and Its Hydrolysates in 1 N HCl at 60 °C for 2 h (A, C) and 6 h (B)

HPLC conditions: A and B; column, YMC-ODS-5-AM (4.6 × 150 mm); elution, 21.6% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. C; column, Puresil™ C_{18} (4.6 × 250 mm); elution, 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. a, NMU-8 (2—7)–OH (H–Phe–Leu–Phe–Arg–Pro–Arg–OH) [retention time (t_R), 6.9 min]; b, [Glu[1]]–NMU-8 (1—7)–OH (H–Glu–Phe–Leu–Phe–Arg–Pro–Arg–OH) (t_R, 8.5 min); c, d-NMU-8 (1—7)–OH (pGlu–Phe–Leu–Phe–Arg–Pro–Arg–OH) (t_R, 21.5 min); d, pGlu–OH (t_R, 9.2 min).

The rates of degradation of d-NMU-8 (1—7)–OH and accumulation of the main hydrolysates in 1 N HCl at 60 °C were next investigated. On incubation for 6 h, about 68% of the starting material was hydrolyzed to give [Glu[1]]–NMU-8 (1—7)–OH, NMU-8 (2—7)–OH, pGlu–OH and H–Glu–OH (Table 1). The internal peptide bonds except at positions 1—2 were not significantly cleaved during the hydrolysis RP-HPLC (Fig. 2B). The half-life ($t_{1/2}$) of the hydrolysis of d-NMU-8 (1—7)–OH was 3.4 h. The main reaction was ring-opening, resulting in an accumulation of [Glu[1]]–NMU-8 (1—7)–OH and the cleavage reaction occurred to a considerably lesser extent. The accumulation curves of the acid hydrolysates (Fig. 3A) showed that the amounts of [Glu[1]]–NMU-8 (1—7)–OH and NMU-8 (2—7)–OH increased almost linearly during the hydrolysis, suggesting that both peptides were quite stable in the acidic solution, and the yield of [Glu[1]]–NMU-8 (1—7)–OH was almost 2.7 times that of NMU-8 (2—7)–OH (in mole percent) during the hydrolysis. As can be seen in Table 1, the amount of pGlu–OH reached a plateau at 4 h incubation; pGlu–OH might decompose gradually to produce H–Glu–OH. Since the Glu–Phe bond of [Glu[1]]–NMU-8 (1—7)–OH was hardly hydrolyzed by 1 N HCl at 60 °C for 6 h, it can be assumed

Table 1. d-NMU-8 (1—7)–OH and Its Hydrolysates during Incubation in 1 N HCl at 60 °C

Amino acid and peptide	Time (h)						
	0	1	2	3	4	5	6
	Mole percent						
d-NMU-8 (1—7)–OH	100	80.0	66.8	55.7	46.2	39.1	32.1
[Glu[1]]–NMU-8 (1—7)–OH	0	10.7	20.3	27.5	33.2	38.7	42.0
NMU-8 (2—7)–OH	0	3.9	7.5	10.1	12.5	14.5	16.2
pGlu–OH	0	2.3	4.8	6.1	7.2	10.2	12.3
H–Glu–OH[a]	0	0	0.8	1.6	2.3	3.6	4.4

a) Analytical data were obtained on an amino acid analyzer system. The values except for H–Glu–OH have an accuracy of about ±1.2%. d-NMU-8 (1—7)–OH (pGlu–Phe–Leu–Phe–Arg–Pro–Arg–OH). [Glu[1]]–NMU-8 (1—7)–OH (H–Glu–Phe–Leu–Phe–Arg–Pro–Arg–OH). NMU-8 (2—7)–OH (H–Phe–Leu–Phe–Arg–Pro–Arg–OH).

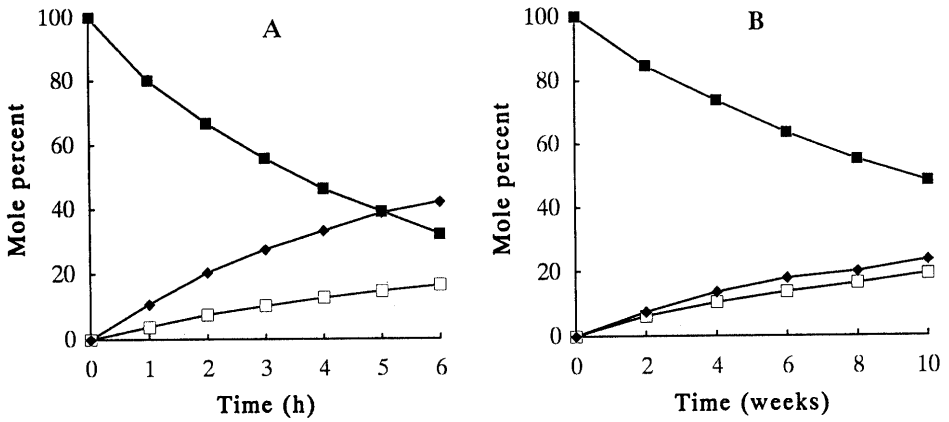

Fig. 3. Time Courses for d-NMU-8 (1—7)-OH and Its Hydrolysates during Incubation in 1 N HCl at 60 °C for 6 h (A) and at 4 °C for Ten Weeks (B)

■, d-NMU-8 (1—7)-OH; □, NMU-8 (2—7)-OH; ◆, [Glu[1]]-NMU-8 (1—7)-OH. The curves (A) are drawn in part from data given in Table 1.

that the most of the H–Glu–OH arose primarily from pGlu–OH. Therefore, the pGlu–Phe linkage in d-NMU-8 (1—7)-OH may be broken directly by acid hydrolysis.

To examine the effect of temperature on the hydrolysis, a solution of d-NMU-8 (1—7)-OH in 1 N HCl was kept at 4 °C for ten weeks. In this experiment, 51.6% of the starting material was hydrolyzed to give [Glu[1]]-NMU-8 (1—7)-OH (23.6%) and NMU-8 (2—7)-OH (19.2%). The accumulation curves (Fig. 3B) of the acid hydrolysates showed that the amount of [Glu[1]]-NMU-8 (1—7)-OH formed was slightly greater than that of NMU-8 (2—7)-OH in mole percent. The results show that the rate of the ring-opening reaction was greatly reduced at 4 °C in comparison with that of the cleavage reaction. The above results are consistent with our preliminary study[2] on the hydrolysis of a model peptide, pGlu–Ala–Phe–OH.

Our study[2] on the model peptide revealed that the hydrolysis of pGlu–Ala–Phe–OH in 1 N HCl at 60 °C for 6 h gave H–Glu–Ala–Phe–OH (40.9%), H–Ala–Phe–OH (35.3%), pGlu–OH (31.3%) and H–Glu–OH (9.5%). The ring-opened product, H–Glu–Ala–Phe–OH, predominated slightly over the cleavage product, H–Ala–Phe–OH, on a mole percent basis. The hydrolysis of H–Glu–Ala–Phe–OH under the same conditions for 6 h produced H–Ala–Phe–OH (1.8%) and regenerated pGlu–Ala–Phe–OH (1.2%). About 96% of the starting material was left in the hydrolysate solution and appreciable cleavage of the Ala–Phe bond of H–Glu–Ala–Phe–OH did not occur. On storage of pGlu–Ala–Phe–OH in 1 N HCl at 4 °C for ten weeks, the cleavage reaction predominated over the ring-opening reaction (molar ratio of approximately 2 : 1).

Next, human big gastrin[6] N-terminal fragment, pGlu–Leu–Gly–Pro–OH, was similarly investigated. When a solution of pGlu–Leu–Gly–Pro–OH in 1 N HCl was allowed to stand at 25 °C for 80 min, two hydrolysates (peaks d and e in Fig. 4) appeared on the RP-HPLC. The decomposition of pGlu–Leu–Gly–Pro–OH was observed on storage for 80 min, whereas that of d-NMU-8 (1—7)-OH was apparent after 20 min. Thus, pGlu–Leu–Gly–Pro–OH seemed to be more stable than d-NMU-8 (1—7)-OH in 1 N HCl at 25 °C. The incubation of pGlu–Leu–Gly–Pro–OH in 1 N HCl at 60 °C gave H–Glu–Leu–Gly–Pro–OH and H–Leu–Gly–Pro–OH[10] as major products (Table 2, Fig. 5). The ring-opening reaction predominated over the cleavage reaction (mole percent of approximately 2 : 1). In addition, the bonds at the amino and carboxyl sides of the Gly residue were cleaved to a considerable extent in 6 h to afford pGlu–Leu–Gly–OH (4.5%), H–Glu–Leu–Gly–OH (4.1%), H–Glu–Leu–OH[11] (6.5%) and H–Gly–Pro–OH[12] (10.4%). The $t_{1/2}$ of the hydrolysis of pGlu–Leu–Gly–Pro–OH was 3.6 h. On the hydrolysis of H–Glu–Leu–Gly–Pro–OH under the same condition, the bonds at the amino and carboxyl sides of the Gly residue were exclusively cleaved[13] to almost the same extent in terms of molar ratio to afford H–Glu–Leu–Gly–OH (12.1%), H–Glu–Leu–OH (11.2%) and H–Gly–Pro–OH (13.7%). The cleavage product of the Glu–Leu linkage, H–Leu–Gly–Pro–OH, and the ring-closure product of the N-terminal Glu residue, pGlu–Leu–Gly–Pro–OH, were not detected in the acid hydrolysate. As the Glu–Leu bond, like the Glu–Phe bond in d-NMU-8 (1—7)-OH, was hardly hydrolyzed in 1 N HCl at 60 °C for 6 h, it can be assumed that the pGlu–Leu linkage in pGlu–Leu–Gly–Pro–OH was cleaved directly by acid hydrolysis to give pGlu–OH, which may generate H–Glu–OH. The results indicated that the cleavage of pGlu–Leu linkage and the ring-opening of the pGlu moiety proceeded faster than the cleavages of the peptide bonds at the amino and carboxyl sides of the Gly residue. Therefore, it is clear that pGlu–peptide structure is extremely labile to dilute acid.

pGlu–His–Pro–OH,[14] which is an enzymatic degradation product of TRH, pGlu–His–Pro–NH_2, was next investigated. The enzymatic degradation mechanism[15] of TRH in blood and various tissues has been examined by using RP-HPLC. When a solution of pGlu–His–Pro–OH in 1 N HCl was allowed to stand at 25 °C for 20 min, two hydrolysates (peak a and c in Fig. 6A) were detected by RP-HPLC. The hydrolysis of pGlu–His–Pro–OH at the same concentration in 1 N HCl at 60 °C produced a complicated mixture of hydrolysates in comparison with the hydrolysis[2] of pGlu–Ala–Phe–OH. HPLC analyses (Fig. 6A, 6B) (Table 3) (Fig. 7) revealed that the major products were the ring-opened product, H–Glu–His–Pro–OH[14] (44.6%), the peptide bond cleavage product, H–His–Pro–OH (20.7%) and pGlu–OH (14.7%), similar-

Table 2. pGlu–Leu–Gly–Pro–OH and Its Hydrolysates during Incubation in 1 N HCl at 60 °C

Amino acid and peptide	Time (h)						
	0	1	2	3	4	5	6
	Mole percent						
pGlu–Leu–Gly–Pro–OH	100	80.6	65.9	53.7	42.7	33.3	26.5
H–Glu–Leu–Gly–Pro–OH	0	8.6	16.0	21.7	25.9	28.7	31.0
H–Leu–Gly–Pro–OH	0	4.3	8.4	11.5	13.6	15.9	17.4
H–Glu–Leu–Gly–OH	0	0	0.6	1.2	2.0	3.1	4.1
H–Glu–Leu–OH	0	0	1.6	3.1	4.2	5.9	6.5
pGlu–Leu–Gly–OH	0	1.4	3.1	3.9	4.4	4.6	4.5
H–Gly–Pro–OH[a]	0	3.4	5.1	6.9	8.0	8.9	10.4
pGlu–OH[a]	0	4.6	7.6	9.0	11.0	12.2	13.6
H–Glu–OH[b]	0	0	1.2	2.1	3.3	4.3	5.4

a) HPLC conditions: column, Puresil™ C_{18} (4.6 × 250 mm); elution, 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. *b*) Analytical data were obtained with an amino acid analyzer system. The values except for H–Glu–OH have an accuracy of about ±2.1%.

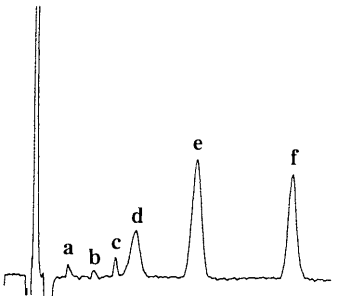

Fig. 4. HPLC Profile of pGlu–Leu–Gly–Pro–OH and Its Hydrolysates in 1 N HCl at 60 °C for 5 h

HPLC conditions: column, YMC-ODS-5-AM (4.6 × 150 mm); elution, 8% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. a, H–Glu–Leu–Gly–OH [retention time (t_R), 6.9 min]; b, H–Glu–Leu–OH (t_R, 9.5 min); c, pGlu–Leu–Gly–OH (t_R, 12.0 min); d, H–Leu–Gly–Pro–OH (t_R, 14.1 min); e, H–Glu–Leu–Gly–Pro–OH (t_R, 20.4 min); f, pGlu–Leu–Gly–Pro–OH (t_R, 31.0 min).

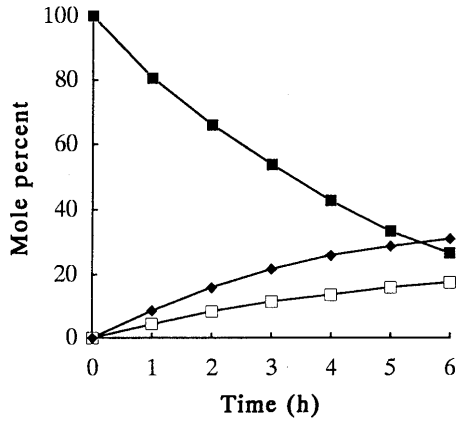

Fig. 5. Time Courses for pGlu–Leu–Gly–Pro–OH and Its Hydrolysates during Incubation in 1 N HCl at 60 °C for 6 h

■, pGlu–Leu–Gly–Pro–OH; ◆, H–Glu–Leu–Gly–Pro–OH; □, H–Leu–Gly–Pro–OH. The curves are drawn in part from data given in Table 2.

ly to the hydrolyses of d-NMU-8 (1—7)–OH and the N-terminal fragment of human big gastrin, together with His–Pro diketopiperazine[16] (4.5%), pGlu–His–OH[17] (2.8%), H–Glu–His–OH[18] (1.9%), and a trace of H–Pro–His–OH[16] (0.4%) during 6 h incubation. The results indicated that hydrolytic cleavage at 60 °C occurred at both peptide bonds of pGlu–His and His–Pro, but the former bond was highly susceptible to dilute HCl compared to the latter. The ring-opening reaction of the pyrrolidone moiety predominated over the cleavage reaction of the pGlu–His bond (molar ratio of about 2 : 1). The ratio of the ring-opening reaction to the cleavage reaction appeared to be almost the same as that of the pGlu–Leu portion of pGlu–Leu–Gly–Pro–OH. Although the ring-opening reaction of the pyrrolidone moiety of pGlu–His–Pro–OH proceeded faster than that of pGlu–Ala–Phe–OH and the cleavage reaction of the pGlu–His bond proceeded more slowly than that of pGlu–Ala bond of pGlu–Ala–Phe–OH, the degradation of pGlu–Ala–Phe–OH ($t_{1/2}$, 3.8 h) was faster than that of pGlu–His–Pro–OH ($t_{1/2}$, 4.5 h). The results indicated that pGlu–His–Pro–OH is stable to acidic solution at 60 °C compared with pGlu–Ala–Phe–OH.

To reconfirm the main pathway of the decomposition of pGlu–His–Pro–OH, H–Glu–His–Pro–OH was hydrolyzed under the same conditions. The Glu–His linkage was extremely resistant to acid hydrolysis, and about 97% of the starting material remained intact after 6 h incubation. As with pGlu–His–Pro–OH, the acid decomposition of H–Glu–His–Pro–OH was complex, affording H–Glu–His–OH (2.4%), H–His–Pro–OH (1.7%), pGlu–His–Pro–OH (1.1%), small amounts of the diketopiperazine (0.2%), pGlu–His–OH (0.2%) and a trace of H–Pro–His–OH (0.2%). In the hydrolyses of pGlu–His–Pro–OH and H–Glu–His–Pro–OH at 60 °C, the amount of H–Glu–His–OH increased almost linearly with time, whereas that of pGlu–His–OH reached a plateau after 4 h incubation, suggesting that the former dipeptide is rather stable in the acidic solution and the latter dipeptide is not. The hydrolysis of pGlu–His–OH in 1 N HCl at 60 °C for 6 h gave H–Glu–His–OH (39.5%), pGlu–OH (10.5%), H–His–OH (9.8%) and H–Glu–OH (1.6%) (Table 4). The ring-opened product predominated over the cleavage product as in the hydrolysis of pGlu–His–Pro–OH. However, there was a significant difference between the ratio of the ring-opened product to the cleavage product between the di- and tripeptide. The $t_{1/2}$ value of the hydrolysis of pGlu–His–OH was 5.2 h, higher than that of pGlu–His–Pro–OH. The results suggested that the ring-opening reaction and the cleavage reaction depend on the chain length of pGlu–peptide.

For further study on the effect of temperature on the acid hydrolysis, a solution of pGlu–His–Pro–OH in 1 N

HCl was kept at 4 °C for 6 weeks; 72.4% of the starting material remained in the hydrolysis solution, which also contained H–Glu–His–Pro–OH (14.8%), H–His–Pro–OH (15.5%) and His–Pro diketopiperazine (0.4%). The RP-HPLC analyses revealed that the ratio of the yield of H–Glu–His–Pro–OH to the sum of those of H–His–Pro–OH + His–Pro diketopiperazine was almost the same in mole percent over the time course of the hydrolysis.

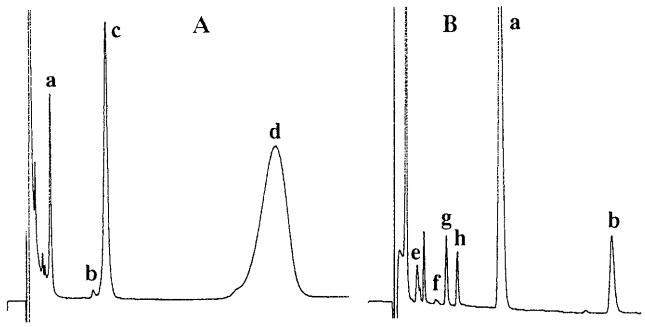

Fig. 6. HPLC Profiles of pGlu–His–Pro–OH and Its Hydrolysates in 1 N HCl at 60 °C for 6 h

HPLC conditions: A; column, YMC-ODS-5-AM (4.6 × 150 mm); elution, 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. B; column, Puresil™ C_{18} (4.6 × 250 mm); elution, 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. a, H–His–Pro–OH [retention time (t_R), 4.9 min]; b, His–Pro diketopiperazine (t_R, 9.8 min); c, H–Glu–His–Pro–OH (t_R, 11.8 min); d, pGlu–His–Pro–OH (t_R, 32.7 min); e, H–Glu–His–OH (t_R, 5.1 min); f, H–Pro–His–OH (t_R, 6.9 min); g, pGlu–His–OH (t_R, 8.1 min); h, pGlu–OH (t_R, 9.3 min).

The ring-opening reaction of the pyrrolidone moiety and the cleavage reaction of the pGlu–His linkage proceeded to approximately the same extent. The results suggested that the rate of the ring-opening reaction was diminished at 4 °C in comparison with that of the cleavage reaction. The results are consistent with the observations on the hydrolysis of d-NMU-8 (1—7)–OH at 4 °C. It was confirmed that the mode of hydrolysis of pGlu–peptide in dilute HCl is affected by reaction temperature.

The present study has revealed that pGlu–peptide is highly susceptible to dilute acid and is hydrolyzed under mild conditions to give not only the ring-opened hydrolysate of the pyrrolidone moiety, but also the cleavage product of the pGlu–peptide linkage. The hydrolysis was greatly affected by change of temperature. The rate of the ring-opening reaction was very much reduced at low temperature. The cleavage of pGlu–peptide linkage and the ring-opening reaction of the pGlu moiety proceeded faster than the cleavage at internal peptide bonds. Thus, the phenomenon that the pGlu–peptide bond is susceptible to diluted HCl as compared to other peptide bonds appears to be a general one.

Experimental

General Synthesis of peptides used in this study was carried out on a Beckman system 990C peptide synthesizer (Beckman Instruments Ltd., U.S.A.). Semi-preparative RP-HPLC was performed on an apparatus

Table 3. pGlu–His–Pro–OH and Its Hydrolysates during Incubation in 1 N HCl at 60 °C

Amino acid and peptide	Time (h)						
	0	1	2	3	4	5	6
	Mole percent						
pGlu–His–Pro–OH	100	86.0	74.5	63.3	53.6	45.6	39.0
H–Glu–His–Pro–OH	0	9.6	19.0	27.5	35.0	39.9	44.6
H–His–Pro–OH	0	5.6	10.3	14.1	17.3	19.4	20.7
His–Pro diketopiperazine	0	0	0.7	1.3	2.7	3.6	4.5
H–Pro–His–OH	0	0	0.1	0.2	0.2	0.3	0.4
H–Glu–His–OH	0	0.3	0.6	1.0	1.4	1.7	1.9
pGlu–His–OH	0	0.9	1.6	2.0	2.4	2.6	2.8
pGlu–OH	0	3.8	7.4	9.6	11.9	12.6	14.7
H–Glu–OH[a)]	0	0	0	0.8	2.2	2.5	4.2

a) Analytical data were obtained with an amino acid analyzer system. The values except for H–Glu–OH have an accuracy of about ± 1.3%.

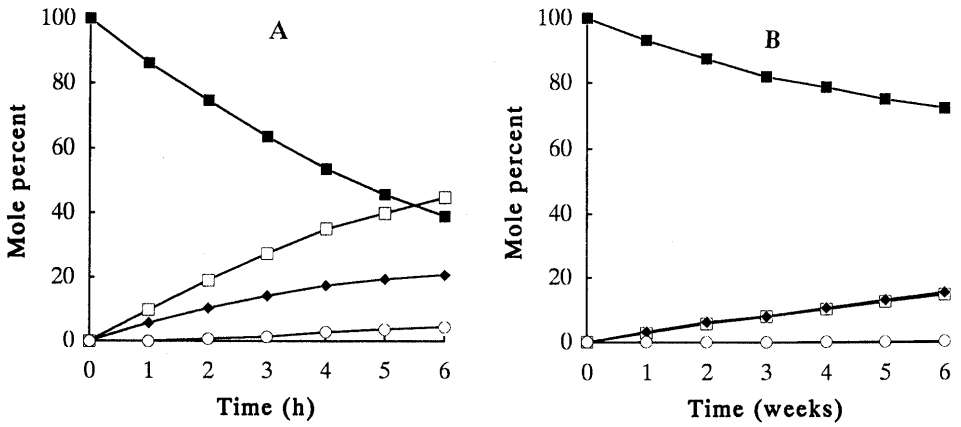

Fig. 7. Time Courses for pGlu–His–Pro–OH and Its Hydrolysates during Incubation in 1 N HCl at 60 °C for 6 h (A) and at 4 °C for Six Weeks (B)

■, pGlu–His–Pro–OH; □, H–Glu–His–Pro–OH; ◆, H–His–Pro–OH; ○, His–Pro diketopiperazine. The curves (A) are drawn in part from data given in Table 3.

Table 4. pGlu–His–OH and Its Hydrolysates during Incubation in 1 N HCl at 60 °C

Amino acid and peptide	Time (h)						
	0	1	2	3	4	5	6
	Mole percent						
pGlu–His–OH[a]	100	89.7	78.7	67.7	58.7	51.2	45.2
H–Glu–His–OH[a]	0	7.3	15.8	23.2	29.5	34.7	39.5
pGlu–OH[a]	0	2.1	4.1	6.5	7.7	9.3	10.5
H–His–OH[b]	0	2.0	3.3	4.5	7.5	8.5	9.8
H–Glu–OH[b]	0	0	0	0	1.6	2.2	1.6

a) HPLC conditions: column, Puresil™ C_{18} (4.6 × 250 mm); elution, 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. *b*) Analytical data were obtained with an amino acid analyzer system. The values except for H–His–OH and H–Glu–OH have an accuracy of about ± 1.2%.

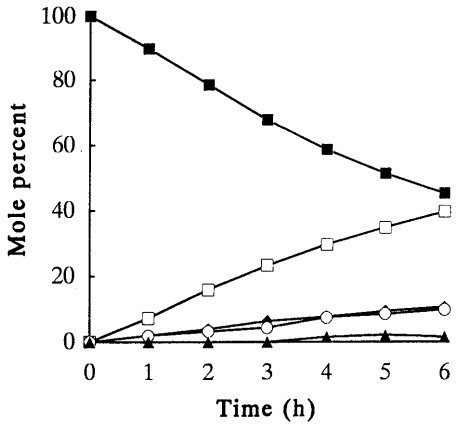

Fig. 8. Time Courses for pGlu–His–OH and Its Hydrolysates during Incubation in 1 N HCl at 60 °C for 6 h

■, pGlu–His–OH; □, H–Glu–His–OH; ◆, pGlu–OH; ○, H–His–OH; ▲, H–Glu–OH. The curves are drawn in part from data given in Table 4.

equipped with a 590 pump (Waters, U.S.A.), a U6K injector (Waters), a S310 model II UV detector (Soma, Japan) and a 561—3003 recorder (Hitachi, Japan), or a 638-30 pump (Hitachi), a Rheodyne 7125 injector (Rheodyne Inc., U.S.A.), a multi-wavelength UV monitor 635 M (Hitachi) and a Unicorder U-228 recorder (Nippon Denshi Kagaku Inc., Japan). Analytical RP-HPLC was accomplished on a system comprising two CCPD pumps (Tosoh Co., Japan), a Rheodyne 7125 injector (Rheodyne) or an Automatic Sample Injector AS-8020 (Tosoh), a UV 8000 or a UV 8011 detector (Tosoh), a Chromatocoder 21 or 12 integrator (System Instruments Co., Ltd. Japan), and a gradient controller PX-8010 (Tosoh) or a 638-30 pump (Hitachi), a Rheodyne 7125 injector (Rheodyne), a multi-wavelength UV monitor 635 M (Hitachi) and a Chromatocoder 12 integrator (System Instruments). Gel chromatography was effected on a Toyopearl HW-40 (super fine) column. Amino acid analysis of the acid hydrolysate was conducted on a 7300 amino acid analyzer (Beckman). HF cleavage reactions were carried out in a Teflon HF apparatus (Peptide Institute Inc., Japan). Fast-atom bombardment mass spectra (FAB-MS) were obtained on a JMS-DX300 mass spectrometer (JEOL Ltd., Japan). Optical rotations of peptides were measured with a DIP-370 digital polarimeter (Nippon Bunko Co., Ltd., Japan). HP-TLC was performed on precoated silica gel plates (Kieselgel 60, E. Merck, Germany).

Reagents Unless otherwise stated, all reagents and solvents were obtained as reagent-grade products from Watanabe Chem. Ind., Ltd. or Wako Pure Chem. Ind. Ltd., Japan, and used without further purification. Boc-protected amino acids were purchased from Peptide Institute Inc., Japan. Boc-amino acid Merrifield resins were obtained commercially from Watanabe Chem. Ind., Ltd.

Peptide Synthesis Peptides were prepared by a standard solid-phase method.[19] All amino acids except Z-pyroglutamic acid were protected as α-Boc derivatives. The protecting groups for the amino acid side chains were *p*-tosyl for the guanidino group of Arg and N^{π}-benzyloxymethyl (Bom) for the imidazole ring of His. Solid-phase peptide synthesis was performed starting from Boc-amino acid Merrifield resin. The elongation of the peptide chain was carried out using Boc-amino acids (2.5 eq) in *N*-methylpyrrolidone with benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate[20] (BOP) (2.5 eq) and *N*-methylmorpholine (2.0 eq) in dimethylacetamide for the first and third coupling reactions, and DCC (2.5 eq)–HOBt (2.5 eq) for the second coupling in DCM and/or DMF. Incorporation of an amino acid was repeated until the Kaiser ninhydrin test[21] became negative. The deprotection of the Boc group during the peptide chain elongation was executed by the use of 33% TFA in DCM for 30 min. After the completion of the peptide chain elongation, the peptide-resin was treated with 33% TFA/DCM, washed with ethanol and dried. Peptides were deblocked and cleaved from the resin with anhydrous liquid HF containing 10% anisole. The reaction mixture was kept at 0 °C for 45 min. After evaporation of HF *in vacuo* under ice-cooling, the residual mixture was washed with ether prior to the extraction of the crude peptide with 12—50% AcOH. The combined extracts were lyophilized. The crude peptide was revealed as a main peak on analytical RP-HPLC.

Peptide Purification The synthetic peptides were highly purified by semi-preparative RP-HPLC on a column (19 × 150 mm) of μ-Bondasphere C_{18} 5—100 A or YMC-pack D-ODS-5-ST S-5 120 A (20 × 150 mm) with 0.1% TFA–MeCN in an isocratic system, followed by gel-filtration on a column (1.5 × 47 cm) of Toyopearl HW-40 (super fine) with 70% MeOH as an eluent.

Peptide Characterization Homogeneity of the purified peptides was ascertained by analytical RP-HPLC with a 0.1% TFA–MeCN system, HP-TLC with two solvent systems, amino acid analysis of acid hydrolysates and FAB-MS. HPLC analysis of the purified peptides was carried out using a YMC-ODS-5-AM (4.6 × 150 mm) or a Puresil™ C_{18} (4.6 × 250 mm) column with isocratic elution (0.1% TFA–MeCN) or linear gradient elution with 4—44% MeCN over a period of 40 min in 0.1% TFA (flow rate, 1 ml/min; UV detection, 210 nm). For amino acid composition analyses, peptides were hydrolyzed with 6 N HCl vapor. Peptide (30—200 mg) was taken in a test tube (6 × 50 mm) and placed in a vial (40 ml), the bottom of which contained 6 N HCl with 3% phenol (0.5 ml). The vial was evacuated under cooling, then closed with a stopper and kept in a block heater at 130 °C for 3 h. The amino acid compositions of the acid hydrolysates were consistent with theoretical values. The analytical data are shown in Table 5.

Optical rotations of peptides were measured with a 3.5 × 50 mm cell. A peptide was dissolved in 2 M AcOH at a concentration of 0.50%. Values shown in Table 6 were obtained by calculation from the means of 3 successive 30-s integrations. *Rf* values in HP-TLC refer to the following solvent systems: Rf^1, *n*-BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24) and Rf^2, *n*-BuOH–AcOEt–AcOH–H_2O (1 : 1 : 1 : 1). The data are shown in Table 6.

When single peaks on HPLC and single spots on HP-TLC in two solvents systems were observed for a peptide, and its amino acid composition and FAB-MS were consistent with the calculated values, the peptide was used for the acid hydrolysis study.

HPLC Analysis of Acid Hydrolysate For the analysis of acid hydrolysates, a solution of a peptide at a concentration of 10^{-3} mol/l in 1 N HCl was prepared in a polypropylene tube (2 ml) with rapid stirring under ice-cooling and divided into seven to twelve aliquots (100 μl each) in polypropylene tubes (2 ml) with tight lids. These were maintained at an appropriate temperature in a thermostated apparatus. Each tube was taken out from the apparatus at 0 h or an appropriate time, and stored at −40 °C until analyzed. All analyses were done within 10 h. An aliquot (15 μl) of this solution was subjected to RP-HPLC analysis to determine

Table 5. FAB-MS Data and Amino Acid Analyses of Synthetic Peptides

Peptide	FAB-MS[a]		Amino acid analysis[b]						
	Found	Formula	Glu	Gly	Pro	Leu	Phe	His	Arg
[Glu[1]]-NMU-8 (1—7)-OH	964	$C_{46}H_{69}N_{13}O_{10}$	1.06 (1)	—	0.97 (1)	1.00 (1)	1.98 (2)	—	1.89 (2)
NMU-8 (2—7)-OH	835	$C_{41}H_{62}N_{12}O_7$	—	—	0.95 (1)	1.00 (1)	2.02 (2)	—	1.98 (2)
pGlu-Leu-Gly-Pro-OH	397	$C_{18}H_{28}N_4O_6$	1.03 (1)	1.01 (1)	1.02 (1)	1.00 (1)	—	—	—
H-Glu-Leu-Gly-Pro-OH	415	$C_{18}H_{30}N_4O_7$	1.04 (1)	1.00 (1)	0.94 (1)	1.00 (1)	—	—	—
pGlu-Leu-Gly-OH	300	$C_{13}H_{21}N_9O_5$	1.04 (1)	0.98 (1)	—	1.00 (1)	—	—	—
H-Glu-Leu-Gly-OH	318	$C_{13}H_{23}N_3O_6$	1.02 (1)	0.98 (1)	—	1.00 (1)	—	—	—
H-His-Pro-OH	253	$C_{11}H_{16}N_4O_3$	—	—	1.01 (1)	—	—	1.00 (1)	—

a) For $[M+H]^+$. *b*) Numbers in parentheses are theoretical values.

Table 6. Characteristics of Synthetic Peptides

Peptide	$[\alpha]_D^{27}$	RP-HPLC[a]	HP-TLC[c]	
	($c=0.5$, 2 M AcOH)	t_R[b] (min)	Rf^1	Rf^2
[Glu[1]]-NMU-8 (1—7)-OH	−51.5°	8.5[d]	0.37	0.46
NMU-8 (2—7)-OH	−69.8°	6.9[d]	0.46	0.48
pGlu-Leu-Gly-Pro-OH	−42.4°	31.0	0.71	0.46
H-Glu-Leu-Gly-Pro-OH	−51.9°	20.4	0.31	0.46
pGlu-Leu-Gly-OH	−63.6°	12.0	0.48	0.46
H-Glu-Leu-Gly-OH	−22.0°	6.9	0.33	0.54
H-His-Pro-OH	−50.7°	4.9[e]	0.16	0.14

a) HPLC conditions: column, YMC-ODS-5-AM (4.6 × 150 mm); elution, 8% MeCN except *d*) 21.6%, *e*) 0% in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. *b*) Retention time. *c*) Rf^1, *n*-BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24); Rf^2, *n*-BuOH–AcOEt–AcOH–H_2O (1 : 1 : 1 : 1).

the amount of the starting material that remained and the amounts of the hydrolysates. The peak areas of the starting material and the hydrolysates were compared with those of standard samples. To identify the hydrolysates, each peak was collected, analyzed for amino acid composition, identified and confirmed by coelution with an authentic sample on the HPLC. RP-HPLC analysis was performed as follows: columns; a YMC-ODS-5-AM (4.6 × 150 mm) or a Puresil™ C_{18} (4.6 × 250 mm); elution, 0—21.6% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. The HPLC analyses of acid hydrolysates of each peptide were carried out in 4—6 experiments. The average values varied within ±2%.

References and Notes

1) Amino acids and their derivatives except glycine mentioned in this paper are of L-configuration unless otherwise indicated. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IBU Commission on Biochemical Nomenclature in *Eur. J. Biochem.*, **138**, 9—37 (1984). Other abbreviations used are: Z, carbobenzoxy; Boc, *tert*-butoxycarbonyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; HOBt, 1-hydroxybenzotriazole; DMF, *N*,*N*-dimethylformamide; TEA, triethylamine; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; MeCN, acetonitrile; AcOH, acetic acid; TFA, trifluoroacetic acid; EtOH, ethyl alcohol; MeOH, methyl alcohol; BuOH, butyl alcohol; AcOEt, ethyl acetate; RP-HPLC, reversed-phase high-performance liquid chromatography; HP-TLC, high-performance thin-layer chromatography.
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