Hydrolytic Cleavage of Pyroglutamyl-Peptide Bond. III. A Highly Selective Cleavage in 70% Methanesulfonic Acid[1,2]

Tadashi HASHIMOTO,* Kazuhiro OHKI, and Naoki SAKURA

Faculty of Pharmaceutical Sciences, Hokuriku University, Kanagawa-machi, Kanazawa 920–11, Japan.
Received May 31, 1996; accepted June 27, 1996

A method for highly selective cleavage of pGlu-peptide linkages in 70% methanesulfonic acid (MSA) is described. When pGlu–Ala–Phe–OH, pGlu–His–Pro–OH and dog neuromedin U-8 (d-NMU-8) (1—7)-OH (pGlu–Phe–Leu–Phe–Arg–Pro–Arg–OH) were hydrolyzed in 70% MSA at 60 °C for 3 d or at 25 °C for 3 d, the pGlu-peptide linkage was predominantly cleaved to give H–Ala–Phe–OH, H–His–Pro–OH and H–Phe–Leu–Phe–Arg–Pro–Arg–OH, in high yields. The results indicated that pGlu-peptide linkages are highly susceptible to 70% MSA, whereas the amide bond of the pyrrolidone moiety of the pGlu residue and other internal peptide bonds are extremely resistant.

A number of native proteins and peptides have been shown to possess a pGlu residue at the N-terminal, which is thought to afford protection against aminopeptidase digestion. Our recent studies[1,3,4] have revealed that pGlu-peptide is sensitive to acid under mild conditions, generating not only the ring-opened product of the pyrrolidone moiety of the pGlu residue, but also the cleavage product of the pGlu-peptide linkage. To develope a method for highly selective cleavage of pGlu-peptide linkages, we studied the stability and susceptibility of pGlu-peptides to aqueous methanesulfonic acid (MSA) at 25 and 60 °C. This paper describes the susceptibility of the pGlu-peptide linkage and the resistance of the amide of the pyrrolidone moiety of the pGlu residue, as well as internal peptide linkages, to aqueous MSA.

Results and Discussion

To develop a simple means of deblocking pGlu-peptides prior to Edman degradation, we examined the susceptibility of the pGlu-peptide bond and the resistance of the pyrrolidone moiety of the pGlu residue, as well as the internal peptide bonds, of pGlu–Ala–Phe–OH, a thyrotropin releasing hormone[5] fragment, pGlu–His–Pro–OH, and dog neuromedin U-8[6] (d-NMU-8) (1—7)-OH (pGlu–Phe–Leu–Phe–Arg–Pro–Arg–OH)[7] to several concentrations of MSA (40—90%) at 60 °C for 90 min (Fig. 1A—C).

The results indicated that the higher the concentration of MSA during hydrolysis, the lower the yield of the ring-opened products, H–Glu–Ala–Phe–OH, H–Glu–His–Pro–OH and H–Glu–Phe–Leu–Phe–Arg–Pro–Arg–OH, respectively. The yields of the cleavage products (H–Ala–Phe–OH, H–His–Pro–OH and H–Phe–Leu–Phe–Arg–Pro–Arg–OH) were highest after hydrolysis in 70% MSA. As the concentration of MSA increased, the rate of the hydrolysis reaction gradually decreased. However, several cleavage products of the internal peptide bonds of d-NMU-8 (1—7)-OH in higher concentration of MSA were evident on HPLC. The optimal concentration of MSA at which to cleave selectively and rapidly the pGlu-peptide linkage seemed to 70%.

Thus, we examined the acid hydrolysis of pGlu–Ala–Phe–OH, pGlu–His–Pro–OH, and d-NMU-8 (1—7)-OH, pGlu–Phe–Leu–Phe–Arg–Pro–Arg–OH, in 70% MSA at 60 °C for 3 h and at 25 °C for 3 d.

When a solution of pGlu–Ala–Phe–OH (10^{-3} mol/l in 70% MSA) was allowed to stand at 60 °C for 3 h, decomposition resulted in the formation of one major and two minor hydrolysates (Fig. 2A). The three peaks and starting material peak on HPLC were quantified and isolated by HPLC, and assigned on the basis of amino acid analysis and direct comparison with authentic samples on RP-HPLC. About 9% of the starting material was left,
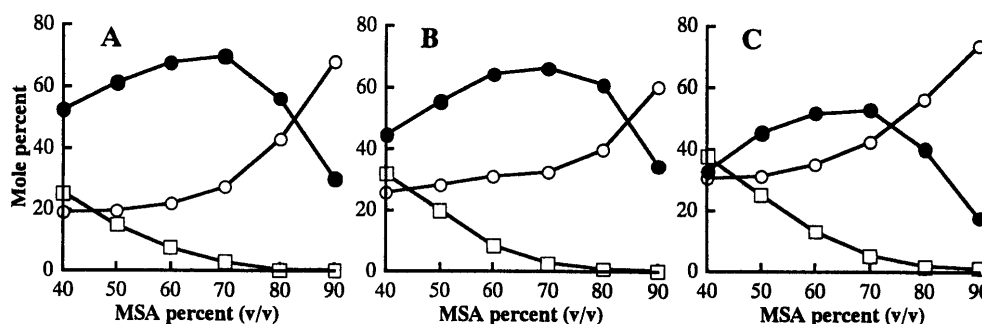

Fig. 1. Relationship between the Concentration of Aqueous Methanesulfonic Acid and Those of pGlu-Peptide [A=pGlu–Ala–Phe–OH, B=pGlu–His–Pro–OH, C=d-NMU-8 (1—7)-OH] and Its Hydrolysates after Incubation at 60 °C for 1.5 h

A: ○, pGlu–Ala–Phe–OH; ●, H–Ala–Phe–OH; □, H–Glu–Ala–Phe–OH. B: ○, pGlu–His–Pro–OH; ●, H–His–Pro–OH; □, H–Glu–His–Pro–OH. C: ○, d-NMU-8 (1—7)-OH (pGlu–Phe–Leu–Phe–Arg–Pro–Arg–OH); ●, NMU-8 (2—7)-OH (H–Phe–Leu–Phe–Arg–Pro–Arg–OH); □, [Glu^1]-NMU-8 (1—7)-OH (H–Glu–Phe–Leu–Phe–Arg–Pro–Arg–OH).

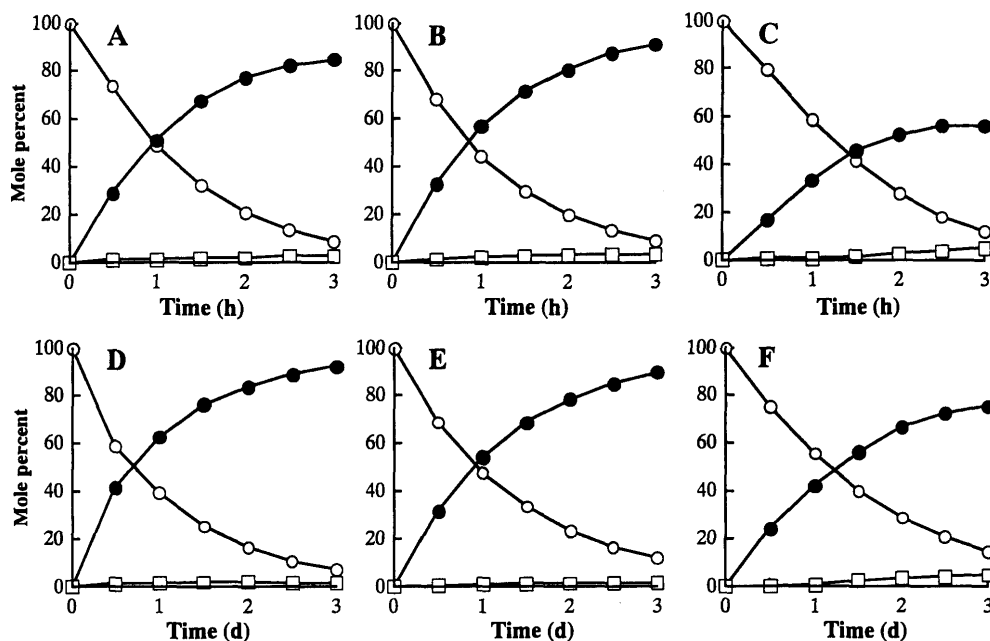* To whom correspondence should be addressed.

Fig. 2. Time Courses of pGlu–Ala–Phe–OH (A, D), pGlu–His–Pro–OH (B, E) and d-NMU-8 (1—7)-OH (C, F) and Their Hydrolysates after Incubation in 70% MSA at 60 °C for 3 h (A—C) and at 25 °C for 3 d (D—F)

A, D: ○, pGlu–Ala–Phe–OH; ●, H–Ala–Phe–OH; □, H–Glu–Ala–Phe–OH. B, E: ○, pGlu–His–Pro–OH; ●, H–His–Pro–OH; □, H–Glu–His–Pro–OH. C, F: ○, d-NMU-8 (1—7)-OH (pGlu–Phe–Leu–Phe–Arg–Pro–Arg–OH); ●, NMU-8 (2—7)-OH (H–Phe–Leu–Phe–Arg–Pro–Arg–OH); □, [Glu^1]-NMU-8 (1—7)-OH (H–Glu–Phe–Leu–Phe–Arg–Pro–Arg–OH).

and the main reaction was the cleavage of the pGlu–Ala linkage, resulting in the production of H–Ala–Phe–OH (85%). A side reaction generated the ring-opened product, H–Glu–Ala–Phe–OH (3%), of the pyrrolidone moiety and the cleavage product, H–Phe–OH (6%), of the Ala–Phe linkage. On incubating this peptide at 25 °C for 3 d, the hydrolysate contained H–Ala–Phe–OH (92%), H–Glu–Ala–Phe–OH (2%) and H–Phe–OH (3%) (Fig. 2D). The accumulation curves (Fig. 2A, D) of the acid hydrolysates showed that the cleavage product predominated over the ring-opened product at a molar ratio of about 31 : 1 at 60 °C for 3 h and about 61 : 1 at 25 °C for 3 d. The results indicated that the pGlu–Ala linkage was highly labile to 70% MSA hydrolysis at 60 and 25 °C, whereas the amide group of the pyrrolidone moiety and the internal peptide bond were resistant. The results also showed that the acid hydrolysis at the low temperature was superior to that at 60 °C for the highly selective cleavage of the pGlu-peptide bond.

The acid hydrolyses[8] of pGlu–Ala–Phe–OH in 1 N HCl at 60 °C for 6 h and at 4 °C for 10 weeks produced two major hydrolysates, H–Ala–Phe–OH (35 and 40%) and H–Glu–Ala–Phe–OH (41 and 19%). The ring-opening reaction of the pyrrolidone moiety predominated over the cleavage of the pGlu–Ala bond at 60 °C. 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. These results indicated that acid hydrolysis in 70% MSA at 25 °C affords a highly selective cleavage of the pGlu-peptide linkage.

The acid hydrolysis of pGlu–His–Pro–OH at 60 and 25 °C for 3 h and 3 d gave similar results. The acid hydrolysates contained the cleavage product, H–His–Pro–OH (91 and 90%) and the ring-opened product, H–Glu–His–Pro–OH (4 and 1%) (Fig. 2B, 2E), respectively. Acid

hydrolyses[3] of pGlu–His–Pro–OH in 1 N HCl at 60 and 4 °C for 6 h and 10 weeks produced H–His–Pro–OH (21 and 16%) and H–Glu–His–Pro–OH (45 and 15%) as major products, respectively. The ring-opening reaction of the pyrrolidone moiety and the cleavage reaction of the pGlu–His linkage proceeded to approximately the same extent in 1 N HCl even at the lower temperature. In contrast to these results, the pGlu–His linkage was highly labile to 70% MSA hydrolysis, and a highly selective, rapid cleavage reaction occurred at the pGlu–His linkage in preference to the amide function of the pyrrolidone moiety.

Next, the degradation of d-NMU-8 (1—7)-OH and the accumulation of its hydrolysates were investigated under the same conditions. Within 3 h at 60 °C, 88% of the starting material was hydrolyzed (Fig. 2C). The main product was NMU-8 (2—7)-OH (H–Phe–Leu–Phe–Arg–Pro–Arg–OH) (56%), and the yield of the ring-opened product, [Glu^1]-NMU-8(1—7)-OH (H–Glu–Phe–Leu–Phe–Arg–Pro–Arg–OH) was 5%. There was considerable cleavage at internal peptide bonds in d-NMU-8 (1—7)-OH, resulting in low yields of the cleavage and ring-opened products. The acid hydrolysis of d-NMU-8 (1—7)-OH at 25 °C for 3 d gave NMU-8 (2—7)-OH (76%) and [Glu^1]-NMU-8 (1—7)-OH (5%), and 15% of the starting material remained intact (Fig. 2F). No cleavage at internal peptide bonds was apparent on HPLC.

As reported,[3] d-NMU-8 (1—7)-OH was hydrolyzed to the extent of 32% in 1 N HCl at 60 °C for 6 h and 48% in 1 N HCl at 4 °C for ten weeks, to give [Glu^1]-NMU-8 (1—7)-OH (42 and 23.6%) and NMU-8 (2—7)-OH (16 and 19%), respectively. The amount of [Glu^1]-NMU-8 (1—7)-OH formed was greater than that of NMU-8 (2—7)-OH in mole percent under both conditions. Our study[4] also revealed that the degradation of d-NMU-8

(1—7)-OH in concentrated HCl at 0 °C for 12 d gave NMU-8 (2—7)-OH (52%) as the main product and [Glu^1]-NMU-8 (1—7)-OH (2%) as a by-product, and that 63.4% of the starting material was hydrolyzed. These results indicated that the acid hydrolysis of the longer peptide fragment in concentrated HCl at low temperature is superior to that in 70% MSA for the highly selective cleavage of the pGlu-peptide bond. However, the former takes a long time and the latter is more practical.

This study has revealed that the pGlu-peptide linkage is highly susceptible to 70% MSA compared with other peptide bonds and the amide bond of the pyrrolidone moiety, and is selectively hydrolyzed at 25 °C to give predominantly the cleavage product of the pGlu-peptide linkage. These results could be helpful in the development of a simple means of deblocking pGlu-peptide prior to Edman degradation.

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 reversed-phase (RP)-HPLC was performed on an apparatus equipped with a 590 pump (Waters, U.S.A.), a 510 pump (Waters), a model 680 gradient controller (Waters), a Rheodyne 7125 injector (Rheodyne Inc., U.S.A.), a UV 8011 detector (Tosoh Co., Japan) and a Data Module 741 (Waters). Analytical RP-HPLC was accomplished on a system comprising a 616 pump (Waters), a Rheodyne 7725i injector (Rheodyne), a 486 Tunable Absorbance Detector (Waters), a 600S Controller (Waters) and data module, Millennium 2010J Chromatography Manager (Waters), and an SDM Degasser (Waters). 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.[9] All amino acids except Z-pyroglutamic acid were protected as α-Boc derivatives. N^x-Benzyloxymethyl (Bom) was used to protect 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[10] (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[11] 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 showed one 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$_{18}$ 5-100A or YMC-pack D-ODS-5-ST S-5 120A (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.

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^{TM} 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 μg) 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.

When single peaks on HPLC and single spots on HP-TLC in two solvent 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.

pGlu-Ala-Phe-OH TLC: Rf (n-BuOH : pyridine : AcOH : H$_2$O = 30 : 20 : 6 : 24) 0.40, Rf (n-BuOH : AcOEt : AcOH : H$_2$O = 1 : 1 : 1 : 1) 0.50. $[\alpha]_D^{27}$ −25.8° (c = 0.5, 2 M AcOH). Amino acid analysis: Glu 1.10 (1), Ala 1.02 (1), Phe 1.00 (1). FAB-MS m/z: 348 [M + H]$^+$.

H-Glu-Ala-Phe-OH TLC: Rf (n-BuOH : pyridine : AcOH : H$_2$O = 30 : 20 : 6 : 24) 0.22, Rf (n-BuOH : AcOEt : AcOH : H$_2$O = 1 : 1 : 1 : 1) 0.42. $[\alpha]_D^{27}$ +4.3° (c = 0.5, 2 M AcOH). Amino acid analysis: Glu 1.09 (1), Ala 0.98 (1), Phe 1.00 (1). FAB-MS m/z: 366 [M + H]$^+$.

HPLC Analysis of the Acid Hydrolysate To analyze acid hydrolysates, a solution of a peptide at a concentration of 10^{-3} mol/l in 40—90% (v/v) MSA was prepared in a polypropylene tube (2 ml) under ice-cooling and divided into seven to twelve aliquots (100 μl each) in polypropylene tubes (2 ml) with tight caps. These were maintained at an appropriate temperature in a thermostated apparatus. Each tube was removed from the apparatus at 0 h or an appropriate time, and stored at −40 °C until analyzed. An aliquot (15 μl) of this solution was examined by RP-HPLC to determine the amount of the starting material that remained and the amounts of the hydrolysates. To identify the hydrolysates, each peak was collected, analyzed for amino acid composition, identified and confirmed by coelution with an authentic sample on HPLC. The peak areas of the starting material and the hydrolysates were compared with those of standard samples. RP-HPLC analysis was performed as follows: column; a YMC-ODS-5-AM (4.6 × 150 mm) or a Puresil^{TM} C$_{18}$ (4.6 × 250 mm); elution, 0—22.4% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. The acid hydrolysates of each peptide were examined by HPLC in triplicate. The average values varied by ±2.8%.

References and Notes

1) Part II. Saito S., Ohki K., Sakura N., Hashimoto T., *Biol. Pharm. Bull.*, **19**, 768—770 (1996).
2) Amino acids and their derivatives mentioned in this paper are of L-configuraiton unless otherwise indicated. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IUB Commission on Biochemical Nomenclature in *Eur. J. Biochem.*, **138**, 9—37 (1984). Other abbreviations used are: BHA, benzhydrylamine; Boc, *tert*-butoxycarbonyl; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluorophosphate; DCC, N,N'-dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DMF, N,N-dimethylformamide; TEA, triethylamime; Z, carbobenzoxyl; DCM, dichloromethane; MeCN, acetonitrile; AcOH, acetic acid; TFA, trifluoroacetic acid; RP-HPLC, reversed-phase high-performance liquid chromatography; HP-TLC, high-performance thin-layer chromatography.
3) Hashimoto T., Ohki K., Sakura N., *Chem. Pharm. Bull.*, **43**, 2068—2074 (1995).
4) Hashimoto T., Saito S., Ohki K., Sakura N., *Chem. Pharm. Bull.*, **44**, 877—879 (1996).
5) Burgus R., Dunn T. F., Desiderio M., Guillemin R., *C. R. Acad. Sci.*, D **269**, 1870—1873 (1969); Schally A. V., Redding T. W., Bowers C. Y., Barrett J. F., *J. Biol. Chem.*, **244**, 4077—4088 (1969).

6) O'Harte F., Bockman C. S., Abel P. W., Conlon J. M., *Peptides*, **12**, 11—15 (1991).

7) Sakura N., Kurosawa K., Hashimoto T., *Chem. Pharm. Bull.*, **43**, 1148—1153 (1995).

8) Ohki K., Sakura N., Hashimoto T., "Peptide Chemistry 1994," ed. by Ohno M., Protein Research Foundation, Osaka, 1995, pp. 185—188.

9) Stewart J., Young J., "Solid Phase Peptide Synthesis," Pierce Chem. Co., Rockford, U.S.A., 1984.

10) Castro B., Dormoy J.R., Evin G., Selve C., *Tetrahedron Lett.*, **14**, 1219—1222 (1975).

11) Kaiser E., Colescott R. L., Bossinger C. S., Cook P. I., *Anal. Biochem.*, **34**, 595—598 (1970).