Hydrolytic Cleavage of Pyroglutamyl-Peptide Bond. IV. Highly Selective Cleavage of Thyrotropin Releasing Hormone (TRH) in Aqueous Methanesulfonic Acid[1,2)]

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The acid hydrolysis of thyrotropin releasing hormone (TRH) (pGlu–His–Pro–NH_2) in various acids under several conditions and the highly selective cleavage of the pGlu–His bond are described. First, the decomposition of TRH in dilute acid was studied. The incubation of TRH in 1 N HCl at 60 °C resulted in the formation of eleven hydrolysates, H–Glu–His–Pro–NH_2, H–Glu–His–Pro–OH, H–His–Pro–NH_2, H–His–Pro–OH, His–Pro diketopiperazine, pGlu–His–Pro–OH, pGlu–His–OH, H–Glu–His–OH, H–Pro–His–OH, pGlu–OH and H–Glu–OH. The results showed that the pGlu–His linkage and pGlu moiety were more sensitive than the C-terminal carboxamide to the acid hydrolysis. Then, a simple means of deblocking pGlu-peptide was applied to TRH. The hydrolysis of TRH in 70% methanesulfonic acid (MSA) at 25 °C produced H–His–Pro–NH_2 and H–His–Pro–OH as major products, and H–Glu–His–Pro–NH_2, the diketopiperazine and pGlu–His–Pro–OH as minor products. The 90% MSA hydrolysis at 25 °C was highly selective for the cleavage product (H–His–Pro–NH_2) of the pGlu–His bond, without affording the ring-opened products (H–Glu–His–Pro–NH_2 and H–Glu–His–Pro–OH). The yield of deamidated products (H–His–Pro–OH and pGlu–His–Pro–OH) was less than 3% after 3 d.

Key words thyrotropin releasing hormone; pGlu–His bond; selective cleavage; dilute HCl; aqueous methanesulfonic acid

Our studies[1,3–5)] have revealed that pGlu-peptides are highly sensitive to acid under mild conditions and that they are hydrolyzed to complex hydrolysates which involve not only the ring-opened product[6)] of the pyrrolidone moiety of the pGlu residue, but also the cleavage product of the pGlu-peptide linkage. We also developed a simple means of deblocking pGlu-peptide, that is, a highly selective cleavage of the pGlu-peptide bond, prior to Edman degradation. The degradation of thyrotropin releasing hormone[7)] (TRH) (pGlu–His–Pro–NH_2) and its metabolites, such as pGlu–His–Pro–OH, H–His–Pro–NH_2 and H–His–Pro–OH, by enzymes endogenous to body fluids and tissues has been investigated in depth.[8)] However, the stability and susceptibility of TRH to acid and the selective cleavage of the pGlu–His linkage have not been reported. We studied the susceptibility of TRH to 1 N HCl at 25 and 60 °C, and to aqueous methanesulfonic acid (MSA) at 25 °C, and analyzed the hydrolysates by RP-HPLC. This paper describes the susceptibility of the pGlu moiety, the internal peptide linkages and the C-terminal α-carboxamide functional group of TRH to 1 N HCl and aqueous MSA, and the development and application of a highly selective means of deblocking the pGlu-residue.

Results and Discussion

We found that the hydrolysis[3)] of the TRH fragment pGlu–His–Pro–OH[9)] in 1 N HCl at 60 °C produced a complicated mixture of hydrolysates, including H–Glu–His–Pro–OH[7)] (44.6%), H–His–Pro–OH[3)] (20.7%), pGlu–OH (14.7%), His–Pro diketopiperazine[10)] (4.5%), pGlu–His–OH[11)] (2.8%), H–Glu–His–OH[12)] (1.9%) and H–Pro–His–OH[10)] (0.4%) during 6 h incubation, and the acid hydrolysis of TRH, owing to the presence of α-carboxamide, is expected to give even more complex hydrolysates. Therefore, prior to application of our simple means of deblocking pGlu-peptide to TRH, we investigated the acid decomposition of TRH in order to perform a highly selective cleavage of the pGlu–His linkage and to minimize the side reactions at the internal peptide bonds, and especially the deamidation of the α-carboxamide.

We first examined the stability of TRH in 1 N HCl at 25 °C. When a solution of pGlu–His–Pro–NH_2 at a concentration of 10^{-3} mol/l in 1 N HCl was allowed to stand for 1 h, decomposition occurred, resulting in the formation of the ring-opened product, H–Glu–His–Pro–NH_2, of the pyrrolidone moiety and the pGlu–His bond cleavage product, H–His–Pro–NH_2,[13)] which were detected as small peaks on HPLC (Fig. 1A). The deamidation product, pGlu–His–Pro–OH also appeared after 6 h. His–Pro diketopiperazine (Fig. 1A, 1B) was detected in the hydrolysate by RP-HPLC after 12 h. The results suggested that the N-terminal portion of TRH is labile to the acid.

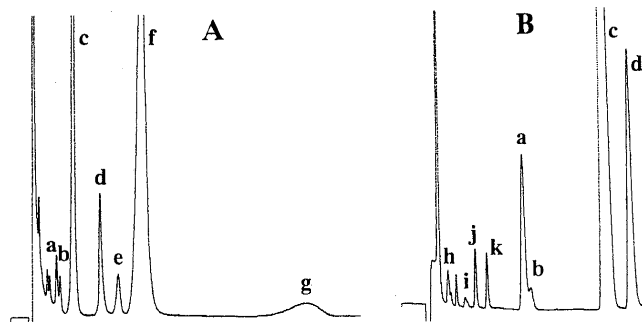

Fig. 1. HPLC Profiles of an Incubation Mixture of TRH in 1 N HCl at 60 °C for 3 h (A) and 6 h (B)

HPLC conditions were as follows. A: column, YMC-ODS-5-AM (4.6 × 150 mm); elution, 0.1% TFA; detection, 210 nm. a, H–His–Pro–OH [retention time (t_R), 4.9 min]; b, H–His–Pro–NH_2 (t_R, 5.3 min); c, H–Glu–His–Pro–NH_2 (t_R, 6.8 min); d, His–Pro diketopiperazine (t_R, 9.8 min); e, H–Glu–His–Pro–OH (t_R, 11.8 min); f, TRH (t_R, 14.5 min); g, pGlu–His–Pro–OH (t_R, 32.7 min). B: column, Puresil™ C_{18} (4.6 × 250 mm); elution, 0.1% TFA; detection, 210 nm. h, H–Glu–His–OH (t_R, 5.1 min); i, H–Pro–His–OH (t_R, 6.9 min); j, pGlu–His–OH (t_R, 8.1 min); k, pGlu–OH (t_R, 9.3 min).

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Table 1. pGlu–His–Pro–NH_2 and Its Hydrolysates during Incubation in 1 N HCl at 60 °C

Peptide	Time (h)						
	0	1	2	3	4	5	6
				Mol percent			
pGlu–His–Pro–NH_2	100	78.1	58.7	42.4	31.9	23.6	18.1
H–Glu–His–Pro–NH_2	0	13.7	25.5	32.9	37.6	41.2	43.7
H–Glu–His–Pro–OH	0	1.1	2.0	4.7	7.1	9.0	10.1
H–His–Pro–NH_2	0	3.7	3.5	2.5	1.7	1.4	1.0
H–His–Pro–OH	0	0.4	1.3	2.4	3.7	4.9	6.1
His–Pro diketopiperazine	0	2.4	7.3	10.6	12.9	14.6	15.5
pGlu–His–Pro–OH	0	2.9	4.7	6.6	6.9	6.9	7.2
pGlu–His–OH[a]	0	1.0	1.8	2.1	2.5	2.7	2.9
H–Glu–His–OH[a]	0	0.2	0.3	0.6	1.1	1.7	2.2
H–Pro–His–OH[a]	0	0.1	0.2	0.3	0.4	0.6	0.8
pGlu–OH[a]	0	3.5	7.0	10.2	12.2	13.9	14.8
H–Glu–OH	0	0.0	0.0	1.5	2.9	3.2	5.4

HPLC conditions: column, YMC-ODS-5-AM (4.6 × 150 mm); elution, 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. *a*) Column, Puresil™ C_{18} (4.6 × 250 mm); elution, 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. The values have an accuracy of about ±1.2%.

Table 2. pGlu–His–Pro–NH_2 and Its Hydrolysates during Incubation in 70% (v/v) Methanesulfonic Acid at 25 °C

Peptide	Time (h)						
	0	12	24	36	48	60	72
				Mol percent			
pGlu–His–Pro–NH_2	100	74.2	54.0	39.0	29.5	22.7	18.4
H–His–Pro–OH	0	2.0	5.2	8.8	11.7	16.2	20.5
H–His–Pro–NH_2	0	23.4	38.0	46.3	47.9	47.8	46.3
H–Glu–His–Pro–NH_2	0	0.5	0.7	1.4	1.9	1.7	2.3
His–Pro diketopiperazine	0	0.9	1.5	2.8	4.7	6.4	7.1
pGlu–His–Pro–OH[a]	0	1.7	2.0	3.1	5.5	4.5	3.0

HPLC conditions: column, Puresil™ C_{18} (4.6 × 250 mm); elution, 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. *a*) Elution, 2.4% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. The values have an accuracy of about ±1.3%.

To analyze the decomposition rate of the starting material and the accumulation rates of the hydrolysates, pGlu–His–Pro–NH_2 in 1 N HCl was incubated at 60 °C for 6 h. The hydrolysates consisted of H–Glu–His–Pro–NH_2, H–Glu–His–Pro–OH, H–His–Pro–NH_2, H–His–Pro–OH, His–Pro diketopiperazine, pGlu–His–Pro–OH, pGlu–His–OH, H–Glu–His–OH, H–Pro–His–OH, pGlu–OH and H–Glu–OH (Table 1). The last was directly determined on an amino acid analyzer. Each peak was assigned on the basis of amino acid analysis and by direct comparison with an authentic sample on HPLC. These peaks were confirmed by coelution with authentic samples on HPLC. After hydrolysis for 6 h, 18.1% of the starting material remained intact. The half-life ($t_{1/2}$) of the hydrolysis was 2.4 h. The results indicated that the ring-opened products, H–Glu–His–Pro–NH_2 and H–Glu–His–Pro–OH, predominated over the pGlu–His bond cleavage products, H–His–Pro–NH_2, H–His–Pro–OH and His–Pro diketopiperazine, with a molar ratio of about 2.4 : 1. The major hydrolysate was the ring-opened product, H–Glu–His–Pro–NH_2, in which the α-carboxamide was intact.

Our recent study[1] revealed that the hydrolysis of pGlu-peptide in 70% MSA produced highly selective cleavage, with a negligible amount of the ring-opened product.

Thus, we examined the acid hydrolysis of TRH in 70% MSA. The hydrolysis of TRH at 25 °C for 3 d produced H–His–Pro–NH_2 (46.3%), H–His–Pro–OH (20.5%), H–Glu–His–Pro–NH_2 (2.3%), His–Pro diketopiperazine (7.1%) and pGlu–His–Pro–OH (3.0%) (Table 2). About 18% of the starting material was left in the hydrolysate solution. The cleavage products (total 73.9%), H–His–Pro–NH_2, H–His–Pro–OH and His–Pro diketopiperazine, greatly predominated over the ring-opened product, H–Glu–His–Pro–NH_2. However, considerable deamidation of the C-terminal occurred to give about 23% yield in total of H–His–Pro–OH and pGlu–His–Pro–OH. The secondary amide of the pyrrolidone was more labile than the primary amide at the C-terminal in 1 N HCl at 25 and 60 °C, whereas the former was more stable than the latter in 70% MSA at 25 °C. The acid hydrolysates of pGlu–His–Pro–OH in 70% MSA at 60 and 25 °C for 3 h and 3 d contained the cleavage product, H–His–Pro–OH (91% and 90%) and the ring-opened product, H–Glu–His–Pro–OH (4% and 1%, respectively).[1] The yield ratio of the cleavage product to the ring-opened product of pGlu–His–Pro–OH was higher than that of TRH under the same conditions.

To minimize the deamidation in TRH hydrolysis, we re-examined the susceptibility of the amide of H–Glu–His–Pro–NH_2 to several concentrations (40%—90%) of MSA at 60 °C for 90 min (data not shown). The results indicated that the higher the concentration of MSA during hydrolysis, the lower the yield of the deamidation product,

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Table 3. pGlu–His–Pro–NH_2 and Its Hydrolysates during Incubation in 90% (v/v) Methanesulfonic Acid at 25 °C

Peptide	Time (h)						
	0	12	24	36	48	60	72
	Mol percent						
pGlu–His–Pro–NH_2	100	90.2	83.9	76.3	67.8	61.0	56.3
H–His–Pro–OH	0	0.4	0.5	0.8	0.9	1.1	1.4
H–His–Pro–NH_2	0	11.4	16.5	24.0	28.1	33.0	37.2
H–Glu–His–Pro–NH_2	0	0.1	0.1	0.2	0.4	0.4	0.6
His–Pro diketopiperazine	0	0.1	0.2	0.3	0.8	0.6	0.6
pGlu–His–Pro–OH[a]	0	0.6	1.2	1.6	2.2	2.3	1.5

HPLC conditions: column, Puresil™ C_{18} (4.6 × 250 mm); elution, 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. *a)* Elution, 2.4% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. The values have an accuracy of about ±2.1%.

H–Glu–His–Pro–OH.

Thus, we examined the selective hydrolysis of TRH in higher concentration of MSA. When a solution of TRH in 90% MSA was allowed to stand at 25 °C for 3 d, the desired product, H–His–Pro–NH_2 (37.2%), was obtained as the major product (Table 3). About 56% of TRH remained intact. During the hydrolysis, no ring-opened product, H–Glu–His–Pro–NH_2, was detectable by HPLC. The total yield of the deamidated products, H–His–Pro–OH (1.4%) and pGlu–His–Pro–OH (1.5%), was less than 3% after 3 d.

The 90% MSA hydrolysis of the native bioactive peptide, TRH, selectively gave the cleavage product (H–His—Pro–NH_2) of the pGlu–His bond without significant cleavage of the amide bond of the pyrrolidone ring, the His–Pro bond or the C-terminal α-carboxamide. The present results should contribute to the development of a simple means of deblocking pGlu-peptides 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 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 Waters 741 data module (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 Millennium 2010J chromatography manager data module (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, TRH and benzhydrylamine (BHA) resin · HCl (1% divinylbenzene copolymer, 100—200 mesh, amino content; 0.5 meq/g) 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.[14] All amino acids except Z-pyroglutamic acid were protected as α-Boc derivatives. The protecting group for the amino acid side chain was N^{π}-benzyloxymethyl (Bom) for the imidazole ring of His. Solid-phase peptide synthesis was performed starting from Boc-amino acid Merrifield resin or BHA resin · HCl. The elongation of the peptide chain was carried out using Boc-amino acids (2.5 eq) in *N*-methylpyrrolidone with benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate[15] (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 *N*,*N*-dimethylformamide (DMF). Incorporation of an amino acid was repeated until the Kaiser ninhydrin test[16] 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} 5-100A 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™ 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), 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 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.

H-Glu-His-Pro-NH_2 TLC: *Rf* (*n*-BuOH : pyridine : AcOH : H_2O = 30 : 20 : 6 : 24) 0.15, *Rf* (*n*-BuOH : AcOEt : AcOH : H_2O = 1 : 1 : 1 : 1) 0.18. $[\alpha]_D^{27}$ −26.3° (*c* = 0.5, 2 M AcOH). Amino acid analysis: Glu 1.00 (1), His 0.93 (1), Pro 0.95 (1), NH_3 1.10 (1). FAB-MS *m/z*: 381 $[M+H]^+$.

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 caps. These were maintained at an appropriate temperature in a thermostated apparatus. Each tube was taken 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 the amount of the starting material that remained and the amounts of

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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 co-HPLC with an authentic sample. RP-HPLC analysis was performed as follows: columns; YMC-ODS-5-AM (4.6×150 mm) or Puresil™ C_{18} (4.6×250 mm); elution, 0—2.4% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm. The HPLC analyses of acid hydrolysates of each peptide were repeated 4—6 times. The average values varied within ±2.1%.

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References and Notes

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