Measurement of Tryptophan in Peptides by Acid Hydrolysis in the Presence of Phenol and its Application to the Amino Acid Sequence of a Sea Anemone Toxin

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The addition of phenol (about 1%) to 6 M HCl largely prevented destruction of tryptophan during hydrolysis of peptides at 110°C for 22 hr. Tryptophan recovery depended on the volume of 6 M HCl containing phenol and the concentration of phenol. The maximum tryptophan recovery was 85% for a standard amino acid mixture. The recovery was slightly lower for proteins. This hydrolytic procedure was advantageous for micro amino acid analysis using a conventional high-performance liquid chromatography with a precolumn labeling technique. The method was used in the amino acid sequence analysis of a minor component of sea anemone toxins isolated from *Anthopleura fuscoviridis*. The toxin consisted of 48 amino acid residues with three tryptophan residues.

Tryptophan in peptides and proteins is destroyed during conventional hydrolysis with 6 M HCl at 110°C for 22~24 hr. Various methods for tryptophan measurement have been reported including the addition of thioglycolic acid to 6 M HCl^{1,2)} and hydrolysis using *p*-toluenesulfonic acid,³⁾ 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole⁴⁾ or 3 M mercaptoethanesulfonic acid^{5,6)} instead of 6 M HCl. Although these methods are successful in total amino acid analysis by ion-exchange chromatography on an automatic amino acid analyzer, the nonvolatility of the solvents makes these methods difficult to use in the newly developed micro amino acid analysis using conventional high-performance liquid chromatography (HPLC) with precolumn labeling,⁷⁾ because the pH of the hydrolysate must be adjusted before derivatization which dilutes the hydrolysate.

In this paper we show that the addition of phenol (about 1%, w/v) to 6 M HCl largely

prevented the destruction of tryptophan during hydrolysis. The acid solvents were evaporated under a vacuum, therefore, the dried hydrolysate could be directly labeled, keeping the reaction mixture to a minimum volume. This hydrolytic procedure was used on the amino acid sequence of a minor component of sea anemone toxins isolated from *Anthopleura fuscoviridis*. Recovery of the 1-dimethylaminonaphthalene-5-sulfonyl chloride (DNS-Cl) derivative of tryptophan from the acid hydrolysis was also described.

MATERIALS AND METHODS

Chemicals. Concentrated HCl (analytical grade), phenol, and a standard amino acid mixture were purchased from Wako Pure Chemicals. Lysozyme (chicken egg white) was obtained from Millipore. 4-Dimethylaminoazobenzene-4'-sulfonyl chloride (DABS-Cl) was from Dojin Chemical. DNS-Cl was from Pierce Chemical. All other chemicals and solvents used were of analytical grade.

Abbreviations: HPLC, high-performance liquid chromatography; DNS-Cl, 1-dimethylaminonaphthalene-5-sulfonyl chloride; DABS-Cl, 4-dimethylaminoazobenzene-4'-sulfonyl chloride; AFT, sea anemone toxin from *Anthopleura fuscoviridis*; TFA, trifluoroacetic acid; CAM, S-carboxamidomethyl; FITC, fluoresceinisothiocyanate; FTH, fluoresceinthiohydantoin.

6 M HCl containing phenol. Six M HCl containing phenol was prepared by double distillation of a mixture of 80 ml of concentrated HCl, 80 ml of distilled water, and 1.6 ml of phenol. The constant boiling fraction was taken, divided into screw capped test tubes, and stored at 4°C. The concentration of phenol in the constant boiling HCl was estimated to be 0.7% by ultraviolet absorbance at 290 nm. The 6 M HCl containing 0.7% phenol was used in this experiment unless otherwise mentioned. Six M HCl containing 2.8% phenol was prepared by double distillation of 6 M HCl containing 4% phenol.

Acid hydrolysis. An acid-washed glass tube with an outer diameter of 5 mm and an inner diameter of 3 mm was flamed to form a bottle 50~60 mm long. Sample solution was placed in the bottom of the tube and then dried *in vacuo*. Six M HCl was added and the tube was evacuated for 10~20 sec and sealed. After hydrolysis the tube was opened and the hydrolysate was dried in a vacuum desiccator warmed at 50°C and then derivatized with DABS-chloride.^{7,16} The dried hydrolysate was dissolved in 10 μ l of 0.1 M sodium bicarbonate buffer (pH 9.0), and 20 μ l of DABS-C1 solution (4 mM in acetone) was added. The tube was heated at 55°C for 20 min with occasional shaking. After derivatization, the sample was diluted with 10 mM sodium phosphate buffer (pH 6.5) to 100 μ l, and 10 μ l of the diluted sample was injected for HPLC analysis after centrifugation.

Hydrolysis of DNS amino acids. Ten microliters of a standard amino acid mixture (500 pmol each) in 0.1 M sodium carbonate buffer (pH 9.0) was mixed with 10 μ l of DNS-C1 solution (7.4 mM in acetone) and heated at 50°C for 15 min with occasional shaking. The reaction mixture was vacuum evaporated and treated with 80 μ l of 6 M HCl containing phenol at 110°C under vacuum. After drying *in vacuo*, the residue was dissolved in 20 μ l of 50% acetone in 10 mM sodium phosphate buffer (pH 7.0) and diluted with 80 μ l of 10 mM sodium phosphate buffer (pH 7.0). Ten microliters of the sample was injected for HPLC analysis.

Chromatographic conditions. Chromatography equipment consisted of a Jasco Tri Rotor SR pump, a Jasco Tri Rotor SR 2 controller, and a Jasco Uvidec IV variable wavelength detector at 436 nm for DABS derivatives or 350 nm for DNS derivatives. The sensitivity of the detector was set at 0.005 AUFS (absorption unit, full scale). Data were processed by a SIC Chromatocorder 11. A saturator column (4.6 $\times$ 50 mm) dry-packed with Wakogel LC ODS-30K (30 μ m particles, Wako Pure Chemicals) and a Hypersil ODS (3 μ m particles, Shadon Southern Products) column (4.6 $\times$ 100 mm) slurry packed in our laboratory were connected through a valve injector, and were housed within an oven compartment maintained at 60°C. The elution solvent systems were (A) 10 mM sodium phosphate buffer, and (B) 10 mM sodium phosphate buffer-acetone (50:50, v/v).⁸ The pH of the phosphate buffer was

adjusted to 6.5 for the separation of DABS derivatives and 7.0 for DNS derivatives. The flow rate was 1.0 ml/min.

Preparation of CAM-AFT-III. Sea anemone toxins were isolated from *Anthopleura fuscoviridis* by extraction with methanol, gel filtration on Sephadex G-50, and ion-exchange chromatography on SP-Sephadex as previously described.⁹ The final purification was achieved by reversed-phase HPLC to give three toxins, AFT-I, AFT-II, and AFT-III. The yields of these toxins were 55.9 mg/kg, 53.7 mg/kg, and 2.1 mg/kg, respectively. In this study, the amino acid sequence of AFT-III was analyzed.

AFT-III was carboxamidomethylated by reduction and alkylation of disulfide bonds by the method of McWherter *et al.*¹⁰ AFT-III (850 μ g) was dissolved in 150 μ l of deoxygenated modification buffer (0.5 M Tris-HCl, pH 8.5, 6 M guanidine-HCl, and 2.5 mM EDTA). One hundred microliters of dithiothreitol solution (21 mg/ml in the modification buffer) was added. The reduction took place at room temperature for 1 hr. At that time, 5.4 mg of monoiodoacetamide was added and the reaction proceeded for 20 min in the dark. The reaction mixture was then injected into a TSK ODS 120T (10 μ m, 4.6 $\times$ 50 mm, Toyo Soda) column equilibrated with 0.1% trifluoroacetic acid (TFA). The column was eluted with 0.1% TFA and then with 80% acetonitrile in 0.1% TFA. S-Carboxamidomethylated (CAM) AFT-III detected by monitoring the absorbance at 280 nm was collected and lyophilized to yield 380 μ g.

Enzymatic digestion of CAM-AFT-III. CAM-AFT-III (150 μ g) was digested with TPCK-trypsin (Worthington) (substrate/enzyme = 100:1, w/w) in 0.2 ml of 0.1 M NH_4HCO_3 (pH 8.0) at 38°C for 16 hr. Fifty micrograms of CAM-AFT-III was digested with chymotrypsin (P-L Biochemicals) (100:1, w/w) in 100 μ l of 0.1 M NH_4HCO_3 at 38°C for 6 hr. The digests were purified by reversed-phase HPLC on a TSK ODS 120T column (10 μ m, 4.6 $\times$ 250 mm) equilibrated with 0.1% TFA. The column was eluted with a linear gradient of acetonitrile at a flow rate of 1.0 ml/min.

Amino acid analysis. One tenth to 1/20 of each fraction was taken in a hydrolysis tube and dried under a vacuum. Peptides were hydrolyzed *in vacuo* with 80 μ l of 6 M HCl containing phenol for 22 hr at 110°C. The hydrolysates were analyzed by the DABS-C1 method as described above.

Amino acid sequence analysis. The amino acid sequences of CAM-AFT-III and peptides obtained by enzymic digestion were determined by the manual fluorescein isothiocyanate (FITC) method.^{11,12} The resulting fluoresceinthiohydantoin (FTH) amino acid was identified by reversed-phase HPLC using a Hypersil ODS (3 μ m, 4.6 $\times$ 50 mm) column.¹³

RESULTS

A standard amino acid mixture (250 pmol) was treated with various volumes of 6 M HCl in the presence and absence of phenol at 110°C for 22 hr and recovery was determined (Table I). No tryptophan was recovered in the absence of phenol. By the addition of phenol to 6 M HCl, tryptophan was recovered, and the recovery increased by increasing the volume of 6 M HCl containing phenol. The maximum recovery (85%) for tryptophan was obtained by 80 μ l of 6 M HCl containing 0.7% phenol. However, 6 M HCl containing phenol gave a lower recovery of cystine and histidine than when 6 M HCl alone was used. The recovery of tryptophan was also dependent on the concentration of phenol in 6 M HCl as shown in Fig. 1. When a lower concentration (0.35%) of phenol was used in the hydrolysis, the recovery was about 30%. On the other hand, increasing the concentration of phenol above 0.7% did not raise the recovery above 85%.

Figure 2 shows the HPLC chromatogram of DABS derivatives of standard amino acids.

Tryptophan eluted between leucine and phenylalanine. For reference, the chromatogram of a blank sample is presented in the lower panel. The blank sample represents the backgrounds that were derived from a hydrolysis tube, 6 M HCl containing phenol, and derivatizing reagents. No significant background peak which

FIG. 1. Effects of the Concentration of Phenol on the Recovery of Tryptophan.

An amino acid mixture (250 pmol) was treated with 80 μ l of 6 M HCl containing various concentration of phenol at 110°C for 22 hr. The recovery of tryptophan was measured by HPLC as described in the text. % Mean values ($\pm$ S.E.) of six dependent experiments were plotted.

TABLE I. EFFECTS OF THE VOLUME OF 6 M HCl ON THE RECOVERY OF AMINO ACIDS BY HYDROLYSIS WITH 6 M HCl IN THE PRESENCE AND ABSENCE OF PHENOL

	6 M HCl				6 M HCl containing 0.7% phenol			
	10 μ l	20 μ l	40 μ l	80 μ l	10 μ l	20 μ l	40 μ l	80 μ l
Asp	91 $\pm$ 9	94 $\pm$ 1	80 $\pm$ 12	101 $\pm$ 1	95 $\pm$ 13	97 $\pm$ 0	92 $\pm$ 5	90 $\pm$ 2
Glu	117 $\pm$ 11	99 $\pm$ 4	109 $\pm$ 5	119 $\pm$ 6	109 $\pm$ 6	114 $\pm$ 6	111 $\pm$ 1	111 $\pm$ 7
Ser	111 $\pm$ 3	105 $\pm$ 5	104 $\pm$ 6	98 $\pm$ 2	99 $\pm$ 3	104 $\pm$ 3	105 $\pm$ 4	93 $\pm$ 2
Thr	102 $\pm$ 5	100 $\pm$ 6	99 $\pm$ 4	92 $\pm$ 3	94 $\pm$ 1	105 $\pm$ 11	100 $\pm$ 4	88 $\pm$ 1
Gly	133 $\pm$ 12	109 $\pm$ 10	119 $\pm$ 4	120 $\pm$ 3	104 $\pm$ 5	125 $\pm$ 12	113 $\pm$ 1	104 $\pm$ 1
Ala	106 $\pm$ 4	104 $\pm$ 7	101 $\pm$ 1	104 $\pm$ 2	100 $\pm$ 6	115 $\pm$ 15	110 $\pm$ 5	103 $\pm$ 2
Pro	108 $\pm$ 5	106 $\pm$ 6	105 $\pm$ 4	103 $\pm$ 2	97 $\pm$ 3	105 $\pm$ 6	103 $\pm$ 4	99 $\pm$ 3
Val	102 $\pm$ 0	96 $\pm$ 3	103 $\pm$ 3	98 $\pm$ 4	100 $\pm$ 2	107 $\pm$ 7	104 $\pm$ 3	100 $\pm$ 3
Arg	98 $\pm$ 1	97 $\pm$ 2	104 $\pm$ 6	103 $\pm$ 5	104 $\pm$ 3	103 $\pm$ 0	109 $\pm$ 1	102 $\pm$ 3
Met	110 $\pm$ 2	112 $\pm$ 4	109 $\pm$ 8	78 $\pm$ 5	96 $\pm$ 1	108 $\pm$ 5	108 $\pm$ 5	92 $\pm$ 5
Ile	104 $\pm$ 4	94 $\pm$ 4	96 $\pm$ 1	92 $\pm$ 4	94 $\pm$ 0	103 $\pm$ 3	103 $\pm$ 1	99 $\pm$ 2
Leu	114 $\pm$ 6	101 $\pm$ 6	100 $\pm$ 3	101 $\pm$ 5	97 $\pm$ 1	106 $\pm$ 4	107 $\pm$ 1	102 $\pm$ 2
Trp	0	0	0	0	27 $\pm$ 7	44 $\pm$ 11	78 $\pm$ 2	85 $\pm$ 2
Phe	95 $\pm$ 2	99 $\pm$ 8	96 $\pm$ 2	93 $\pm$ 3	94 $\pm$ 1	102 $\pm$ 5	105 $\pm$ 3	99 $\pm$ 2
(Cys) ₂	75 $\pm$ 1	78 $\pm$ 6	66 $\pm$ 2	53 $\pm$ 5	67 $\pm$ 5	46 $\pm$ 5	32 $\pm$ 5	21 $\pm$ 3
Lys	69 $\pm$ 9	86 $\pm$ 19	73 $\pm$ 2	85 $\pm$ 12	89 $\pm$ 3	78 $\pm$ 19	81 $\pm$ 11	84 $\pm$ 5
His	43 $\pm$ 5	61 $\pm$ 12	42 $\pm$ 12	82 $\pm$ 12	59 $\pm$ 9	60 $\pm$ 20	62 $\pm$ 12	56 $\pm$ 4
Tyr	65 $\pm$ 1	73 $\pm$ 22	72 $\pm$ 22	83 $\pm$ 8	101 $\pm$ 5	110 $\pm$ 13	112 $\pm$ 13	105 $\pm$ 3

% Mean values of three experiments. $\pm$ S.E.

TABLE II. AMINO ACID COMPOSITIONS OF CAM-AFT-III AND LYSOZYME HYDROLYZED WITH 6 M HCl CONTAINING PHENOL

	AFT-III		Lysozyme			
	Expected values	Experimental results	Expected values	Experimental results		
		I		I	II	III
Asp	4	4.2	21	20.9	20.8	19.7
Glu	1	1.4	5	5.5	6.3	5.9
Cys	6	4.4 ^a	8	0.8	0.4	0.6
Ser	4	3.2	10	9.0	9.2	8.7
Thr	2	1.9	7	6.5	6.8	6.9
Gly	9	9.0	12	11.4	11.4	10.4
Ala	2	2.3	12	12.0	12.0	12.0
Pro	4	4.3	2	2.5	2.5	2.3
Val	2	2.1	6	6.5	6.5	6.3
Arg	1	1.1	11	12.3	12.3	12.2
Met	0	0	2	2.2	2.1	2.3
Ile	3	2.4	6	6.0	6.2	6.1
Leu	3	3.4	8	8.8	9.1	8.9
Trp	3	2.5	6	4.1	3.7	4.4
Phe	0	0	3	3.6	3.8	3.6
Lys	2	1.5	6	6.8	6.9	6.8
His	2	1.0	1	1.4	1.5	1.3
Tyr	0	0	3	3.7	3.7	3.6

I: Hydrolysis was done in 80 μ l of 6 M HCl containing 0.7% phenol. II: Hydrolysis was done in 160 μ l of 6 M HCl containing 0.7% phenol. III: Hydrolysis was done in 80 μ l of 6 M HCl containing 2.8% phenol.

^a Measured as S-carboxymethylcysteine.

interfered the analysis of DABS amino acids was observed in this experiment.

Table II shows the amino acid compositions of CAM-AFT-III and lysozyme after hydrolysis with 80 μ l of 6 M HCl containing phenol for 22 hr at 110°C. One microgram of sample was hydrolyzed and derivatized with DABS-C1. One tenth of the derivatized sample was enough to give an adequate response in this HPLC system (Fig. 3). Tryptophan was recovered from CAM-AFT-III and lysozyme in a yield of 83% and 68%, respectively. Neither a larger volume of 6 M HCl containing 0.7% phenol nor a higher concentration of phenol in 6 M HCl improved the recovery of tryptophan from lysozyme. The yield of cystine was very low.

Recovery of tryptophan from acid hydrolysis in the presence of phenol encouraged us to examine the recovery of DNS-tryptophan. As can be seen from Table III, DNS-

tryptophan gave recoveries of 40% and 28% after hydrolysis for 4 hr and 16 hr, respectively. This result indicates that DNS-tryptophan is less stable than free tryptophan in these conditions.

Figure 4 shows the separation of three sea anemone toxins, AFT-I, AFT-II, and AFT-III, on reversed-phase HPLC. AFT-II and AFT-III were separated by the solvent system of 10 mM ammonium acetate (pH 6.5) and 2-propanol, but not by the solvent system of 0.1% TFA and acetonitrile. After carboxamidomethylation, AFT-II and AFT-III could not be resolved even with the 10 mM ammonium acetate (pH 6.5)-2-propanol system.

The manual amino terminal sequencing of 5 nmol of CAM-AFT-III yielded the sequence of residues 1 through 17 and showed one potential tryptic cleavage site between 15 and 16. The FITC derivative from the 10th residue eluted at the position of alanine, but the amino

FIG. 2. HPLC Chromatogram of DABS Amino Acids.

An amino acid mixture (250 pmol) was derivatized as described in the text and 10 μ l (25 pmol) of the derivatized sample was chromatographed. Column: Hypersil ODS (3 μ m), 4.6 $\times$ 100 mm. Gradient elution, mobile phase: A = 10 mM phosphate buffer (pH 6.5); B = 10 mM phosphate buffer, pH 6.5-acetone (50:50), flow-rate 1.0 ml/min. The gradient profile is shown by the broken line. The one-letter code for the amino acids is used except for the following abbreviations: C = cystine; O = sulfonic acid; Z = amide.

acid analysis of the tryptic peptide containing the sequence showed the residue was a derivative of aspartic acid (Fig. 5), and the FITC derivative regenerated FTH-aspartic acid by heating at 50°C for 1 hr in 6 M HCl.

In order to determine the amino acid sequence of CAM-AFT-III, the peptide was digested with trypsin and separated by reversed-phase HPLC (Fig. 6). Seven major peptides were isolated and analyzed using the hydrolytic procedure described here. Five of them contained tryptophan (Table IV). Chymotryptic digestion of CAM-AFT-III yielded six major peptides which could be separated and purified by HPLC as shown in Fig. 7. Four of them contained tryptophan (Table V). The sequence of the tryptic and chymotryptic peptides were analyzed by the manual FITC method.

The amino acid sequence of AFT-III is

FIG. 3. HPLC Chromatogram of DABS Amino Acids Generated after Hydrolysis and Derivatization of CAM-AFT-III.

CAM-AFT-III (1 μ g) was hydrolyzed with 80 μ l of 6 M HCl containing phenol at 110°C for 22 hr *in vacuo*. The hydrolyzate was derivatized with DABS-Cl as described in the text, and one tenth of the sample was injected. Chromatographic conditions were the same as those in Fig. 2. C = S-carboxymethylcysteine.

shown in Fig. 8 together with the details of the peptides from which it was deduced. In most cases, quantitative amino acid analysis using our hydrolytic procedure supported the sequences obtained.

DISCUSSION

To determine the amino acid sequence of a protein or peptide, the amino acid compositions of many peptide fragments must be analyzed rapidly from small quantities of sample. Thus, micro amino acid analysis using a single hydrolysis is desirable. As examples, Tables IV and V show amino acid compositions of tryptic and chymotryptic peptides obtained from CAM-AFT-III. In general, a single amino acid analysis using the method described here gave acceptable results with a good recovery of tryptophan. Although the addition of phenol to 6 M HCl is common to

get a good yield of tyrosine,¹⁴⁾ no paper has described the protective effect of phenol for tryptophan. The concentration of phenol used in acid hydrolysis is usually 0.1~0.2%. Recently, gas-phase hydrolysis using 6 M HCl

TABLE III. RECOVERY OF DNS-AMINO ACIDS BY HYDROLYSIS WITH 6 M HCl CONTAINING 0.7% PHENOL

	4 hr	16 hr
Asp	109	98
Glu	102	89
Ser	84	60
Gly	111	74
Thr	110	90
Ala	95	80
Arg + Pro	78	47
Val	106	97
Met	93	90
Ile	110	90
Leu	106	97
Trp	40	28
Phe	107	97
(Cys) ₂	26	6
Lys	97	82
His	9	1
Tyr	109	102

% Mean values of two analyses.

FIG. 4. HPLC Chromatograms of Anemone Toxins.

Column, TSK ODS 120T (10 μ m), 4.6 $\times$ 250 mm; flow rate, 1.0 ml/min. In the upper panel, the column was eluted with a linear gradient of 2-propanol–10 mM ammonium acetate (pH 6.5) system and in the lower panel the column was eluted with a linear gradient of acetonitrile–0.1% TFA system. Ten micrograms of each peptides was injected. I, AFT-I; II, AFT-II; III, AFT-III.

FIG. 5. HPLC Chromatograms from the Sequencing of 5 nmol of CAM-AFT-III.

Column, Hypersil ODS (3 μ m), 4.6 $\times$ 50 mm; column temperature, 60°C; gradient elution, mobile phase, A = 10 mM sodium phosphate buffer (pH 7.0); B = 10 mM sodium phosphate buffer (pH 7.0)–acetone (80 : 20); a 30 min linear gradient from 15 to 70% solvent B. Panel 1 is a chromatogram of authentic FTH amino acids. The cycle number (2~10) of each step is given in each chromatogram. Percentages of extracts of FTH amino acids used for HPLC analysis at each cycle are indicated in parentheses. B=internal marker; C=S-carboxamidomethylcysteine; X=unknown derivative.

TABLE IV. AMINO ACID COMPOSITIONS OF TRYPTIC PEPTIDES OBTAINED FROM CAM-AFT-III

	T1	T2	T3	T4	T5	T6	T7
Asp		1.0 (1) ^a	2.1 (2)			1.0 (1)	1.8 (2)
Glu							
Cys ^b	1.5 (2)	1.4 (2)	1.6 (2)	1.3 (2)			1.6 (2)
Ser		0.8 (1)	1.6 (2)			0.9 (1)	1.5 (2)
Thr				1.0 (1)	0.9 (1)	0.9 (1)	0.9 (1)
Gly		1.9 (2)	2.4 (3)	1.9 (2)	1.9 (2)	2.0 (2)	4.1 (4)
Ala		1.0 (1)		0.9 (1)	1.1 (1)		1.0 (1)
Pro		1.1 (1)	2.0 (2)	1.0 (1)	1.1 (1)		1.1 (1)
Val			1.9 (2)				
Arg			1.0 (1)				
Met							
Ile				1.0 (1)	1.0 (1)	1.1 (2)	1.2 (2)
Leu		0.9 (1)	1.0 (1)			1.2 (1)	2.3 (2)
Trp		0.7 (1)		0.7 (1)	0.9 (1)	0.8 (1)	2.0 (2)
Phe							
Lys	1.0 (1)	0.8 (1)		0.7 (1)			0.8 (1)
His		0.7 (1)		0.5 (1)	0.4 (1)		0.6 (1)
Tyr							

^a The numbers of parentheses are from the sequence analysis.

^b Measured as S-carboxymethylcysteine.

FIG. 6. HPLC Separation of Tryptic Peptides of CAM-AFT-III.

Tryptic peptides from the digestion of 150 μ g of CAM-AFT-III were dissolved in 0.1% TFA, and put on a TSK ODS 120T (10 μ m, 4.6 $\times$ 250 mm) column equilibrated with 0.1% TFA and eluted with a linear gradient of acetonitrile in 0.1% TFA.

containing 1% phenol of peptides and proteins has been reported for micro amino acid analysis coupled with precolumn labeling with phenylisothiocyanate.¹⁵⁾ We recovered tryptophan from liquid-phase hydrolysis with dou-

FIG. 7. HPLC Separation of Chymotryptic Peptides of CAM-AFT-III.

Chymotryptic peptides from the digestion of 50 μ g of CAM-AFT-III were dissolved in 0.1% TFA, and put on a TSK ODS 120T column as described in Fig. 6.

ble distilled 6M HCl containing 1% phenol. The recovery depended on the volume of the 6M HCl. Therefore, it is not surprising if tryptophan can not be recovered from gas-

FIG. 8. The Amino Acid Sequence of AFT-III.

Although good recovery of tryptophan was obtained in the hydrolysis of free amino acids and peptides, the same conditions resulted in large losses of tryptophan when applied to proteins, such as lysozyme. This could be due to the fact that the destruction of tryptophan when in peptide form is different from that

when it is present as a free amino acid in the hydrolysis mixture. It is also likely that elements that are not removed from the protein during purification catalyze degradation reactions.¹⁷⁾ We do not know exactly how phenol prevents the destruction of tryptophan during acid hydrolysis. Phenol may act as a scavenger of the residual oxidizing agent that might be present in the hydrolysis tube.

DNS-C1 is a well characterized fluorogenic reagent for the identification on amino acids, peptides, and proteins. The DNS-C1 method¹⁸⁾ is one of the powerful methods for end-group and sequence analysis of peptides and proteins. The stability of DNS-amino acids to acid hydrolysis has been evaluated.^{19,20)} The destruction of tryptophan which occurs, even after a short 4-hr period of hydrolysis at 110°C, represents a limitation of the DNS-Edman method for the sequencing of peptides. Although the introduction of 4 M methanesulfonic acid containing 0.2% 3-(2-aminoethyl)-indole²¹⁾ or 3 M mercaptoethane sulfonic acid²²⁾ has made it possible to recover DNS-tryptophan, the DNS derivative must be recovered by extraction from the hydrolysate after neutralization, and some hydrophilic amino acid derivatives can not be extracted.²²⁾ On the other hand, as 6 M HCl containing phenol is volatile, the hydrolysate can be dried under a vacuum and identified directly. Thus, this hydrolytic procedure can be coupled to the amino terminal and DNS-Edman analyses. The reason for the low recovery of DNS-histidine is not known.

Neurotoxins have been isolated from a variety of sea anemone species and have become useful tools in physiological, pharmacological, and biochemical studies. The amino acid sequences of many of these toxins are known.^{23~29)} We have isolated three cardiotoxic polypeptides from *Anthopleura fusco-iridis* and determined the amino acid sequences of two major toxins, AFT-I and AFT-II.⁹⁾ AFT-I consists of 47 amino acid residues, while AFT-II consists of 48 amino acid residues. There are four sequence differences between AFT-I and AFT-II: at residue 3 (Ala

for Pro), 12 (Arg for Ser), 21 (Thr for Ile), and an additional Gly at the N-terminal position on AFT-II. The yield of AFT-III was less than 5% of those of the major toxins. AFT-II was the most toxic to mice and showed a minimum lethal dose of 100 µg/kg by intravenous injection. The minimum lethal dose of AFT-I was 300 µg/kg and of AFT-III was 500 µg/kg. The toxicities of the three polypeptides to *Hemigrapsus sanguineus* crabs were similar and were in the range of 100 to 150 µg/kg. In this study, we not only determined the amino acid sequence of AFT-III to investigate the structure-function relationship of AFTs but also applied the hydrolytic procedure described here to this analysis to demonstrate its utility in the amino acid sequence analysis of peptides. The result shows that the amino acid sequence of AFT-III is the same as that of AFT-II except for residue 10. The β -carboxyl group of aspartic acid-10 in AFT-III is probably modified to be nonionizable. The characteristic mobilities of AFT-II and AFT-III on HPLC by changing the pH of the solvents can be accounted for by such modification. In addition, the separation between AFT-II and AFT-III may be achieved by the conformational transition of the molecule which occurs concomitant with protonation of the β -carboxyl group of the aspartic acid, because AFT-II and AFT-III can not be resolved even at pH 6.5 after S-carboxamidomethylation. The lysine-47-glutamine-48 linkage commonly found in sea anemone toxins has been reported to resist tryptic digestion,^{9,29)} but the linkage of AFT-III was readily cleaved. This indicates that the interaction between aspartic acid-10 and the carboxyl-terminal region in the molecule was disrupted by the modification of the β -carboxyl group. Comparison of the structures and toxicities of AFT-II and AFT-III shows that aspartic acid-10 is not essential but is important for the expression of the toxic properties of the sea anemone toxin. The study of the structure and biological significance of the derivative of aspartic acid-10 is in progress.

REFERENCES

- 1) H. Matsubara and R. M. Sasaki, *Biochem. Biophys. Res. Commun.*, **35**, 175 (1969).
 - 2) Y. Yokote, K. M. Arai and K. Akahane, *Anal. Biochem.*, **152**, 245 (1986).
 - 3) T.-Y. Liu and Y. H. Chang, *J. Biol. Chem.*, **246**, 2842 (1971).
 - 4) R. J. Simpson, M. R. Neuberger and T.-Y. Liu, *J. Biol. Chem.*, **251**, 1936 (1976).
 - 5) B. Penke, R. Ferenczi and K. Kovacs, *Anal. Biochem.*, **60**, 45 (1974).
 - 6) K. Maeda, J.-J. Scheffler and A. Tsugita, *Hoppe-Seyler's Z. Physiol. Chem.*, **365**, 1183 (1984).
 - 7) J.-Y. Chang, R. Knecht and D. G. Braun, *Methods, in Enzymology*, **91**, 41 (1983).
 - 8) K. Muramoto and H. Kamiya, *Bull. Japan. Soc. Sci. Fish.*, **51**, 817 (1985).
 - 9) S. Sunahara, K. Muramoto, K. Tenma and H. Kamiya, *Toxicon*, **25**, 211 (1987).
 - 10) C. A. McWherter, T. W. Thannhauser, R. A. Fredrickson, M. T. Zagotta and H. A. Scheraga, *Anal. Biochem.*, **141**, 523 (1984).
 - 11) K. Muramoto, H. Kawauchi and K. Tuzimura, *Agric. Biol. Chem.*, **42**, 1559 (1978).
 - 12) K. Muramoto and H. Kamiya, *Biochim. Biophys. Acta*, **874**, 285 (1986).
 - 13) K. Muramoto, H. Kamiya and H. Kawauchi, *Anal. Biochem.*, **141**, 446 (1984).
 - 14) F. Sanger and E. O. P. Thompson, *Biochim. Biophys. Acta*, **71**, 471 (1963).
 - 15) B. A. Bidlingmeyer, S. A. Cohen and T. L. Tarvin, *J. Chromatogr.*, **336**, 93 (1984).
 - 16) R. Knecht and J.-Y. Chang, *Anal. Chem.*, **58**, 2375 (1986).
 - 17) A. S. Inglis, *Methods in Enzymology*, **91**, 26 (1983).
 - 18) W. R. Gray, *Methods in Enzymology*, **25**, 333 (1972).
 - 19) C. Gros and B. Labouesse, *Eur. J. Biochem.*, **7**, 463 (1969).
 - 20) B. S. Hartley, *Biochem. J.*, **119**, 805 (1970).
 - 21) R. Flengsrud, *Anal. Biochem.*, **76**, 547 (1976).
 - 22) J. R. Giglio, *Anal. Biochem.*, **82**, 262 (1977).
 - 23) G. Wunderer, H. Fritz, E. Wachter and W. Machleidt, *Eur. J. Biochem.*, **68**, 193 (1976).
 - 24) M. Tanaka, M. Haniu, K. T. Yasunobu and T. R. Norton, *Biochemistry*, **16**, 204 (1977).
 - 25) G. Wunderer and M. Eulitz, *Eur. J. Biochem.*, **89**, 11 (1978).
 - 26) T. R. Norton, *Fed. Proc.*, **40**, 21 (1981).
 - 27) H. Schweitz, J.-N. Bidard, C. Frelin, D. Pauron, H. P. M. Vijverberg, D. M. Mahasneh, M. Lazdunski, F. Vilbois and A. Tsugita, *Biochemistry*, **24**, 3554 (1985).
 - 28) S. Nishida, S. Fujita, M. Satake and N. Tamiya, *Eur. J. Biochem.*, **150**, 171 (1985).
 - 29) N. S. Reimer, C. L. Yasunobu, K. T. Yasunobu and T. R. Norton, *J. Biol. Chem.*, **260**, 8690 (1985).
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