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# Solid-phase C-terminal sequencing of peptides

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**Summary.** C-terminal amino acid sequence analysis seemed to be established procedure, as the counterpart of Edman's N-terminal sequencing method. However, poor recovery of the C-terminal amino acids in the reaction in homogeneous solution suggested further improvement of the method. In the present study, N-terminal amino acid was fixed covalently to the controlled pore glass (CPG) beads and the C-terminal amino acid was activated (by treating with acetic anhydride), coupled with thiocyanate to form thiohydantoin (TH) ring at the C-terminus. Then, the C-terminal amino acid was split off as the corresponding TH derivative, and analyzed by HPLC. Hydrolysis of the TH derivative was achieved at 60°C in the presence of 2 M HCl for 2 h. Solid phase fixed peptide was washed simply with acetone, and dried for the next cycle of the reaction. So far obtained results in the heterogeneous mixture are not satisfactory in terms of the recovery of the C-terminal TH, and improvement of the recovery and further steps are under progress.

**Keywords:** Amino acids – C-terminal peptide – Thiohydantoin derivatives – C-terminal sequencing – Solid phase peptide sequencing

## Introduction

We reported some results concerning a sequential amino acid analysis of small peptides. The most recent paper is concerned about the method of hydrolysis of a modified C-terminal amino acid; a thiohydantoin (TH) ring is formed at the C-terminus to convert the sec. amide (peptide) to the corresponding tert. amide, which is more susceptible to hydrolysis (Yamashita et al., 1990). In that paper, we used microwave (2540 MHz) energy to heat for the hydrolysis of the TH ring from the C-terminus of the peptide.

We found that somewhat impractical yield of the amino acid derived TH ring might be due to the extraction step, where TH is extracted from an aqueous mixture of a peptide (minus one amino acid at the first step) by organic solvent.

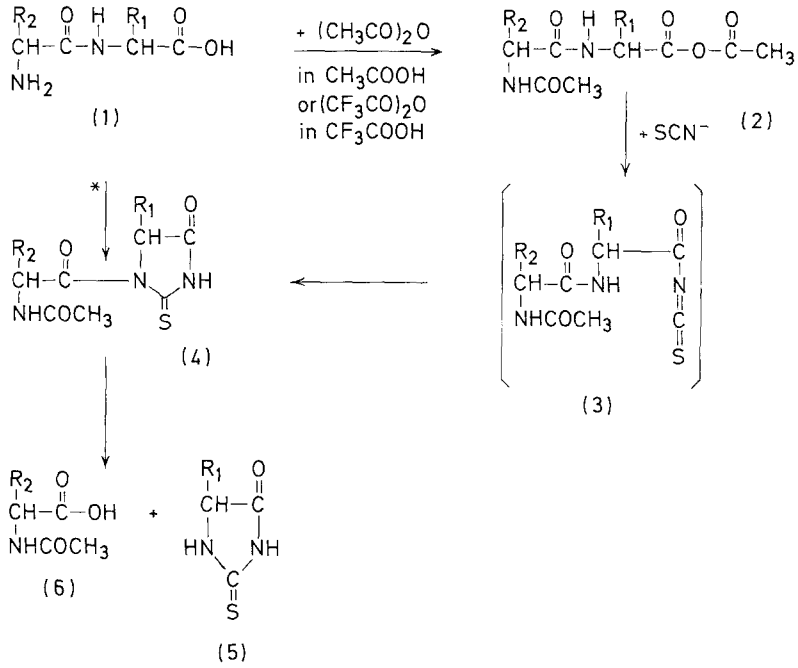


**Fig. 1.** Overall steps for the formation of the thiohydantoin (TH) derivative of amino acids and of the C-terminal amino acid of a peptide

Thus, heterogeneous system was explored to separate the TH ring more efficiently from the mixture with the parent peptide, which is bound to heterogeneous solid phase.

The N-terminus of a given peptide was thus fixed on "the organic solvent resistant" solid phase, and then the routine procedure of C-terminal TH formation was attempted (Fig. 1). Then, the tertiary amide bond, which is discriminated from other peptide bonds (—C(=O)—N(H)—, mostly sec. amide), was hydrolyzed by rather milder conditions than the conventional ones, with 2 M HCl at 60° for 3 h. Split amino acid TH of the C-terminal origin was extracted with ethyl acetate (EtOAc), leaving the parent peptide (minus one amino acid after the 1st step) on the solid phase.

## Materials and methods

### *Peptides*

Schizophrenia related peptide (Thr-Val-Leu). Peptide T (Ala-Ser-Thr-Thr-Thr-Asn-Thr-Thr). Bovine adrenal medulla dodecapeptide (BAM-12 P) (Thr-Gly-Gly-Phe-Met-Arg-Arg-Val-Gly-Arg-Pro-Glu). Tyrosyl bradykinin (Thr-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg). Gamma-Endorphin (Trp-Gly-Gly-Phe-Met-Thr-Ser-Lys-Ser-Gln-Thr-Pro-Leu-Pro-Leu-Val-Thr-Leu).

### *Solid phase supports*

AF-tresyl TOYOPEAL 650 M (TOSHO). Tresyl-activated Sepharose 4 B (agarose) (Pharmacia). Hydrazide beads (polystyrene) (PIERCE). Aminopropyl CPG (glass) (CPG Inc.).

*HPLC columns*

Waters $\mu$ Bondasphere 5 $\mu$ $C_{18}$-100Å. Waters $\mu$ Bondasphere 15 $\mu$ $C_{18}$-100Å.

*HPLC detectors*

UV-Vis Detector/S-3702 (Soma Optical Co.). UV-Detector 3000 B (Senshu Scientific Co.). Electronic pressure gauge: NPG-500 E (Nippon Seimitsu Kagaku Co.). Pump: NSP 800-505 (Nippon Seimitsu Kagaku Co.). Flow System 3100 (Senshu Sci. Co.). Sample Injector: Rheodyne 7125 (Rheodyne). Recorder: Model FBR 251 A Toa Denpa Co.). Chromatocorder 12 (System Instruments).

*i) Synthesis of reference amino acid thiohydantoins (TH')*
*ii) Formation of the C-terminal TH ring*

Fig. 1 illustrates a whole cycle of chemical reactions of preparing reference standard of TH derivatives of amino acids when N-terminal aminoacyl group is replaced by hydrogen, and conditions of each steps may be found in the preceeding paper (Yamashita et al., 1990).

*Fixation of N-terminal amino group to a solid phase supports*

In a typical experiment, 50 mg of aminopropyl-controlled pore glass beads (CPG) was placed in a test tube. To the test tube, cold 1% glutaraldehyde was added, and after removing gas in the mixture under reduced pressure, it was mixed at 4° for 30 min, and then the solid phase support was washed with water and phosphate buffer, pH 7.5. Then, 180.2 nmol Leu-Enkephalin in 0.1 M phosphate buffer, pH 7.5, was added together with sodium cyanoborate, and the mixture was stirred at 4° overnight. After the reaction, the mixture was washed with 2 M NaCl. Any amino group capturing activity of the solid phase was blocked by the treatment with 0.05 M Tris-HCl buffer, pH 8, stirring for 1 h. As the bridge material, thiophosgen-$CHCl_3$ solution, or N-hydroxy-succinimide was used (Fig. 2).

*Sequential degradation of a peptide on a solid phase support*

The procedures were essentially the same as those preparing reference amino acid TH's except the N-terminal amino group is linked to a solid phase support by covalent bond.

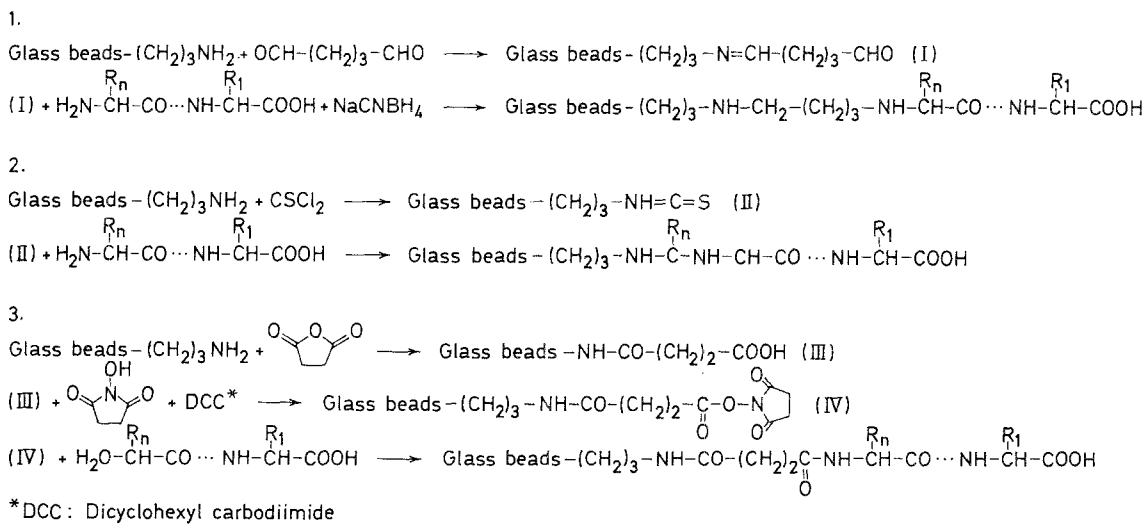


**Fig. 2.** Chemical reactions to bind the N-terminal amino group by means of various bridging (spacing) materials

**Table 1.** Conditions for separating and identifying amino acid derived thiohydantoins (TH) on HPLC

| |
|---|
| Columns: |
| Waters uBondasphere 5 $\mu$ $C_{18}$-100 Å, 4.0 × 150 mm. |
| Mobile phases: |
| 10 mM, 50 mM or 100 mM $AcONH_4$ (pH 6.8) : MeCN = 70 : 30 (v/v). |
| 10 mM $AcONH_4$ (pH 3.5, 4.0, 5.0, 6.0, 8.0, 9.0) : MeCN = 70 : 30 (v/v). |
| 10 mM $AcONH_4$ (pH 6.8) : MeCN = 70 : 30 (v/v) + 0.002%, 0.005%, 0.01% or 0.02% SDS. |
| 10 mM $AcONH_4$ (pH 6.8) : MeCN : THF = 70 : 28 : 2 |
| 70 : 25 : 20 |
| or 70 : 20 : 10 (v/v). |
| 10 mM $AcONH_4$ (pH 4) : MeCN : THF = 70 : 20 : 10 (v/v) + 0.005% SDS. |
| Flow rate: 1.0 ml/min. |
| Detection of TH : 254 nm. |

After the reaction, as shown in Fig. 1, heterogeneous solid phase was recovered by centrifuging at 2,000 rpm for 3 min. Acid hydrolyzed C-terminal amino acid TH was extracted with EtOAc twice. Solvent was evaporated, and the residue was dissolved in the mobile phase of HPLC. Residual solid phase + peptide (minus one amino acid moiety) was washed twice with acetone, and dried for the next cycle of the TH formation.

*Separation and identification of amino acid derived TH's*

Thus obtained C-terminal amino acid originated TH was identified on HPLC using various mobile phases (Table 1).

## Results

Procedures of preparing reference amino acid TH's are illustrated in Fig. 1.

HPLC conditions for separation and analysis of amino acid TH's are shown in Table 1.

HPLC characteristics of amino acid TH's are shown in Tables 2–6. Properties of the solid phase supporting materials and amount of peptide attached to aminopropyl-CPG are shown in Tables 7 and 8. Hydrolysis of the peptidyl-TH on solid phase by various conditions are shown in Table 9.

## Discussion

Thiohydantoin (TH) derivatives of the C-terminal amino acid origin seem to be good method for sequential analysis of peptides as the counterpart of the Edman's N-terminal sequencing. In a solution, extraction of formed TH was incomplete, so that the yields of TH after the 2nd step might not be quantitative. In addition, recovery of the residual parent peptide from the solution may be incomplete, and during a series of extraction procedures, some residual or contaminated material may disturb the complete recovery and the subsequent steps would be confused. To improve such shortcomings, a peptide was attached to a solid phase material, so that the extraction of TH and recovery of the parent peptide (on a solid phase support) became much simpler. Various conditions

**Table 2.** HPLC characteristics of thiohydantoin derivatives of amino acids

| Thiohydantoin derivatives of | [$AcONH_4$ (pH 6.8) : MeCN = 70 : 30 (v/v)] 10 mM* | 50 mM* | 100 mM* |
|---|---|---|---|
| Histidine | 1.65 | 1.68 | 1.69 |
| Glycine | 1.67 | 1.68 | 1.70 |
| Lysine | 1.69 | 1.71 | 1.72 |
| Alanine | 1.75 | 1.83 | 1.90 |
| Threonine | 1.95 | 2.00 | 2.11 |
| Glutamic acid | 2.03 | 2.23 | 2.27 |
| Arginine | 2.11 | 2.25 | 2.31 |
| Asparagine | 2.14 | 2.30 | 2.37 |
| Tyrosine | 2.17 | 2.26 | 2.43 |
| Valine | 2.34 | 2.40 | 2.51 |
| Proline | 2.56 | 2.48 | 2.63 |
| Leucine | 3.86 | 3.87 | 4.03 |
| Phenylalanine | 4.22 | 4.58 | 4.56 |
| Methionine | 5.71 | 6.31 | 6.44 |
| Isoleucine | 12.43 | 13.23 | 13.50 |
| Tryptophan | 14.22 | 14.85 | 16.25 |
| Norleucine** | 3.98 | 4.18 | 4.36 |
| | | (retention time, min) | |

Concentrations of $AcOHN_4$ are changed
* Concentration of $AcONH_4$.
** Used as the internal standard.

**Table 3.** HPLC characteristics of thiohydantoin derivatives of amino acids

| Thiohydantoin derivatives of | [10 mM $AcONH_4$ : MeCN = 70 : 30 (v/v)] pH 3.5* | pH 4.0* | pH 5.0* | pH 6.0* | pH 8.0* | pH 9.0* |
|---|---|---|---|---|---|---|
| Histidine | 1.66 | 1.62 | 1.62 | 1.61 | 1.53 | 1.28 |
| Glycine | 1.64 | 1.62 | 1.62 | 1.59 | 1.54 | 1.37 |
| Lysine | 1.83 | 1.79 | 1.78 | 1.72 | 1.55 | 1.39 |
| Alanine | 1.92 | 1.91 | 1.88 | 1.84 | 1.68 | 1.17 |
| Threonine | 2.16 | 2.14 | 2.02 | 2.01 | 1.89 | 1.73 |
| Glutamic acid | 5.27 | 5.23 | 2.65 | 2.38 | 1.90 | 1.73 |
| Arginine | 3.38 | 3.38 | 2.74 | 2.47 | 1.95 | 1.74 |
| Asparagine | 3.48 | 3.34 | 2.84 | 2.57 | 1.90 | 1.76 |
| Tyrosine | 2.35 | 2.36 | 2.24 | 2.22 | 2.13 | 2.03 |
| Valine | 2.51 | 2.43 | 2.39 | 2.35 | 2.30 | 2.17 |
| Proline | 2.58 | 2.60 | 2.56 | 2.56 | 2.36 | 2.18 |
| Leucine | 3.95 | 3.91 | 3.88 | 3.87 | 3.71 | 3.30 |
| Phenylalanine | 4.43 | 4.36 | 4.34 | 4.26 | 4.21 | 3.63 |
| Methionine | 8.63 | 8.40 | 7.89 | 7.43 | 3.51 | 1.85 |
| Isoleucine | 17.86 | 17.86 | 16.55 | 15.89 | 9.04 | 2.98 |
| Tryptophan | 19.99 | 18.83 | 18.21 | 17.63 | 10.01 | 3.29 |
| Norleucine** | 4.22 | 4.19 | 4.18 | 4.08 | 3.96 | 3.53 |
| | | | | | (retention time: min). | |

pH's of $AcONH_4$ are changed. pH was adjusted by 10 mM $CH_3COOH$ at pH 3.5–6.0 and by 10 mM $NH_4OH$ at pH 8.0–9.0.
* pH 3.5–6.0: pH adjusted by 10 mM $AcONH_4$ and 10 mM $CH_3COOH$
pH 8.0–9.0: pH adjusted by 10 mM $AcONH_4$ and 10 mM $NH_4OH$
** Used as the internal standard.

**Table 4.** HPLC characteristics of thiohydantoin derivatives of amino acids

| Thiohydantoin derivatives of | [10 mM $AcONH_4$ (pH 6.8) : MeCN = 70 : 30 (v/v) + SDS] 0.002% | 0.005% | 0.01% | 0.02% (SDS) |
|---|---|---|---|---|
| Histidine | 2.58 | 2.66 | 2.75 | 2.84 |
| Glycine | 1.65 | 1.58 | 1.56 | 1.54 |
| Lysine | 1.66 | 1.65 | 1.63 | 1.59 |
| Alanine | 1.68 | 1.66 | 1.67 | 1.63 |
| Threonine | 1.88 | 1.88 | 1.87 | 1.82 |
| Glutamic acid | 1.80 | 1.72 | 1.63 | 1.57 |
| Arginine | 1.81 | 1.72 | 1.66 | 1.62 |
| Asparagine | 1.82 | 1.75 | 1.67 | 1.67 |
| Tyrosine | 2.15 | 2.07 | 2.00 | 1.93 |
| Valine | 2.29 | 2.27 | 2.24 | 2.23 |
| Proline | 2.36 | 2.30 | 2.31 | 2.28 |
| Leucine | 3.65 | 3.60 | 3.51 | 3.22 |
| Phenylalanine | 4.18 | 4.41 | 4.07 | 3.45 |
| Methionine | 5.33 | 5.13 | 5.03 | 4.26 |
| Isoleucine | 12.42 | 11.89 | 11.35 | 8.99 |
| Tryptophan | 13.57 | 12.95 | 12.45 | 8.48 |
| Norleucine* | 3.89 | 3.80 | 3.76 | 3.32 |
| | | | (retention time, min). | |

Various amounts of SDS are added as detergent.
* Used as the internal standard.

**Table 5.** HPLC characteristics of thiohydantoin derivatives of amino acids

| Thiohydantoin derivatives of | [10 mM $AcONH_4$ (pH 6.8) : MeCN : THF] 70 : 28 : 2 | 70 : 25 : 5 | 70 : 20 : 10 (v/v) |
|---|---|---|---|
| Histidine | 1.52 | 1.77 | 1.78 |
| Glycine | 1.52 | 1.79 | 1.83 |
| Lysine | 1.57 | 1.84 | 1.84 |
| Alanine | 1.88 | 2.41 | 2.94 |
| Threonine | 2.05 | 2.53 | 2.89 |
| Glutamic acid | 1.99 | 2.24 | 2.33 |
| Arginine | 2.00 | 2.25 | 2.34 |
| Asparagine | 2.01 | 2.26 | 2.36 |
| Tyrosine | 2.30 | 2.79 | 3.24 |
| Valine | 2.44 | 2.92 | 3.33 |
| Proline | 2.39 | 2.69 | 2.72 |
| Leucine | 4.03 | 4.73 | 4.93 |
| Phenylalanine | 4.74 | 5.27 | 5.44 |
| Methionine | 6.21 | 6.45 | 6.72 |
| Isoleucine | 14.56 | 15.63 | 15.99 |
| Tryptophan | 16.23 | 16.21 | 18.91 |
| Norleucine* | 4.46 | 4.87 | 5.59 |
| | | (retention time, min). | |

Various amounts of THF are added.
* Used as the internal standard.

**Table 6.** HPLC characteristics of thiohydantoin derivatives of amino acids

| Thiohydantoin derivatives of | [10 mM $AcONH_4$ (pH 4) : MeCN : THF] 70 : 20 : 10 (v/v) + 0.005% SDS |
|---|---|
| Histidine | 4.40 |
| Glycine | 1.59 |
| Lysine | 1.64 |
| Alanine | 3.19 |
| Threonine | 2.63 |
| Glutamic acid | 6.72 |
| Arginine | 4.03 |
| Asparagine | 4.11 |
| Tyrosine | 3.09 |
| Valine | 2.87 |
| Proline | 2.53 |
| Leucine | 5.22 |
| Phenylalanine | 6.22 |
| Methionine | 11.38 |
| Isoleucine | 21.69 |
| Tryptophan | 25.11 |
| Norleucine* | 5.68 |
| | (retention time, min). |

THF and SDS are added to the mobile phase.
* Used as the internal standard.

**Table 7.** Tested solid phase supporting materials and their properties

| Solid phase support | Material | Properties |
|---|---|---|
| AF-Tresyl TOYOPEAL 650 M (TOSHO) | | Not swollen in anhydrous condition |
| Tresyl-activated Sepharose 4B (Pharmacia) | Agarose | Not swollen in anhydrous condition<br>Dissolved in HCl |
| Hydrazide Beads (PIERCE) | Polystyrene | Dissolved in organic solvents |
| Aminopropyl-CPG (CPG Inc.) | Glass | Any solvents can use |

**Table 8.** Amount of peptide bound to aminopropyl-CPG with various bridging materials

| Method of linkage | Amount of peptide* (nmol) | Amount of aminopropyl-CPG (mg) | Amount of peptide bound to aminopropyl-CPG (nmol) | (%) |
|---|---|---|---|---|
| Glutaraldehyde method | 180.2 | 10 | 66.9 | 37.2 |
| | | 20 | 78.9 | 43.8 |
| | | 30 | 134.6 | 74.8 |
| | | 40 | 128.1 | 71.1 |
| | | 50 | 140.3 | 77.9 |
| | | 100 | 121.1 | 67.3 |
| Thiophosgene method | 180.2 | 50 | 62.3 | 34.6 |
| N-Hydroxysuccinimide method | 180.2 | 50 | 84.3 | 46.8 |

* Peptide: Leucine-Enkephalin (Tyr-Gly-Gly-Phe-Leu)

**Table 9.** Hydrolysis of the solid-phase bound peptide C-terminal TH under various conditions

| HCL normality (N) | Duration of hydrolysis (h) | Recovery in the first step (nmol) | Yield (%) | Recovery in the second step (nmol) | Yield (%) |
|---|---|---|---|---|---|
| 2 | 1 | 83.8 | 13.9 | 47.9 | 7.9 |
| 2 | 2 | 79.0 | 13.1 | 69.7 | 11.5 |
| 2 | 3 | 348.7 | 57.7 | 329.8 | 54.6 |
| 4 | 1 | 48.5 | 8.0 | 43.4 | 7.2 |
| 4 | 2 | 131.2 | 21.7 | 124.1 | 20.5 |
| 4 | 3 | 305.2 | 50.5 | 151.1 | 25.0 |
| 6 | 1 | 114.2 | 18.9 | 89.9 | 14.9 |
| 6 | 2 | 265.1 | 43.9 | 228.7 | 37.9 |
| 6 | 3 | 312.5 | 51.7 | 28.0 | 2.9 |

Peptide: Schizophrenia related peptide (Thr-Val-Leu), 0.2 mg (604 nmol)
Temperature for hydrolysis: 60°
Amount of aminopropyl-CPG: 100 mg

were examined, as shown in Tables, and the following conditions were found to be the most adequate in the present method, although there are many things to be improved. Peptide is best attached to aminopropyl-CPG by means of glutaraldehyde. Once a peptide was attached to the solid phase support, C-terminal TH could be hydrolyzed under milder conditions, by 2 N HCl at 60° for 3 h.

Side reactions might be avoided to recover the parent peptide, since it was just washed with acetone and dried at lower temperature, preparing for the next step within a shorter time.

A series of our trials are intended to develop quantitative analysis of a given peptide. We used volatile solvents and reagents for the purpose, and the present solid phase support maybe favorable for the purpose. However, we must find out conditions to obtain better yield of TH and better recovery of the parent pepticle.

Automatic instrument may be developed from the present work to attain rapid C-terminal sequencing with a minute amount of sample.

## Reference

Yamashita S, Miyashita M, Tsubokawa K (1990) Jpn J Clin Chem 19: 315–321

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