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A Sensitive Method for the Fluorometric Analysis of Phenylthiohydantoin Amino Acids using Hypochlorite-Thiamine Reagent[1]

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A sensitive method for the detection and determination of phenylthiohydantoin amino acids using hypochlorite–thiamine reagent is described. Phenylthiohydantoin amino acids were developed on a Kieselgel plate and N-chlorinated on the plate by spraying sodium hypochlorite. Excess hypochlorite was removed by aeration and the plate was sprayed with the alkaline thiamine reagent. The chlorinated thiohydantoin gave an intensely fluorescent spot. An application of this method to Edman degradation is also described.

Keywords——phenylthiohydantoin amino acid; fluorometric detection; fluorometric determination; thiamine; sodium hypochlorite; Edman degradation; cycloheptaamylose; peptide; sequence analysis

Edman degradation has been widely used for the elucidation of the primary structures of peptides.[3] Although an automatic sequence analyzer based on this principle is now available, thin–layer chromatography (TLC) still plays an important role in this field because it is suitable for the detection of micro amounts of phenylthiohydantoin (PTH) amino acids produced by the Edman degradation. The samples of peptides available for analysis are often small and difficult to replace, so a sensitive detection method for PTH-amino acids in TLC is very desirable.

Tamura *et al.*[4] and Maeda *et al.*[5] reported degradation methods using isothiocyanate derivatives having intrinsic fluorescence. However, these methods require specific conditions for derivatization and separation.

In the preceding papers[6,7] we have reported the micro determination of peptides by means of N-chlorination followed by the oxidation of thiamine with the N-chloropeptides to give fluorescent thiochrome. This paper describes the application of this reaction to the micro-scale detection and determination of PTH-amino acids on thin–layer chromatograms.

Experimental

PTH-amino acids, phenylisothiocyanate (PTC), N,N-dimethylallylamine (DMAA), trifluoroacetic acid (TFA), cycloheptaamylose (C7A), and thiamine hydrochloride were obtained from Tokyo Kasei Kogyo Co. (Tokyo). Antiformin® was obtained from Koso Chemical Co. PTH-amino acids were dissolved in ethylene chloride to give 1 mg/ml. PTH-Asp and PTH-Glu were dissolved in minimum amounts of hot water and further diluted with ethylene chloride to make 1 mg/ml. Thin–layer plates 0.25 mm thick were prepared

1) This paper constitutes part IX of a series entitled "Microanalysis of Proteins and Peptides." Preceding paper, Part V: ref. 5. A preliminary account of this work has been presented: T. Kinoshita, J. Murayama, K. Murayama, and A. Tsuji, *Chem. Pharm. Bull.*, **28**, 641 (1980).
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from Kieselgel G without indicator (E. Merck, Darmstadt) and activated at 110° for 30 min. Fluorescence intensity was measured with a Yamato-Asuka scanning fluorometer, model SFR-21, equipped with a Hitachi recorder, model 056.

Solvents for Thin-Layer Chromatography[8]——Solvent I: *n*-heptane–*n*-propionic acid–ethylene chloride (59: 17: 25). Solvent II: *n*-heptane–*n*-butanol–75% formic acid (50: 30: 9).

Hypochlorite Reagent——Aqueous sodium hypochlorite solution containing 3 w/v % available chlorine was prepared daily by diluting Antiformin® with redistilled water.

Thiamine Reagent——Thiamine hydrochloride (3 g) was added to 100 ml of water, then 0.1 g of sodium carbonate was further added immediately before use.[6]

Procedure——A Kiselgel, plate is spotted with sample solutions and developed with either solvent I or solvent II. After the development, the solvent is removed by heating the plate at 110° for 1 hr. The plate is sprayed with the hypochlorite reagent (25 $\mu g/cm^2$) and allowed to stand for 5 min. Excess chlorine is removed by blowing cold air from a hair dryer on the plate for 20 min. The plate is then sprayed with the thiamine reagent (25 $\mu g/cm^2$) and allowed to stand for 5 min. The plate is viewed using a long wave (365 nm) ultraviolet light source. The fluorescence intensity is measured with a scanning fluorometer.

Edman Degradation——Edman degradation was carried out according to Iwanaga and Samejima[9] and the ethyl acetate extract of PTH-amino acids finally obtained was subjected to TLC as described above.

Results

The fluorescence intensity increased with the concentration of hypochlorite reagent and reached a plateau at 2 to 5 w/v % available chlorine. The fluorescence intensity also increased with the concentration of thiamine reagent and was maximum within the range of 2.2 to 2.5

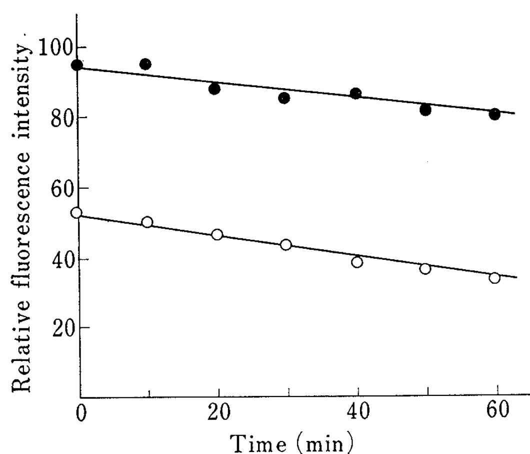

Fig. 1. Stability of Fluorescence Intensity of 1 μg/spot of PTH-Pro (●) and PTH-Leu (○)

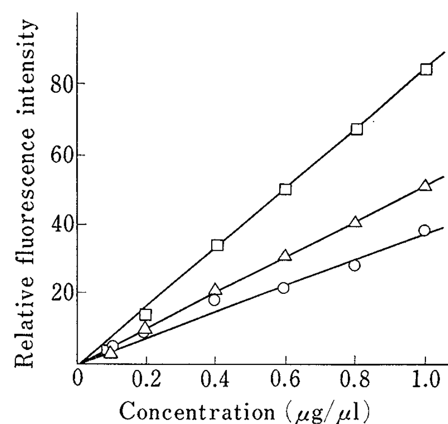

Fig. 2. Standard Curves for PTH-Lys (□), PTH-Leu (△), and PTH-Phe (○)

Table I. Limits of Detection for PTH-amino Acids and Their *Rf* Values

PTH-amino acid	Limit of detection (nmol)	*Rf* value
Leu	0.57	0.85
Ileu	0.44	0.87
Val	0.20	0.77
Met	0.40	0.69
Pro	0.18	0.67
Trp	0.39	0.31
Ala	1.17	0.45
Gly	4.63	0.12

Solvent: *n*-heptane–*n*-butanol-ethylene chloride (58: 17: 25).

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9) S. Iwanaga and Y. Samejima, *Protein, Nucleic Acid and Enzyme* (Japanese), **15**, 1038 (1970).

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w/v% of thiamine. Oxidation of thiamine was accelerated by addition of 0.1 w/v% sodium carbonate to the thiamine reagent. C7A was previously found to enhance and stabilize the fluorescence of thiochrome.[6] The thiamine reagent was therefore enriched with 1.8 w/v% C7A. The same reaction time as that previously used for proteins[6] was also appropriate for PTH-amino acids. The reaction conditions in the standard procedure were accordingly fixed as described above.

Figure 1 shows the stability of fluorescence of PTH-proline and PTH-leucine developed with solvent I. A slow decrease in the fluorescence intensity was observed, whereas the fluorescence of thiochrome was highly stable on Kieselgel layer.[6] However, PTH-amino acids separated better on Kieselgel plates, and the quenching was negligible when the fluorescence intensity was measured within 10 min after spraying the thiamine reagent in the presence of C7A.

The limits of detection for PTH-amino acids in nmol/spot and their *Rf* values are listed in Table I. Excellent sesitivity was observed for all PTH-amino acids tested except PTH-Gly.

Standard curves for PTH-Leu, PTH-Phe and PTH-Lys are displayed in Fig. 2. The curves were linear in the range of 0.1—1.0 μg/spot, and passed through the origin. The coefficient of variation for 4 nmol/spot of PTH-Leu was 5.3% ($n=11$) when each assay was carried out on a different occasion. The difference among the slopes of standard curves may partly be due to the different modes of adsorption of the PTH-amino acids on the Kieselgel layer.

Discussion

Many workers have proposed methods for the detection of PTH-amino acids. A method for their separation and identification on Kieselgel plates has been established by Jeppson and Sjöquist.[8] This method is simple, rapid and reliable, but the detection under UV-light requires large amounts of samples. The iodine–azide test[10] is more sensitive than UV irradiation, but is less sensitive than the present method. The iodine-starch test[11] is specific and applicable to less than 0.5 nmol of PTH-amino acids, but the intensely colored background prevents quantitative estimation of the spotted PTH-amino acids. Chromatography on a fluorescent silica gel plate permits the detections of 0.1 μg of the derivatives.[12] Nevertheless, this method is neither specific nor quantitative. Although an Edman dansyl procedure[13] has recently been proposed as a sensitive tool for sequence analysis of peptides, it is more tedious than the present method.

The present method is highly sensitive for PTH-amino acids other than PTH-Gly. It is not only useful for identification of the terminal amino acid residue of peptides but is also applicable to their quantitation, being suitable for the sequence analysis of branched peptides. The conditions for the separation of PTH-amino acids on silica gel layers, which have been investigated previously, can be utilized without any modification in conjunction with the reagent herein described. Moreover, the determination of proteins is often necessary in laboratories in which the Edman degradation is carried out. The present reagent and technique can be advantageously applied to both microassay of proteins[6] and to Edman degradation.

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