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Note

Fluorescence detection of picomol amounts of the phenylthiohydantoin derivative of histidine in thin-layer chromatography

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The phenythiohydantoin (PTH) derivatives of amino acid residues are employed in protein sequence determination. However, the identification of PTH-histidine, even in high-performance liquid chromatography, can be difficult[1,2]. During the identification of PTH derivatives of amino acids on silica thin-layer plates for manual sequencing of peptides[3] it was noted that when a lamp emitting ultra-violet light at 366 nm was used a bright blue fluorescence was seen at the expected position for PTH-histidine. In fact, the fluorescence was observed at the point of application of an ethyl acetate extract of the PTH derivatives corresponding to sequence cycles in which histidine residues were otherwise identified. Only small amounts of PTH-histidine are extracted into the organic solvent and its identification often depends on chemical reactions, *e.g.*, with Pauly's reagent[4].

In the present report a thin-layer chromatographic (TLC) method for the detection of as little as 60 pmol of the PTH derivative of histidine is described.

MATERIALS

PTH derivatives of amino acids were purchased from Sigma. Thin-layer plates, either high-performance silica gel plates, HP-KF (20 × 10 cm), with a layer thickness of 200 μm and incorporating a fluorescence indicator (Whatman, Clivetown, NJ, U.S.A.) or high-performance silica gel plates without a fluorescence indicator, Merck Art. 13748 (Merck, Darmstadt, F.R.G.), were used. Solvents: acetic acid, propionic acid, *n*-heptane, 1,2-dichloroethane, methanol and ethanol, all pro analysis grade from Merck. A Desaga (Heidelberg, F.R.G.) MinUVIS UV lamp was used.

RESULTS AND DISCUSSION

The initial observations were made on plates developed in a mixture of *n*-heptane, 1,2-dichloroethane and priopionic acid[5]. However, by applying these solvents separately to PTH-histidine, on the thin-layer plates containing a fluorescence indicator, it was found that only treatment with propionic acid was necessary for the development of the fluorescence. Since acetic acid is a satisfactory substitute for the malodorous propionic acid, it was used as the organic acid in the subsequent ex-

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periments. In order to concentrate the PTH derivative it was decided to develop the plates for a short distance, *e.g.*, 1–2 cm, using ethanol–glacial acetic acid (70:30), Fig. 1. The alcohol (ethanol was used instead of the toxic methanol) served to slow down the migration of the PTH-histidine so that it did not appear in the solvent front.

Non-derivatized histidine did not fluoresce under conditions where PTH-histidine exhibited a bright, blue fluorescence; and PTH-histidine did not fluoresce when plates without fluorescein were used (data not shown). No other PTH amino acid derivative, except PTH-histidine, showed any fluorescence when exposed to radiation

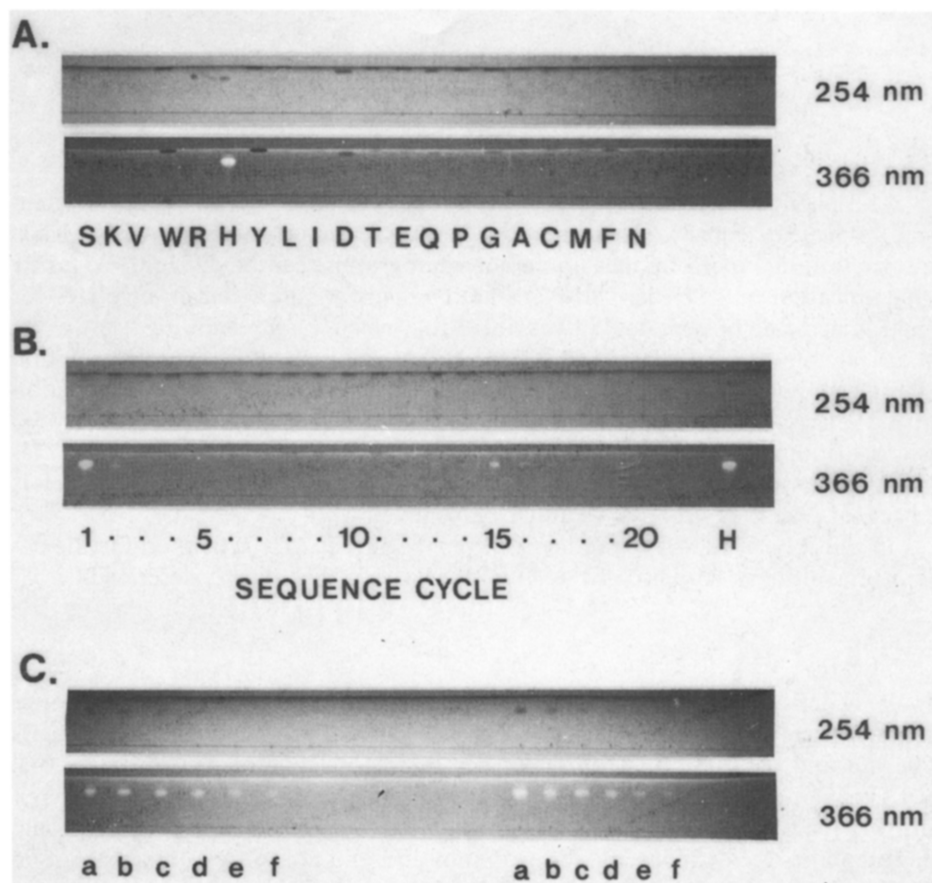

Fig. 1. Visualization of the PTH derivative of histidine on silica gel thin-layer plates containing fluorescein and developed in ethanol–glacial acetic acid (70:30). A, Comparison of the PTH derivatives, each 2 nmol, of different amino acids normally occurring in proteins. The one-letter code for the amino acids is indicated below the spots. Photographs of the same plate were taken during exposure to light of wavelength 254 nm (upper, green fluorescent background) and during exposure to light of wavelength 366 nm (lower, dark blue background). Note that PTH-His (H) is the only PTH amino acid derivative that gives a bright fluorescence when exposed to 366-nm light. B, Identification of PTH-histidine during sequential Edman degradation of an icosapeptide having histidine residues at positions one and fifteen, performed on a Beckman 89OC spinning-cup sequencer. The amount of PTH-His in sequence cycle one was approximately 1 nmol. At the far right 2 nmol of PTH-His standard were spotted. C, Dilution series of PTH-histidine standards: a, 2 nmol; b, 1 nmol; c, 500 pmol; d, 250 pmol; e, 125 pmol; f, 63 pmol.

with the UV light at 366 nm (Fig. 1A). Some PTH amino acid derivatives actually adsorbed such light (Fig. 1A). In fact the PTH-histidine also absorbed this light; this first became apparent when the plates were photographed with a normal black and white film (Agfapan 25) which turned out not to be sensitive to the emitted light and yielded a dark spot for the PTH-histidine. The fluorescence can be fixed on, *e.g.*, Agfachrome R 100S film for colour slides and black and white prints can be obtained therefrom by use of, *e.g.*, Agfaortho 25 Document film (Fig. 1).

It has not been determined whether the fluorescence is caused by the PTH derivative of histidine itself or by some contaminating derivative of histidine. Importantly, however, the fluorescence is observed whenever PTH-histidine is formed during the Edman degradation of peptides. In Fig. 1B the PTH amino acid derivatives from the sequencing of human pancreatic icosapeptide are illustrated; histidyl residues are found in this peptide both at positions one and fifteen[6]. Thus, whether or not the fluorescence is caused by PTH-histidine *per se*, it is nevertheless a potentially useful method for specific identification of histidine residues during sequence analysis.

One potential application of the method is in the identification of peptides with NH_2-terminal histidine such as the glucagon-like peptides that are synthesized together with glucagon[7]. One cycle of the manual Edman degradation could be performed on aliquots from selected chromatographic fractions before spotting them on thin-layer plates. The sensitivity of the method is illustrated in Fig. 1C; it was found that the bluish fluorescence could be used to detect as little as 60 pmol of PTH-histidine. The actual detection limit of the method might well be improved, *e.g.*, through a more elegant spotting technique.

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