Journal of Chromatography, 79 (1973) 344–346

CHROM. 6635

Note

Identification of phenylthiohydantoins of amino acids by thin-layer chromatography

The identification of amino acids as their phenylthiohydantoins (PTHs) is of major importance in sequence determinations of peptides and proteins by the EDMAN degradation technique. The need for a rapid procedure has increased recently because of the desirability of matching the output from the protein sequenator which releases one amino acid derivative from the peptide chain every 90 min[1]. EDMAN[2] recommends thin-layer chromatography for this purpose and runs ten samples simultaneously on thin layers of silica gel on glass plates, with reference mixtures in alternate lanes to assist identification. As a rule, two plates are used, one for chromatography in system H for the resolution of the more polar amino acid derivatives, and one in system D to resolve PTHs with very similar mobilities, in particular isoleucine from leucine and valine from phenylalanine.

Likewise, JEPPSON AND SJÖQUIST[3] recommend a thin-layer procedure for identification of the PTH-amino acids but use successive solvent systems to effect resolution of all the derivatives. This method is advantageous when the amount of sample is limited because only one plate is required for the identifications. The purpose of this note is to report an improved EDMAN H system[2] which, in conjunction with ninhydrin colour reactions of the PTH-amino acids, allows identification to be made after only one chromatographic step.

The recommended system is as follows. The ratio of ethylene chloride to acetic acid in the solvent is 60:7 (*cf.* EDMAN[2], 60:14). In preference to coated glass plates, the chromatography is carried out on silica gel containing a fluorescent dye (Merck, Kieselgel 60F 254*) on an aluminium plate, which is developed for a distance of 12.5 cm. The pre-coated aluminium plates give smaller, more discrete spots but the most important improvement is to the relative R_F values of PTH-isoleucine and PTH-leucine. A small but reproducible difference, equivalent to that in the EDMAN D system[2], is obtained for these derivatives. We have also found the pre-coated aluminium plates to have other advantages over the glass plates prepared in the laboratory; they are more resistant to abrasion, the PTH mixtures exhibit more reproducible R_F values across the plate, and the colour reactions are more permanent.

Fig. 1 illustrates the results obtained with the modified system and shows the separations in systems D and H of EDMAN[2] for comparison. It shows that PTH-isoleucine and PTH-phenylalanine are resolved from PTH-leucine and PTH-valine, respectively. PTH-aspartic acid, PTH-glutamic acid and PTH-tyrosine show increased separations. Samples from our sequenator generally behave normally in the system but occasionally the non-polar derivatives run slightly slower than the standards. This effect was not observed when fresh PTH standard mixtures were used. We have found that loading the samples in ruled lanes[2] gives more compact spots and hence

* The original plates (Kieselgel F 254) were found to be unsatisfactory for PTH separations.

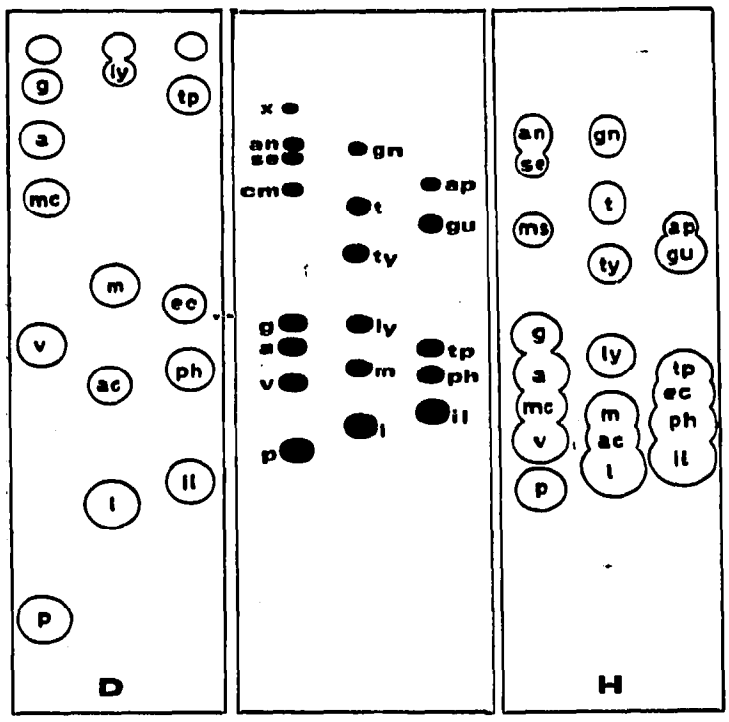

Fig. 1. Chromatography of PTH-amino acids on a pre-coated aluminium plate* using ethylene chloride–acetic acid solvent with the EDMAN[2] systems D and H** for comparison. PTH derivatives of S-carboxymethylcysteine product (X), asparagine (an), glutamine (gn), serine (se), aspartic acid (ap), S-carboxymethylcysteine (CM), glutamic acid (gu), methionine sulphone (ms), tyrosine (ty), glycine (g), lysine (ly), alanine (a), tryptophan (tp), S-ethylcysteine (ec), S-methylcysteine (mc), methionine (m), phenylalanine (ph), valine (v), S-allylcysteine (ac), isoleucine (il), leucine (l) and proline (p).

more sensitive detection of the fastest moving derivatives, that is, PTH-leucine, PTH-isoleucine and PTH-proline. The hydrolysis products of phenyl isothiocyanate—phenylthiourea (PTU) and diphenylthiourea (DPTU)—are usually well resolved from the amino acid derivatives; PTU appears in a clear area behind PTH-glycine and DPTU between PTH-proline and PTH-leucine. The increased separation of PTH-aspartic acid and PTH-glutamic acid is also useful in identification because these acids are formed from their amides during conversion to the phenylthiohydantoins.

A comparison of the two H systems in Fig. 1 indicates that a reduction in the proportion of acetic acid in the solvent markedly affects the relative positions of some of the PTH derivatives and these data could therefore be used to alter the separation of such amino acids if desired. The most useful application is to the identification of PTH-(S-carboxymethyl)cysteine (PTH-CMC), which is difficult to distinguish from PTH-aspartic acid and PTH-glutamic acid in the EDMAN system[2]. PTH-CMC runs just ahead of PTH-aspartic acid in our recommended system, but it can be completely resolved if the proportion of acetic acid in the mixture is increased. However,

* Plates should be stored over P_2O_5 to remove excess moisture.

** System D: xylene. Plate soaked in formamide–acetone (1:3) and air-dried. System H: ethylene chloride–acetic acid (30:7). Paper lining soaked with solvent.

during sequencing, it is common to obtain an additional spot from the CMC residue, and this second spot (X in Fig. 1), which runs between the origin and PTH-asparagine in the present system, simplifies the identification of PTH-CMC.

To eliminate ambiguities and/or to provide confirmatory evidence for certain residues from the thin-layer chromatogram we have used a ninhydrin–collidine spray as recommended by ROSEAU AND PANTEL[4]. With the aluminium-backed plates, the heating time can be reduced to 8 min at 107° (*cf.*, 15 min at 110°), and the reactions for the non-polar PTH-amino acids are more sensitive. Hence, in contrast to the findings of ROSEAU AND PANTEL[4] (and our own on coated glass plates), the PTHs of valine, phenylalanine, leucine and isoleucine gave strong blue–violet colorations. Presumably the aluminium plates permit a more rapid heat transfer, which minimizes thermal decomposition and allows maximum colour development. PTH-proline is least sensitive but gives a white spot with a blue halo at twice the concentration of the other amino acid derivatives. PTH-CMC gives an intense pink colour (with a yellow centre at high concentrations), which distinguishes it from the weaker pink colour of PTH-aspartic acid. The remaining amino acid derivatives can usually be identified by their differences in colour.

After the colours have been developed with ninhydrin, an additional spray with a 0.1 % solution of copper nitrate in absolute ethanol and brief heating (3 min at 107°) changes the colours of most of the spots.

For example, the rather weak PTH-asparagine spot can be positively identified at low concentrations because it takes on a more intense yellow colour. This treatment is particularly useful for confirming the identification of either PTH-valine or PTH-phenylalanine. The copper spray produces pink colorations with the valine, leucine and isoleucine derivatives, whereas PTH-phenylalanine turns yellow.

The procedures described here have facilitated the identification of amino acid derivatives from the sequenator and have greatly decreased the need for confirmation by supporting techniques such as gas chromatography and amino acid analysis.

The authors gratefully acknowledge the skilful technical assistance of Mr. P. M. STRIKE.

Division of Protein Chemistry, CSIRO, A. S. INGLIS
Parkville (Melbourne), Victoria 3052 (Australia) P. W. NICHOLLS

1 P. EDMAN AND G. BEGG, *Eur. J. Biochem.*, 1 (1967) 80.
2 P. EDMAN, in S. B. NEEDLEMAN (Editor), *Protein Sequence Determination*, Springer-Verlag, Berlin, 1970.
3 J. O. JEPPSON AND J. SJÖQUIST, *Anal. Biochem.*, 18 (1967) 264.
4 G. ROSEAU AND P. PANTEL, *J. Chromatogr.*, 44 (1969) 392.

Received January 8th, 1973