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Note

Improved thin-layer chromatography technique for the identification of phenylthiohydantoin amino acids

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Automated amino acid sequencing[1,2] has been refined[3] sufficiently for investigators to anticipate the successful completion of 10–25 cycles on larger peptides or proteins and near completion of smaller peptides. To obtain quantitative as well as qualitative results, gas chromatography (GC)[4] is a frequently employed tool but some ambiguities remain. This is especially true with respect to phenylthiohydantoin (PTH) amino acids having nearly identical retention times; for example, threonine and glycine, proline and threonine, δ-carboxymethyl cysteine and serine. In addition, the deamidation of glutamine or asparagine may result in their incorrect identification as the acid, due to a lowered sensitivity of the GC system. An alternate, sensitive method for the verification of PTH amino acids is important.

For these reasons, we improved existing techniques for thin-layer chromatography (TLC). We utilized the information of Inagami and Murakami[5] and designed a rapid, color-coded system for the complete resolution of all of the PTH amino acids commonly encountered. In this system, only the PTH derivatives of dehydrothreonine, valine, leucine and isoleucine fail to produce a characteristic color. This analytical system is completed within 1 h for the identification of at least seven steps of an automated Edman degradation.

MATERIALS AND METHODS

Plastic-backed, precoated silica gel TLC plates without fluorescent indicator were purchased from Eastman-Kodak (Rochester, N.Y., U.S.A.; Cat. No. 13179). These plates were cut in half making them 10 × 20 cm, and the uncut surface was used as the lower plate edge. Solvents for chromatography were purchased as analytical or Sequanal grade solvents. Reference PTH amino acids were purchased from Pierce (Rockford, Ill., U.S.A.).

Each plate was scored to give twenty 9-mm channels; a 1-cm edge was left on each side of the TLC plate. The borders were scraped with a razor blade to remove approximately 4 mm of silica gel from each side, thus protecting the outside channels from distortions. The origin was placed 3 cm from the lower edge of our plates.

The samples from each Edman cycle were converted to their PTH derivatives[2], extracted with ethyl acetate, dried under nitrogen and reconstituted in a fixed volume

of ethyl acetate. These samples were scanned on a Beckman DK-2A to quantitate yields[6] and to approximate sample concentrations for their application to the TLC plates. Typical scans for the PTH derivatives of serine, lysine, threonine and proline[2] were used in the final identification of these residues. PTH derivatives of arginine and histidine remained in the aqueous phase and were identified by specific spot tests[7,8].

TLC was carried out with seven PTH steps per plate. Approximately 2.5 nmoles of each step were applied to their respective channel on the left-hand side of the plates and the three reference samples were spotted next to a blank channel on this side of the plate. The blank channels were used for labeling each of the reference PTH derivatives. The last seven channels were spotted with 5 nmoles each of the same seven steps. After completion of the chromatography and drying of the plates, these latter seven channels were separated from the reference side by cutting the plate with scissors. Two plates were identically prepared for each set of seven steps. One plate was developed in a xylene–methanol (80:10) system (XM)[5] and the other was developed in heptane–ethylene dichloride–propionic acid (45:25:30) system (VM)[9]. Ascending chromatography required approximately 20–25 min at ambient temperature. The plates were removed, dried at 90° and examined under UV light. The PTH derivatives of proline, phenylalanine, tyrosine, tryptophan, aspartic acid, serine and δ-carboxymethyl cysteine gave positive UV spots. PTH derivatives of tyrosine and tryptophan were yellow prior to staining. The portions of the plates including the references were sprayed with iodine azide[2], while the other sections (5 nmoles) were sprayed with a solution of 1.7% ninhydrin in methanol–collidine–acetic acid (15:2:5)[10,11]. The ninhydrin plate was again heated at 90° for 20 min. The ninhydrin colors developed and certain characteristic changes could be detected by blowing a saturated ammonia atmosphere over the ninhydrin plate.

Automated Edman degradations were carried out with a Beckman 890C sequencer and the resulting PTH derivatives were identified by GC using a programmed temperature increase from 180° to 285° (initial time, 2 min; final time, 3 min; temperature increase, 7.5°/min) and by the described TLC method.

RESULTS AND DISCUSSION

The resolution of the described solvent systems is shown in Fig. 1. In the above systems, leucine–isoleucine must be resolved by an alternate method, usually GC. The protein or peptide material being studied was reduced and alkylated[12] using

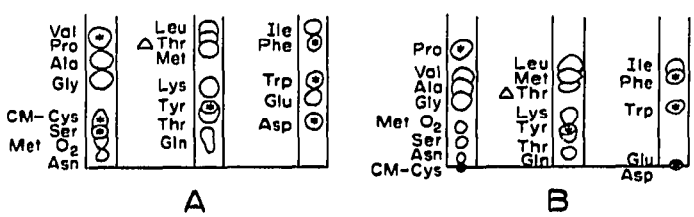

Fig. 1. Ascending TLC chromatography profile at ambient temperature for PTH amino acids. (A) Solvent VM. (B) Solvent XM. Fresh, unlined tanks were prepared after three runs; solvents older than three days were also replaced. The spots with an asterisk indicate UV-positive PTH derivatives. Δ Thr = dehydrothreonine; CM-Cys = δ-carboxymethyl cysteine.

tritiated iodoacetic acid and the labeled δ-carboxymethyl cysteine could be distinguished from aspartic acid on the basis of radioactivity. A major serine breakdown product having the same mobility as lysine in solvent VM, but a slightly greater R_F value than lysine in solvent XM, gave a different color on the ninhydrin plate, especially in an ammonia atmosphere. All other residues were resolved, either on the basis of R_F value or color reaction or both. Minor quantities of glutamine or asparagine still produce intense, characteristic colors. PTH derivatives which had no color reaction after ninhydrin spray were analyzed by GC (Table I).

TABLE I

CHARACTERISTICS OF PTH AMINO ACIDS FOLLOWING NINHYDRIN SPRAY

PTH derivative	*Color properties*	*NH_4OH color change*
Valine	colorless; use GC	
Proline	UV; colorless	light blue after heating
Alanine	purple	deeper color
Glycine	orange	
Serine	UV; purple	
Serine breakdown	faint orange	weak red
Asparagine	yellow	more intense
δ carboxymethy Cysteine	UV; purple	
Methionine sulfone	light tan	
Leucine	colorless; use GC	
Methionine	faint tan	
Lysine	very faint pink	weak blue after heating
Tyrosine	UV; yellow before spray	intense yellow
Threonine	colorless	light tan
Dehydrothreonine	colorless*	
Glutamine	dark green	dark blue
Isoleucine	colorless; use GC	
Phenylalanine	UV; colorless	faint yellow
Tryptophan	UV; yellow before spray	deep yellow
Aspartic acid	UV; pink	darker
Glutamic acid	grey	dark blue

* Characteristic scan at 320 nm.

The colors produced in this system easily permit the identification of those amino acids having nearly identical R_F values; for example, lysine and a serine degradation product, alanine, methionine and phenylalanine, and tyrosine and threonine. In addition, GC is an inadequate tool for identifying the PTH derivatives serine, lysine, glutamic and aspartic acids and their respective amides. The TLC system described here consistently gave positive identification of these residues after the GC became ambiguous. The formation of trimethylsilyl derivatives[4] of glutamine and asparagine usually results in their partial deamidation, making their positive identification more difficult. The colors produced by aspartic acid (pink) and asparagine (yellow) permit their positive identification, even when partial deamidation has occurred. The degeneration of serine following repetitive exposure to acid in the sequence procedure yields a PTH derivative which is not identified by GC, but can be distinguished both by its color and its multiple spot pattern on the iodine azide plate. The sensitivity of the technique is greatest for those PTH derivatives which fail to be resolved by GC.

As our system is currently designed, we can adequately identify seven steps from an automated Edman degradation in 1 h by TLC and have the residue identified on the basis of its color and R_F value from the iodine azide developed portion of the plate. In this same time interval, only two samples can be identified by GC. We have recently eliminated the iodine azide spray to permit the identification of fourteen automated degradation steps on one TLC plate, but feel that this procedure is best left to the discrimination of the individual investigator. We have utilized this TLC technique to complete the sequence of the non-thrombin portion of bovine prethrombin[13].

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