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Note

Spray reagents for the detection of *p*-phenylazophenylthiohydantoins of amino acids on silica gel plates

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The standardisation of a suitable method for identifying *p*-phenylazophenylthiohydantoins (PAPTHs) of amino acids is essential because they are degraded stepwise from a portein in the determination of N-terminal amino acid sequences[1,2]. We have already reported a thin-layer chromatographic (TLC) technique for identifying PAPTHs of amino acids in only 30 min[3]. Although the procedure is rapid and requires only two solvent systems with the same components in different concentrations, the R_F values of some of the PAPTHs are very close and it may therefore sometimes be difficult to identify such PAPTHs satisfactorily when determining the N-terminal amino acid sequence of a protein.

For the detection of colourless amino acids or colourless phenylthiohydantoins of amino acids on paper or TLC plates, various spray reagents have been used[4–7]. In this study we investigated several spray reagents for revealing PAPTHs of amino acids separated by ascending TLC.

EXPERIMENTAL

Preparation of p-phenylazophenylthiohydantoins (PAPTH) of amino acids

PAPTHs of amino acids were prepared and identified by TLC according to Datta and Datta[3]. An amino acid was dissolved in water and the pH was adjusted to 11.8. *p*-Phenylazophenyl isothiocyanate was dissolved in dioxan and added to the amino acid solution. The pH was again adjusted to 11.8 and the solution kept in the cold for 2 h. It was then extracted with benzene and the aqueous layer was freeze-dried. The residue was dissolved in hydrochloric acid–glacial acetic acid mixture and kept overnight. Next day the solution was freeze-dried and the residue extracted with ethyl acetate. The ethyl acetate extract was evaporated to dryness under vacuum to give crystals of the PAPTH of the amino acid.

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Identification on TLC plates

After TLC, the plates were sprayed with nine reagents.

Reagent I. (A) The TLC plates were dried and exposed to the vapour of diethylamine for 30 min, sprayed with a 0.25% solution of ninhydrin in *n*-butanol and heated for 15 min at 80°[4].

(B) A mixture of diethylamine (20 ml) and a 0.25% solution of ninhydrin in *n*-butanol (10 ml) was sprayed on the TLC plates, which were then dried at 80° for 15 min. The plates were subsequently sprayed with a 0.25% solution of ninhydrin in *n*-butanol and dried at 80° for 20 min.

TABLE I

TLC PLATES SPRAYED WITH REAGENTS IA AND IB

PAPTH of amino acid	*Colour*	
	Reagent IA	*Reagent IB*
dl-Glycine	Brownish yellow, yellow ring followed by cream ring	Violet, brown ring followed by light yellow ring
dl-Alanine	Grey with blue tinge, white ring	Deep violet, brown ring followed by pale yellow ring
l-Threonine	Brown	Pink, violet ring followed by cream ring
l-Proline	Light green, cream ring followed by violet ring	Deep violet, light violet ring followed by deep brown ring
l-Tyrosine	Pink, deep yellow ring	Pink, violet ring followed by creamish yellow ring
dl-Valine	Violet, pink ring followed by violet, brown and orange rings	Pink, violet ring followed by yellow ring
l-Glutamine	Crimson, yellow ring	Yellow, violet ring
dl-Serine	Bluish pink, white ring followed by very light pink and violet rings	Pink, violet ring followed by brown, cream rings
l-Leucine	Greyish brown, pinkish cream ring	Deep violet brown ring followed by pale brownish yellow ring
l-Isoleucine	Violet, white ring followed by light pink ring	Violet, pink ring followed by grey, yellowish white rings
dl-Aspartic acid	Brown, cream ring followed by yellow and brown rings	Violet, brown ring followed by deep yellow ring
l-Hydroxyproline	Cream, violet ring	Light pink, cream ring
l-Asparagine	Yellow	Purple
l-Glutamic acid	Violet, creamish pink ring followed by violet ring	Deep violet, pink ring followed by violet, grey and cream rings
l-Phenylalanine	Violet, cream ring followed by violet and light pink rings	No change
dl-Methionine	Brown, yellow ring	Violet, orange ring followed by pale yellow ring
l-Cystine	Light violet, brown ring followed by cream ring	Brownish violet, brownish yellow
l-Lysine	Pink with grey tinge, white ring	Violet, cream ring
l-Tryptophan	Yellow, brown ring	Violet, yellow ring
l-Arginine	Light pink, light violet ring followed by cream ring	No change
l-Histidine	Grey, brown ring followed by cream ring	Deep pinkish brown, cream ring

Reagent II. (A) The spots on the TLC plates were sprayed with a 1% solution of phenylnaphthylamine in *n*-butanol, then dried at 60°.

(B) The spots on the TLC plates were sprayed with reagent IIA, then dried at 100°. The colours of the spots were observed against a pink background.

(C) The TLC plates after treatment with reagent IIB were sprayed with a 0.25% solution of ninhydrin in *n*-butanol, then dried at 110°. The colours of the spots were observed against a dirty yellow background.

Reagent III. The spots on the TLC plates were sprayed with cyclohexylamine reagent, then dried at 110° for 1 h. All of the spots turned yellow. The plates were

TABLE II

TLC PLATES SPRAYED WITH REAGENTS IIA, IIB AND IIC

PAPTH of amino acid	*Colour*		
	Reagent IIA	*Reagent IIB*	*Reagent IIC*
dl-Glycine	Brown	Light brown	Orange
dl-Alanine	Purplish pink, grey ring	Pink, white ring followed by blue and brown rings	Crimson, purple ring
l-Threonine	Pink, light yellow ring	No change	No change
l-Proline	Light sea green	Deep sea green	Brown, violet blue ring
l-Tyrosine	Pink, lemon yellow ring	Very light pink, grey ring followed by yellow ring	Light sea green, light brown ring followed by deep blue and light green rings
dl-Valine	Pink, violet ring followed by yellow ring	Very light pink, lemon yellow ring	Orange, blue ring
l-Glutamine	No change	No change	Purple
dl-Serine	Light grey, light pinkish mauve ring	Pink, white ring followed by blue and brown rings	Light sea green, light brown ring followed by deep blue and light green rings
l-Leucine	Buff	Light pink, grey ring followed by yellow ring	Grey, blue ring followed by sea green ring
l-Isoleucine	Light buff, lemon ring	Very light pink, greyish green ring followed by yellow ring	Greenish yellow, deep blue ring
dl-Aspartic acid	Crimson, red ring followed by golden yellow ring	Light crimson, light red ring followed by light golden yellow ring	Deep pink, purple ring followed by orange and green rings
l-Hydroxyproline	Light yellow	No change	No change
l-Asparagine	Greyish black	Grey	Cream
l-Glutamic acid	Pink, yellow ring	Pink, violet ring followed by lemon yellow ring	Orange, blue ring
l-Phenylalanine	Light sky blue	Light green	Parrot green
dl-Methionine	Pale pink	Pink, lemon yellow ring	Yellow, pinkish green
l-Cystine	Pink, violet ring followed by brown and yellow rings	Bluish green	Pink, deep blue ring
l-Tryptophan	Green	Blue, violet ring	Lemon yellow
l-Arginine	Light green	Bluish green	Yellow, blue ring
l-Histidine	Pinkish grey	Light pink, green ring	Pink, green ring

subsequently sprayed with a 0.25% solution of ninhydrin in *n*-butanol, then dried at 80° for 15 min.

Reagent IV[5]. A mixture of a 0.2% solution of ninhydrin in absolute ethanol (25 ml), glacial acetic acid (5 ml) and 2,4,6-collidine (1 ml) was mixed with a 1% solution of copper(II) nitrate trihydrate in absolute ethanol in the proportions 25:1.5 just before use, then sprayed on the TLC plates.

Reagent V. (A) Isatin (1 g), zinc acetate (1.5 g), pyridine (1 ml) and isopropanol (100 ml) were mixed according to Barrollier *et al.*[6] and sprayed on the TLC plates.

(B) Isatin (1 g), zinc acetate (1.5 g), glacial acetic acid (1 ml), isopropanol (95 ml) and water (5 ml) were mixed and sprayed on the TLC plates[6].

RESULTS AND DISCUSSION

The colours of the spots of PAPTHs after spraying with the different reagents are given in Tables I–IV.

TABLE III

TLC PLATES SPRAYED WITH REAGENT III

PAPTH of amino acid	*Colour*
dl-Glycine	Greenish green, yellow ring followed by violet ring
dl-Alanine	Brownish purple, light pink ring followed by violet ring
dl-Threonine	Grey, pink ring
l-Proline	Greenish grey, pink ring followed by cream and violet rings
l-Tyrosine	Pink, violet ring followed by yellow ring
dl-Valine	Pink, violet ring followed by yellow ring
l-Glutamine	Yellow, white ring
dl-Serine	Greenish purple, violet ring followed by whitish pink and purple rings
l-Leucine	Blue, pink ring followed by light violet ring
l-Isoleucine	Pink, yellow ring
dl-Aspartic acid	Green, deep yellow ring followed by pink and purple rings
l-Hydroxyproline	Pink, violet ring followed by orange ring
l-Asparagine	Purple
l-Glutamic acid	Lemon yellow with violet tinge
l-Phenylalanine	Pink, light purple ring followed by pink ring
dl-Methionine	Light purple, orange ring followed by violet ring
l-Cystine	Greenish purple, orange ring followed by violet ring
l-Lysine	Bluish grey, pink ring followed by violet ring
l-Tryptophan	Lemon yellow, violet ring
l-Arginine	Orange, purple ring
l-Histidine	Light grey, pink ring

It can be seen that the original red colour was chagned. Moreover, the colours of the derivatives of the various amino acids were different with every spraying agent in all but a few instances. When reagent VA was sprayed on the TLC plates, the PAPTHs of proline and aspartic acid gave blue and reddish pink colours, respectively. The original colour of other PAPTHs vanished. With reagent VB, the colours given by the PAPTHs of proline and aspartic acid were deep violet and red, respectively. The colours given by the PAPTHs of histidine, hydroxyproline, serine and glutamic acid were light pink. The original colour of other PAPTHs vanished.

TABLE IV

TLC PLATES SPRAYED WITH REAGENT IV

PAPTH of amino acid	*Colour*	
	When hot	*When cooled to room temperature*
dl-Glycine	Deep yellow	Light yellow
dl-Alanine	Deep greyish purple	Grey
dl-Threonine	Deep mauve	Deep mauve
l-Proline	Very light orange	Violet
l-Tyrosine	White	Yellow
dl-Valine	Light purple	Light purple
l-Glutamine	Deep mauve	Deep mauve
dl-Serine	White	Deep mauve, purple ring
l-Leucine	Pinkish purple	Pinkish purple
l-Isoleucine	White	Very light pink
dl-Aspartic acid	Grey, turning black	Yellow
l-Hydroxyproline	Light pink	Light pink
l-Asparagine	Buff	White, lemon yellow ring
l-Glutamic acid	Deep purple	Yellow
l-Phenylalanine	No change	No change
dl-Methionine	Light purple	Deep purple
l-Cystine	No change	No change
l-Lysine	Light pink	Purplish pink
l-Tryptophan	Deep violet	Yellow, with violet ring
l-Arginine	No change	No change
l-Histidine	Pinkish orange	Pinkish orange

In previous work[3] we used a second solvent system to separate the PAPTH of threonine from aspartic acid, that of serine from histidine and that of valine from phenylalanine. When spray reagents are used the colours of these derivatives differ. Thus the need for a second solvent system[3] can easily be overcome. With every spray reagent the colours of the PAPTHs of some amino acids are the same, and in such instances more than one spraying agent should be used.

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