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SEPARATION OF TWO TYPES OF DIPHENYLINDENONE DERIVATIVES OF AMINO ACIDS (ITH- AND DIS-AMINO ACIDS) APPLICABLE TO SEQUENCING OF PROTEINS ON POLYAMIDE SHEETS

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SUMMARY

Several chromatographic systems are proposed that provide the possibility of determining all amino acids commonly found in proteins as diphenylindenone thiohydantoins and diphenylindenone sulphonamides at the picomole level using two-dimensional thin-layer chromatography on polyamide sheets.

INTRODUCTION

2-*p*-Isothiocyanophenyl-3-phenylindenone or diphenylindenonyl isothiocyanate (DIITC) was proposed as a coloured reagent for sequencing proteins and peptides[1]. With the corresponding sulphochloride 2-*p*-chlorsulphophenyl-3-phenylindenone[2] or diphenylindenonesulphonyl chloride (DIS-Cl), a highly sensitive fluorescent method for the detection of N-terminal groups of peptides and proteins was developed[3].

Using DIITC, the N-terminal amino acid was identified on a thin layer of silica gel G as a coloured (diphenyl)indenonylthiohydantoin (ITH) derivative[4] or as a fluorescent isobenzofuran derivative[5]. With DIS-Cl, the N-terminal amino acid was identified on a thin layer of silica gel G as a coloured diphenylindenonesulphonyl (disyl or DIS) amino acid or as a fluorescent isobenzofuran derivative[3] using solvent systems described in an earlier paper[6].

These two types of diphenylindenone derivatives, ITH-amino acids and DIS-amino acids, can be used in a double checking technique in the sequence analysis of proteins. The purpose of this work was to find suitable solvent systems for the separation on polyamide sheets of ITH and DIS derivatives of the amino acids found in native proteins.

EXPERIMENTAL

Polyamide sheets

Cheng-Chin polyamide sheets (15 × 15 cm) from BDH Chemicals, Poole, Great Britain, were cut into 5 × 5 cm squares.

Preparation of ITH derivatives

ITH-amino acids were synthesized, purified and characterized as described earlier[7,8]. These compounds were obtained in micromole amounts in the following way[1]. To a solution of 3 μmole of amino acid in 0.3 ml of 0.4 *M* dimethylallylamine buffer (pH 9.6) was added 0.3 ml of a pyridine solution of 3.39 mg (10 μmole) of DIITC. The reaction was carried out at 40° under a nitrogen atmosphere. After 2 h, the solution was evaporated to dryness *in vacuo* over phosphorus pentoxide and potassium hydroxide. The residue was dissolved in 0.2 ml of a mixture of acetic acid and 6 *M* hydrochloric acid (5:1) and the solution was warmed for 10 min at 80° under nitrogen. The solvents were evaporated over potassium hydroxide. On thin-layer chromatograms the spot of ITH derivative was easily distinguished from the other spots, due mainly to the unreacted reagent and bisdiphenylindenonylthiourea, by its colour and R_F value.

Preparation of DIS derivatives

The preparation, purification and IR characterization of the DIS-amino acids were described earlier[9,10]. For chromatographic purposes, as described earlier[6], DIS-amino acids can be prepared by adding DIS-Cl dissolved in acetone (1 mg/ml) to an equal volume of a 0.1 *M* sodium hydrogen carbonate solution of 5 nmole of amino acid or of an amino acid mixture (the concentration of each amino acid being 5 nmole/ml). After leaving the solution for 3 h in a closed tube at room temperature, it was evaporated to dryness at low pressure. The residue was dissolved in methanol and aliquot volumes were applied on the chromatogram.

The solvents *n*-pentane, benzene, toluene, *n*-butanol, acetic acid, propionic acid and ethylene chlorhydrin were re-distilled.

Chromatography

ITH-amino acids in acetone or methanol solutions and DIS-amino acids in methanol solutions were applied on polyamide sheets (5 × 5 cm) using a microcapillary tube. The samples were spotted at a distance of 0.5 cm from the edge of the sheet. For optimal separation, the diameter of the spots should not exceed 2 mm. The chromatograms were developed in a 250-ml closed glass chamber, containing 10 ml of solvent system and filter-paper for saturation of the atmosphere. A pre-equilibration of about 10–15 min was used before immersion of the chromatogram. When the solvent front reached the edge of the sheet the latter was removed and dried in a stream of hot air. For the development of a two-dimensional chromatogram the sheet was chromatographed in two solvent systems in perpendicular directions with intermediate drying. The same solvent system was used for about five chromatograms, then replaced with fresh solvent.

ITH-amino acids and DIS-amino acids are coloured compounds and, when spotted in amounts greater than 0.1 nmole, are detected on polyamide sheets as yellow or yellow-orange spots. To detect smaller amounts, the polyamide chromatogram was observed under UV light (365 nm). Diphenylindenone derivatives were detected as dark violet spots on light blue background as a result of the increased absorption of UV light due to the aromatic system (diphenylindenone residue). On treatment of the polyamide sheet with sodium ethoxide solution (5 g of sodium per 100 ml of 96% ethanol) diphenylindenone derivatives were detected as yellow-green fluorescent spots under UV light[3,5].

For repeated use of the polyamide sheets, the layers were washed with the solvents suggested by Wang and Wu[11]: acetone–85% formic acid (9:1) and acetone–29% ammonia (9:1). The chromatogram must be washed immediately after chromatography and location of the spots in order to avoid irreversible adsorption on the polyamide. If the sheet has been treated with sodium ethoxide for UV detection, preliminary washing with water is necessary before dipping it in the above rinse solution. Under these conditions of washing, the sodium ethoxide detection does not decrease the possibility of repeated use of the polyamide sheet.

RESULTS AND DISCUSSION

Separation of ITH-amino acids

Using published data for the separation of phenylthiohydantoin amino acids[12,13] and on the basis of our own investigations, the following solvent systems can be proposed as suitable for the separation of ITH-amino acids: I, toluene–*n*-pentane–glacial acetic acid (60:30:15); II, 60% aqueous acetic acid. The running time in solvent I is 15 min and in solvent II 60 min.

The two solvent systems can be used separately for one-dimensional and in combination for two-dimensional chromatography of ITH-amino acids. A two-dimensional chromatogram for the separation of ITH derivatives of 22 amino acids, DIITC and its by-products in sequencing [mono(diphenyl)indenonylthiourea (MITU) and bis(diphenyl)indenonylthiourea (BITU)] is presented in Fig. 1. On the starting point, 0.5 cm from both edges of the sheet, a mixture containing 0.5 nmole of each shown compound, dissolved in acetone-methanol (1:1), was applied. After consec-

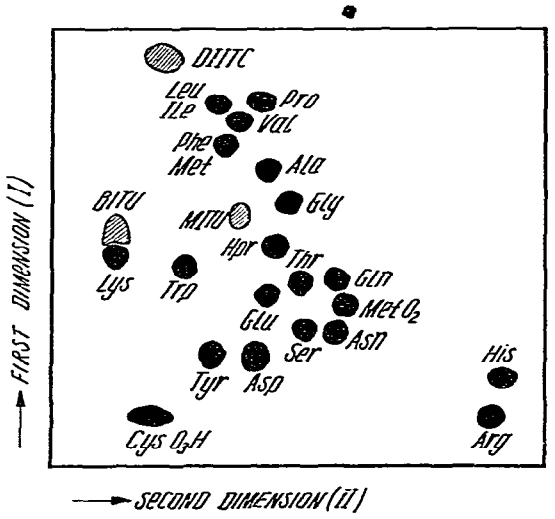

Fig. 1. Two-dimensional separation of 22 (diphenyl)indenonylthiohydantoin (ITH) amino acids, 2-*p*-isothiocyanophenyl-3-phenylindenone (DIITC), mono(diphenyl)indenonylthiourea (MITU) and bis(diphenyl)indenonylthiourea (BITU) on Cheng-Chin polyamide sheet. Solvents: first dimension, solvent I [toluene–*n*-pentane–glacial acetic acid (60:30:15)], 4.5 cm, 15 min; second dimension, solvent II (60% aqueous acetic acid), 4.5 cm, 60 min. ITH derivatives are indicated by abbreviations for the corresponding amino acids; Cys O_3H = cysteic acid; Met O_2 = methionine sulphone.

utive development in two perpendicular directions in systems I and II, 23 coloured spots were detected on the chromatogram. Only the ITH-derivatives of leucine and isoleucine and those of phenylalanine and methionine remained unseparated. ITH-methionine and ITH-phenylalanine can readily be separated in solvent III: *n*-butanol–glacial acetic acid (9:1) (R_F values 0.63 ± 0.01 and 0.71 ± 0.01, respectively). Also in solvent III ITH-leucine and ITH-isoleucine have similar R_F values of 0.52 ± 0.01 and 0.55 ± 0.01, respectively, but their relative positions are very reproducible and their differentiation is reliable.

The spots of ITH-lysine and BITU are close together, but they differ in colour and shape. On the other hand, BITU is usually extracted completely in sequencing. The difference between the colour of the ITH-amino acids (yellow and yellow-orange) and the colour of the reagent and its thiourea products (pink-red) facilitate the identification of the N-terminal amino acid as a coloured spot.

R_F values of the ITH-amino acids, DIITC and its by-products in the proposed solvent systems are presented in Table I (average values from 21 determinations).

The sensitivity of the colour and UV detection of the ITH-amino acids is shown in Fig. 2. On starting points 1–6 of a one-dimensional chromatogram were

TABLE I

R_F VALUES OF ITH-AMINO ACIDS, 2-*p*-ISOTHIOCYANOPHENYL-3-PHENYLINDENONE (DIITC) AND MONO- AND BIS(DIPHENYL)INDENONYLTHIOUREAS (MITU AND BITU) ON POLYAMIDE SHEETS

Distance: 4.5 cm.

ITH-amino acid	*Solvent system*		
	I	*II*	*III*
Alanine	0.61 ± 0.02	0.37 ± 0.02	–
Arginine	0.03 ± 0.01	0.88 ± 0.02	–
Asparagine	0.18 ± 0.02	0.48 ± 0.02	–
Aspartic acid	0.12 ± 0.01	0.32 ± 0.02	–
Cysteic acid	0.00	0.06 ± 0.02	–
Glutamine	0.29 ± 0.03	0.50 ± 0.02	–
Glutamic acid	0.25 ± 0.03	0.35 ± 0.02	–
Glycine	0.52 ± 0.02	0.41 ± 0.02	–
Histidine	0.12 ± 0.02	0.90 ± 0.02	–
Hydroxyproline	0.35 ± 0.03	0.39 ± 0.02	–
Isoleucine	0.75 ± 0.02	0.26 ± 0.02	0.55 ± 0.01
Leucine	0.75 ± 0.02	0.24 ± 0.02	0.52 ± 0.01
Lysine	0.31 ± 0.01	0.01 ± 0.00	–
Methionine	0.65 ± 0.02	0.27 ± 0.03	0.63 ± 0.01
Methionine sulphone	0.23 ± 0.02	0.50 ± 0.02	–
Phenylalanine	0.67 ± 0.03	0.27 ± 0.02	0.71 ± 0.01
Proline	0.84 ± 0.03	0.32 ± 0.03	–
Serine	0.18 ± 0.03	0.44 ± 0.02	–
Threonine	0.28 ± 0.02	0.44 ± 0.02	–
Tryptophan	0.29 ± 0.03	0.17 ± 0.02	–
Tyrosine	0.11 ± 0.02	0.23 ± 0.02	–
Valine	0.72 ± 0.02	0.30 ± 0.02	–
DIITC	0.93 ± 0.03	0.14 ± 0.01	–
MITU	0.44 ± 0.03	0.14 ± 0.01	–
BITU	0.39 ± 0.01	0.00	–

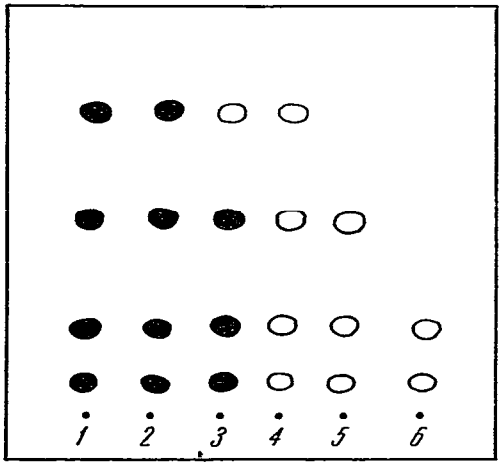

Fig. 2. Sensitivity of detection of a mixture of ITH-amino acids applied in the following amounts: (1) 0.4 nmole of each derivative; (2) 0.2 nmole; (3) 0.1 nmole; (4) 0.05 nmole; (5) 0.02 nmole; (6) 0.01 nmole. The identities of the spots, in order of increasing R_F, are: ITH-tyrosine, ITH-glutamic acid, ITH-glycine, ITH-proline. Chromatography was carried out in a single dimension with solvent I [toluene–*n*-pentane–glacial acetic acid (60:30:15)]. Distance, 4.5 cm. ●, Coloured spot; ○, spot visible only under UV light (directly as a dark violet spot or as a fluorescent spot after treatment with sodium ethoxide).

applied decreasing amounts of the following ITH-amino acids with different R_F values in solvent system I: ITH-tyrosine, ITH-glutamic acid, ITH-glycine and ITH-proline. The amounts spotted were as follows: on starting point 1, 0.4 nmole from each derivative; on starting point 2, 0.2 nmole; on starting point 3, 0.1 nmole; on starting point 4, 0.05 nmole; on starting point 5, 0.02 nmole; and on starting point 6, 0.01 nmole. As can be seen in Fig. 2, the sensitivity of the colour detection is 0.1–0.2 nmole. When the same chromatogram was inspected under UV light the spots originally indicated only by a contour were detected additionally as dark violet spots and the sensitivity increases to 0.01–0.05 nmole. The application of fluorescence detection with sodium ethoxide under UV light does not increase this sensitivity further. This fact can be explained by the slight fluorescence of the polyamide layer, which decreases the contrast of the fluorescent spots and the detection limit is 0.01–0.05 nmole. Therefore, the visual detection of coloured spots and of quenched spots under UV light is recommended.

In this way, the sensitivity of detection of the ITH-amino acids on a polyamide layer as coloured spots and under UV light (0.1–0.01 nmole) is 10–100 times higher than the corresponding sensitivity when the same derivatives are chromatographed on silica gel G (1 nmole)[4]. By inspection of the polyamide sheet under UV light the high sensitivity of the sodium ethoxide detection of ITH-amino acids on silica gel G (0.01–0.04 nmole)[5] was achieved. On the other hand, the sensitivity of detection of ITH-amino acids on a polyamide sheet (0.01–0.05 nmole) under UV light is therefore higher than that for the corresponding phenylthiohydantoin derivatives by quenching the fluorescence of the polyamide layer (0.05–0.2 nmole)[12].

Coloured thiohydantoins for sequencing work and amino acid identification have also been used by Chang *et al.*[14]. The azo group permits the detection of 4-N,N-

dimethylaminoazobenzene-4'-thiohydantoins of amino acids as red spots directly on the polyamide sheet with high sensitivity (1 pmole).

Separation of DIS-amino acids

The solvent systems for the separation of dansyl derivatives according to Woods and Wang[15] on polyamide sheets were unsuitable for the disyl amino acids. We suggest the following solvent systems for their separation: (1) 60% aqueous acetic acid; (2) benzene–propionic acid (1:1); (2a) benzene–propionic acid (8.5:1.5); (3) toluene–ethylene chlorohydrin–25% ammonia (3:5:2)[16]. Solvents 1 and 3 were used without a filter in the chamber for saturation of the atmosphere. The running time in solvent systems 1 and 3 is 60 min, while in systems 2 and 2a it is 30 min.

The R_F values of the disyl amino acids for solvent systems 1 and 2 are given in Table II (averages from ten determinations). Solvent system 2a is suggested for the separation of disylleucine and disylisoleucine. To distinguish disylcysteic acid and disylsulphonic acid, solvent system 3 is suitable.

The reliable detection of most of the disyl derivatives can be achieved ty two-dimensional chromatography (5 × 5 cm) in solvent systems 1 (first dimension) and 2 (second dimension) (Fig. 3). It can be seen that the disyl derivatives of the pairs serine–methionine sulphone and threonine–asparagine are not separated. The sepa-

TABLE II

R_F VALUES OF DISYL AMINO ACIDS, DISYL CHLORIDE, DISYLAMIDE AND DISYL-SULPHONIC ACID ON POLYAMIDE SHEETS

Distance: 4.5 cm.

Disyl amino acid	*Solvent system*			
	1	*2*	*2a*	*3*
Alanine	0.41 ± 0.03	0.62 ± 0.02	—	—
Arginine	0.84 ± 0.02	0.72 ± 0.03	—	—
Asparagine	0.53 ± 0.02	0.37 ± 0.02	—	—
Aspartic acid	0.41 ± 0.01	0.20 ± 0.02	—	—
Cysteic acid	0.00	0.00	0.00	0.79 ± 0.01
Glutamine	0.42 ± 0.01	0.47 ± 0.01	—	—
Glutamic acid	0.42 ± 0.01	0.31 ± 0.01	—	—
Glycine	0.04 ± 0.02	0.41 ± 0.02	—	—
Histidine	0.84 ± 0.02	0.37 ± 0.02	—	—
Isoleucine	0.33 ± 0.02	0.81 ± 0.03	0.66 ± 0.01	—
Leucine	0.32 ± 0.02	0.78 ± 0.01	0.57 ± 0.01	—
ε-Lysine	0.84 ± 0.01	0.27 ± 0.02	—	—
Bis-lysine	0.08 ± 0.01	0.52 ± 0.02	—	—
Methionine sulphone	0.53 ± 0.01	0.28 ± 0.01	—	—
Phenylalanine	0.30 ± 0.02	0.66 ± 0.01	—	—
Proline	0.52 ± 0.02	0.81 ± 0.03	—	—
Serine	0.52 ± 0.02	0.25 ± 0.02	—	—
Threonine	0.53 ± 0.02	0.38 ± 0.02	—	—
Bis-tyrosine	0.00	0.69 ± 0.02	—	—
Valine	0.39 ± 0.02	0.79 ± 0.02	0.58 ± 0.01	—
DIS-amide	0.43 ± 0.02	0.59 ± 0.02	—	—
DIS-chloride	0.30 ± 0.02	0.95 ± 0.01	—	—
DIS-sulphonic acid	0.00	0.00	0.00	0.09 ± 0.02

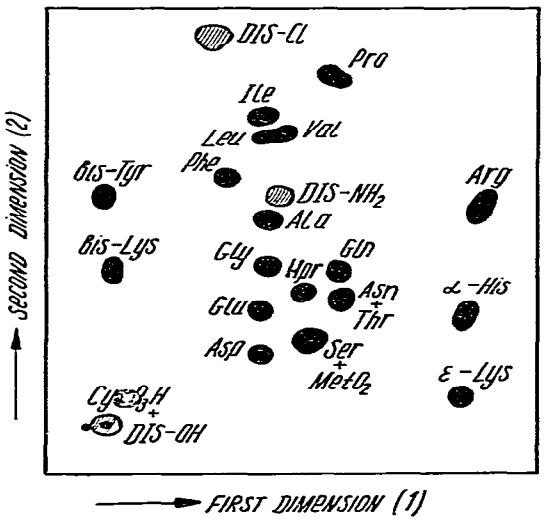

Fig. 3. Two-dimensional chromatogram of disyl amino acids on polyamide sheet (5 × 5 cm). First dimension, 60% aqueous acetic acid; second dimension, benzene–propionic acid (1:1). The starting point is 5 mm from both edges of the polyamide sheet. Disyl derivatives are indicated by abbreviations for the corresponding amino acids.

ration of threonine from asparagine is not necessary, as after hydrolysis one would expect the appearance of a more intense spot, corresponding to disylaspartic acid, the R_F value of which differs considerably from that of disylasparagine. When a single spot appears, it should be due to disylthreonine. Disylcysteic acid and disylsulphonic acid remain at the start. They can be separated with solvent system 3, even on 3 × 3 cm sheets. All disyl amino acids, as well as the artefacts disylamide and disyl chloride, move with the front in solvent 3, whereas disylsulphonic acid remains at the start.

The sensitivity of the technique is shown in Fig. 4. On starting points 1–8 increasing amounts of a mixture of three disyl amino acids (aspartic acid, glutamine and valine) with different R_F values were spotted. The chromatogram was developed with solvent system 2. The amounts spotted were as follows: on starting point 1, 0.0075 nmole (of all disyl amino acids); on starting point 2, 0.01 nmole; on starting point 3, 0.025 nmole; on starting point 4, 0.05 nmole; on starting point 5, 0.075 nmole; on starting point 6, 0.1 nmole; on starting point 7, 0.25 nmole; and on starting point 8, 0.5 nmole (Fig. 4). The results indicate that amounts from 0.25 to 0.1 nmole of the disyl derivatives can be detected as coloured spots. Amounts from 0.1 to 0.05 nmole can be detected by ultraviolet irradiation (365 nm) due to the absorption of the diphenylindenonesulphonyl residue. Amounts from 0.01 to 0.0075 nmole are detected after treatment with sodium ethoxide, following the transformation of the disyl derivatives into fluorescent isobenzofuran derivatives[3]. These results indicate that the sensitivity of detection of the disyl derivatives on polyamide sheets (0.1 nmole) is considerably higher than the corresponding colour detection of the same derivatives on silica gel G (1 nmole). The sensitivity of detection of the disyl amino acids as fluorescent spots is lower than the corresponding fluorescent detection on silica gel G (1 pmole)[6], owing to the fluorescence of the polyester foil of the polyamide sheet,

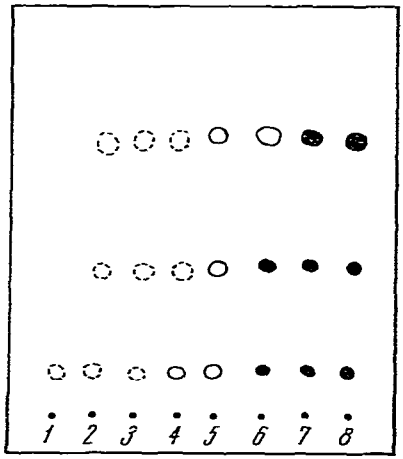

Fig. 4. One-dimensional chromatogram of a mixture of disyl derivatives of aspartic acid, glutamine and valine in increasing amounts from 0.01 to 0.5 nmoles. Solvent system 2: benzene–propionic acid (1:1). ●, Coloured spot; ○, spot visible under UV light; ◌, fluorescent spot visible under UV light after treatment with sodium ethoxide.

which decreases the contrast between the spots and the background. This effect was observed by Zimmer *et al.*[17] in the detection of dansyl amino acids. They established that when the polyester foil is replaced with an aluminium one, which does not fluoresce, the detection limit increases. Under analogous conditions (polyamide layer on the aluminium foil) the detection limit increases to 3 pmole (as a result of the absorption under UV light) and to 1 pmole after treatment with sodium ethoxide.

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