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SEPARATION OF AMINO SUGARS AND RELATED COMPOUNDS BY TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Separation of amino compounds, including galactosamine and glucosamine, found in lipopolysaccharide and peptidoglycan, can be accomplished by two-dimensional thin-layer chromatography on cellulose layers. The solvent systems used are: 2-propanol–90% formic acid–water (80:4:20, v/v) in the first dimension and lutidine–water (65:35, v/v) in the second dimension. Compounds were detected using a chromogenic ninhydrin spray.

INTRODUCTION

As part of a detailed study on the characterization of lipopolysaccharide (LPS)-defective mutants of *Pseudomonas aeruginosa* we required a rapid, sensitive and inexpensive technique to screen the LPS preparations for amino compounds. LPS from *P. aeruginosa* strains contain glucosamine, glucosamine phosphate, galactosamine, alanine and usually fucosamine (2-amino-2,6-dideoxy-DL-galactose) and/or quinovosamine (2-amino-2.6-dideoxy-D-glucose)[1–3]. Using thin-layer chromatography (TLC), on cellulose and silica, we had been unable to achieve adequate separation of galactosamine from glucosamine even though many solvent systems, proported to give separation of these compounds, have been tried.

In this paper we describe a two-dimensional TLC technique which in addition to separating galactosamine and glucosamine also resolves all amino compounds commonly found in the LPS and peptidoglycan layers of the bacterial cell walls.

MATERIALS AND METHODS

TLC plates and solvents

Aluminum plates (20 × 20 cm) pre-coated with cellulose (0.1 mm; without fluorescent indicator) were obtained from BDK Chemicals (Toronto, Canada; Cat. 5537). The developing solvents were: (I) 2-propanol–90% formic acid–water (80:4:20, v/v)[4] and (II) lutidine (2,6-dimethylpyridine)–water (65:35, v/v)[5].

It was found that TLC sheets from the supplier contained a large amount of

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impurity which manifest itself as a fairly wide yellow band at the solvent front. This material interfered with the even ascension of solvent II. In an attempt to overcome this each plate was developed in solvent I and then dried prior to future use.

Spray reagent[6]

Solution A: 10 ml of acetic acid and 2 ml of collidine were added to a solution of 1 g of ninhydrin (Sigma, St. Louis, MO, U.S.A.) in 50 ml of absolute ethanol. Solution B: 0.5 g of cupric nitrate–trihydrate was dissolved in 50 ml of absolute ethanol.

Prior to use solutions A and B were mixed in the ratio of 50:3 (v/v). We have found that the ten-fold higher concentration of ninhydrin used here gave better definition to the spots following heating (105°C, 2–3 min).

Standards

All the amino compounds were from commercial sources except for rhamnosamine (2-amino-2,6-dideoxy-L-mannose), quinovosamine and fucosamine which were kindly provided by Dr. M. Perry (National Research Council of Canada, Ottawa, Canada). Since the latter two compounds were received as the N-acetyl derivatives they were converted to the hydrochloride salts by hydrolysis with 2 *M* HCl at 100°C for 2 h (ref. 7) (M. Perry, personal communication). Stock solutions (10 mg/ml) were prepared in distilled water and frozen until used.

Lipopolysaccharide and cell walls

Samples of *Arthrobacter glacialis* cell walls, kindly provided by Dr. G. Greer, Max Planck Institute für Immunbiologie (Freiberg, G.F.R.) and LPS purified from *P. aeruginosa* KCIIR were hydrolyzed in 6.1 *M* HCl (Sequinal grade, Pierce, Rockford, IL, U.S.A.) for 4 h at 105°C. Following this period the hydrolysates were extracted with hexane prior to drying.

Procedure

Samples (1–2 μg) were spotted at one corner of the TLC plates and these were developed in solvent I for 5–6 h at 30°C. The plates were then dried and developed at right angles to the first solvent in solvent II (*ca.* 6 h). Following drying the compounds were visualized using a ninhydrin–cupric nitrate chromogenic spray.

RESULTS

Our objective was to develop a TLC system which would resolve constituents of LPS and peptidoglycan. Although a considerable number of solvent systems were examined by us, and also by Ho[8], none adequately resolved galactosamine from glucosamine[9–17]. Their resolution has subsequently been achieved by two dimensional TLC employing 2-propanol–formic acid–water (80:4:20)[4] in the first dimension and lutidine–water (65:35) in the second dimension. The separation of these two compounds together with other amino sugars and amino acids found in bacterial cell envelopes is illustrated in Fig. 1. The difference in colour with the chromogenic ninhydrin spray reagent (Table I) also aids in the differentiation of glucosamine from

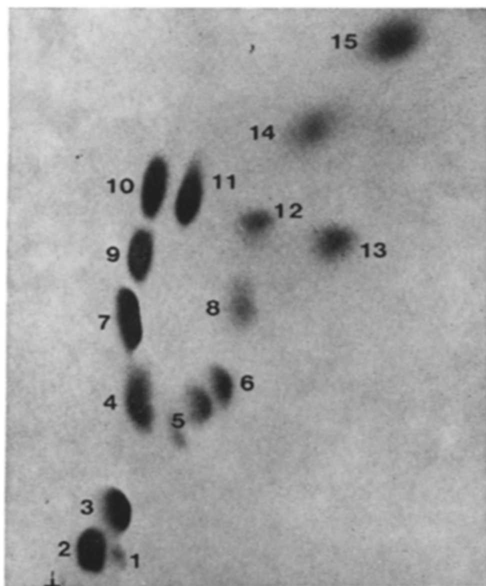

Fig. 1. Resolution of a mixture of fifteen ninhydrin-positive compounds by two-dimensional TLC on cellulose sheets. 1 = Glucosamine phosphate; 2 = diaminopimelic acid; 3 = ethanolamine phosphate; 4 = glycine; 5 = galactosamine; 6 = glucosamine; 7 = glutamic acid; 8 = fucosamine; 9 = β-alanine; 10 = γ-aminobutyric acid; 11 = α-aminobutyric acid; 12 = muramic acid; 13 = quinovosamine; 14 = phenylalanine; 15 = leucine. For actual R_F values see Table I.

galactosamine. The ninhydrin spray reagent was that described by Stahl[6] and calls for 0.1 g of ninhydrin (solution A; Materials and methods). We observed that this amount gave very faint spots, the different colours of which could not be clearly differentiated photographically. Increasing the ninhydrin concentration ten fold greatly increased the staining intensity.

A total of 41 amino acids, polyamines and amino sugars were chromatographed separately in the two solvent systems and their mobilities and colour responses to the spray reagent are recorded in Table I. One of the few drawbacks to this system was its inability to resolve basic compounds, such as lysine and arginine. These amino compounds possess low mobilities in both solvents and tend to spread out upon chromatography in the second dimension.

The application of this technique to separating amino compounds in LPS and cell wall hydrolysates can be seen in Figs. 2 and 3, respectively. Good separation of all expected components was obtained in both cases. All LPS hydrolysates tested (*Salmonella typhimurium* and *P. aeruginosa*) showed a smear near the origin which may mask the identification of compounds which run in that area.

The TLC plates, which we obtained commercially, appeared to contain a high amount of impurities (see Materials and methods) and our early studies employed plates pre-run in solvent I. More recently, however, it has been noted that the wash did not, in any way, improve the resolution of the compounds, and therefore in order to keep the procedure as simple as possible this step may be eliminated.

TABLE I

R_F VALUES OF AMINO ACIDS, AMINO SUGARS AND RELATED COMPOUNDS ON CELLULOSE PLATES USING TWO SOLVENT SYSTEMS

Each value is the average of duplicate determination.

No.	*Compound*	R_F		*Colour with ninhydrin–cupric nitrate spray*
		2-Propanol–formic acid–water (80:4:20)	*Lutidine–water (65:35)*	
1	Alanine	0.465	0.265	Purple
2	β-Alanine	0.48	0.18	Green–blue
3	α-Aminobutyric acid	0.60	0.30	Purple
4	γ-Aminobutyric acid	0.63	0.195	Purple
5	Arginine	0.19	0.04	Purple
6	Asparagine	0.155	0.15	Orange
7	Aspartic acid	0.235	0.24	Blue–purple
8	Cadaverine	0.26	0.065	Grey–purple
9	Citrulline	0.235	0.16	Purple
10	Cysteine	0.245	0.14	Grey
11	α-ε-Diaminopimelic acid	0.08	0.006	Grey-purple
12	Ethanolamine	0.42	0.11	Brown
13	Ethanolamine phosphate	0.14	0.135	Purple–blue
14	Fucosamine	0.46	0.39	Orange–yellow
15	Galactosamine	0.27	0.25	Yellow
16	Glucosamine	0.26	0.37	Yellow–brown
17	Glucosamine phosphate	0.011	0.045	Orange–yellow
18	Glutamic acid	0.345	0.26	Purple
19	Glutamine	0.185	0.22	Purple
20	Glycine	0.29	0.19	Red
21	Histidine	0.15	0.18	Greyish
22	γ-Hydroxylysine	0.098	0.0125	Grey
23	Isoleucine	0.795	0.48	Purple
24	Leucine	0.765	0.48	Purple
25	Lysine	0.195	0.043	Grey–blue
26	Mannosamine	0.35	0.34	Yellow–grey
27	Methionine	0.64	0.415	Light purple
28	Muramic acid	0.50	0.455	Grey
29	Norleucine	0.79	0.50	Blue–purple
30	Norvaline	0.75	0.37	Purple
31	Ornithine	0.16	0.035	Purple
32	Phenylalanine	0.745	0.55	Green
33	Proline	0.425	0.27	Bright-yellow
34	Putrescine	0.25	0.055	Purple
35	Quinovosamine	0.525	0.525	Yellow–orange
36	Rhamnosamine	0.405	0.46	Yellow
37	Serine	0.255	0.24	Purple
38	Threonine	0.37	0.24	Greyish
39	Tyrosine	0.55	0.46	Grey–yellow
40	Tryptophan	0.525	0.47	Grey–yellow
41	Valine	0.70	0.365	Purple

The system reported here gives good resolution of glucosamine and galactosamine together with other amino compounds commonly found in bacterial cell envelopes.

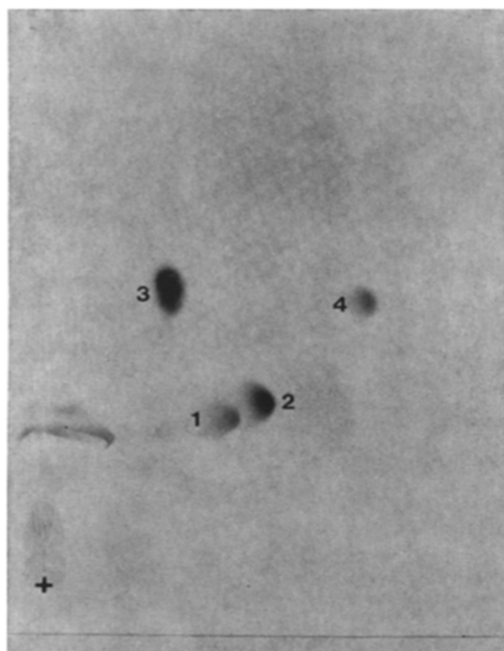

Fig. 2. Chromatographic separation of amino compounds in hydrolysates of *P. aeruginosa* lipopolysaccharide. 1 = Galactosamine; 2 = glucosamine; 3 = alanine; 4 = quinovosamine.

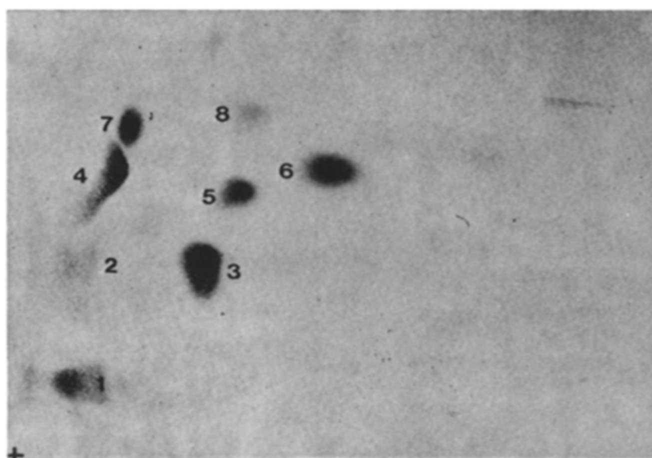

Fig. 3. TLC separation of amino compounds in *A. glacialis* cell wall hydrolysates. 1 = Diaminopimelic acid; 2 = glycine; 3 = glucosamine; 4 = glutamic acid; 5 = fucosamine; 6 = quinovosamine; 7 = alanine; 8 = muramic acid.

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