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AN IMPROVED TECHNIQUE FOR THE ANALYSIS OF AMINO ACIDS AND RELATED COMPOUNDS ON THIN LAYERS OF CELLULOSE

X. THE CHARACTERIZATION OF SOME METHIONYL, PHENYLALANYL, TYROSYL AND OTHER PEPTIDES BY THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

J. G. HEATHCOTE, R. J. WASHINGTON and B. J. KEOGH
Department of Biochemistry, The University of Salford, Salford M5 4WT (Great Britain)
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SUMMARY

This paper is a continuation of previous work (Parts VI, VIII, and IX) designed to identify small peptides in biological fluids by a combination of ion-exchange and thin-layer chromatography. Several series of peptides, mainly dipeptides, with methionine, phenylalanine, tyrosine, aspartic acid, serine, or glutamic acid as the N-terminal amino acid, have been examined.

INTRODUCTION

In Parts VI[1], VIII[2] and IX[3] it was shown that many peptides can be erroneously identified as amino acids, if the only criterion adopted is that of elution time on ion-exchange chromatography. The concurrent use of thin-layer chromatography (TLC) for the analysis of a given series of peptides prevents errors of identification which can readily occur if either method is used alone[4].

In the present paper, a total of thirty-two peptides with methionine, phenylalanine, tyrosine, aspartic acid, serine or glutamic acid forming the N-terminal amino acid residue have been examined and their behaviour on both ion-exchange and TLC have been compared.

MATERIALS AND EQUIPMENT

The TLC equipment, glass tanks and 1-μl "microcaps" capillary pipettes were supplied by Shandon (London, Great Britain). The automatic ion-exchange chromatographic system for the analysis of amino acids (Model NC-1 Auto Analyzer; Technicon, Houndmills, Basingstoke, Great Britain) was used throughout the work. The column was filled with "Chromobeads" (8% cross-linked ion-exchange resin "Type B").

Densitometer

The instrument used was the "Chromoscan", a double-beam densitometer with a thin-layer attachment (Joyce-Loebl, Gateshead-on-Tyne, Great Britain).

Cellulose powder

The cellulose powder used in this investigation was MN-300, without binder (Macherey, Nagel and Co.; Agents: Camlab, Cambridge, Great Britain). Before use this powder was purified as described previously[2].

Solvents for chromatographic development

These solvents were obtained from Hopkin and Williams (Chadwell Heath, Great Britain). The 2-methyl-2-butanol and the *n*-butanol were of GPR grade and all other solvents were of the "AnalaR" grade.

Detection reagent

The reagent was the ninhydrin–cadmium acetate reagent previously described[2]. It consisted of cadmium acetate (0.5 g), water (50 ml), glacial acetic acid (20 ml) and propanone (to 500 ml). To each portion required for use, solid ninhydrin was added to a final concentration of 0.20% (w/v).

Peptides

These di- and tripeptides were obtained commercially from Sigma (Kingston-upon-Thames, Great Britain). All amino acid residues other than glycyl were of the L configuration. Stock solutions (0.025 *M*) of these peptides were prepared in aqueous 2-propanol (10% v/v) with the addition of the minimum amount of dilute hydrochloric acid, required to give complete solution. These solutions were kept refrigerated when not in use. No hydrolysis or decomposition was noticed during the period in which these stock solutions were in use.

Preparation of the cellulose layers

The purified cellulose powder (15 g) was spread as a slurry over five plates (20 × 20 cm) at an initial thickness of 400 μm. The coated plates were allowed to dry overnight in a horizontal position before use[3].

Chromatographic solvent systems

The solvent systems used for bi-dimensional TLC of the peptides were as follows: For the first dimension (solvent No. 1), 2-propanol–butanone–1 *N* hydrochloric acid (60:15:25) and, for the second dimension (solvent No. 2), 2-methyl-2-butanol–butanone–propanone–methanol–water–0.88 ammonia solution (50:20:10:5:15:5). These solvents are identical with those reported previously[2]. Peptides of the phenylalanyl and tyrosyl series which were not satisfactorily resolved by solvent No. 1, had adequate separation when solvent No. 3 was used in the first dimension. The composition of solvent No. 3 is that previously reported[2], *viz.*, *n*-butanol–butanone–water–0.88 ammonia solution (80:5:17:3). Solvent No. 2 was retained as the solvent for the second dimension throughout.

Ion-exchange chromatography

Sodium citrate buffers of pH 2.875, 3.800 and 5.00, as well as ninhydrin reagent were prepared as described in the Technicon manual for use at a flow-rate of 30 ml/h over a normal elution time of about 19 h; in some cases, however, this time was extended up to a maximum time of 25 h.

METHODS

Application of samples and developments of plates[3]

After application of the sample solution (1 μl) (equivalent to 2.5×10^{-2} μmoles) development was carried out in the first dimension with either solvent No. 1 or No. 3 (100 ml) until the solvent had travelled 13 cm from the origin. The plate was removed, dried in a stream of cold air for at least 15 min and then heated in a convection oven at 60° for 15 min. The plate was then cooled before development in the second dimension with solvent No. 2 (170 ml) in a direction at right angles to the first dimension. After development the solvent was removed by heating at 60° for 15 min. The plate was finally cooled in a current of cold air before spraying.

Detection of the peptides[2,3]

The plates were sprayed with the ninhydrin–cadmium acetate reagent until they appeared translucent. After heating at 60° for 30 min, the R_F value and initial colour of each peptide complex were noted. Estimation of the final colour of each complex was made after allowing the sprayed plates to stand overnight (about 18 h) in an ammonia-free atmosphere. The amount of each spot was then determined by quantitative densitometry.

Densitometry

The instrument was used with slit No. 1005 (10×0.5 mm) at wavelengths of 490 nm and 405 nm. The area (mm^2) under the densitometric curve was obtained for each wavelength from the product of the peak height and the width at half-height. From this the colour yield (area in mm^2 per μmole of peptide) was calculated.

Ion-exchange chromatography

A suitable amount of peptide was dissolved in 0.1 *N* hydrochloric acid and then applied to the column, and the quantitative response of the eluted peak obtained at wavelengths of 440 and 570 nm. The colour yield was calculated in arbitrary units of area per μmole. The position of elution of each peptide was expressed as a fraction of the time interval between the aspartic acid and histidine standards. This R_{AH} value or retention factor, has been defined previously[1].

RESULTS AND DISCUSSION

Thin-layer chromatography

The R_F values for the peptides are given in Table I. The initial and final colours as well as the colour yields at 405 and 490 nm after bi-dimensional chromatography, are also quoted in this table. The numbering of the peptides follows on from that given in Part IX[3].

In solvent No. 1 each of the peptides phenylalanyl–alanine (P91), phenyl-

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF PEPTIDES ON THIN LAYERS OF CELLULOSE

Peptide	*No.*	*R_F value × 100*			*Colour yield ($mm^2/\mu mole$) × 10^{-4}*		*Ratio of area at 490 nm to area at 405 nm*	*Colour of complex (initial/final)*
		Solvent No. 1	*Solvent No. 2*	*Solvent No. 3*	*405 nm*	*490 nm*		
Asp–Gly	P77	48	2	–	8.4	13.7	1.6	yellow/red
Glu–Glu	P78	85	0	–	12.3	30.4	2.5	red/red
Glu–Gly	P79	64	3	–	11.1	29.2	2.6	red/red
Glu–Val	P80	86	4	–	14.8	42.4	2.8	yellow/red
Glutathione (oxidised)	P81	35	0	–	7.0	19.9	2.8	pink/red
Glutathione (reduced)	P81a	68	0	–	3.8	17.1	4.5	pink/red
Met–Ala	P82	79	50	36	1.9	5.4	2.8	red/red
Met–Ala-Ser	P83	64	40	21	0.47	3.6	7.6	pink/pink
Met–Glu	P84	79	13	0	2.6	5.7	2.2	red/red
Met–Gly	P85	64	44	28	4.2	8.8	1.7	red/red
Met–Leu	P86	97	71	69	1.7	6.6	3.9	red/red
Met–Met	P87	91	65	51	2.8	9.5	3.4	red/red
Met–Phe	P88	95	70	62	4.6	8.9	1.9	red/red
Met–Pro	P89	73	52	38	1.9	4.2	2.2	orange/red
Met–Ser	P90	67	43	25	2.9	4.7	1.6	red/red
Phe–Ala*,**	P91	91/100	45	58	13.9	34.0	2.4	orange/red
Phe–Gly*,**	P92	81/100	52	54	9.4	26.6	2.8	orange/red
Phe–Leu*	P93	100	85	84	13.3	27.8	2.1	orange/red
Phe–Phe*	P94	100	83	86	15.6	47.0	3.0	orange/red
Phe–Pro*	P95	91	75	62	13.0	16.0	1.2	yellow/orange
Phe–Trp*,**	P96	100/100	72	79	5.9	15.5	2.6	pink/pink
Phe–Tyr*,**	P97	100/100	78	65	22.6	47.0	2.1	red/red
Phe–Val*	P98	100	81	77	14.0	47.2	3.4	orange/orange red
Ser–Ala	P99	54	26	7	3.9	4.5	1.1	orange/orange
Ser–Leu	P100	87	63	39	0.49	0.38	0.8	orange/orange
Tyr–Ala*,***	P101	79/100	40	38	15.4	36.8	2.4	orange/pink
Tyr–Glu*,****	P102	95/80	23/75	5/75	–	–	–	orange/red
Tyr–Gly*,**	P103	68/100	34	27	10.3	31.2	3.0	orange/red
Tyr–Leu*,***	P104	93/100	60	80	16.3	50.9	3.1	orange/red
Tyr–Phe*,***	P105	92/100	58	70	15.6	45.1	2.9	orange/red
Tyr–Tyr*,***	P106	91/100	68	52	16.2	33.2	2.0	red/red
Tyr–Val*	P107	92	72	58	26.5	52.4	2.0	orange/red

* For these peptides, colour yields were determined following TLC using solvent No. 3 in the first dimension and solvent No. 2 in the second.

** Major/minor spot R_F values and colour yields.

*** Two spots of similar concentrations even in different solvents. No colour yields possible.

alanyl–glycine (P92), phenylalanyl–tryptophan (P96), phenylalanyl–tyrosine (P97), tyrosyl–alanine (P101), tyrosyl–glutamic acid (P102), tyrosyl–glycine (P103), tyrosyl–leucine (P104), tyrosyl–phenylalanine (P105) and tyrosyl–tyrosine (P106) formed two spots on the plate. The peptide tyrosyl–glutamic acid (P102) also gave two spots in both solvent No. 2 and No. 3 and was therefore the only peptide to show two spots after bi-dimensional chromatography. Although the area under the densitometric curve corresponding to each spot could be measured for peptide (P102) there was no way of determining the amount of peptide responsible and hence no colour yield could be calculated.

TABLE II

ELUTION PATTERN OF PEPTIDES ON ION-EXCHANGE RESIN (TECHNICON)

Peptide	*No.*	$R_{AH} \times 100$	*Colour yield (area per μmole)*		*Ratio of area at 570 nm to area at 440 nm*
			440 nm	*570 nm*	
Glu–Glu	P78	0	3.9	30.0	7.6
Glu–Gly*	P79	0	Similar-sized peaks		
Glu–Gly*	P79	9.2			
Asp–Gly*	P77	12.2	—	Minor peak	—
Asp–Gly*	P77	14.6	—	Major peak	—
Glutathione (oxidised)	P81	22.8	19.2	88.0	4.6
Met–Ala–Ser	P83	39.3	8.5	46.0	5.4
Glu–Val	P80	46.2	13.2	52.8	4.0
Met–Ser	P90	51.7	12.1	66.4	5.5
Met–Glu	P84	60.0	7.2	83.4	11.6
Met–Gly	P85	64.3	9.6	56.0	5.8
Met–Ala	P82	67.2	8.8	56.0	6.4
Ser–Leu	P100	70.0	11.2	68.0	6.1
Tyr–Glu	P102	72.9	10.8	54.8	5.1
Met–Pro	P89	73.5	4.9	19.2	3.9
Met–Met	P87	77.0	Coincides with ammonia peak		
Tyr–Gly	P103	81.5	11.1	60.3	5.4
Lys–Asp*****	P63	82.2	—	—	—
Tyr–Ala	P101	83.6	10.8	51.8	4.8
Lys–Asp*****	P63	84.5	—	—	—
Met–Leu	P86	85.6	12.4	74.4	6.0
Phe–Gly	P92	86.0	9.7	56.6	5.8
Phe–Ala	P91	89.2	9.5	52.5	5.5
Tyr–Val	P107	90.5	11.6	60.3	5.2
Phe–Pro	P95	91.7	7.3	14.6	2.0
Phe–Val	P98	94.8	11.7	84.3	7.2
Met–Phe	P88	97.8	9.0	56.4	6.3
Tyr–Leu	P104	100.0	10.2	64.2	6.3
Phe–Leu	P93	106.6	10.4	62.5	6.0
Tyr–Tyr	P106	109.1	16.0	81.6	5.1
Phe–Tyr	P97	115.9	11.1	63.2	5.7
Tyr–Phe	P105	116.5	14.0	60.6	4.3
Phe–Phe	P94	116.9	8.8	50.3	5.7
Phe–Trp**	P96	—	—	—	—

* The peptide gives double peaks and no quantitative values are possible.

** Not eluted from the column even after 25 h (that is an $R_{AH} \times 100$ value of about 150).

*** Mentioned before in Part IX[3].

The formation of two spots in solvent No. 1 was not due to double-fronting, as other peptide samples on the same plate were not affected. The location of the double spots did not correspond to that expected for either of the component amino acids if these were free. The resolution of the peptides on TLC was comparable to that obtained by Munier *et al.*[5] or by Elson *et al.*[6], who used a combination of chromatography and electrophoresis[7].

Ion-exchange chromatography

The position of elution from the ion-exchange column recorded as R_{AH} values $\times$ 100 for most peptides, as well as the colour yields in arbitrary units of area per μmole, are given in Table II. The peptide phenylalanyl–tryptophan (P96), because it was not eluted, and those peptides, aspartyl–glycine (P77) and glutamyl–glycine (P79), which formed double peaks had no colour yields recorded.

Our results are in broad agreement with those reported by King and Wainer[7] in their extensive survey of methionyl peptides.

The peptides lysyl–aspartic acid (P63)[3], aspartyl–glycine (P77) and glutamyl–glycine (P79) were eluted as close double peaks and hence no quantitative colour yields could be obtained. The second peak of aspartyl–glycine and of lysyl–aspartic acid was judged visually to be the major component, while glutamyl–glycine had both peaks of equal size. Although Kasai and Sakamura[8] have shown that dipeptides γ-glutamyl–Y are eluted before, and α-glutamyl–Y eluted after the position occupied by the C-terminal amino acid (Y), this cannot be the explanation for the behaviour of lysyl–aspartic acid and aspartyl–glycine as these peaks are too close together.

The peptide phenylalanyl–tryptophan (P96) was not eluted from the ion-exchange column even on prolonging the elution. We reported similar behaviour for peptides histidyl–phenylalanine (P59), lysyl–lysine (P66), and lysyl–phenylalanine (P67) recently[3]. All these peptides, which caused difficulties on ion-exchange chromatography, were located easily on TLC as single spots, yet the converse was true for some phenylalanyl and tyrosyl peptides (P91, P92, P97, P101, P102, P103, P104, P105, and P106). This behaviour re-inforces what was stated previously[3], *viz.*, "the importance of using at least two different and independent methods in the analysis of complex mixtures".

The techniques that have been described compare favourably with those advocated by other authors[9].

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