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

IX. THE CHARACTERIZATION OF SOME HISTIDYL, PROLYL AND LYSYL DIPEPTIDES BY THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

This paper is a continuation of previous work (Parts VI and VIII), designed to identify small peptides in biological fluids by a combination of ion-exchange and thin-layer chromatography. Three series of dipeptides, one having proline, another histidine and a third lysine as the N-terminal amino acid, have been examined.

INTRODUCTION

In Parts VI[1] and VIII[2] 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[3].

In the present paper, twenty-one peptides with proline, histidine or lysine as the N-terminal residue have been examined and their behaviour on both ion-exchange and TLC have been compared.

MATERIALS AND EQUIPMENT

Chromatographic 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-I Auto Analyzer; Technicon Instruments, Houndmills, Basingstoke, Hants., 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 MN300, 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, Essex, 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 reagents

The main reagent was the ninhydrin–cadmium acetate reagent previously described[2], and 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). Another reagent, isatin–cadmium acetate[4], formulated as above but with isatin replacing ninhydrin, was also used for the characterization of the prolyl-peptides.

Dipeptides

These were obtained commercially from Sigma (Kingston-upon-Thames, Surrey, 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) and these were kept refrigerated when not 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[4].

Chromatographic solvent systems

The solvent systems used for bi-dimensional TLC of the dipeptides 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]. Prolyl–isoleucine, prolyl–leucine, prolyl–methionine and prolyl–valine, however, were not satisfactorily resolved by solvent No. 1, and solvent No. 4, which has not been previously reported, was used for the first dimension in determining colour yields of these peptides. The composition of solvent No. 4 was 2-methyl-2-butanol–butanone–propanone–methanol–0.88 ammonia solution (25:20:35:5:20).

Solvent No. 2 was retained as the solvent for the second dimension as before.

Ion-exchange chromatography

Sodium citrate buffers of pH 2.875, 3.800 and 5.00 and 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 for the basic peptides up to a maximum time of 25 h.

METHODS

Application of samples and development of plates[2]

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. 4 (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

The plates were sprayed with the ninhydrin–cadmium acetate (or isatin–cadmium acetate) reagent until they appeared translucent. After heating at 60° for 30 min (90° for 30 min in the case of the isatin reagent), the R_F value and initial colour of each peptide complex was 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 of each spot (in mm^2) was recorded as 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 dipeptides are given in Table I together with the colour yields obtained at 405 and 490 nm after bi-dimensional chromatography. The

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF DIPEPTIDES 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*
		Solvent No. 1	*Solvent No. 2*	*Solvent No. 4*	*405 nm*	*490 nm*	
His-ala	P56	20	26	29	9.9	16.7	1.7
His-gly	P57	24	12	20	17.6	35.2	2.0
His-leu	P58	70	54	63	6.0	18.2	3.1
His-phe	P59	57	50	63	11.1	14.9	1.3
His-ser	P60	12	15	18	11.8	13.0	1.1
His-tyr	P61	57	52	40	8.5	12.5	1.5
Lys-ala	P62	35	9	40	12.5	30.0	2.4
Lys-asp	P63	28	2	2	14.4	23.4	1.6
Lys-gly	P64	19	8	27	19.5	35.6	1.8
Lys-leu	P65	80	42	75	5.6	19.6	3.5
Lys-lys	P66	14	9	48	15.0	31.0	2.1
Lys-phe	P67	66	42	75	13.9	20.6	1.5
Lys-val	P68	54	28	70	22.4	32.0	1.4
Pro-hyp	P69	69	21	19	1.6	3.9	2.4
Pro-ile*	P70	100	53	74	2.2	6.0	2.7
Pro-leu*	P71	100	57	77	1.2	2.1	1.7
Pro-met	P72	100	50	72	3.3	10.3	3.1
Pro-phe	P73	92	52	75	1.1	2.9	2.6
Pro-trp	P74	91	50	67	1.1	1.8	1.6
Pro-tyr	P75	93	47	65	2.6	6.0	2.3
Pro-val*	P76	100	42	66	3.6	11.5	3.2

* For these peptides, colour yields were determined following TLC using solvent No. 4 in the first dimension and solvent No. 2 in the second.

TABLE II

COLOUR OF PEPTIDE–REAGENT COMPLEXES AFTER TLC ON CELLULOSE

Peptide	*No.*	*Ninhydrin/Cd*		*Isatin/Cd*	
		Initial	*Final*	*Initial*	*Final*
Pro-hyp*	P69	Orange	Red-purple	Blue	Blue
Pro-leu	P71	Red	Red-purple	Blue	Blue
Pro-tyr	P75	Red	Red-purple	Blue	Blue
Lys-ala**	P62	Orange	Red	No response	
Lys-leu	P65	Purple	Purple	No response	
Lys-phe	P67	Purple	Purple	No response	
Lys-val	P68	Pink	Purple	No response	
His-ala***	P56	Yellow	Red	No response	
His-ser	P60	Yellow	Orange	No response	
His-phe	P59	Yellow	Orange	No response	

* The colours obtained for pro-hyp were also found for P70, P72, P74, and P76.
** The colours obtained for lys-ala were also found for P63, P64, and P66.
*** The colours obtained for his-ala were also found for P57, P58, and P61.

numbering of the peptides follows on from that given in Part VIII (ref. 2). The initial and final colours obtained with the staining reagents are shown in Table II.

Ion-exchange analysis

The colour yields and the eluted position for each peptide in terms of its R_{AH} value $\times 100$ are given in Table III. The prolyl-series had, as expected, very low colour

TABLE III

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

Peptides his–phe (P59), lys–lys (P66) and lys–phe (P67) were not eluted from the column even after 25 h (*i.e.* at an $R_{AH} \times 100$ value of about 150).

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*	
Pro–hyp	P69	28.1	0.5	0.4	0.8
Pro–val	P76	56.2	0.8	0.5	0.6
Pro–met	P72	65.5	2.4	1.4	0.6
Pro–ile	P70	73.2	0.7	<0.1	—
Pro–leu	P71	74.9	1.0	0.1	0.1
Pro–tyr	P75	85.1	0.6	0.4	0.7
Pro–phe	P73	88.3	0.5	0.3	0.6
His–ser	P60	100.0	7.0	23.2	3.3
His–gly	P57	108.1	7.8	31.6	4.1
Lys–ala	P62	108.2	15.5	55.0	3.6
His–ala	P56	109.5	5.4	22.9	4.2
Lys–gly	P64	110.0	18.0	63.6	3.5
Lys–val	P68	112.5	18.0	66.5	3.7
His–leu	P58	124.5	6.6	27.0	4.1
Lys–leu	P65	126.5	20.7	76.0	3.7
Pro–trp	P74	131.0	0.5	0.4	0.8
His–tyr	P61	146.0	6.0	25.0	4.2

yields at the given wavelengths of 440 and 570 nm. It may be recalled that we reported previously that prolyl–glycine (P24) gave no response at these wavelengths. The peptides histidyl–phenylalanine, lysyl–lysine and lysyl–phenylalanine were not eluted from the column even on prolonging the elution with normal buffers up to a maximum of 25 h. The latter compounds would therefore not be detected during ion-exchange analysis alone and this reinforces the importance of using at least two different and independent methods in the analysis of complex biological mixtures.

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