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

PART VIII. THE CHARACTERIZATION OF LEUCYL AND ISOLEUCYL DIPEPTIDES BY THIN-LAYER AND ION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

In this paper, which is a continuation of previous work (Part VI), two series of dipeptides, one with leucine and the other with isoleucine as the N-terminal residue, have been examined by both ion-exchange and thin-layer chromatography.

The possibility of identifying these compounds in biological fluids by a combination of these techniques has been demonstrated.

INTRODUCTION

In Part VI (ref. 1), we showed that many peptides can easily be erroneously identified as amino acids if the only criterion is that of elution time on ion-exchange chromatography.

Dipeptides which differ from each other only in the sequence of amino acid residues are often resolved with difficulty by thin-layer chromatography (TLC), yet may be adequately resolved by ion-exchange chromatography. Therefore, the concurrent use of both thin-layer and ion-exchange chromatography prevents the errors of identification which can readily occur if either method is used alone.

In this paper, which follows on from Part VI, seventeen dipeptides with either isoleucyl or leucyl N-terminal residues have been examined and their behaviour has been compared with that of amino acids and peptides examined previously[1].

MATERIALS AND EQUIPMENT

Chromatographic equipment

The TLC equipment, glass tanks and 1-μl "microcaps" capillary pipettes were supplied by Shandon Scientific Co. Ltd. (65 Pound Lane, London N.W.10, Great Britain). The automatic ion-exchange chromatographic system for the analysis of amino acids (AutoAnalyzer, Model NC-I, Technicon Instruments Co. Ltd., Hamilton Close, Houndmills, Basingstoke, Hants., Great Britain) was used throughout the work and 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 & Co. Ltd., Gateshead-on-Tyne, Great Britain).

Cellulose powder

The cellulose powder used in this investigation was MN 300, without binder (Macherey, Nagel & Co. Ltd.; Agents, Camlab (Glass) Ltd., Cambridge, Great Britain). Before use, it was purified as follows. Cellulose power (50 g), as purchased, is slurried with a mixture of methanol and water (4:1, 200 ml) and the slurry is poured into a large Buchner funnel and washed in the funnel with solvents (a)–(e) in the following order: (a) 2-propanol–water–acetic acid (60:20:20, 300 ml); (b) methanol–water (25:75, 200 ml); (c) methanol–1 *N* hydrochloric acid (60:40, 200 ml); (d) water (200 ml); (e) methanol (200 ml). The powder is then dried overnight *in vacuo* before use. The purified powder is stable for one month.

Solvents for chromatographic development

These solvents were obtained from Hopkin and Williams Ltd., (Freshwater Road, Chadwell Heath, Essex, Great Britain). The 2-methyl-2-butanol and the *n*-butanol were of G.P.R. grade, and all other solvents were of AnalaR grade.

Detection reagent

This reagent was the ninhydrin–cadmium acetate reagent previously described[2], 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 (weighed to $\pm$ 1 mg) to give a final concentration of 0.2 % (w/v).

Peptides and amino acids

The dipeptides were obtained commercially (Sigma (London) Chemical Co. Ltd., 12 Lettice Street, London, S.W.6., Great Britain) and were found to be chromatographically homogeneous: all amino acid residues except for glycyl were of the L-configuration. Stock solutions (0.025 *M*) of the common amino acids and of the peptides, for TLC, were prepared in aqueous 2-propanol (10 %) 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[2].

Chromatographic solvent systems

The solvent systems of HEATHCOTE AND HAWORTH[2] were used for the separation of some of the dipeptides on TLC. For the first dimension, the solvent system (solvent No. 1) was 2-propanol–butanone–1 *N* hydrochloric acid (60:15:25), and for development in the second dimension, the solvent system (solvent No. 2) was 2-methyl-2-butanol–butanone–propanone–methanol–water–0.88 ammonia solution

(50:20:10:5:15:5). Some peptides, which moved with the solvent front in solvent No. 1, were resolved by the use of another solvent system (solvent No. 3) in the first dimension, consisting of *n*-butanol–butanone–water–0.88 ammonia solution (80:5:17:3).

Ion-exchange chromatography

Sodium citrate buffers of pH 2.875, 3.80 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 total elution time of about 19 h.

METHODS

Application of samples and development of plates

The conditions given previously[2] were followed. After application of the sample solution (1 μl) (equivalent to 2.5×10^{-2} μmoles), development in the first dimension with either solvent No. 1 or No. 3 (100 ml) was allowed to proceed until the solvent front had travelled 13 cm from the origin. The plate was removed, dried in a stream of cool air for at least 15 min, and then heated in a convection oven at 60° for 15 min so as to remove final traces of hydrogen chloride or of ammonia, respectively. 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 in a convection oven 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 cadmium acetate–ninhydrin reagent until they appeared translucent. After heating at 60° for 30 min and cooling, 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 in an ammonia-free atmosphere, away from light. This step was necessary because the final colour and maximum colour yield of the ninhydrin–peptide complexes were not attained until after about 18 h.

Densitometry

The instrument was used with slit No. 1005 (10 × 0.5 mm) and a gear ratio of 1:2 to scan each coloured complex after the maximum colour intensity had been attained. The filters, which had nominal wavelengths of 490 and 405 nm, were chosen so as to give the maximum response to the red and yellow–orange peptide complexes, respectively. 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. The maximum colour yield of each peptide was expressed as the calculated area (mm^2) per μmole of peptide. The ratio of the colour yield at 490 nm to that at 405 nm was also recorded for each peptide.

Ion-exchange chromatography

The standard Technicon AutoAnalyzer was used with interference filters of wavelengths 570 and 440 nm. After preliminary trials, a convenient amount of pep-

tide was dissolved in 0.1 *N* hydrochloric acid, and 1 ml of this solution, containing an amount of peptide sufficient to give the optimal response, was applied to the column.

Norleucine could not be used as an internal standard because its peak obscured those of several peptides. Accordingly, 0.1 μmole of aspartic acid and 0.1 μmole of histidine were added to each experiment as internal standards. These standards were eluted outside the positions occupied by most of the peptides. The position of elution of each peptide was expressed as a fraction of the time interval between the standards, this being the retention factor, R_{AH}, as previously defined[1].

RESULTS AND DISCUSSION

Thin-layer chromatography

The R_F values obtained for the dipeptides are given in Table I together with the colour yields at 490 and 405 nm. The initial and final colours of all complexes were red, except for leucyl–tyrosine (orange to red) and isoleucyl–proline (purple).

The numbering of the peptides in the following tables follows on from that given in Part VI (ref. 1); some values taken from that paper are included for the purpose of comparison.

Those peptides with high R_F values in solvent No. 1, which were not resolved by two-dimensional chromatography using solvents No. 1 and No. 2 as previously described, were resolved by using solvent No. 3 followed by solvent No.2, as can be

TABLE I

CHROMATOGRAPHIC BEHAVIOUR OF DIPEPTIDES ON THIN LAYERS OF CELLULOSE

Peptide	*No.*	R_F *value* × 100			*Colour yield* ($nm^2/\mu mole$) × 10^{-4}		*Ratio of area at 490 nm to area at 405 nm*
		Solvent No. 1	*Solvent No. 2*	*Solvent No. 3*	*405 nm*	*490 nm*	
Ile-ala	P39	90	68	56	3.5	7.8	2.2
Ile-gly	P40	84	53	46	3.7	5.2	1.4
Ile-glu	P41	94	13	13	10.1	23.0	2.3
Ile-leu	P42	100	88	86	4.6	14.6	3.2
Ile-lys	P43	58	50	41	8.6	22.5	2.6
Ile-met	P44	94	79	80	3.7	8.4	2.3
Ile-phe	P45	100	81	89	4.5	13.6	3.0
Ile-pro	P46	89	60	63	2.9	3.5	1.2
Ile-ser	P47	87	53	38	5.8	13.7	2.3
Ile-trp	P48	100	83	83	2.6	10.8	4.1
Ile-val	P49	100	80	83	5.8	14.5	2.5
Leu-ala[a]	P21	97	58	—	2.9	5.1	1.8
Leu-gly[a]	P22	82	52	—	2.9	5.6	1.9
Leu-leu	P50	99	66	83	3.8	8.5	2.2
Leu-met	P51	100	71	75	9.6	15.1	1.6
Leu-phe	P52	99	73	79	6.9	10.5	1.5
Leu-ser	P53	88	61	41	4.5	9.8	2.2
Leu-trp	P54	100	73	77	7.7	13.0	1.7
Leu-tyr	P55	97	67	66	8.9	10.8	1.2
Leu-val[a]	P23	100	77	—	3.1	5.0	1.6

[a] Taken from Part VI (ref. 1).

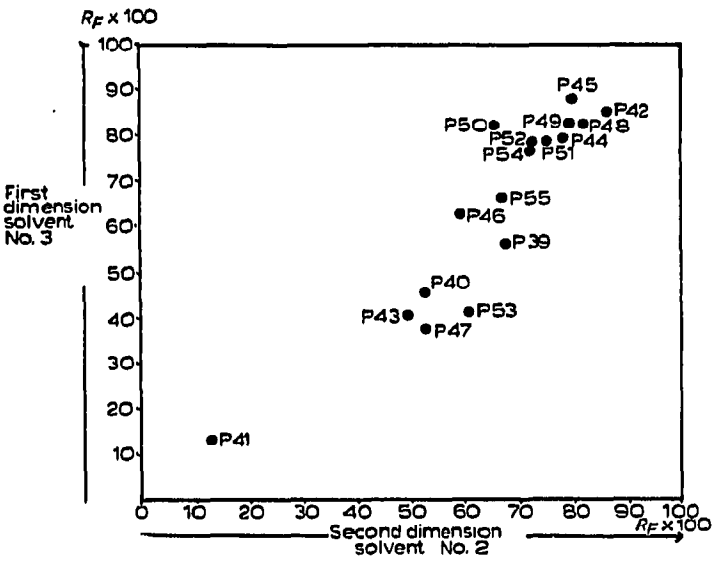

Fig. 1. Map of dipeptides after chromatography on thin layers of cellulose.

seen from the map (Fig. 1). The colour yields of the peptide complexes on TLC are comparable with those of the corresponding complexes for amino acids, thus making this technique suitable for small amounts of these peptides.

Ion-exchange analysis

The eluted position for each peptide, in terms of its R_{AH} value × 100, is given in Table II. The isoleucyl peptides are compared and contrasted with those of the corresponding leucyl series.

It can be seen that the R_{AH} × 100 values for most of the isomeric pairs of dipeptides are closer than those for the amino acids, isoleucine (54.6) and leucine

TABLE II

ELUTED POSITIONS OF DIPEPTIDES ON ION-EXCHANGE ANALYSIS

Peptide	*No.*	R_{AH} × 100	*Peptide*	*No.*	R_{AH} × 100
Ile–ala	P39	67.8	Leu–ala[a]	P21	66.0[b]
Ile–gly	P40	70.7	Leu–gly[a]	P22	70.2
Ile–glu	P41	61.0			
Ile–leu	P42	80.5	Leu–leu	P50	82.7
Ile–lys	P43	125.0			
Ile–met	P44	74.5	Leu–met	P51	76.0
Ile–phe	P45	98.0	Leu–phe	P52	98.9
Ile–pro	P46	69.3			
Ile–ser	P47	44.4	Leu–ser	P53	54.8
Ile–trp	P48	135.0	Leu–trp	P54	137.0
			Leu–tyr	P55	91.7
Ile–val	P49	70.0	Leu–val[a]	P23	66.5

[a] Taken from Part VI (ref. 1).
[b] Recalculated value.

(57.5), and hence these substances would not be resolved from each other or from amino acids. For example, if one considers the $R_{AH} \times 100$ values for isoleucyl–lysine (125) and arginine (122), then it is obvious that these compounds would not be clearly resolved.

As leucine is eluted from the ion-exchange column after isoleucine, it might be expected that the R_{AH} values of the leucyl-peptides would be greater than those of the isoleucyl series, but this was not always the case.

Apart from those peptides which contain proline, there are considerable variations in colour yield between similar isoleucyl and leucyl peptides. For example, leucyl–leucine and isoleucyl–leucine have almost identical elution positions (R_{AH} values of 82.7 and 80.5, Table II), yet the magnitudes of the colour yields at 570 nm are 35.0 and 3.0, respectively (Table III).

TABLE III

COLOUR YIELDS OF DIPEPTIDES ON ION-EXCHANGE ANALYSIS

Peptide	*No.*	*Colour yield (Area/μmole)*		*Ratio of area at 570 nm to area at 440 nm*
		440 nm	*570 nm*	
Ile–ala	P39	0.7	3.1	4.4
Ile–gly	P40	1.0	4.0	4.0
Ile–glu	P41	0.3	3.3	11.0
Ile–leu	P42	0.8	3.0	3.8
Ile–lys	P43	1.5	8.3	5.5
Ile–met	P44	0.6	3.2	5.3
Ile–phe	P45	0.4	2.3	5.8
Ile–pro	P46	5.9	40.5	6.8
Ile–ser	P47	0.6	2.9	4.8
Ile–trp	P48	0.3	2.6	8.6
Ile–val	P49	0.4	4.0	10.0
Leu–ala[a]	P21	2.6	19.2	7.4
Leu–gly[a]	P22	8.1	59.0	7.3
Leu–leu	P50	5.4	35.0	6.5
Leu–met	P51	2.7	17.5	6.5
Leu–phe	P52	3.2	19.8	6.2
Leu–ser	P53	10.4	78.2	7.5
Leu–trp	P54	2.5	43.8	17.5
Leu–tyr	P55	11.5	81.0	7.0
Leu–val[a]	P23	5.0	16.0	3.2

[a] Taken from Part VI (ref. 1).

On thin-layer chromatography, the colour yields of the leucyl peptides do not differ greatly from those of the corresponding isoleucyl compounds. On ion-exchange analysis, the reverse is true and there is a remarkable contrast in colour yield (area per μmole) between the peptides of the isoleucyl series and those of the leucyl series. Values for the colour yields of both series of dipeptides at wavelengths of 440 and 570 nm are given in Table III.

The low colour yields at 570 nm that were obtained on ion-exchange analysis of the isoleucyl peptides were similar to those previously noted for the valyl peptides[1].

Comparative values[1] for the colour yields of amino acids and selected alanyl, glycyl, isoleucyl, leucyl and valyl peptides are given in Table IV.

It can be seen from Table IV that the colour yield of a peptide with ninhydrin, at 570 nm, decreases markedly as the N-terminal amino acid residue moves along the series from glycine to valine.

TABLE IV

COLOUR YIELD AT 570 nm OF PEPTIDES AND AMINO ACIDS ON ION-EXCHANGE ANALYSIS

Amino acid	*Colour yield*	*Dipeptide*	*Colour yield*	*Tripeptide*	*Colour yield*
Glycine	92	(P10) Gly-ala	74.0	(P13) Gly-gly-gly	36.8
Alanine	80	(P1) Ala-ala	44.6	(P5) Ala-gly-gly	18.8
Valine	79	(P26) Val-ala	2.4	(P28) Val-gly-gly	1.2
Isoleucine	79	(P39) Ile-ala	4.6		
Leucine	83	(P21) Leu-ala	19.2		

It is evident that the reaction between the peptide and ninhydrin is increasingly inhibited as the size of the N-terminal amino acid increases. This effect may be due partly to steric hindrance caused by this amino acid residue, but there is the possibility that the alkyl side-chains also affect electron availability at the reaction centre. Support for the latter suggestion arises from the observation that the colour yields, at 570 nm, of the leucyl peptides are appreciably greater than those of the corresponding isoleucyl peptides. The interposition of a saturated carbon atom between the alkyl groups and the reaction centre would tend to diminish any electronic effect due to the presence of these groups.

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