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CHROMATOGRAPHIC BEHAVIOUR OF SMALL PEPTIDES ON LAYERS OF AMMONIUM TUNGSTOPHOSPHATE

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SUMMARY

Ammonium tungstophosphate was used in the thin-layer chromatography of 96 peptides, using ammonium nitrate and nitric acid solutions as eluents. The retention of the dipeptides is mainly governed by an ion-exchange mechanism, whereas adsorption appears to determine the affinity of most tripeptides towards the stationary phase. The affinity sequence of the dipeptides can be predicted from that of the corresponding C-terminal amino acids. The retention of glycine and alanine oligomers increases with increase in the number of amino acid residues. Interesting separations of hydrophilic peptides and, particularly, of the oligomers of glycine and alanine are reported.

INTRODUCTION

Ammonium tungstophosphate (AWP) has recently been employed as a stationary phase in the thin-layer chromatography (TLC) of nitrogenous organic compounds[1–3]. The high sensitivity and the unusual sequence of the affinities for amino acids[1], primary aromatic amines[2] and DNP-amino acids[3] have suggested the use of this inorganic exchanger in the study of peptides. Many peptides exhibit marked hydrophilic characteristics, such as glycine and alanine oligomers, for which both reversed-phase and the soap TLC fail[4–6].

Also in column chromatography, the separation of hydrophilic peptides and the separation of glycine and alanine oligomers are fairly difficult[7–9]. Some glycine oligomers have been separated on columns of copper(II)-modified silica gel[7] or cellulose-based anion exchangers using their copper(II) complexes in alkaline solution[8]. The oligomers of alanine can be separated on a column of silanized silica gel (C_{18}) with phosphate buffer (pH 2.1) as the eluent at 70°C[9]. Under these conditions, however, Ala and Ala–Ala are not well separated.

EXPERIMENTAL

Standard solutions of peptides and amino acids were prepared by dissolving the compounds in water–methanol (1:1, v/v). The peptides were detected by spraying

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the wet layers with a solution of 1 % ninhydrin in pyridine–glacial acetic acid (5:1, v/v) and then heating the layers at 100°C for 5 min.

The amount of substance deposited on the layer was 0.5 μg for peptides, 0.2 μg for glycine and alanine and 2 μg for hexaglycine.

Ammonium tungstophosphate was obtained as described in a previous paper[2]; the precipitate was rinsed with a 1 *M* ammonium nitrate solution, washed with water and then air-dried. The layers were prepared according to a previous report[2]. The measurements were carried out at 25°C. The migration distance was 10 cm unless otherwise stated.

RESULTS AND DISCUSSION

Aqueous eluents

Table I lists the chromatographic characteristics of 96 peptides, glycine and alanine on layers of AWP–$CaSO_4 \cdot \frac{1}{2}H_2O$ (4:2), eluting with aqueous solutions of ammonium nitrate at different concentrations. The pH of the solutions is slightly acidic (*e.g.*, the pH of 4 *M* ammonium nitrate solution is 5.70); with all the eluents the formation of a double front is observed; the first front merges with the second at high salt concentrations.

The affinity sequence of peptides is completely different from that observed on silanized silica gel alone or impregnated with anionic and cationic detergents[4–6] and does not change with increase in the salt concentration in the eluent. Exceptions are some dipeptides that contain a basic amino acid residue (His or Arg), whose affinity towards the stationary phase decreases more than that of the other compounds as the ammonium nitrate concentration is increased.

The sequence of the dipeptides can be predicted from that of the corresponding C-terminal amino acids, as the curves a, b and c in Fig. 1 show.

In contrast to the results on silanized silica gel impregnated with anionic and cationic detergents, the retention of the glycyldipeptides is more marked than that of the corresponding C-terminal amino acids, and this effect can be used for the separation of the two groups of compounds. Further, on this exchanger, the alkyl-α-amino acids (from Gly to Leu) can be separated from all of the peptides studied (eluent: 1 *M* ammonium nitrate solution) and the dipeptides containing leucine as the terminal residue are less retained than those containing phenylalanine. These last series of dipeptides can be separated by soap TLC only in an alkaline medium[4,5].

The sequence of the affinities of the hydrophilic tripeptides is Gly–Gly–Gly = Gly–Gly–Ala > Gly–Ala–Gly ≈ Gly–Ala–Ala > Ala–Ala–Ala. On the basis of this sequence can be concluded, in contrast to hydrophilic dipeptides, that the C-terminal amino acid does not affect the retention of the above-mentioned tripeptides (*cf.*, Gly–Gly–Gly with Gly–Gly–Ala and Gly–Ala–Gly with Gly–Ala–Ala), whereas the replacement of the starting or intermediate glycine residue with alanine leads to a decrease in retention. These trends can be explained on the basis of the steric hindrance due to the methyl group of alanine and to its distance from the $-NH_3^+$ group involved in the exchange reaction.

Glycine and alanine oligomers exhibit unusual behaviour: their retention increases with increase in the number of amino acid residues in the molecule. Such behaviour is thought to be due to the increasing distance between the $-NH_3^+$ group of

TABLE I

R_F VALUES OF PEPTIDES ON LAYERS OF AWP–$CaSO_4 \cdot \frac{1}{2}H_2O$ (4:2)

Compound	*1 M NH_4NO_3*	*2 M NH_4NO_3*	*3 M NH_4NO_3*	*4 M NH_4NO_3*
Gly	0.66	0.75	0.88	0.91
Gly_2	0.44	0.59	0.70	0.75
Gly_3	0.25	0.36	0.51	0.60
Gly_4	0.13	0.22	0.35	0.43
Gly_5	0.05	0.11	0.22	0.31
Gly_6	0.02	0.05	0.10	0.15
Gly–Ala	0.46	0.61	0.72	0.77
Gly–Ser	0.48	0.63	0.74	0.80
Gly–Leu	0.46	0.58	0.70	0.75
Gly–Ile	0.41	0.53	0.65	0.71
Gly–Val	0.46	0.61	0.73	0.78
Gly–Thr	0.52	0.66	0.77	0.83
Gly–Met	0.27	0.39	0.48	0.53
Gly–Trp	0.10	0.19	0.24	0.30
Gly–Glu	0.39	0.53	0.63	0.70
Gly–His	0.08	0.18	0.27	0.35
Gly–Pro	0.28	0.42	0.50	0.56
Gly–Tyr	0.35	0.48	0.57	0.62
Gly–Phe	0.28	0.40	0.48	0.53
Ala	0.68	0.78	0.91	0.93
Ala_2	0.52	0.68	0.80	0.85
Ala_3	0.34	0.50	0.65	0.72
Ala_4	0.31	0.45	0.57	0.63
Ala_5	0.21	0.31	0.45	0.50
Ala–Gly	0.49	0.64	0.76	0.82
Ala–Ser	0.56	0.71	0.83	0.89
Ala–Thr	0.57	0.72	0.83	0.89
Ala–Trp	0.16	0.26	0.31	0.36
Ala–Val	0.50	0.64	0.76	0.81
Ala–Ile	0.45	0.58	0.68	0.73
Ala–Tyr	0.40	0.53	0.63	0.68
Ala–His	0.11	0.22	0.33	0.41
β-Ala–Ala	0.36	0.48	0.59	0.65
β-Ala–His	0.07	0.13	0.18	0.23
Ser–Gly	0.51	0.65	0.77	0.83
Ser–Ala	0.52	0.66	0.78	0.84
Ser–Leu	0.51	0.64	0.76	0.80
Ser–Phe	0.36	0.48	0.57	0.62
Ser–His	0.11	0.23	0.34	0.42
Val–Gly	0.50	0.65	0.77	0.83
Val–Ala	0.52	0.66	0.78	0.84
Val–Val	0.47	0.61	0.72	0.77
Val–Leu	0.51	0.64	0.73	0.78
Val–Phe	0.33	0.45	0.53	0.59
Val–Tyr	0.40	0.53	0.62	0.69

(Continued on p. 342)

TABLE I *(continued)*

Compound	*1 M NH_4NO_3*	*2 M NH_4NO_3*	*3 M NH_4NO_3*	*4 M NH_4NO_3*
Leu–Gly	0.47	0.61	0.72	0.77
Leu–Ala	0.49	0.64	0.75	0.79
Leu–β-Ala	0.36	0.49	0.59	0.65
Leu–Ser	0.54	0.69	0.80	0.86
Leu–Val	0.49	0.64	0.74	0.78
Leu–Leu	0.53	0.63	0.68	0.71
Leu–Ile	0.49	0.62	0.71	0.74
Leu–Tyr	0.31	0.46	0.55	0.61
Leu–Trp	0.11	0.19	0.23	0.27
Leu–Phe	0.28	0.40	0.47	0.52
Ile–Gly	0.44	0.58	0.70	0.76
Thr–Gly	0.55	0.70	0.81	0.88
Met–Gly	0.26	0.40	0.53	0.59
Met–Val	0.25	0.36	0.51	0.57
Met–Leu	0.28	0.38	0.52	0.58
Met–Met	0.13	0.23	0.34	0.41
Met–Phe	0.13	0.22	0.32	0.37
Met–Tyr	0.16	0.26	0.39	0.46
Met–His	0.03	0.09	0.17	0.23
Met–Arg	0.02	0.08	0.17	0.25
Trp–Gly	0.10	0.20	0.32	0.40
Trp–Ala	0.14	0.24	0.35	0.42
Trp–Leu	0.12	0.22	0.32	0.38
Trp–Phe	0.04	0.09	0.14	0.18
Phe–Gly	0.28	0.40	0.52	0.59
Phe–Ala	0.29	0.41	0.54	0.61
Phe–Ser	0.34	0.47	0.59	0.64
Phe–Val	0.31	0.42	0.54	0.60
Phe–Trp	0.03	0.06	0.11	0.13
Phe–Phe	0.14	0.24	0.33	0.37
Phe–Tyr	0.19	0.29	0.40	0.46
Tyr–Gly	0.32	0.45	0.57	0.64
Tyr–Ala	0.36	0.49	0.61	0.69
Tyr–Phe	0.14	0.23	0.33	0.37
Asp–Gly	0.47	0.62	0.76	0.83
Asp–Ala	0.48	0.63	0.77	0.84
Arg–Gly	0.07	0.16	0.30	0.38
Arg–Phe	0.03	0.07	0.14	0.19
Arg–Asp	0.07	0.18	0.32	0.42
His–Gly	0.10	0.20	0.34	0.40
His–Ala	0.12	0.22	0.36	0.43

TABLE I *(continued)*

Compound	*1 M NH_4NO_3*	*2 M NH_4NO_3*	*3 M NH_4NO_3*	*4 M NH_4NO_3*
His–Ser	0.12	0.22	0.36	0.42
His–Leu	0.10	0.20	0.32	0.37
His–Pro	0.05	0.11	0.21	0.29
His–Met	0.05	0.11	0.18	0.24
His–Phe	0.04	0.09	0.15	0.19
His–Tyr	0.05	0.11	0.18	0.25
Gly–Gly–Ala	0.25	0.36	0.51	0.60
Gly–Ala–Gly	0.29	0.42	0.57	0.66
Gly–Ala–Ala	0.31	0.44	0.59	0.67
Gly–Gly–Phe	0.16	0.21	0.30	0.34
Gly–Leu–Tyr	0.17	0.26	0.36	0.42
Leu–Gly–Phe	0.12	0.17	0.25	0.29
1st front	0.75	0.83	0.92	0.94

peptides and the carboxylic group. This assumption also accounts for the higher retention of the glycyldipeptides than that of the corresponding C-terminal amino acids.

The degree of protonation of the carboxylic group also affects the retention; on eluting with 0.5 *M* nitric acid, the retention of the protonated oligomers is more marked than with 0.5 *M* ammonium nitrate and reversal of the affinity sequence is observed for the alanine oligomers [Ala 0.55, Ala_2 0.26, Ala_4 0.11, Ala_3 0.07, Ala_5 0.04].

The quantitative influence of the number of residues on the R_M values of glycine and alanine oligomers can be seen from Fig. 2. In Fig. 2a only glycine does not fit the straight lines. It should be noted that with increase in the salt concentration in the eluent, the slopes change from 0.32 to 0.37. For alanine oligomers the experimental points do not fall on straight lines (see Fig. 2b); their slopes, however, are in

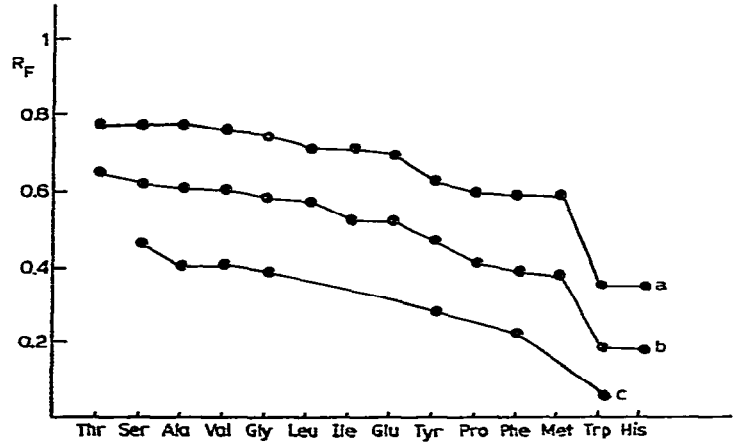

Fig. 1. Affinity sequence of (a) amino acids, (b) glycyl dipeptides and (c) phenylalanyl dipeptides on layers of AWP–$CaSO_4 \cdot \frac{1}{2}H_2O$ (4:2). Eluent: 2 *M* NH_4NO_3.

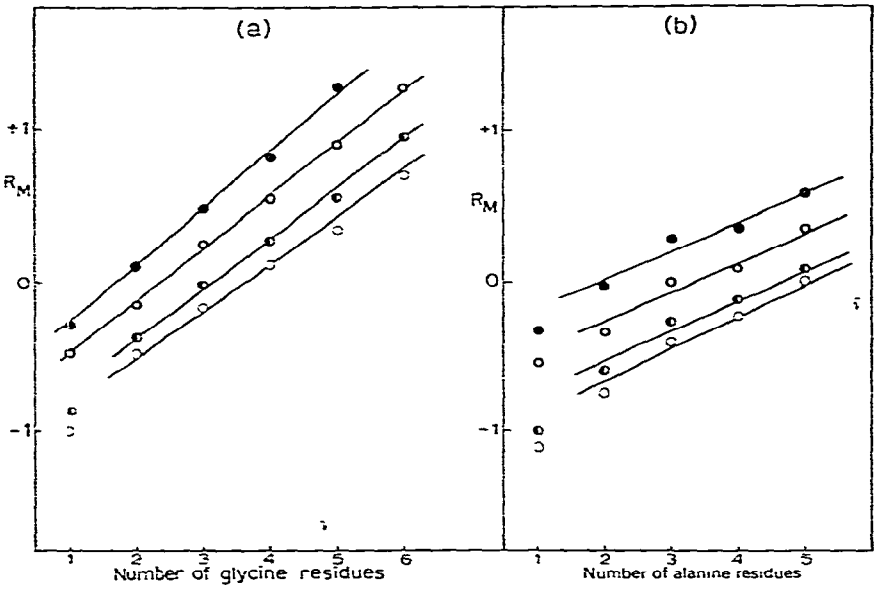

Fig. 2. (a) R_M *versus* number of residues in glycine oligomers. (b) R_M *versus* number of residues in alanine oligomers. Eluents from the top to bottom: 1, 2, 3 and 4 *M* NH_4NO_3.

the range 0.18–0.22. This result suggests that the increment in the R_M value correlated with the –NH–CH_2CO– residue is roughly twice as large as that of the –NH–CH(CH_3)CO– residue. The differences in the slopes do not depend on the presence of a double front on the layer. In fact, referring the R_M values to the first solvent front, straight lines similar to those of Fig. 2a and b are obtained with slopes in both instances 0.03–0.05 units higher.

The retention order of glycine and alanine oligomers is Ala < Gly < Ala_2 < Gly_2 < Ala_3 < Ala_4 < Gly_3 < Ala_5 < Gly_4 < Gly_5 < Gly_6. With respect to the theoretical order predicted from the number of residues, there are two exceptions: Ala_2–Gly_3 and Ala_5–Gly_4. Such reversals in the sequence indicate that the number of residues is not the only parameter determining the retention when different oligomers are compared. Other factors, such as the conformation of the peptide and its tendency to interact with the stationary phase, must also be taken into account.

As regards the retention mechanism of peptides, when the R_M values are plotted against the logarithm of the ammonium nitrate activity, straight lines are obtained for more than half the compounds. If the R_M value is calculated with respect to the first solvent front, the number of peptides that give rise to straight lines increases considerably. Further, the slopes of the lines relative to dipeptides formed by neutral amino acid residues approach the theoretical value[10], as they decrease from a mean value of 1.46 to 1.16. A similar effect is shown by the dipeptides with two positively charged groups in the molecule (hystidyldipeptides); their slopes change from a mean value of 1.96 to 1.72 (first solvent front). It should be noted that with inorganic divalent cations, experimental slopes of 1.4–1.7 were observed[11].

The values of the slopes, notwithstanding the above-mentioned restrictions (double front of the solvent, non-linear trends for many compounds), show that in

the retention process of many dipeptides on AWP–$CaSO_4 \cdot \frac{1}{2}H_2O$ layers an ion-exchange mechanism prevails.

Most polypeptides (tri-, tetra-, penta- and hexapeptides) exhibit a curvilinear R_M *versus* $\log a_{NH_4NO_3}$ plot, even if the R_M values are calculated with respect to the first solvent front, indicating that the influence of the ion-exchange process for these compounds is smaller than with dipeptides. Thus, "desorption" appears to determine the chromatographic behaviour for most polypeptides. This finding has already been observed with aromatic amines[2].

Aqueous–organic eluents

On these layers, aqueous–organic eluents containing more than 50% of organic solvent cannot be be used as they migrate so slowly that the time required for a run is prohibitively long.

Elution with water–organic solvent (methanol, ethanol or glacial acetic acid) (70:30) reduces the analysis time to about 2 h for a 10-cm migration distance. The peptides, however, are strongly retained and therefore these eluents are unsuitable for analytical purposes.

Analytical applications

The layers of AWP can be usefully employed in the separation of hydrophilic peptides, hystidyldipeptides and glycine and alanine oligomers with better results than in reversed-phase and soap TLC[4–6]. On these layers, all pairs of isomeric dipeptides reported in Table I were well separated when eluted with ammonium nitrate and

TABLE II

SEPARATION OF MIXTURES OF HYDROPHILIC PEPTIDES AND PAIRS OF ISOMERIC DI- AND TRIPEPTIDES ON LAYERS OF AWP–$CaSO_4 \cdot \frac{1}{2}H_2O$ (4:2)

Mixture	*Eluent*
Ala–Ala–Ala/Gly–Ala–Ala/Gly–Gly–Ala	2, 3 or 4 *M* NH_4NO_3
Gly–Gly–Ala/Gly–Ala–Gly	2, 3 or 4 *M* NH_4NO_3
Gly–Gly–Ala/Gly–Ala–Ala	2, 3 or 4 *M* NH_4NO_3
His–Ala/His–Leu/His–Pro/His–Met/His–Phe	4 *M* NH_4NO_3
Gly–Thr/Ala–Thr	1 *M* NH_4NO_3
Gly–Ser/Gly–Thr	1 *M* NH_4NO_3
His–Ala/Gly–His	4 *M* NH_4NO_3
Ser–Gly/Gly–Ser	2 *M* NH_4NO_3
Gly–Leu/Gly–Ile	2 or 3 *M* NH_4NO_3
Gly–Ile/Ile–Gly	2 or 3 *M* NH_4NO_3
His–Gly/Gly–His	4 *M* NH_4NO_3
Phe–Gly/Gly–Phe	3 or 4 *M* NH_4NO_3
Trp–Leu/Leu–Trp	4 *M* NH_4NO_3
Ala–Ser/Ser–Ala	3 *M* NH_4NO_3
Gly–Ala/Ala–Gly	3 *M* NH_4NO_3
Gly–Thr(0.27)/Ala–Thr(0.34)	0.5 *M* HNO_3
Ala–Gly(0.19)/Ala–Ala(0.26)/Ala–Ser(0.32)	0.5 *M* HNO_3
Asp–Gly(0.19)/Asp/Ala(0.24)	0.5 *M* HNO_3
Gly–Ala(0.17)/Ala–Ala(0.26)	0.5 *M* HNO_3
Gly–Thr(0.27)/Thr–Gly(0.31)	0.5 *M* HNO_3

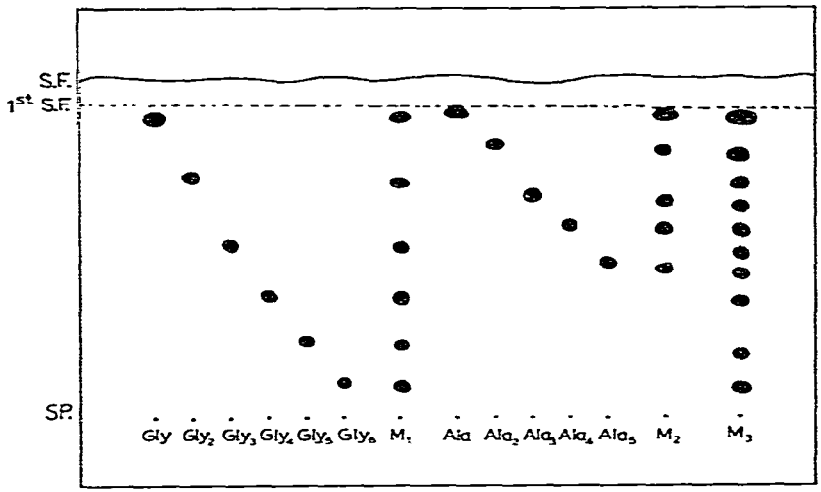

Fig. 3. Thin-layer chromatogram of glycine and alanine oligomers on AWP–$CaSO_4 \cdot \frac{1}{2}H_2O$ (4:2). Eluent: 3 M NH_4NO_3. M_1 = Mixture of glycine oligomers; M_2 = mixture of alanine oligomers; M_3 = mixture of glycine and alanine oligomers. S.P. = start point; 1st S.F. = first solvent front; S.F. = solvent front.

nitric acid solutions, with the exception of His–Ala/Ala–His, His–Ser/Ser–His, His–Met/Met–His, Val–Phe/Phe–Val, Ser–Phe/Phe–Ser and Ala–Val/Val–Ala. The separation of hydrophilic tripeptides (Ala–Ala–Ala/Gly–Ala–Ala/Gly–Gly–Ala and Gly–Gly–Ala/Gly–Ala–Gly) is very important from an analytical point of view. The best experimental conditions for the separations of these compounds are reported in Table II.

It should be noted that even pairs of isomeric dipeptides with marked hydrophobic characteristics (Trp–Leu/Leu–Trp) were separated on AWP layers; this separation cannot be achieved by either reversed-phase or soap TLC[4,5].

The oligomers of glycine and alanine were separated by eluting with 3 M ammonium nitrate (see Fig. 3). The separation of glycine from alanine can be effected with 0.5 M nitric acid as the eluent.

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