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SEPARATION OF PEPTIDES ON A POLYSTYRENE RESIN COLUMN

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SUMMARY

A high-performance liquid chromatographic column of porous spherical polystyrene—divinylbenzene copolymer (Hamilton PRP-1) was found to be useful for reversed-phase resolution of a wide range of peptides. Recoveries and resolution were comparable with those from more widely used alkyl silica-based columns. Tests involving more than 40 peptides, ranging from 2 to 34 residues, and 3 proteins, indicated wide applicability of this column. The retention times of peptides of known composition are predicted. The stability of the resin at high pH permits protocols of separation involving successive chromatograms at widely different pH values, and offers a second dimension to the resolving power of a single column.

INTRODUCTION

In the past several years, reversed-phase high-performance liquid chromatography (HPLC) on alkyl-modified silica columns has become one of the most important techniques for separation of peptide mixtures. However, alkyl-modified silica lacks chemical stability outside the pH range of 1.5 to 8. Solvation of large denatured peptides may require more extreme pH values, particularly in the alkaline range. Neutral porous polystyrene—divinylbenzene copolymer columns, e.g. Amberlite XAD-2, have been known for years to be stable over a wide range of pH [1, 2]. Recently Lee and Kindsvater [3] introduced a 10-μm spherical preparation of this adsorbent, the Hamilton PRP-1 column, which is designed for HPLC. The column has been successfully used to separate nucleosides [3], other small molecules [4], and peptides [5],

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although the latter study encompassed only peptides of six residues or less. We now show that complex mixtures of larger peptides can be separated on a porous polystyrene column (Hamilton PRP-1) at both high and low pH.

EXPERIMENTAL

Materials

Almost all peptides were obtained by tryptic degradation of sperm whale myoglobin, hen lysozyme (Sigma), or bovine brain calmodulin (a gift from Dr. Brad Olwin, University of Washington). Other oligopeptides were purchased from the indicated sources: Tyr-Gly-Gly-Phe-Met (Pierce); Gly-Phe (California Biomedical Research); Gly-Trp, Phe-Phe (Mann Research Labs.); Trp-Tyr-NH_2 (Vega Biochemicals). TPCK trypsin was purchased from Worthington. Sperm whale myoglobin was further purified before use [6]. Reagents were obtained as follows: acetonitrile (Burdick and Jackson Labs.); trifluoroacetic acid (Pierce).

The PRP-1 column was obtained from Hamilton (Reno, NV, U.S.A.).

Methods

Chromatography was carried out on a Hamilton PRP-1 column using a Varian Model 5000 liquid chromatograph. The column (150 × 4.1 mm) contains spherical, uniform, 10-μm particles of a polystyrene—divinylbenzene copolymer with a pore volume of 0.76 ml/g and a large surface area (415 m^2/g). Mobile phases comprised of linear gradients from 0.1% aqueous trifluoroacetic acid (pH 2) to acetonitrile containing 0.07% trifluoroacetic acid, or from 5 m*M* ammonium bicarbonate (adjusted to pH 8, 9.6 or 11 with ammonium hydroxide) to acetonitrile. These are referred to as pH 2, 8, 9.6, or 11 systems, respectively. The concentration of acetonitrile was increased linearly from 0 to 60% over 30 min (2%/min) at a flow-rate of 2 ml/min. Amino acid analyses were performed with a Dionex amino acid analyzer (Model D-500).

Retention times (t_{Ri}) of peptides were recorded and fitted [7] to the relationship $t_{Ri} = A \ln (1 + \Sigma D_j n_{ij}) + C$, where n_{ij} is the number of residues of amino acid j in peptide i, D_j is a retention constant for residue j, and A and C are constants. Non-linear multiple regression analyses were done on a PDP-12 computer with a floating-point processor to compute values for A and C and to obtain best fits for the D_j values in 46 peptides. Initial values of D_j were those for C_{18} columns [7]. Matrix inversion was performed using double-precision arithmetic.

RESULTS

Separation of tryptic peptides from myoglobin and lysozyme

Myoglobin was digested with trypsin (100:2, w/w, in 0.1 *M* NH_4HCO_3 for 6 h at 37°C), and an aliquot of the digest (30 nmol) was chromatographed (Fig. 1A) on a column of PRP-1 using the mobile-phase system at pH 2. Peak fractions were collected and subjected to amino acid analysis after hydrolysis to check purity and recovery. Unresolved mixtures (fractions 3, 5, 9 in Fig. 1A)

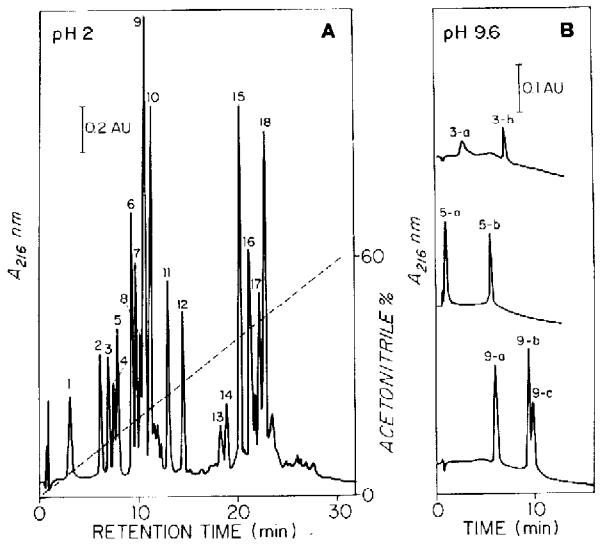

Fig. 1. (A) Chromatogram of 30 nmol of a tryptic digest of myoglobin on a column of PRP-1 in the system at pH 2. (B) Unresolved peaks 3, 5, and 9 were rechromatographed under the same conditions but using the pH 9.6 system. Purified peptides are identified in Table I.

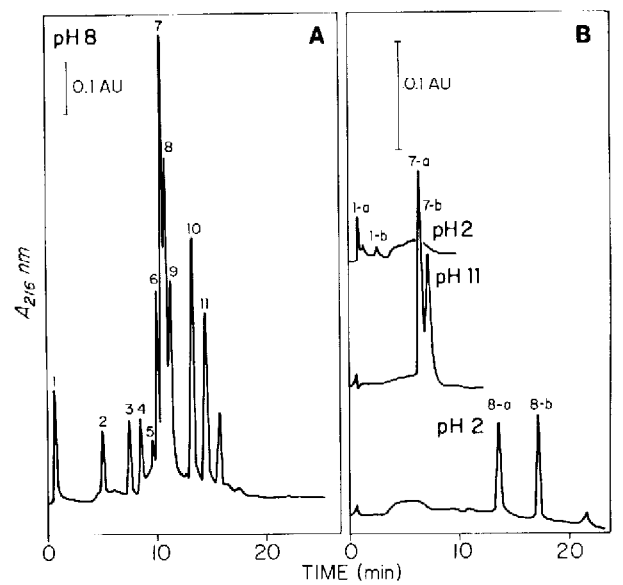

Fig. 2. (A) Chromatogram of 10 nmol of tryptic digest of reduced and S-carboxymethylated lysozyme on a PRP-1 column at pH 8 as in Fig. 1A. (B) Unresolved peaks were rechromatographed under the same conditions but using either the pH 2 or pH 11 system. Peaks: 1-a = NR, 1-b = GCR, 2 = TPGSR, 3 = CELAAMKR, 4 = HGLDNYR, 5 = KVFGR, 6 = FESNFNTQATNR, 7-a = WWCNDGR, 7-b = NTDGSTDYGILQINSR, 8-a = GTDVQAWIR, 8-b = NLCNIPESALLSSDITASVNCAK, 9 = GTDVQAWIR, 10 = GYSLGNWVCAAK, 11 = IVSDGDGMNAWVAWR.

TABLE I

AMINO ACID COMPOSITIONS OF TRYPTIC PEPTIDES PURIFIED FROM MYOGLOBIN

The peptides were purified as described in Fig. 1. In each case, the identity of the peptide was established by comparison of its amino acid composition with that (in parentheses) of a segment of myoglobin [8].

Amino acid	Peptide No. (Fig. 1)						
	1	2	3-a	3-b	4	5-a	5-b
Residues	146—147	48—50	141—145	46—47	57—63	51—56	43—45
Asp			1.0 (1)		1.0 (1)		1.0 (1)
Thr						1.0 (1)	
Ser					0.9 (1)		
Glu					1.1 (1)	2.0 (2)	
Pro							
Gly							
Ala			1.7 (2)		1.0 (1)	1.0 (1)	
Val							
Met						0.8 (1)	
Ile			0.8 (1)				
Leu		0.8 (1)			1.0 (1)		
Tyr	0.7 (1)						
Phe				0.7 (1)			0.9 (1)
His		0.9 (1)					
Lys	1.0 (1)	1.0 (1)	1.1 (1)	1.0 (1)	1.8 (2)	1.2 (1)	
Arg							1.0 (1)
Trp							
Length (residues)	2	3	5	2	7	6	3
Yield (%)	94	100	66	67	97	83	93

Amino acid	Peptide No. (Fig. 1)						
	6	7	8	9-a	9-b	9-c	10
Residues	35—42	97—102	32—34	148—153	79—96	78—96	119—133
Asp							2.8 (3)
Thr	0.9 (1)				1.0 (1)	1.0 (1)	
Ser	1.0 (1)				1.1 (1)	1.1 (1)	
Glu	2.0 (2)			2.0 (2)	3.3 (3)	2.9 (3)	1.3 (1)
Pro	1.0 (1)	1.1 (1)			1.0 (1)	1.1 (1)	0.9 (1)
Gly				2.0 (2)	1.2 (1)	1.3 (1)	3.0 (3)
Ala					3.0 (3)	3.0 (3)	3.0 (3)
Val							
Met							0.9 (1)
Ile		2.0 (2)					
Leu	1.0 (1)		1.0 (1)	1.0 (1)	2.0 (2)	2.0 (2)	
Tyr				0.8 (1)			
Phe			0.8 (1)				0.8 (1)
His	1.0 (1)	1.1 (1)			3.0 (3)	3.0 (3)	1.0 (1)
Lys	1.2 (1)	2.2 (2)	1.2 (1)		2.6 (3)	3.4 (4)	1.3 (1)
Arg							
Trp							
Length (residues)	8	6	3	6	18	19	15
Yield (%)	100	91	98	86	43	21	100

TABLE I (continued)

Amino acid	Peptide No. (Fig. 1)						
	11	12	13	14	15	16	18
Residues	17—31	134—139	64—78	64—77	1—16	103—118	1—31
Asp	1.8 (2)						2.0 (2)
Thr			1.7 (2)	2.0 (2)			
Ser					1.0 (1)	1.5 (2)	1.2 (1)
Glu	1.9 (2)	1.2 (1)			3.0 (3)	2.0 (2)	5.0 (5)
Pro							
Gly	2.0 (2)		2.0 (2)	2.0 (2)	1.1 (1)		3.0 (3)
Ala	2.3 (2)	1.1 (1)	2.2 (2)	2.1 (2)	1.2 (1)	1.0 (1)	3.0 (3)
Val	1.8 (2)		2.0 (2)	2.0 (2)	3.1 (3)	1.6 (1)	5.0 (5)
Met							
Ile	1.9 (2)		1.0 (1)	0.9 (1)		1.7 (3)	2.2 (2)
Leu	1.4 (1)	2.0 (2)	3.0 (3)	2.9 (3)	2.7 (3)	2.0 (2)	3.9 (4)
Tyr						0.8 (1)	
Phe		0.9 (1)				0.8 (1)	
His	0.9 (1)		1.1 (1)	1.2 (1)	1.0 (1)	1.8 (2)	2.1 (2)
Lys			2.4 (2)	1.5 (1)	1.5 (1)		2.0 (1)
Arg	0.9 (1)	1.0 (1)				0.8 (1)	1.0 (1)
Trp					ND (2)		ND (2)
Length (residues)	15	6	15	16	14	16	31
Yield (%)	81	98	33	36	42	74	18

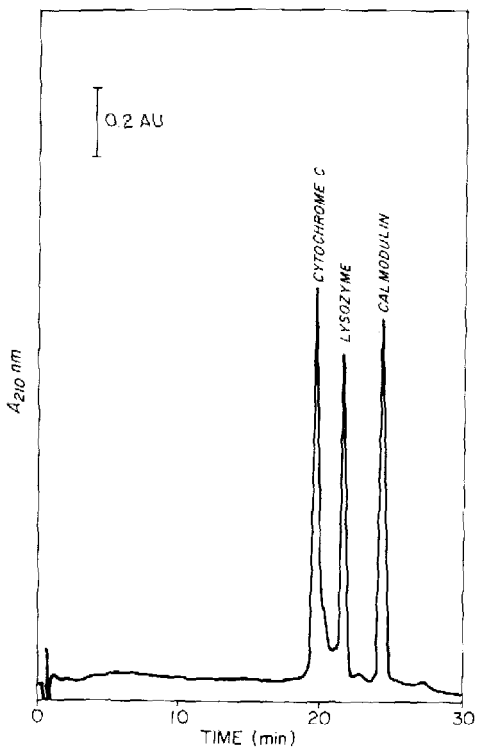

Fig. 3. Separation of a mixture of 1 nmol each of three proteins using the same protocol as in Fig. 1A.

were rechromatographed under the same conditions but using the mobile-phase system at pH 9.6. Twenty-one peptides were isolated in pure form. Ten of the 12 peptides shorter than 10 residues were recovered in better than 80% yield. Two of the longer peptides (Nos. 10 and 16 in Table I) were recovered in 100

TABLE II

RETENTION TIMES FOR VARIOUS PEPTIDES ON THE PRP-1 COLUMN

Retention times were measured on the Hamilton PRP-1 column using the gradient described in Methods, then compared with predicted retention times calculated from the amino acid compositions and computed D_j values from Table III.

No.	Sequence*	Retention time			
		pH 2		pH 8	
		Observed	Predicted	Observed	Predicted
1	YK	3.1	4.0	2.3	3.9
2	TPGSR	5.6	5.0	5.1	4.2
3	HLK	6.2	5.9	5.8	7.3
4	DIAAK	7.0	6.8	1.6	1.5
5	FK	7.0	7.1	6.5	7.6
6	ASEDLKK	7.5	6.9	1.3	0.8
7	TEAEMK	7.9	8.3	0.9	1.1
8	FDR	7.9	7.9	5.0	5.5
9	GF	8.7	9.1	8.6	8.2
10	DTDSEEEIR	9.1	8.6	0.4	0.2
11	SHPETLEK	9.3	9.6	5.0	4.7
12	EAFR	9.5	9.3	5.5	6.9
13	HKIPIK	9.7	9.6	10.7	9.8
14	HGLDNYR	10.0	9.3	8.5	7.1
15	LFK	10.2	11.1	11.0	9.9
16	GHHEAELKPLAQSHATK	10.5	10.3	9.7	10.4
17	ELGYQG	10.5	10.5	6.0	6.6
18	GW	10.5	10.4	10.5	9.4
19	FESNFNTQATNR	11.2	11.4	10.1	9.9
20	HPGNFCADAQGAMNK	11.2	11.0	9.2	9.0
21	WY-NH_2	11.2	11.2	13.8	13.8
22	CELAAMKR	11.8	11.3	6.5	5.8
23	ELGTVMR	11.8	13.4	8.8	10.2
24	DGNGYISAAELR	12.7	13.1	8.6	9.1
25	VEADVAGHGQDILIR	13.0	15.6	10.2	11.3
26	FF	13.5	12.6	11.7	11.1
27	NTDGSTDYGILQINSR	13.5	13.6	10.7	10.6
28	WWCNDGR	13.6	13.6	10.7	10.5
29	YGGFM	13.6	13.5	11.2	11.9
30	GTDVQAWIR	14.0	13.7	11.3	11.3
31	ALELFR	14.5	14.8	12.9	11.1
32	VFDKDGNGYISAAELR	14.6	15.6	11.0	11.8
33	Ac-ADQLTEEQIAEFK	14.9	14.9	7.2	7.2
34	EAFSLFDKDGDGTITTK	15.0	15.9	10.3	10.6
35	GYSLGNWVCAAK	15.8	15.1	13.3	12.8
36	HVMTNLGEK*LTDEEVDEMIR	16.3	16.3	9.2	9.2
37	NLCNIPESALLSSDITASVNC	17.2	17.9	11.0	11.8
38	NKALELFRKDIAAKYKELGYQG	17.4	17.2	17.7	13.8
39	IVSDGDGMNAWVAWR	17.6	18.3	14.5	15.7
40	EADIDGDGQVNYEEFVQM	18.2	16.6	9 0	7.5
41	HGVTVLTALGAILKK	18.3	17.4	—	15.0
42	EADIDGDGQVNYEEFVQMMTAK	18.3	17.6	11.0	10.0
43	HGVTVLTALGAILKKK	19.0	18.0	—	15.0
44	VLSEGEWQLVLHVWAK	20.2	20.2	17.2	17.5
45	YLEFISEAIIHVLHSR	21.3	19.7	19.7	17.6
46	SLGQNPTEAELQDMINEVDADGNGTIDFPEFLTM	22.0	22.6	11.6	13.7
47	VLSEGEWQLVLHVWAKVEADVAGHQDILIR	22.7	23.2	17.8	19.5

Single letter abbreviations for amino acid residues are listed in Table III. In addition, the following abbreviations are used: C, S-carboxymethyl cysteine; K, trimethyllysine; Ac-, N-acetyl; -NH_2, carboxyl-terminal amide.

and 74% yield, respectively. The other longer peptides (14 to 29 residues) were each products of incomplete cleavage, but the sum of yields within each redundant set indicated overall recoveries ranging from 60% (residues 1—16, the sum of yields of peptides 15 and 18) to 99% (residues 17—31, peptides 11 and 8).

Similar experiments with 10 nmol of S-carboxymethyl lysozyme (Fig. 2) used the pH 8 system for the first chromatography and either the pH 2 system or the pH 11 system to resolve mixtures. Although fractions 7 and 8 each contained two unresolved peptides, other peptides ranging in length from 2 residues (fraction 1-a in Fig. 2) to 23 residues (fraction 8-b) were resolved in narrow symmetrical peaks with no indication of distortion at the higher molecular weights.

Separation of a mixture of proteins

A mixture of horse cytochrome c, hen lysozyme, and bovine brain calmodulin (1 nmol each) was separated on a PRP-1 column (Fig. 3) using the system at pH 2. Recoveries were estimated to be 82, 87, and 89% respectively for cytochrome c (11,702 daltons), lysozyme (14,314 daltons), and calmodulin (16,705 daltons).

TABLE III

COMPUTED RETENTION CONSTANTS (D_j) OF AMINO ACID RESIDUES IN PEPTIDES

Amino acid		C_{18} Silica* pH 2	Polystyrene PRP-1		No. of residues considered
			pH 2	pH 8	
Tryptophan	(W)	2.34	1.79	2.48	12
Phenylalanine	(F)	1.71	1.37	1.89	21
Isoleucine	(I)	1.38	1.06	1.28	26
Leucine	(L)	1.34	1.23	1.09	39
Tyrosine	(Y)	1.23	0.77	0.67	12
Methionine	(M)	0.85	0.97	1.31	13
Proline	(P)	0.48	0.55	—0.32	8
Valine	(V)	0.38	0.73	0.85	27
Threonine	(T)	0.12	0.21	—0.01	23
Histidine	(H)	0.34	—0.12	0.66	16
Alanine	(A)	0.13	0.15	—0.03	45
Glutamine	(Q)	0.36	—0.39	—0.22	18
Glutamic acid	(E)	0.27	0.17	—0.55	41
Glycine	(G)	0.22	0.04	0.27	43
Serine	(S)	0.18	—0.16	0.60	21
Arginine	(R)	0.26	—0.21	0.32	18
Aspartic acid	(D)	0.10	—0.08	—1.04	35
Asparagine	(N)	—0.45	—0.17	—0.25	22
Lysine	(K)	0.05	—0.50	0.04	25
Carboxymethylcysteine	(CMC)	1.57	0.09	—0.86	6
Trimethyllysine	(TML)	—1.38	—2.42	—1.69	1
Acetyl-	(Ac-)	0.81	0.69	0.71	1
-Amide	(-NH_2)	—0.56	—0.42	2.92	1

*On a Waters μBondapak C_{18} column (Sasagawa et al. [7]).

Retention behavior of peptides

Retention times (t_{Ri}) were measured in both the pH 2 and the pH 8 systems for 47 peptides of known structure (Table II). Computed best fit individual residue retention constants (D_j) are listed in Table III. The predicted retention time of each peptide was then calculated, using the computed D_j values, and compared with the observed t_{Ri} in Table II. The correlation of observed and predicted retention times at pH 2 is 0.98 (Fig. 4A). The mean percent deviation of retention times is only 4.9%. The analogous values at pH 8 (Fig. 4B) are 0.96 and 11.6%, respectively.

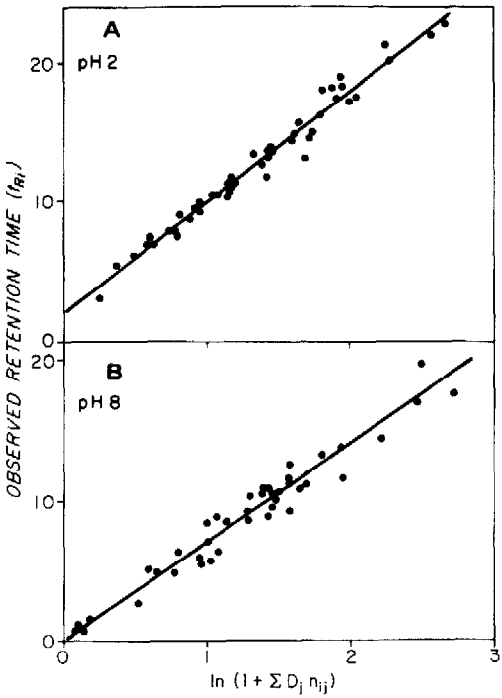

Fig. 4. Relationship between the amino acid composition of a peptide and its retention time on the PRP-1 column at pH 2 (A) and pH 8 (B). The observed retention times were plotted against ln (1 + $\Sigma D_j n_{ij}$), where D_j is the computed retention constant of amino acid j (Table III) and n_{ij} is the number of residues of amino acid j in peptide i.

DISCUSSION

The present experiments with the polystyrene-based PRP-1 column indicate that both the resolving power and the recovery of peptides are comparable with those of the more commonly used silica-based reversed-phase columns. Whereas elution of cationic peptides from the silica-based columns is occasionally perturbed by negative charges on exposed silica surfaces, polystyrene-based columns should resolve peptides exclusively by hydrophobic interactions. Previous studies with very small peptides had suggested that good resolution on the PRP-1 column was possible [5], and the present studies demonstrate resolution of complex mixtures at pH values between 2 and 11

(Figs. 1, 2) with peptides ranging from 2 to 34 residues (Table II). Similar conditions separated three small proteins (Fig. 3) although it is probable that such chromatography will generally result in denaturation and loss of biological activity.

Recoveries of peptides were generally better than 80%. Of the 21 peptides documented in Table I, 9 were recovered in better than 90% yield in a single chromatographic step. Five, which required a second purification step (Fig. 1B), were obtained in 66—93% yield. Those which were recovered from the myoglobin digest in less than 50% yield were each the result of incomplete enzymatic digestion either at residues 77—79 (Lys-Lys-Lys) or at residues 16—17 (Lys-Val), where nearby acidic residues apparently retard cleavage.

The principal advantage of the polystyrene-based column is its utility over a wide range of pH. The same column is used at pH 2, 8, 9.6, and 11 in Figs. 1 and 2. With silica-based columns, the higher values would not be possible. Inclusion of the alkaline range facilitates resolution of a mixed fraction from initial chromatography by rechromatography at a very different pH. Yang et al. [9] recognized the value of such rechromatography but were restricted on their silica-based column to pH 6.0 and 2.15. Without such restrictions on the PRP-1 column, one can optimize a first separation at one pH, then rechromatograph an unresolved fraction at a widely disparate elution pH. For example, peptides 9-b and 9-c, which differ only by an extra amino-terminal lysine in 9-c, co-elute at pH 2 (Fig. 1A) but separate at pH 9.6 (Fig. 1B) where the hydrophobic character of the lysyl residue is not countered by its positive charge. Conversely, by changing the pH from 8 to 2 two co-eluting peptides, 8-a and 8-b, separate because three more carboxylates are neutralized in 8-b

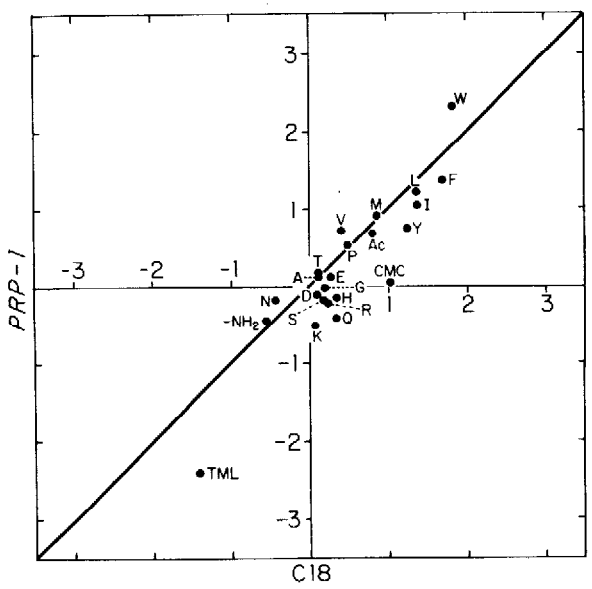

Fig. 5. Correlation of the specific retention constants (D_j) at pH 2 for each amino acid residue (see code in Table III) on the polystyrene (PRP-1) column and on the alkyl silica (C_{18}) column. Points falling on the diagonal line have identical retention constants in the two systems.

than in 8-a (Fig. 2). We have recently reported other two-step purification schemes involving the analogous principle, but with successive chromatograms interrupted by chemical modification of the hydrophobicity of a specific amino acid residue [10, 11]. Other selective procedures and newer matrices should facilitate even further the isolation of peptides from complex mixtures.

Previous studies with silica-based columns [7] indicated that the retention behavior of a few peptides can serve as the basis for predicting the mobility of others on the same column. Table II presents such retention data for 47 peptides on the PRP-1 column and Table III lists individual retention constants for each amino acid residue, as computed from these data. The retention constants for the polystyrene column differ significantly from the corresponding values for an octadecyl silica column (Fig. 5). This allows for further resolution of difficult mixtures by successive combinations of columns.

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A Fortan IV program for calculating predicted retention time from amino acid composition is available on request.

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