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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS AND POLYPEPTIDES ON POLYSTYRENE-COATED SILICA SUPPORTS

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SUMMARY

A new type of hydrolytically stable reversed-phase packing material prepared by multi-point covalent binding of polystyrene chains onto the surface of porous silica was examined in the high-performance liquid chromatography of proteins and polypeptides. Whereas wide-pore material was shown to give a rapid and efficient resolution of proteins, packings with smaller pores provided better selectivities for peptides.

INTRODUCTION

The separation of proteins and polypeptides by reversed-phase high-performance liquid chromatography (HPLC) has been the focus of much of the effort of several research groups during the last decade. Theoretical considerations of the problem and an overview of the achievements and practical applications of the technique can be found in the literature[1,2].

Concerning the column packing materials for the reversed-phase (RP) chromatography of proteins and polypeptides, the main interest has been devoted to the structure of the hydrophobic interface layer[3,4], the pore structure of the silica matrix[5–7] and the hydrolytic stability of the packing[8–11]. According to many experimental findings, the length of the alkyl chains on the surface of brush-type RP packings exerts no significant effect on the retention of proteins, unlike that of small organic molecules, but still influences the total quality of resolution. An average pore diameter of the support matrix of *ca.* 30 nm was found to give an optimum match to the free diffusion requirements of large protein molecules, and even wide-pore materials with pores as large as 100 nm were demonstrated to give good resolutions[12]. The hydrolytic stability of alkyl-modified silicas at acidic pH of aqueous–organic eluents used in the RP chromatography of proteins was shown to be insufficient in many instances, although it improves with increasing chain length of the alkyl groups graft-

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ed to the surface. Branched alkylsilanes usually produce packings of enhanced hydrolytical stability[10].

We have described[13] the surface modification of porous silica with a copolymer of styrene with methylvinyldichlorosilane. The polystyrene chains were then converted into a chiral ligand-exchanging phase. When used at high temperatures and under severe hydrolytic conditions, the polymer-modified packings were found to be much more resistant than analogous monomerically bonded chiral phases. Similarly, hydrolytically stable ion-exchange phases could be prepared starting from the polystyrene-grafted material. More detailed information on the polystyrene grafting procedure, the porous structure of the material obtained and its chromatographic properties will be given elsewhere[14].

This paper addresses the examination of polystyrene-coated silicas as packings for the RP chromatography of proteins and polypeptides with special concern for the selectivity and resolving power of this hydrolytically stable material.

EXPERIMENTAL

Chemicals

Zorbax PSM 60, PSM 500 and PSM 1000, all of particle diameter (d_p) 5 μm, were supplied by DuPont (Wilmington, DE, U.S.A.); silica with a mean pore diameter (p_d) of 200 nm and d_p = 10 μm was obtained from E. Merck (Darmstadt, F.R.G.).

Proteins and peptides used in chromatographic experiments included ribonuclease A from bovine pancrease (Rna), myoglobin from horse skeletal muscle (Myo), hen egg albumin (Ova), cytochrome *c* (Cyt), chicken egg white lysosyme (Lys), gramiciding C (Grm), conalbumin (Con), human transferrin (Tyn), His–Gly dipeptide (dp), Tyr–Gly–Gly–Phe–Met pentapeptide (pp) and Ser–Arg–Val–Tyr–Ile–His–Pro–Leu octapeptide (op) (all from Servam, Heidelberg, F.R.G.) and also catalase from bovine pancrease (Cat) (E. Merck).

Chromatographic experiments

The chromatograph consisted of two LKB 2150 pumps, an LKB 2152 controller, a BT 3030 variable-wavelength detector (Biotronic, F.R.G.) operated at 220 nm and a Model 2210 potentiometric recorder (LKB). All experiments were performed on 125 × 4.6 mm I.D. stainless-steel columns (Bischoff, F.R.G.).

Linear gradients from 5% B in A to 100% B were used in all instances, where A is 0.1% aqueous trifluoroacetic acid and B is acetonitrile–0.1% aqueous trifluoroacetic acid (2:1, v/v).

When determining the capacity factor, k', the void volume of the column was estimated from the negative peak of pure water which was injected into the aqueous–organic eluent.

RESULTS AND DISCUSSION

Polystyrene-coated RP materials

Table I presents data on the pore structure of the initial silica materials and that of the final RP packings prepared by grafting styrene–methylvinyldichlorosilane copolymers onto the silica surface. It is obvious that the mesoporous silica, Zorbax

TABLE I

CHARACTERISTICS OF THE INITIAL SILICAS AND POLYSTYRENE-COATED REVERSED-PHASE PACKINGS

Silica	*Specific surface area*[a] *(m^2/g)*	*Carbon content (%, w/w)*	*Ligand density ($\mu mol/m^2$)*	*Specific surface area*[b] *(m^2/g)*
Zorbax PSM 60	384	13.4	3.6	250
Zorbax PSM 500	29	2.4	9.3	25
Zorbax PSM 1000	17	1.4	8.4	17
Silica (p_d 200 nm)	16.7	2.7	15.5	17

[a] Before modification.
[b] After modification.

PSM 60, displays the highest carbon content of 13.4% (w/w) after modification, whereas the highest density of 15.5 μmol of styrene units per m^2 of the surface is attained on the wide-pore material, silica, with p_d = 200 nm. The internal surface area of Zorbax PSM 60 decreased markedly, from 384 to 250 m^2/g, on binding of polystyrene, to a similar extent to when the material was treated with *n*-octyl- and *n*-octadecylsilanes. Simultaneously, its specific pore volume decreased from 0.7 to 0.4 cm^3/g. In contrast, no changes in the specific surface area of silica (p_d = 200 nm) or of Zorbax PSM 1000 was observed after grafting it with the polystyrene chains. Hence it is important to bear in mind that the coating procedure only alters the shape of small pores and probably blocks these finest pores of the initial packing material, whereas the wide pores appear to remain unaffected.

Isocratic elution

Fig. 1 shows the retention of the test peptides and proteins as a function of the acetonitrile content, φ, in the mobile phase (0.1% in trifluoroacetic acid). The small peptides (di- and penta-) are retained to different extents on the columns. Their respective capacity factors in pure water as mobile phase were 0.5 and 8.5 on Zorbax PSM 60 (carbon content 13.4%, w/w), 0 and 0.7 on Zorbax PSM 500 (carbon content 2.4%, w/w) and both unretained on Zorbax PSM 1000 (carbon content 1.4%, w/w). It is concluded that the retention of the two solutes is proportional to the carbon content of the packing.

There is a sharp decrease in the retention of all proteins examined with increase in the acetonitrile content, φ, ranging from 0.2 to 0.5. At higher acetonitrile contents ($0.55 < \varphi < 0.7$), the proteins elute in the exclusion volume of the column ($-0.3 < k' < 0$), with the exception of the highly hydrophobic gramicidin. The retention curves on polystyrene-coated packings (Fig. 1) show essentially the same behaviour as on conventional *n*-octyl and *n*-octadecyl reversed-phase packings[15]. Still, there is a difference in that only a relatively small (Fig. 1A) or no (Fig. 1B and (C) enhancement of retention of proteins can be observed on the polystyrene–coated packings at higher concentrations of acetonitrile, whereas on conventional RP packings the k' values usually increase again at $\varphi > 0.5$ owing to silanophilic interactions[15].

As has been repeatedly confirmed in numerous publications[15–19], the logarithm of the capacity factors, log k', of proteins is linearly correlated with the content of

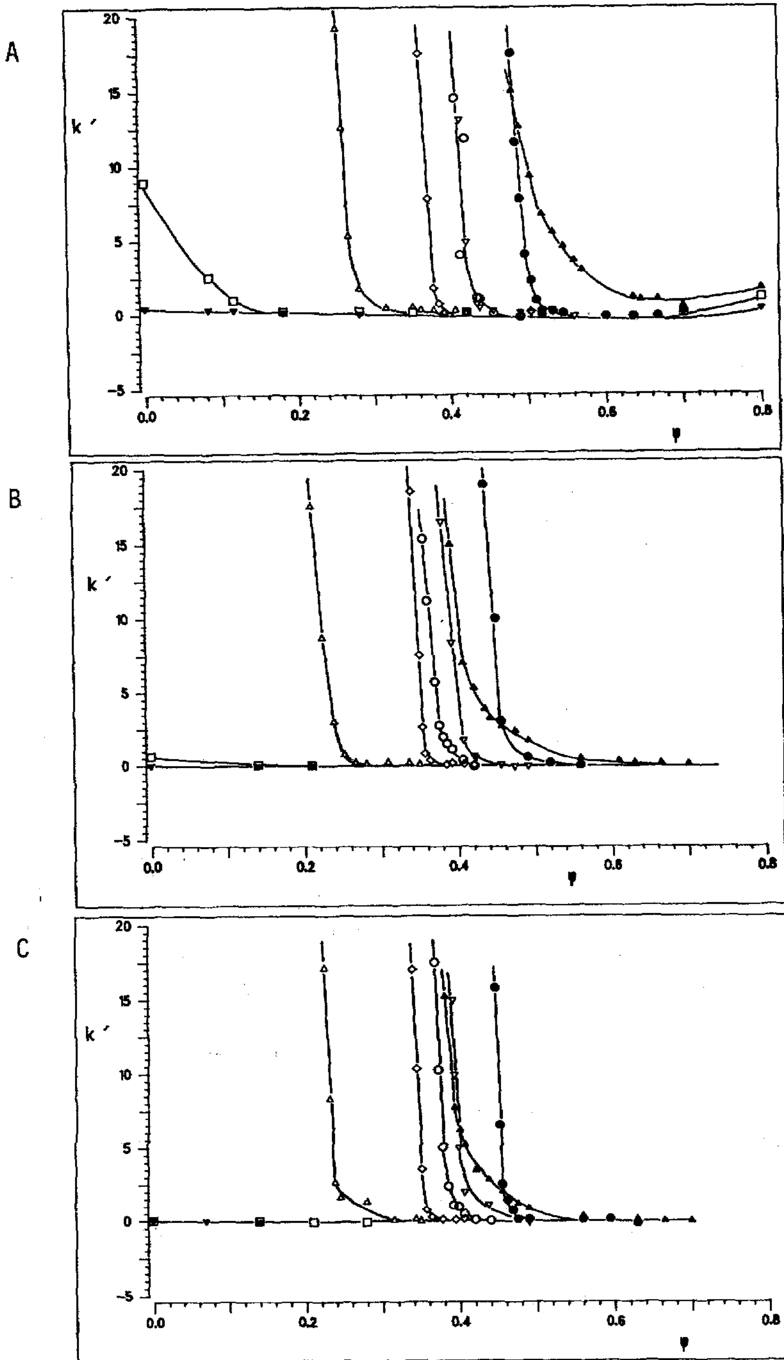

Fig. 1. Plot of capacity factors of proteins and peptides against the fraction of acetonitrile in the eluent for the polystyrene-coated silicas: (A) Zorbax PSM 60; (B) PSM 500; (C) PSM 1000. Eluent: acetonitrile–aqueous 0.1% TFA. ▼ = Dipeptide; ▽ = catalase; ○ = myoglobin; ● = ovalbumin; □ = pentapeptide; △ = ribonuclease; ▲ = gramicidin; ◇ = transferrin.

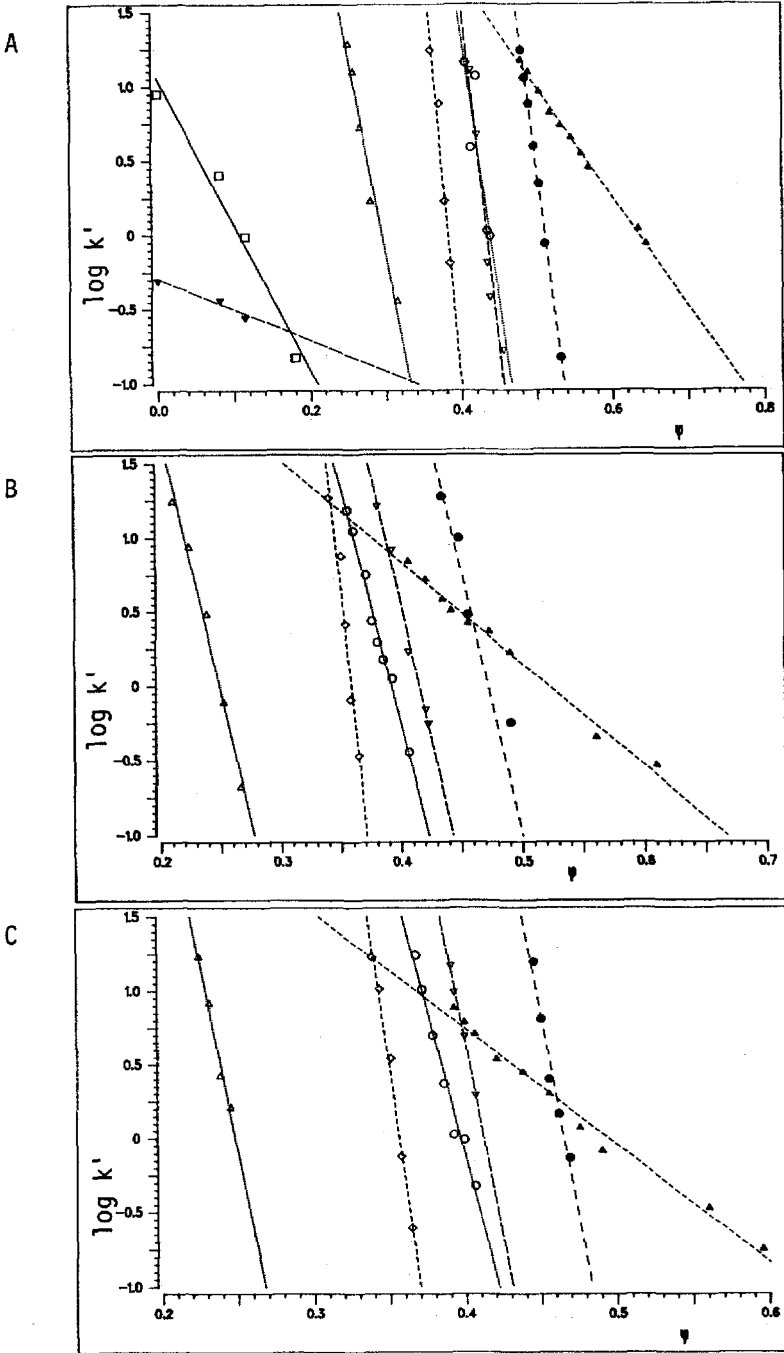

Fig. 2. Plot of log k' of peptides and proteins against the fraction of acetonitrile in the eluent. Conditions and symbols as in Fig. 1.

TABLE II

SOLVENT STRENGTH PARAMETERS, S_i, FOR PEPTIDES AND PROTEINS ON POLYSTYRENE-COATED SILICAS IN A MODEL PHASE WITH ACETONITRILE–MOBILE AS THE ORGANIC SOLVENT

Silica	*dp*	*pp*	*Rna*	*Grm*	*Ova*	*Tyn*	*Myo*	*Cat*
Zorbax PSM 60	2	10	27	5	40	59	36	46
Zorbax PSM 500	–	–	35	7	35	77	39	40
Zorbax PSM 1000	–	–	51	8	54	73	35	53

organic modifier in accordance with the equation given by Snyder[20], *i.e.*, $\log k' = \log k'_{i>w} - S_i \varphi$, where $k'_{i>w}$ is the capacity factor of solute i in pure water ($\varphi = 0$) and S_i is the solvent strength parameter. The latter depends on the type of the solute molecule i and its molecular weight, and on the organic modifier. The above linear relationship was found to hold for the polystyrene-coated packings (Fig. 2). Table II gives the solvent strength parameter values S_i for acetonitrile in combination with various solutes and packings as determined from the slopes of the straight lines in Fig. 2.

There are three main contributions to the retention of proteins on RP silica materials[18] arising from polar solute–surface interactions, size exclusion and hydrophobic interactions. With increasing pore diameter of the initial support material, the size-exclusion phenomena should diminish gradually. The polar interaction of solutes with the surface silanol groups should also decrease because of the enhanced density of polystyrene chains on the surface in large pores. Therefore, the retention of protein solutes on the macroporous materials should be mainly governed by hydrophobic interactions. Accordingly, the highest solvent strength values in Table II are found for combinations of large and hydrophobic protein solutes such as transferrin and polystyrene-coated macroporous packings. Here, the hydrophobic adsorption–desorption mechanism is largely operative[18].

Gradient elution

The polystyrene-coated RP materials easily resolve mixtures of peptides and proteins under gradient elution conditions (Fig. 3).

The elution sequence of peptides and proteins on the new RP phase was found to be essentially the same as that on conventional brush-type RP packings[1,2]. The separation of various pairs of proteins expressed as the difference in retention times (Table III) does not change much on passing from one macroporous material to another, but differs dramatically from that on the mesoporous polystyrene-coated Zorbax PSM 60. In the latter instance, the peaks of lysosyme and conalbumin coelute and the peaks of catalase and myoglobin appear in the reverse order compared with the macroporous packing.

Hearn and Aquilar[16] pointed out some specific factors that limit the solute permeability on small-pore n-alkylsilicas, including geometric effects, hydrodynamic drag on the diffusing solute due to the proximity of pore walls, and aggregation of proteins in solution or at the solid–liquid interface. Any of the above specific hindrance factors can be involved in changing the retention of such large protein molecules as catalase (240 000 daltons) or conalbumin (78 000 daltons).

Peptides and proteins are retained on the basis of their hydrophobic character

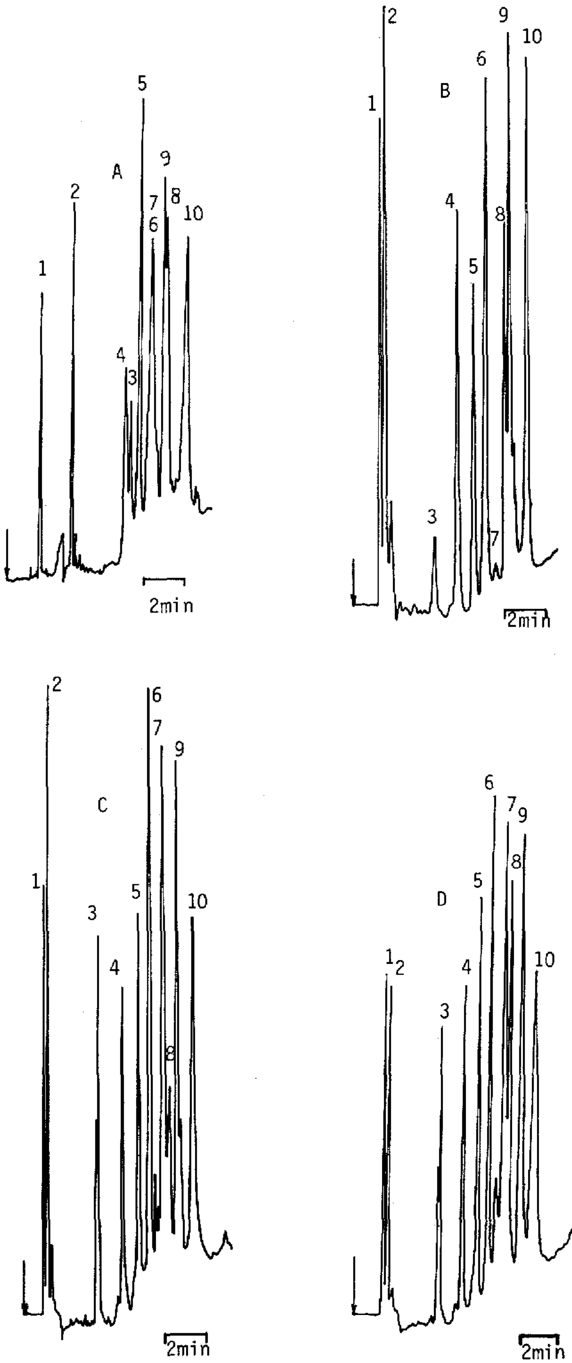

Fig. 3. Separation of a peptide–protein mixture on the polystyrene-coated silicas: (A) Zorbax PSM 60; (B) PSM 500; (C) PSM 1000; (D) silica (p_d = 200 nm). Flow-rate, 1 ml/min; gradient from 5% to 100% B in 10 min. 1 = Dipeptide; 2 = pentapeptide; 3 = octapeptide; 4 = ribonuclease; 5 = cytochrome *c*; 6 = lysosyme; 7 = conalbumin; 8 = myoglobin; 9 = catalase; 10 = ovalbumin.

TABLE III

DIFFERENCES IN THE RETENTION TIMES OF PROTEINS AND PEPTIDES ON POLYSTYRENE-COATED SILICAS

Silica	*Difference in retention times (min)*								
	dp − pp	*op − pp*	*Rna − op*	*Cyt − Rna*	*Lys − Cyt*	*Con − Lys*	*Myo − Con*	*Cat − Myo*	*Cat − Ova*
Zorbax PSM 60	−1.58	2.89	−0.26	0.48	0.66	0.00	0.90	−0.18	−1.12
Zorbax PSM 500	−0.25	2.47	1.10	0.85	0.55	0.47	0.48	0.20	−0.94
Zorbax PSM 1000	−0.17	2.55	1.19	0.59	0.68	0.68	0.38	0.30	−0.82
Silica (p_d = 200 nm)	−0.25	2.59	1.12	0.71	0.60	0.68	0.25	0.60	−0.72

Flow-rate, 1 ml/min; gradient from 5% to 100% B in 10 min; for other conditions and abbreviations, see Experimental.

TABLE IV

RETENTION TIMES FOR PEPTIDES AND PROTEINS ON POLYSTYRENE-COATED SILICAS

Silica	*Retention time (min)*										
	dp	*pp*	*op*	*Rna*	*Cyt*	*Lys*	*Con*	*Cat*	*Myo*	*Ova*	$t_{Ova} - t_{Rna}$
Zorbax PSM 60	1.65	3.23	6.12	5.86	6.34	7.00	7.00	7.72	7.90	8.84	2.98
Zorbax PSM 500	1.36	1.61	4.08	5.18	6.03	6.58	7.05	7.73	7.53	8.67	3.49
Zorbax PSM 1000	1.02	1.19	3.74	4.93	5.52	6.20	6.88	7.56	7.26	8.38	3.45
Silica (p_d = 200 nm)	1.53	1.78	4.37	5.49	6.20	6.80	7.48	8.33	7.73	9.05	3.56

Flow-rate, 1 ml/min; gradient from 5% to 100% B in 10 min; for other conditions and abbreviations, see Experimental.

TABLE V

PEAK CAPACITIES FOR PEPTIDES AND PROTEINS ON POLYSTYRENE-COATED SILICAS FOR DIFFERENT GRADIENT TIMES t_G

Silica	*Gradient*[a]	*Peak capacity*									
		dp	*pp*	*op*	*Rna*	*Cyt*	*Lys*	*Con*	*Cat*	*Myo*	*Ova*
Zorbax PSM 60	A	96	122	−56	48	56	52	–	30	38	44
	B	84	79	52	27	48	21	–	–	–	21
	C	84	76	35	31	47	26	–	–	–	28
	D	34	30	2	11	19	13	–	–	–	10
Zorbax PSM 500	A	58	–	34	48	54	46	–	38	50	44
	B	61	–	35	48	44	42	–	32	50	42
	C	64	–	34	40	47	44	–	–	45	33
	D	49	–	33	41	37	51	–	–	40	30
Zorbax PSM 1000	A	134	–	75	67	75	–	–	52	–	26
	B	134	–	75	56	56	–	42	52	–	39
	C	130	–	56	53	37	–	34	42	–	29
	D	123	–	35	50	34	–	26	35	–	27
Silica (p_d = 200 nm)	A	103	58	67	58	46	67	45	42	42	27
	B	67	67	46	54	54	58	29	45	48	27
	C	56	44	44	43	44	52	40	35	40	27
	D	47	35	33	40	47	37	28	31	39	23

[a] A, 0.5 ml/min, t_G = 20 min; B, 1.0 ml/min, t_G = 10 min; C, 2.0 ml/min, t_G = 5 min; D, 3.0 ml/min, t_G = 3.3 min.

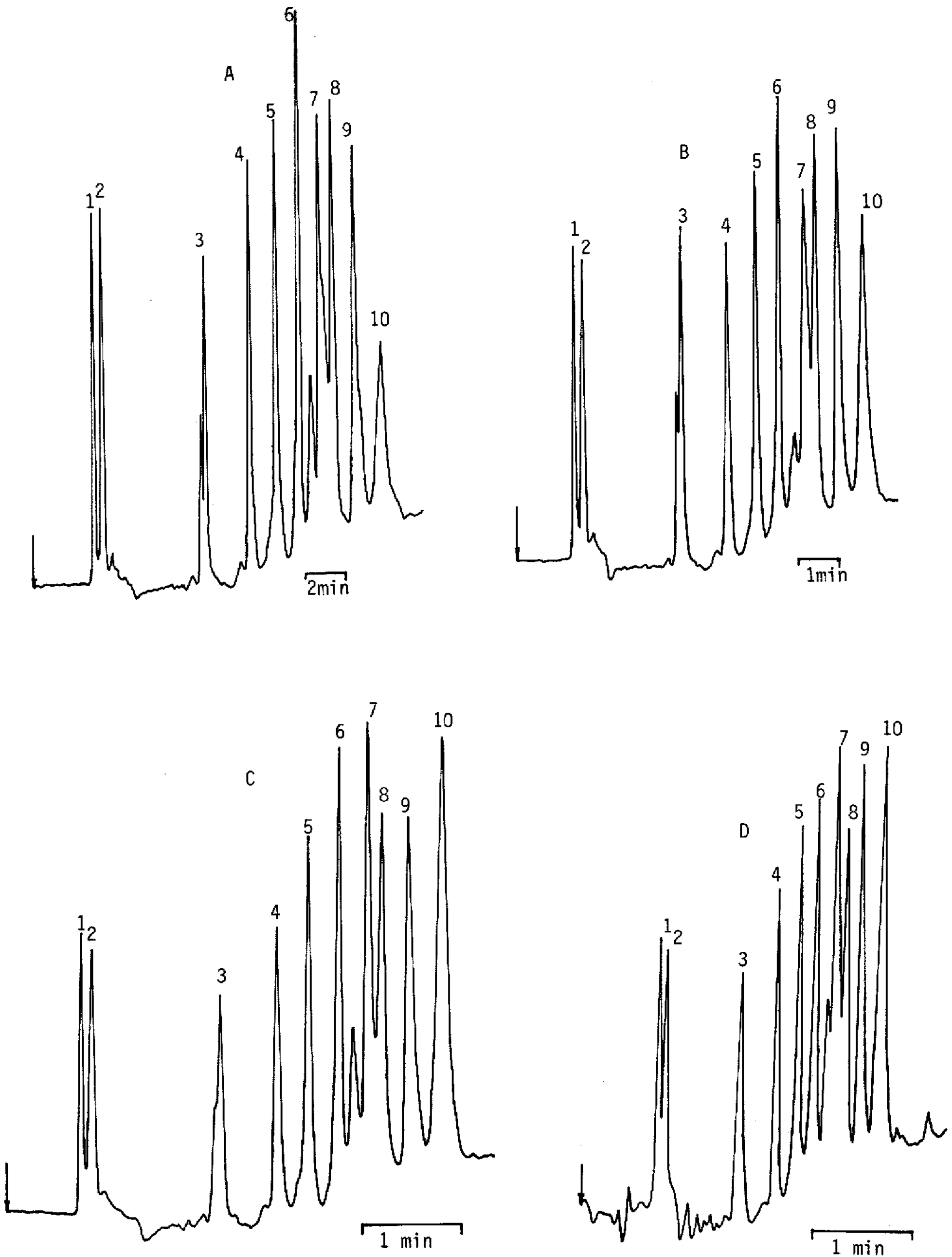

Fig. 4. Separation of a peptide–protein mixture on polystyrene-coated silica (p_d = 200 nm) at different gradient times from 5% to 100% B: (A) in 20 min at 0.5 ml/min; (B) in 10 min at 1 ml/min; (C) in 5 min at 2 ml/min; (D) in 3.3 min at 3 ml/min. Other conditions and peak numbers as in Fig. 3.

and relative to a certain volume fraction of the organic modifier when $\varphi < 0.6$. The retention of the three peptides on the Zorbax PSM columns is seen to decrease with decreasing carbon content and is highest with the coated Zorbax PSM 60 packing. In contrast to peptides, the retention of proteins remains fairly independent of the carbon content (see Table IV). A possible explanation is that the proteins are unable to penetrate the pores of the coated Zorbax PSM 60 packing, and hence solute–surface interactions take place exclusively at the external surface of the particles. As a result, the elution window, *i.e.*, the retention time difference between ovalbumin (last-eluting peak) and ribonuclease (first-eluting peak), is smaller than that observed on the macroporous coated silicas. The wider range of elution differences on the macroporous silicas is probably due to the fact that the internal surface area is totally accessible for solute–surface interactions.

In gradient elution of proteins, the peak capacity, *PC*, is a measure of resolution. Table V gives the values of peak capacities calculated according to the equation $PC = t_G/4\sigma$, where t_G is gradient time and σ is the band width in time units[20]. The gradient volume in this series of experiments was kept constant, but the flow-rate varied from 0.5 to 3.0 ml/min with the gradient time changing from 20 to 3.3 min, respectively. There is a distinct trend that the peak capacity values decrease with increasing flow-rate, which agrees with observations reported earlier[21]. An improved peak capacity at lower flow-rates can also be seen in Fig. 4, where the polystyrene-coated silica (p_d = 200 nm) was examined. Here, mixtures of the octapeptide and conalbumin are well separated from the main components at a flow-rate of 0.5 ml/min, but not at flow-rates of 2 ml/min or higher.

As demonstrated for the coated silica of p_d = 200 nm (Fig. 4), the peak height increases with decreasing flow-rate for all solutes except ovalbumin. The same observation was made with PSM 1000 coated silica but not with PSM 500 and PSM 60. It is conceivable that the longer the residence time of the ovalbumin in the column, the greater is the probability of irreversible adsorption, as observed by Nice *et al.*[21] with *n*-alkyl-bonded silicas.

REFERENCES

1 W. S. Hancock and J. T. Sparrow, in Cs. Horváth (Editor), *High Performance Liquid Chromatography —Advances and Perspectives*, Vol. 3, Academic Press, New York, 1983, p. 49.
2 M. T. W. Hearn, in Cs. Horváth (Editor), *High Performance Liquid Chromatography —Advances and Perspectives*, Vol. 3, Academic Press, New York, 1983, p. 87.
3 J. D. Pearson and F. E. Regnier, *J. Liq. Chromatogr.*, 6 (1983) 497.
4 F. L. DeVosh, D. M. Robertson and M. T. W. Hearn, *J. Chromatogr.*, 392 (1987) 17.
5 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 282 (1983) 541.
6 M. V. Levis, A. Follon, S. Stein, K. D. Gibson and S. Udenfriend, *Anal. Biochem.*, 104 (1980) 153.
7 W. C. Machoney, *Biochim. Biophys. Acta*, 104 (1980) 153.
8 J. Köhler, D. B. Chase, R. D. Farlee, A. J. Vega and J. J. Kirkland, *J. Chromatogr.*, 352 (1986) 275.
9 J. L. Glajch and J. J. Kirkland, *J. Chromatogr.*, 384 (1987) 81.
10 J. Köhler and J. J. Kirkland, *J. Chromatogr.*, 385 (1987) 125.
11 N. Sagliano, R. Hartwick, J. DiBussolo and N. Miller, *J. Chromatogr.*, 443 (1988) 155.
12 H. Engelhardt and H. Müller, *Chromatographia*, 19 (1984) 77.
13 A. Kurganov, A. Tevlin and V. Davankov, *J. Chromatogr.*, 261 (1983) 223.
14 A. Kurganov, O. Kuzmenko, V. Davankov, K. Unger, B. Eray and U. Trüdinger, *J. Chromatogr.*, submitted for publication.
15 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 255 (1983) 125.

16 M. T. W. Hearn and M. J. Aquilar, *J. Chromatogr.*, 392 (1987) 33,
17 C. T. Mant, T. W. Burke and R. S. Hodges, *Chromatographia*, 24 (1987) 565.
18 M. T. W. Hearn and B. Grego, *J. Chromatogr.*, 266 (1983) 75.
19 L. R. Snyder, in Cs. Horváth (Editor), *High Performance Liquid Chronatograpohy —Advances and Perspectives*, Vol. 1, Academic Press, New York, 1980, p. 207.
20 L. R. Snyder, M. A. Stadalius and M. A. Quarry, *Anal. Chem.*, 55 (1981) 1412A.
21 E. C. Nice, M. W. Capp, N. Cooke and N. J. O'Mare, *J. Chromatogr.*, 218 (1981) 569.