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Note

Chromatographic fractionation of proteins at high organic solvent modifier concentrations

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A skewed U-shaped (or bimodal) dependency exists between retention times and concentration of organic solvent during reversed-phase chromatography of some small organic compounds, peptides and proteins[1–5]. Recently, we described a reversed-phase high-performance liquid chromatography (RP-HPLC) procedure (inverse-gradient RP-HPLC), based upon this phenomenon, for recovering subnanomole amounts of protein from sodium dodecylsulfate polyacrylamide gel electroeluates in a form suitable for microsequence analysis[5–9]. Using small pore size (600–120 Å) reversed-phase packings with large surface areas (200–400 m^2/g) and high carbon content (7–15%) proteins can be retained at high concentrations of 1-propanol (90–100%). Under these conditions sodium dodecylsulfate and acrylamide gel-related contaminants are not retained and are washed through the column. Retained proteins can be recovered from the column by the addition of an ion-pairing agent (*e.g.* trifluoroacetic acid) into the mobile phase and elution with a gradient of decreasing 1-propanol concentration (*i.e.*, an "inverse-gradient"). Proteins recovered from gel electroeluates by this method are free of high concentrations of sodium dodecylsulfate and acrylamide-related artifacts. Such artifacts interfere with the Edman chemistry, HPLC-based phenythiohydantion-amino acid analysis and peptide mapping. Inverse-gradient RP-HPLC has been successfully employed to recover a wide variety of proteins, many of which are not amenable to conventional RP-HPLC, from sodium dodecylsulfate gel electroeluates in a form suitable for N-terminal sequence analysis in the 10–500 pmol range[5,8,10]. Recently, the utility of this method was extended to recover proteins from the detergent mixtures (*e.g.* 2% sodium dodecylsulfate–1% Triton X-100) used to elute electroblotted Coomassie blue-stained proteins from poly(vinylidine difluoride) membranes[7].

As a continuation of these studies, we present in this paper an evaluation of other commercially-available silica-based reversed-phase supports which exhibit U-shaped (or bimodal) behavior (*i.e.*, protein retention at high organic modifier concentrations). In addition we demonstrate that protein mixtures can be fractionated by inverse-gradient RP-HPLC.

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EXPERIMENTAL

The proteins employed in this study were purchased from Sigma (St. Louis, MO, U.S.A.) and were of the highest available commercial grade. Trifluoroacetic acid (99+% grade) was from Pierce (Rockford, IL, U.S.A.). Deionised water, obtained from a tandem Milli-RO and Milli-Q system (Millipore, MA, U.S.A.) was used for all buffers. HPLC-grade organic solvents were purchased from Mallinckrodt (Melbourne, Australia). The HPLC system used has been described elsewhere[5,11]. The following reversed-phase supports were used in this study (a) Brownlee VeloSep Octyl (C_8) or Octadecyl (C_{18}) cartridges (3 μm, 100 Å, 40 × 3.2 mm I.D.) obtained from Applied Biosystems (Foster City, CA, U.S.A.). (b) ODS-Hypersil (C_{18}) columns (5 μm, 120 Å, 100 × 2.1 mm I.D.; 3 μm, 120 Å, 60 × 4.6 mm I.D.; or cartridges (5 μm, 120 Å, 20 × 2.1 mm I.D.) were obtained from Hewlett-Packard (Waldbronn, F.R.G.). ODS-Hypersil (C_{18}) microbore columns (3 μm, 120 Å, 50 × 2.1 mm I.D. or 50 × 1.0 mm I.D.) were packed as previously described[5]. Brownlee RP-300 Octyl (C_8) cartridges 7 μm, 300 Å, 100 × 2.1 mm I.D.) were obtained from Applied Biosystems. (d) LiChrospher Diol (Merck, Darmstadt, F.R.G.) columns (5 μm, 75 × 4.6 mm I.D.) were packed as previously described[5].

RESULTS AND DISCUSSION

Details of the silica-based packings employed in this study are summarized in Table I. Of the supports examined, the small pore size (100-120 Å) large surface area (170–200 m^2/g) supports (*e.g.* ODS-Hypersil and Brownlee C_8 VeloSep) exhibited comparable efficiences for a number of proteins chromatographed in the inverse-gradient elution mode (Fig. 1). These supports are commonly utilized for the RP-HPLC of low-molecular-weight compounds (*e.g.* peptides)[6,8,9,12]. Interestingly, the large pore size (300 Å) support used in this study (Brownlee RP-300) was not considered useful in the inverse-gradient mode since proteins were recovered in unacceptably large volumes (600–1500 μl) (Fig. 1).

Table II shows the retention times obtained for ten proteins of known primary structure on different columns operated in the classical reversed-phase or inverse-gradient reversed-phase elution mode. Unlike the chromatographic behavior of small peptides where it is well documented that a clear relationship exists between the

TABLE I

DATA FOR SPHERICAL POROUS SILICA SUPPORTS USED IN THIS STUDY

Obtained directly from the manufacturer. NA = Not available.

Support	*Carbon content (%)*	*Surface area (m^2/g)*	*Surface coverage ($\mu mol/m^2$)*	*Particle size (μm)*	*Pore volume (ml/g)*	*Designations*
ODS-Hypersil	9.5–10.0	170	2.06	3–5	0.7	C_{18}-120Å
Brownlee VeloSep C_8	7.4–8	200	2.2	3	0.8	C_8-100Å
Brownlee VeloSep C_8	12–13.2	200	1.9	3	0.8	C_{18}-100Å
Brownlee RP-300	7	80–110	8.7	7	0.5–0.6	C_8-300Å
Merck Diol	NA	250	NA	5	NA	Diol-100Å

TABLE II

RETENTION TIMES, t_R (min), FOR STANDARD PROTEINS ON DIFFERENT COLUMNS OPERATED IN EITHER REVERSED-PHASE OR INVERSE-GRADIENT REVERSED-PHASE ELUTION MODE

Columns: 1 = ODS-Hypersil, 20 × 2.1 mm I.D.; 2 = ODS-Hypersil, 100 × 2.1 mm I.D.; 3 = Brownlee RP-300, 30 × 2.1 mm I.D.; 4 = Brownlee C_8 VeloSep 40 × 3.2 mm I.D.; 5 = Brownlee C_{18} VeloSep, 40 × 3.2 mm I.D. Chromatographic conditions: reversed-phase, linear 50-min gradient from 0 to 100% B where solvent A = 0.1% (v/v) aqueous trifluoroacetic acid and solvent B = *n*-propanol–water (50:50) containing 0.1% (v/v) trifluoroacetic acid; inverse-gradient reversed-phase, linear 50-min gradient from 0 to 100% B where solvent A = 100% *n*-propanol and solvent B = *n*-propanol–water (50:50) containing 0.4% (v/v) trifluoroacetic acid (values in parenthesis were for solvent B = *n*-propanol–water (50:50) containing 0.1% (v/v) trifluoroacetic acid). Flow-rate: columns 1–3, 200 μl min^{-1}; columns 4 and 5, 400 μl min^{-1}. Detection, UV at 280 nm. Column temperature, 40°C. Sample load: 10 μg protein in 20 μl water. Data were obtained in duplicate with at least two independent sample preparations and averaged with resulting precision of 1–2%.ND = not determined.

Protein	M_r *(kDa)*	*HI*[a]	t_R *(min)*											
			Reversed-phase elution mode					*Inverse-gradient reversed-phase elution mode*						
			1	*2*	*3*	*4*	*5*	*1*	*2*	*3*	*4*		*5*	
Insulin	5.8	1180	21.67	26.46	20.96	20.62	19.91	12.57	8.14	6.29	7.35	(11.83)	5.67	(8.24)
Cytochrome *c*	11.6	1110	25.71	30.57	23.21	23.85	22.85	15.67	11.80	11.35	9.93	(16.73)	7.36	(11.26)
Ribonuclease B	13.5	870	20.29	25.37	19.24	19.08	17.89	15.32	11.47	11.56	9.62	(16.18)	7.38	(13.78)
α-Lactalbumin	14.1	1150	29.04	33.97	26.54	27.32	26.07	15.24	11.42	8.01	9.65	(15.78)	7.33	(10.95)
Lysozyme	14.7	970	26.59	31.84	24.13	24.70	23.52	15.43	11.45	10.78	9.71	(16.00)	7.33	(10.96)
Trypsin inhibitor	20	1040	32.98	38.77	28.54	30.35	28.68	15.86	12.17	8.53	10.12	(17.16)	7.51	(11.67)
Carbonic anhydrase	30	1060	35.78	41.25	30.70	32.63	30.47	17.51	13.68	11.56	11.06	(19.53)	8.17	(12.92)
Ovalbumin	45	1110	42.14	48.05	34.60	37.95	35.73	16.86	13.72	9.48	11.06	(19.62)	8.06	(12.93)
α-Amylase	52	1070	36.67	41.06	29.87	32.10	30.12	17.65	14.83	11.05	11.79	(21.14)	8.64	(13.82)
Transferrin	90	ND	33.04	38.58	26.63	28.73	26.59	18.10	14.79	14.80	11.77	(21.33)	8.42	(11.17)

[a] Average hydrophobicity, obtained from ref. 14.

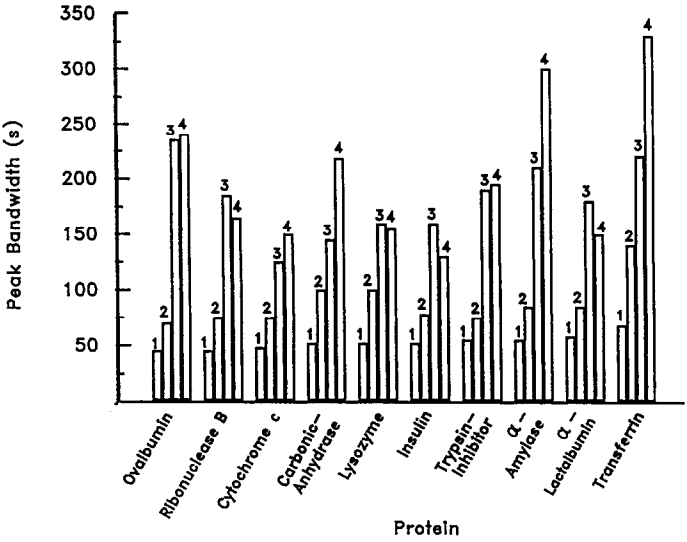

Fig. 1. Plot of the peakwidth (seconds) of eluted proteins from various columns operated in the inverse-gradient reversed-phase elution mode. Chromatographic conditions are given in Table II. Columns: (1) Brownlee VeloSep C_8 (40 × 3.2 mm I.D.); (2) ODS-Hypersil (100 × 2.1 mm I.D.); (3) Brownlee RP-300 (100 × 2.1 mm I.D.); Merck LiChrosorb Diol (75 × 4.6 mm I.D.). Proteins: ovalbumin; ribonuclease B; cytochrome *c*; carbonic anhydrase; lysozyme; insulin; trypsin inhibitor; α-amylase; α-lactalbumin transferrin.

polarity of a peptide and its retention order[13], an examination of the chromatographic behavior of the panel of proteins used in this study reveals no clear correlation between the calculated hydrophobicities[14] of these proteins (summarized in Table II) and their elutions times with 1-propanol.

The data presented in Table II clearly shows that the order of protein retention on the columns studied is largely independent of the chromatographic mode employed. Thus, for a particular support the protein retention order is essentially the same in both the conventional reversed-phase and inverse-gradient reversed-phase elution mode. However, upon close scrutinization of the retention data in Table II, some reversals in protein selectivity pattern are apparent. For example, the retention order of insulin and ribonuclease as well as ovalbumin and transferrin (Table II) are reversed in the two chromatographic modes. These findings are in accord with previously reported observations, that multiple retention processes may be involved in the binding of organic compounds[1], peptides and proteins[2,3] to silica-based reversed-phase supports. This change in selectivity pattern is suggestive of normal (or polar) phase chromatographic behavior in the inverse-gradient mode and is due, presumably, to residual silanol groups remaining on reversed-phase supports[1–5] (*i.e.*, a silanophilic mechanism[15]). This notion is supported by the work of Bij *et al.*[1] who demonstrate that the addition of *n*-butylamine to the eluent attenuated silanophilic interactions which resulted in regular retention behaviour for peptides. However, the observation by others[2,3,5] that other alkylamines (*e.g.*, triethylamine) do not dramatically influence this irregular U-shaped behaviour for peptides suggests that processes other than silanophilic interactions may be involved.

It is well known from the literature[16–19] that conformational transitions in

proteins can be induced by apolar compounds such as detergents and organic solvents. For instance it has been recently demonstrated (using circular dichroism, fluorescence and visible spectroscopy) that 1-propanol can induce a reversible conformational change in proteins to an apparently ordered helical form[20]. Consistent with this hypothesis is the recent report[17] that protein conformation can have a marked influence on protein retention behavior on reversed-phase supports. In these studies Benedek *et al.*[17] established that "native" and "denatured" forms of proteins can be clearly resolved on reversed-phase packings and that the kinetics of protein unfolding is a function of both the organic modifier employed and the incubation time that a protein spends on the bonded-phase surface prior to development of the column[17]. In the case of small peptides it has been demonstrated that their retention behavior on reversed-phase supports can be strikingly influenced if the peptide can be induced to form an amphipathic helix[21,22]. Hence, at high organic solvent concentrations protein structures may be disrupted to produce periodic but dispersed polar/apolar helical exteriors[23] which, in turn, may influence the interaction between protein and

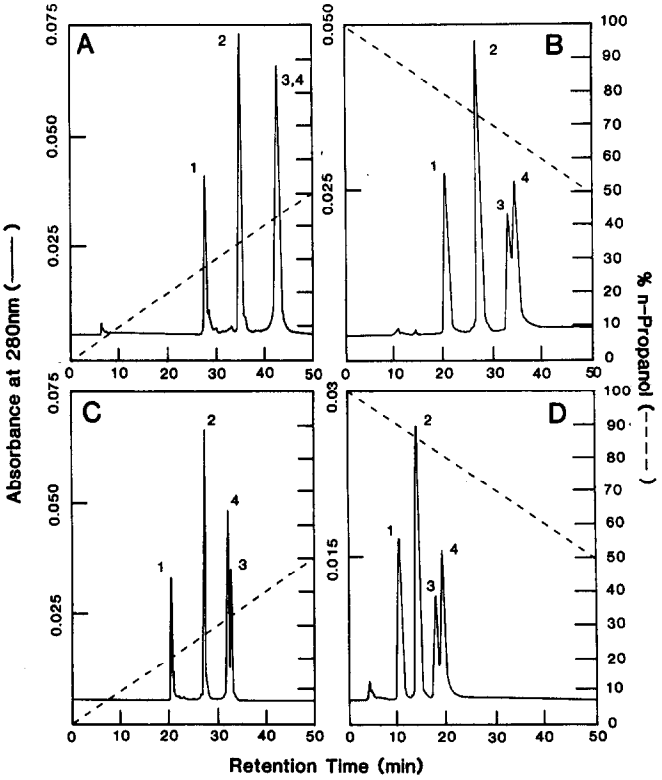

Fig. 2. Separation of proteins using reversed-phase and inverse-gradient reversed-phase elution modes. Chromatographic conditions: (A) ODS-Hypersil (100 × 2.1 mm I.D.); reversed-phase elution mode (linear 50-min gradient from 0 to 100% B, where solvent A = 0.1% (v/v) trifluoroacetic acid, solvent B = 50% (v/v) aqueous *n*-propanol containing 0.1% (v/v) trifluoroacetic acid). (B) ODS-Hypersil (100 × 2.1 mm I.D.). Inverse-gradient reversed-phase elution mode (linear 50-min gradient from 0 to 100% B, where solvent A = 100% *n*-propanol, solvent B = 50% aqueous *n*-propanol containing 0.1% (v/v) trifluoroacetic acid. (C) Brownlee C_8 VeloSep (40 × 3.2 mm I.D.). Reversed-phase elution mode (same as in Table II). (D) Brownlee C_8 VeloSep (40 × 3.2 mm I.D.). Inverse-gradient reversed-phase elution mode (same as in Table II). Flow-rate 400 μl min^{-1}. Column temperature, 40°C. Proteins: 1 = bovine insulin; 2 = α-lactalbumin; 3 = carbonic anhydrase; 4 = α-amylase. Sample load: 10 μg in 20 μl water.

chromatographic support. Thus, the selectivity changes may result from proteins assuming different conformations in the conventional reversed-phase and inverse-gradient reversed-phase elution modes; *i.e.*, different proteins conformations may be induced by the mobile phase interactions of the different chromatographic modes.

As described earlier[5], the ion-pairing agent trifluoroacetic acid modulates protein retention behavior in the "inverse-gradient" chromatographic mode (see also Table II) as well as chromatographic efficiencies and protein recoveries. For practical purposes, we routinely use 0.1–0.4% (v/v) trifluoroacetic acid in the second mobile phase in order to minimise peak bandwidth; under these conditions proteins were typically recovered in 100–300 μl using 2.1 or 3.2 mm I.D. columns[24].

The chromatographic efficiency of proteins in the inverse-gradient reversed-phase elution mode, approximately 90% of that achieved in the conventional reversed-phase elution mode, permits protein fractionations. Indeed, for the panel of proteins employed in this study α-amylase and carbonic anhydrase are better resolved in the inverse-gradient mode than the reversed-phase mode (Fig. 2). Thus the inverse-gradient RP-HPLC procedure described here offers the potential for resolving preparative amounts of proteins at high organic solvent concentrations within the confines of a chromatographic column.

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