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Note

High-performance liquid chromatography of polypeptides and proteins on a reversed-phase support

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The separation of peptides and proteins by high-performance liquid chromatography (HPLC) is limited by the instability of the ion-exchange and molecular-sieve-gel support materials under pressure. Pressure-stable porous glass beads suffer from the disadvantage of irreversible adsorption of proteins to the silanol-groups of the glass[1]. Recent results have shown that HPLC on a reverse-phase support is suitable for separating peptides[2,3,4], and, by using gradient elution with phosphate buffer solution and methanol, we have been able to separate peptides of molecular weight up to 1000 (ref. 4). In continuation of this work, we have now developed an elution system suitable for separating proteins on a reversed-phase support.

MATERIALS AND METHODS

Calibration proteins [Combithek (Boehringer, Mannheim, G.F.R.)], insulin (Serva, Heidelberg, G.F.R.), potassium dihydrogen phosphate and phosphoric acid (p.a.; Merck, Darmstadt, G.F.R.), isopropanol (p.a., Merck, redistilled before use), and ethyleneglycol monomethyl ether (2-methoxyethanol) (LAB, Merck) were used. Water was purified as described earlier[4].

The chromatograph assembly (Waters Assoc., Königstein, G.F.R.) consisted of two pumps (Model 6000), a programmer (Model 660) and an injector (Model U6K). The column (300 mm × 4 mm I.D.) was packed by the slurry method at 6000 p.s.i. with 10-μm octadecylsilane (ODS) coated particles (Nucleosil 10 C-18, Macherey & Nagel, Düren, G.F.R.) and was maintained at 47° during chromatography. The linear gradient used was established from two solvents (*A* and *B*). Solvent *A* consisted of 0.05 *M* potassium dihydrogen phosphate–2-methoxyethanol adjusted to pH 2 with phosphoric acid (95:5), and solvent *B* of isopropanol–2-methoxyethanol adjusted to pH 2 with phosphoric acid (95:5). The initial concentration of the gradient was 90% of solvent *A* with 10% of solvent *B*; the final concentration was 50% of each solvent. The gradient was terminated after 30 min, the flow-rate being 2 ml/min and the pressure increasing from 1900 to 3500 p.s.i. The sample volume was 25 μl of protein solution (1 mg/ml), and proteins were detected in the eluate at 280 nm (Perkin-Elmer detector LC 55). Equilibration of the column to the initial conditions took about 5 min.

RESULTS

The chromatographic separation of five proteins is shown in Fig. 1; the individual proteins are listed in Table I according to their retention times (R_t values). It can be seen that the R_t values and the peak areas (determined by planimetry) are reproducible for most of the proteins. There was a rectilinear relationship between the amount of albumin injected and the peak area on the chromatogram (see Fig. 2).

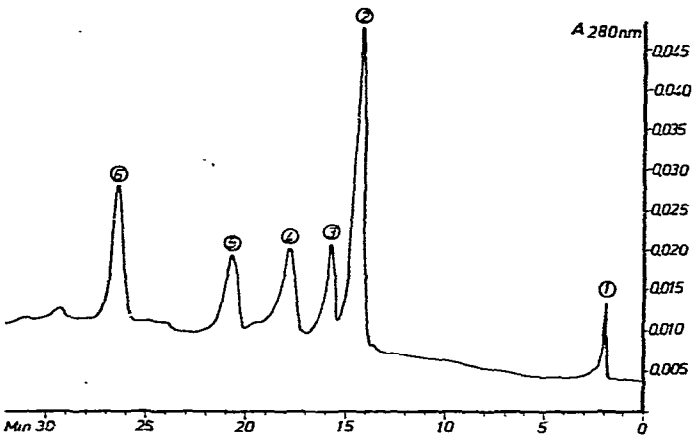

Fig. 1. Separation of proteins on reversed phase; for conditions of separation, see Materials and methods. Peaks: 1, impurity; 2, insulin; 3, cytochrome c; 4, bovine albumin; 5, catalase; 6, ovalbumin.

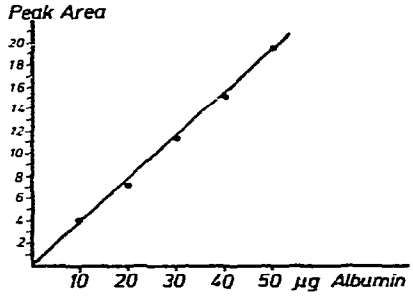

Fig. 2. Relationship between amount of bovine albumin injected and peak area in planimeter units.

Aldolase is not shown on the chromatogram, as it has the same R_t value as catalase. Chymotrypsinogen A and ferritin are eluted from the column at the given R_t values (Table I), but, besides the main peak, these materials show broad peaks arising probably from impurities. The reproducibilities of the peak areas of these proteins, and that of insulin (which tended to be precipitated from the stock solution) were not evaluated.

TABLE I

REPRODUCIBILITY OF R_t VALUES AND OF QUANTITATIVE DETERMINATIONS

Protein	*Molecular weight*	*Reproducibility**			
		R_t value, min ± S.D. (%)		*Peak area, planimeter units ± S.D. (%)*	
Insulin	5400	14.40 ± 1.3	(5)	—	
Cytochrome *C*	12,900	16.10 ± 1.8	(5)	1.80 ± 0	(4)
Bovine albumin	67,000	17.90 ± 1.6	(18)	1.95 ± 3	(4)
Aldolase	158,000	20.05 ± 0.5	(4)	0.69 ± 3.6	(4)
Catalase	240,000	20.80 ± 0.5	(6)	0.83 ± 4.8	(4)
Chymotrypsinogen A	25,000	21.80 ± 1.3	(3)	—	
Ferritin	450,000	25.90 ± 0.4	(4)	—	
Ovalbumin	43,000	26.70 ± 2.2	(8)	1.38 ± 2.2	(4)

* Figures in parentheses are the numbers of determinations.

DISCUSSION

Octadecylsilane, chemically bound to silica gel (ODS, reversed phase) is a non-polar phase. Adsorbed molecules are eluted by organic solvent mixtures, *e.g.*, methanol–water. Proteins are strongly bound to this reversed phase when they are applied in aqueous solution, as the non-polar groups of the proteins interact with the octadecylsilane groups. This interaction can be destroyed by non-polar organic solvents, but proteins are poorly soluble in such solvents and tend to be precipitated and thus not eluted. The difficulty is to find a solvent mixture sufficiently polar enough to leave the proteins in solution, yet sufficiently non-polar to break the interaction of the proteins with the reversed phase. We have solved this problem by adding 2-methoxyethanol to the initial aqueous solvent as well as to the final organic eluent. 2-Methoxyethanol is a molecule with both hydrophobic and hydrophilic groups and therefore behaves like a surface-active agent. It is adsorbed on the reversed phase, rendering this less hydrophobic; under these conditions, proteins are less strongly adsorbed and can be eluted more readily. 2-Methoxyethanol could probably be replaced by other compounds with similar properties.

Our results suggest that the chromatographic behaviour of proteins is determined not only by the polarity of the system, as has been shown for peptides[4], but at least to some extent by the molecular weight. Most of the proteins are eluted according to their molecular weight (but not ovalbumin or chymotrypsinogen A). The use of isopropanol and phosphate buffer solution at acid pH proved to be advantageous, as the proteins were soluble in this mixture and gave narrow peaks on the chromatogram.

To summarize, our results clearly show that polypeptides and proteins up to a molecular weight of 450,000 can be separated by reversed-phase HPLC; R_t values and quantitative measurements are highly reproducible. The chromatographic conditions, however, can be adjusted to suit particular separation problems, *e.g.*, by changing the solvent composition and the gradient. The method is suitable for rapid analytical separations of proteins and might offer a convenient supplement to electrophoresis, gel chromatography and ion-exchange chromatography.

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