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EVALUATION OF ADVANCED SILICA PACKINGS FOR THE SEPARATION OF BIOPOLYMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

VI. DESIGN, CHROMATOGRAPHIC PERFORMANCE AND APPLICATION OF NON-POROUS SILICA-BASED ANION EXCHANGERS

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SUMMARY

The linear solvent strength model of Snyder was applied to describe fast protein separations on 2.1-μm non-porous, silica-based strong anion exchangers. It was demonstrated on short columns packed with these anion exchangers that (i) a substantially higher resolution of proteins and nucleotides was obtained at gradient times of less than 5 min than on porous anion exchangers; (ii) the low external surface area of the non-porous anion exchanger is not a critical parameter in analytical separations and (iii) μg-amounts of enzymes of high purity and full biological activity were isolated.

INTRODUCTION

The replacement of soft organic packings by microparticulate rigid macroporous organic polymers and wide-pore bonded silicas has substantially improved the separation performance in all modes of interactive chromatography of proteins and polynucleotides[1,2]. However, the results obtained so far indicate that the enhancement in resolution, speed, and biorecovery was not as high as expected. Several factors responsible for this observation are related to two intrinsic properties of porous packings: the tortousity and connectivity of pores and the heterogeneity of the surface. As a consequence, the kinetics of mass transfer of analytes can be slow, due to restricted intraparticle diffusion of polymers, and the remaining active surface sites can give rise to undesired interactions. All together, the effects cause additional peak dispersion and often considerable losses in recovery of biological activity.

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The logical solution to this problem is the use of non-porous packings, which offer a number of advantages, such as (i) fast mass transfer kinetics due to lack of restricted pore diffusion; (ii) absence of the enthalpic and entropic exclusion of solutes occurring in porous packings; (iii) maximum surface accessibility; (iv) maximum ligand utilization by appropriate ligand design and adjustment of ligand density and topography; (v) preservation of biological activity, due to short residence time of solutes in the column and (vi) fast column regeneration, due to the absence of an internal column volume.

The use of non-porous supports has some disadvantages also. Due to the lack of porous and internal surface, the external surface area of non-porous particles is extremely low. Assuming a solid density of 2.2 g/ml, the external surface area is calculated to 5.5 m^2/g at d_p (particle diameter) = 0.5 μm, 2.7 m^2/g at d_p = 1.0 μm, and 0.55 m^2/g at d_p = 5 μm. It is seen that the surface area of non-porous packings is about two orders of magnitude lower than that of porous packings. As a result, the solute retention and the mass loadability of packings decreases in the same proportion. To compensate for this drawback, the use of non-porous particles of 1–2 μm is recommended. They have a surface area of 2–4 m^2/ml of packing, comparable to that of a 200-nm pore-size packing. Particles much smaller than 1 μm are difficult to pack into columns and to operate with a conventional high-performance liquid chromatography (HPLC) system.

In 1984 we introduced 1.5-μm non-porous, monodisperse silica beads as packings for affinity chromatography with binding capacities for proteins comparable to those of 100- to 200-nm pore-size silicas[3]. Phillips *et al.*[4] have described the use of non-porous glass beads with $d_p > 10$ μm in affinity chromatography. Since that time, a number of non-porous packings have been developed and their outstanding chromatographic properties have been demonstrated[5–12].

In this paper, we describe the investigation of the potential of 2-μm silica-based non-porous strong anion exchangers in fast high-resolution separation and micropreparative isolation of biopolymers.

EXPERIMENTAL

Materials and methods

Non-porous monodisperse 2.1-μm silica and LiChrospher Si-300 -1000 and -4000 (10 μm) silicas were from Merck (Darmstadt, F.R.G.). Polyethylenimine-6 (PEI-6; molecular weight *ca.* 600) was purchased from Polysciences (Warrington, PA, U.S.A.). Glyceryltriglycidyl ether and pentaerythritol triglycidyl ether for crosslinking were from Grillonit, Ems-Werke AG (Domat/Ems, Switzerland). Methyl iodide, inorganic salts and solvents were of analytical grade or comparable quality.

Samples

Pure proteins, soybean trypsin inhibitor (STI), bovine serum albumin (BSA), catalase (CAT) and alcoholdehydrogenase (ADH) were from Merck; conalbumin (CON), ovalbumin (OVA) and human transferrin (TRA) from Serva (Heidelberg, F.R.G.); human serum albumin (HSA) from Behring-Werke (Marburg, F.R.G.) and horse heart myoglobin from Sigma (St. Louis, MO, U.S.A.). All 5'-mono, di- and triphosphate nucleotides were also purchased from Sigma. The DNA restriction

fragments were a gift of Prof. Müller (Merck). The enzyme F_1-ATPase from *Micrococcus luteus* was prepared in our laboratory.

Synthesis of anion exchangers

The method of Alpert and Regnier[13], modified by Kopaciewicz *et al.*[14], was applied for polyethylenimine coating and crosslinking of the porous and non-porous silicas with a minor variation. A 3-g amount of the non-porous silica was weighed into a 100-ml round-bottom flask, and 50 ml of a 10% (w/v solution of PEI-6 in methanol were added. The flask was sonicated, degassed briefly and kept at room temperature for 48 h. The coated silica was collected in a sintered glass funnel with a G4 frit and dried under vacuum for 2 h. Crosslinking was performed by suspending the coated silica in 50 ml of a solution of 10% (v/v) glyceryldiglycidyl ether (pentaerythritol triglycidyl ether) in methanol. The flask was sonicated, evacuated shortly and left at room temperature for 72 h. The product was isolated on a sintered glass funnel, washed with methanol and water and dried under vacuum. The porous particles were modified as described by Kopaciewicz *et al.*[14]. For quaternization the PEI-coated silicas were methylated using methyl iodide[15].

As the PEI loading of porous as well as non-porous silicas could not be assessed by elemental analysis due to the low nitrogen content, the solute retention served as a relative measure of mass of the load.

Packing procedure

The PEI-coated packing materials were slurry-packed into columns of 33 × 8 mm I.D. (Bischoff Analysentechnik und -geräte, Leonberg, F.R.G.) with 2-propanol–cyclohexanol (3:2, v/v) mixtures at constant flow-rates under maximum pressures of 60 MPa for the 2.1-μm non-porous particles and 40 MPa for the porous silicas. Alternatively, the anion exchangers were also packed with a 0.01 *M* Tris–HCl buffer (pH 8), yielding the same column performance as those packed with 2-propanol/cyclohexanol mixtures. The end fittings were made of paper filters (porosity ≤ 0.2 μm), supplied by Schleicher & Schüll (Dassel, F.R.G.) and supported by metal (porosity < 2 μm) frits from Bischoff Analysentechnik und -geräte.

HPLC instrumentation

All chromatographic procedures were performed with a high-pressure gradient LC system, consisting of two Model 2150 HPLC pumps and a Model 2152 HPLC controller (Pharmacia-LKB Biotechnology, Uppsala, Sweden). All measurements were monitored at 280 nm for proteins and 254 nm for nucleotides using a Shimadzu SPD 6VA detector (Kyoto, Japan) with a 0.6-μl cell. The response time of 50 ms of the detector was sufficient to monitor fractions eluted a few seconds apart. The dwell time at a flow-rate of 1 ml/min amounted to 16.2 s. Two manual injection systems from Rheodyne (Cotati, CA, U.S.A.) were employed, equipped with a 1-μl and a 20-μl sample loop. Dead volume of all connecting tubing (0.125 mm I.D.) was kept at a minimum. The mobile phases were filtered through 0.45-μm porosity filters and degassed. For proteins and DNA fragments linear gradient elution was carried out with 0.01 *M* Tris–HCl buffer at pH 8 (eluent A) and 0.5 and 1 *M* sodium chloride in Eluent A (eluent B). The 5′-phosphate nucleotides were chromatographed by gradient elution, using a potassium phosphate buffer (0.01–0.5 *M*, pH 8).

Protein mass recovery

For the assessment of the mass recovery the column was loaded with a solution of 1 or 5 mg of pure protein in 0.01 *M* Tris–HCl (pH 8) and then eluted with 0.4 *M* sodium chloride in eluent A. In a second experiment the sample was directly injected into the detector. The collected volumes of samples eluted with and without the column were measured spectrophotometrically at 280 nm. Mass recovery of the following proteins were examined: OVA, BSA, CON, TRA, CAT, ADH, HSA and STI.

Recovery of the enzymatic activity

The activity of CAT[16] was determined by monitoring the decrease of the hydrogen peroxide concentration at 240 nm (UV) during the first 2 min of the reaction. CAT was injected at four different concentrations (2364 – 26 000 U) and eluted with 0.5 *M* in eluent A. Again, the experiments were repeated without the column, *i.e.*, by direct connection of the injector with the detector.

RESULTS AND DISCUSSION

Application of the linear solvent strength (LSS) model of Snyder to anion-exchange chromatography of proteins on non-porous packings

For the application of non-porous packings in interactive HPLC of proteins it is useful to predict operating conditions and to optimize the separation with respect to resolution, speed, and recovery. Recently, Snyder and co-workers[17–20] have developed a general model applicable to the chromatographic separations of large molecules in size-exclusion, reversed-phase, hydrophobic interaction and ion-exchange chromatography. To check the general validity of the linear solvent strength model of Snyder, it was applied to fast protein separations on the non-porous 2.1-μm anion exchangers. First isocratic retention parameters were calculated by means of two gradient runs. In isocratic ion-exchange chromatography the solute capacity factor is a function of salt concentration, c, of the mobile phase as

$$\log k' = \log K - m \log c \tag{1}$$

where K is the ion-exchange distribution constant and m the effective charge of the solute. Furthermore the average k' value of the solute during elution can be calculated by

$$\bar{K} = 1/1.15 \cdot b \tag{2}$$

where b is the gradient steepness parameter (eqn. 4). $\bar{K}$ corresponds to the k' value of the migrating band at the halfway point along the column. The concentration, $\bar{c}$, at the column midpoint is obtained by

$$\bar{c} = c_0 + [t_g - t_0 - t_D - 0.3(t_0/b)]\Delta c/t_G \tag{3}$$

where c_0 and c_f are the initial and final salt concentrations ($\Delta c = c_f - c_0$), t_g the retention time in gradient elution, t_0 the column dead-time, t_D the dwell-time and t_G the gradient time. The gradient steepness parameter, b, is given by

$$b = V_m \log(c_f/c_0)/t_G f_v \tag{4}$$

where f_v is the volume flow-rate and V_m the column dead-volume. It should be emphasized that b is not constant over the entire gradient. Hence, an average value of $b(b_0)$ is defined by

$$b_0 = rb \tag{5}$$

where r is

$$r = \Delta c/2.3\bar{c} \log(c_f/c_0) \tag{6}$$

A more reliable method for calculating isocratic retention data is based on the equation

$$b_1 = (t_0 \log \beta)/[t_{g1} - (t_{g2}/\beta) - (t_0 + t_D)(\beta - 1)/\beta] \tag{7}$$

when b_2 is equal to

$$b_2 = b_1/\beta \tag{8}$$

with

$$b_2/b_1 = \beta = t_{G2}/t_{G1} \tag{9}$$

Values of $\bar{K}$ *vs.* $\bar{c}$ are calculated from gradient elution data for proteins and compared with the experimental results obtained isocratically (k' *vs.* c).

Furthermore, the chromatographic performance, can be expressed by the resolution, R_s, as

$$R_s = 1.176 \frac{t_{g2} - t_{g1}}{[w_{0.5}]_1 + [w_{0.5}]_2} \tag{10}$$

where t_{g1} and t_{g2} are the retention times of the proteins and $[w_{0.5}]_1$ and $[w_{0.5}]_2$ the bandwidths at half-height of the peak. From the experimental data the b values were calculated for the 0.5- and 1.0-min gradients at a flow-rate of 4 and 5 ml/min and for the 1-, 2- and 5 min gradient at a flow-rate of 1.5 ml/min. Values of $\bar{K}$ against $\bar{c}$ were plotted for TRA, OVA and STI and compared with the experimental data, obtained isocratically from the plots of k' against c. Fig. 1 shows a relatively good agreement between the data obtained from isocratic elution and gradient runs. Note that the average $\bar{K}$ values are mostly $\ll 1$, caused by the smaller differences between the retention times of proteins on the non-porous anion exchanger. It was shown that the LSS model is applicable to fast separations on non-porous packings.

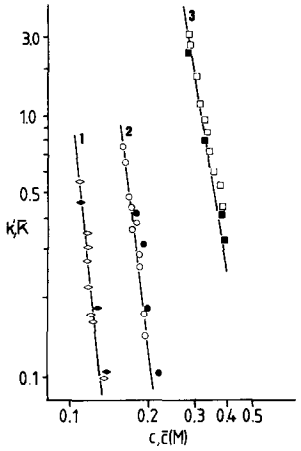


Fig. 1. Correlation of retention data from isocratic (◆, ●, ■) and gradient elution (◇, ○, □). Ion-exchange chromatography of (1) transferrin, (2) ovalbumin and (3) soybean trypsin inhibitor on a non-porous strong anion-exchange column; conditions and equations as described in text.

Fast high-resolution separations of proteins and polynucleotides

Tests have been performed to compare the resolution of proteins in anion-exchange chromatography on non-porous 2.1-μm packings and 10-μm silicas of graduated pore diameter. All products were PEI coated and quaternized.

In Fig. 2 the chromatographic resolution R_s for OVA–STI is plotted against the gradient time, t_G, obtained from separations of proteins on non-porous and porous anion exchangers. It is obvious that the porous anion exchanger based on LiChrospher

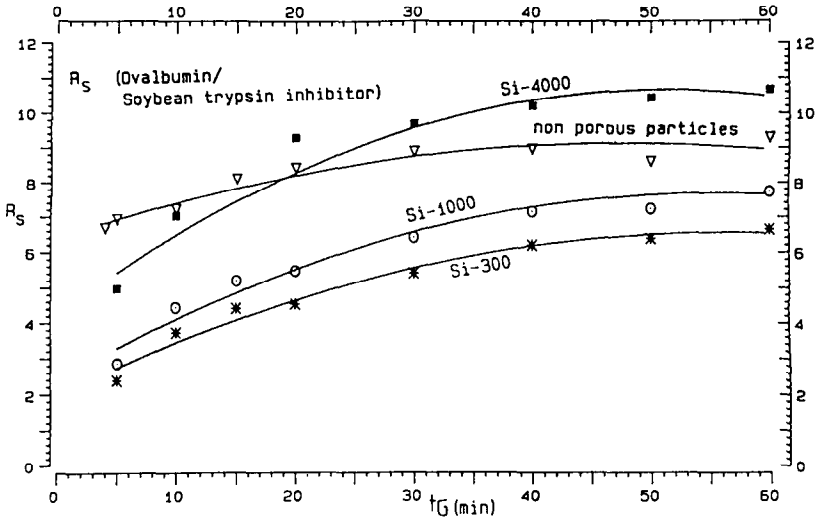


Fig. 2. Chromatographic resolution, R_s, of OVA–STI on PEI-coated porous and non-porous strong anion-exchange columns as a function of gradient time, t_G, at a constant flow-rate of 1.5 ml/min. Gradient: 0–0.5 *M* sodium chloride.

Si-4000 provides a better resolution at larger gradient times of about 20 min than the non-porous anion exchangers. Higher R_s values are the result of larger retention differences between t_{g1} and t_{g2} for the porous packing. However, the highest resolutions at $t_G < 20$ min were achieved on columns packed with the non-porous anion exchangers. Although there is a difference in particle size between 2.1-μm (non-porous) and 10-μm (porous), all porous anion exchangers gave a higher band-broadening due to the intraparticulate mass transfer. Nearly the same results were achieved by Duncan *et al.*[7], comparing 10-μm polymer-based, porous and non-porous ion exchangers. The difference in resolution for high-speed separations becomes much more pronounced at high flow-rates and very short gradient times (see Fig. 3A and B). But it must be noticed, that the linear velocity, u, is nearly double as high for the non-porous particles as for porous packings, caused by the absence of the pore volume for the non-porous anion exchanger. So the difference between the curves of Lichrospher Si-4000 and the non-porous packing decreases, when the resolution is plotted against the linear velocity (not shown). A comparison of the chromatograms

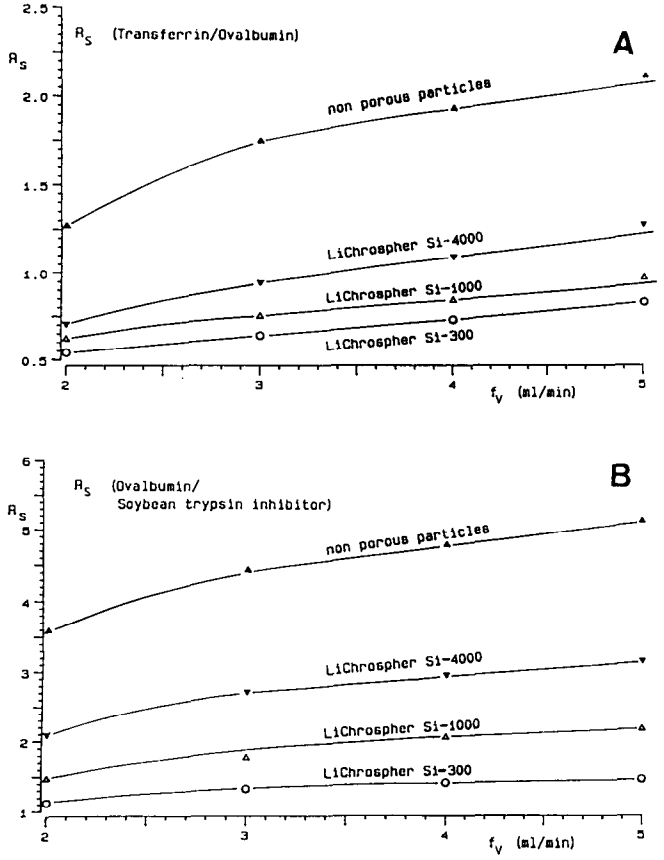


Fig. 3. Chromatographic resolution, R_s, of (A) TRA–OVA and (B) OVA–STI on porous and non-porous strong anion-exchange columns as a function of volume flow-rate, f_v. Gradient: 1 min, linear (0–0.5 *M* sodium chloride).

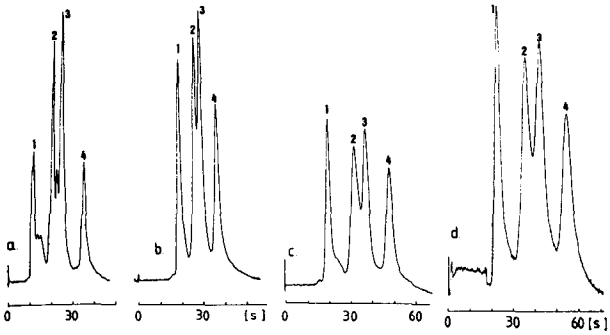


Fig. 4. Fast separation of (1) MYO, (2) TRA, (3) OVA and (4) STI on PEI-coated silica-based strong anion-exchange columns (33 mm × 8 mm I.D.). (a) Non-porous 2.1-μm particles, (b) LiChrospher Si-4000, (c) LiChrospher Si-1000, (d) LiChrospher Si-300. Gradient time: 0.5 min, linear (0–0.5 *M* sodium chloride). Flow-rate; 5 ml/min.

obtained with different anion exchangers demonstrate the advantage of the non-porous material (Fig. 4). In case of the non-porous anion exchanger (Fig. 4a) the prepeak of OVA (see arrow) was detected. Furthermore, the retention times and peak width of proteins are seen to become larger with decreasing the pore size (Fig. 4b–d).

Protein mass and enzymatic activity recoveries

Mass recoveries of $\geqslant$97% were achieved for OVA, BSA, CON, TRA, CAT, ADH, HSA and STI on the non-porous anion exchanger (Table I). With the exception of CON and TRA, recoveries were generally superior to those obtained from the porous packings under identical conditions. Especially with LiChrospher Si-300 larger amounts of proteins were irreversibly adsorbed on the surface of the silica.

TABLE I

PROTEIN MASS RECOVERIES USING POROUS AND NON-POROUS STRONG ANION EXCHANGERS

Columns (33 mm × 8 mm I.D.): (a) non-porous particles, (b) LiChrospher Si-4000, (c) LiChrospher Si-1000 and (d) LiChrospher Si-300. For conditions, see text.

Proteins	*Mass recovery (%)*			
	Column			
	a	*b*	*c*	*d*
OVA	98	97	97	96
BSA	98	97	97	94
CON	99	99	98	99
TRA	99	99	99	99
CAT	98	97	97	89
ADH	98	98	98	94
HSA	98	96	95	93
STI	97	96	95	89

A similar result was achieved for the recovery of the enzymatic activity of CAT. Although CAT is a tenaciously retained protein, with the non-porous anion exchanger the activity recovery was 97–98% for all concentrations. With porous packing the values were lower, *i.e.*, between 90 and 95% were obtained. Possibly the decrease of the biological activity of catalase on porous anion exchangers is caused by the multisite solute surface interactions in the pores of the silica.

Temperature effects

While shorter retention times of proteins have been observed at elevated column temperatures in reversed-phase chromatography, the retention times for strong anion exchangers increased with increasing temperature. Raising the temperature from 298 to 333 K resulted in an increase in the retention time of DNA-fragments with a non-porous anion exchanger by 70%. According to Helfferich[21], it is assumed that with increasing temperature the diffusion coefficient in ion exchangers increases, the matrix becomes more flexible and the diameter of the ions decreases because solvation is reduced. The resolution of protein separation was nearly doubled at a temoerature of 328 K, caused mainly by longer retention times and, to a minor extent, by a small decrease in peak bandwidth at higher temperatures. But above 328 K, resolution could no more be calculated because strong band broadening and dramatic losses of mass recovery occurred. To avoid denaturation at higher temperatures, it is necessary to minimize the contact time between protein and stationary phase. Only non-porous particles, allow to separate proteins with a very short contact time due to their capacity, the low column dead-volume and the absence of pore diffusion.

High-resolution separations of 5'-phosphate nucleotides

Significant differences in resolution between non-porous and porous anion exchangers were also observed for smaller molecules like 5'-mono, di- and tri-phosphate nucleotides. A comparison of the chromatograms in Fig. 5 shows, that all 12 nucleotides were well separated on the non-porous anion exchanger within 2 min. Resolution on porous packings is decreased by pore diffusion and larger particle sizes. Best results in nucleotide separations were obtained with LiChrospher Si-4000 especially for longer gradient times. But there were no significant differences between the porous materials varying the pore size. Fig. 6 demonstrates that all nucleotides can actually be resolved in less than 90 s on the non-porous (2.1-μm) anion exchanger.

Furthermore, on the non-porous anion exchanger the nucleotides can also be resolved isocratically on the non-porous anion exchanger. The plate numbers calculated from separations of 5'-mono- and diphosphate nucleotides using the non-porous packing show that they do not change significantly. This is probably due to the lack of pore diffusion, which minimizes the mass transfer effects, *e.g.*, expressed in the Van Deemter *C* Term. The highest plate numbers of nearly 140 000 plates/m were achieved at a flow-rate of 2.0 ml/min.

Fast micropreparative fractionation of crude F_1-ATPase

A crude extract of F_1-ATPase (molecular weight *ca.* 400 000) from *Micrococcus luteus* was fractionated by anion-exchange chromatography using the non-porous strong anion-exchange packing. Fig. 7 shows a 10-min gradient (0–1 *M* sodium chloride) of the sample at a load of *ca.* 200 μg. Six subfractions were collected. Fraction

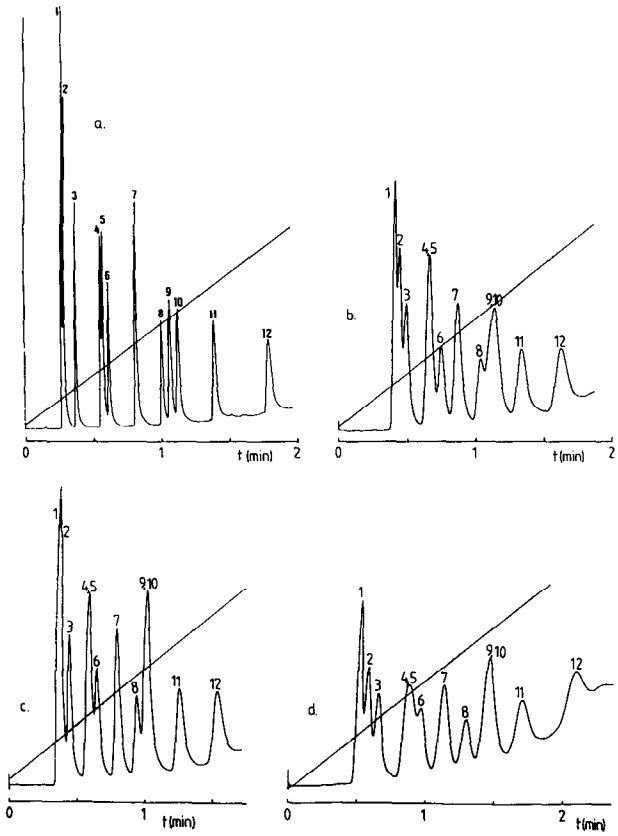


Fig. 5. Separation of 5′-mono-, di- and triphosphate nucleotides. Columns: PEI-coated strong anion exchangers. (a) Non-porous particles, (b) LiChrospher Si-4000, (c) LiChrospher Si-1000, (d) LiChrospher Si-300. Gradient time: 2 min, linear, 0.01–0.5 *M* KH_2PO_4 (pH 6.3). Flow-rate: 5 ml/min. Peak identification: 1 = UMP; 2 = CMP; 3 = AMP; 4 = GMP; 5 = UDP; 6 = CDP; 7 = ADP; 8 = UTP; 9 = CTP; 10 = GDP; 11 = ATP; 12 = GTP.

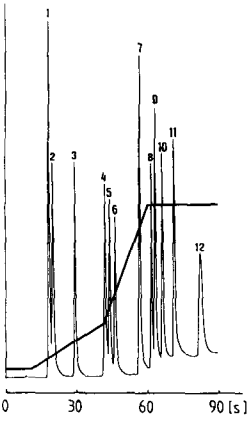
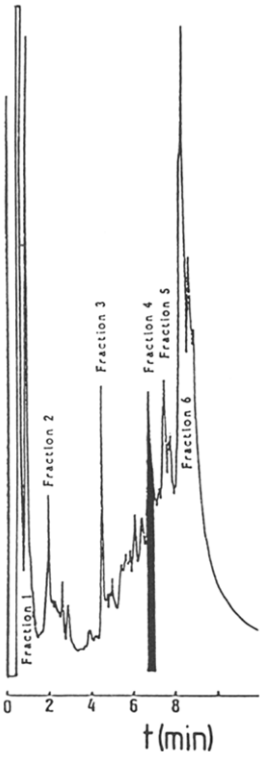


Fig. 6. Fast high-resolution separation of 5′-mono-, di- and triphosphate nucleotides. Column: non-porous (2.1-μm) packing, PEI-coated strong anion exchanger. Flow-rate; 5 ml/min. Gradient: 0 min (0.01 *M* KH_2PO_4, pH 6.3), 0.2 min (0.01 *M* KH_2PO_4), 0.7 min (0.14 *M* KH_2PO_4), 1.0 min (0.5 *M* KH_2PO_4). The elution sequence of the nucleotides is given in Fig. 5.

4 contained the pure protein (*ca.* 60 μg). The purity was checked by electrophoretic monitoring (Fig. 8). The sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of the six subfractions showed a superior purity compared to the reference purified by Shephadex A-25 (Pharmcia-LKB Biotechnology). The enzymatic activities of subfraction 4 and the reference sample were comparable. Limited loadability of the non-porous anion exchanger due to the low surface area, hence, low ion-exchange capacity became notable when the mass load exceeded *ca.* 200 μg of crude extract. At higher loads, the purity as well as the recovery of enzymatic activity decreased.



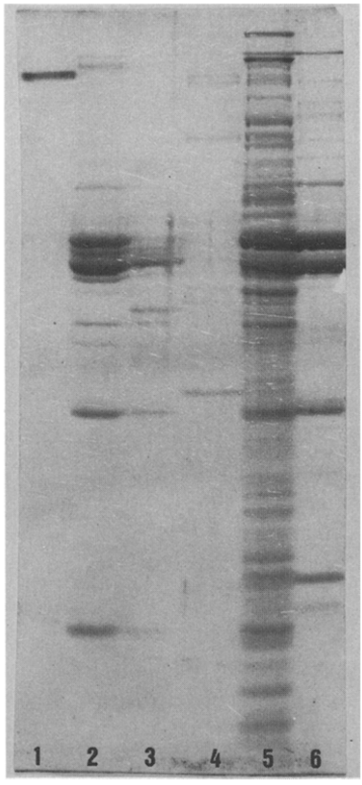


Fig. 7. Fractionation of F_1-ATPase crude extract from *Micrococcus luteus* (molecular weight *ca.* 400 000) on a PEI-coated, non-porous, strong anion-exchange column (33 mm × 8 mm I.D.). Buffer: 0.01 *M* Tris–HCl (pH 8). Gradient time: 10 min (0–1.0 *M* sodium chloride). Flow-rate: 1.5 ml/min. Sample volume: 20 μl. Detection: UV at 280 nm. Six subfractions were taken. Fraction 4 contains the pure protein (*ca.* 60 μg/injection).

Fig. 8. SDS-PAGE electrophoretic monitoring of the chromatographic separation shown in Fig. 7. Lane 1, fractions 1–3; lane 2, fraction 4; lane 3, fraction 5; lane 4, fraction 6; lane 5, crude extract; lane 6, reference sample purified by DEAE-Sephadex A-25.

CONCLUSION

We have demonstrated that non-porous (2.1-μm) silica-based anion exchangers are well suited for fast high-resolution analytical separations and for the fast micropreparative isolation of proteins and polynucleotides. It was also shown that low-molecular-weight substances such as nucleotides, can be separated in *ca.* 1 min on short columns of 33 mm × 8 mm I.D. packed with non-porous anion exchanger.

Furthermore, the linear solvent strength model of Snyder has been applied to the non-porous anion exchanger, separating proteins with very short gradient times and high flow-rates. Comparison of the calculated isocratic retention for TRA, OVA and STI from gradient runs shows a good agreement with measured isocratic retention data.

The column temperature also plays a significant role in anion-exchange chromatography of proteins. With increasing temperature the solvation decreases, the protein retention times increase strongly and the resolution becomes better. But the longer contact time between protein and anion-exchange surface leads to denaturation of biologically active products.

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