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AGAROSE-BASED MEDIA FOR HIGH-RESOLUTION GEL FILTRATION OF BIOPOLYMERS

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SUMMARY

Some chemical, physical and chromatographic properties of microparticulate gel filtration media based on cross-linked agarose are described. The new cross-linking procedure results in increased rigidity and chemical and physical stability. Linear flow-rates of around 75 cm/h are obtained for 6% agarose, 13-μm particles packed in 30 × 1 cm I.D. columns at around 1 MPa (10 bar, 150 p.s.i.). The chromatographic function is unaffected after storage for 2 weeks at 40°C in 0.1 *M* HCl or 0.1 *M* NaOH. The high stability also allows for column rinsing with, *e.g.*, 70% formic acid and 30% acetonitrile. The fractionation range for the 6% agarose medium (Superose 6) is linear in the range $5 \cdot 10^3$–$5 \cdot 10^6$ daltons and for the 12% agarose medium (Superose 12) in the range $1 \cdot 10^3$–$5 \cdot 10^5$ daltons. Typical plate numbers for low-molecular-weight solutes are $>3 \cdot 10^4$ and $>4 \cdot 10^4$ m^{-1} for the 6 and 12% agarose media, respectively, with typical reduced plate heights around 2.5. The effect of particle diameter on the resolution of globular proteins is presented for 110-, 33- and 14-μm cross-linked 6% agarose gel filtration media. Typical elution profiles for proteins, peptides, polysaccharides and nucleic acids are shown.

INTRODUCTION

Ever since its introduction 25 years ago[1], gel filtration chromatography has retained the position as the most widely used technique for the separation and purification of biological macromolecules. In the ideal gel filtration medium, solute partitioning between the mobile phase and the stationary liquid in the gel phase takes place without any interaction with the gel-forming polymer matrix. In this respect, gels based on cross-linked polysaccharides and polyacrylamide have been regarded as close to ideal and most media on the market today are based on these materials. However, even if neutral hydrophilicity and inertness are necessary properties of a functional gel filtration medium, there are a number of other requirements, such as adequate pore size distribution, rigidity and physical and chemical stability, which also have to be fulfilled. Media based on cross-linked dextran and agarose (a physi-

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cally cross-linked, spontaneously gel-forming galactan) share most of these properties and are the dominating classical gel filtration media. Traditionally, however, they are, both regarded as too soft to be applicable in high-performance liquid chromatography (HPLC) systems, where modified silica is the dominating matrix, and it was only recently reported that HPLC-compatible agarose gels could, in fact, be prepared by adequate cross-linking[2].

Cross-linking of soluble, non-gelling polysaccharides such as dextran takes place in an aqueous solution of random coil polymers. The final pore size distribution is dependent on the concentration both of dextran and of the cross-linker. Gels suitable for the separation of macromolecules are prepared at low concentrations of both and are therefore inherently soft, irrespective of the type of cross-linker used. Agarose, in contrast, undergoes spontaneous and reversible sol–gel transitions on heating and cooling of aqueous solutions. This phenomenon is based on physical cross-linking of the helical structures formed by the agarose polymer. The physical cross-linking gives rise to a three-dimensional network of aggregated polymer molecules, the pore size distribution of which is dependent of the agarose concentration of the starting solution only. By covalently cross-linking the aggregated polymers in an aqueous environment, the chemical and physical stability of agarose gels can be improved considerably[3].

With some cross-linkers, such as divinyl sulphone (DVS), a dramatic improvement in rigidity is obtained, but at the expense of the chemical stability[4]. In our laboratories we have developed a new cross-linking procedure, not only giving unsurpassed rigidity and chemical and physical stability, but which also retains the well known advantages of agarose as a matrix for the chromatography of biological macromolecules. The cross-linking procedure[5] is performed in two steps. In the first step the agarose is reacted with a mixture of long-chain di- and polyfunctional epoxides in the presence of an aprotic solvent and in the second the partially cross-linked gel is reacted with short-chain bifunctional cross-linkers in an aqueous solvent. Two products based on this material have recently been made available by Pharmacia as pre-packed Superose 6 HR 10/30 and Superose 12 HR 10/30 columns, made from 6 and 12% agarose beads, respectively. This paper describes the physical, chemical and chromatographic properties of these media for high-performance gel filtration chromatography.

EXPERIMENTAL

Bead size distribution

The particle size distribution analysis was performed according to a previously described procedure[6]. The Coulter Counter was calibrated using particle-diameter distributions determined by microscopy. The particle size, d_p, reported here is equal to the median of the volume-size distribution.

Matrix rigidity

The improvement in rigidity of the cross-linked agarose beads was analysed by running a linear flow gradient and continuously recording the generated pressure drops over 30 × 1 cm I.D. columns.

The columns were connected to a high-pressure pump, which was controlled

TABLE I

DATA FOR PROBE MOLECULES AND TYPICAL YIELDS ON SUPEROSE 6 AND SUPEROSE 12

Probe	*Source**	*Lot No.*	*Molecular weight (monomer)***	*Yield (%)*	
				Superose 6	*Superose 12*
Thyroglobulin	A	C 047	669 000	91	98
Ferritin	A	C 171	440 000	79	99
Catalase	A	C 063	232 000	91	88
IgG	E	1064	160 000		
Aldolase	A	C 060	158 000		
Bovine serum albumin (BSA)	A	C 166	67 000	90	100
Ovalbumin	A	C 168	43 000	88	84
β-Lactoglobulin	B	F8040	35 000		
Chymotrypsinogen A	A	C 165	25 000	90	84
Myoglobin	B	F7036	18 800		
Ribonuclease A	A	C 167	13 700	96	91
Cytochrome *c*	B	F7050	12 400	97	96
Cytidine	C		243		
Serum (pooled)	D				

* Sources: A = Pharmacia (Uppsala, Sweden); B = Sigma (St. Louis, MO, U.S.A.); C = Merck (Darmstadt, F.R.G.); D = University Hospital of Uppsala (Uppsala, Sweden); E = Kabi (Stockholm, Sweden).

** Data from the manufacturer.

by a gradient programmer to run a linear flow gradient from 0 to 3 ml min in 3 h[6]. The analogue signal from a pressure sensor (part No. 19-4331-01; Pharmacia) was fed to a recorder (Rec-481; Pharmacia) and the back-pressure was related to the flow-rate by means of the chart speed. The result was compared with the theoretical pressure drop calculated from the Blake–Kozeny equation with the experimentally determined value of the void fraction of 0.3[6].

Chromatographic performance

The chromatographic performance of the pre-packed Superose 6 HR 10/30 and Superose 12 HR 10/30 columns was analysed by running mixtures of the model proteins and other probe molecules listed in Table I. The standard eluent was 0.05 *M* phosphate in 0.15 *M* NaCl solution adjusted to pH 7.0. The columns were attached to a Pharmacia FPLC system composed of a P-500 high-precision pump, a V-7 sample injector with a 100-μl loop, a UV-1 UV monitor (280 nm, HR 10 cell) and a Rec-481 recorder. The 33- and 110-μm cross-linked agarose media Superose 6 Prep grade and Sepharose CL-6B, respectively, were packed into 500 × 16 mm I.D. glass columns as described previously[7].

RESULTS AND DISCUSSION

Matrix rigidity

The theoretical pressure drop as a function of particle size for rigid particles is illustrated in Fig. 1. The new cross-linking procedure for agarose used in the pro-

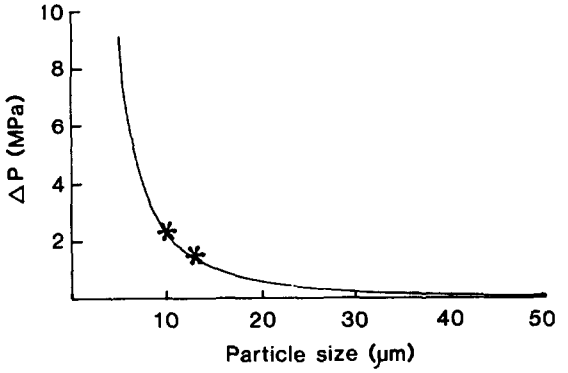


Fig. 1. Pressure drop as a function of particle size. Theoretically derived curve for a 30-cm column packed with rigid particles with a void fraction of 0.30 and at a linear flow-rate of 100 cm/h. The two stars indicate the experimentally determined positions of the Superose 6 HR 10/30 and Superose 12 HR 10/30 columns, respectively.

duction of Superose 6 and Superose 12 enabeled us to reduce the particle diameter to around 10 μm for the 12% agarose medium and to around 13 μm for the 6% gel, giving back-pressures that were very close to the theoretical values at the linear flow-rate (100 cm/h) used. When analysing the pressure–flow relationships for the Superose 6 HR 10/30 and Superose 12 HR 10/30 pre-packed columns, the results shown in Figs. 2 and 3, respectively, were obtained. The broken lines represent the theoretical pressure drops for rigid gels, calculated as mentioned previously. The Superose 12 HR 10/30 column displays a linear pressure–flow behaviour at linear flow-rates up to approximately 185 cm/h, corresponding to a pressure drop of 4.0 MPa. For the less rigid Superose 6 there is a more pronounced deviation from the

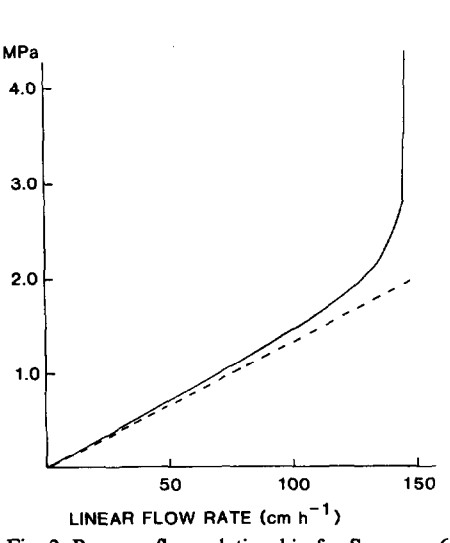


Fig. 2. Pressure-flow relationship for Superose 6 HR 10/30 (No. 321586; V_0/V_c = 0.30).

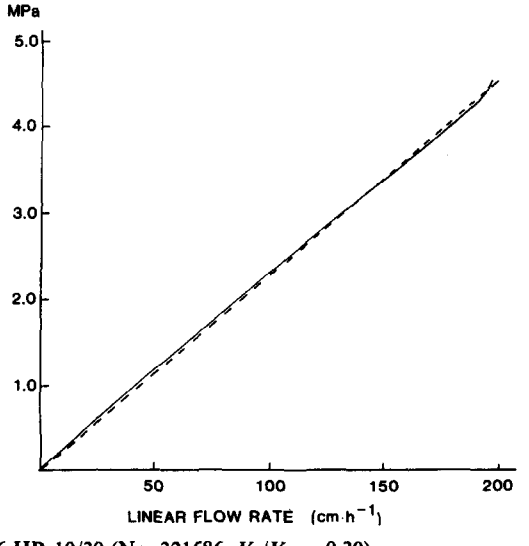
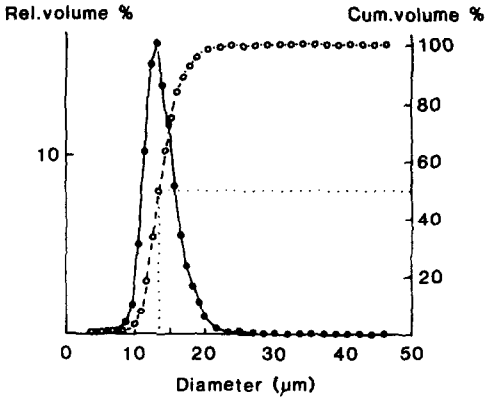


Fig. 3. Pressure-flow relationship for Superose 12 HR 10/30 (No. 38084B; V_0/V_c = 0.30).



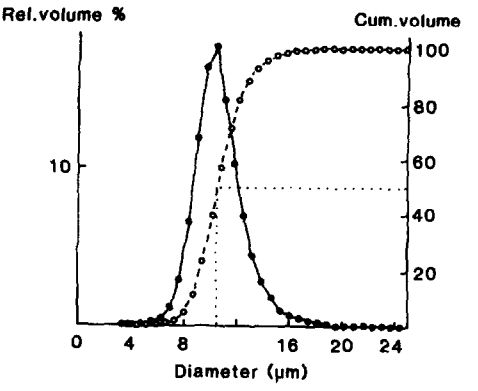


Fig. 4. Particle size distribution of Superose 6 (lot 38117).

Fig. 5. Particle size distribution of Superose 12 (lot 35251).

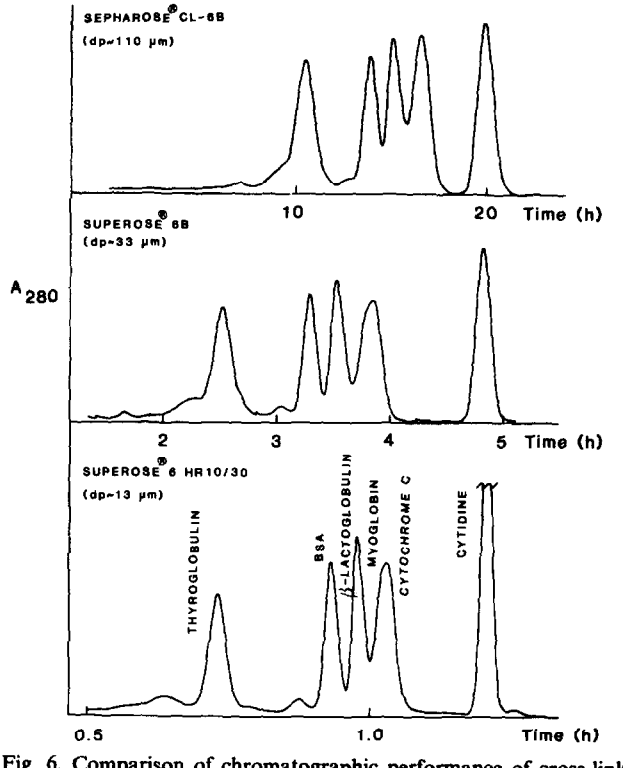


Fig. 6. Comparison of chromatographic performance of cross-linked agarose gels of different particle diameters (as indicated). Sample: 0.5 mg/ml of thyroglobulin + 0.8 mg/ml of bovine serum albumin + 0.25 mg/ml of β-lactoglobulin + 0.1 mg/ml of myoglobin + 0.1 mg/ml of cytochrome *c* + 0.01 mg/ml of cytidine. Sample volume and linear flow-rate: 1000 μl, 2.5 cm/h; 100 μl, 10 cm/h; and 50 μl, 38 cm/h from top to bottom, respectively.

theoretical behaviour and the linearity is extended to a linear flow of approximately 110 cm/h, corresponding to a pressure drop of approximately 1.5 MPa. At higher flow-rates, the bottom part of the column probably rapidly becomes compressed, with a dramatically reduced void fraction, which leads to a sudden increase in pressure drop. For the column tested in Fig. 2 this happened when the flow-rate approached 150 cm/h. Compared with silica-based materials, cross-linked agarose can therefore be regarded as semi-rigid. However, the flow-rates at which compression occurs far exceed those recommended for the separation of high-molecular-weight solutes, which are in the region of 25–50 cm/h.

Bead size distribution

The particle size distribution of Superose 6 and of Superose 12 are shown in Figs. 4 and 5, respectively. The mean particle sizes were 13.5 and 10.5 μm, respectively, and the relative widths of the distributions were 7–8%. The expected efficiency of a 10-μm material, *i.e.*, of $3.5 \cdot 10^4$–$4.5 \cdot 10^4$ plates/m, as measured with acetone as solute, is easily achieved with the Superose HR 10/30 columns.

Chromatographic performance

Reducing the particle diameter of chromatographic media may either be utilized to improve of the resolution and hence the separation and purification of the components in a sample mixture or to shorten the separation time by increasing the flow-rate. The latter possibility is illustrated in Fig. 6, where the separations of a protein mixture on media of bead diameter 110, 33 and 13 μm are compared. A reduction in the particle size by a factor of three permits an increase in flow-rate

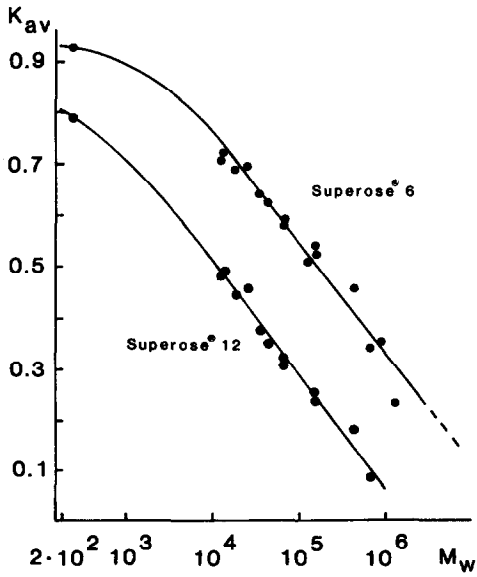


Fig. 7. Calibration graphs for proteins on Superose 6 (lot 33790) and Superose 12 (lot 33620). The dots represent cytidine, cytochrome *c*, ribonuclease A, myoglobin, chymotrypsinogen A, β-lactoglobulin, ovalbumin, human serum albumin, bovine serum albumin, aldolase, IgG, ferritin, thyroglobulin, dimer of ferritin and dimer of thyroglobulin.

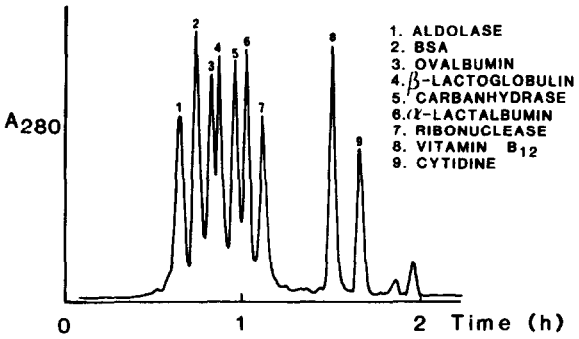


Fig. 8. Separation of a protein mixture on two Superose 12 HR 10/30 columns coupled in series. Sample volume: 200 μl containing 2.65 mg of protein mixture. Flow-rate: 0.3 ml/min. (Courtesy A. Domicelj, Pharmacia.)

corresponding to a decrease in separation time by a factor of four, with retention of resolution. Fig. 6 also shows that the selectivity of Superose 6 is very similar to that of Superose 6B (now renamed Superose 6, Prep grade) and Sepharose CL-6B, which facilitates scaling-up transformations of process separations between these chromatographic materials.

The selectivity curves for Superose 6 and Superose 12 are shown in Fig. 7. The working range for Superose 6 is approximately $5 \cdot 10^3$–$5 \cdot 10^6$ daltons. This is also illustrated in Fig. 6. For Superose 12, the corresponding range is $5 \cdot 10^2$–$5 \cdot 10^5$

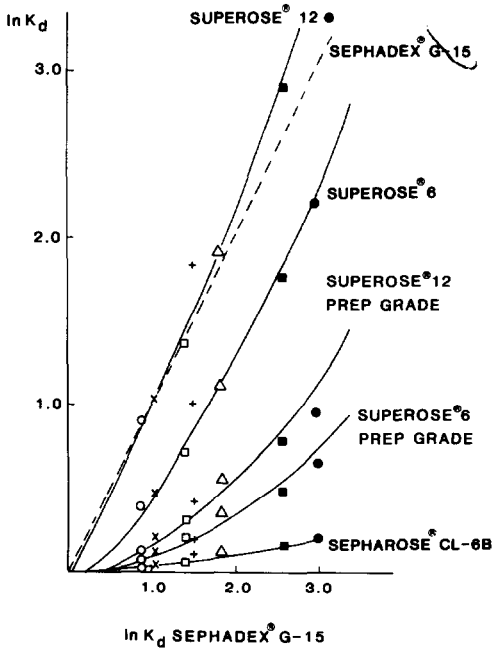


Fig. 9. Relationship between ln K_d for octanol (○), decanol (+), benzene (×), phenol (□), 1,3-dihydroxybenzene (△), 3-bromophenol (■) and iodophenol (●) on cross-linked agarose gels (as indicated) and ln K_d for the same solutes on Sephadex G-15. (Courtesy Å. Haglund[8].)

daltons and in Fig. 8 a mixture of globular model proteins is shown. Compared with TSK 4000 SW and TSK 3000 SW columns, the selectivity curves are less steep, thus covering a wider molecular weight range. However, the lower selectivity is compensated for by a smaller V_0 and a larger V_i. The void fraction, V_0/V_c, of the Superose columns are invariably close to 0.30. The corresponding values for TSK and other silica-based columns are in the range 0.37–0.40. The pore fraction, V_i/V_c, is another essential parameter in gel filtration chromatography. For Superose 6 it is 0.65 and for Superose 12 0.55, significantly higher than for silica-based columns.

Agarose inherently contains small amounts of charged groups, mainly sulphate. There is also a slight risk of the formation of carboxylic groups during the manufacturing process. To suppress ionic interactions between these groups and positively charged solutes, it is recommended to add neutral salts to the eluting buffer; 0.15 *M* NaCl has proved to be sufficient in all tested cases. The high rigidity of Superose has been achieved by extensive cross-linking as described in the Introduction. As a result, Superose is less hydrophilic than, *e.g.*, Sepharose CL. However, no influence on elution behaviour was observed for high-molecular-weight solutes such as proteins and nucleic acids. For low-molecular-weight solutes, on the other hand, the effect is significant, as illustrated in Fig. 9. The elution behaviour in distilled water of octanol, decanol, benzene, phenol, 1,3-dihydroxybenzene, 3-bromophenol and 3-iodophenol was investigated for Superose 12, Superose 6, Superose 12 Prep grade, Superose 6 Prep grade, Sepharose CL-6B and Sephadex G-15. The graphs are based on a study of a total of more than 25 compounds and the results of this comprehen-

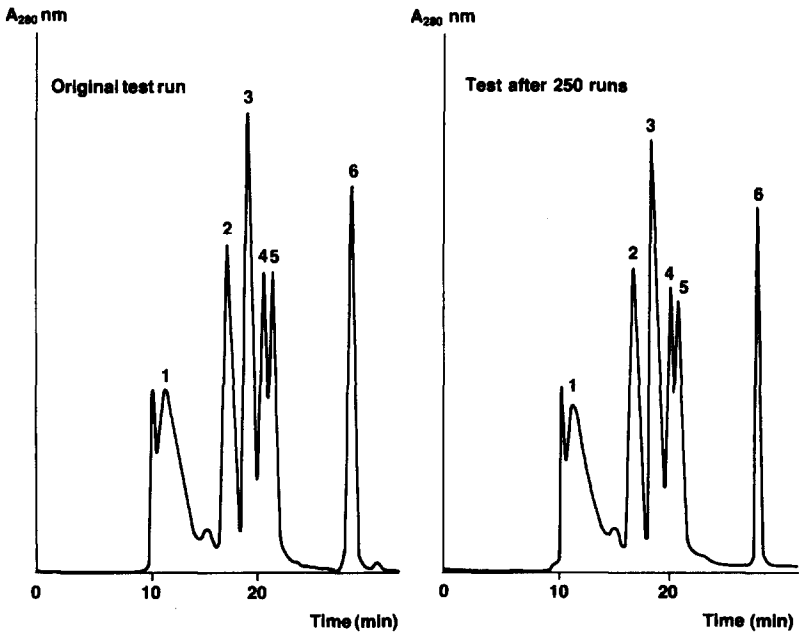


Fig. 10. Test of the reproducibility of the separation of a protein mixture on Superose 12 HR 10/30. The same protein mixture was used as in Fig. 6. Sample volume: 100 µl. Flow-rate: 0.75 ml/min. The 250 runs were a consecutive application of the following cycle of samples: 1, 10% serum; 2, 5% egg-white; 3, 50% cultured milk whey; 4, 1% cytochrome *c* tryptic digest. (Courtesy G. Glad, Pharmacia.)

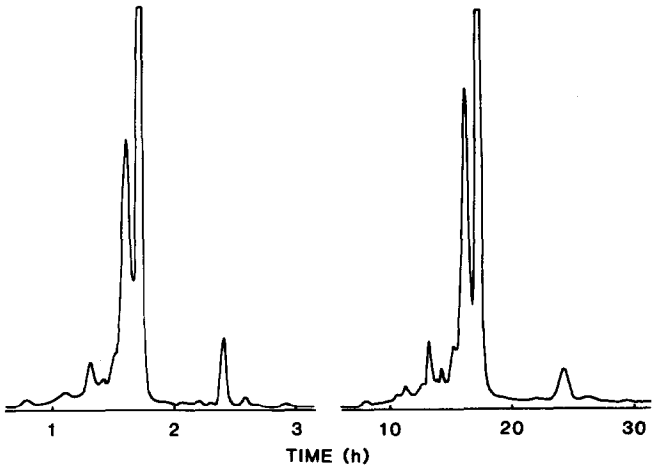


Fig. 11. Separation of normal human serum on Superose 6 HR 10/30 (two columns in series) at two different flow-rates. A 100-μl volume of serum diluted 1:3 was run at 20 ml/h (left) and at 2.0 ml/h (right).

sive study will be published elsewhere[8]. From the results of this investigation, we can conclude that the degree and nature of hydrophobic and aromatic interactions with Superose 12 and Superose 6 are similar to those of Sephadex G-15. As a consequence, one might expect retardation of smaller hydrophobic and/or aromatic peptides. In some instances this retardation might be utilized to advantage.

Chemical and physical stability

One of the major advantages of cross-linked agarose-based media over those based on silica is their high chemical stability. Superose columns can be used over

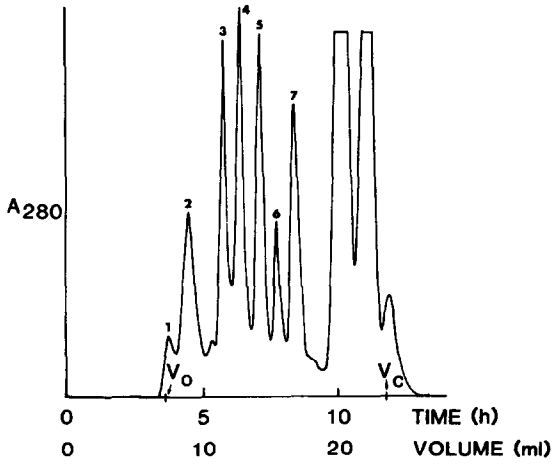


Fig. 12. Separation of a protein mixture on Superose 6 HR 10/30 equilibrated in 6 *M* guanidinium hydrochloride. Sample: 50 μl of a solution of a reduced and alkylated protein mixture of (1) thyroglobulin dimer, (2) thyroglobulin, (3) β-galactosidase, (4) bovine serum albumin, (5) ovalbumin, (6) chymotrypsinogen and (7) ribonuclease. Flow-rate: 2 ml/h. V_e = Geometrical column volume. (Courtesy B. Hedman and A. Medin.)

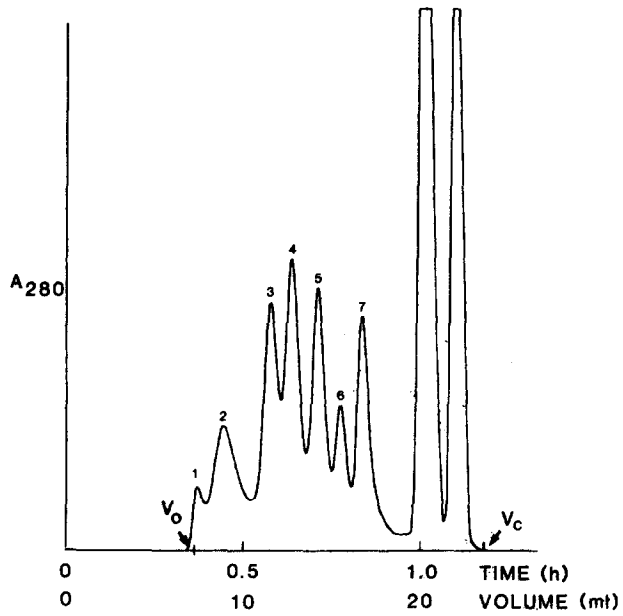


Fig. 13. As for Fig. 12, but with a 10-fold higher flow-rate (20 ml/h).

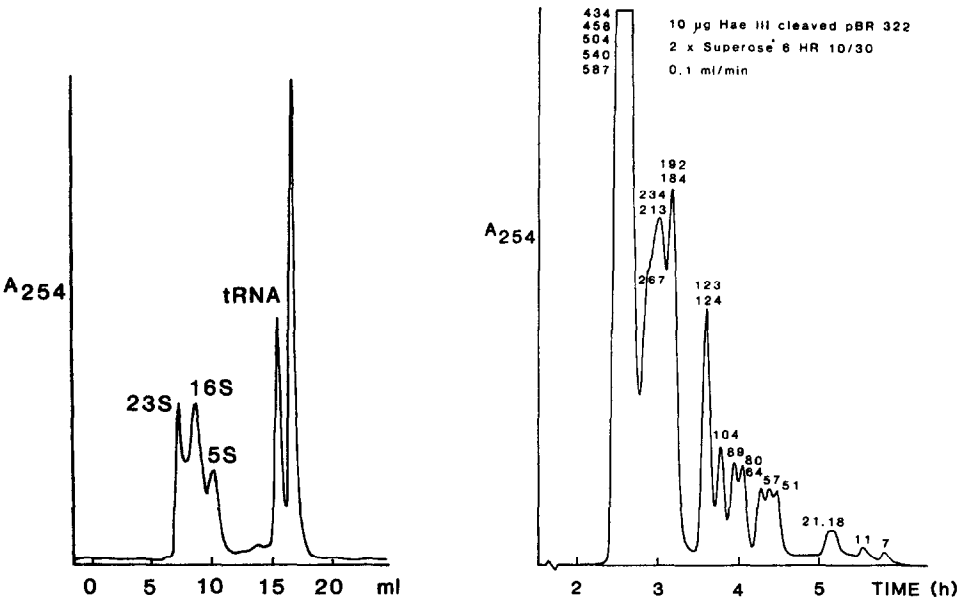


Fig. 14. Separation of a mixture of RNA molecules on Superose 6 HR 10/30 equilibrated in 0.1 *M* sodium acetate solution (pH 7.0) containing 0.75 *M* NaCl and 1% methanol. 23S, 16S and 5S ribosomal RNA from *E. coli* MRE-600 and tRNA from wheat germ; 10 µg of each type in a sample volume of 50 µl. Flow-rate: 0.1 ml min. The last large peak probably contains degraded RNA. (Courtesy C. Bengtsson, Pharmacia.)

Fig. 15. Separation of DNA restriction fragments obtained by cleavage of pBR 322 with Hae III; 10 µg in 100 µl applied on Superose 6 HR 10/30 (two columns coupled in series); 0.05 *M* Tris–HCl, 1 m*M* EDTA, pH 8.0. Flow-rate: 0.1 ml/min. (Courtesy C. Bengtsson, Pharmacia.)

the whole pH range (1–14). Hence they can be stored in 0.1 *M* HCl or 0.1 *M* NaOH at 40°C for at least 2 weeks without any change in functional chromatographic properties. The columns can be used in high concentrations of guanidine hydrochloride, urea, chaotropic ions, SDS and other detergents, 70% formic acid (Superose 12) and 30% acetonitrile. Higher concentrations of organic solvents might cause some shrinking, which may, however, be compensated for by adjusting the column adaptor.

The excellent reproducibility, even after 250 runs, is illustrated in Fig. 10.

Scaling-up

The maximum loading capacity of the Superose 6 HR 10/30 and Superose 12 HR 10/30 columns is in the range 5–10 mg of protein in a sample volume of 200–300 μl. For the best results, the sample volume should not exceed 50 μl. When larger loadings are required, the preparative grades based on 33-μm diameter particles should be used packed in HR 16/50 columns (Pharmacia). Here sample loads in the range 50–100 mg/ml are applicable with a resolution similar to that of 10-μm particles, but with an approximately four-fold increase in separation time.

For still higher capacities, 110-μm particles are recommended (Sepharose CL-6B) (*cf.*, Fig. 6).

Examples of applications

To illustrate the chromatographic capability of the Superose HR 10/30 columns, a number of applications covering representative areas have been studied. Fig. 11 shows the results of two runs with normal human serum on Superose 6 HR 10/30 in order to compare the effect of flow-rate on the resolution of the major components. Denaturating conditions, such as 6 *M* guanidine hydrochloride, lead reduced and alkylated protein subunits to adopt the same conformation, regardless of composition[9]. Thus it is possible to calibrate a column, equilibrated in this eluent, with respect to the number of amino acids in the polypeptide chains. From this value, and knowing the average weight of its amino acids (from amino acid analysis), it is possible to determine the molecular weights of unknown polypeptides with excellent precision and accuracy[10]. Figs. 12 and 13 show the results of the separation of a protein

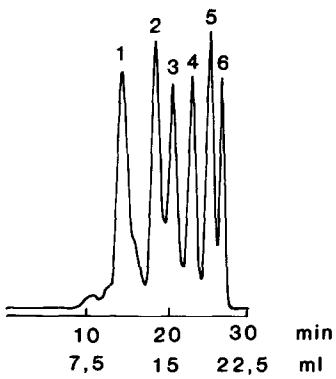


Fig. 16. Separation of a mixture of polyethylene glycols on Superose 12 HR 10/30 in distilled water. Mixture of 1.6 mg in 50 μl of (1) PEG 20 000, (2) PEG 6000, (3) PEG 3000, (4) PEG 1000, (5) PEG 200 and (6) ethylene glycol (MW, 62). Flow-rate: 0.75 ml/min. (Courtesy A. Domicelj, Pharmacia.)

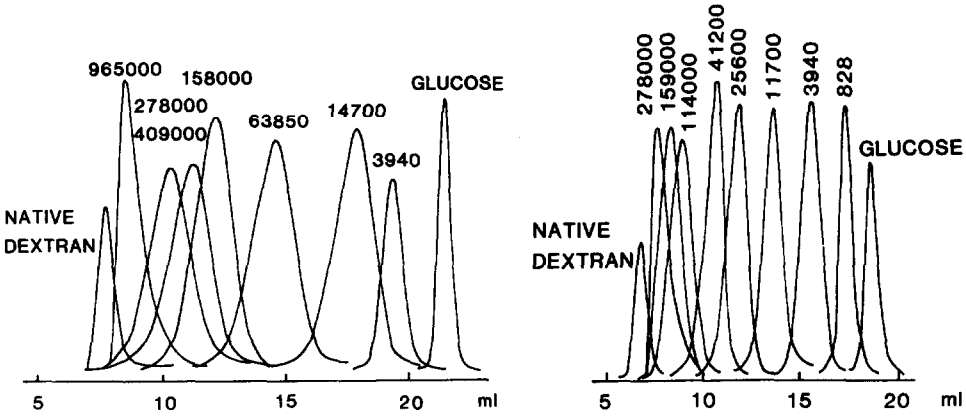


Fig. 17. Elution profiles of narrow dextran fractions on Superose 6 HR 10/30 equilibrated in 0.25 *M* NaCl. Each fraction was run separately. Sample volume: 50 µl containing 250 µg of dextran. (Courtesy Å. Öhrlund, Pharmacia.)

Fig. 18. Elution of narrow dextran fractions on Superose 12 HR 10/30. Conditions as in Fig. 17.

mixture (reduced and alkylated components) on Superose 6 HR 10/30, equilibrated in 6 *M* guanidine hydrochloride, at two different flow-rates. Fig. 14 demonstrates the separation of a mixture of RNAs and Fig. 15 the separation of a mixture of DNA restriction fragments obtained from digestion of the plasmid pBR 322 with restriction endonuclease Hae III.

Fig. 16 shows the separation of polyethylene glycol fractions on Superose 12 HR 10/30 in distilled water, and Figs. 17 and 18 show the elution profiles of narrow dextran fractions analysed on Superose 6 HR 10/30 and Superose 12 HR 10/30, respectively.

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