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New family of high-resolution ion exchangers for protein and nucleic acid purifications from laboratory to process scales

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ABSTRACT

A new family of polymer-based ion exchangers was tested for the purification of acidic and basic proteins on both the analytical and preparative scales. Protein-Pak™ HR series packings are available as strong cation (SP) and weak anion (DEAE) exchangers, allowing the development of a purification method regardless of the isoelectric point of protein. Three particle sizes, 8, 15 and 40 μm, are offered in scalable Advanced Purification (AP) glass columns or as bulk packings. The lower back pressures of the 15- and 40-μm packings compared to that of the 8-μm material allow rapid throughput of large volumes without exceeding the pressure limitations of the resin or the column. The capacity of the AP1 (100 mm × 10 mm) glass columns, containing these ion-exchange packings, is comparable to other ion-exchange columns. The resolution of mouse serum, plasmids, and a standard protein mixture was demonstrated and compared with the results obtained with other resin-based ion exchangers of similar particle size. Proteins were purified without significant loss of biological activity or mass.

INTRODUCTION

High-performance ion-exchange chromatography is one of the most popular separation techniques in biochemistry. Proteins can usually be separated based on the electrostatic interactions between the zwitterionic protein surface and the charged stationary phase [1,2]. Nucleic acids with negatively charged backbones can also be separated by ion-exchange chromatography. High resolution is possible with this technique, because it is capable of separating such molecules with only small differences in charge on either the surface or the backbone.

Ion-exchange chromatography has been used extensively for protein purification for a long time. The first hydrophilic ion exchangers for proteins were developed in the 1950's and were based on cellulose [3]. These soft gel materials, diethylaminoethyl (DEAE) and carboxymethyl (CM) derivatives of cellulose are still used today; the softness and shrinking or swelling properties of these gels do not allow the optimum separation to be obtained. Porath *et al.* [4] in 1971 attempted to solve this problem with hard hydrophilic ion exchangers by developing cross-linked agarose ion-exchange gels. Other groups developed hydrophilic vinyl polymer ion-exchange gels [5]. In order to improve the resolution by the ion exchangers, packing materials with small particle

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sizes became available in both silica gels and hydrophilic polymer gels [6,7]. Most polymeric packings today occur as either analytical or preparative, but do not offer the particle size range that allows one to scale-up their separation from test tube to process with the same resin.

The new packing material discussed in this report, the Protein-Pak™ HR series, is similar to the currently available high-performance ion-exchange packings; they give a high separation efficiency and a high speed of separation. In addition, one chemistry is available for both preparative and analytical chromatography since three particle sizes are available (8, 15 and 40 μm).

Protein-Pak DEAE HR and SP HR materials are made from a rigid, spherical, porous, hydrophilic, polymeric, methacrylate gel. The 1000-Å pore size particles permit proteins with up to 10^6 molecular weight to penetrate the pores and interact with the functional groups. The packing materials have covalently bonded functionalities of diethylaminoethyl (DEAE) and propylsulfonic acid (SP), respectively. Here, we report the characteristics, performance and applications of the new family of packings and compare it with other commercial ion exchangers.

EXPERIMENTAL

Three tests were performed on the Protein-Pak HR materials and, for comparison, with Protein-Pak 5PW packings: the separation of a standard protein mixture, mass recovery and protein binding capacity. The protein binding capacity was determined at several different pH values. The system used for these assays was a Waters 840 chromatography data station with a Waters 600E solvent delivery system, Model 490 detector, 712 WISP autoinjector and a Wescan conductivity meter. The Protein-Pak HR materials were packed in the Waters Advanced Purification (API) 100 mm × 10 mm glass columns, while the Protein-Pak 5PW glass column dimensions were 75 mm × 8 mm. All proteins and buffer salts were purchased from

TABLE I

PROTEINS USED TO EVALUATE THE PERFORMANCE OF ION-EXCHANGE COLUMNS

Protein	Molecular weight	Isoelectric point	Concentration (%)
Protein-Pak DEAE HR series protein mixture			
Adenosine	267	–	0.3
Carbonic anhydrase	28 000	7.3	12.5
Human transferrin	77 000	6.0–6.5	31.1
Ovalbumin	44 500	4.7	25.0
Soybean trypsin inhibitor	21 500	4.5	31.1
Protein-Pak SP HR series protein mixture			
Myoglobin	17 500	7.0	8.0
Ribonuclease A	13 500	8.8	33.0
Chymotrypsinogen A	25 000	9.0	17.0
Cytochrome *c*	12 400	9.4	25.0
Lysozyme	14 400	11.0	17.0

Sigma (St. Louis, MO, U.S.A.), unless otherwise noted. Assay Kit 345-B from Sigma was used to measure biological activity of glucose-6-phosphate dehydrogenase and Coomassie protein assay reagent (Pierce) was used to determine protein concentrations. The column efficiencies were measured with acetone as the marker.

Protein-Pak DEAE HR packing in 8-, 15- and 40-μm particle sizes and Protein-Pak DEAE-5PW (nominally 10 μm) were evaluated with 20 m*M* Tris–HCl (pH 8.2) (eluent A), and with 20 m*M* Tris–HCl (pH 8.2) with 1 *M* sodium chloride (eluent B) for all three assays. The proteins in the standard protein mixture were carbonic anhydrase, human transferrin, ovalbumin and soybean trypsin inhibitor with adenosine (molecular weight 267) as the void-volume marker. These proteins were chosen because they cover a wide range of molecular weights and isoelectric points and represent a variety of protein characteristics. The molecular weights and isoelectric points are given in Table I. The DEAE HR series columns were eluted with a gradient of 0 to 25% eluent B over 38 min at a flow-rate of 1.56 ml/min. The gradient for the DEAE-5PW packing was 0 to 25% eluent B over 30 min at a flow-rate of 1.0 ml/min; the flow-rate and gradient duration were adjusted for differences in the column dimensions (see Appendix 1 for scale-up equations). The sample injection volume was 100 μl, containing 0.5 mg protein.

Protein-Pak SP HR in 8-, 15- and 40-μm particle sizes and the Protein-Pak SP-5PW (nominally 10 μm) packings were evaluated by gradient elution with 20 m*M* sodium phosphate (pH 7.0) (eluent A), and 20 m*M* sodium phosphate (pH 7.0) with 1 *M* sodium chloride (eluent B) in all three assays. The molecular weights and isoelectric points of the proteins in the standard protein mixture are given in Table I. The injection volume was 100 μl, containing 0.35 mg protein. The gradient for the SP HR series packings was from 0 to 50% eluent B over 74 min at a flow-rate of 1.56 ml/min. The gradient of the SP-5PW packing was 0 to 50% eluent B over 60 min at a flow-rate of 1.0 ml/min. Flow-rate and gradient duration were adjusted to account for differences in column dimensions and gave equivalent linear velocities.

The ten proteins used for mass recovery experiments with the DEAE 8HR and 5PW columns, are given in Table II. The mass recoveries of these proteins at 100-μg loads (20 μl) were determined from the ratio of peak areas with and without the column in-line.

A similar mass recovery study was performed with 100-μg loads of ten proteins on the Protein-Pak SP 8HR and SP-5PW columns (Table III).

The protein binding capacities of the weak anion exchangers (DEAE) were determined on-line by pumping a bovine serum albumin solution (4 mg/ml) into the column until breakthrough occurred. The column was then flushed with eluent A until the absorbance at 280 nm decreased to less than 0.02 units. The bound protein was then removed with eluent B and quantitated (extinction coefficient equal to 0.65 absorbance units per mg/ml). The binding buffer for this study was 20 m*M* Tris–HCl (eluents A and B), except at pH 10, where the buffer was 20 m*M* 2-(N-cyclohexylamino)-ethanesulphonic acid (CHES).

The protein binding capacities of the strong cation exchangers (SP) were measured with cytochrome *c* (4 mg/ml) at 280 nm (extinction coefficient equal to 1.9 absorbance units per mg/ml). The binding buffers were 20 m*M* sodium acetate and 20 m*M* MES 2-(N-morpholino)ethanesulphonic acid (MES) for pH 5.0, and 20 m*M* sodium phosphate for pH 6.5, 7.0 and 7.5.

TABLE II

MASS RECOVERY OF 100 μg OF VARIOUS PROTEINS USING PROTEIN-PAK DEAE 8HR COMPARED TO DEAE-5PW

Conditions: eluent A: 20 m*M* Tris–HCl, pH 8.2; eluent B: eluent A + 1 *M* sodium chloride; injection volume: 20 μl in eluent A (100 μg); flow-rate: 1 ml/min; gradient: isocratic in 100% eluent B.

Proteins, 100 μg (0.03% of capacity)	Molecular weight	Isoelectric point	Recovery (%)	
			DEAE 8HR	DEAE-5PW
Bovine serum albumin	67 500	4.9	100	95
Carbonic anhydrase	28 000	7.3	100	99
Conalbumin	76 600	6.8	98	97
Cytochrome *c*	12 400	9.5	98	77
Human transferrin	77 000	6.0–6.5	98	100
β-Lactoglobulin	35 000	5.1	99	90
Ovalbumin	44 500	4.7	100	100
Rabbit immunoglobulin G	155 000	6.0–7.0	100	90
Rat immunoglobulin G	155 000	6.0–7.0	100	95
Soybean trypsin inhibitor	21 500	4.5	80	86

The recovery of biological activity was detemined on both types of the Protein-Pak HR material. A Waters 650 Advanced Protein Purification System with a non-metallic 484 detector, 712 WISP, FOXY fraction collector and a Wescan conductivity meter (Santa Claire, CA, U.S.A.) were used for this study.

The recovery of applied biological activity of pure glucose-6-phosphate dehydrogenase, from yeast, was determined on the Protein-Pak DEAE 15HR glass column. The buffers used were 20 m*M* Tris–acetate (pH 8.0) as eluent A and eluent B

TABLE III

MASS RECOVERY OF 100 μg OF VARIOUS PROTEINS USING PROTEIN-PAK SP 8HR COMPARED TO SP-5PW

Conditions: eluent A: 20 m*M* sodium phosphate, pH 7.0; eluent B: eluent A + 1 *M* sodium chloride; injection volume: 20 μl in eluent A (100 μg); flow-rate: 1 ml/min; gradient: isocratic in 100% eluent B.

Proteins, 100 μg (0.03% of capacity)	Molecular weight	Isoelectric point	Recovery (%)	
			SP 8HR	SP-5PW
Chymotrypsin	21 600	8.8	100	96
Chymotrypsinogen A	25 000	9.0	92	96
Cytochrome *c*	12 400	9.4	98	90
Hemoglobin	64 500	7.0	84	65
β-Lactoglobulin	35 000	5.1	90	90
Lysozyme	14 400	11.0	98	95
Myoglobin	17 500	7.0	90	86
Ovalbumin	44 500	4.7	95	90
Ribonuclease A	13 500	8.8	100	95
Soybean trypsin inhibitor	21 500	4.5	93	91

was 20 m*M* Tris–acetate (pH 8.0) plus 0.5 *M* sodium acetate. Glucose-6-phosphate dehydrogenase (100 μg) was injected and eluted over 44 min with a gradient from 0 to 100% eluent B at a flow-rate of 1.25 ml/min. The fractions were collected at 1-min intervals. The glucose-6-phosphate dehydrogenase activity of each fraction was measured by monitoring the reduction of NADP at 340 nm.

The purification of lysozyme from egg white was used to determine recovery of biological activity from a crude preparation on a Protein-Pak SP 8HR glass column. The rate of lysis of *Micrococcus lysodeikticus* was monitored spectrophotometrically at 450 nm [8,9]. The buffers were 20 m*M* sodium phosphate (pH 7.0) (eluent A) and 20 m*M* sodium phosphate (pH 7.0) plus 1 *M* sodium chloride (eluent B). The proteins in egg white (50 mg load) were separated by a linear gradient from 0 to 50% eluent B over 84 min at a flow-rate of 1.56 ml/min. Fractions were collected at 1-min intervals.

APPLICATIONS

Scale-up studies

Sample load was scaled up on a Protein-Pak DEAE 8HR AP1 glass column under the conditions outlined above for the DEAE HR standard protein mixture. The protein resolution assay load of 0.5 mg in 100 μl, which is about 0.2% of the dynamic column capacity, was scaled up to 22 mg protein in 4.4 ml, a 44-fold increase, which is equivalent to 6.2% of the dynamic column capacity.

A second study demonstrated a large-scale preparative purification which involved scale-up from an AP1 (100 mm × 10 mm) to an AP5 (100 mm × 50 mm) glass column. The protein mixture was injected in 110 ml of eluent A containing 550 mg. The same conditions were used as described above for the AP1 glass column, except the flow-rate was 39 ml/min (to maintain a constant linear velocity) and the initial isocratic hold in the gradient was for 25 min (to allow time for sample load onto the column).

Mouse serum

The proteins in mouse serum were separated using identical conditions to those used for the standard protein mixture of the DEAE HR packings. The sample load was 4 mg in 75 μl eluent A.

Plasmid purification

Another application was the separation of pRSVcat plasmid from an RNase-treated and alkaline-lysed preparation on the Waters Protein-Pak DEAE 8HR glass column. Eluent A was 25 m*M* Tris–HCl (pH 8.0) with 1 m*M* EDTA and eluent B was 25 m*M* Tris–HCl (pH 8.0) with 1 m*M* EDTA plus 1 *M* sodium chloride. The preparation (20.8 mg total mass) was loaded at 40% eluent B, pumped isocratically for 40 min, and then increased linearly to 60% eluent B over 40 min at a flow-rate of 1.5 ml/min. This separation was carried out at room temperature.

RESULTS AND DISCUSSION

The average efficiency of the AP1 column, with acetone as the marker, was 2000 theoretical plates for both the 8HR and 15HR packing materials.

The chromatograms for the standard protein mixture for the Protein-Pak DEAE

8HR, 15HR and 40HR are shown in Figs. 1–3 and demonstrate the high resolution capabilities of the 8HR and 15HR using the standard protein mixture. The resolution of a seven-protein mixture at 100 μg load by 8 and 15HR did reflect the two-fold difference in particle size. In addition, the 40HR column gave significantly broader peaks, as expected. The protein resolution was greater on the DEAE-8HR and 15HR column than on the DEAE-5PW column; the DEAE-5PW chromatogram is in Fig. 4. The resolution (R_s) values were measured for the ovalbumin and soybean trypsin inhibitor separation; DEAE 8HR was 24, DEAE 15HR was 20 and DEAE-5PW was 17. The DEAE 15HR column gave better resolution than the DEAE-5PW which has a smaller particle size, 15 *versus* 10 μm, respectively. The separation factors (α) also reflected the improved resolution of the HR materials over the DEAE-5PW column.

The chromatography for the standard protein mix on the SP-8HR, 15HR and 40HR materials are shown in Figs. 5–7. Again, the resolution between the 8HR and 15HR materials was similar and the 40HR had broader peaks. The Protein-Pak SP 8HR and 15HR columns had better resolution of cytochrome *c* and lysozyme than the SP-5PW column, R_s of 33 and 31 *versus* 20, respectively; the chromatogram for the SP-5PW is in Fig. 8.

The figures for the standard protein mixtures show that the proteins are not eluted on the anion exchangers and cation exchangers based on their isoelectric points, p*I*. The ion-exchange process is determined primarily by electrostatic interactions, but

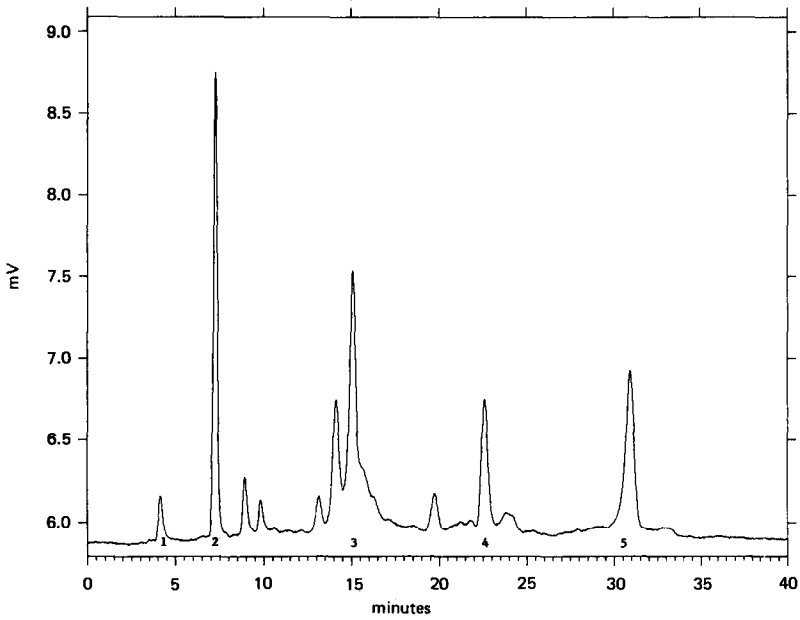

Fig. 1. Protein resolution on Protein-Pak DEAE 8HR anion-exchange column. Column: API (100 mm × 10 mm) glass column; eluent A: 20 m*M* Tris–HCl at pH 8.2; eluent B: eluent A + 1 *M* sodium chloride; flow-rate: 1.56 ml/min; detector: 280 nm; gradient: 0 to 25% eluent B over 38 min; sample load: 0.5 mg protein. Peaks: 1 = adenosine; 2 = carbonic anhydrase; 3 = human transferrin; 4 = ovalbumin; 5 = soybean trypsin inhibitor.

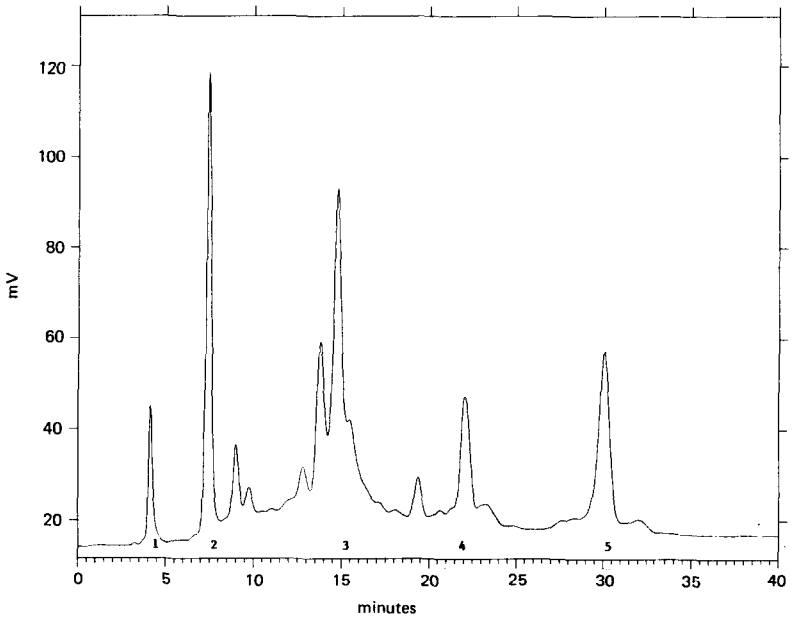

Fig. 2. Protein resolution on Protein-Pak DEAE 15HR anion-exchange column. Conditions and peaks as in Fig. 1.

protein retention is the result of the interaction between the surface charge (not the net charge) of the proteins (Z-value) and that of the stationary phase [10]. Therefore, the elution order of the proteins in the standard mixtures for the SP and DEAE columns (Figs. 1–8) did not follow the order of the pI values.

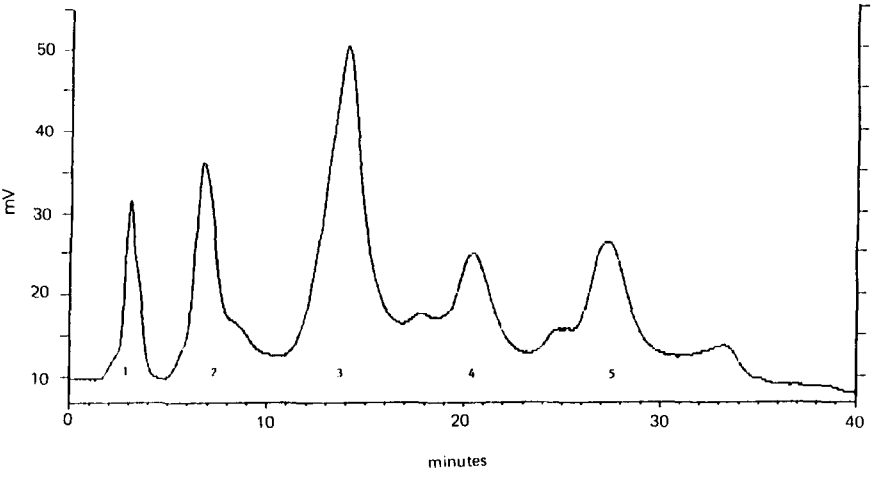

Fig. 3. Protein resolution on Protein-Pak DEAE 40HR anion-exchange column. Conditions and peaks as in Fig. 1.

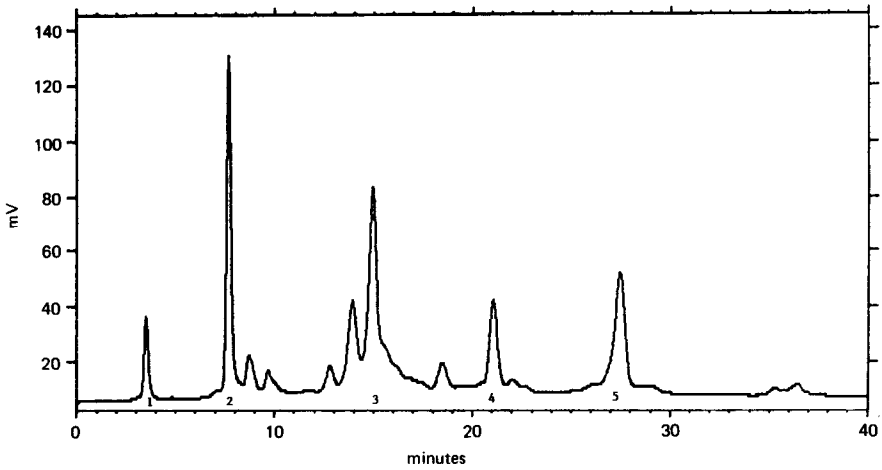

Fig. 4. Protein resolution on Protein-Pak DEAE-5PW anion-exchange column. Column: 75 mm × 8 mm glass column; eluent A: 20 m*M* Tris–HCl at pH 8.2; eluent B: eluent A + 1 *M* sodium chloride; flow-rate: 1 ml/min; detector: 280 nm; gradient: 0 to 25% eluent B over 30 min; sample load: 0.5 mg protein. Peaks: 1 = adenosine; 2 = carbonic anhydrase; 3 = human transferrin; 4 = ovalbumin; 5 = soybean trypsin inhibitor.

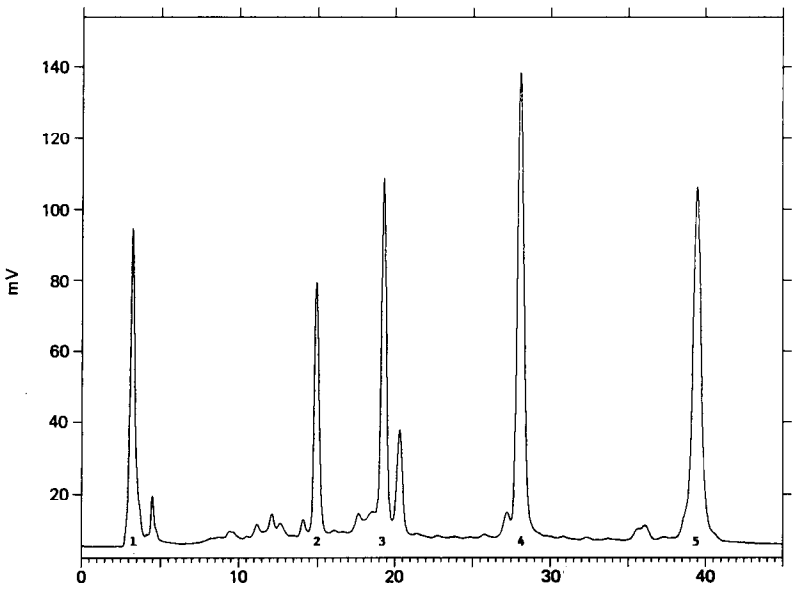

Fig. 5. Protein resolution on Protein-Pak SP 8HR cation-exchange column. Column: API (100 mm × 10 mm) glass column; eluent A: 20 m*M* sodium phosphate at pH 7.0; eluent B: eluent A + 1 *M* sodium chloride; flow-rate: 1.56 ml/min; detector: 280 nm; gradient: 0 to 50% eluent B over 74 min; sample load: 0.35 mg protein. Peaks: 1 = myoglobin; 2 = ribonuclease A; 3 = chymotrypsinogen A; 4 = cytochrome *c*; 5 = lysozyme.

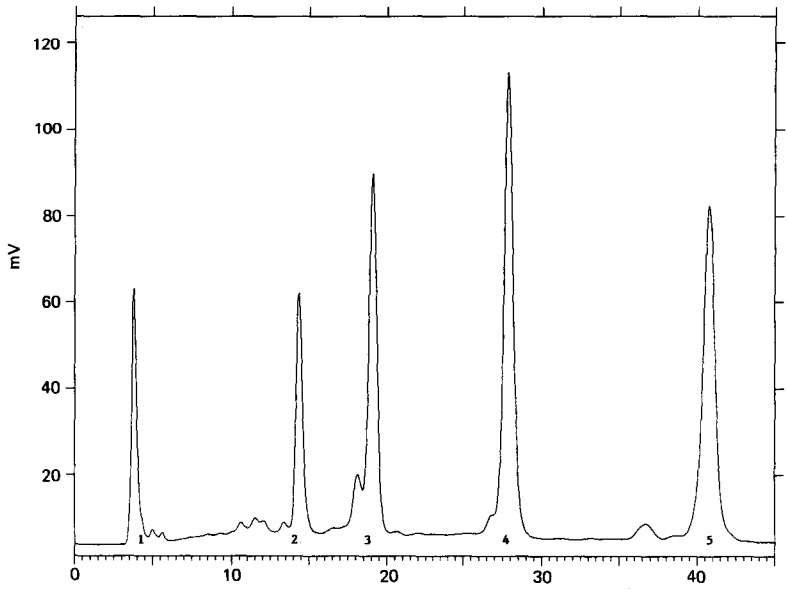

Fig. 6. Protein resolution on Protein-Pak SP 15HR cation-exchange column. Conditions and peaks as in Fig. 5.

The hydrophilic nature of the polymer surface resulted in high recovery of basic and acidic proteins regardless of the charge of the packing; the 1000-Å pore size led to excellent recovery for a full range of molecular weights. The mass recovery data for the Protein-Pak DEAE 8HR and DEAE-5PW columns are summarized in Table II. All of the recoveries on the DEAE 8HR were 98% or greater except for the soybean trypsin

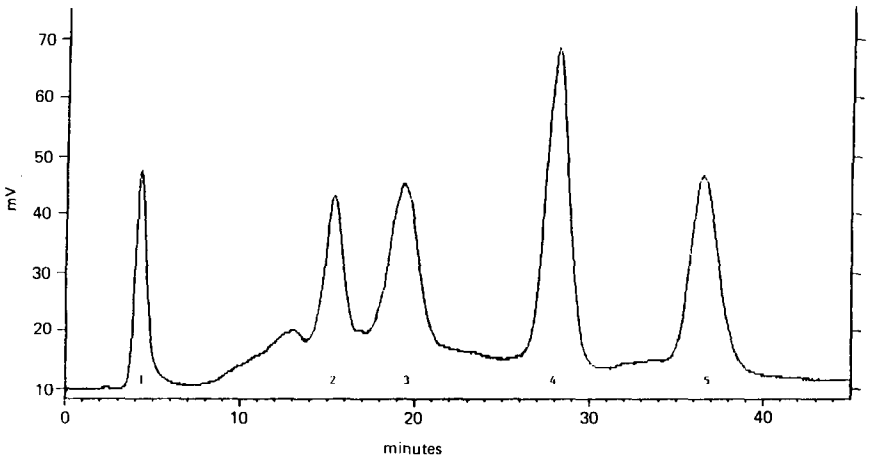

Fig. 7. Protein resolution on Protein-Pak SP 40HR cation-exchange column. Conditions and peaks as in Fig. 5.

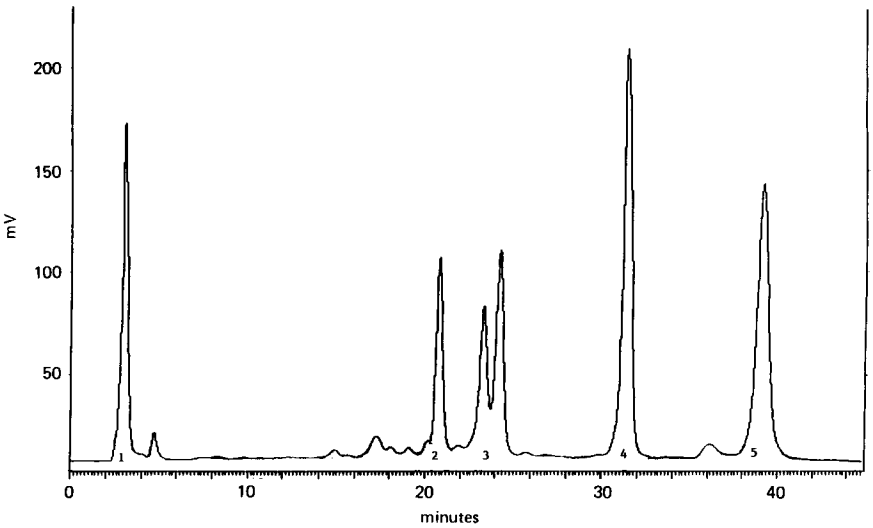

Fig. 8. Protein resolution on Protein-Pak SP-5PW cation-exchange column. Column: 75 mm × 8 mm glass column; eluent A: 20 m*M* sodium phosphate at pH 7.0; eluent B: eluent A + 1 *M* sodium chloride; flow-rate: 1 ml/min; detector: 280 nm; gradient: 0 to 50% eluent B over 60 min; sample load: 0.35 mg protein. Peaks: 1 = myoglobin; 2 = ribonuclease A; 3 = chymotrypsinogen A; 4 = cytochrome *c*; 5 = lysozyme.

inhibitor which had an 80% recovery. The packing has low non-specific binding using cytochrome *c*, and the more hydrophobic proteins, such as ovalbumin, did not interact with the polymer at pH 8.0. The recovery of cytochrome *c* was 77% on the DEAE-5PW column. Despite its high isoelectric point of 9.5, cytochrome *c* was partially retained on the DEAE-5PW as expected; 43% of the recovered protein was in a retained peak. The difference in behavior of cytochrome *c* on the two DEAE packings suggests that there may be some residual carboxyl groups on the DEAE-5PW polymer backbone; the presence of negative charges is further supported by the fact that the retained cytochrome *c* was recovered with high salt. The mass recoveries of the remaining proteins on the DEAE-5PW were greater than 90%, except for soybean trypsin inhibitor which was 86%.

The protein mass recoveries for the Protein-Pak SP HR and the SP-5PW are summarized in Table III. The recoveries on the SP HR packings were 90% or greater except for that of hemoglobin which had an 84% recovery. The recoveries on the Protein-Pak SP-5PW column were all 86% or greater, except for hemoglobin which had only 65% recovery. The SP-5PW was reported to give only 73% recovery of hemoglobin at pH 6.0 and that the recovery of hemoglobin is dependent on the gradient steepness and the pH. Hemoglobin is comprised of four subunits and is difficult to recover as an intact protein, resulting in possible mass loss on the ion-exchange surface. Alternatively, if the conformational changes in hemoglobin during chromatography affect the extinction coefficient at 280 nm, then the resulting error in the spectrophotometric analysis could result in low percent recoveries.

The degrees of substitution of ion exchangers as determined by titration (total

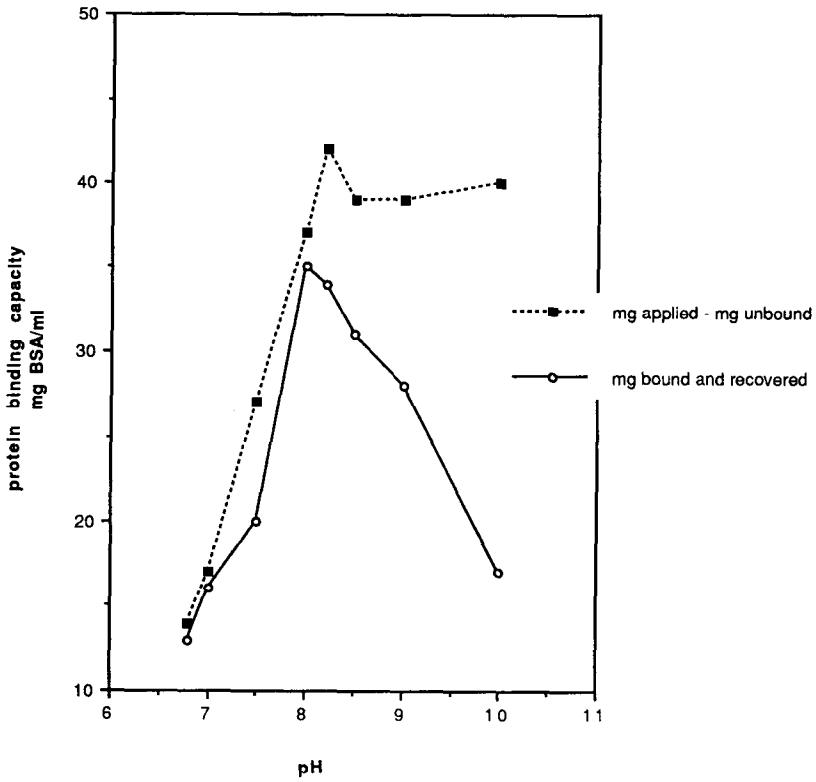

Fig. 9. Protein-binding capacity as a function of pH on Protein-Pak DEAE 8HR. Eluent A (for pH 6.8–9.0): 20 m*M* Tris–HCl; eluent B (for pH 6.8–9.0): eluent A + 1 *M* sodium chloride; eluent A (for pH 10.0): 20 m*M* CHES; eluent B (for pH 10.0): eluent A + 1 *M* sodium chloride; flow-rate: 1 ml/min; detector: 280 nm; sample: bovine serum albumin in eluent A.

ionic capacity) are 100 to 500 μmol/ml of packing [11]. The average degree of substitution of the Protein-Pak HR series is 250 μmol/ml. For Mono Q and Mono S the corresponding figure is 300 μmol/ml. The available capacity or protein-binding capacity of an ion-exchange packing is defined as the amount of protein that can be bound. Only part of the ionic groups are available to bind proteins. The capacity depends on both the operating conditions and the given protein.

The capacity for bovine serum albumin (BSA) was studied as a function of pH on the DEAE 8HR packing with BSA. Fig. 9 shows that there was an increase in capacity as the pH was increased. A plateau was seen after pH 8.0 when the capacity was measured by the amount retained (measured as the difference between total mg BSA applied and mg BSA unbound). However, a decrease in capacity was noticed if the protein binding capacity was only from the amount of bound BSA that was recovered. In all cases the mg bound relative to the amount recovered was used to measure percent recovery of BSA.

The above results may be interpreted from a conclusion reached by Gooding and Schmuck [12]. They noted an increase in protein-binding capacity when they compared

TABLE IV

STUDY OF PROTEIN-BINDING CAPACITY *VERSUS* pH ON THE PROTEIN-PAK DEAE 8HR

Conditions: eluent A: 20 m*M* Tris–HCl (pH 6.8–9.0); eluent B: eluent A + 1 *M* sodium chloride; eluent A: 20 m*M* CHES (pH 10.0); eluent B: eluent A + 1 *M* sodium chloride. Sample: bovine serum albumin in eluent A.

pH	Capacity $\left(\frac{\text{mg applied} - \text{mg bound}}{\text{column volume}}\right)$	Capacity $\left(\frac{\text{mg bound and recovered}}{\text{column volume}}\right)$	Recovery (%)
6.8	14	13	94
7.0	17	16	97
7.5	27	20	86
8.0	37	35	95
8.2	42	34	88
8.5	39	31	80
9.0	39	28	72
10.0	40	17	44

weak and strong anion exchangers. In addition, they noted that the backbone of the polymeric resin became more hydrophobic as the pH increased. Therefore, they concluded that the capacity measured was a combination of both ionic and hydrophobic interactions. The recovery data for the Protein-Pak DEAE HR packing supports their observation since when the pH was increased to 10.0, the protein-binding capacity measured by the amount retained remained on a plateau of 40 mg BSA/ml, but the recovery of BSA dropped to 44%; data are shown in Table IV. The BSA bound by hydrophobic interactions did not elute, therefore the capacity measured by the amount of protein recovered in high salt instead of the amount retained, dropped to only 17 mg BSA/ml at pH 10.0 from the 34 mg/ml at pH 8.2. From these data, the best pH range to determine protein-binding capacity for the DEAE packings was pH 8.0 to 8.5.

The Protein-Pak DEAE 8HR had an average capacity of 45 mg BSA/ml while the DEAE 15HR and 40HR had capacities of 40 mg BSA/ml. The DEAE-5PW had a protein-binding capacity of 68 mg/ml of BSA. When these values were compared on a per column basis, the DEAE-HR (AP1 glass column 100 × 10 mm) columns had more capacity than the DEAE-5PW (75 × 8 mm); 8HR, 15HR and 40HR had capacities of 310–350 mg BSA/column and DEAE-5PW was 262 mg BSA/column, as shown in Table V. For reference, a Pharmacia Mono Q 5/5 HR (1 ml volume) column has a capacity of 100 mg BSA, as measured by the same protein-binding capacity procedure. Since the capacity is extremely sensitive to the availability of charged groups and thus to pore size, differences in capacity are expected for the various ion exchangers, even those with similar degrees of substitution [11].

A pH *versus* protein-binding capacity curve was also examined for the Protein-Pak SP 8HR packing. The capacity was highest at pH 5, 30 mg cytochrome *c*/ml, dropped to 13.6 mg cytochrome *c* at pH 6.5 and remained relatively constant between pH 6.5 to 7.5. The capacity at pH 5.0 was higher in 25 m*M* MES buffer (pK_a 6.15), 39 mg cytochrome *c*/ml, than in 20 m*M* sodium acetate buffer, 30 mg cytochrome *c*.

TABLE V

PROTEIN-BINDING CAPACITY OF THE PROTEIN-PAK HR SERIES AND THE PROTEIN-PAK 5PW PACKINGS

Conditions: *DEAE*: eluent A: 20 m*M* Tris–HCl, pH 8.2; eluent B: eluent A + 1 *M* sodium chloride; sample: bovine serum albumin in eluent A. *SP*: eluent A: 25 m*M* MES, pH 5.0; eluent B: eluent A + 1 *M* sodium chloride; sample: cytochrome *c* in eluent A. *Column HR series*: API (100 mm × 10 mm) = 7.85 ml. *Column 5PW*: (75 mm × 8 mm) = 3.80 ml.

	mg BSA/ml	mg BSA/column	mg cyto-chrome *c*/ml	mg cyto-chrome *c*/column
Anion exchangers				
Protein-Pak DEAE 8HR	45	353		
Protein-Pak DEAE 15HR	40	314		
Protein-Pak DEAE 40HR	40	314		
Protein-Pak DEAE-5PW	69	262		
Cation exchangers				
Protein-Pak SP 8HR			40	314
Protein-Pak SP 15HR			40	314
Protein-Pak SP 40HR			22	173
Protein-Pak SP-5PW			50	190

The average protein-binding capacities of the SP 8HR and 15HR were greater than that of the SP 40HR, 40 mg/ml *versus* 22 mg cytochrome *c*/ml. The SP-5PW column had a protein-binding capacity of 50 mg/ml of cytochrome *c*. When these values were compared on a per column basis, the SP 8HR and 15HR (AP 100 × 10 mm) glass columns have more capacity than the SP-5PW (75 × 8 mm) column: the values for SP 8HR and 15HR were 314 mg cytochrome *c*/column and SP-5PW was 190 mg cytochrome *c*/column, as given in Table V. The corresponding value for a Pharmacia Mono S 5/5 HR column (1 ml volume) was 60 mg cytochrome *c* as measured by the outlined procedure.

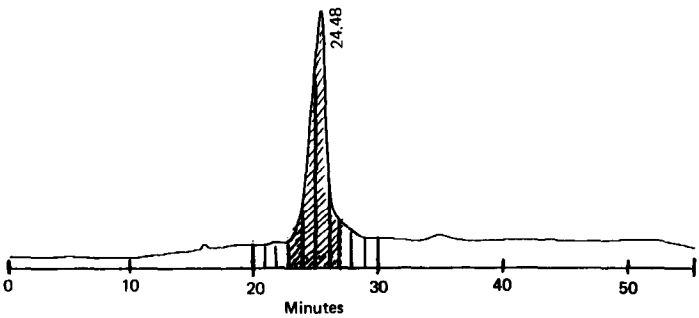

Fig. 10. Recovery of mass and biological activity on Protein-Pak DEAE 15HR. Column: API (100 mm × 10 mm) glass column; eluent A: 20 m*M* Tris–acetate at pH 8; eluent B: eluent A + 0.5 *M* sodium acetate; flow-rate: 1.25 ml/min; detector: 280 nm; gradient: 0 to 100% eluent B over 44 min; sample: glucose-6-phosphate dehydrogenase from yeast (1 mg/ml); injection volume: 100 μl (100 μg).

TABLE VI

RECOVERY OF MASS AND BIOLOGICAL ACTIVITY

Conditions: *Protein-Pak DEAE 15HR*: eluent A: 20 m*M* Tris–acetate, pH 8.0; eluent B: eluent A + 0.5 *M* sodium acetate; sample: glucose-6-phosphate dehydrogenase from yeast (1 mg/ml); reference: Fig. 11. *Protein-Pak SP 8HR*: eluent A: 20 m*M* sodium phosphate, pH 7.0; eluent B: eluent A + 1 *M* sodium chloride; sample: hen egg whites (25 mg/ml); reference: Fig. 12.

	DEAE 15HR	SP 8HR
Mass applied	100 μg	50 mg
Mass recovered	115 μg	44.9 mg
Mass recovery (%)	115	90
Units activity applied	18.5	94 400
Units activity recovered	16.8	76 673
Units activity recovery (%)	91	81
Initial specific activity (units/mg)	185	1888
Specific activity over 4 fractions (units/mg)	183 (fractions 23–27)	44 838 (fractions 41–45)
Purification factor	0.99	24

Biological activity

Glucose-6-phosphate dehydrogenase from yeast was assayed for recovery of biological activity and protein mass on the Protein-Pak DEAE 15HR material; the data are given in Fig. 10 and Table VI. The specific activity of the pooled four main fractions was 183 units/mg, which shows that the protein was not denatured or altered on the column because the specific activity prior to chromatography was the same, 185 units/mg. There was a 91% recovery of biological activity units and a 115% recovery of total protein.

The Protein-Pak SP 8HR material was used in a purification of lysozyme from chicken egg white; data are given in Table VI and chromatography is in Fig. 11. The majority of the components in egg white are acidic, with p*I* values between 3.9 and 6.6;

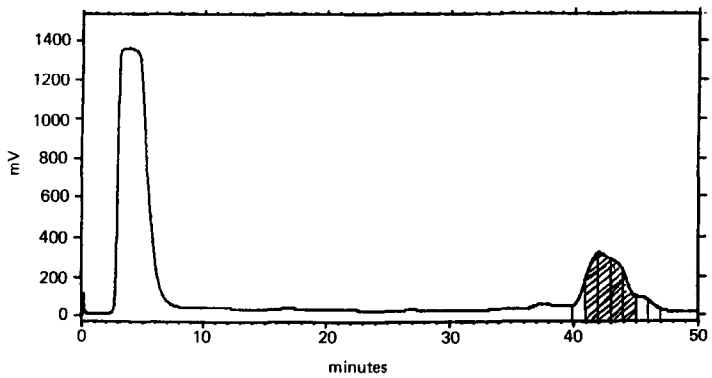

Fig. 11. Purification of lysozyme from egg white on Protein-Pak SP 8HR. Column: AP1 (100 mm × 10 mm) glass column; eluent A: 20 m*M* sodium phosphate at pH 7.0; eluent B: eluent A + 1 *M* sodium chloride; flow-rate: 1.56 ml/min; detector: 280 nm; gradient: 0 to 50% eluent B over 84 min; sample: egg white from hen (25 mg/ml); injection volume: 50 mg.

only lysozyme (p*I* 10.5–11.0) and avidin (p*I* 9.5–10.0) are on the basic side [13]. The initial specific activity of lysozyme, before chromatography, was 1888 units/mg. The specific activity of the pooled four main fractions was 44 838 units/mg which shows a 24-fold purification factor of the lysozyme. There was a 90% recovery of total protein and an 81% recovery of biological activity units.

APPLICATIONS

Scale-up studies

The goal of the scale-up was to demonstrate scalability between the AP1 and AP5 glass columns utilizing 5–10% of the total dynamic capacity of the column. The protein mixture was baseline resolved at the 0.5 mg protein load on the Protein-Pak DEAE 8HR glass column, as previously shown in Fig. 1. The chromatography was repeated without the adenosine, the unretained marker, in the mixture (Fig. 12). When the sample load on this column was scaled-up from 0.5 mg to 22 mg (from 0.2% to 6.2% of total column dynamic capacity), the proteins were still well resolved. The retention times of the peaks were within 5% of each other, as shown in Fig. 13. To demonstrate the scalability of the Advanced Purification columns, an AP5 (100 × 50 mm) glass column was packed with the DEAE-8HR material. The AP5 column was loaded with the protein mix at 6% of total column capacity or 550 mg in 110 ml, the same % column capacity as shown on the AP1 column but a twenty-five times greater amount of protein. The equations that were used to scale-up the separation are given in Appendix 1. The separation on the AP5 column demonstrates the ability to scale to larger diameter columns with the same particle size packing and obtain equivalent separations, as shown in Figs. 13 and 14.

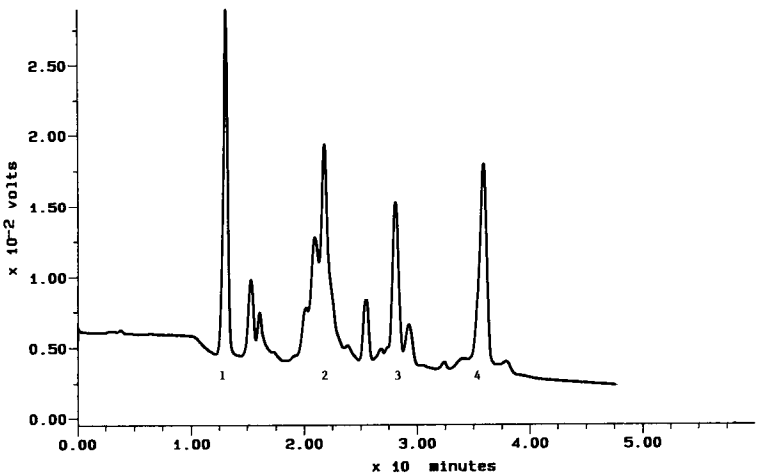

Fig. 12. Scale-up study: 0.5 mg protein mixture load on AP1 glass column. Column: AP1 (100 mm × 10 mm) glass column; eluent A: 20 m*M* Tris–HCl at pH 8.2; eluent B: eluent A + 1 *M* sodium chloride; flow-rate: 1.56 ml/min; detector: 280 nm; gradient: 0 to 25% eluent B over 38 min; sample load: 0.5 mg protein in 100 μl eluent A (0.2% of dynamic column capacity). Peaks: 1 = carbonic anhydrase; 2 = human transferrin; 3 = ovalbumin; 4 = soybean trypsin inhibitor.

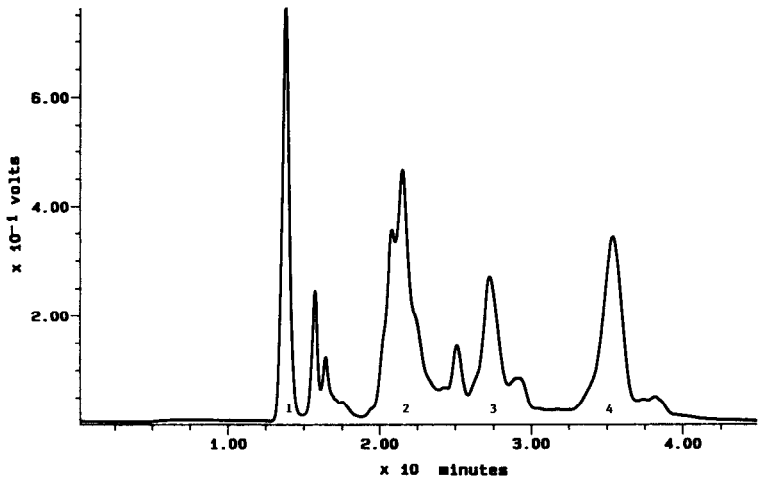

Fig. 13. Scale-up study: 22 mg protein mixture load on AP1 glass column. Column: AP1 (100 mm × 10 mm) glass column; eluent A: 20 m*M* Tris–HCl at pH 8.2; eluent B: eluent A + 1 *M* sodium chloride; flow-rate: 1.56 ml/min; detector: 280 nm; gradient: 0 to 25% eluent B over 38 min; sample load: 22 mg protein in 4.4 ml eluent A (6.2% of dynamic column capacity). Peaks: 1 = carbonic anhydrase; 2 = human transferrin; 3 = ovalbumin; 4 = soybean trypsin inhibitor.

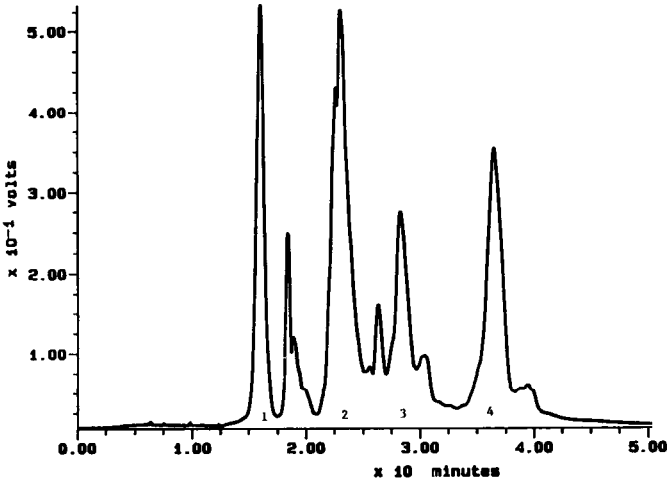

Fig. 14. Scale-up study: 550 mg protein mixture load on AP5 glass column. Column: AP5 (100 mm × 50 mm) glass column; eluent A: 20 m*M* Tris–HCl at pH 8.2; eluent B: eluent A + 1 *M* sodium chloride; flow-rate: 39 ml/min; detector: 280 nm; gradient: 0 to 25% eluent B over 38 min; sample load: 550 mg protein in 110 ml eluent A (6% of dynamic column capacity). Peaks: 1 = carbonic anhydrase; 2 = human transferrin; 3 = ovalbumin; 4 = soybean trypsin inhibitor.

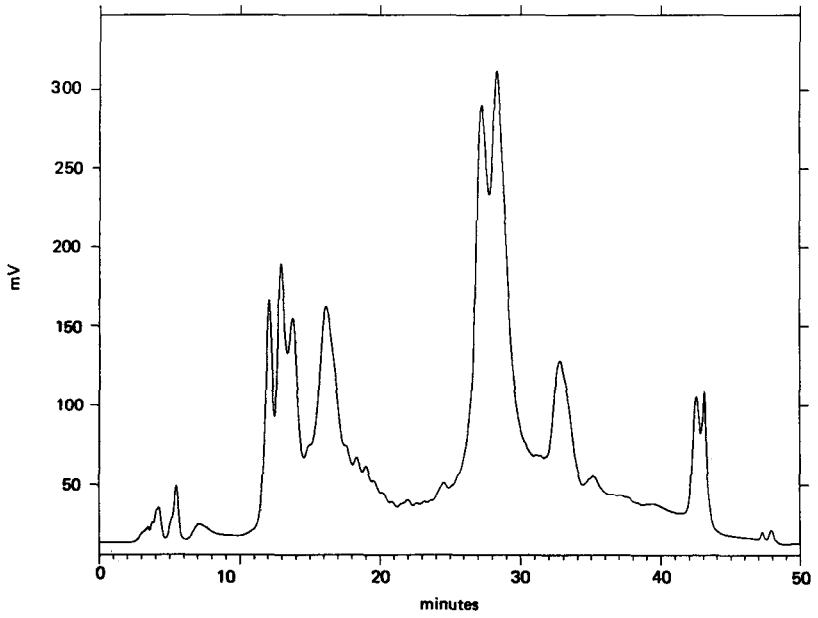

Fig. 15. Resolution of mouse serum proteins on Protein-Pak DEAE 15HR anion-exchange column. Column: API (100 mm × 10 mm) glass column; eluent A: 20 m*M* Tris–HCl at pH 8.2; eluent B: eluent A + 1 *M* sodium chloride; flow-rate: 1.56 ml/min; detector: 280 nm; gradient: 0 to 25% eluent B over 38 min; sample load: 75 μl (4 mg).

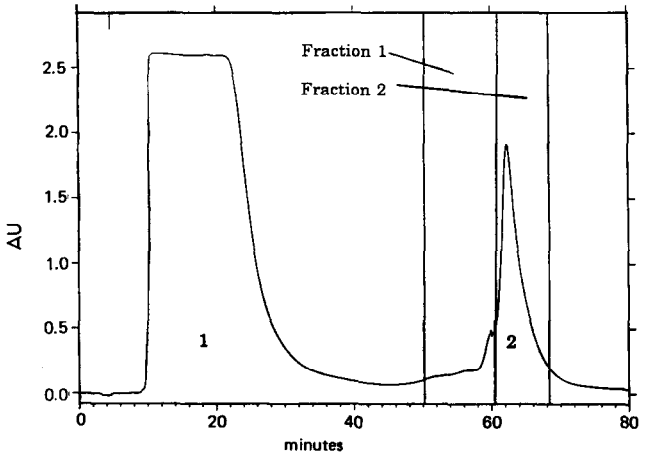

Fig. 16. Plasmid separation on Protein-Pak DEAE 8HR anion-exchange column. Column: API (100 mm × 10 mm) glass column; eluent A: 25 m*M* Tris–HCl with 1 m*M* EDTA at pH 8; eluent B: eluent A + 1 *M* sodium chloride; flow-rate: 1.5 ml/min; detector: 280 nm; gradient: isocratic at 40% eluent B for 40 min and then increased linearly to 60% eluent B over 40 min; sample load: 20.8 mg of a partially purified preparation containing pRSVcat plasmid. Peaks: 1 = unretained peak; 2 = plasmid.

Mouse serum

Mouse serum contains polyclonal immunoglobulin G, transferrin and serum albumin as major proteins. Mouse ascites fluid for monoclonal antibody production has a similar protein composition. The identity of the peaks were not determined, but the overall resolution of the serum proteins was noted. The DEAE 8HR and 15HR resolved the mouse serum proteins into 14 and 12 peaks. The performance of DEAE 8HR is similar to that of the DEAE-5PW which resolved the mouse serum into 16 peaks. The chromatogram for mouse serum on the DEAE 15HR is shown in Fig. 15.

Plasmid purification

Traditionally, purification of plasmid DNA from crude cell lysates containing proteins, RNA and chromosomal DNA was performed using cesium chloride density gradient ultracentrifugation in the presence of ethidium bromide. This technique is time-consuming and does not yield a totally pure plasmid fraction. The isolation of the pRSVcat plasmid on the Protein-Pak DEAE 8HR material gave a high purity fraction; the chromatography is in Fig. 16. The isolation of the plasmid was complete in less than 60 min and the purified fraction possessed excellent biological activity.

CONCLUSION

The Protein-Pak HR series of resin-based, high-performance ion-exchange packings provide high resolution, excellent recovery and high capacity. The Protein-Pak HR series performed comparable and in some cases better than the Protein-Pak 5PW columns. The range of particle sizes and column configurations facilitates full scalability from mg to gram quantities for chromatographic separations of biomolecules such as proteins and nucleic acids. The scale-up strategy shown here demonstrates that the new Protein-Pak HR series successfully employs the same particle size and chemistry to optimize the separation and loading at the analytical scale and to obtain the same resolution at the preparative scale after adjusting the load and linear velocity for column diameter.

APPENDIX 1

Scale-up equations

Sample load. Scale the sample load according to the internal volumes of the columns, as follows:

$$m_1 = m_2 \cdot \frac{d_1^2 l_1}{d_2^2 l_2}$$

where m = sample mass (mg), d = internal diameter of the AP column (cm), l = length of the AP column (cm); in all equations, subscripts 1 and 2 refer to the preparative and analytical columns, respectively.

Flow-rate. Scale-up the flow-rate (F) to maintain the same linear velocity in the preparative column as in the previously developed analytical separation, as follows:

$$F_1 = F_2 \cdot \frac{d_1^2}{d_2^2}$$

Gradient duration. Scale-up the duration of the gradient so the preparative gradient occurs over the same number of column volumes as the analytical gradient, as follows:

$$t_{g_1} = \frac{V_1 t_{g_2} F_2}{V_2 F_1}$$

where V = void volume of the AP column (ml) and t_g = gradient duration (min).

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