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# Schemes for efficient protein purification on a family of polymeric ion exchangers in glass columns

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ABSTRACT

The Protein-Pak HR series is a family of anion (DEAE and Q) and cation (SP and CM) exchange packings for protein and nucleic acid purifications. The characteristics of the weak anion-(DEAE) and the strong cation-(SP) series were reported in an earlier paper. The weak cation (CM) and strong anion (Q) series are new members of the Protein-Pak HR family. All four ion exchangers are available in 8-, 15- and 40-$\mu$m particle sizes. In this study we demonstrated that the 15- and 40-$\mu$m bulk packings are suited for the early states of large scale-protein purifications. The Protein-Pak 8HR series of pre-packed Advanced Purification (AP) glass columns with 8-$\mu$m particles gave the high resolution required in the latter stages of the purification or analysis. These packings were used in the AP-1 (100 mm × 10 mm) glass columns to demonstrate their performance at several flow-rates (0.5–4 ml/min), sample loads and pH values. Both CM and Q 8HR packings were shown to have good recovery of both protein mass and biological activity. Protein mixtures used to evaluate these packing materials included those of mouse serum, egg white, and papain. The Protein-Pak HR series has also been shown to be stable to operation at 4°C and to regeneration (sodium hydroxide and acetic acid) which is important for use of these packings in a purification scheme.

## INTRODUCTION

The efficiency of large-scale protein purifications is becoming more important as the new therapeutic proteins move from research to manufacturing. The purification of proteins from crude mixtures normally consist of three stages: the preliminary or initial isolation stage, the purification stage and the final polishing stage [1]. The purification stage often requires at least four steps involving two or more chromatographic modes. The two most frequently used modes are ion-exchange and affinity chromatography [2]. Naveh and Siegel [3] explained that the scheme for large-scale purification of therapeutic monoclonal antibodies requires a combination of chromatographic steps to remove all contaminants, each being resolved under different conditions or on different principles. The choice of certain chromatographic techniques is dictated by several parameters: (1) sample volume; (2) protein concentration and viscosity of the sample; (3) the desired degree of purity of the protein product; (4) the presence and level of contaminants such as nucleic acids, pyrogens and proteolytic enzymes; and (5) the ease with which different packings can be regenerated or washed free of contaminants and denatured proteins. The ability to regenerate a chromatographic material and the purchase price sets the material cost of a given purification step [1]. Burnouf [4] stressed that the scheme for an industrial purification process must yield a safe and efficient product and provide the highest possible purity and yield at the lowest cost.

The purification scheme frequently begins with the use of an open column containing an inexpensive soft gel and ends with the use of high-resolution liquid chromatographic separations. The low cost and ability to regenerate the softer gels have made them attractive for the early purification step. These

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early hydrophilic ion exchangers which were first developed in the 1950s are still used today [5]. However, the swelling and shrinking properties of the soft gels make them tedious to use. The long time period required to complete the chromatography has the potential to give low recoveries (often due to proteolytic digestion).

A solution to these problems is the use of hard hydrophilic packings for ion-exchange chromatography which are either cross-linked agarose gels or hydrophilic vinyl polymers [6,7]. These ion exchangers are normally rugged materials (chemically and physically stable) that are available in bulk. The large-particle ($\geqslant 15$ $\mu$m), rigid-polymeric packings are applicable in the initial steps of the purification scheme; in many cases they can be regenerated like the softer gels. However, these materials allow rapid throughput because the flow-rate limit is orders of magnitude higher than for soft gels. The significant reduction in time and improvements in recoveries over the soft gels often outweigh the increase in packing material cost. The higher resolving power of the rigid packings than the softer gel leads to higher degrees of purification in the early stages. This also reduces the number of steps required for purification which gives an additional improvement in mass recovery and reduction in process cost.

The latter stage of the purification uses the smaller particle size packings ($\leqslant 10$ $\mu$m) which are now available as both polymeric and silica-based materials. Therefore, it is possible to have a cost-effective purification scheme which uses high-performance liquid chromatography (HPLC) throughout the process.

The Protein-Pak HR family of anion and cation exchangers discussed in this report are useful throughout the purification scheme. The family includes 15- and 40-$\mu$m bulk packings for concentrating the sample and separating the desired protein in the early stages of purification. The Protein-Pak HR family also includes 8-$\mu$m materials in prepacked glass columns for the high resolution required in the final stages of the purification. The Protein-Pak HR series are rigid, spherical, porous hydrophilic, polymeric methacrylate gels. The particles have 1000 Å pores which allow proteins up to $10^6$ molecular weight to penetrate and interact with the functional groups. The strong anion-exchange (Q) packings have quaternary methylamine functional groups. The weak cation-exchange (CM) packings have carboxymethyl functional groups. This paper discusses the characteristics of and utility for the Protein-Pak Q HR and CM HR series ion-exchange packings for protein purification and analysis. The characteristics and performance of the Protein-Pak DEAE HR and SP HR series have been reported previously [8].

## EXPERIMENTAL

The Protein-Pak HR materials were characterized by a standard procedure for protein resolution, mass recovery and protein binding capacity [8]. The protein resolution was also determined at different flow-rates and over a range of protein loads. The Protein-Pak HR materials were packed in Waters Advanced Purification glass columns (AP-1) with dimensions of 100 mm × 10 mm.

All proteins and buffer salts were from Sigma (St. Louis, MO, USA). Acetone was the marker for measuring the column plate counts or efficiencies. The biological activity of glucose-6-phosphate dehydrogenase was measured with the assay kit 345-B from Sigma.

These studies used three different solvent delivery systems, including the Waters 600E multisolvent delivery system, the 650 Advance Protein Purification (APP) System and the 625 non-metallic LC System. The other components of the HPLC systems were either the Model 490 programmable multiwavelength detector or 484 tunable absorbance Detector, the 712 WISP automatic sample injector and a Wescan conductivity meter. A Foxy fraction collector was connected to the Model 650 APP system. A Waters Model 845 computer controlled the HPLC systems and contained the System Suitability software to analyze the data.

The eluents used for the protein resolution, capacity, flow-rate, sample load, and mass recovery studies on Protein-Pak Q HR packings were 20 m*M* Tris–HCl (pH 8.2, eluent A) and 20 m*M* Tris–HCl (pH 8.2) with 1 *M* sodium chloride (eluent B). The proteins in the standard mixture were carbonic anhydrase, human transferrin, ovalbumin and soybean trypsin inhibitor with adenosine as the void volume marker. The isoelectric points and molecular weights are in Table I. The gradient for protein

TABLE I

PROTEINS USED TO EVALUATE THE PERFORMANCE OF ION-EXCHANGE COLUMNS

| Protein | Molecular weight | Isoelectric point | Concentration (%, based on mg/ml) |
|---|---|---|---|
| *Protein-Pak Q 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 CM 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 |

resolution was 0–25% eluent B at flow-rates of 0.5, 1.0, 1.56, 2 and 4 ml/min. The gradient duration was adjusted at each flow rate to cover 7.8 column volumes.

The protein resolution on Protein-Pak Q 8HR was also evaluated at pH 6.0 using 20 m*M* piperazine and 20 m*M* piperazine with 1 *M* sodium chloride. The standard mixture in Table I was used.

The Protein-Pak CM HR packings were evaluated 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). The eluents were used for sample load, recovery of mass and biological acitivity (pH 6.0) studies. The proteins in the standard mixture were myoglobin, ribonuclease A, chymotrypsinogen A, cytochrome *c* and lysozyme. The isoelectric points and molecular weights are in Table I.

The protein resolution as a function of load was determined on both the Protein-Pak Q and CM 8 HR AP-1 glass columns. The Waters Model 490 programmable multiwavelength detector was modified with a prep cell in order to detect the large protein loads. The 2-ml injection loop was put in the Waters Model 712 WISP automatic sample injector to accomodate the large sample volumes. The sample (mixture in Table I) was applied to the Protein-Pak CM 8HR AP-1 glass column at five protein loads. Injection volumes ranged from 10 to 1600 μl for 0.2-, 2-, 8-, 20- and 32-mg protein loads. The gradient was 0–50% eluent B in 20 min. A mixture of conalbumin (29.8%), cytochrome *c* (0.8%), ovalbumin (39.6%) and soybean trypsin inhibitor (29.8%) was injected on the Protein-Pak Q 8HR AP-1 glass column. The sample loads were 0.53, 5.3, 26, 53, and 106 mg of total protein. The gradient was 0–25% buffer B in 18 min.

Tables II and III list the proteins for the mass recovery studies performed on the Protein-Pak Q and CM 8HR columns, respectively. The protein load for both columns was 150 μg per injection. The proteins were eluted from the CM 8HR column using a steep linear gradient from 0 to 100% eluent B (20 m*M* sodium phosphate buffer, pH 7.0, with 1 *m* sodium chloride) in 2 min at 1 ml/min.The mass recovery was done at both pH 7.0 (20 m*M* sodium phosphate) and pH 8.2 (20 m*M* Tris–HCl) on the Protein-Pak Q 8HR column. The gradient was 0–50% eluent B (each eluent A with 1 *M* sodium chloride) in two min at 1 ml/min. The mass recoveries for these proteins were determined from the ratio of peak areas with and without the column in line.

The protein-binding capacities of the packings were determined by pumping the appropriate protein solution into the column until breakthrough occurred. The columns (75 mm × 8 mm steel column) were then flushed with an eluent A until the absorbance at 280 nm decreased below 0.02 absorbance units. The bound protein eluted with an eluent B and the amount was determined based on

TABLE II

MASS RECOVERY OF VARIOUS PROTEINS USING PROTEIN-PAK Q 8HR

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 (150 μg); flow-rate, 1 ml/min; gradient, 0–50% B in 2 min.

| Proteins, 150 μg (0.03% of capacity) | Molecular weight | Isoelectric point | Recovery (%) |
|---|---|---|---|
| Bovine serum albumin | 67 500 | 4.9 | 84 |
| Carbonic anhydrase | 28 000 | 7.3 | 92 |
| Conalbumin | 76 600 | 6.8 | 82 |
| Cytochrome *c* | 12 400 | 9.4 | 86 |
| Ferritin | 445 000 | 3 | 91[a] |
| Human transferrin | 77 000 | 6.0–6.5 | 84 |
| Goat immunoglobulin G | 155 000 | 6.0–7.0 | 90 |
| β-Lactoglobulin A | 35 000 | 5.1 | 70 |
| Ovalbumin | 44 500 | 4.7 | 87 |
| Soybeen trypsin inhibitor | 21 500 | 4.5 | 87 |

[a] Ferritin: eluent A, 20 m*M* sodium phosphate, pH 7.0; eluent B, eluent A + 1 *M* sodium chloride.

its extinction coefficient. Bovine serum albumin (4 mg/ml, extinction coefficient equal to 0.65 absorbance units per mg/ml) was used to measure the capacity of the strong anion exchanger. The capacity study for the Protein-Pak CM 8HR column employed cytochrome *c* (4 mg/ml, extinction coefficient of 1.9); eluents A and B were 25 m*M* 2-(N-morpholino)ethanesulfonic acid (MES) (pH 5.0) and 25 m*M* MES (pH 5.0) with 1 *M* sodium chloride, respectively.

The chicken egg white was separated from the yolk, diluted (1:4 with eluent A) and then filtered (0.45-μm Millex Filter, Millipore) prior to analysis. The diluted egg white (100 μl) was applied to the Protein-Pak CM 8HR AP-1 glass column at pH 6.0. The most retained peak (containing lysozyme) was collected in five 1-ml fractions and assayed for activity. A commercial preparation of lysozyme was also analyzed on the CM 8HR column and checked for biological acitivity after chromatography. The

TABLE III

MASS RECOVERY OF VARIOUS PROTEINS USING PROTEIN-PAK CM 8HR

Conditions: eluent A, 20 m*M* sodium phosphate, pH 7.0; eluent B, eluent A + 1 *M* sodium chloride; injection volume, 15 μl in eluent A (150 μg); flow-rate, 1 ml/min; gradient, 0–100% B in 2 min.

| Proteins, 150 μg (0.08% of capacity) | Molecular weight | Isoelectric point | Recovery (%) |
|---|---|---|---|
| Chymotrypsin | 21 600 | 8.8 | 94 |
| Chymotrypsinogen A | 25 000 | 9 | 92 |
| Cytochrome *c* | 12 400 | 9.4 | 98 |
| Hemoglobin | 64 500 | 7 | 73 |
| Immunoglobulin G | 155 000 | 6.0–7.0 | 92 |
| β-Lactoglobulin A | 35 000 | 5.1 | 89 |
| Lysozyme | 14 400 | 11 | 92 |
| Myoglobin | 17 500 | 7 | 89 |
| Ovalbumin | 44 500 | 4.7 | 88 |
| Soybean trypsin inhibitor | 21 500 | 4.5 | 91 |

enzyme assay was discussed previously [8].

Glucose-6-phosphate dehydrogenase (G6-PDH) was isolated from yeast enzyme concentrate (2 mg) using the Protein-Pak Q 8HR AP-1 glass column. The mobile phases were 20 m*M* Tris acetate, pH 8.0 (eluent A) and eluent A with 0.5 *M* sodium acetate (eluent B). Ten fractions (2 min each or 3.12 ml) were collected and assayed for protein concentration and G6-PDH activity. The fractions were collected based on the retention time of a commercial preparation of G6-PDH. The crude yeast enzyme concentrate was also checked for G6-PDH activity. The G6-PDH assays was discussed in an earlier publication [8]. The assay used to measure the protein concentrations was also reported earlier [8].

*Regeneration*

The Protein-Pak Q and CM 8HR AP-1 glass columns were regenerated by washing with (four column volumes each) water, 0.1 *M* sodium hydroxide, water, 30% acetic acid, water, and finally 1 *M* sodium chloride. The washings were carried out at 0.5 ml/min on both columns. The resolution of the standard protein mixture (Table I) was determined before and after the regeneration.

*Natural mixtures*

Egg white (prepared above) and mouse serum were applied to the Protein-Pak Q 8HR column. The separation of mouse serum and egg white proteins was done at pH 8.2 using the Tris–HCl buffers mentioned above.

Papain from papaya latex was analyzed on the Protein-Pak CM 8HR column using the sodium phosphate buffers at pH 7.0.

## RESULTS AND DISCUSSION

The column backpressure was monitored to determine the flow-rate range over which the packing can be used. The pressure across the packed bed is proportional to the inverse square of the mean particle size. Therefore, the backpressure is expected to decrease with an increase in particle size. The pressure across the Protein-Pak Q 8HR AP-1 glass column was 2.6–4.8-fold greater than that of the 15HR column between 0.5 and 2.0 ml/min (for example, at 2 ml/min, 260 p.s.i. *versus* 100 p.s.i.). The 15HR column and the 40HR column differed the most in pressure at 4 ml/min where the latter column was 4-fold lower in pressure (292 p.s.i. *versus* 72 p.s.i.). The Protein-Pak CM HR AP-1 glass columns also showed a decrease in pressure across the packed bed as particle size increased. At 4 ml/min the pressures were 338, 116 and 100 p.s.i. for the 8, 15 and 40 HR AP-1 glass columns, respectively.

The protein resolutions of the standard mixture in Table I by the Protein-Pak Q HR series are shown in Figs. 1–3. The proteins are baseline resolved on each packing. The difference in the 8-$\mu$m and 15-$\mu$m packings in Figs. 1 and 2 is noted by the higher resolution on the smaller particle packing. The 40-$\mu$m packing in Fig. 3 gave much broader peaks as expected and as supported by its lower efficiency than the 8HR AP-1 column (1000 *versus* 4000 plates at 1 ml/min). The retention times of the proteins are slightly less on the 40HR column because a lower dead volume solvent delivery system (Model 625) was used for its analysis. The Q 8HR and 15HR packings were analysed on the larger delay volume system (Model 600E).

The selectivities were constant (within the error limits of the measurement) with an increase in particle size for both the anion- and cation-exchange columns. Therefore, a separation can be scaled-up to larger columns using either the same packing or one with a greater particle size.

The 40HR bulk packings are applicable at the early purification stage where rapid throughput of large sample volumes may be required. The selectivity ($\alpha$) did not change significantly with an increase in flow-rate. As an example, the change in the selectivity with flow-rate is presented in Fig. 4 for Protein-Pak Q 40HR packing. The peak widths do change with flow-rate as expected which leads to a decrease in resolution. At the early stages of purification throughput is more important than high resolution.

Ion-exchange packings separate proteins based on both their isoelectric point and surface charge ($Z$ value) [9,10]. The strength of the electrostatic interaction between the packing and the protein determines its retention on the column. Therefore, the proteins in the standard mixtures interacted with the CM and Q packings to various degrees resulting in their separation. Since a wide range of molecular weight and isoelectric point proteins are separated on both the Q and CM HR packings, they should

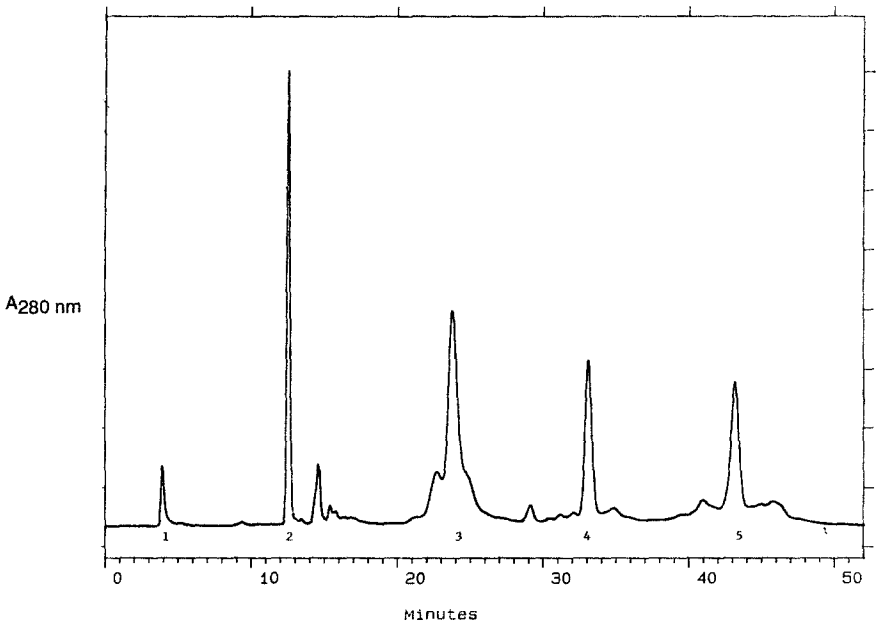


Fig. 1. Protein resolution on Protein-Pak Q 8HR anion-exchange column. Column, AP-1 (100 mm × 10 mm) glass column; eluent A, 20 m*M* Tris–HCl (pH 8.2); eluent B, eluent A + 1 *M* sodium chloride; flow-rate, 1 ml/min; detector, 280 nm; gradient, 0–25% eluent B over 38 min; sample load, 0.5 mg protein; system, Model 600E multisolvent delivery system. Peaks: 1 = adenosine; 2 = carbonic anhydrase; 3 = human transferrin; 4 = ovalbumin; 5 = soybean trypsin inhibitor.

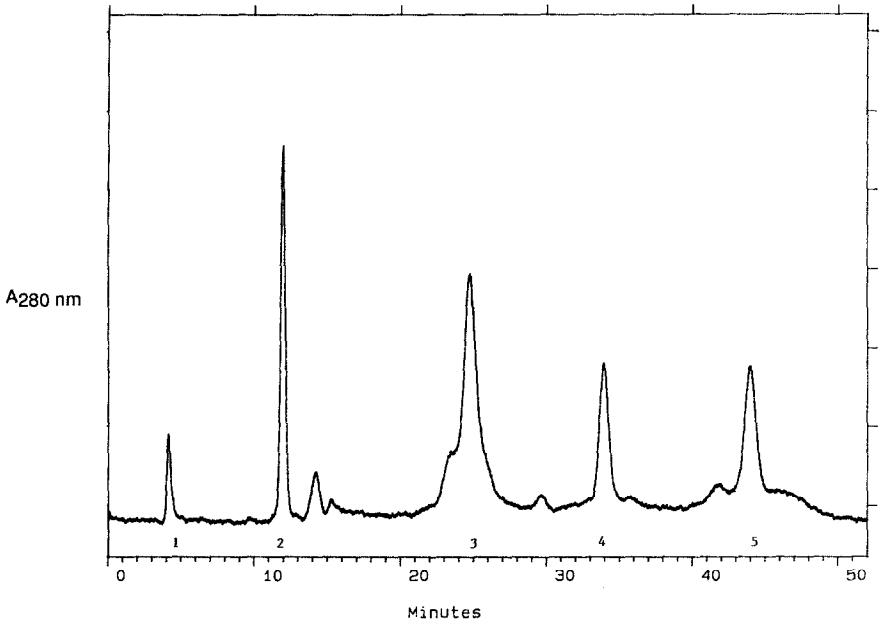


Fig. 2. Protein resolution on Protein-Pak Q 15HR anion-exchange column. For conditions see Fig. 1.

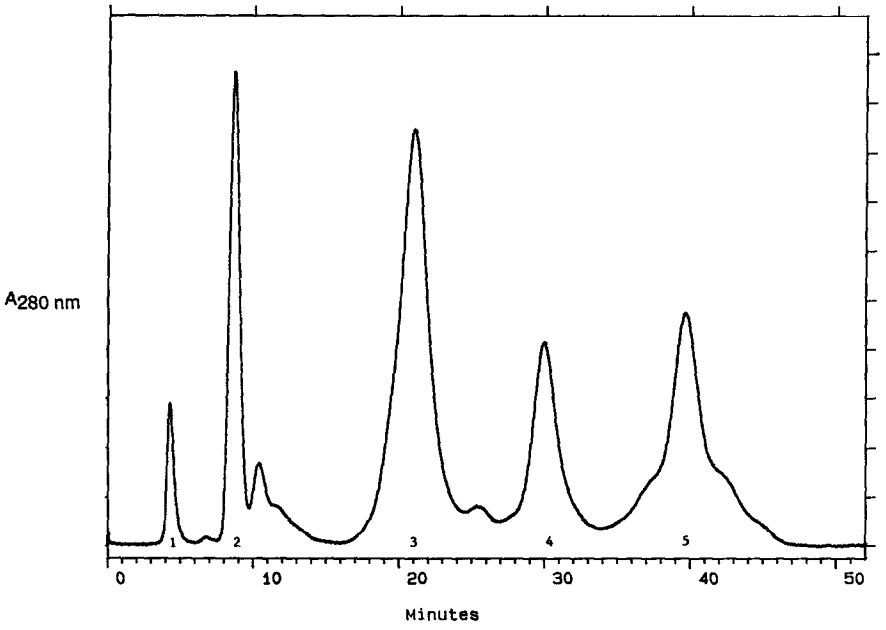


Fig. 3. Protein resolution on Protein-Pak Q 40HR anion-exchange column. System: Model 625 non-metallic LC. For all other conditions see Fig. 1.

be applicable for the purification of many different proteins.

The separation of the desired protein can be optimized by changing the operating pH. The operating pH may also be determined by the pH range where the biological acitivity is maintained. Therefore, it is important to show that a given packing is operational over a pH range. A protein mixture was well resolved on the Protein-Pak Q HR column at both pH 8.2 and pH 6.0 when comparing the upper (A) and lower chromatogram (B) in Fig. 5. The major difference is that carbonic anhydrase is not retained at pH 6.0 since its isoelectric point is pH 7.0. In anion-exchange chromatography a protein is normally retained if the operating pH is above its isoelectric point [9]. As discussed earlier, the number and the distribution of charges on the protein surface also impacts upon its retention [9].

Another property of ion-exchange packings which determines their applicability for purification is the protein-binding capacity. The capacity is influenced by the physical properties of the given protein, pH, buffer strength and buffer type [1]. The flow-rate was shown not to have an effect on the protein-binding capacity in the normal operating range. The capacity of the Protein-Pak Q and CM HR series are given in Table IV. The capacity of the Protein-Pak Q 8HR AP-1 column (510 mg BSA) is competitive with that of other commercial materials. For instance, the 1-ml Pharmacia Mono Q column has a capacity of 100 mg BSA and the Toso-Haas Progel-TSK DEAE 5PW column (75 mm × 7.8 mm) has a capacity of 272 mg BSA. The capacity is consistent across particle size for both CM and Q HR packings which facilitates scale-up.

Most ion-exchange columns give good protein resolution up to at least 5% and sometimes as high as 20% of absolute protein binding capacity. The upper level (20%) is referred to as the dynamic capacity and represents the amount of protein that can be adsorbed and desorbed rapidly (about 30 s) [1]. The amount of protein loaded on the column also impacts upon the resolution. Most preparative chromatography conditions will set the loading level at the dynamic capacity.

Fig. 6 illustrates chromatography at three loads on the Protein-Pak Q 8HR AP-1 column. The three levels represent 0.1% (0.53 mg), 5% (26 mg) and 20% (106 mg) of capacity (capacity data in Table IV), respectively. The selectivity between conalbu-

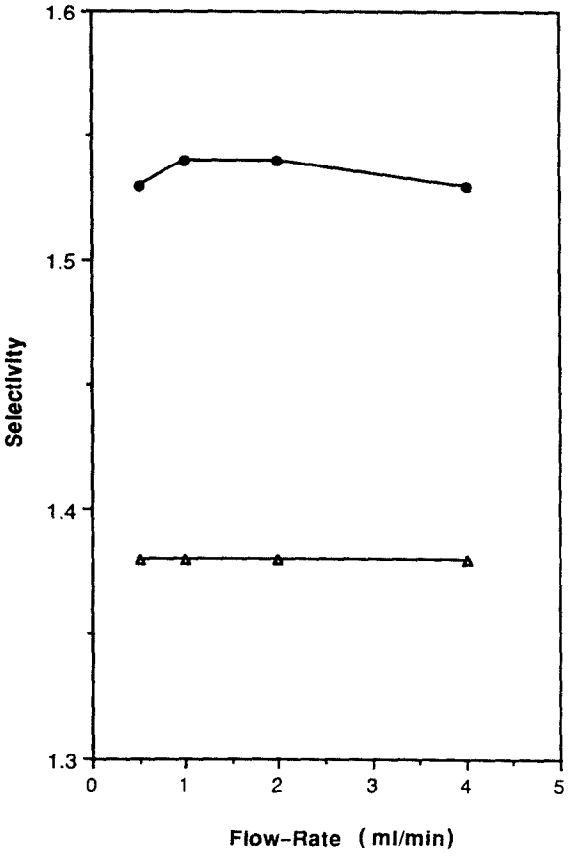


Fig. 4. Flow-rate *versus* selectivity on Protein-Pak Q 40HR column. Flow-rates, 0.5, 1, 2 and 4 ml/min; gradient, 0–25% eluent B. For all other conditions see Fig. 1. Selectity ($\alpha$): ● = ovalbumin–human transferrin; △ = soybean trypsin inhibitor–ovalbumin.

min and ovalbumin did not change as the protein load increased; however, both proteins eluted slightly earlier at the highest loading. The shift in ovalbumin did cause an increase in the selectivity (1.03 at 5% *vs.* 1.24 at 20%) between this protein and soybean trypsin inhibitor. The minor component eluting with the soybean trypsin inhibitor became more pronounced at the high load.

Fig. 7 presents the effect of sample load on the Protein-Pak CM 8HR column. Chromatography is shown at 0.10% (0.2 mg), and 20% (32 mg) of capacity. The five proteins are still baseline resolved at 5% of capacity which is desired (data not shown). The proteins are separated at 20% loading but there is a noticeable decrease in resolution. The decrease in resolution between neighboring proteins was due more to the increases in peak widths than to changes in selectivity. For example, the width of both ribonuclease A and chymotrypsinogen A increased three-fold between the highest and lowest load but the selectivity changed by only 9%. The Protein-Pak Q and CM 8HR columns can definitely be used up to 5% of capacity for protein resolution and in most cases at loads equivalent to the dynamic capacity.

The Protein-Pak HR series of packings contain a hydrophilic polymeric material with 1000-Å pores which allows a wide range of proteins to be recovered at high yield. Excellent protein recoveries have been reported for the strong cation- (SP 8HR) and weak anion- (DEAE 8HR) exchange packings [8]. Tables II and III give the mass recoveries for various proteins on Protein-Pak Q 8HR and CM 8HR, respectively. The proteins cover a wide range of molecular weights and isoelectric points. The recoveries on the Protein-Pak Q 8HR were greater than 80% for all proteins except $\beta$-lactoglobulin A. $\beta$-Lactoglobulin A is very strongly retained on an anion exchanger due to the available surface charges. This strong retention by the anion exchanger probably leads to some irreversible binding, especially at the low load (150 $\mu$g) used in this study. The recovery of $\beta$-lactoglobulin was not improved by changing the operating conditions to pH 7.0. The mass recovery of ferritin ws significantly greater at pH 7.0 than at pH 8.2 (91% *versus* 57%). The low isoelectric point of ferritin (pI 3) and its high molecular weight may have contributed to its low recovery at the higher pH. Several of the other proteins also gave higher recoveries at pH 7.0 than at pH 8.2 (for example, conalbumin, 104%, bovine serum albumin, 113%). When the operating pH is closer to the isoelectric point of a protein its net charge is less and consequently it may bind less strongly to the ion-exchange packings [11].

The mass recoveries of 10 proteins on Protein-Pak CM were greater than or equal to 88%. The eleventh protein analyzed, hemoglobin, was recovered at 73%. Hemoglobin recoveries were lower than those of the other proteins on Protein-Pak SP 8HR (84%) and SP 5PW (65%) [8]. The possible reasons given for the low recovery of hemoglobin on the SP cation exchange packings were the dependence of recovery on the gradient steepness and

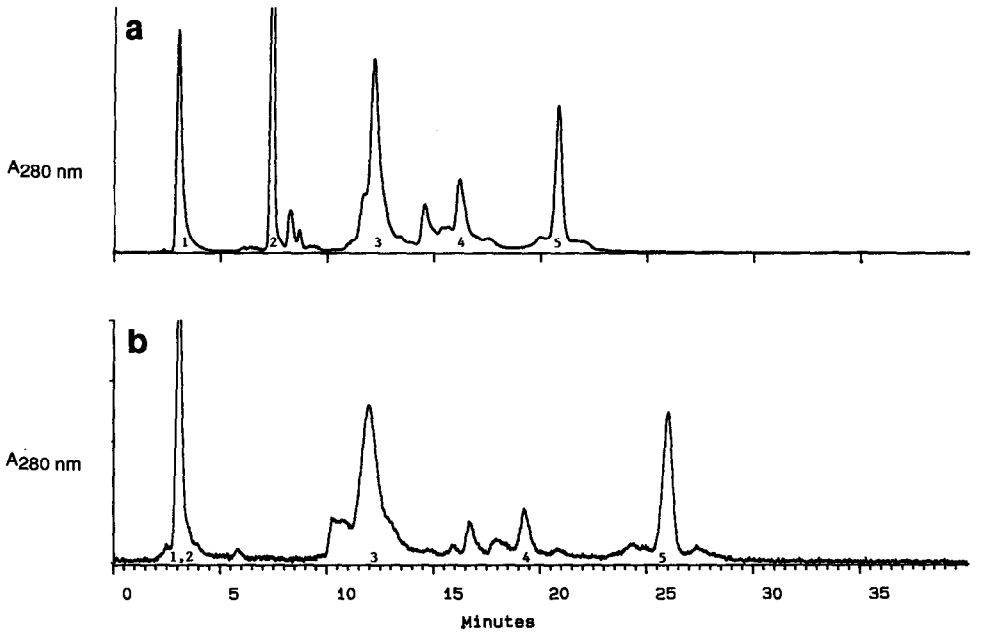


Fig. 5. Protein resolution *versus* pH on Protein-Pak Q 8HR column. Flow-rate, 2 ml/min; gradient, 0–40% eluent B in 40 min. (a) injection volume, 200 $\mu$l (1 mg); for all other conditions see Fig. 1; (b) eluent A, 20 m*M* piperazine–HCl, pH 6.0; eluent B: eluent A + 0.5 *M* sodium chloride; injection volume, 90 $\mu$l (0.43 mg). For identity of peaks see Fig. 1.

the pH. In addition, it was suggested that since hemoglobin consists of four subunits it may be difficult to recover as an intact protein [8].

The large-scale processes used in manufacturing must be able to give good recovery of both mass applied and the biological activity. An increase in specific acitivity should occur at each purification step. Protein purification by ion-exchange chromatography in contrast to reversed-phase seldom causes loss of biological activity. However, conformational changes during ion-exchange chromatography which result in loss of mass (irreversible binding) or biological activity have been addressed [12,13]. These conformational changes occur due to either protein–protein or protein–surface interactions during adsorption and desorption. If the pro-

TABLE IV

PROTEIN-BINDING CAPACITY OF THE PROTEIN-PAK HR SERIES PACKINGS

Conditions: Anion: eluent A, 20 m*M* Tris–HCl, pH 8.2; eluent B, eluent A + 1 *M* sodium chloride; sample, bovine serum albumin (BSA) in eluent A; cation: eluent A, 25 m*M* MES, pH 5.0; Eluent B, eluent A + 1 *M* sodium chloride; sample, cytochrome *c*; column, AP-1 (100 mm × 10 mm) glass column.

| Capacity | Anion exchangers: Protein-Pak Q | | | Cation exchangers: Protein-Pak CM | | |
|---|---|---|---|---|---|---|
| | 8HR | 15HR | 40HR | 8HR | 15HR | 40HR |
| BSA (mg/ml) | 65 | 75 | 74 | | | |
| BSA (mg/column) | 510 | 590 | 581 | | | |
| Cytochrome *c* (mg/ml) | | | | 23 | 25 | 32 |
| Cytochrome *c* (mg/column) | | | | 181 | 196 | 250 |

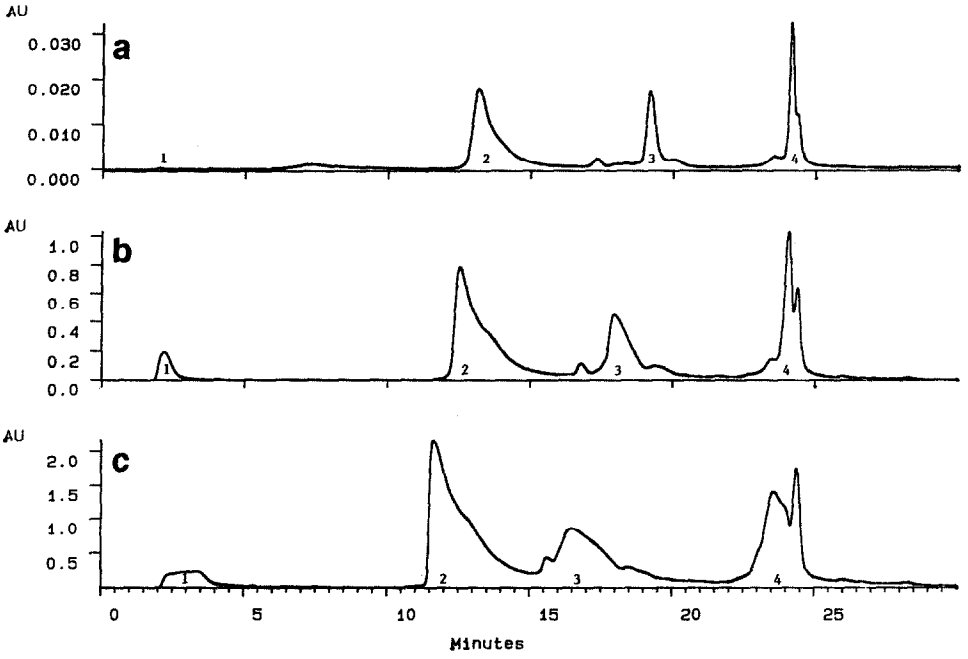


Fig. 6. Protein load study on Protein-Pak Q 8HR column. Gradient, 0–25% eluent B in 18 min; for all other conditions see Fig. 2. Peaks: 1 = cytochrome *c*; 2 = conalbumin; 3 = ovalbumin; 4 = soybean trypsin inhibitor; sample loads. (a) 0.53 mg; (b) 26 mg; (c) 106 mg.

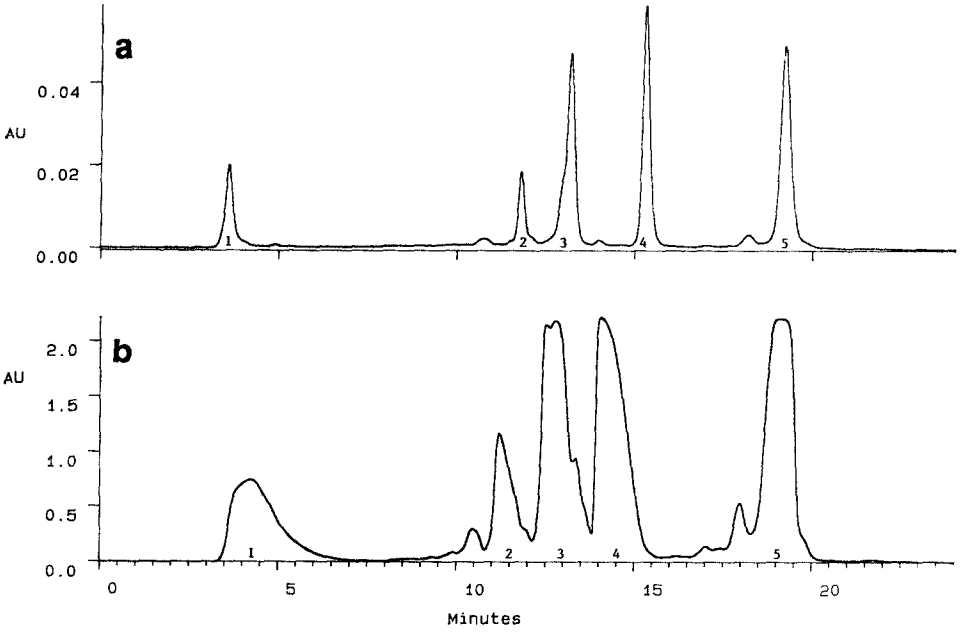


Fig. 7. Protein load study on Protein-Pak CM 8HR column. Column, AP-1 (100 mm × 10 mm) glass column; eluent A, 20 m*M* sodium phosphate at pH 7.0; eluent B, eluent A + 1 *M* sodium chloride; flow-rate, 1.56 ml/min; gradient, 0–50% eluent B in 20 min; system: Model 625 non-metallic LC. Peaks: 1 = myoglobin; 2 = ribonuclease A; 3 = chymotrypsinogen; 4 = cytochrome *c*; 5 = lysozyme. (a) Injection volume, 10 μl (0.2 mg); (b) injection volume, 1600 μl (32 mg).

tein conformation changes during the adsorption/desorption process, then intermediates can form which bind irreversibly or become biologically inactive. Biologically active lysozyme and glucose-6-phosphate dehydrogenase were purified on Protein-Pak 8HR AP-1 glass columns as shown in Table V and Figs. 8 and 9. There was no evidence that irreversible intermediates formed or the presence of any problems associated with protein-surface interactions.

The lysozyme peak was isolated from chicken egg white at pH 6.0 on the Protein-Pak CM 8HR AP-1 column. The biological activity was found in fraction 4 (25–26 min). The retention times for the lysozyme isolated from egg white and that of a commercial lysozyme preparation were the same, as shown in Fig. 8. Both lysozyme peaks contained biological activity.

Glucose-6-phosphate dehydrogenase was isolated from yeast enzyme concentrate on the Protein-Pak Q 8HR column with a 18-fold purification. The specific activity for G6-PDH increased from 7.9 units/mg in the crude mixture to 138 units/mg for material collected from the column. The shaded area in Fig. 9 shows that G6-PDH was one of the more retained yeast enzymes and composed about 4% of the total sample mass. The broad area with biological activity may be related to the many variants of G6-PDH (a tetrameric enzyme with molecular weight between 206 000 and 240 000).

TABLE V

PURIFICATION OF BIOLOGICALLY ACTIVE PROTEINS

Conditions: Protein-Pak Q 8HR: eluent A, 20 m*M* Tris acetate, pH 8.0; eluent B, eluent A + 0.5 *M* sodium acetate; sample, yeast enzyme concentrate, 200 μl (10 ml/ml) ref. Fig. 9. Protein-Pak CM 8HR: eluent A, 20 m*M* sodium phosphate, pH 6.0, eluent B, eluent A + 1 *M* sodium chloride; sample, chicken egg white; ref. Fig. 8.

| Column | Q 8HR |
|---|---|
| Protein | Glucose-6-phosphate dehydrogenase from yeast enzyme concentrate (YEC) |
| Mass applied (mg) | 2 |
| *Fractions 19–23 (38–46 min)* | |
| Mass (mg) | 0.075 |
| Units | 10.4 |
| Specific activity (units/mg) | 138 |
| Crude specific activity (units/mg) | 7.9 |
| Purification factor | 18 |
| **Column** | **CM 8HR** |
| Protein | Lysozyme from chicken egg white |
| Amount applied (1:4 dilution) (μl) | 100 |
| *Fraction 4 (25–26 min)* | |
| Mass in lysozyme peak (μg) | 27 |
| Mass in enzyme reaction (μg) | 2.7 |
| Units | 40 |
| Specific acitivity (units/mg) | 14 814 |
| *Commerical enzyme* | |
| Mass in enzyme reaction (μg) | 4 |
| Units | 18 |
| Specific acitivity (units/mg) | 4500 |
| Ratio laboratory: commercial preparation | 3.3 |

*Stability*

The use of the ion-exchange columns for large-scale purification processes is affected by the stability of the columns to regeneration, and temperature. The ability to regenerate a packing is critical to its utilization in the early stage with crude mixtures. The lifetime of the column can be extended by regeneration. The Protein-Pak Q 8HR and CM 8HR AP-1 glass columns withstood regeneration with 0.1 *M* sodium hydroxide followed by 30% acetic acid. In most cases the columns were regenerated by washing with four column volumes of each eluent as determined by testing the standard protein mixtures. There were no changes in backpressures nor in protein resolution after regeneration. The backpressures of the Q 8HR column were 250 p.s.i. before and 180 p.s.i. after regeneration at 1 ml/min. It is interesting to note that the Protein-Pak HR columns can withstand washing with 50% aqueous ethanol to remove lipids.

The ability to perform a gradient analysis at 4°C is important for enzymes that may decrease in activity when purified at room temperature. The Protein-Pak Q 8HR AP-1 column could be operated up to 1.5 ml/min at 4°C without the backpressure exceeding the recommended 500 p.s.i. The pressure was 180 p.s.i. at room temperature and 330 p.s.i. at 4°C (1 ml/min flow-rate). The backpressure decreased by about 50 p.s.i. when the column was switched to a high-salt buffer. The larger particle

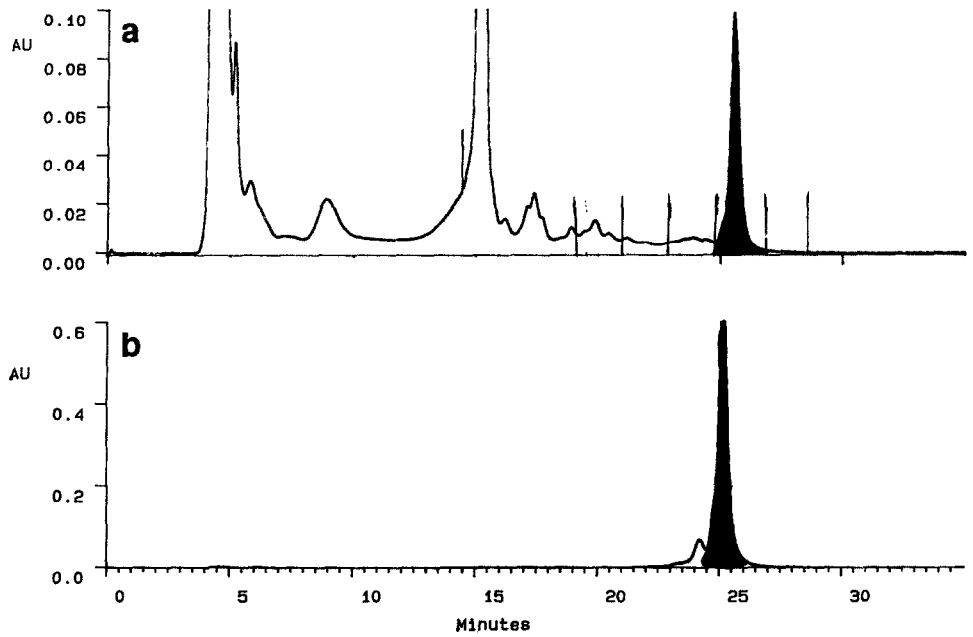


Fig. 8. Purification of egg white lysozyme on Protein-Pak CM 8HR. Column, AP-1 glass column (100 mm × 10 mm); eluent A, 20 m*M* sodium phosphate, pH 6.0; eluent B, eluent A + 1 *M* sodium chloride; flow-rate, 1 ml/min; gradient, 0–50% buffer B in 15 min, hold for 15 min. (a) Lysozyme from egg white; injection volume, 100 μl of 1:4 dilution with eluent A; (b) commercial lysozyme, injection volume, 100 μl (0.5 mg). Shaded peak areas contain lysozyme activity.

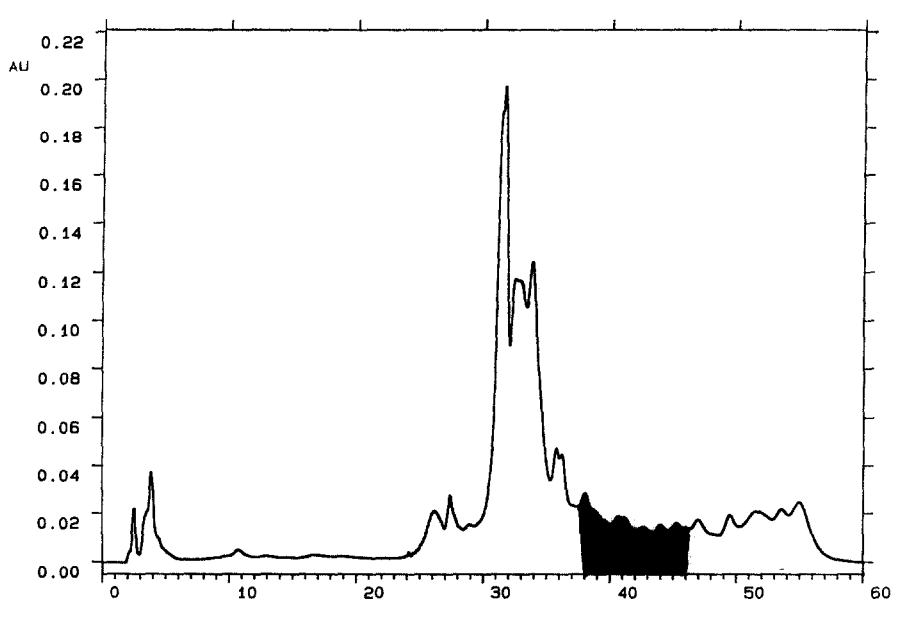


Fig. 9. Purification of glucose-6-phosphate dehydrogenase on Protein-Pak Q 8HR. Column, AP-1 (100 mm × 10 mm) glass column; eluent A, 20 m*M* Tris acetate at pH 8.0; eluent B, eluent A + 0.5 *M* sodium acetate; flow-rate, 1.56 ml/min; detector, 280 nm; gradient, 17 min hold, 0–100% eluent B over 30 min; sample load, 200 μl (2 mg) yeast enzyme concentrate in eluent A. Shaded area contains enzyme activity.

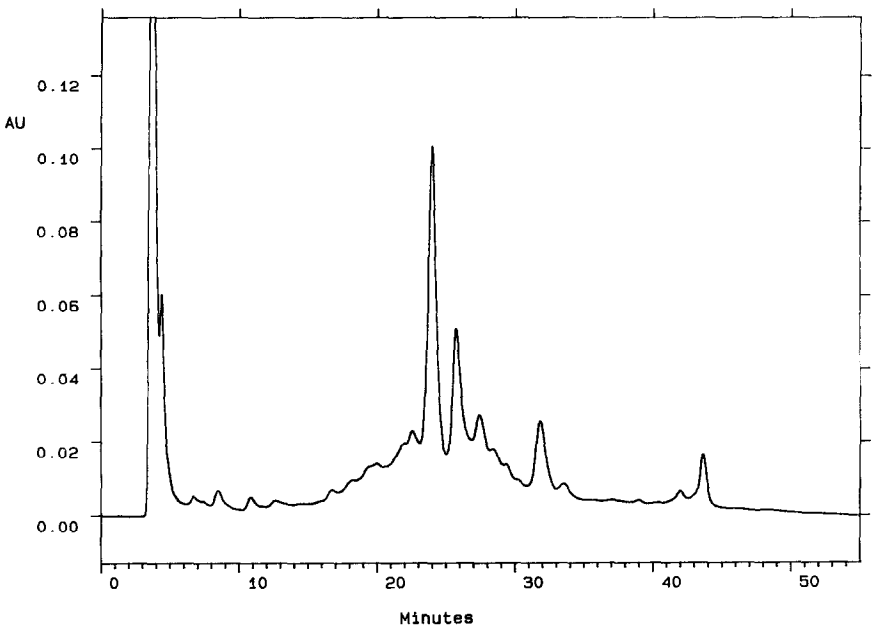


Fig. 10. Chromatogram of papain on Protein-Pak CM 8HR. Injection volume, 200 $\mu$l (400 $\mu$g) papain from Papaya latex; for all other conditions see Fig. 7.

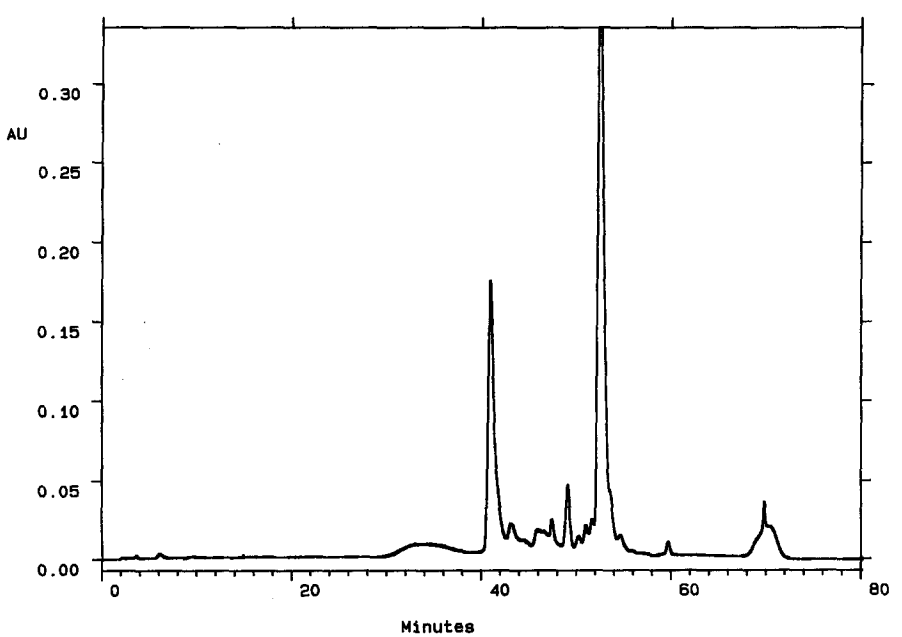


Fig. 11. Chromatogram of egg white proteins on Protein-Pak Q 8HR column. Flow-rate, 1.56 ml/min; gradient, 22 min in eluent A then 0–25% eluent B in 40 min; injection volume, 100 $\mu$l of chicken egg white diluted 1:4 with eluent A. For all other conditions see Fig. 1.

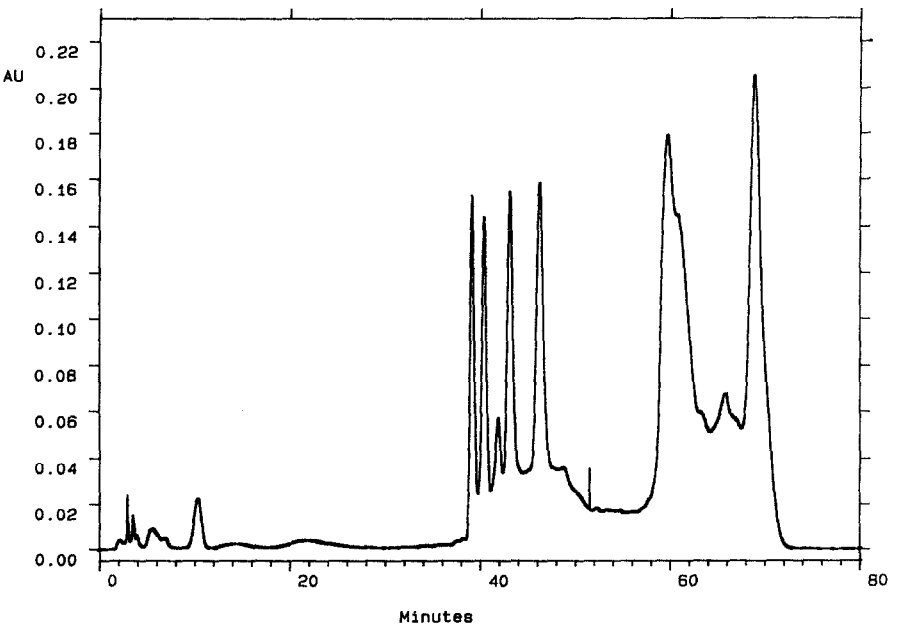


Fig. 12. Chromatogram of mouse serum proteins on Protein-Pak Q 8HR. Flow-rate, 1.56 ml/min; gradient, 22 min in eluent A then 0–25% eluent B in 40 min; injection volume, 75 μl of mouse serum. For all other conditions see Fig. 1.

packings (15 and 40HR) should operate in the cold without difficulty since the backpressure will be lower for these materials than the 8HR packing as discussed earlier.

*Natural protein mixtures*

The industrial processes require the separation of crude mixtures of proteins with a variety of different properties. The Protein-Pak HR series performed well for separating natural protein mixtures. Fig. 10 shows the separation of papain on the Protein-Pak CM 8HR column which is competitive with that shown in the product bulletin for another commercial CM column [14]. The egg white proteins were resolved on Protein-Pak CM 8HR column as shown in Fig. 8a; ovalbumin is not retained. Conalbumin appears at about 15 min and lysozyme is the most retained peak.

The Protein-Pak Q 8HR column was used to separate the proteins in egg white and mouse serum at pH 8.2. Both mixtures contain acidic proteins (isoelectric point less than 7.0) which are separated on the anion-exchange column as shown in Figs. 11 and 12. The two retained peaks in Fig. 11 are probably conalbumin and ovalbumin. Some of the proteins in mouse serum in Fig. 12 which are retained and separated are albumin, transferrin and immunoglobulins.

## CONCLUSIONS

The Protein-Pak HR ion-exchange series meets the challenges of industrial large-scale purification schemes. The Protein-Pak HR series packings can be used at any stage since they (1) perform well at fast flow-rates for rapid throughput, (2) have competitive protein-binding capacities, (3) are usable at low temperatures (4°C) and (4) can be regenerated. The Protein-Pak 8HR series should work well in a protein purification scheme. Proteins were recovered from the 8HR packing in high yield and with retention of biological activity. The Protein-Pak HR packings also performed well at loads equivalent to dynamic capacity; the amount of crude material applied in preparative chromatography is often about 20% (dynamic capacity) or greater of the absolute capacity. The packings can be used for the separation of crude mixtures such as mouse serum

and egg white. Since both anion and cation materials are available which operate over a wide pH range the Protein-Pak HR series should be included in the purification scheme for many therapeutic proteins.

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