A New Family of High Resolution Ion Exchangers for Protein and Nucleic Acid Purifications from Laboratory to Process Scales

Donna M. Dion, Kevin O'Connor, Dorothy Phillips, George J. Vella, William Warren

Waters Chromatography Division, Milford, MA 01757
ABSTRACT

A new family of polymer-based ion exchangers is now available for the purification of acidic and basic biomolecules 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 or pH limitations of the protein. Three particle sizes, 8, 15 and 40 micron, are offered in scalable Advanced Purification (AP) glass columns or as bulk packings, thus, making high resolution purifications convenient at any scale. The approximately two fold lower backpressures of the 15 and 40HR packings than the 8HR material allow rapid throughput of large volumes without exceeding the pressure limitations of the resin or the column. The AP1 (10 x 100 mm) glass columns containing these ion exchange packing are cost competitive on a capacity per column basis. High resolution of mouse serum, snake venom components, monoclonal antibodies, plasmids and a standard protein mix has been demonstrated and compared to those on other resin-based ion exchangers of similar particle size. Protein were purified without loss of biological activity or mass.
INTRODUCTION

The Protein-Pak™ HR series ion exchange packing materials are designed to deliver high resolution, excellent recovery, and full scalability in the chromatographic separation of biomolecules such as proteins, peptides and nucleic acids.

Available in two chemistries:

Protein-Pak DEAE-HR anion exchange packing materials with covalently bonded diethylaminoethyl functionalities on the hydrophilic polymeric support.

Protein-Pak SP-HR cation exchange packing materials with covalently bonded propyl sulfonic acid functionalities on the hydrophilic polymeric support.

Available in three particle sizes:

The packing materials are made from a rigid spherical, fully porous polymeric gel with nominal particle diameters of 8, 15 or 40 microns.

Available in different column sizes for scale-up:

These packing materials are available in scalable pre-packed glass columns (8μ and 15μ) or in bulk (15μ and 40μ) to meet all analytical or preparative needs.
<table>
<thead>
<tr>
<th>COLUMNS</th>
<th>SIZES</th>
<th>SUGGESTED FLOW RATE RANGES</th>
<th>PRESSURE LIMITS</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP1</td>
<td>10 mm X 100 mm</td>
<td>1-3 ml/min.</td>
<td>10 MPa (1500 psi or 100 atm)</td>
</tr>
<tr>
<td>AP2</td>
<td>20 mm x 100 mm</td>
<td>4-12 ml/min.</td>
<td>6.8 MPa (1000 psi or 68 atm)</td>
</tr>
<tr>
<td>AP5</td>
<td>50 mm x 100 mm</td>
<td>25-75 ml/min.</td>
<td>3.4 MPa (500 psi or 34 atm)</td>
</tr>
</tbody>
</table>
## MASS RECOVERY OF PROTEIN-PAK™ SP-8HR COMPARED TO SP-5PW

<table>
<thead>
<tr>
<th>PROTEINS</th>
<th>100μg (0.03% of capacity)</th>
<th>MOLECULAR WEIGHT</th>
<th>ISOELECTRIC POINT</th>
<th>PERCENT SP-8HR</th>
<th>RECOVERY SP-5PW</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHYMOTRYPSIN</td>
<td></td>
<td>21600</td>
<td>8.8</td>
<td>100</td>
<td>96</td>
</tr>
<tr>
<td>CHYMOTRYPSINOGEN A</td>
<td></td>
<td>25000</td>
<td>9.0</td>
<td>92</td>
<td>96</td>
</tr>
<tr>
<td>CYTOCHROME C</td>
<td></td>
<td>12400</td>
<td>9.4</td>
<td>98</td>
<td>90</td>
</tr>
<tr>
<td>HEMOGLOBULIN</td>
<td></td>
<td>64500</td>
<td>7.0</td>
<td>84</td>
<td>65</td>
</tr>
<tr>
<td>β-LACTOglobulin</td>
<td></td>
<td>35000</td>
<td>5.1</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>LYSOZYME</td>
<td></td>
<td>14400</td>
<td>11.0</td>
<td>98</td>
<td>95</td>
</tr>
<tr>
<td>MYOGLOBIN</td>
<td></td>
<td>17500</td>
<td>7.0</td>
<td>90</td>
<td>86</td>
</tr>
<tr>
<td>OVALBUMIN</td>
<td></td>
<td>44500</td>
<td>4.7</td>
<td>95</td>
<td>90</td>
</tr>
<tr>
<td>RIBONUCLEASE A</td>
<td></td>
<td>13500</td>
<td>8.8</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>SOYBEAN TRYPSIN INHIBITOR</td>
<td></td>
<td>21500</td>
<td>4.5</td>
<td>93</td>
<td>91</td>
</tr>
<tr>
<td>PROTEINS</td>
<td>MOLECULAR WEIGHT</td>
<td>ISOELECTRIC POINT</td>
<td>%RECOVERY DEAE-8HR</td>
<td>%RECOVERY DEAE-5PW</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------------</td>
<td>-------------------</td>
<td>--------------------</td>
<td>--------------------</td>
<td></td>
</tr>
<tr>
<td>BOVINE SERUM ALBUMIN</td>
<td>67500</td>
<td>4.9</td>
<td>100</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>CARBONIC ANHYDRASE</td>
<td>28000</td>
<td>7.3</td>
<td>100</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>CONALBUMIN</td>
<td>76600</td>
<td>6.8</td>
<td>98</td>
<td>97</td>
<td></td>
</tr>
<tr>
<td>CYTOCHROME C</td>
<td>12400</td>
<td>9.5</td>
<td>98</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>HUMAN TRANSFERRIN</td>
<td>77000</td>
<td>6.0-6.5</td>
<td>98</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>ß-LACTOglobulin</td>
<td>35000</td>
<td>5.1</td>
<td>99</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>OVALBUMIN</td>
<td>44500</td>
<td>4.7</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>RABBIT IgG</td>
<td>155000</td>
<td>6.0-7.0</td>
<td>100</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>RAT IgG</td>
<td>155000</td>
<td>6.0-7.0</td>
<td>100</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>SOYBEAN TRYPsin INHIBITOR</td>
<td>21500</td>
<td>4.5</td>
<td>80</td>
<td>86</td>
<td></td>
</tr>
</tbody>
</table>
# PROTEIN BINDING CAPACITY ON A PER COLUMN BASIS

## ANION EXCHANGERS

**Prepacked Columns**

<table>
<thead>
<tr>
<th>Column Type</th>
<th>Glass Column Dimensions</th>
<th>mg BSA/Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein-Pak™ DEAE 8-HR</td>
<td>AP1 (10 x 100mm)</td>
<td>353</td>
</tr>
<tr>
<td>Protein-Pak™ DEAE 15-HR</td>
<td>AP1 (10 x 100mm)</td>
<td>314</td>
</tr>
<tr>
<td>Protein-Pak™ DEAE-5PW</td>
<td>8 x 75mm</td>
<td>262</td>
</tr>
</tbody>
</table>

**Bulk Material**

<table>
<thead>
<tr>
<th>Column Type</th>
<th>Glass Column Dimensions</th>
<th>mg BSA/Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein-Pak™ DEAE 15-HR</td>
<td>AP1 (10 x 100mm)</td>
<td>314</td>
</tr>
<tr>
<td>Protein-Pak™ DEAE 40-HR</td>
<td>AP1 (10 x 100mm)</td>
<td>314</td>
</tr>
</tbody>
</table>

## CATION EXCHANGERS

**Prepacked Columns**

<table>
<thead>
<tr>
<th>Column Type</th>
<th>Glass Column Dimensions</th>
<th>mg BSA/Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein-Pak™ SP 8-HR</td>
<td>AP1 (10 x 100mm)</td>
<td>314</td>
</tr>
<tr>
<td>Protein-Pak™ SP 15-HR</td>
<td>AP1 (10 x 100mm)</td>
<td>314</td>
</tr>
<tr>
<td>Protein-Pak™ SP-5PW</td>
<td>8 x 75mm</td>
<td>190</td>
</tr>
</tbody>
</table>

**Bulk Material**

<table>
<thead>
<tr>
<th>Column Type</th>
<th>Glass Column Dimensions</th>
<th>mg BSA/Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein-Pak™ SP 15-HR</td>
<td>AP1 (10 x 100mm)</td>
<td>314</td>
</tr>
<tr>
<td>Protein-Pak™ SP 40-HR</td>
<td>AP1 (10 x 100mm)</td>
<td>173</td>
</tr>
</tbody>
</table>

*PROTEIN-BINDING CAPACITY CONDITIONS DEAE-HR: Bovine Serum Albumin, 20mM Tris/HCl pH 8.2
*PROTEIN-BINDING CAPACITY CONDITIONS SP-HR: Cytochrome C, 25mM MES pH 5.0
FIGURE 1:

Purification of Lysozyme from Egg White

Protein-Pak™SP 8HR

Initial Specific Activity: 1,888 units/mg
Specific Activity over 4 fractions (time: 41-45 minutes): 44,838 units/mg
Purification Factor: 24
% mass recovery = 90
% units biological activity recovery = 81

Column: AP 1 glass column (10 x 100mm)
Buffer: 20mM Sodium Phosphate +/- 1M NaCl, pH 7.0
Sample: Egg White from chicken eggs (Sigma # E-0500), 25 mg/mL
Gradient: 0-50%B over 84 min. at 1.56 mL/min
Injection Amount: 50 mg
Recovery of Mass and Biological Activity

Protein-Pak™ DEAE 15HR

Specific Activity over 4 main fractions (time: 23-27 minutes)
% mass recovery = 115
% units biological activity recovery = 91

Column: AP 1 glass column (10 x 100 mm)
Buffer: 20mM Tris +/- 0.5M Na Acetate, pH 8.0
Sample: Glucose-6-phosphate dehydrogenase from Yeast (Sigma # G-6378), 1 mg/mL
Gradient: 0-100%B over 44 min. at 1.25 mL/min
Injection Amount: 100μg
PROTEIN RESOLUTION ON PROTEIN-PAK™ DEAE-8HR
ANION EXCHANGE COLUMN

COLUMN: AP1(10 mm x 100 mm) GLASS COLUMN
BUFFER A: 20 mM TRIS-HCl pH 8.2;
BUFFER B: BUFFER A WITH 1M SODIUM CHLORIDE.
FLOW RATE: 1.56 ML/ MINUTE. DETECTOR: 280 nm
GRADIENT: 0 to 25% BUFFER B OVER 38 MINUTES
LOAD: 0.5 MG PROTEIN
PEAKS: 1) ADENOSINE, 2) CARBONIC ANHYDRASE,
3) HUMAN TRANSFERRIN, 4) OVALBUMIN,
5) SOYBEAN TRYSIN INHIBITOR.
**FIGURE 4:**

PROTEIN RESOLUTION ON PROTEIN-PAK™ DEAE-15HR ANION EXCHANGE COLUMN

COLUMN: AP1(10 mm x 100 mm) GLASS COLUMN
BUFFER A: 20 mM TRIS-HCl pH 8.2;
BUFFER B: BUFFER A WITH 1M SODIUM CHLORIDE.
FLOW RATE: 1.56 ML/MINUTE. DETECTOR: 280 nm
GRADIENT: 0 to 25% BUFFER B OVER 38 MINUTES
LOAD: 0.5 MG PROTEIN
PEAKS: 1) ADENOSINE, 2) CARBONIC ANHYDRASE,
3) HUMAN TRANSFERRIN, 4) OVALBUMIN,
5) SOYBEAN TRYSIN INHIBITOR.
COLUMNS:  AP1 (10 mm x 100 mm) GLASS COLUMN
BUFFER A: 20 mM TRIS-HCl pH 8.2;
BUFFER B: BUFFER A WITH 1M SODIUM CHLORIDE.
FLOW RATE: 1.56 ML/ MINUTE. DETECTOR: 280 nm
GRADIENT: 0 to 25% BUFFER B OVER 38 MINUTES
LOAD: 0.5 MG PROTEIN
PEAKS: 1) ADENOSINE, 2) CARBONIC ANHYDRASE,
3) HUMAN TRANSFERRIN, 4) OVALBUMIN,
5) SOYBEAN TRYPsin INHIBITOR.
RESOLUTION OF MOUSE SERUM PROTEINS ON PROTEIN-PAK™ DEAE-15 HR ANION EXCHANGE COLUMN

COLUMN: AP1(10 mm x 100 mm) GLASS COLUMN
BUFFER A: 20 mM TRIS-HCl pH 8.2;
BUFFER B: BUFFER A WITH 1M SODIUM CHLORIDE.
FLOW RATE: 1.56 ML/ MINUTE.
GRADIENT: 0 to 25% BUFFER B OVER 38 MIN.
DETECTOR: 280 nm
LOAD: 75 µL MOUSE SERUM OR 4 MG
PROTEIN RESOLUTION ON PROTEIN-PAK™ SP-8HR
CATION EXCHANGE COLUMN

COLUMN: AP1(10 mm x 100 mm) GLASS COLUMN
BUFFER A: 20 mM SODIUM PHOSPHATE pH 7.0;
BUFFER B: BUFFER A WITH 1M SODIUM CHLORIDE.
FLOW RATE: 1.56 ML/MINUTE. DETECTOR: 280 NM
GRADIENT: 0 to 50% BUFFER B OVER 60 MINUTES.
LOAD: 0.35 MG PROTEIN
PEAK IDENTIFICATION: 1) MYOGLOBIN,
2) RIBONUCLEASE A, 3) CHYMOTRYPSINOGEN A,
4) CYTOCHROME C, 5) LYSOZYME.
FIGURE 7:
PROTEIN RESOLUTION ON PROTEIN-PAK\textsuperscript{TM} SP-15HR CATION EXCHANGE COLUMN

COLUMN: AP1(10 mm x 100 mm) GLASS COLUMN
BUFFER A: 20 mM SODIUM PHOSPHATE pH 7.0;
BUFFER B: BUFFER A WITH 1M SODIUM CHLORIDE.
FLOW RATE: 1.56 ML/MINUTE. DETECTOR: 280 NM
GRADIENT: 0 to 50% BUFFER B OVER 60 MINUTES.
LOAD: 0.35 MG PROTEIN
PEAK IDENTIFICATION: 1) MYOGLOBIN,
2) RIBONUCLEASE A, 3) CHYMOTRYPSINOGEN A,
4) CYTOCHROME C, 5) LYSOZYME.
COLUMNS: AP1 (10 mm x 100 mm) GLASS COLUMN  
BUFFER A: 20 mM SODIUM PHOSPHATE pH 7.0;  
BUFFER B: BUFFER A WITH 1M SODIUM CHLORIDE.  
FLOW RATE: 1.56 ML/MINUTE. DETECTOR: 280 NM  
GRADIENT: 0 to 50% BUFFER B OVER 60 MINUTES.  
LOAD: 0.35 MG PROTEIN  
PEAK IDENTIFICATION: 1) MYOGLOBIN,  
2) RIBONUCLEASE A, 3) CHYMOTRYPSINOGEN A,  
4) CYTOCHROME C, 5) LYSOZYME.
SCALE-UP EQUATIONS

Sample Load - scale the sample load according to the internal volumes of the columns, as follows:

\[
\text{load}_{\text{prep}} = \frac{\text{load}_{\text{analytical}} \times (D_1)^2 L_1}{(D_2)^2 L_2}
\]

where: 
- \(D_1\) = internal diameter of the preparative AP column (cm)
- \(L_1\) = length of the preparative Ap column (cm)
- \(D_2\) = internal diameter of the analytical AP column (cm)
- \(L_2\) = length of the analytical AP column (cm)

Flow Rate - scale up the flow rate to maintain the same linear velocity in the preparative column as in the previously developed analytical separation, as follows:

\[
Q_{\text{prep}} = \frac{Q_{\text{analytical}} \times (D_1)^2}{(D_2)^2}
\]

where: 
- \(Q\) = flow rate (mL/min)
- \(D_1\) = internal diameter of the preparative AP column (cm)
- \(D_2\) = internal diameter of the analytical AP column (cm)

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:

\[
GD_{\text{prep}} = \frac{(V_p)(GD_a)(Q_a)}{(V_a)(Q_p)}
\]

where: 
- \(V_p\) = void volume of the preparative AP column (mL)
- \(GD_a\) = gradient duration of the analytical method (min)
- \(Q_a\) = flow rate of analytical method (mL/min)
- \(V_a\) = void volume of the analytical column (mL)
- \(Q_p\) = flow rate of preparative method (mL/min)
**FIGURE 9:**

**HUMAN MILK WHEY SEPARATION ON PROTEIN-PAK™ DEAE-8HR ANION EXCHANGE COLUMN**

**COLUMN:** AP1(10 mm x 100 mm) GLASS COLUMN

**BUFFER A:** 100 mM TRIS, pH 7.9;

**BUFFER B:** BUFFER A WITH 1M SODIUM CHLORIDE.

**FLOW RATE:** 0.5 ML/MINUTE. DETECTOR: 254 NM

**GRADIENT:** LOAD AT 100%A AND HOLD FOR 2 MINUTES, THEN TO 11%B OVER 14 MINUTES AND HOLD FOR 10 MINUTES; FOLLOWED BY 11%B TO 30%B OVER 15 MINUTES, THEN TO 100%B OVER 1 MINUTE.

**LOAD:** 75 µL OF HUMAN MILK WHEY (NEAT)

**PEAK IDENTIFICATION:** 1) LACTOFERRIN.
FIGURE 10:
PLASMID SEPARATION ON PROTEIN-PAK™ DEAE-8HR
ANION EXCHANGE COLUMN

Separation of 20.8mg of a partially purified prep containing the pRSVcat plasmid on the Waters Protein-Pak DEAE 8HR anion exchange chemistry in AP-1 hardware (1 x 10 cm). Buffer A: 25mM TRIS/Cl, pH 8.0 with 1mM EDTA. Buffer B: A + 1.0M NaCl. Gradient: Load at 40% B and hold for 40 minutes then to 60% B over 40 minutes, linear. Flow 1.5mL/min. Temperature: Ambient
Analysis of Void Fraction. A 60μL injection of void peak from Figure 1 was applied on the Waters Protein-Pak DEAE 8HR anion exchange chemistry in AP-1 hardware (1 x 10 cm). Buffer A: 25mM TRIS/Cl, pH 8.0 with 1mM EDTA. Buffer B: A + 1.0M NaCl. Gradient: Load at 40% B then to 60% B over 40 minutes, linear. Flow 1.5mL/min. Temperature: Ambient
Optimization of Sample Load

Column: Protein-Pak™ DEAE 8HR (1 x 10 cm)
Sample: C18 purified H241 IgG2a (@36mg) diluted 1:6 in Buffer A.
Total volume injected = 60 ml
Buffer A: 20 mM Tris/Cl, pH 8.5
Buffer B: 20 mM Tris/Cl + 0.3 M NaCl, pH 7.0
Flow Rate: 1.56 ml/min
Gradient: 100% A hold for 40 min then 0 - 100% B in 40 min

Graph: Peaks for Albumin, IgG, and Transferrin.
Optimization of Sample Load

Column: Protein-Pak™ DEAE 8HR (1 X 10 cm)
Sample: C18 purified H241 IgG2a (@9mg) diluted 1:6 in Buffer A.
Total volume injected=15ml
Buffer A: 20mM Tris/Cl, pH 8.5
Buffer B: 20mM Tris/Cl + 0.3M NaCl, pH 7.0
Flow Rate: 1.56 ml/min
Gradient: 100% A hold for 10 min then 0 - 100% B in 40 min

---

Graph showing peaks for Albumin, IgG, and Transferrin.
FIGURE 14
Large Scale Preparative Purification

Column: Protein-Pak™ DEAE 8HR (5 X 10 cm)
Sample: C18 purified H241 IgG2a (@450mg)
diluted 1:6 in Buffer A.
Total volume injected=750ml
Buffer A: 20mM Tris/Cl, pH 8.5
Buffer B: 20mM Tris/Cl + 0.3M NaCl, pH 7.0
Flow Rate: 39 ml/min
Gradient: 100% A hold for 25 min then 0 - 100% B in 40 min
Separation of Snake Venom Components

On Protein-Pak™ DEAE-8HR Anion Exchange Column

Column: AP1 (10 X 100 mm) Glass Column
Buffer A: 20mM TRIETHANOLAMINE, pH 7.5
Buffer B: BUFFER A + 0.35 M NaCl
Flow Rate: 4 mL/Min   Detector: 280nm
Gradient: 0-100% BUFFER B over 38 min
Gradient Delay: 7.5 minutes
Load: 100 µL (0.7 grams)
SEPARATION OF SNAKE VENOM COMPONENTS
ON MONO Q HR 5/5 ANION EXCHANGE COLUMN

COLUMN: 5 x 50 mm
BUFFER A: 20mM TRIETHANOLAMINE, pH 7.5
BUFFER B: BUFFER A + 0.35 M NaCl
FLOW RATE: 1 mL/MIN    DETECTOR: 280nm
GRADIENT: 0-100% BUFFER B over 20 min
LOAD: 100 µL (0.7 grams)
SEPARATION OF SNAKE VENOM COMPONENTS
ON PROTEIN-PAK™ DEAE-5PW ANION EXCHANGE COLUMN

COLUMN: 8 x 75 mm
BUFFER A: 20 mM TRIETHANOLAMINE, pH 7.5
BUFFER B: BUFFER A + 0.35 M NaCl
FLOW RATE: 2.5 mL/MIN   DETECTOR: 280 nm
GRADIENT: 0-100% BUFFER B over 58 min
GRADIENT DELAY: 6.0 minutes
LOAD: 100 µL (0.7 grams)
CONCLUSIONS

Ion exchange is the most widely used HPLC separation technique in protein purification. There is increasing demand for the isolation of large quantities of proteins with maximum recovery of product and activity.

Scalability is an important factor in any purification which requires the isolation of milligram to gram quantities of protein. It is, therefore, essential to employ a scale-up strategy which enables the prediction of the chromatographic performance in a large scale purification. 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 for load and linear velocity.