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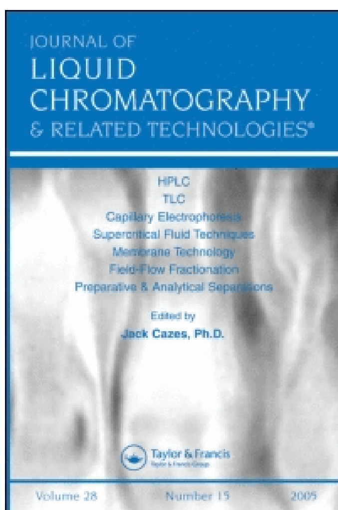

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M. Patricia Strickler[a]; M. Jude Gemski[a]
[a] Waters Chromatography Division, Millipore Corporation Life Science Application Laboratory, Rockville, Maryland

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PROTEIN PURIFICATION ON A NEW PREPARATIVE ION EXCHANGER

M. Patricia Strickler and M. Jude Gemski
Waters Chromatography Division
Millipore Corporation
Life Science Application Laboratory
Rockville, Maryland 20852

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ABSTRACT

The ACCELL chromatographic media is a new packing designed specifically for the isolation and purification of proteins. The anionic and cationic functional groups are bonded to an encapsulated 40μ silica particle which can be readily packed into any size column. These columns can be operated on both high performance and medium performance liquid chromatographic equipment. The optimization of the separation conditions on the anion exchange media for the preparative purification of a monoclonal antibody (Mab) from ascites will be discussed as well as a multi-step purification of the enzyme prostatic acid phosphatase (PAP) from seminal fluids on the anion and cation exchangers.

INTRODUCTION

The isolation and recovery of proteins from ion exchange chromatographic media is affected by many parameters such as the pH

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and nature of the buffer; the ionic strength and nature of the counter ion, the length of the gradient, flow rate and temperature at which the separation is carried out. Methods development to choose the best conditions for purification involves investigating some or all of these parameters. However, evaluation of chromatographic conditions on soft gel ion exchangers is time consuming because of the low flow rates at which they are run. These low flow rates often make thorough methods development impractical. The introduction of a new generation of rigid ion exchangers makes methods development practical for protein separations. The ACCELL chromatographic media permits the optimal separation conditions of a protein to be determined with the same ease and convenience available for small molecule separations.

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The ACCELL chromatographic media is a rigid 40µ particle which can be easily packed into both small analytical and large preparative columns. Many different buffers and gradients can be easily evaluated with 30 to 45 minute gradients at flow rates of 1-2ml/min. Also, only microgram amounts of protein samples or standards need be used to develop the optimal separation. Once the best method is chosen the ACCELL ion exchanger can be packed into preparative columns. Both the analytical and preparative columns can be operated at much higher flow rates than soft gels reducing significantly the overall separation time. These columns can be operated on both medium performance (MPLC) and high performance liquid chromatographs (HPLC).

The methods development and scale up of a single step purification of a monoclonal antibody (Mab) from ascites and the multi-step purification of an enzyme, prostatic acid phosphatase (PAP), on the ACCELL media ion exchangers will be discussed.

MATERIALS AND METHODS

Reagents

The murine ascites, E2-2C9-5, was obtained from Dr. B. P. Doctor, Division of Biochemistry, Walter Reed Army Institute of Research. The ascites fluid was diluted 1/20 with initial buffer and filtered using a 0.45μ Millex-HV filter (Millipore Corp., Bedford, MA). Immunological recovery was determined by a solid-phase RIA (1). The seminal plasma was donated by Dr. S. Leto, Washington Fertility Clinic, Washington, D.C. The substrate for testing phosphatase activity was obtained from Sigma Chemical Co., St. Louis, MO. The water was MilliQ grade (Millipore Corp., Bedford, MA) and the buffers and salts were of the highest analytical grade available.

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Instrumentation

A liquid chromatograph consisting of two M510 solvent delivery systems, a U6K injector or 710B autosampler, a M481 variable wavelength detector, and a 840 data and chromatography station were used to do the gradient separations. The gel filtration separations utilized an isocratic system consisting of a M510 solvent delivery system, a 710B autosampler, a M441 detector and a 840 data and

chromatography station (Waters Chromatography Division, Millipore Corp., Milford, MA). Enzymatic activity was determined using a Spectronic 20 (Bausch & Lomb, Inc., Rochester, NY).

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Anion Exchange Separation of Monoclonal Antibodies

A 7.5mm x 7.5cm stainless steel column was dry packed with ACCELL-QMA (Waters). After packing the column was pumped at 5ml/min with water for 15 minutes. The column was re-opened and topped off with additional packing and re-run in water for 5 minutes. A glass column (25mm x 25cm) used for the preparative separation was packed using a slurry of the media in the initial buffer. Buffers were filtered before use through a 0.45μ filter. For methods development 20mM tris and sodium phosphate buffers in the pH range 8.5 to 7.0 (tris) and pH 7.8 to 7.0 (phosphate) were used with 30 minute linear
sodium chloride gradients to 0.3M NaCl (tris) and 0.4M NaCl (phosphate) at a flow rate of 1ml/min. The optimized analytical conditions for the Mab separation were 20mM sodium phosphate, pH 7.0 to 20mM sodium phosphate, pH 7.0 with 0.4M NaCl, 45 minute linear gradient at 0.5ml/min. The preparative separation used the same buffers with an 165 minute linear gradient at 2ml/min. Columns were monitored at 280nm at room temperature.

Anion Exchange Separation of Prostatic Acid Phosphatase

The same columns used for the Mab separations were employed for the PAP separation. Tris buffers in the pH range of 8.0 to 7.0 and

phosphate buffers in the pH range of 7.8 to 7.0 with 30 minute linear NaCl gradients to 0.5M NaCl at flow rates of 1ml/min were used for methods development. The optimized analytical conditions were 20mM sodium phosphate, pH 7.0 to 20mM phosphate, pH 7.0 with 0.5M NaCl, 45 minute linear gradient at 0.5ml/min. The preparative conditions employed the 165 minute linear gradient at 2ml/min. Columns were monitored at 280nm at room temperature.

Cation Exchange Separation of Prostatic Acid Phosphatase

A 7.5mm x 7.5cm stainless steel column was dry packed with the ACCELL-CM (Waters). Sodium acetate, pH 4.0 to sodium acetate, pH 4.0 with 1M NaCl were the buffers. An initial 10 minute hold at the starting buffer was followed by a 15 minute linear gradient at 1.0ml/min. Final conditions were held for 20 minutes at 1ml/min. The column was monitored at 280nm at room temperature.

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Sample Preparation for the Prostatic Acid Phosphatase Separations

The seminal fluid was diluted 1:2 with the sodium phosphate, pH 7.0 buffer and filtered through a 0.45μ HA membrane with an AP prefilter (Millipore). Aliquots of 5ml from the pooled active fractions from the QMA separation were diluted 1:4 with 20mM sodium acetate, pH 4.0 and brought to a pH of 4.0 with glacial acetic acid. Samples were applied to the column through the pump.

Determination of Enzymatic Activity and Protein Concentration

Prostatic acid phosphatase activity was determined using the method of Bergmeyer (2) and the protein concentration was determined using the Lowry method (3).

Gel Filtration HPLC

Gel filtration of fractions from the ion exchange separations was carried out on one PROTEIN PAK 300SW (7.8mm x 30cm) column (Waters) using a 0.1M sodium phosphate, pH 6.8 at a flow rate of 1ml/min. The column was monitored at 214nm.

Anion Exchange Analysis of Fractions from Mab Separation

Anion exchange analysis of Mab separation fractions was carried out on a PROTEIN PAK DEAE-5PW (7.5mm x 7.5cm) column (Waters) using a 30 minute linear gradient of 20mM tris, pH 8.5 to 20mM tris, pH 7.0 with 0.3M NaCl at a flow rate of 1ml/min. The column was monitored at 280nm.

RESULTS AND DISCUSSION

Physical Properties of ACCELL Chromatographic Media

The ACCELL ion exchange media is a 40μ silica particle with a 500A° pore. The wide pore permits the efficient separation and recovery of proteins. The silica is encapsulated with diol and an acrylamide polymer. The functional groups, quartenary methylamine

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(QMA) and carboxymethyl (CM) are bonded to the acrylamide layer. The encapsulation protects the silica from dissolving at high pH. The ACCELL exchangers can be run routinely in the pH range of 2-9. A variety of wash buffers compatible with the ACCELL media are listed in Table 1. The media can be dry or slurry packed in small columns and slurry packed into preparative columns.

Methods Development of a Single Step Monoclonal Antibody Separation

The hybidoma E2-2C9 grown in murine ascites had previously been purified to homogeneity in a single step on a microparticulate anion exchange HPLC column (4). Using this 10μ material it was possible to remove not only transferrin and albumin but mouse IgG as well. A method to purify this IgG_1 Mab to the same degree of homogeneity was developed on the new media. Microgram amounts of ascites were injected onto an analytical column packed with the ACCELL QMA and a variety of different separation conditions were explored. Thirty minute linear gradients at 1ml/min were used to screen a variety of buffer salts and pH. Phosphate buffers were found to give better resolution and recovery of the Mab from transferrin and albumin than tris buffers. Phosphate buffer at pH 7.8 did not resolve transferrin from Mab while phosphate buffer from pH 7.6 to 7.0 removed transferrin and albumin from the Mab. Mouse IgG present in the ascites could only be resolved from the Mab at pH 7.0. In determining the optimum buffer pH a continuous linear ionic strength gradient of increasing amounts of sodium chloride up to 0.4M NaCl was generated on the HPLC. Better resolution of transferrin, mouse

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Table 1

Chemical Compatibility of Wash Buffers and Anti-Microbials with ACCELL Media

10% acetic acid
2.5M sodium acetate
20% aqueous propanol
0.1% trifluoroacetic acid
5.0M urea
0.5% Hibitane (Chlorhexidine)
1.0% Triton X-100
1.0% Brij - 35
0.1M sodium hydroxide*
0.05% sodium azide

* Limited volumes only, use only if other washing buffers do not clean sufficiently

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IgG and albumin from the Mab was achieved by extending the duration of the gradient to 45 minutes at a flow rate of 0.5ml/min. The optimized separation is shown in Figure 1. The increase in the duration of the gradient at a slower flow rate not only improves resolution but increases the amount of ascites that can be fractionated at one time. Maximum loading is determined

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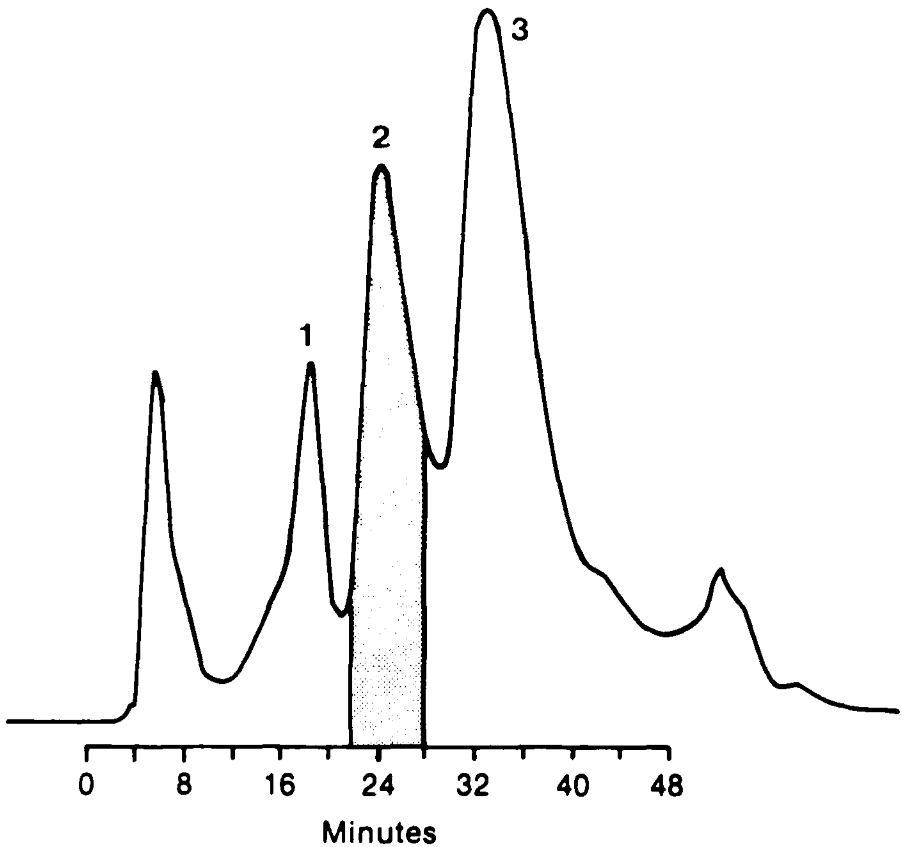

Figure 1: Separation of 0.025ml of ascites, diluted 1/20, on ACCELL QMA packed in a 7.5mm x 7.5cm column using a 45 minute linear gradient of 20mM sodium phosphate, pH 7.0 to 20mM sodium phosphate, pH 7.0 with 0.4M NaCl at a flow rate of 0.5ml/min. The column was monitored at 280nm, 0.2AUFS at room temperature. (1) transferrin, (2) IgG_1 Mab, (3) albumin.

experimentally by applying increasing amounts of ascites to the column until resolution of the Mab from the contaminating proteins is lost. Because a column will appear overloaded due to overloading of the ultraviolet detector, fractions are collected from these loading runs and analyzed by gel filtration HPLC. This technique will show the actual resolution achieved at various sample loading levels because fractions containing homogeneous Mab will be identified. The amount of homogeneous Mab recovered at various levels of sample loading can also be determined by quantitating the peak area or height of Mab from the gel filtration data. For example, a 0.3ml sample of ascites (10mg protein) allows recovery of greater than 80% of the Mab homogeneous. If only 60% of homogeneous Mab is required 0.5ml can be loaded. The amount of purified protein and the criteria of purity should be decided on prior to the loading study.

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Preparative Separation of Mab

The optimized conditions established on the analytical column are transferred to the preparative column. The sample amount applied to the preparative column can be calculated using the following equation:

$$\text{prep sample amt.} = \text{anal sample amt.} \times \frac{(D_2)^2 \times L_2}{(D_1)^2 \times L_1}$$

where D_2 = diameter of prep column

L_2 = length of prep column

D_1 = diameter of anal column

L_1 = length of anal column

Therefore, 12ml of ascites (30mg/ml) or 360mg of protein can be applied to a 25mm x 25cm column packed with ACCELL QMA.

The flow rate at which the preparative column should be run for direct scale up is calculated to be 5.5ml/min as follows:

$$\text{Flow rate prep} = \text{flow rate anal} \times \frac{(D_2)^2}{(D_1)^2}$$

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Since the analytical columns and preparative columns are not of equal length the duration of the gradient must be calculated so that equivalent column volumes of buffer will be applied to the preparative column.

gradient duration prep =

$$\frac{(D_2)^2 \times L_2 \times \text{gradient duration}_{\text{anal}} \times \text{flow rate}_{\text{anal}}}{(D_1)^2 \times L_1 \times \text{flow rate prep}}$$

The direct scale up of analytical conditions to the preparative column would employ a 150 minute gradient.

The maximum sample amount applied to this preparative column, however, can be increased significantly by increasing the length of the gradient and reducing the flow rate. Increasing the duration of the gradient to 165 minutes and lowering the flow rate to 2ml/min doubles the column capacity as evidenced by the resolution of Mab from transferrin and albumin shown in Figure 2. This 2ml sample is 80 times the sample amount (0.025ml) shown in Figure 1. If the gradient duration were not increased from 150 to 165 minutes and the flow rate lowered from 5.5 to 2ml/min only 1ml of ascites (40 times the sample amount) could be loaded on the column while maintaining the equivalent resolution of the separation in Figure 1.

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Gel filtration HPLC and SDS gel analyses of these fractions indicate that the Mab is recovered with a high degree of homogeneity. The removal of mouse IgG from the Mab is determined by anion exchange analysis of these fractions on a microparticulate ion exchanger as shown in Figure 3. In this separation mouse IgG elutes at 16 minutes while the IgG_1 Mab elutes at 22 minutes. Greater than 95% of the immunological activity was recovered as determined by solid phase radioimmunoassay (1).

Methods Development of the Purification of Prostatic Acid Phosphatase

Prostatic acid phosphatase (PAP) is an enzyme, molecular weight of 102,000, which is 5% of the soluble protein in seminal fluid. This glycoprotein is most often purified by a combination of anion

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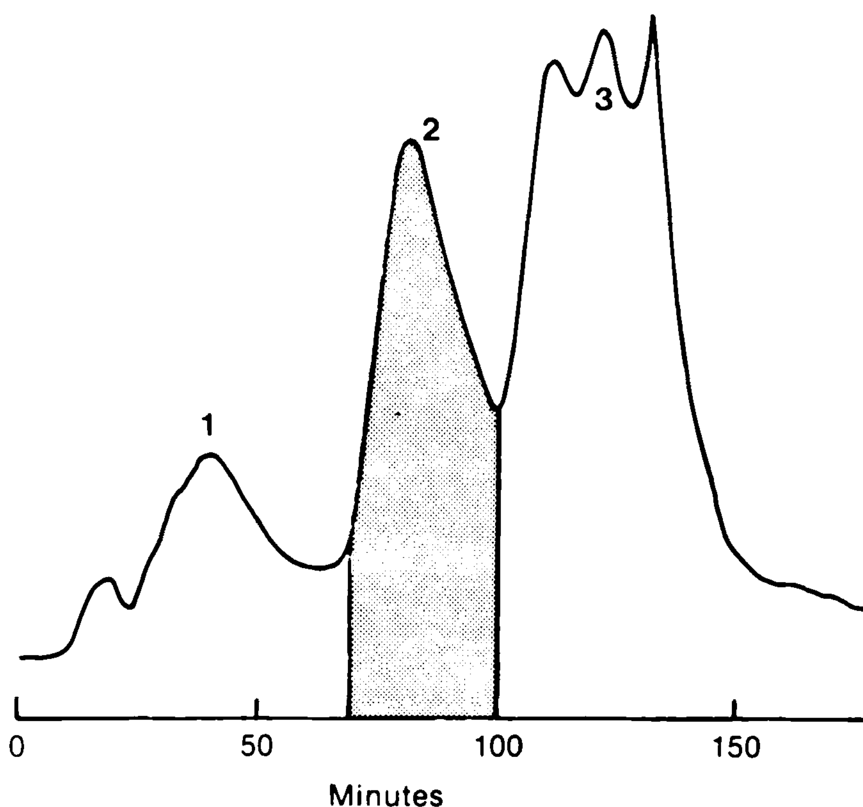

Figure 2: Separation of 2ml of ascites, diluted 1/20, on ACCELL QMA packed in a 25mm x 25cm column using a 165 minute linear gradient of 20mM sodium phosphate, pH 7.0 to 20mM sodium phosphate, pH 7.0 with 0.4M NaCl at a flow rate of 2.0ml/min at room temperature. The column was monitored at 280nm, 0.2AUFS. (1) transferrin, (2) IgG_1 Mab, (3) albumin.

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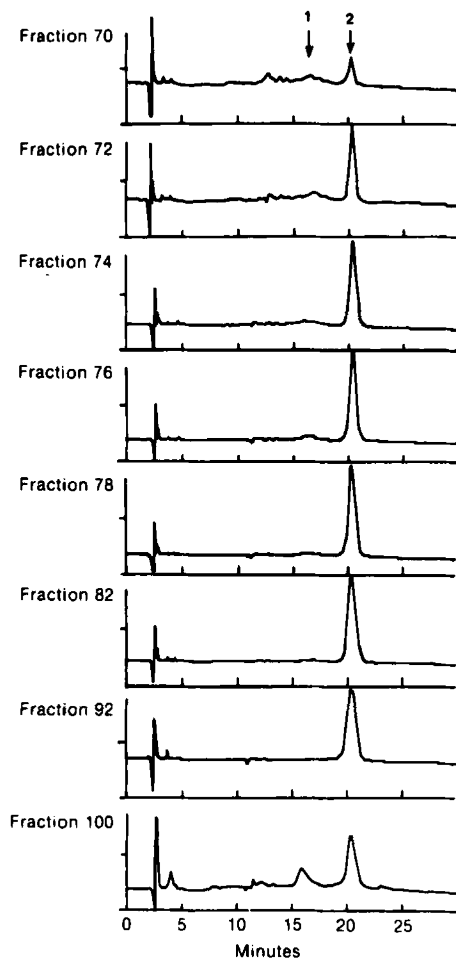

Figure 3: Ion exchange analyses of fractions from the preparative ACCELL QMA separation of ascites.

Chromatography was carried out on a PROTEIN PAK DEAE-5PW (7.5mm x 7.5cm) column using the conditions described in Materials and Methods. 0.01ml of the fractions containing Mab were injected. The column was monitored at 280nm, 0.08AUFS. Polyclonal mouse IgG elutes at position (1) and Mab at (2).

exchange and cation exchange chromatography on soft gels (5). A method of separation of this enzyme was developed on the ACCELL chromatographic media starting with a tris, pH 8.0 linear salt gradient which had been employed on a DEAE cellulose column (5). Aliquots of 0.025 ml were applied to the column and the fractions examined for enzymatic activity. The enzymatic activity was found in 10 fractions resolved from the major concentrations of protein. Tris in a pH range of 8.5 to 7.0 did not appreciably alter the resolution. Phosphate buffers in the pH range of 7.8 to 7.0, however, eluted the enzymatic activity in less fractions, therefore, with greater efficiency than elution with the tris buffer. Phosphate buffer, pH 7.0 eluted the enzyme in five fractions.

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Preparative Separation of Prostatic Acid Phosphatase

The optimized conditions were applied to the preparative column with a gradient duration of 165 minutes at a flow rate of 2ml/min. so that 20ml or 80 times the amount of sample could be applied to the column as shown in Figure 4. All fractions were assayed for enzymatic activity. The peak of enzymatic activity eluting at the beginning of the run was lost when the fractions were re-tested 60 minutes later. Because the presence of enzymatic activity is no indication of the composition the active fractions were also screened by gel filtration HPLC. Only active fractions without major contamination and with detectable protein (fractions 92-132) at a retention time corresponding to 100,000 molecular weight were pooled for separation on the ACCELL CM column.

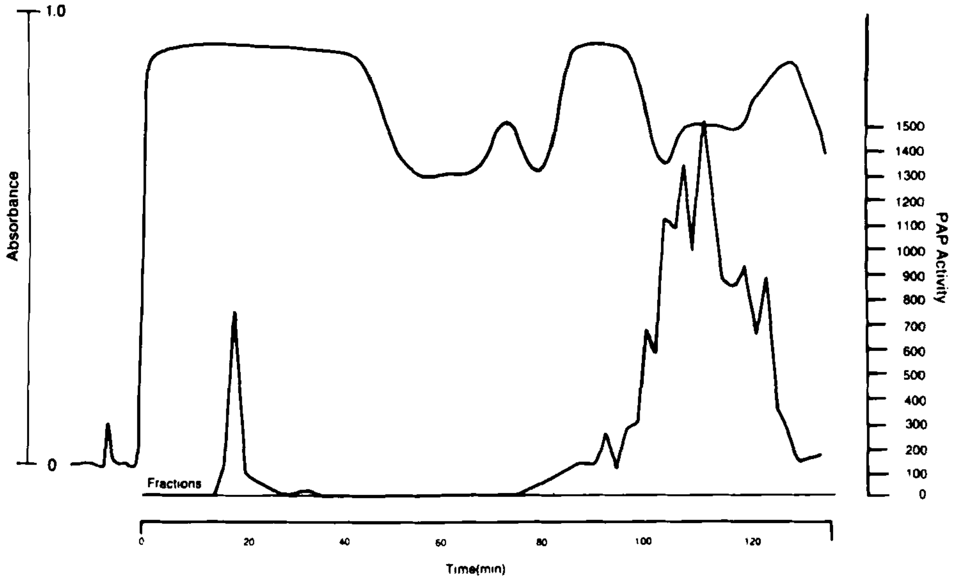

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Figure 4: Preparative separation of 20ml of seminal fluid, diluted 1:2, on a 25mm x 25cm column packed with ACCELL QMA using a 165 minute linear gradient of 20mM sodium phosphate, pH 7.0 to 20mM sodium phosphate, pH 7.0 with 0.5M NaCl at a flow rate of 2ml/min. The column was monitored at 280nm, 1.0AUFS at room temperature. All fractions were tested for prostatic acid phosphatase activity and the activity of each fraction is superimposed on the uv trace.

Following a 10 minute hold at initial conditions a 15 minute linear gradient is initiated at a flow rate of 1ml/min. The column was monitored at 280nm, 0.5AUFS. Nucleic acid (1) and PAP (2).

The major contaminant in the pooled fractions from the ACCELL QMA separation is nucleic acid which is easily removed by cation exchange chromatography. A sodium acetate buffer at pH 4.0 with a linear salt gradient gave excellent resolution of PAP from contaminating proteins and nucleic acids as shown in Figure 5. An initial hold for 10 minutes was used so that large volumes (5ml of pooled active fractions diluted 1:4) could be applied to the analytical column. As with all the preceding separations, samples were applied to the column without dialysis into the initial buffer. Samples are diluted to sufficiently lower the salt concentration so that the proteins would bind to the ion exchanger. Since the ACCELL media is a rigid particle large sample volumes can be pumped onto the column at high flow rates. This method of sample preparation and application is very reproducible and eliminates a dialysis step. In the case of applying the pooled active fractions from the QMA separation the pH also had to be adjusted from pH 7 to 4, otherwise the proteins would not bind to the column. The chromatograms of twelve 5ml aliquots of pooled material are superimposable. A summary of the recovery of enzymatic activity from this multi-step isolation is shown in Table 2. An overall 48% recovery of enzymatic activity is consistent with that achieved by open column procedures, however, the specific activity of 1020 units/mg of protein is significantly greater (5).

Gel filtration HPLC and SDS gel analyses of the fractions from the CM separation showed the material to be homogeneous. Analysis of

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Table 2

Recovery of Prostatic Acid Phosphatase Activity

	Total Units Activity	% Recovery	Total Protein (mg)	Units Activity / mg Protein
Seminal Plasma Diluted 1:2 with 20mM $NaPO_4$ pH 7.0, and Filtered	24,080		880	27.4
Accell™ QMA Separation Pooled Active Fractions	19,565	81%	91	215
5ml Aliquot of Active Fraction Diluted 1:4 with 20mM NaOAc, pH 4.0 and Final pH Adjusted to pH 4.0 with Acetic Acid	1,540		7.1	218
Accell CM Separation Pooled Active Fractions	918	60%	0.9	1020
Overall Recovery		48%		
Purification Factor		37X		

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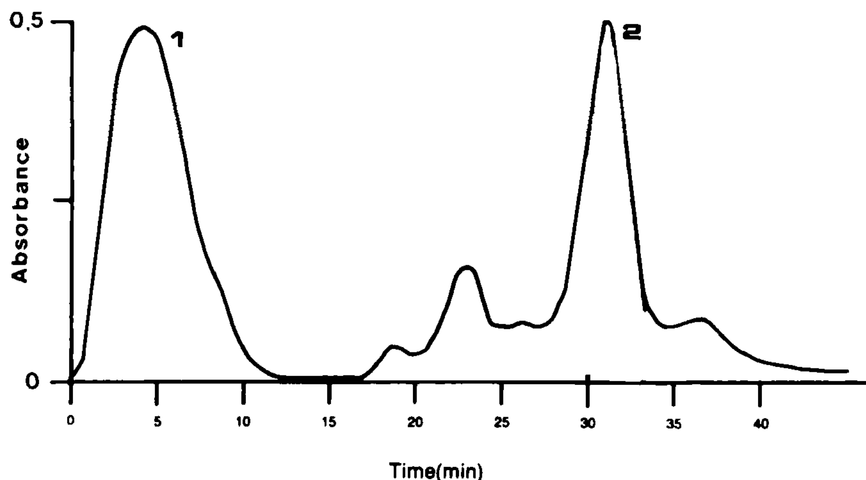

Figure 5: Cation exchange separation of 5ml, diluted 1:4; of the pooled active fractions from the anion exchange fractionation of seminal fluid, Figure 4.

Chromatography was carried out on a 7.5mm x 7.5cm column packed with ACCELL CM using 20mM sodium acetate, pH 4.0 to 20mM sodium acetate, pH 4.0 with 1.0M NaCl.

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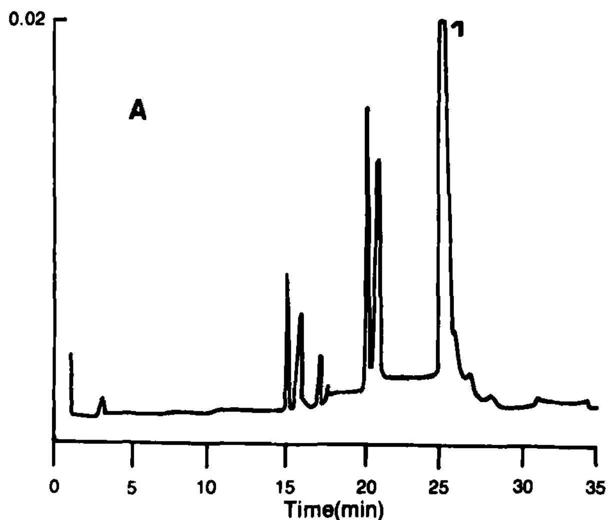
this material on reverse phase HPLC, however, is more definitive. A method previously reported in this journal shows that the enzyme, PAP, can be isolated directly from seminal fluid on a reverse phase HPLC column (6). The fractions from the ACCELL media were examined using this method. Figure 6 shows the pooled active fractions from the cation exchange separation (Panel B) compared to PAP isolated from soft gel exchangers (Panel A). This figure clearly shows that the increased specific activity is indeed due to the recovery of a more homogeneous protein.



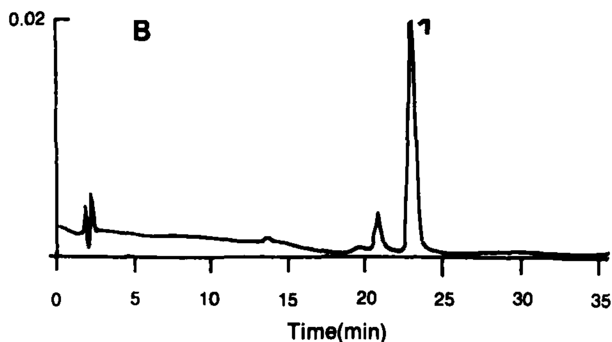


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Figure 6: Reversed phase HPLC separation of PAP (1) purified on soft gel ion exchange media, Panel A, and on ACCELL chromatographic media, Panel B.

Chromatography was carried out on a μBondapak C_{18} (3.9mm x 30cm) column using a 30 minute linear gradient of 12%B / 88%A to 70%B / 30%A at a flow rate of 1.5ml/min. Buffer A is 100% 0.1% aqueous trifluoroacetic and buffer B is acetonitrile with 0.1% trifluoroacetic acid. The column was monitored at 280nm, 0.2AUFS.

SUMMARY

The use of the ACCELL chromatographic media in the purification of both the monoclonal antibody and prostatic acid phosphatase improved and accelerated the entire separation procedure. The ACCELL media, because it is based on a rigid particle, was packed into analytical size columns and methods were developed using microgram amounts of sample. The maximum sample loading was determined by a loading study on the analytical column using only small amounts of sample. The preparative separation was then carried out using the same packing in a larger column. The rigidity of the particle allows the total separation time to be greatly reduced compared to separations performed on soft gels because the ACCELL media can be operated at higher flow rates. Dialysis into the initial buffer is unnecessary because samples can be diluted with sufficient initial buffer to lower the salt concentration and the pH adjusted if required. Large sample volumes can be easily applied to the column at flow rates that a soft gel would not tolerate. In conclusion, the ACCELL chromatographic media gives protein isolation methods development and scale-up separations the speed and convenience of small molecule separations.

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