NOTES
PROTEIN PURIFICATION

94-0407

A FREE TECHNICAL PUBLICATION ISSUED TO PROTEIN CHEMISTS THE WORLD OVER VOL. 1, NO. 1

Announcing! A Brand New Technical Publication for the Protein Chemist.

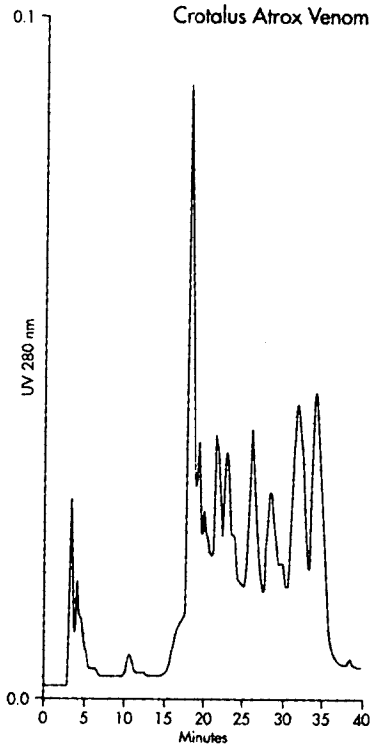
Protein purification is of great importance in the study of biological processes and in the production of pharmaceuticals by recombinant techniques. The researcher's aim is to obtain a protein with high specific activity as quickly and as easily as possible. Increasingly, protein chemists are turning to FPLC® to purify proteins for several important reasons.

High Resolution. High performance ion exchange, affinity, hydrophobic interaction and gel filtration chromatography materials are available in bulk and as prepacked columns and offer excellent recovery of biomolecules with high specific activity.

Speed. The short separation times and the ability to switch quickly between various separation techniques used in biochemistry, saves you time and ensures the maintenance of biological activity.

Biocompatibility. Columns and instrumentation are made of totally-inert materials and are gentle to proteins ensuring you high recoveries, minimal maintenance and long column lifetimes.

Fast Protein LC for High Resolution Fractionation of Complex Biological Mixtures



Sample: 100 µl of Crotalus atrox (snake) venom
Column: Protein-Pak DEAE 8HR 10 x 100 mm
Method: Auto•Blend
Initial Conditions: 20 mM Tris buffer pH 8.1
Final Conditions: 20 mM Tris buffer pH 8.1 with 0.4 M NaCl
Gradient: Linear, 30 min
Flow Rate: 1.2 ml/min

The fractionation of the components contained in Crotalus atrox (snake) venom demonstrates the ability of fast-protein LC to provide the high resolution necessary for fractionation of complex samples. Protein Purification Notes reports on novel purification methods which have proven to increase the utility of FPLC.

Protein Purification Notes (PPN) conveys information on methods and instrumentation for purifying biomolecules. PPN carries technical articles on new advances in protein isolation techniques, with an emphasis on improving speed, resolution, and the recovery of biologically-active species.

To Continue Receiving *Protein Purification Notes* – Act Now!

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To get a free copy, check the appropriate box on the attached reply card and mail today!

FPLC is a registered trademark of Pharmacia Fine Chemicals

High Performance Glass Columns and Economical Bulk Packings.

The 8 µm and 15 µm Protein-Pak HR packings are supplied in prepacked Advanced Purification (AP) glass columns. AP glass columns are designed for use with both small and large scale purification protocols. These metal-free columns are composed of borosilicate glass and have high pressure ratings enabling you to use faster flow rates for rapid separations. All prepacked columns are 100 mm in 10 mm, 20 mm or 50 mm. Protein-Pak HR 15 µm and 40 µm packings are also available in bulk quantities. Waters offers a wide range of empty AP Glass Columns making it possible to pack a bed volume that is optimized for a specific application. This choice of column volumes provides greater flexibility in methods optimization and process scaling.

To learn more about the Protein-Pak HR product offering check the appropriate box on the reply card.

*Empty AP Glass Columns are also available in 200 mm, 300 mm and 600 mm lengths.

Approximate Protein Binding Capacity Per Prepacked Column*.

Prepacked Column	8HR DEAE**	8HR SP**	15HR DEAE	15HR SP	40HR DEAE	40HR SP
10 mm x 100 mm	300 mg	300 mg	300 mg	300 mg	N/A	N/A
20 mm x 100 mm	1250 mg	1250 mg	1250 mg	1250 mg	N/A	N/A
50 mm x 100 mm	7800 mg	7800 mg	7800 mg	7800 mg	N/A	N/A

Protein Binding Capacity of Bulk Packings (mg/ml):

Bulk Packings	8HR DEAE**	8HR SP**	15HR DEAE	15HR SP	40HR DEAE	40HR SP
	N/A	N/A	40 mg/ml	40 mg/ml	40 mg/ml	20 mg/ml

* For best results, use only 5-20% of the column's protein binding capacity.

** Bovine serum albumin in 20 mM Tris/Cl pH 8.2 was used to measure protein binding capacity of Protein-Pak DEAE HR series. Cytochrome C in 25 mM MES pH 5.0 was used to measure protein binding capacity of Protein-Pak SP HR series.

Ordering Information.

Column	Column Dimension	Particle Size	Part Number	Price
Protein-Pak DEAE 8HR	10 mm x 100 mm	8 µm	35650	$ 695
Protein-Pak DEAE 8HR	20 mm x 100 mm	8 µm	38560	2,980
Protein-Pak DEAE 8HR	50 mm x 100 mm	8 µm	38561	14,040
Protein-Pak DEAE 15HR	10 mm x 100 mm	15 µm	38564	550
Protein-Pak DEAE 15HR	20 mm x 100 mm	15 µm	38565	1,840
Protein-Pak DEAE 15HR	50 mm x 100 mm	15 µm	38566	6,300
Protein-Pak SP 8HR	10 mm x 100 mm	8 µm	35655	695
Protein-Pak SP 8HR	20 mm x 100 mm	8 µm	38562	2,980
Protein-Pak SP 8HR	50 mm x 100 mm	8 µm	38563	14,040
Protein-Pak SP 15HR	10 mm x 100 mm	15 µm	38567	550
Protein-Pak SP 15HR	20 mm x 100 mm	15 µm	38568	1,840
Protein-Pak SP 15HR	50 mm x 100 mm	15 µm	38569	6,300
Bulk Ion Exchange Packings:				
Protein-Pak DEAE 15HR	50 grams	15 µm	35688	$ 650
	500 grams	15 µm	35689	5,500
Protein-Pak DEAE 40HR	50 grams	40 µm	35691	125
	500 grams	40 µm	35692	1,250
Protein-Pak SP 15HR	50 grams	15 µm	35694	650
	500 grams	15 µm	35695	5,500
Protein-Pak SP 40HR	50 grams	40 µm	35697	125
	500 grams	40 µm	35698	1,250

Your comments and suggestions are welcome. Address them to:

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34 Maple Street
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(508) 478-2000 Ext. 2750

Waters
Division of MILLIPORE

New Protein Purification Videotape Available.

Shows How to Optimize an Ion Exchange Protein Purification of a Liver Homogenate.

Protein chemists can now receive free, a 15-minute videotape on fast protein LC. *Optimizing Protein Purifications* shows how to fractionate a crude protein mixture and recover a highly active protein. The videotape includes instructional segments on the principles of ion exchange for protein purification and discusses how to systematically change buffer pH and gradient slope using the Auto•Blend Method to improve resolution and increase the specific activity of purified proteins.

The videotape is notable for its step-by-step approach explaining the protein purification process for a liver homogenate from crude fractionation to high-resolution purification. A limited number of videotapes are available for distribution. To get your free copy, check the appropriate box on the reply card and mail today.

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Gammabind-G is a trademark of Genex Corporation. FPLC is a registered trademark of Pharmacia Fine Chemicals.

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Ion Exchange Packings for Protein Purification at Any Scale.

Ion exchange chromatography is the most frequently used method for the separation of biological molecules. Since most biomolecules (e.g. proteins, polysaccharides, and nucleic acids) consist of charged molecules, ion exchange chromatography is useful for recovering, and retaining the biological activity of, large amounts of proteins, enzymes, immunoglobulins and nucleic acids.

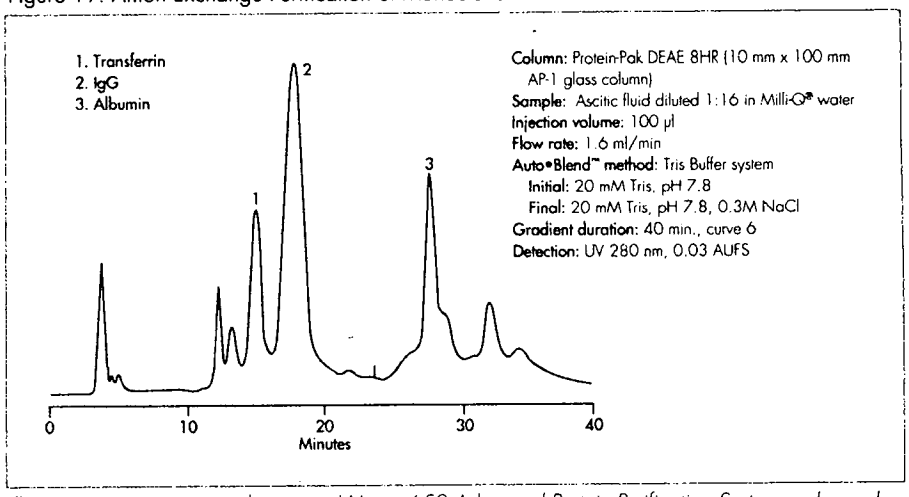
New Waters Protein-Pak HR Ion Exchangers.

Waters Protein-Pak HR chromatography packings feature a macroporous (1000Å) polymeric base material with a high capacity for biomolecular binding and a rigid structure for high linear velocities. The hydrophilic nature of the resin ensures minimal nonspecific adsorption. Protein-Pak HR ion exchangers are compatible with aqueous acids, bases and buffers in the pH range of 2 to 12. Protein-Pak HR ion exchangers are available with DEAE, a weak anion exchanger, or SP, a strong cation exchanger.

Three Particle Sizes Offer a Wide Range of Resolution.

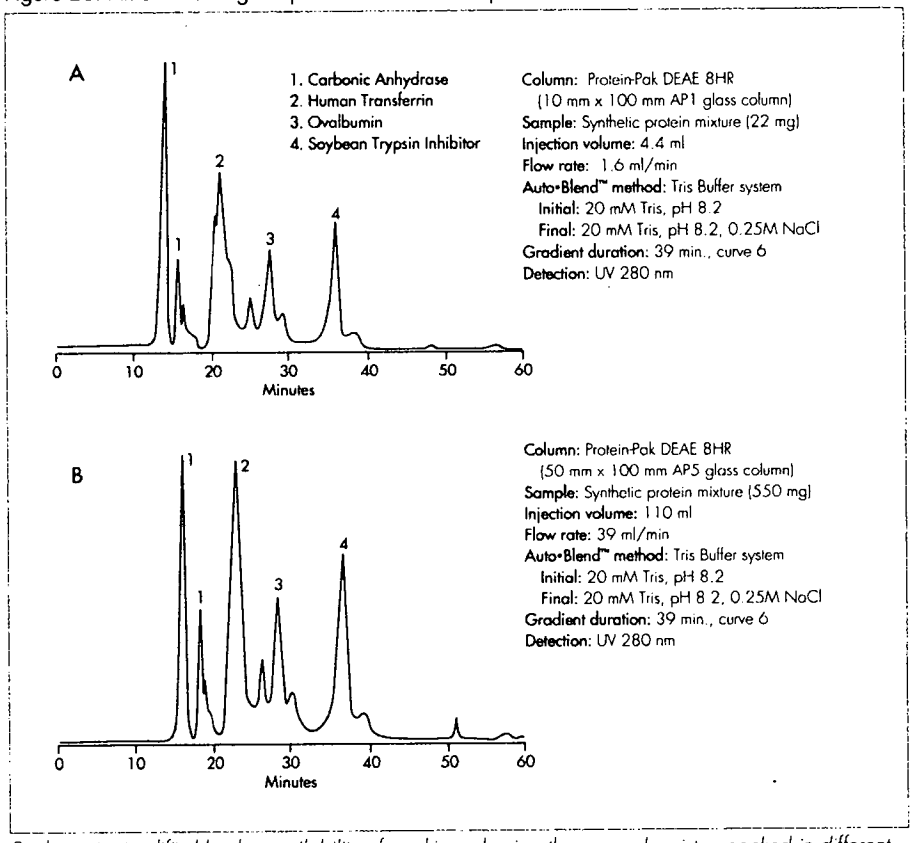
Three different particle sizes (8 µm, 15 µm and 40 µm) offer the range of resolution necessary for analysis and purification of biomolecules. Unlike other ion exchangers for protein purification, Protein-Pak HR packings are synthesized with the same chemistry on all particle sizes. The buffer system used on the 8 µm (8HR) packing can be used on the 15 µm (15HR) and 40 µm (40HR) packing yielding identical separation selectivity. Predictability of results makes scale up of isolation procedures easy and convenient.

Figure 19: Anion Exchange Purification of Monoclonal Antibodies.



This separation was carried out on a Waters 650 Advanced Protein Purification System and a scalable Protein-Pak DEAE 8HR glass column (10 mm x 100 mm). Within 40 minutes, monoclonal IgG was purified from other proteins typically found in ascitic fluid. Fractions were collected and peaks were assigned based cn relative migration to authentic standards by SDS PAGE.

Figure 20: Anion Exchange Separation and Scale Up of a Protein Mixture.



Scale up is simplified by the availability of packings, having the same chemistry, packed in different size columns. The chromatograms demonstrate the ease and reproducibility of the scale up process. An analytical separation, Figure 20A was first optimized for load, flow rate and gradient duration and represents a 22 mg load of a synthetic protein mixture on a 10 mm x 100 mm glass column packed with 8 µm Protein-Pak DEAE 8HR. Figure 20B illustrates a separation of a 25-fold increase in sample load (550 mg) on a 50 mm x 100 mm glass column packed with the same packing.

Binds to a Variety of Ligands of Your Choice.

Many ligands have been successfully bound to the Waters Protein-Pak Epoxy-Activated material and the resulting packings have been used to purify many different biomolecules. For example, recombinant proteins A and G have been used for the purification of human and mouse IgG. Also, N-acetyl-D-glucosamine was immobilized and used for the purification of wheat germ hemagglutinin.

Biospecific Purification of Microgram-to-Gram Quantities.

Waters Protein-Pak affinity packing is available both in microcolumns and as bulk packing material. Protein-Pak microcolumns are perfect for scouting coupling conditions or for use with small quantities of sample. Bulk affinity packings from Waters can be packed in any column size to meet purification needs at any scale. Also, preparative scale runs can be performed at rapid flow rates keeping run times significantly shorter than those for soft gels.

Fast Purification With High Recovery of Biological Activity.

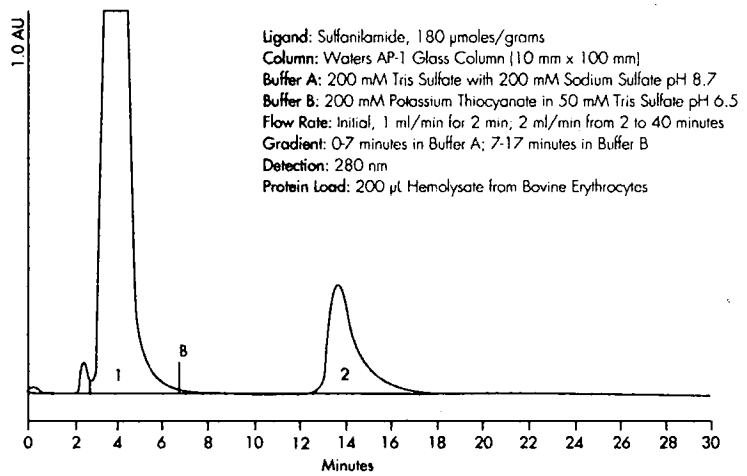
Carbonic anhydrase was purified using Protein-Pak Epoxy-Activated affinity material packed in a Waters AP glass column. This enzyme is an esterase found in erythrocytes that helps control the transport of molecular CO_2 through the bloodstream. Sulfanilamide, an inhibitor of carbonic anhydrase, was reacted with the epoxy-activated material at 45°C for 25 hours.

A bovine hemolysate (200 µl of 24 mg/ml solution) was applied to the column equilibrated with Tris sulfate at pH 8.7 (Figure 18.) After washing out the unretained protein (peak 1) the buffer was switched to Tris sulfate pH 6.5 containing potassium triocyan-ate to elute the carbonic anhydrase (peak 2). Esterase activity in peak 2 was measured by the hydrolysis of nitrophenyl-acetate to nitrophenol. Carbonic anhydrase was purified eight-fold with a 100% recovery of biological activity (Table 2) in less than 16 minutes.

Table 1: Protein-Pak™ Epoxy-Activated Affinity Applications.

Immobilized Ligands	Applications
Gammabind-G™ and Protein A	Human IgG Mouse IgG In Serum
Sulfanilamide	Carbonic Anhydrase
N-Acetyl-D-Glucosamine	Wheat Germ Lectin
Heparin	Lysozyme; Antithrombin III
Concanavalin A	Ribonuclease B, Horseradish Peroxidase
Cibacron Blue	Bovine Serum Albumin
Aminophenylboronic Acid	Uridine, Adenosine, Sorbital

Figure 18: Protein-Pak Epoxy-Activated Affinity Products: Carbonic Anhydrase Purification.



Fast purification of carbonic anhydrase resulted in an eight-fold increase in specific enzyme activity with 100% recovery of biological activity.

Table 2: Purification of Carbonic Anhydrase from Hemolysate

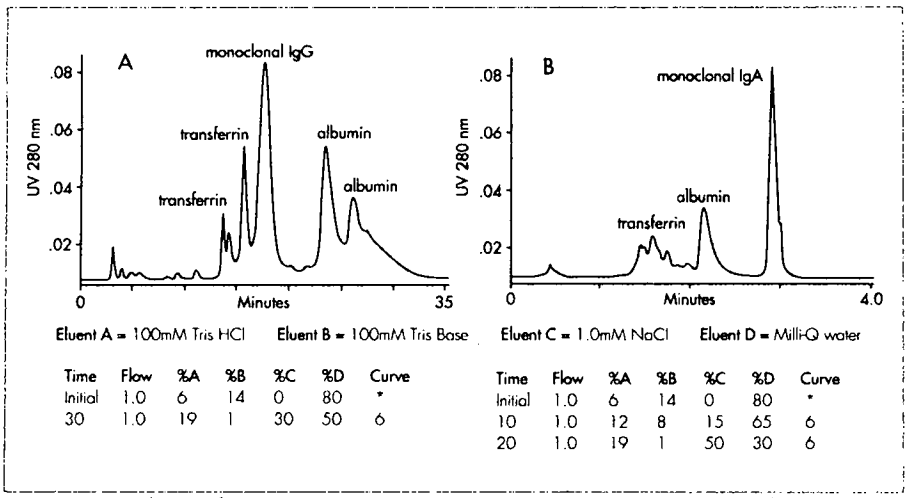
	Protein Concentration	Total Volume	Total Protein	Total Biological Activity	Specific Activity	Purification Factor	% Recovery of Biological Activity
Crude	23.8 mg/ml	1 ml	23.8 mg	8.75 units	0.37 U/mg	1	100%
Affinity-Purified Peak 2	.06 mg/ml	10 ml	0.59 mg	1.75 Units	2.96 U/mg	8	100%

The 200 µl of bovine hemolysate (24 mg protein/ml) applied to the column.
*Carbonic anhydrase activity was measured by converting nitrophenyl acetate to nitrophenol at pH 7.5.

Ordering Information

Waters Protein-Pak Epoxy-Activated Affinity Material	Part No.	Price
25 gram bottle (50 ml)	30653	$ 195
100 gram bottle (200 ml)	30654	550
Protein-Pak Affinity microcolumns (10/pkg)	35955	49

Figure 15: Purification of Monoclonal Antibodies.



Time	Flow	%A	%B	%C	%D	Curve
Initial	1.0	6	14	0	80	*
30	1.0	19	1	30	50	6

Time	Flow	%A	%B	%C	%D	Curve
Initial	1.0	6	14	0	80	*
10	1.0	12	8	15	65	6
20	1.0	19	1	50	30	6

A: Separation of 2 ml of mouse ascites containing monoclonal IgG using an optimized concurrent pH and ionic strength gradient profile on a Protein-Pak DEAE 5PW anion exchange column.

B: Auto•Blend optimized method for isolation of monoclonal IgA from mouse ascites.

Note: Peaks were collected and identified by SDS-PAGE under reducing conditions.

The Best System for Fast Protein LC.

The Waters 650 is the only system optimized for fast protein LC with built-in Auto•Blend capability. Use the 650 at any step of your protein purification protocol. Transfer your open column separations to an automated instrument and improve reproducibility as well as initial recovery and resolution. Perform final polishing using high resolution chemistry on the same instrument. Purify as much protein as you need by taking advantage of system and column capacity up to the multigram scale. Experience fast, error-free programming and editing of methods with instant, real time monitoring of system operating parameters and status.

References

1. Warren, W., et al., (1989) American Biotechnology Laboratory, 7(6), 34-40
2. Gemski, M.J., et.al.,(1985) BioTechniques 3(5), 378-384.

Rapid Biomolecule Purifications Using a New Epoxy-Activated Affinity Chemistry.

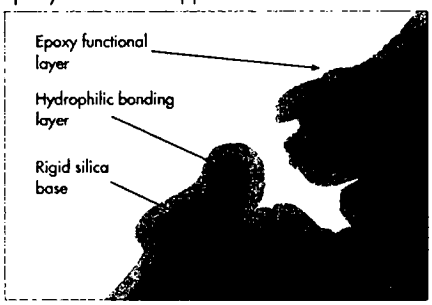
Affinity chromatography is a technique for purifying biomolecules based on bioselective interactions and typically results in high purification and recovery of biological activity. Throughput using affinity techniques however, has been limited due to the structure of conventional agarose-based affinity materials. These packings compress easily and can not be used at the high flow rates necessary for fast purification. Waters new Protein-Pak Epoxy-Activated Affinity packing overcomes this restriction through the use of a unique silica-based particle treated to produce a highly stable rigid hydrophilic surface which retains its structural integrity at high flow rates. Protein-Pak Epoxy-Activated packing therefore offers advantages not available in other affinity packings.

Particle Structure and Chemistry.

Waters Protein-Pak Epoxy-Activated Affinity packings have a rigid non-compressible 40 µm silica base with a 500Å pore size. The porous silica base gives the packing high available capacity for large biopolymers while the rigid structure allows high flow rates without the compression typically associated with conventional agarose based gels. A polymerization process encapsulates the silica base with a hydrophilic bonding layer. Active sites of the silica are blocked by the hydrophilic bonding layer to produce low non-specific binding properties (Figure 16).

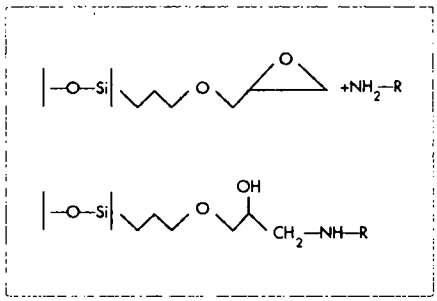
Waters Protein-Pak Epoxy-Activated affinity matrix contains a glycidoxypropyl moiety that results in a seven-atom spacer arm (Figure 17). The spacer arm allows the binding sites to be more accessible to the ligand which allows for high binding capacity of sample. The epoxy-activated groups covalently bond small and large ligands (Table 1, see next page) via amino, hydroxyl or sulfhydryl groups resulting in a stable linkage which is less likely to be susceptible to hydrolysis and ion-specific interactions.

Figure 16: Waters Protein-Pak™ Epoxy-Activated Support.



A polymerization process encapsulates all active sites of the rigid wide-pore silica base to produce low non-specific binding properties.

Figure 17: Chemistry Of Epoxy-Activated Products.



Assessing the Effect of Changes in Buffer pH on Ion Exchange Separations of Proteins.

Protein Separations Respond to Subtle pH Changes.

Complex biological samples typically contain many proteins with varying isoelectric points or surface charge properties. The pH of the buffer selected for the separation of these samples can dramatically affect both the recovery and resolution achieved. This is illustrated by examining the effect of pH on the protein mass and enzymatic activity profile for a crude mouse liver extract separated on a Waters Protein-Pak DEAE 8HR anion exchange column (Fig 14). By systematically varying the pH from 7.2 to 8.6, significant changes in the resolution of the total protein are seen, and various isozymes of lactate dehydrogenase are separated. There is a particularly large difference in the retention of the major activity peak (LDH-A_4) relative to the bulk of the protein between pH 8.4 and pH 8.6.

Maximize Recovery and Specific Activity.

Waters exclusive Auto•Blend Method offers a systematic approach to optimizing protein separations for maximum recovery and resolution. The Waters 650 Advanced Protein Purification System incorporates this technology into an instrument designed for fast protein liquid chromatography. This system is capable of precise, reproducible delivery and accurate blending of four buffers. The Auto•Blend Method automatically produces a buffer of the desired pH and ionic strength. By quickly screening a wide pH and ionic strength range, conditions that provide the best resolution and recovery are achieved.

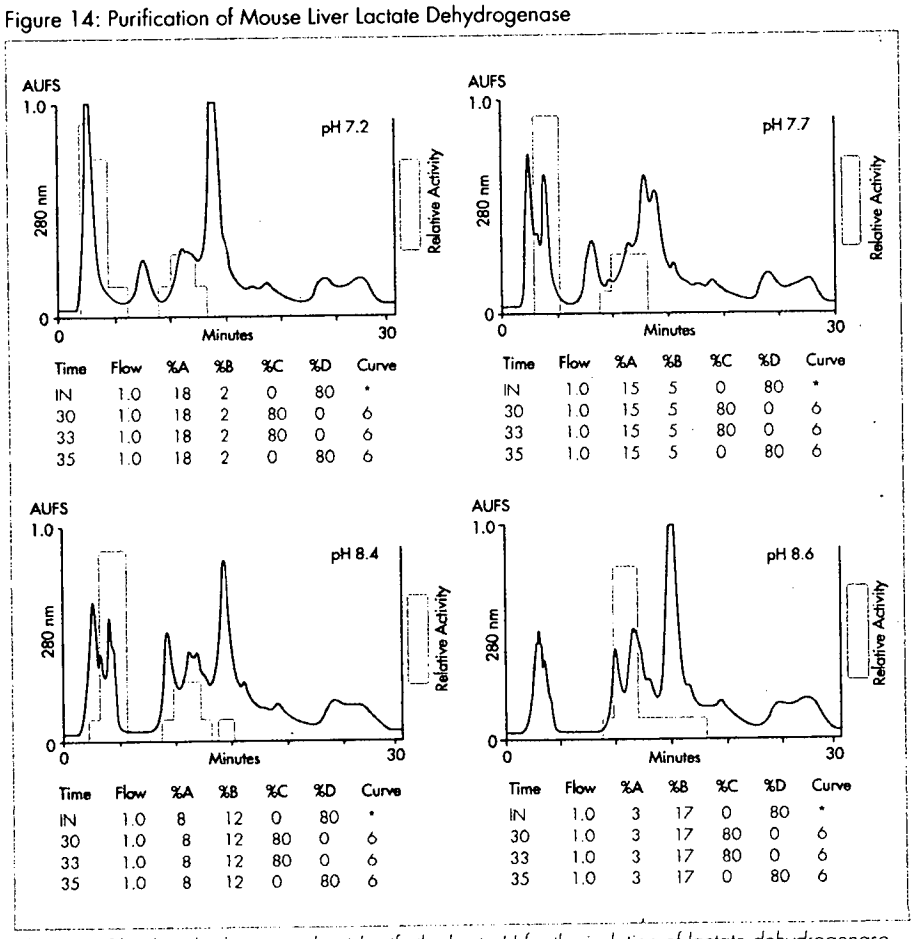
Easily Program the Desired pH.

Waters has developed Auto•Blend tables to help get you started. These tables are described in detail in the article, "A New Strategy for Protein Isolation" on page 4 of this issue of PPN. Generated by varying integral ratios of acidic and basic buffers, the tables provide a reference point for adjusting pH to the desired level. By simply programming different proportions of acidic and basic buffer, the 650 system automatically blends buffers of a desired pH. The ionic strength of the final buffer is controlled independently by varying the proportions of 1.0 M NaCl and water.

Simultaneous pH and Ionic Strength Gradients.

Additional selectivity in ion exchange separations can be achieved by independent pH and ionic strength gradient profiles. For example, the purification of monoclonal antibodies present in ascitic fluids, tissue culture or serum-free media is best performed using a concurrent buffer pH and ionic strength gradient. The Auto•Blend Method simplifies this purification procedure as shown by the separation of monoclonal IgG from mouse ascites (Figure 15A). Due to the diversity of monoclonal subclasses and sample sources, modifications in the established separation protocol may be required. Independent programming of buffer pH and ionic strength on the 650 system significantly improves the separation and recovery of a monoclonal IgA from ascites (Figure 15B).

Figure 14: Purification of Mouse Liver Lactate Dehydrogenase



Time	Flow	%A	%B	%C	%D	Curve
IN	1.0	18	2	0	80	*
30	1.0	18	2	80	0	6
33	1.0	18	2	80	0	6
35	1.0	18	2	0	80	6

Time	Flow	%A	%B	%C	%D	Curve
IN	1.0	15	5	0	80	*
30	1.0	15	5	80	0	6
33	1.0	15	5	80	0	6
35	1.0	15	5	0	80	6

Time	Flow	%A	%B	%C	%D	Curve
IN	1.0	8	12	0	80	*
30	1.0	8	12	80	0	6
33	1.0	8	12	80	0	6
35	1.0	8	12	0	80	6

Time	Flow	%A	%B	%C	%D	Curve
IN	1.0	3	17	0	80	*
30	1.0	3	17	80	0	6
33	1.0	3	17	80	0	6
35	1.0	3	17	0	80	6

The Auto•Blend method was used to identify the best pH for the isolation of lactate dehydrogenase (LDH) from mouse liver. The pH was systematically adjusted by programming changes within the gradient table.

the alkaline phosphatase separation shows, Waters Auto•Blend Method is much more convenient than conventional binary gradient systems. What's more, the Auto•Blend Method's ability to blend stock buffers precisely and reproducibly, greatly improves the chromatography.

Using concentrated stock buffers, one can perform separations over a range of useful pH's without having to invest a lot of time and effort preparing various buffer pairs of predetermined pH and ionic strength. Coupled with high performance, biocompatible chemistries and instrumentation, the Auto•Blend Method will further increase laboratory productivity when purifying biological molecules.

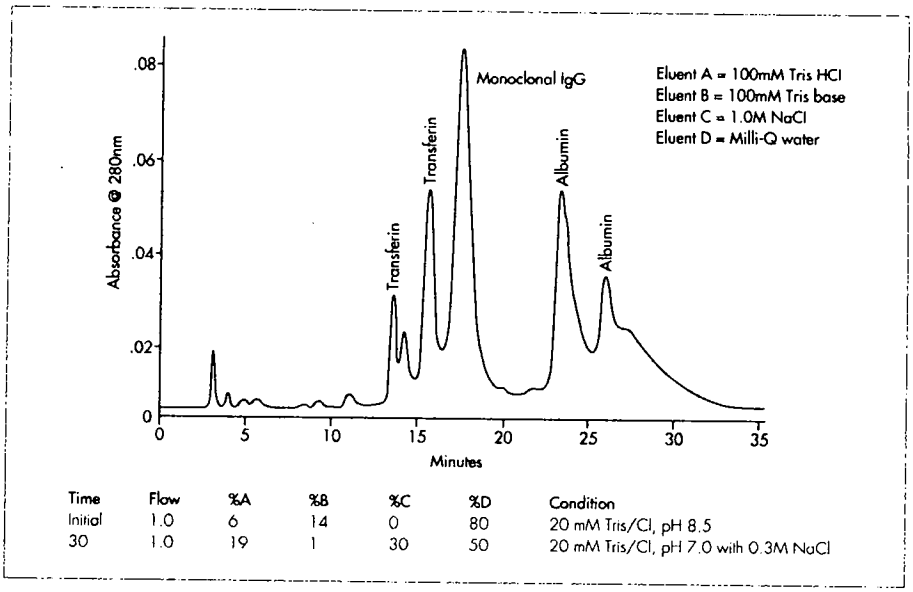
For More Information.

For information about Auto•Blend and Waters 650 Advanced Protein Purification System, check the appropriate box on the attached reply card and return it to us today.

References

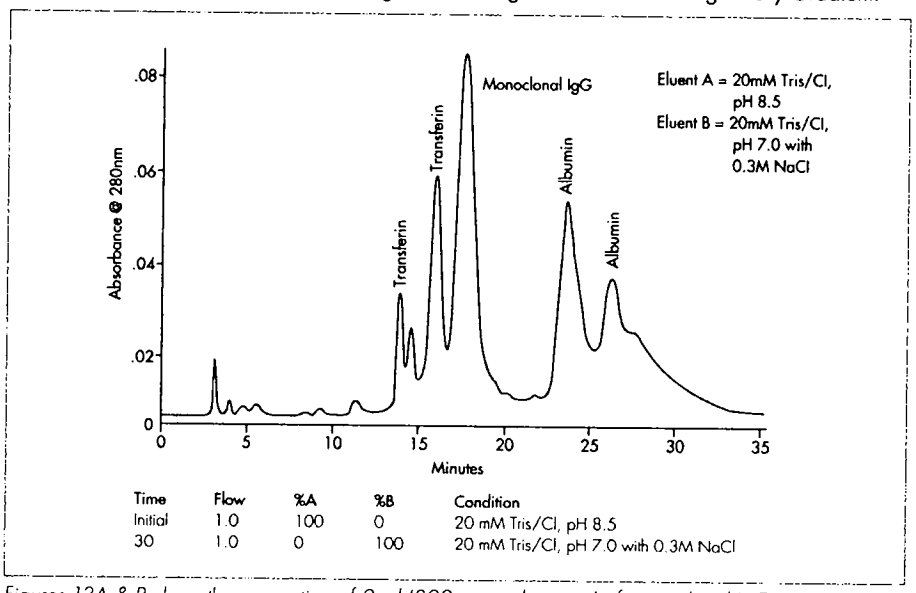
1. Hua, J.C. et al., "Purification and partial sequencing of bovine liver alkaline phosphatase," Arch. of Biochem. and Biophys. 241, 380-385 (1985).
2. Besman, M. and Coleman, J.E., "Isozymes of bovine intestinal phosphatase," J. Biol. Chem. 260, 11190-11193 (1985).
3. Regnier, F.E., "Liquid chromatography of immunoglobulins," LC•GC 5, 965-967 (1985).
4. Gemski, M.J. et al., "Single step purification of monoclonal antibody from murine ascites and tissue culture fluids by anion exchange high performance liquid chromatography," Biotechniques 3, 378-384 (1985).

Figure 13A: Separation of Monoclonal IgG Containing Mouse Ascites Using the Auto•Blend Method.



Time	Flow	%A	%B	%C	%D	Condition
Initial	1.0	6	14	0	80	20 mM Tris/Cl, pH 8.5
30	1.0	19	1	30	50	20 mM Tris/Cl, pH 7.0 with 0.3M NaCl

Figure 13B: Separation of Monoclonal IgG Containing Mouse Ascites Using Binary Gradient.



Time	Flow	%A	%B	Condition
Initial	1.0	100	0	20 mM Tris/Cl, pH 8.5
30	1.0	0	100	20 mM Tris/Cl, pH 7.0 with 0.3M NaCl

Figures 13A & B show the separation of 2 ml (300 µg total protein) of monoclonal IgG containing mouse ascites (diluted 1:20 in 20 mM Tris/Cl, pH 8.5) on Protein-Pak DEAE anion exchange column comparing the Auto•Blend Method (13A) with conventional binary gradient (13B) formation. Peaks were collected and identified by SDS-PAGE under reducing conditions (data not shown).

Using Auto•Blend to Quickly Adjust Elution Conditions.

The second chromatographic run was performed at pH 8.5 using the same NaCl gradient. A 20 mM Tris/Cl, pH 8.5 buffer was utilized throughout the separation by simply programming the LC system to deliver 6% eluant A and 14% eluant B. There was no need to prepare new buffer solutions. Both a greater total recovery of alkaline phosphatase activity (86%) was achieved as well as an increase in specific activity (7.7X), compared to the pH 9.5 separation.

The buffer pH was again reduced by programming the system to deliver 17% eluant A and 3% eluant B (i.e., 20 mM Tris/Cl, pH 7.5). The sample was separated with the same NaCl gradient. Recovery of alkaline phosphatase approached 100% with an even greater increase in enzyme specific activity (12.3X) compared to the previous runs.

Figure 11: Effect of pH on Recovery of Alkaline Phosphatase Activity from a Bovine Intestinal Mucosa.

pH of Separation	% Alkaline Phosphatase Recovered[1]	Specific Activity[2]
9.5	77	925
8.5	86	1551
7.5	96	2475

1. Alkaline phosphatase activity of the collected fractions and control was determined using a colorimetric assay performed at pH 9.5 with the chromogenic substrate para-nitrophenyl phosphate. The controls were bovine intestinal mucosa preparations at various pH values and were not subjected to chromatography.

2. Specific activity = units of alkaline phosphatase activity per milligram of total protein (BCA Protein Assay Reagent Kit with BSA standard, Pierce Chemical Co., Rockford, IL)

The summary of the results from three chromatographic separations using different pH buffers indicated that buffer pH affects both enzymatic activity and resolution of protein. By lowering the isolation buffer pH to 7.5 close to 100% of the alkaline phosphatase was recovered with a 12.3 - fold increase in enzyme-specific activity. Waters Auto•Blend Method enabled the quick and easy optimization of the isolation without the need to prepare numerous paired buffers of various pH strengths.

Figure 12

PROGRAM GRADIENT TABLE #: ■

TIME	FLOW	%A	%B	%C	%D	CURVE
INITIAL	1.00	1	19	0	80	•
10.00	1.00	1	19	0	80	11
50.00	1.00	1	19	50	30	6
51.00	1.00	1	19	80	0	11

CLEAR LINE CLEAR TABLE SAVE HELP

The CRT screen on Waters 650 Advanced Protein Purification System displays the Auto•Blend program for a pH 9.5/NaCl gradient separation of a bovine intestinal mucosa preparation.

Using the Auto•Blend Method, the authors were able to optimize the partial purification without first having to prepare numerous paired buffers of various pH and ionic strengths.

Note: Post-run analysis of the chromatography and collected fractions revealed which pH adjustments to make to increase enzyme recovery and specific activity even further.

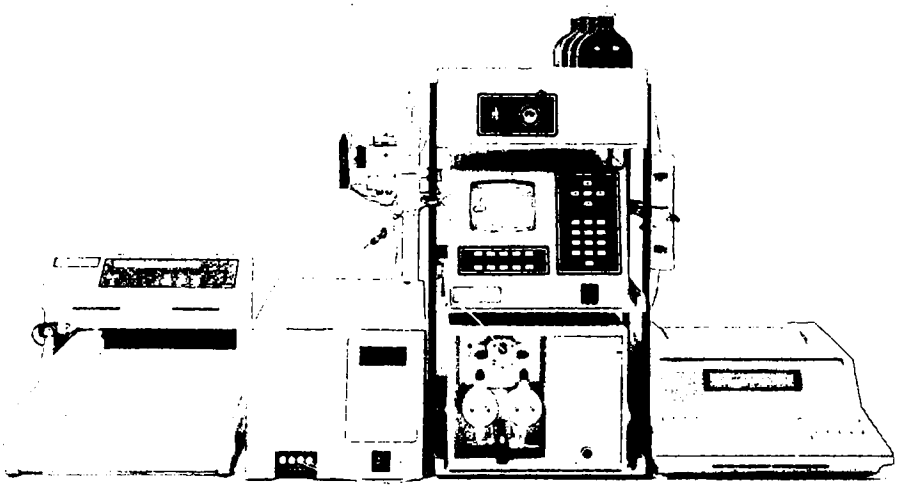
Waters Auto•Blend Method - For Easier Purifications.

The Auto•Blend Method eliminates the need to make up buffers by hand. To show that a binary gradient method can be directly transferred to a quaternary gradient method using Auto•Blend, the authors elected to separate a monoclonal IgG antibody contained in mouse ascites using the Waters 650 Advanced Protein Purification System. Concurrent pH and ionic strength gradients were performed on a Protein-Pak DEAE anion exchange column utilizing a 30-minute linear gradient (Curve 6) from 20 mM Tris/Cl, pH 8.5 to 20 mM Tris/Cl, pH 7.0 containing 0.3M NaCl at a flow rate of 1.0 ml/min.[3,4]

As shown in Figures 13A and 13B, there are no significant differences between the Auto•Blend gradient (13A) and the conventional binary gradient (13B). It should be noted that the pH and ionic strength gradients may be programmed independently using the Auto•Blend Method. (This is not possible using a binary gradient system.)

Conclusion.

Developing protein purification methods on rigid, biocompatible, chemistries and on advanced instrumentation is far easier than developing the same methods on compressible gels in large open column supports eluted by gravity flow or peristaltic pump systems. As

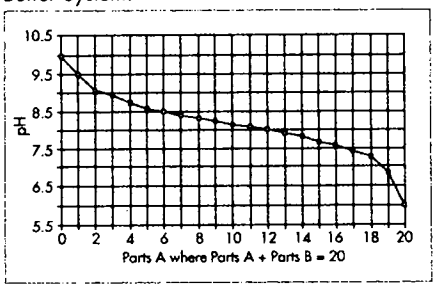


Waters 650 Advanced Protein Purification System was developed for the protein chemist who wants a purification system that offers fast, high performance protein separations with a metal-free fluid path. The 650 System has four buffer inlets and is the only fast protein LC system that allows you to run the Auto•Blend Method. The microprocessor based controller and integral CRT screen make programming easy. The wide flow range of the Waters 650 System (0.1 - 45 ml/min. or 0.5 - 80 ml/min.) make it the perfect instrument for lab scale or pilot scale protein purification.

Selecting Buffers With the Auto•Blend Method.

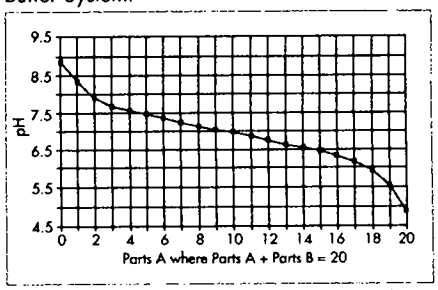
Figure 7 shows an Auto•Blend table for a 20 mM Tris buffer and shows how Auto•Blend mixes 100 mM stock Tris solutions to obtain buffers of a specific pH and ionic strength. If a 20 mM Tris/Cl, pH 8.0 buffer is required, the system can be programmed to deliver 12 parts eluant A, 8 parts eluant B, and 80 parts eluant D. Auto•Blend tables for sodium phosphate (Figure 8), sodium acetate (Figure 9), and sodium citrate (Figure 10) were produced in a similar fashion.

Figure 7: Auto•Blend Table for Tris Buffer System.



Data generated by precise blending of 100 mM stock Tris solutions with Milli-Q water to create 20 mM Tris/Cl buffers of different pH values. Eluant line A = 100 mM Tris HCl, Eluant line B = 100 mM Tris base, Eluant line C = 1.0M NaCl, Eluant line D = Milli-Q water.

Figure 8: Auto•Blend Table for Phosphate Buffer System.



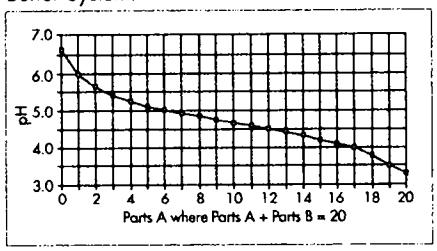
Data generated by precise instrument blending of 100 mM stock sodium phosphate solutions with Milli-Q water to create 20 mM sodium phosphate buffers of various pH values. Eluant line A = 100 mM NaH_2PO_4, Eluant line B = 100 mM Na_2PO_4, Eluant line C = 1.0M NaCl, Eluant line D = Milli-Q water.

Optimizing an Alkaline Phosphatase Separation.

The purification of a bovine intestinal mucosa preparation (Sigma Chemical Co., St. Louis, MO) containing numerous proteins and enzymes including alkaline phosphatase illustrates just one of the many applications for Auto•Blend.[1,2] A sample of this preparation containing 13 mg of total protein was solubilized in water obtained with a Millipore Milli-Q® Water System filtered through a 0.45 µm Millex™ Filter Unit. Repetitive chromatography on a Protein-Pak DEAE-8HR anion exchange column equilibrated (at 10 column volumes) in 20 mM Tris/Cl at various pH values provides the information needed to select optimum elution conditions.

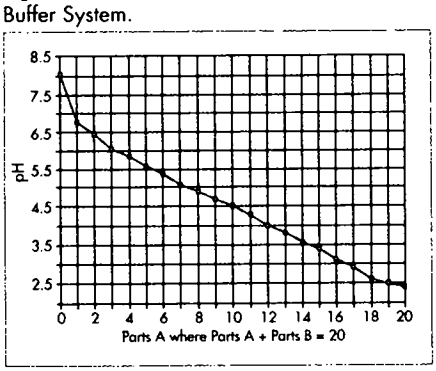
Proteins retained on the column were eluted using a linear gradient from 0 to 0.5 M NaCl over 40 min. Buffer pH was held constant throughout each separation. Fractions were collected and assayed for total protein (BCA Protein Assay Reagent Kit with BSA standard, Pierce Chemical Co., Rockford, Illinois) as well as total alkaline phosphatase activity (chromogenic para-nitrophenyl phosphate assay[1]). As a control, the filtered bovine intestinal mucosa preparation was assayed for total protein and alkaline phosphatase activity. With the data from each chromatographic run, comparisons of enzyme recovery can be made.

Figure 9: Auto•Blend Table for Acetate Buffer System.



Data generated by precise instrument blending of 100 mM stock acetate solutions with Mill-Q water to create 20 mM sodium acetate buffers of various pH values. Eluant line A = 100 mM acetic acid, Eluant line B = 100 mM Na-acetate, Eluant line C = 1.0 M NaCl, Eluant line D = Milli-Q water.

Figure 10: Auto•Blend Table for Citrate Buffer System.



Data generated by precise instrument blending of 100 mM stock citrate solutions with Milli-Q water to create 20 mM sodium citrate buffers of various pH values. Eluant line A = 100 mM citric acid, Eluant line B = 100 mM Na_3-citrate, Eluant line C = 1.0 M NaCl, Eluant line D = Milli-Q water.

Measuring the Effect of Buffer pH on Recovery Values.

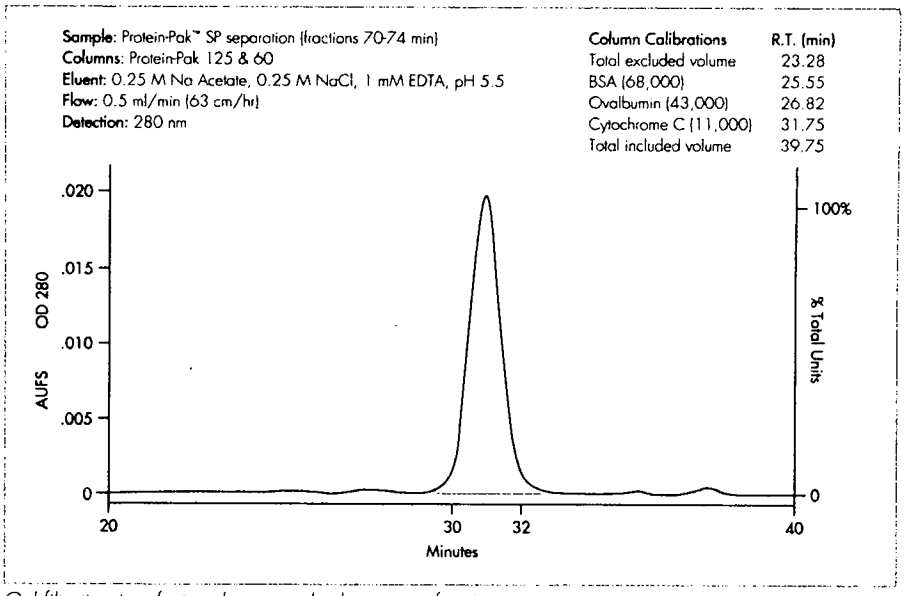
Figure 11 (next page) summarizes the results of three chromatographic runs. These runs tested the effect of buffer pH on the maintenance of enzymatic activity (% alkaline phosphatase recovered) as well as the effect of buffer pH on the resolution of alkaline phosphatase from contaminating proteins, which is measured by the degree of alkaline phosphatase specific activity. The first chromatographic separation was performed at pH 9.5, the pH optimum for measuring alkaline phosphatase enzyme activity.[1,2] Figure 12 (next page) is a photo of the gradient program used for this separation. An initial 10-minute hold was included during which the sample was loaded onto the column. At the end of the gradient, the column was washed with 0.8M NaCl to completely remove all bound material. Specific activity (total enzyme units/total protein) increased more that four-fold compared to the control (described above), and 77% of the applied enzymatic activity was recovered. While these values may be acceptable for the first step of a protein purification, improvements in resolution and recovery were sought.

Increase Specific Activity While Purifying Bioactive Materials in Less Than a Day.

Waters 650 Advanced Protein Purification System and high performance cation exchange columns facilitated the purification of acid phosphatase activity from bovine liver with a 179-fold increase in specific activity. The entire purification took less than one day because the procedure could be quickly and easily optimized on the Waters 650 system. Gel filtration chromatography was shown to be a fast and easy method to assess fraction purity.

For additional information on either Waters 650, Waters Protein-Pak glass columns, or Waters Accell CM bulk media, be sure to check the appropriate box on the attached reply card.

Figure 5: Gel Filtration Analysis.



Gel filtration is a fast and easy method to assess fraction purity.

A New Strategy for Protein Isolation.

Waters Auto•Blend Method Automatically Blends Buffers for Simpler Separation Optimization and Purification.

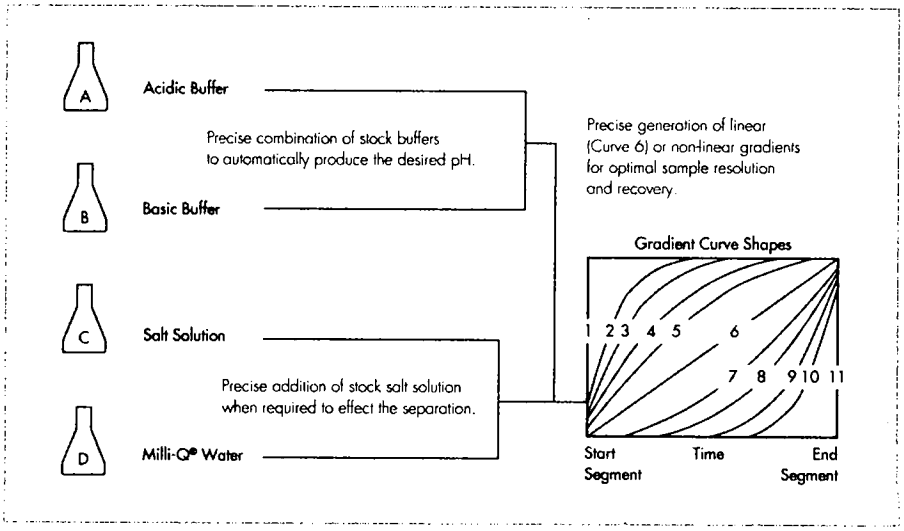
Selecting Buffers for Protein Isolation.

The ionic strength and pH of buffers are the two most important parameters for controlling the selectivity of protein separations on ion exchangers. The conventional approach to gradient formation and optimization requires the careful preparation of buffers of predetermined pH and ionic strength. A binary gradient chromatography system can be used to run buffer pH and ionic strength gradients, however, methods development historically requires the manual preparation of numerous buffer pairs.

The Waters Auto•Blend Method was developed for easy and systematic buffer blending. Using a set of four stock buffers (Figure 6), the Auto•Blend Method automatically generates pH and ionic strength gradient profiles for resolving and recovering biologically-active molecules.

This article describes the use of the Auto•Blend Method for optimizing the purification of bovine alkaline phosphatase by ion exchange chromatography.

Figure 6: Auto•Blend Method.



By generating precise linear or non-linear gradients using four stock buffer solutions, Waters Auto•Blend Method allows the biochemist to develop rapid protein isolation methods without having to repeatedly premix buffers.

Waters 650 system, one can quickly determine the optimal elution buffers, gradient profile, and gradient duration. The system has four buffer inlets that permit the blending of eluents, column regeneration and sample injection in large volumes. The system's interactive CRT screen allows fast, error-free programming and real-time monitoring of the isolation process.

Purifying the Enzyme.

The next step in the separation development strategy was to purify a sufficient amount of enzyme for activity and structural analysis. The separation was scaled up to nearly one gram of crude sample injected onto a 50 mm I.D. x 160 mm glass column (Figure 2). Flow rate was increased to 41 ml/min to maintain a constant linear velocity compared to the smaller column. The wide operating flow rate range of the Waters 650 system facilitates the efficient separation of gram quantities of crude samples on large glass columns without having to transfer the method to a second system. The large sample volume was loaded onto the column automatically by using one of the 650 system's four buffer inlets.

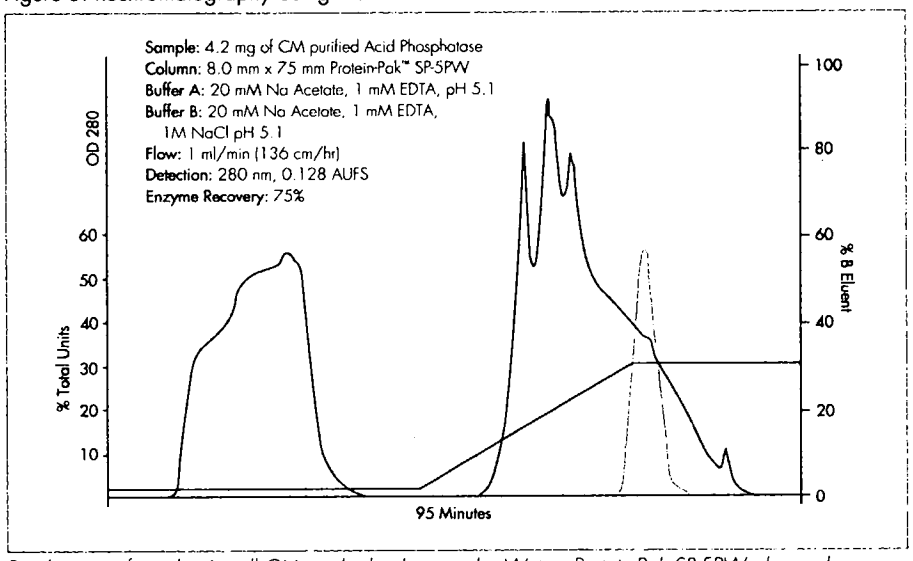
Increasing Protein Specific Activity.

Enzyme activity assays conducted on the fractions collected from the preparative purification step indicated which fractions should be pooled for further processing. To achieve higher specific activity, a second purification step was performed using a 10 µm, polymeric, high performance cation exchange glass column (Protein-Pak SP-5PW). Cation exchange chromatography permits the continued use of elution buffers having a pH within the range of enzyme stability. A prepacked, small particle sulfopropyl column was chosen because it has a higher efficiency and different selectivity from Accell CM media and was shown to further purify acid phosphatase from the pooled active fractions (Figure 3).

Assessing Fraction Purity.

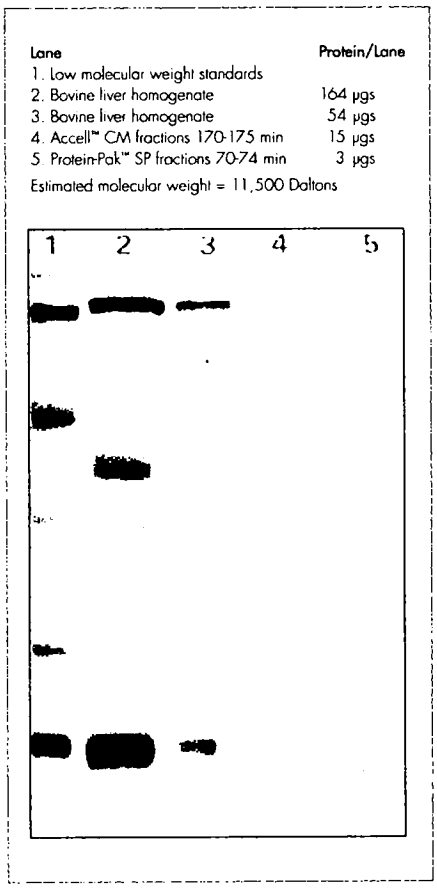
Fraction purity was assessed by both SDS-PAGE and gel filtration chromatography. The silver-stained reducing SDS-polyacrylamide gel shows one major band for the active fraction obtained from the Protein-Pak SP-5PW column (Figure 4). This fraction was also analyzed by gel filtration chromatography on the 650 system and was found to have one major peak (Figure 5). Both techniques estimated the molecular weight of the enzyme to be 11,500 Daltons.

Figure 3: Rechromatography Using Protein-Pak™ SP-5PW.



By changing from the Accell CM packed column to the Waters Protein-Pak SP-5PW glass column one is able to change selectivity and use a smaller particle size packing to increase specific activity of the starting sample by 179-fold.

Figure 4: Reducing 12.5% SDS-PAGE Analysis (Silver-Stained).



Lane	Protein/Lane
1. Low molecular weight standards	
2. Bovine liver homogenate	164 µgs
3. Bovine liver homogenate	54 µgs
4. Accell™ CM fractions 170-175 min	15 µgs
5. Protein-Pak™ SP fractions 70-74 min	3 µgs

Estimated molecular weight = 11,500 Daltons

A Fast Method for Purifying Highly Active Proteins from Crude Mixtures.

Isolating a Freely-Soluble Enzyme With Waters™ 650 Advanced Protein Purification System.

The purification of acid phosphatase from bovine liver is an example of a freely-soluble protein isolation procedure. Before choosing the chromatographic separation mode, the stability of the enzyme was tested over a pH range of 4.0 to 8.5. It was found that the optimal enzyme activity in a crude liver homogenate was achieved at pH 5.1. At this pH, acid phosphatase is positively charged which dictates the use of cation exchange chromatography as the appropriate ion exchange mode in the purification scheme.

Choice of Cation Exchange Packing.

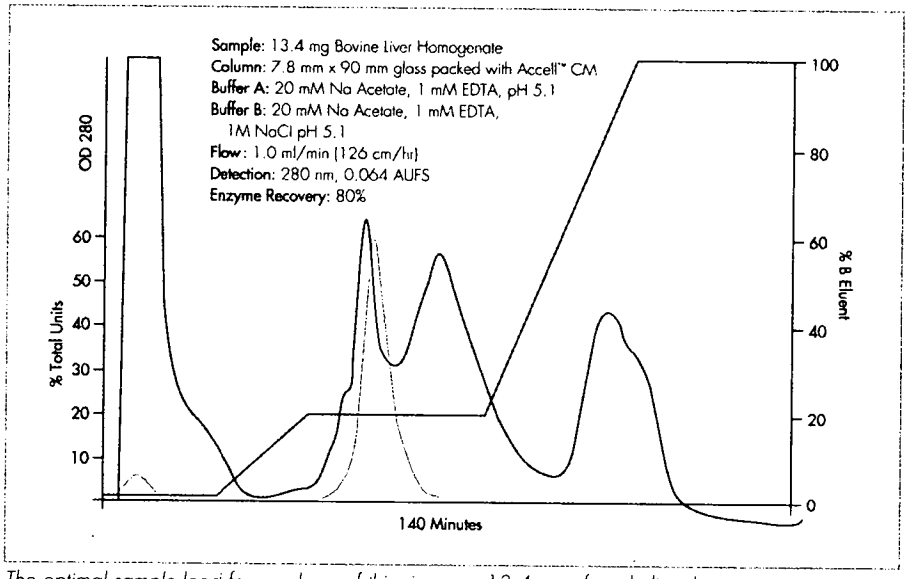
Waters offers the protein chemist two families of bulk cation exchange packings which can be used for the isolation of proteins from crude mixtures prior to final high performance purification. Protein-Pak™ SP 40HR is a 40 µm, 1000Å rigid polymer-based material that is a strong cation exchanger and is stable at pH conditions of 2 to 12. The same chemistry is also offered in a 15 µm and 8 µm particle size with identical selectivity and higher performance than the 40 µm packing. (Refer to "Ion Exchange Packing for Protein Purification at Any Scale" found on page 11 for more information on Protein-Pak HR packings.)

Waters Accell™ CM is a 40 µm, 500Å polymer-encapsulated silica media that is a weak cation exchanger. A copolymerization process encapsulates the silica base with a hydrophilic bonding layer making the media stable to reuse with aqueous buffers in the pH range of 2 to 9. Accell CM was chosen for this work because it enabled inexpensive processing of large volumes of crude sample while providing good fractionation of the crude liver homogenate.

Optimizing the Separation.

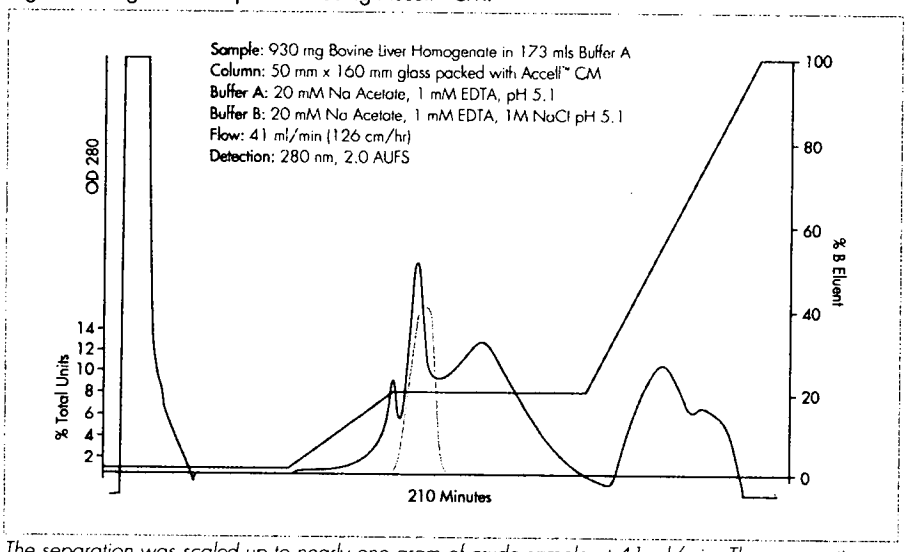
To optimize isolation conditions, a 7.8 mm I.D. x 90 mm glass column was packed with Accell CM media. The optimal sample load for a column of this size was 13.4 mg of crude liver homogenate per injection (Figure 1). The method was developed quickly and simply on Waters 650 Advanced Protein Purification System. By performing quick, small scale runs on the

Figure 1: Loading Study Using Accell™ CM.



The optimal sample load for a column of this size was 13.4 mg of crude liver homogenate per injection.

Figure 2: Large Scale Separation Using Accell™ CM.



The separation was scaled up to nearly one gram of crude sample at 41 ml/min. The preparative chromatogram shows an identical separation as the analytical chromatogram and retains the same level of biological activity. The sample was loaded through one of the four buffer inlets on the Waters 650.

NOTES PROTEIN PURIFICATION

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THIS ISSUE'S TOPICS INCLUDE ...

- **A Fast Method for Purifying Highly Active Proteins from Crude Mixtures** — Page 2

  Multi-Step Isolation of a Freely-Soluble Enzyme. Most protein chemists still start their protein purification protocols by using open columns to fractionate and concentrate crude protein mixtures prior to using FPLC™ for final purification. Now there's a fast-protein LC system for purifying crude mixtures as well as for final polishing. PPN reports on this approach and describes a new method for purifying acid phosphatase from crude bovine liver.

- **Automated Buffer Blending** — Page 4

  A New Strategy for Protein Isolation. Whether developing gradient conditions for the best separation or running established conditions, preparing buffers for protein isolation is time consuming and inconvenient. PPN describes a new method known as Auto•Blend™ that eliminates this tedious task.

- **Assessing the Effect of Changing Buffer pH on Ion Exchange Protein Separations** — Page 8

  Protein Separations Respond to Subtle pH Changes. Ionic strength and pH are the two most important parameters for controlling the selectivity of protein isolations on ion exchangers. A systematic approach to method optimization can dramatically improve the recovery of specific activity. PPN explains how you can accomplish this quickly using the Auto•Blend Method.

- **Rapid Biomolecule Purifications Using a New Epoxy-Activated Affinity Chemistry** — Page 9

  Affinity chromatography typically results in high purification and recovery of biological activity. PPN describes a new epoxy-activated affinity packing material that is easily and efficiently coupled with your specific affinity ligand for rapidly purifying biomolecules.

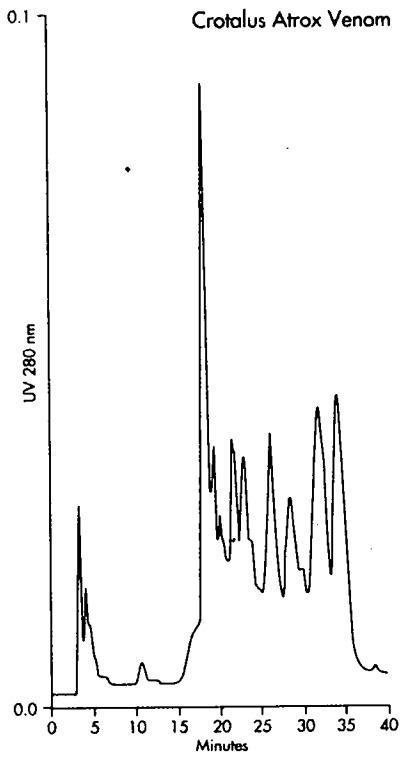
- **Ion Exchange Packings for Protein Purification at Any Scale** — Page 11

  PPN describes a new family of ion exchange packings for protein purification. A choice of particle sizes within the same family of chemistries enables easy, predictable scale up of protein isolations.

- **Now Available on Videotape: Optimizing Protein Purifications** — Page 12

  If you're a protein chemist, Waters would like to send you a new videotape on protein purification, free of charge. See inside for details.

Fast-Protein LC for High Resolution Fractionation of Complex Biological Mixtures



Sample: 100 µl of Crotalus atrox (snake) venom
Column: Protein-Pak DEAE 8HR 10 x 100 mm
Method: Auto•Blend
Initial Conditions: 20 mM Tris buffer pH 8.1
Final Conditions: 20 mM Tris buffer pH 8.1 with 0.4 M NaCl
Gradient: Linear, 30 min
Flow Rate: 1.2 ml/min

The fractionation of the components contained in Crotalus atrox (snake) venom demonstrates the ability of fast-protein LC to provide the high resolution necessary for fractionation of complex samples. Protein Purification Notes reports on novel purification methods which have proven to increase the utility of FPLC.

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5 ☐ Advanced Purification Glass Columns
6 ☐ Accell Bulk Packings
7 ☐ Waters Auto•Blend Method
8 ☐ Protein-Pak HR Columns and Packings
9 ☐ Protein-Pak Epoxy-Activated Affinity Packings

10 ☐ Please send me a copy of the videotape *Optimizing Protein Purifications*

11 ☐ I presently own one or more FPLC systems. (Please specify number of systems _____)

I plan to add the following capability to my lab:

Type of System	in 0-3 Mos	4-6 Mos	7-12 Mos	+1 year
FPLC	12 ☐	13 ☐	14 ☐	15 ☐
Analytical HPLC	16 ☐	17 ☐	18 ☐	19 ☐
Ion Chromatography	20 ☐	21 ☐	22 ☐	23 ☐
Prep HPLC	24 ☐	25 ☐	26 ☐	27 ☐
GC	28 ☐	29 ☐	30 ☐	31 ☐
CE	32 ☐	33 ☐	34 ☐	35 ☐

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