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Membrane Convective Liquid Chromatography (MCLC) for the Ion Exchange Separation of Bio-Molecules

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Membrane Convective Liquid Chromatography

Membrane-Based Chromatography Columns for the High Performance Separation of Biomolecules.

by Richard Hamilton and Robert Barry

ABSTRACT

Ion exchange chromatography is performed everyday in the biotech laboratory. Columns packed with soft gel and porous particle media used in most ion exchange separations is often characterized by slow flow rates, low resolution, and low throughput. These limits are often due to the mass transport of molecules inherent in diffusion dominant systems.

Membrane Convective Liquid Chromatography (MCLC) is a new approach to the separation of biomolecules. Unlike beads or traditional soft gels, mass transport of the molecules being separated is achieved through convective, rather than diffusive, flow.

This allows the flow to be increased without band broadening and loss of resolution caused by small pore diffusion that is typical of conventional porous particles. High resolution separations of biomolecules can be achieved in minutes, not hours.

This study focuses on the application of membrane-based convective chromatography devices for the rapid, high resolution separation of proteins, enzymes and other biomolecules by ion exchange.

INTRODUCTION

MEMBRANE CONVECTIVE LIQUID CHROMATOGRAPHY (MCLC)

Membrane-Based Chromatography Columns for the High Performance Separation of Biomolecules.

The extensive application of liquid chromatography for bioseparations began with the introduction of soft gels in the late 1950s. These natural polymers, based on cellulose, agarose and dextrans, made it possible to form supports that are hydrophilic, easy to functionalize, economical and sufficiently porous for the separation of large biomolecules. However, these packing materials also suffered from various chromatographic limitations.

In the middle 1970s the introduction of high performance polymer particles helped eliminate several limitations. The HPLC materials were strong and could be made in a variety of particle and pore sizes for most applications. These new columns could be used at faster flow rates with smaller particles to improve resolution an throughput. Surface chemistries could be created for a full range of separation techniques. Although these new HPLC separation materials solved a number of the problems associated with soft gels, they created several new limitations. These new high pressure columns required special equipment to pack and operate. Resolution was improved, but capacity was low and throughput reduced.

Many of the factors that limit resolution in chromatography are associated with the efficiency of molecular transport to and from the active binding sites on the solid support. The application of wide pore particles produced a significant improvement in resolving power. Most importantly however, it is the diffusion of the molecules in and out of those pores that physically limits the flow rate and therefor the throughput.

The key to increasing resolution AND increasing throughput is to eliminate the mass transport limitations characteristic of conventiona chromatography.

The key to high performance liquid chromatography is to reduce the contribution of different band-broadening processes to column plate height, H [2].

$$H = Au^{0.33} + B/u + Cu + Du \qquad \text{Equ. 1.0}$$

where:
- Au = Eddy diffusion and mobile phase transfer
- B/u = Longitudinal diffusion
- Cu = Stagnant mobile phase transfer
- Du = Stationary phase mass transfer

Mass transport in a chromatography column occurs primarily by two mechanisms: (1) diffusive mass transport through the stagnant liquid in the pore and (2) convective mass transport in the flowing mobile phase between the particles in the column. There are other factors that effect resolution such as eddy diffusic longitudinal diffusion and stationary phase mass transport, but the ratio of these other mass transport effects are insignificant. The "stagnant mobile phase mass transfer" has been recognized as one of the major causes of band spreading in liquid chromatography. There have been many approaches to solving this problem short of eliminating the pore entirely with nonporous beads.

We have taken a new approach-
we have eliminated the beads and kept the pores.

Membrane Convective Liquid Chromatography (MCLC) is a new approach to high resolution separations. By flowing through the pores and not past them, we eliminate the diffusive mass transport limitation characteristic of conventional bead columns. By placing the active surface as close to the flowing buffer as possible, transport of the molecule to and from the active surface is by convective mass transport. Resolution is increased as peaks narrow, even at very high flow rates. Equilibration, separation and re-equilibration are faster, yet capacity and throughput are high.

[1] E.A. Peterson and H.A. Sober, J. Am. Chem. Soc. 78, (1956), 751.

[2] L.R. Snyder and J.J. Kirkland, 'Introduction to Modern Liquid Chromatography', 1979.

EXPERIMENTAL PROTOCOL / RESULTS

All separation were performed on a CM MemSep-1000 (1.4 ml bed volume) using a Waters 650 Advanced Protein Purification System and the Auto-Blend* method for rapid optimization of separations. All chromatographic runs were conducted at pH 5.25. The loading buffer was 20 mM sodium acetate. The protein were eluted using a 20 bed volume linear gradient from 0 to 0.25M NaCl in 20 mM sodium acetate.

Flow rates were varied from 1.4 ml/min (1 bed volume/min) to 14.0 ml/min (10 bed volumes/min). The three protein test mixture consisted of α-chymotrypsinogen A, cytochrome c (bovine heart), and lysozyme. A total of 1 mg of the protein mix was applied to the MemSep for each run.

Membrane-based chromatography columns introduce a fundamentally new approac to eliminating a number of the limitations in the separation of biomolecules. By utilizing unique membrane systems and optimized device design, **an innovative chromatographic system has been developed that dramatically decreases separation times and increases resolution.**

Three plots were constructed from the resulting data (Fig. 1,2 & 3).

Figure 1. Peak volume vs Flow Rate: In a typical porous particle column, peak volume increases as flow increases due to the mismatch in the convective and the diffusive mass transport rates.

Figure 2. Separation Efficiency (H) vs Flow Rate: Separation efficiency is relate to several mass transport factors as described in equation 1.0.

Figure 3. Resolution (Rs) vs Flow Rate: In a porous bead system, as flow rate increases, resolution decreases. Complete separation between two peaks is a number greater than 1. Resolution is calculated by using the equation:

$$Rs = \left[(t_2 - t_1) \,/\, 1/2\,(w_1 + w_2)\right]$$

Figure 4. Separation of the three protein mix at 1.4 ml/min (1 bed volume/min
Total run time = 32 minutes.

Figure 5. Separation of the three proteins mix at 2.8 ml/min (2 bed volume/min
Total run time = 16 minutes.

Figure 6. Separation of the three protein mix at 5.6 ml/min (4 bed volume/min)
Total run time = 8 minutes.

Figure 7. Separation of the three protein mix at 9.8 ml/min (8 bed volumes/min
Total run time = 4 minutes.

Figure 8. Separation of the three protein mix at 14.0 ml/min (10 bed volume/m
Total run time = 3 minutes.

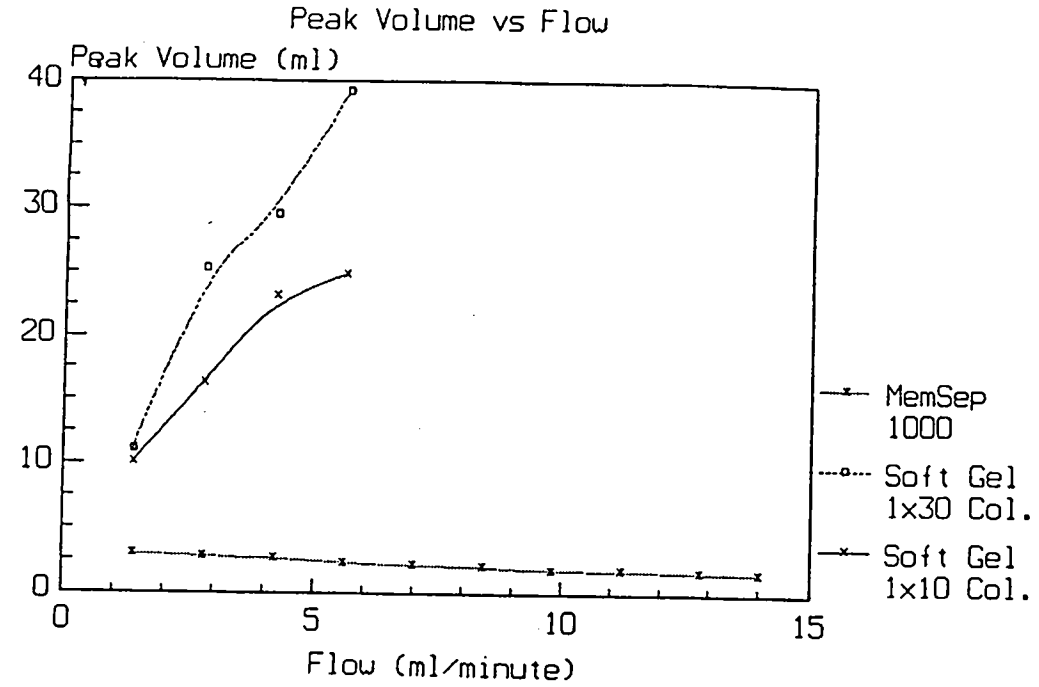


FIG. 1

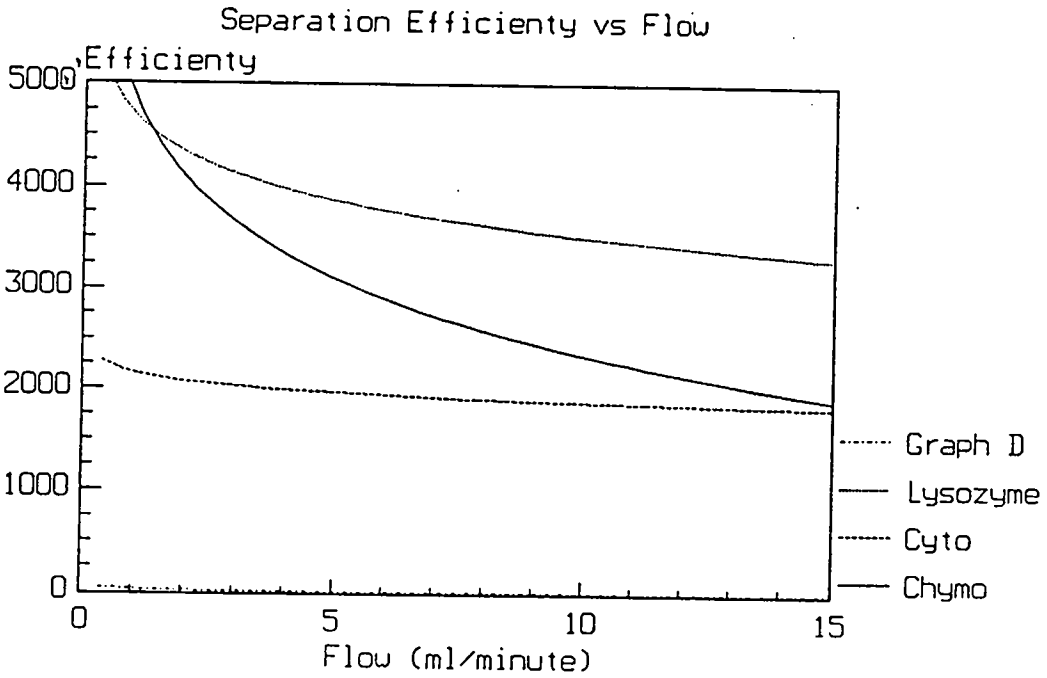


FIG. 2

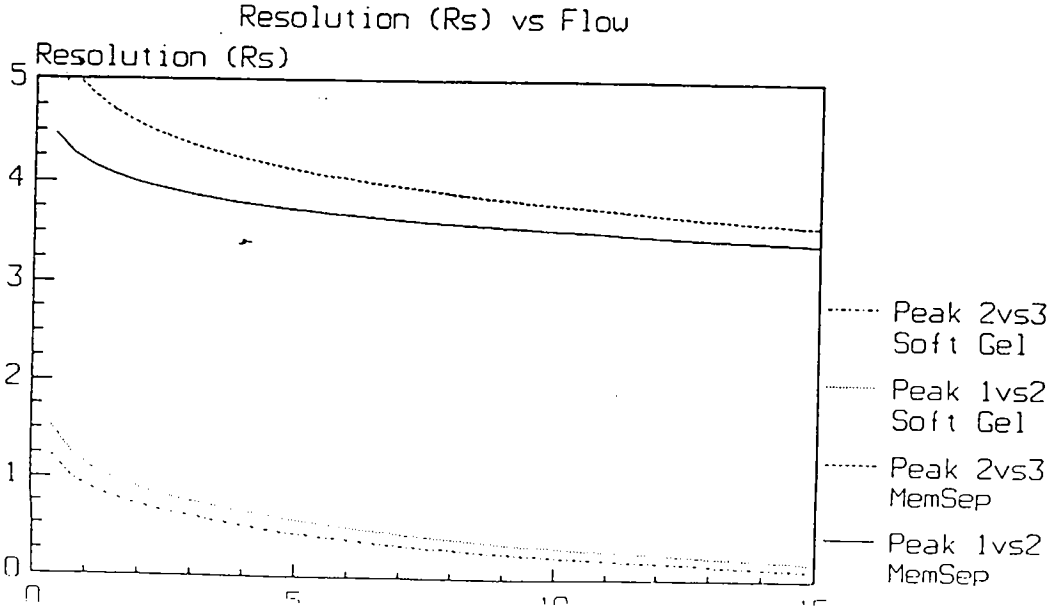


FIG. 3

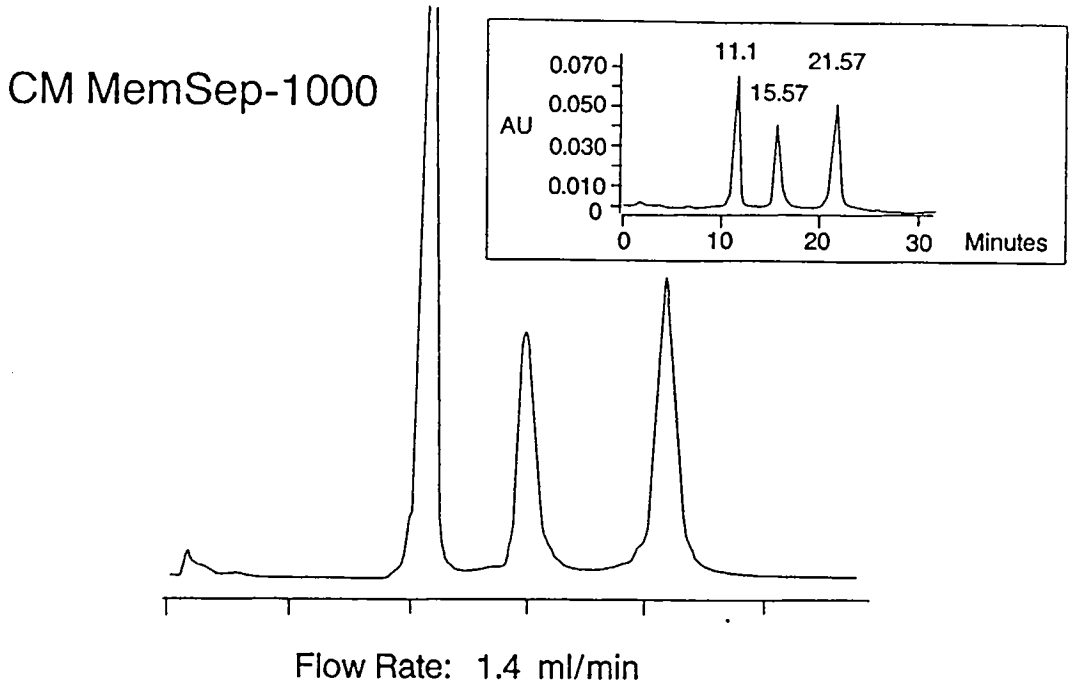

CM MemSep-1000
AU
0.070
0.050
0.030
0.010
0
11.1
15.57
21.57
0
10
20
30
Minutes
Flow Rate: 1.4 ml/min

FIG. 4

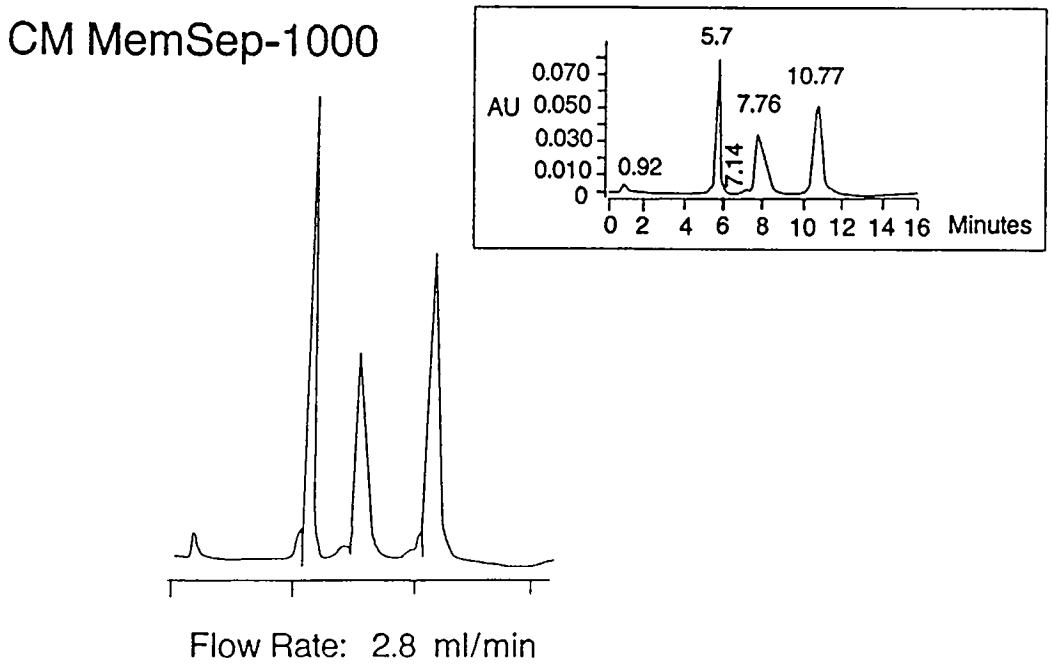
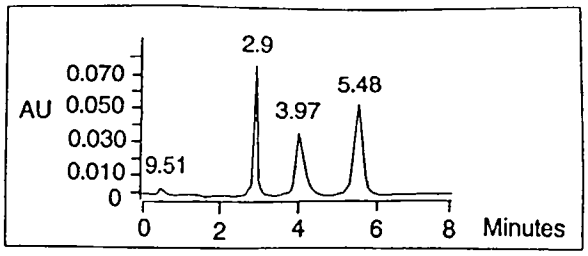
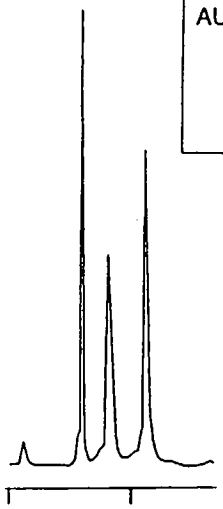

CM MemSep-1000
AU
0.070
0.050
0.030
0.010
0
5.7
7.76
10.77
0.92
7.14
0 2 4 6 8 10 12 14 16
Minutes
Flow Rate: 2.8 ml/min

FIG. 5

CM MemSep-1000

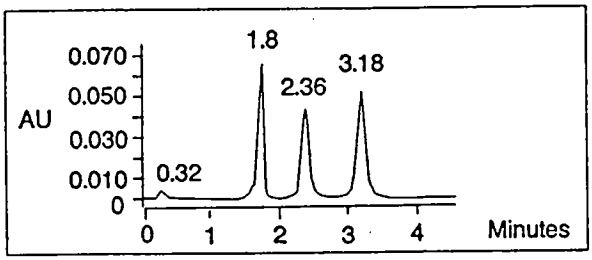


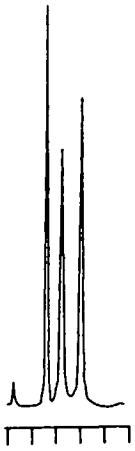


Flow Rate: 5.6 ml/min

FIG. 6

CM MemSep-1000

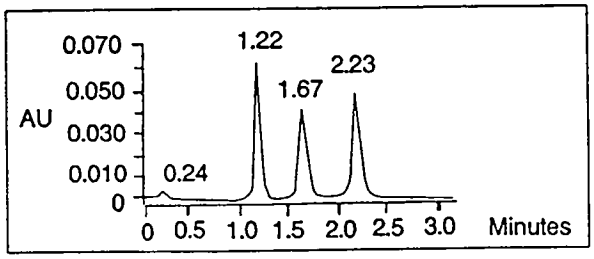




Flow Rate: 9.8 ml/min

FIG. 7

CM MemSep-1000



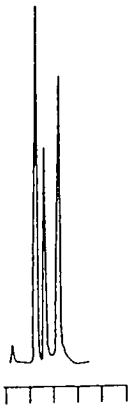


FIG. 8

High Performance Alternative To Conventional Soft Gel Chromatography

INTRODUCTION

Ion exchange chromatography provides a powerful separation tool that finds application in virtually all branches of the life sciences. The value of its utility on the practical level is defined in terms of the speed and resolution of the separation, and the purity and biological activity of the recovered product.
MemSep® (Membrane Separation) Chromatography Cartridges form Millipore Corporation combine the resolution capabilities of conventional chromatography media with the speed and throughput capabilities of membrane systems. Here, the utility of the MemSep anion and cation exchanger in protein separation is examined by comparing them to the separation of the same protein test mixture using conventional soft gel chromatography.

EXPERIMENTAL PROTOCOL / RESULTS

Separation of the indicated protein mixtures was examined first on high performance agarose columns at flow rates of 2.8, 4.2 and 5.6 ml/min. Within a reasonable run time the resolution exhibited at 2.8 and 4.2 ml/min were comparable, whereas a significant decrease in the resolving power of the agarose column was observed at the higher flow rate of 5.6 ml/min. The protein elution profiles obtained at a flow rate of 4.2 ml/min is shown in Figure 1a & 2a.

The elution sequence of the two protein mixture employing cation (CM) exchange chromatography (Fig. 1) is:
1. α-chymotrypsinogen A
2. lysozyme

The elution sequence of the three protein mixture employing anion (DEAE) exchange chromatography (Fig. 2) is:
1. human transferrin
2. ovalbumin
3. ß-lactglobulin A

The agarose column was then removed and replaced with the corresponding MemSp-1000 cartridge to obtain the chromatograms shown in Fig. 1b & 2b. The flow rate, gradient, run time and sample quantity (1 mg of total protein) all remained the same as indicated for the agarose column.

In Figure 1c & d, and Figure 2c & d, the chromatographic separation of the corresponding protein mixtures was examined at considerably shorter run times, under modified gradient conditions and increased flow rates of 5.6 ml/min (Fig. 1c; 2c) and 11.2 ml/min (Fig. 1d; 2d) using the MemSep-1000 ion exchangers.

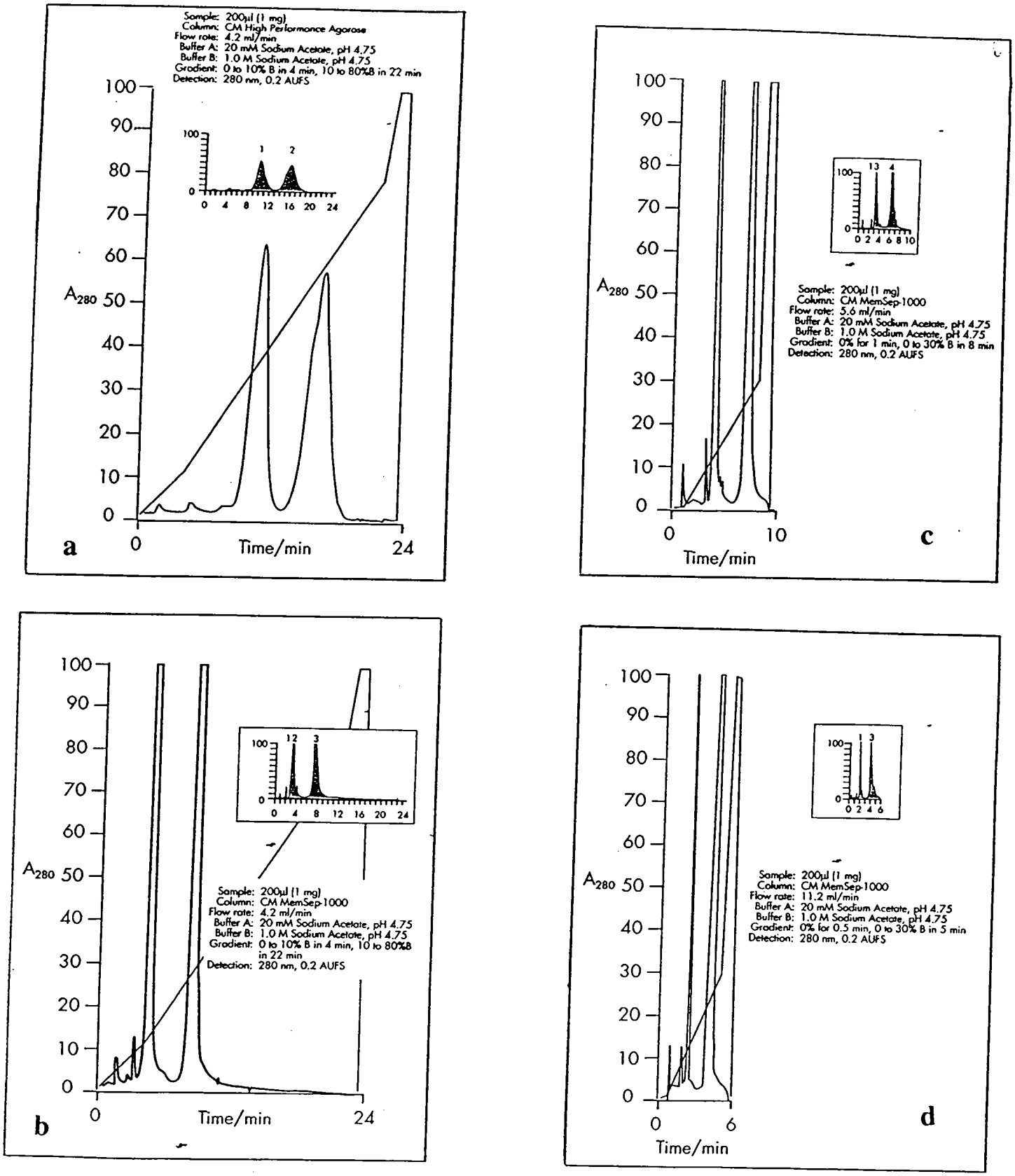


FIGURE 1. Separation of a two protein mixture (α-chymotrypsinogen A and lysozyme) on: a. High Performance CM Agarose column (1X10 cm); b, c, & d. DEAE MemSp-1000 Chromatography Cartridge.

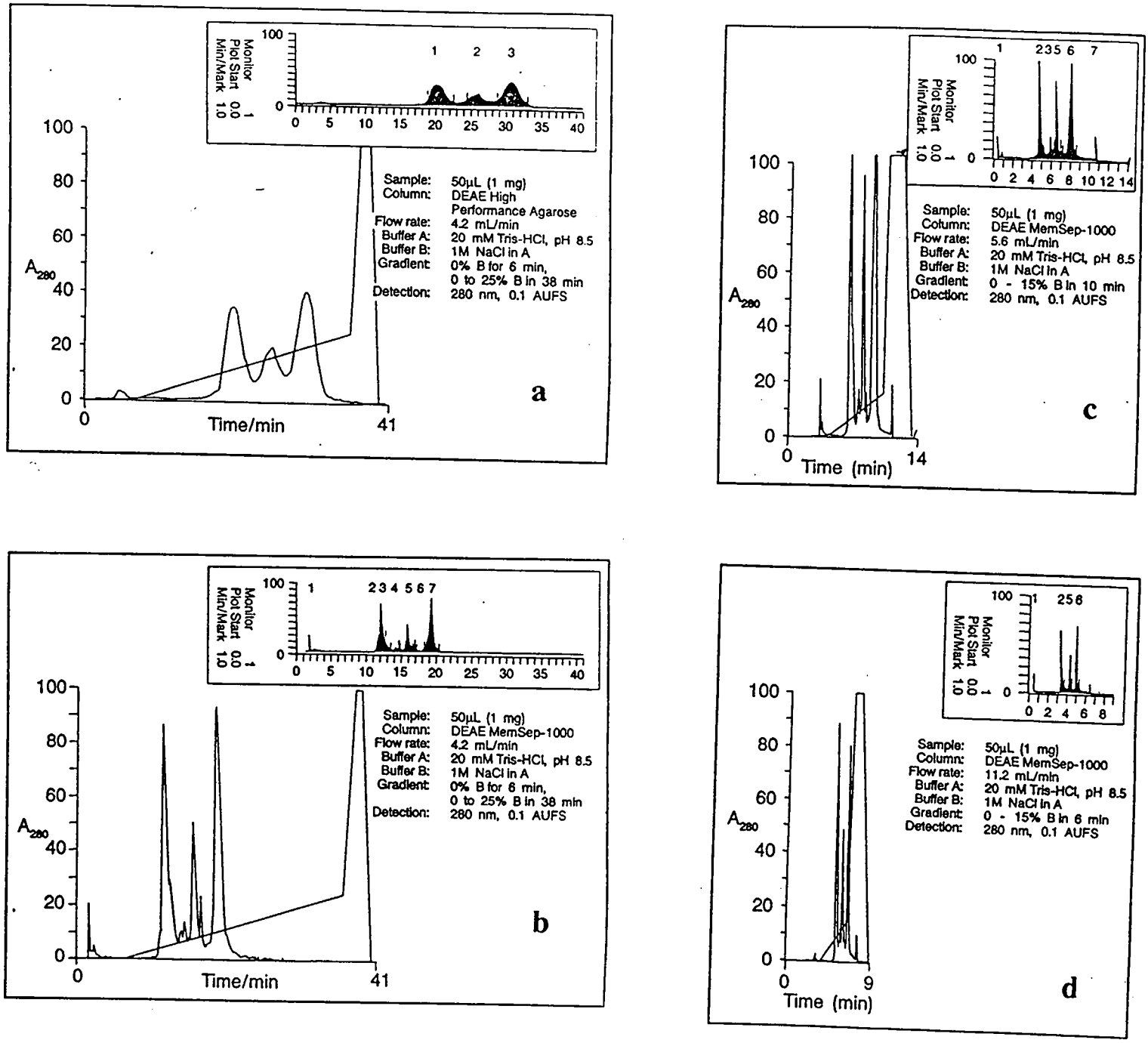


FIGURE 2. Separation of a three protein mixture (human transferrin, ovalbumin, ß-lactoglobulin A) on: a. High Performance DEAE Agarose column (1X10 cm); b, c, & d. DEAE MemSp-1000 Chromatography Cartridge.

Rapid Separation of Glucose-6 Phosphate Dehydrogenase From Crude Yeast Extract

INTRODUCTION

The goal in all purification protocols is the isolation of the desired product in the purest form possible, in the shortest period of time, while maintaining the highest degree of biological activity. Here, the utility of MemSep anion exchange chromatography is examined in the rapid separation of G6P-DH from yeast.

EXPERIMENTAL PROTOCOL / RESULTS

All chromatographic separations were performed using Waters 650 Advanced Protein Purification System. The flow rate was 5 or 10 ml/min with a 10 min linear gradient from 0 to 0.2M NaCl in 20 mM Tris buffer. The DEAE MemSep-1000 (1.4 ml bed vol.) and the larger DEAE MemSep-1010 (4.9 ml bed vol.) have protein binding capacities of 10-20 mg and 60-80 mg respectively.

Separation of glucose-6-phosphate dehydrogenase (G6P-DH) from yeast extract was first evaluated at low concentrations (~5 mg) on the DEAE MemSep-1010 chromatography cartridge employing the Waters Auto-Blend* method. Because of the speed advantage offered by MemSep cartridges, injections could be made ever 15 minutes, examining separations at nine different pH values, from pH 7.25 to pH 9.1, in just over 2.5 hours (Figure 1).

Protein detection is at 280 nm. Enzymatic activity was determined using a spectrophotometric assay monitoring the reduction of $NADP^+$ at 340 nm.

$$\text{Glucose-6-P} + NADP^+ \xrightarrow{\text{G6P-DH}} \text{Gluconate -6-P} + NADPH + H^+$$

Using the information provided by the Auto-Blend pH study, the yeast extract was then fractionated at considerably higher sample concentrations (20 - 50 mg) on the MemSep-1010 under optimal conditions (Fig. 2a). The active peak was collecte desalted, concentrated and applied to a DEAE MemSep-1000 chromatography cartridge. The resulting chromatogram demonstrates a single peak which co-chromatographs with authentic enzyme (Fig. 2b).

The purity of the final chromatographic fraction was examined using SDS-PAGE which showed that the isolated G6P-DH is free of major protein contaminants. Recovery of the target enzyme was greater than 80%, demonstrating activity on the order of ~340 U/mg.

*Warren et al., 'A New Strategy for Rapid Optimization of Protein Separations', American Biotechnology Laboratory, June 1989.

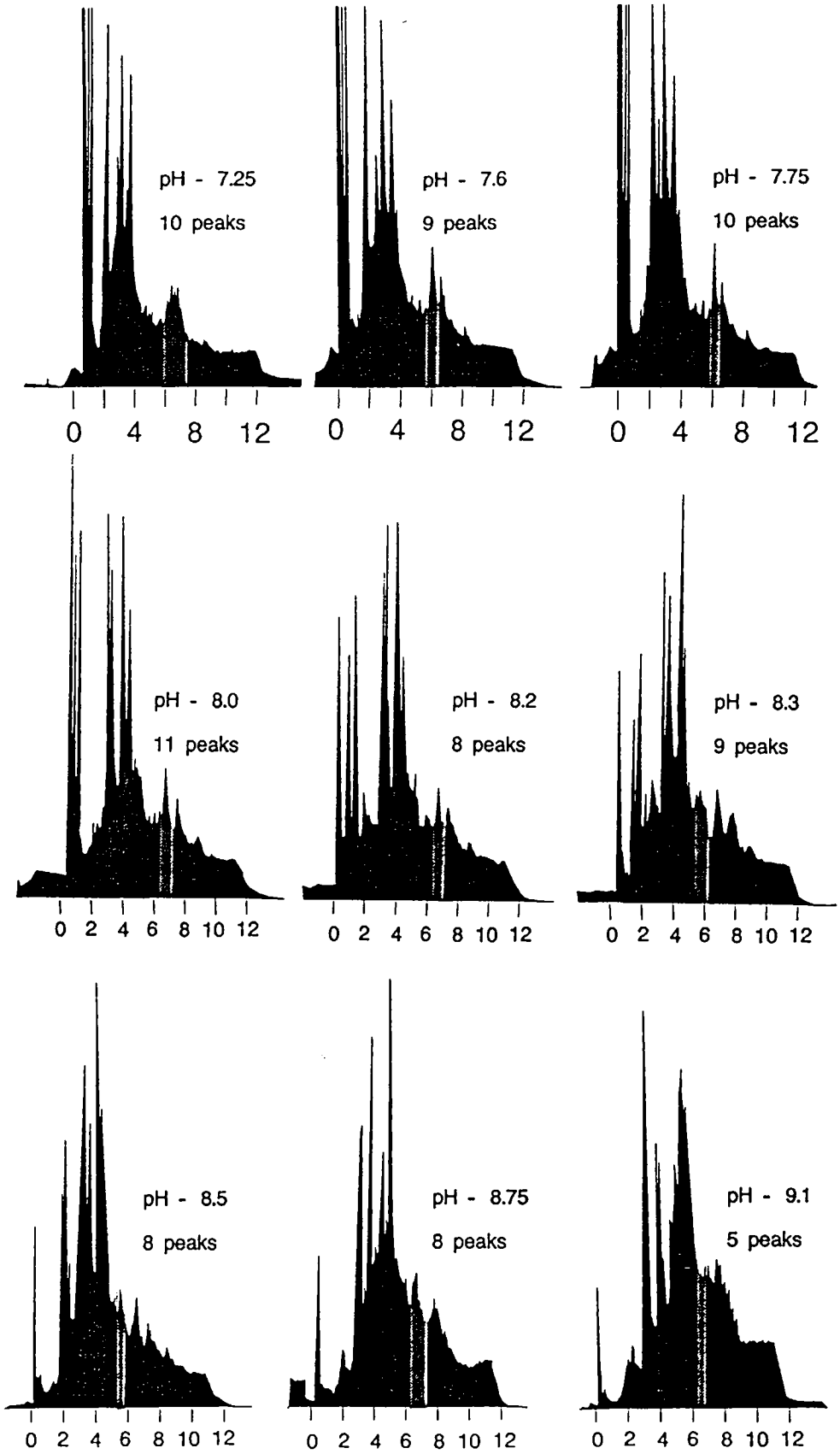


FIGURE 1. Fractionation of crude yeast extract (~5 mg) on a DEAE MemSep-1010 cartridge at nine different pHs from 7.25 to 9.1 using the Waters Auto-Blend* method. G6P-DH containing fractions are indicated by the grey areas.

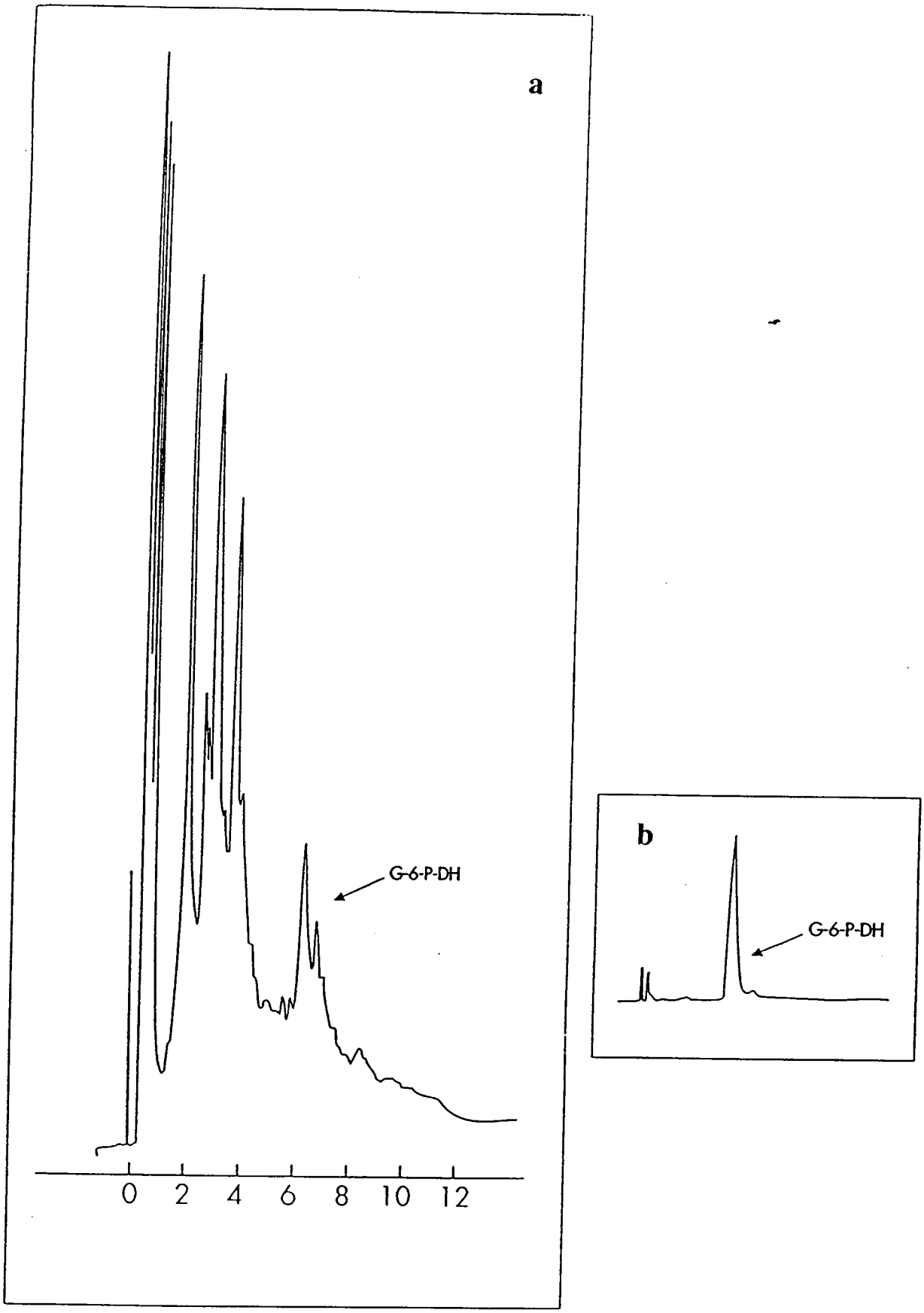


FIGURE 2. a. Separation of ~35 mg of yeast enzyme concentrate at pH 7.6 on a DEAE MemSep-1010 Chromatography Cartridge. b. Rerun of the isolated G6P-DH fraction on a DEAE MemSep-1000.

Single-Step Purification of Mab from Ascites

INTRODUCTION

No two monoclonal antibodies (Mab) are alike. When considering purification strategies, the variation in the physio-chemical nature of monoclonals necessitates a variety of separation techniques to draw upon. The considerations for an isolation method includes: purity requirements, which depend upon the intended use and application of the purified Mab; the scale-up potential of the method; and the stability and bioactivity of the recovered material. Here, the utility of the DEAE MemSep anion exchanger is examined in a single-step purification of a Mab (anti-scorpion toxin II-3C5) from murine ascites.

EXPERIMENTAL PROTOCOL / RESULTS

Employing the membrane-based MemSep anion exchange (DEAE) cartridge, variations in chromatographic conditions such as the pH of the loading buffer, and the gradient elution conditions were examined in the isolation of anti-scorpion toxin antibody from murine ascites.

The loading buffer (A) was comprised of 20 mM Tris-HCl, at pH 7.2 or pH 8.0 as indicated. The elution buffer (B) was made up of 20 mM Tris-HCl and 1.0 M NaCl, pH 8.0. The mouse ascites used in this study was developed according to the published procedure (Bahraui, E., et al., J. Immunology, 141, 214-220, 1988), and was the kind gift of the author.

Before applying the ascites to the DEAE MemSep, 200 ul was filtered through a Millex-GV 0.22 um filter unit, followed by a four-fold dilution with buffer A. For all chromatographic runs, the MemSep was equilibrated with buffer A at 3 ml/min for at least 5 minutes prior to sample loading. A linear gradient and/or step gradient was employed as indicated (see Fig. 1 & 2). A flow rate of 3 ml/min was used with detection at 280 nm.

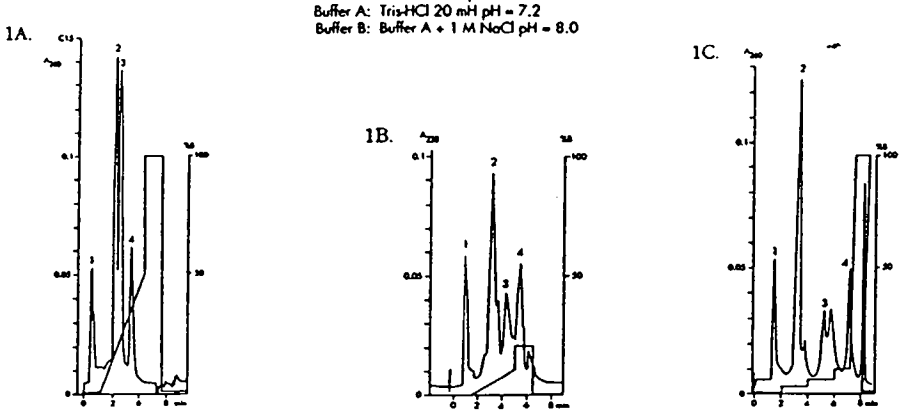
Each of the major peaks eluting from the MemSep anion exchanger was collected and subjected to further fractionation by gel filtration chromatography using a Waters Protein-Pak 300SW column. This column was first equilibrated with the running buffer which was comprised of 50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4. The column was then calibrated using High Molecular Weight Markers (Sigma).

The peak designated as number 2 in Figure 1 & 2 consistently resulted in a single peak on the gel filtration column which co-chromatographed with the molecular weight of the scorpion toxin II Mab. The purity of the recovered Mab was further examined and verified employing SDS-PAGE.

The purification of Mabs from ascites using DEAE MemSep is an attractive alternative to affinity techniques for several reasons. Near physiological pH conditions may potentially enhance the stability and activity of the recovered Mab. The ion exchange procedure is directly scalable. In addition, unlike protein-A, ion exchange may be used to purify any monoclonal, and may purify monoclonal from host IgG.

Figure 1. Separation of anti-scorpion toxin II Mab (3C5) from mouse ascites with loading buffer at pH 7.2 under various gradient elution conditions. The target Mab eluted in peak number 2.

Sample: Mouse Ascites 200 µL (1:24)
Column: DEAE MemSep 1000
Buffer A: Tris-HCl 20 mM pH = 7.2
Buffer B: Buffer A + 1 M NaCl pH = 8.0



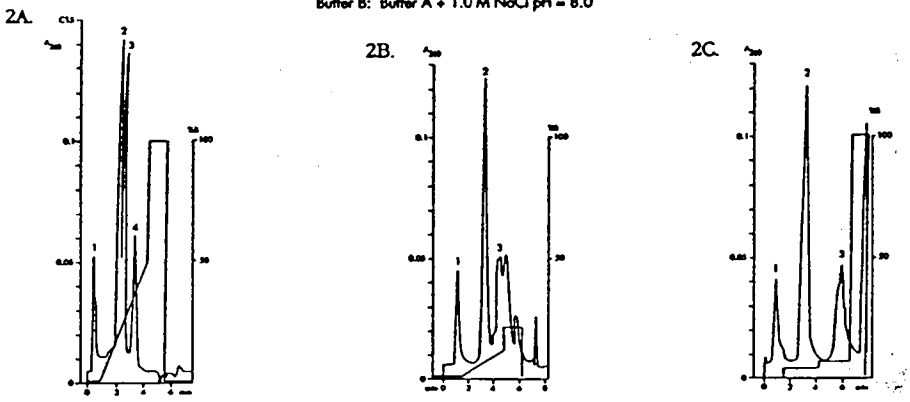
0% B for 1 min.
0-50% B for 5 min.

0% B for 1.75 min.
0-10%B for 5 min.

0% B for 2 min.
2.5% B for 2 min.
5% B for 2 min.
10% B for 1 min.

Figure 2. Separation of anti-scorpion toxin II Mab (3C5) from mouse ascites with the loading buffer at pH 8.0 under the given elution conditions. The anti-scorpion toxin II Mab is contained in peak number 2.

Sample: Mouse Ascites 200 µL (1:4)
Column: DEAE MemSep 1000
Buffer A: Tris-HCl 20 mM pH = 8
Buffer B: Buffer A + 1.0 M NaCl pH = 8.0



0% B for 1 min.
0-50% B for 5 min.

0% B for 1.75 min.
0-10% B for 5 min.

0% B for 1.5 min.
2.5% B for 2.5 min.
5% B for 2 min.

SEPARATION OF CYTOCHROME C FROM FOUR DIFFERENT SPECIES

INTRODUCTION

The separation of complex protein mixtures always presents a challenge to liquid chromatography. If the proteins are very similar in amino acid composition. shape and size the task becomes even greater. These separations require the highest column efficiency and resolution.

In this study MemSep Chromatography was examined in the separation of cytochrome c from four different species. A mixture of these cytochromes are difficult to separate on any chromatography system. There are three amino acids out of 104, and a single charge difference between cytochrome c from chicken, cow, pigeon and horse. Their molecular weights differ by 111 units. The separation of these four proteins require the column to perceive the very small difference in charge density and composition.

EXPERIMENTAL PROTOCOL / RESULTS

All separations were performed using the Waters 650 Advanced Protein Purification System and Auto-Blend* method for rapid optimization of protein separations. The three chromatograms were run in 33 minutes (11 minutes each) under the following conditions.

The three separate chromatographic runs were conducted at pH 5.0, 5.25 and 5.6 as indicated (Fig. 1, 2 and 3). The loading buffer in each run was 20 mM sodium acetate. The proteins were eluted using a linear gradient from 0 to 0.15 M NaCl in 20 mM sodium acetate buffer. The flow rate was 5.6 ml/min with detection at 390 nm. A total og 5 mg of the protein mix was applied to the column for each run

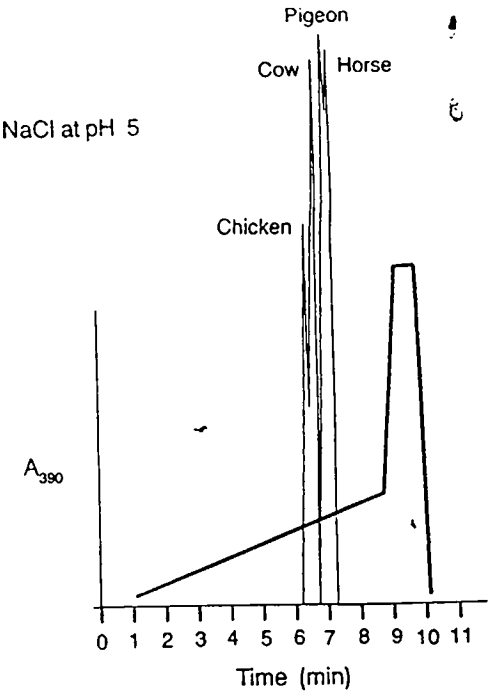
Figure 1. Fractionation of the cytochrome mix at pH 5.0 on the CM MemSep-1000

Figure 2. Fractionation of the cytochrome mix at pH 5.25 on the CM MemSep-1000

Figure 3. Fractionation of the cytochrome mix at pH 5.6 on the CM MemSep-1000

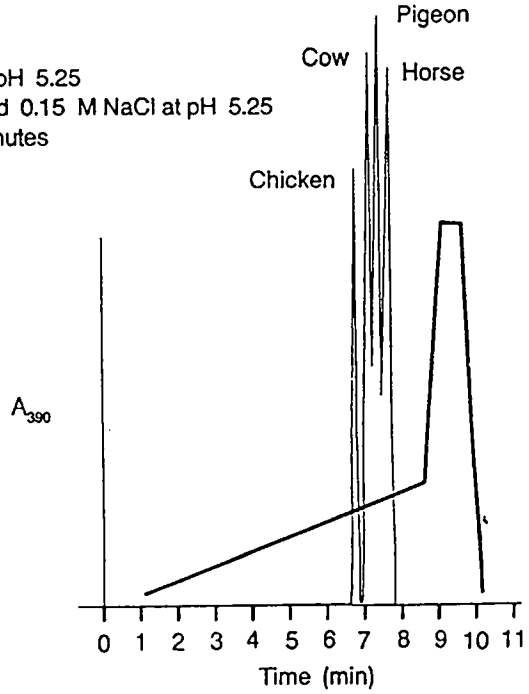
Initial Conditions: 20 mM NaAc at pH 5
Final Conditions 20 mM NaAc and 0.15 M NaCl at pH 5
Gradient: Linear in 8.5 minutes
Flow Rate: 5.6 ml/minute
Detection: 390 nm

FIG. 1



Initial Conditions: 20 mM NaAc at pH 5.25
Final Conditions 20 mM NaAc and 0.15 M NaCl at pH 5.25
Gradient: Linear in 8.5 minutes
Flow Rate: 5.6 ml/minute
Detection: 390 nm

FIG. 2



Initial Conditions: 20 mM NaAc at pH 5.6
Final Conditions 20 mM NaAc and 0.15 M NaCl at pH 5.6
Gradient: Linear in 8.5 minutes
Flow Rate: 5.6 ml/minute
Detection: 390 nm

FIG. 3