A Simple, Two-Component Buffer Enhances Use of Chromatofocusing for Processing of Therapeutic Proteins

Kathleen A. Logan,[1] Inger Lagerlund,[2] Steven M. Chamow[3]

[1]Natural Sciences Department, College of Notre Dame, Belmont, California
[2]Amersham Pharmacia Biotech, Uppsala, Sweden
[3]Department of Recovery Sciences, Genentech, Inc., South San Francisco, California

Received 22 January 1998; accepted 9 July 1998

Abstract: To extend the feasibility of chromatofocusing to industrial use, we have developed a simple chromatofocusing buffer system capable of generating a smooth pH gradient without the use of an external gradient maker. Using two cationic buffering components, an internal pH gradient is produced on appropriate chromatography media over a broad pH range (9.5 to 5.0). The utility of this buffer system is demonstrated with PBE94 and DEAE Sepharose fast flow ion-exchangers, as well as with experimental fast flow chromatofocusing gels. Using a rapid flow rate, we evaluated this buffer system for recovery of a therapeutic protein from a bacterial cell extract. The simplicity of the buffer system requiring no external gradient maker, coupled with the use of fast flow chromatographic media to produce broad-range pH gradients, improves the scalability of chromatofocusing for processing of therapeutic proteins. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* **62**: 208–215, 1999.

Keywords: chromatofocusing; pH gradients; preparative chromatography; therapeutic proteins; multicomponent buffers; biochemical manufacturing

INTRODUCTION

Ion-exchange chromatography is widely used in the biotechnology industry for the purification of proteins from complex mixtures. The recent development of mechanically rigid chromatography media that are capable of withstanding high flow rates (e.g., highly crosslinked fast flow agarose) has enhanced performance in large-scale protein chromatography in which kilograms of protein are produced. Isoelectric focusing (IEF) is the electrophoretic separation of proteins based on isoelectric points and is a powerful tool for analysis of a complex mixture of proteins. Combining elements of both methods, chromatofocusing extends the approach of analytical IEF to ion-exchange column chromatography (Giri, 1990; Hutchens, 1989; Sluyterman, 1982; Sluyterman and Elgersma, 1978; Sluyterman and Wijdenes, 1978).

In chromatofocusing, a pH gradient is formed internally as the mobile phase migrates through the column. Bound protein species separate and generally elute in order of their isoelectric points. Because of its potential for resolving species with only slightly different charge characteristics, this method is often employed in the isolation and characterization of closely related protein species such as isozymes (Giri, 1990; Hutchens, 1989). The chromatofocusing operation is performed typically as follows: the column is equilibrated in a low ionic strength buffer at alkaline pH and the protein sample is applied. The pH gradient is then produced internally by application of ''Polybuffer'' or ''buffer cocktail'' adjusted to a pH lower than that used for equilibration. Each protein elutes generally near its isoelectric point; however, a protein may elute as much as 1 or 2 pH units from its pI. (See Hutchens [1989] for a helpful discussion of protein surface charge and comparison of chromatofocusing with isoelectric focusing.)

Performance is most predictable when a smooth pH gradient is produced. Uniform buffering capacity in both mobile and stationary phases is required to achieve a smooth pH gradient (Hutchens, 1989; Sluyterman and Elgersma, 1978). Polybuffer (Amersham), the commercially available mobile phase most often employed, is a proprietary mixture of polyampholytes (Righetti et al., 1988) developed specifically for use in chromatofocusing. A significant disadvantage of Polybuffer for preparative use is that removal of polyampholytes from eluted proteins is not straightforward (Hutchens, 1989; Hutchens et al., 1986a; Righetti, 1990). Also commercially available are Buffalyte (Pierce) cocktails, solutions of simple amphoteric and nonamphoteric reagents. However, the gradients reported with Buffalyte are not as smooth as with polyampholytes (Hearn and Lyttle, 1981; Liu and Anderson, 1997). Although these commercial preparations have found wide use analytically, their value in preparative applications is limited (Strong and Frey, 1997). To realize the full potential of chromatofocusing for use in biochemical manufacturing, buffer systems that contain nonproprietary and inexpensive buffering components that can be easily removed from protein solutions are needed. To this end, several laboratories have developed alternative chromatofocusing buffer systems employing multicompo-

Correspondence to: S. M. Chamow, Scios, Inc., 2450 Bayshore Parkway, Mountain View, CA 94043; telephone: (650)-940-6672; fax (650)-962-5900; e-mail: chamow@sciosinc.com.

CCC 0006-3592/99/020208-08

nent buffers (Hearn and Lyttle, 1981; Hutchens et al., 1986a, b; Janeck et al., 1991; Liu and Anderson, 1997; Strong and Frey, 1997); however, most are too complex to be easily adapted for industrial-scale use.

In combination with these mobile phase buffers, a variety of stationary phase media have been employed (Giri, 1990; Hearn and Lyttle, 1981; Hutchens, 1989) that provide buffering over the desired range. The polybuffer exchangers PBE94 and PBE118 (Amersham) are agarose-based media produced specifically for use in chromatofocusing. These are weak anion exchangers containing a mixture of tertiary and quaternary amines as ligands immobilized on Sepharose CL-6B. The compressible agarose matrix of PBE, which is only moderately crosslinked, limits the flow rate at which it can be operated to approximately 100 cm/h (Amersham, 1994). Indeed, a highly crosslinked fast flow alternative to PBE would improve scalability.

In this work, we have focused on extending the use of chromatofocusing for application in biochemical manufacturing. We report here the development of a two-component mixture of simple buffering species that produces a smooth gradient over a broad range of pH (9.5 to 5.0). We evaluated the performance of this buffer system with PBE94 and with several fast flow chromatographic media that meet the pressure and flow-rate needs of biochemical manufacturing. We demonstrate that this two-component buffer system gives excellent performance. Combined with highly crosslinked media such as DEAE Sepharose fast flow, this simplified buffer system has the potential to extend chromatofocusing to industrial-scale operation.

MATERIALS AND METHODS

Mobile Phase Buffers

Polybuffer 96 and Pharmalyte 8-10.5 were purchased from Amersham (Piscataway, NJ); diethanolamine 99%, ethanolamine 99+%, imidazole 99%, *N*-methyldiethanolamine 99%, and piperazine 99% from Aldrich (Milwaukee, WI); imidazole, Tris free base, and guanidine HCl from Research Organics (Cleveland, OH); and triethanolamine from Sigma Chemical Co. (St. Louis, MO). All other reagents were analytical grade. Equilibration and elution buffers included 20% (w/v) glycerol. Multicomponent buffers (multibuffers) were usually prepared from 100 m*M* stock solutions of the appropriate components. Buffers were adjusted to the designated pH with HCl, 0.2-μm filtered, and degassed for approximately 5 min. The equilibration buffers were made fresh as needed or stored under nitrogen to maintain the pH at or above 9.5. The pH of fractions was measured immediately upon completion of the experiment to minimize CO_2 absorption. Titration of the mobile phase with 0.20 *M* HCl was performed on 100-mL aliquots of each buffer (minus glycerol) with NaCl added to 50 m*M*.

Stationary Phase Media

All chromatography media were from Amersham (Piscataway, NJ and Uppsala, Sweden). Three anion exchangers included in this study are Q Sepharose fast flow, DEAE Sepharose fast flow (high capacity), and ANX Sepharose 4 fast flow (low sub). In addition, four chromatofocusing gels were studied: Polybuffer Exchanger PBE94 (Lot # 06281, average buffer capacity 31 μmol/pH unit · mL, based on Sepharose CL-6B), and three experimental gels prepared for this study (average buffer capacities 25, 42, and 53 μmol/pH unit · mL, based on Sepharose 6 fast flow).

Sepharose and Polybuffer are trademarks owned by Amersham; Buffalyte is a trademark owned by Pierce Chemical Co.

Chromatographic Equipment and Procedures

Columns were packed in 15.0 × 1.0 cm i.d. columns with flow adapters (Omnifit) from Western Analytical Products Inc. (Temecula, CA); buffer and sample were delivered using a peristaltic pump (Masterflex, Cole-Palmer, Niles, IL); eluent was monitored using a 2138 Uvicord-S detector (Amersham). pH of fractions was measured using a Radiometer PHM82 standard pH meter or a Beckman Φ45 pH meter, and absorbance at 280 nm was measured with a Hewlett Packard 8452A diode-array spectrophotometer. Chromatography was performed at room temperature. Columns were prepared as follows: they were (a) packed at 75 or 225 cm/h in 20% ethanol and washed with water; (b) charged with chloride using 2 *M* NaCl, 10 m*M* Tris HCl (pH 8) (3 CV); and (c) equilibrated with 100 m*M* diethanolamine or piperazine (4 CV), followed by equilibration buffer. Flow rate and pH are indicated in the figure legends. We used a shorter column height than the approximate 20 to 30 cm most often used in chromatofocusing and a high linear flow rate in all experiments (Amersham, 1981). The protein sample (in equilibration buffer) was loaded and the column washed with 2 CV of equilibration buffer. The pH gradient was generated and the proteins were eluted using 15 CV of multibuffer or Polybuffer. Fractions (3 mL) were collected during elution and the pH measured immediately upon completion of the experiment. Columns were regenerated using one of three procedures: (a) 2 CV of 2 *M* NaCl, 10 m*M* Tris-Cl (pH 8); (b) 1 CV of 50% (v/v) acetic acid followed by 4 CV of 2 *M* NaCl, 100 m*M* Tris-Cl (pH 8); or (c) 3 CV of 6 *M* guanidine HCl, 20 m*M* Tris-HCl (pH 8). Columns were stored in regeneration buffer. Peak resolution, R_s, was calculated as: $R_s = (V_2 - V_1)/(w1/2_1 + w1/2_2)$, where V is the elution volume of each peak at its maximum and $w1/2$ is the peak width at one-half height (Amersham, 1994). V and $w1/2$ were measured from Uvicord S-generated chromatograms.

Purification of Recombinant Human Growth Hormone

E. coli frozen cell paste was thawed and suspended in 10 m*M* Tris (pH 8); the periplasmic fraction was released by

osmotic shock. This fraction, containing recombinant human growth hormone (rhGH), was clarified by centrifugation and 0.45-μm filtration and exchanged into equilibration buffer. A sample (12 mL containing 1.5 mg of rhGH) was loaded on a CF_{53}-FF column (9.0 × 1.0 cm) equilibrated in 10 m*M* piperazine (pH 10.3). The column was eluted using Multibuffer-2 (pH 5.0). Buffers contained 20% glycerol. The column was regenerated using 6 *M* guanidine HCl, 10 m*M* Tris-Cl (pH 8). The flow rate was 225 cm/h; fractions (3 mL) were collected during elution and regeneration. Recovery of total protein was monitored by A_{280}, and rhGH was quantified by HPLC. Fold purification was calculated as milligrams of rhGH per milligram of protein in the column pool (fractions 39 to 46) relative to the column load.

Proteins

Horse skeletal muscle myoglobin (pI 7.3 and 6.8, isozymes not resolved in this study), bovine pancreas ribonuclease A type IIIA (pI 9.3), and bovine erythrocyte carbonic anhydrase (pI 6.8) were from Sigma; rhGH (Nutropin, pI 6.0) and CD4-IgG (pI 9.0) were produced at Genentech (South San Francisco, CA). Individual stock solutions of proteins (5 mg/mL in water) were stored at −20°C until the time of use. Protein stock solutions were combined and transferred into equilibration buffer using prepacked Sephadex G-25 columns (PD-10, Amersham). Each model protein sample was prepared in a 3-mL volume for loading onto chromatofocusing columns.

Gel Electrophoresis

Samples were analyzed using 10% or 16% SDS-PAGE (Laemmli; 16% precast gels were purchased from Novex, San Diego, CA) and stained with Coomassie blue.

RESULTS

Multicomponent Buffers

Initially, we undertook to develop a five-component buffer system (Multibuffer-5) for use with PBE94 as a downstream manufacturing step in the purification of the recombinant protein, CD4 immunoadhesin (CD4-IgG) (Byrn et al., 1990). Buffer components were chosen such that: (i) the pK_a values of all components differed by about 0.5 pH unit; and (ii) only cationic species provided the buffering (see Table I, part A). Each buffering component was present at 4 m*M;* 20% glycerol was included to enhance protein solubility at low ionic strength and at pHs near the pI. Chloride was used as the counterion. Thus, a chromatofocusing system using chemically defined buffer components was achieved. By avoiding the use of anionic buffering species, the pH gradient was not affected by mobile phase buffering components adsorbing to the column (Liu and Anderson, 1997; Strong and Frey, 1997).

Table I. Composition of the multicomponent buffers.

	pK_a[a]	ΔpH
A. Multibuffer-5 and Multibuffer-6 (components at 4 m*M*)		
Ethanolamine[b]	9.50	—
Diethanolamine	8.88	0.62
N-methyldiethanolamine	8.52	0.36
Tris	8.06	0.46
Triethanolamine	7.76	0.30
Imidazole	6.95	0.81
B. Multibuffer-2 (components at 15 m*M*)		
Piperazine	9.81 (pK_2)	—
Triethanolamine	7.76	2.05
Piperazine	5.55 (pK_1)	2.21

[a]At 25°C (Perrin and Dempsey, 1974).
[b]In Multibuffer-6 only.

To compare the performance of Multibuffer-5 with that of a commercial mobile phase preparation, CD4-IgG was eluted from a column of PBE94 and fractions analyzed by SDS-PAGE. CD4-IgG is a recombinant, homodimeric glycoprotein (Harris et al., 1990). Representative profiles using Polybuffer 96/Pharmalyte 8-10.5, the mixture recommended (Amersham, 1985) for operation from pH 9 to 7, and Multibuffer-5 on PBE94 are shown (Fig. 1). Elution profiles were similar with both buffer systems. Both Polybuffer and Multibuffer-5 generated smooth pH gradients. To illustrate the presence of polyampholytes found in Polybuffer, which would have to be removed before further processing of a therapeutic protein, SDS-PAGE analysis was performed on untreated column fractions. SDS-PAGE shows the high background that results from fixing and staining the polyampholytes found in Polybuffer (Fig. 1A, lanes 2–8). In contrast, no background staining is observed in similar samples containing the small molecule buffering species in Multibuffer-5 (Fig. 1B, lanes 10–14). Thus, for large-scale preparative applications, Multibuffer-5 would be preferable to Polybuffer.

To optimize the multibuffer further, modifications to Multibuffer-5 were explored. A sixth component (ethanolamine) was added to increase buffering at higher pH values (Table I, part A). Although this Multibuffer-6 worked well, we sought a simpler mixture containing fewer buffering components. Indeed, we found that a similar gradient could be generated using fewer components with more widely spaced pK_a values. Theory predicts that a linear gradient can be achieved with pK_a values separated by 1 pH unit (Righetti, 1990). Nevertheless, a two-component, wide-pH-interval multibuffer (Multibuffer-2) was developed in which the component pK_as differed by approximately 2 pH units (Table I, part B). In Multibuffer-2, piperazine with two pK_as provides buffering at both high and low pH, whereas triethanolamine buffers near neutral pH. Note that Multibuffer-2 likewise contains only small molecule buffering species thus providing the same advantage as Multibuffer-5 and -6.

To characterize these mobile phase systems, titration curves for the three multibuffers were compared with a Polybuffer mixture (Fig. 2). Somewhat surprisingly, Mul-

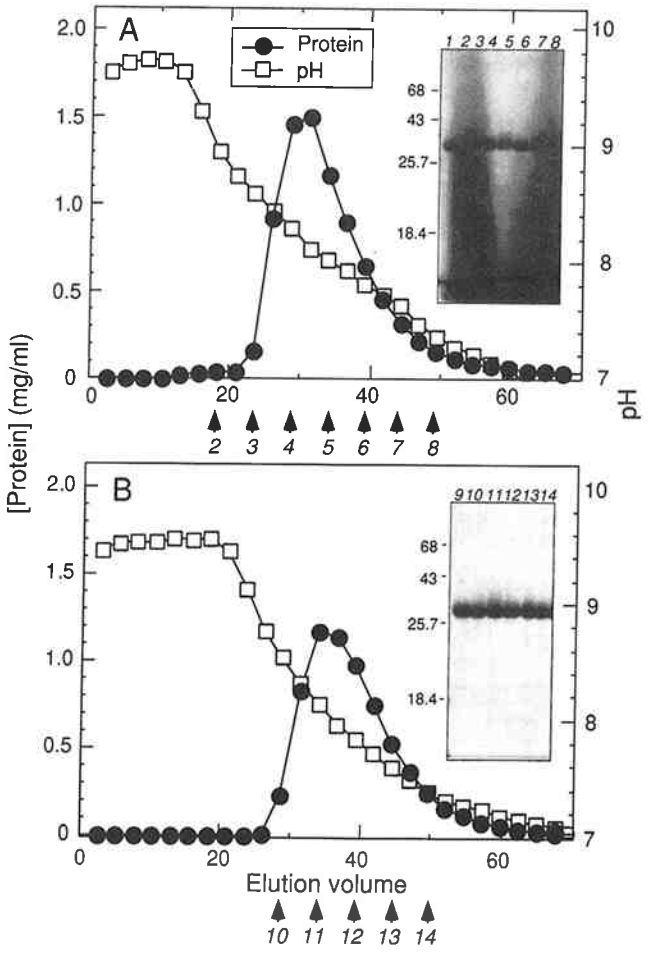

Figure 1. Comparison of Polybuffer and Multibuffer-5 in chromatofocusing on PBE94. (A) Polybuffer. A 6.4 × 1.0 cm (5 mL) PBE94 column was equilibrated with 25 m*M* diethanolamine (pH 9.6). Purified CD4-IgG (24 mg) was applied in equilibration buffer and eluted at 100 cm/h with Polybuffer (pH 6.8). Protein concentration (from A_{280}) and pH are plotted vs. elution volume. (B) Multibuffer-5. Column as in (A), except equilibration was done with 10 m*M* diethanolamine (pH 9.5). Twenty milligrams was loaded and the gradient was generated with Multibuffer-5 (pH 7.0). The insets are Coomassie blue-stained 10% SDS-PAGE of untreated column fractions: load, lanes 1 and 9; Polybuffer peak fractions, lanes 2–8; Multibuffer peak fractions, lanes 10–14. Gel lanes corresponding to peak fractions are indicated below the chromatograms.

tibuffer-2 showed uniform buffering over a range of 5 pH units (approx. 10 to 5). Polybuffer displayed a buffering range slightly less than that of Multibuffer-2 (approx. 10 to 6). Multibuffer-2 produced a superior gradient at the acidic end (pH < 7), relative to Multibuffers-5 and -6. It is possible that the biprotic nature of piperazine can partially explain the uniform buffer capacity seen with Multibuffer-2 (see Rilbe's [1996] comparison of titration curves of biprotic bases compared with two monoprotic species with the same pK_as). Performance of these multibuffers in chromatofocusing is described and compared in what follows.

Characterization of Fast Flow Chromatofocusing Media

To optimize the utility of these multicomponent buffers for chromatofocusing at large scale, a fast flow stationary phase

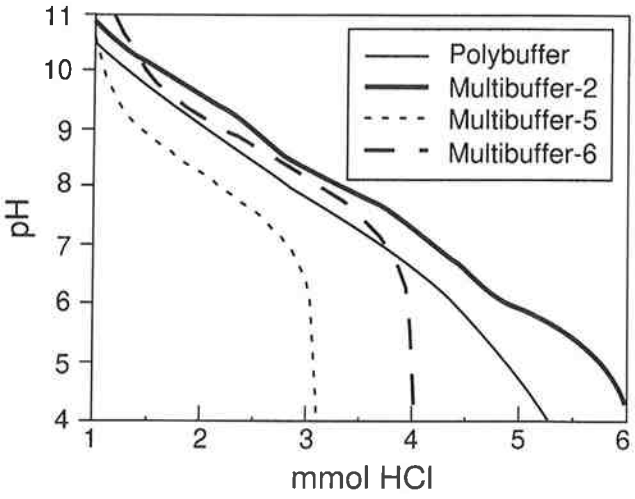

Figure 2. Titration of mobile phases. The indicated buffer mixture in 50 m*M* NaCl (100 mL) was adjusted to pH 11.8 with 1.0 *M* NaOH, then titrated with 0.20 *M* HCl. Polybuffer contained 5.2 mL of Polybuffer 96 and 1.0 mL of Pharmalyte 8-10.5. In Multibuffer-2, each component was at 15 m*M* (see Table I, part B); in Multibuffer-5 and Multibuffer-6, each component was at 4 m*M* (see Table I, part A). pH is plotted vs. millimoles of HCl added.

is needed. Therefore, for this study we prepared three experimental fast flow chromatofocusing gels as high-throughput alternatives to PBE94. Comprised of tertiary and quaternary amines linked to Sepharose 6 fast flow, these gels differed in average buffer capacity due to differences in ligand density. The average buffer capacity was 25, 42, and 53 μmol/pH unit · mL (not shown) for the experimental media, whereas that of PBE94 was 31 μmol/pH unit · mL. We refer to these chromatofocusing fast flow media as CF_{25}-FF, CF_{42}-FF, and CF_{53}-FF, respectively.

A single mobile phase condition was selected to evaluate these chromatographic media. Quality of pH gradients and peak resolution of a mixture of two proteins were evaluated for the three experimental chromatofocusing media and PBE94 using Multibuffer-6 (Fig. 3). Gradient quality for the four media varied with their buffering capacity; the lowest buffering gel (CF_{25}-FF) produced a short, steep pH gradient similar to PBE94, whereas the higher buffering gels (CF_{42}-FF and CF_{53}-FF) produced pH gradients over a broader range. With regard to resolution, the best resolution of the two model proteins was achieved with CF_{53}-FF (R_s = 2.3 with CF_{53}-FF vs. 1.6 with CF_{42}-FF, 1.7 with CF_{25}-FF, and 1.9 with PBE94). Thus, with this six-component buffer, these experimental fast flow media performed comparably to PBE94, and in one case (CF_{53}-FF) performance was improved. Of the experimental media, only CF_{53}-FF was evaluated further.

Performance of Two-Component Multibuffer with Various Media

Having evaluated stationary phases using Multibuffer-6, we focused on Multibuffer-2 for further development. Multibuffer-2 (described earlier and in Table I, part B) was

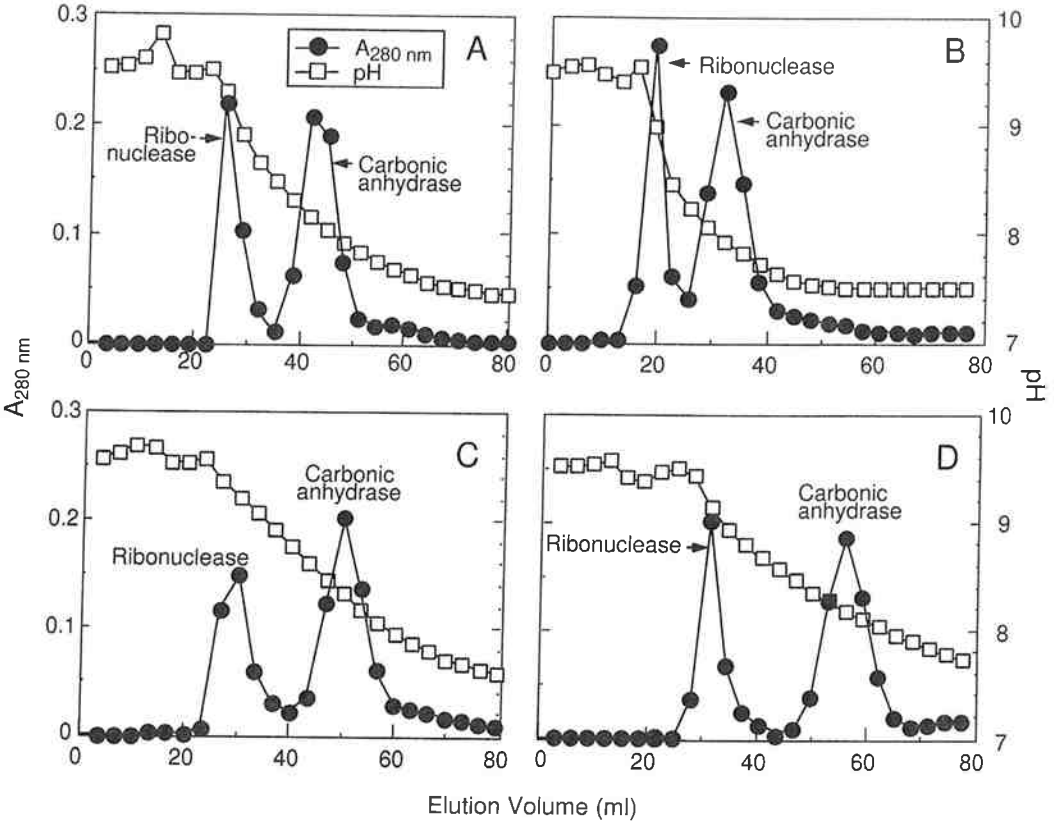

Figure 3. Performance of experimental fast flow chromatofocusing gels using Multibuffer-6. Columns (9.0 × 1.0 cm, 7 mL) packed with (A) PBE94, (B) CF_{25}-FF, (C) CF_{42}-FF, or (D) CF_{53}-FF were equilibrated with 10 m*M* diethanolamine (pH 9.5). Five milliliters of a protein mixture (ribonuclease 3.2 mg, carbonic anhydrase 1.8 mg) was prepared in equilibration buffer and loaded. Columns were operated at 80 cm/h. The pH gradient was generated with Multibuffer-6 (pH 7.5). A_{280} and pH are plotted vs. elution volume.

evaluated on PBE94 and the experimental fast flow gel, CF_{53}-FF, using a mixture of three proteins. Performance was judged on the basis of chromatograms and SDS-PAGE of collected fractions (Fig. 4A and B). For both PBE94 and CF_{53}-FF we obtained a smooth gradient over a pH range of 9.5 to 5.0. Compared with PBE94, CF_{53}-FF (Fig. 4B) produced a shallower pH gradient and resulted in better resolution of the proteins. (For example, for separation of ribonuclease from myoglobin, R_s = 1.5 for CF_{53}-FF and 0.9 for PBE94.) Recovery was approximately 95% for both separations.

The combination of Multibuffer-2 and CF_{53}-FF performed very well at 75 cm/h. To test a higher flow rate, we evaluated this same system at 225 cm/h (Fig. 4C). No effect on the quality of the pH gradient was observed, and the gradient was highly reproducible. Protein recovery again was approximately 95%. There was some loss in protein resolution at 225 cm/h compared with 75 cm/h (for separation of ribonuclease from myoglobin, R_s = 1.1 and 1.5, respectively). Consequently, a slower flow rate was evaluated in one experiment in which the pH gradient was eluted at approximately 25 cm/h, whereas all other column steps were carried out at 225 cm/h. A corresponding increase in peak resolution was achieved (data not shown).

The excellent performance of Multibuffer-2 with CF_{53}-FF prompted us to explore the use of Multibuffer-2 with other fast flow ion exchange media. We tested the ability of Multibuffer-2 to generate internal pH gradients with several commercial anion exchangers: DEAE Sepharose fast flow, ANX Sepharose 4 fast flow, and Q Sepharose fast flow, in addition to PBE94 and CF_{53}-FF (Fig. 4D). Multibuffer-2 was effective in producing a smooth, wide-range, internally generated pH gradient with DEAE Sepharose fast flow, PBE94, and the experimental CF_{53}-FF, consistent with the presence of heterogeneous functional groups on these media. The slope of the gradient was most shallow with CF_{53}-FF, followed by PBE94, then by DEAE Sepharose fast flow. Thus, Multibuffer-2 can be used with any of these media for chromatofocusing. In contrast, ANX and Q Sepharose fast flow produced sharp transitions in pH, consistent with the presence of homogeneous functional groups (tertiary and quaternary amines, respectively).

Purification of a Recombinant Protein from Bacterial Cells Using Multibuffer-2 and CF_{53}-FF

To demonstrate the utility of Multibuffer-2 for chromatofocusing using a fast flow media, we chose, as a test case, purification of a recombinant protein, rhGH, expressed in *E. coli* (Fig. 5). rhGH is a 22-kDa single-chain polypeptide (Gray et al., 1985; Olson et al., 1981) Correctly processed rhGH was recovered from the periplasmic space of bacterial

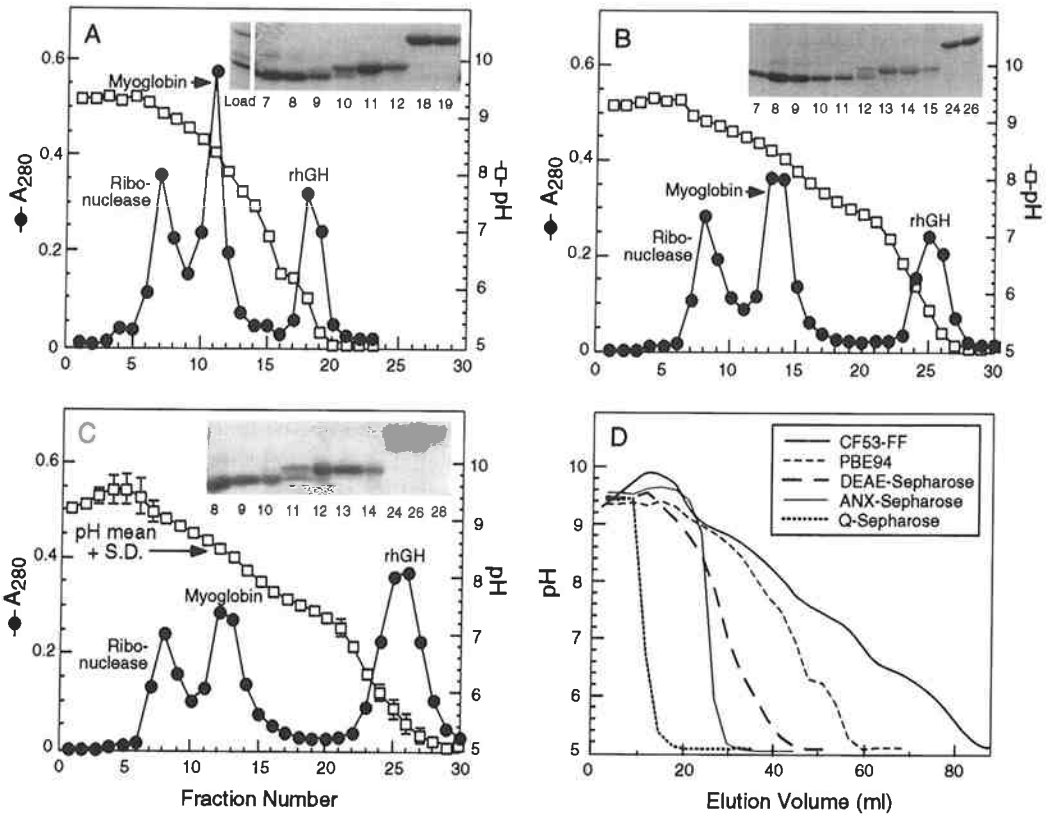

Figure 4. Performance of Multibuffer-2 with different anion exchangers. (A) PBE94 operated at 75 cm/h; (B) CF_{53}-FF operated at 75 cm/h; (C) CF_{53}-FF operated at 225 cm/h. Columns (9.0 × 1.0 cm, 7 mL) were equilibrated with 10 m*M* piperazine (pH 9.4). A mixture of proteins (5 mg ribonuclease, 2 mg myoglobin, and 5 mg rhGH) in equilibration buffer was loaded onto the columns. During the elution phase, the pH gradient was generated using Multibuffer-2 (pH 5.0). A_{280} and pH were measured for each 3-mL fraction. SDS-PAGE analysis (16%) for the indicated fractions is shown in insets; the initial protein mixture (Load) is in the first lane of (A), inset. Note: In (B) and (C), alternate fractions are loaded for the rhGH peak. In (C), the protein mixture that was loaded contained twice the amount (10 mg) of rhGH; the average pH and standard deviation from three experiments are plotted. (D) pH gradients produced on the indicated anion exchangers in columns (9.0 × 1.0 cm) equilibrated with 10 m*M* piperazine (pH 9.5). Elution phase was performed at 225 cm/h (PBE94, 75 cm/h) using Multibuffer-2 (pH 5.0). pH is plotted vs. elution volume.

cells by osmotic shock. This complex mixture was buffer exchanged into 10 m*M* piperazine (pH 10.3) and applied to CF_{53}-FF equilibrated in the same buffer. The column was eluted in a pH gradient generated using Multibuffer-2, adjusted to pH 5.0. All column steps were performed at a flow rate of 225 cm/h. pH 10.3 was used for equilibration and sample loading to test the upper limit of buffering with Multibuffer-2 on CF_{53}-FF. A smooth gradient from pH 9.2 to 5.0 was observed. rhGH eluted in a peak centered at pH 5.5 (fractions 39 to 46); the proteins eluting earlier in the gradient were not identified. A 40-fold purification with 70% recovery of rhGH was achieved in a total run time of 120 min (equilibration through regeneration). Thus, substantial enrichment of rhGH from *E. coli* periplasmic extract was achieved using Multibuffer-2 and CF_{53}-FF, suggesting that this chromatographic system could be applied as an initial purification step. (It may be more optimally used as a polishing step; see Discussion). The parameters that can be varied to optimize this system are discussed next.

DISCUSSION

Chromatofocusing offers internal generation of a pH gradient on a weak anion exchange column in which the functional groups are heterogeneous. This ability to generate a gradient in situ represents a marked advantage of chromatofocusing in designing column operations for the manufacture of therapeutic proteins. Despite its potential, several factors have limited the use of chromatofocusing in industrial environments, including the expense of polyampholytes and other proprietary mobile phases, the potential difficulty in removing these substances from protein solutions, and the limited availability of chromatofocusing media suitable for large-scale operation.

Previous attempts have been made to circumvent the mobile phase problem by designing multicomponent buffer systems. For example, buffers employing 14 to 17 components and consisting of common amphoteric and nonamphoteric buffers were described by Hearn and Lyttle (1981). Hutchens et al. (1986a and b) used up to 31 components to produce linear gradients. Janeck et al. (1991) employed four to six components to produce ascending gradients; only acidic or basic buffering species were used. Strong and Frey (1997), using two or three buffering components, purposefully produced step gradients to concentrate the eluted protein. We have focused our work, as did Strong and Frey, on the potential of chromatofocusing as a scalable manufactur-

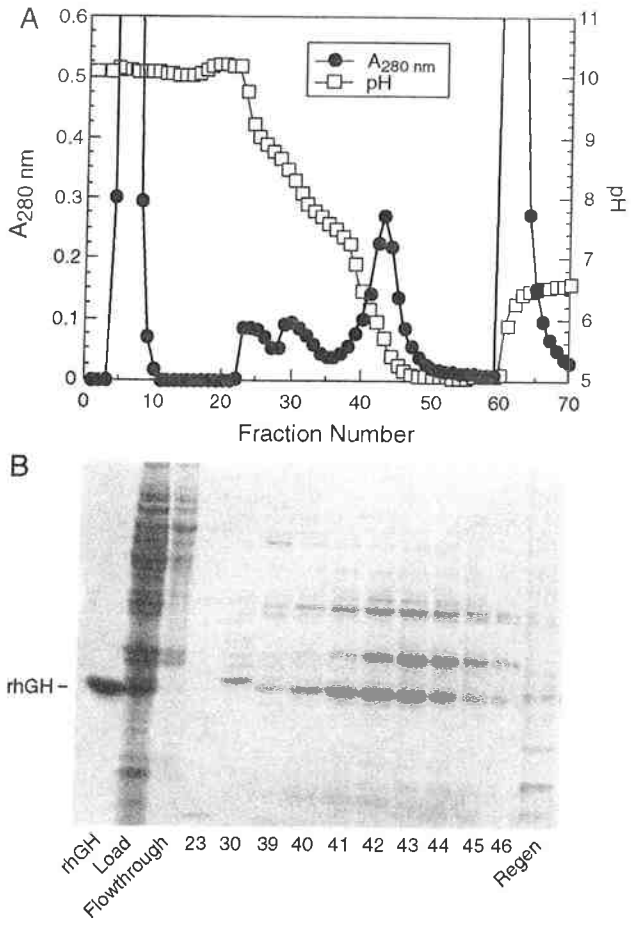

Figure 5. Purification of a recombinant protein from a bacterial periplasmic extract using Multibuffer-2 with CF_{53}-FF. A sample (12 mL containing 1.5 mg of rhGH) was loaded on a CF_{53}-FF column (9.0 × 1.0 cm) equilibrated in 10 m*M* piperazine (pH 10.3). The column was eluted using Multibuffer-2 (pH 5.0). The flow rate was 225 cm/h; fractions (3 mL) were collected during elution and regeneration. (A) Chromatogram: A_{280} and pH are plotted vs. fraction number. (B) SDS-PAGE analysis (16%) shows column load, flow-through (pooled fractions 4 to 9), the indicated pH gradient fractions (23, 30, 39 to 46), and regeneration (fraction 61). Purified rhGH is included as a standard to identify the product band.

ing operation. We have designed a mobile phase containing just two simple buffering species, which produce a linear gradient suitable for use in large-scale production of therapeutic proteins.

For large-scale operation of a chromatofocusing column (e.g., 20- to 100-L column volume), several variables can be manipulated to optimize performance. These variables include the range and slope of the pH gradient, the nature of the counterion, and the flow rate. Although our aim here was to test the broadest possible pH range, in practice it may be desirable to operate in a narrower range. A narrower pH range with a shallower gradient could improve resolution of closely eluting proteins. Preliminary experiments suggest that this is feasible. In one rhGH purification study, we achieved a shallower gradient using triethanolamine (pH 7.8) to equilibrate and Multibuffer-2 at half-concentration to elute (data not shown). Thus, it is possible to manipulate the gradient slope by altering the concentration of the multibuffer. In addition, by equilibrating the column at a lower pH (e.g., 8.0 or 7.0), protein degradation from exposure to extremes of pH (Patel and Borchardt, 1990) can be minimized.

For the cationic multibuffers in this study, chloride was used as counterion. In manufacturing practice, less corrosive acids may be desirable. Other potential counterions include acetate, formate, and sulfate. Initial experiments employing acetate in Multibuffer-2 (pH 5.0) gave unsatisfactory results, presumably due to participation of acetate as a buffering species (pK_a 4.8). Because ion effects can be quite empirical (Kopaciewicz and Regnier, 1983), counterion selection must be tailored to the application.

Column throughout is limited by flow rate, which in turn is limited primarily by the rigidity of the chromatography media and by system constraints (Sofer and Nystrom, 1989). A variety of ion-exchange media have been employed for chromatofocusing of proteins, including DEAE cellulose (Hearn and Lyttle, 1981), Bakerbond PEI (Hutchens et al., 1986b), PBE94 (Giri, 1990), and Q Sepharose fast flow (Strong and Frey, 1997). Our results confirm the following for a given mobile phase: the higher the buffering capacity of the stationary phase, the shallower the gradient and the better the resolution. PBE94 works well for chromatofocusing due to its buffer capacity; however, it is not easily scalable because it is not a rigid medium. In contrast, use of fast flow media allows for a higher flow rate and minimal run time, without exceeding pressure limitations. Thus, a medium that combines rigidity with buffer capacity over a broad pH range is preferred for chromatofocusing to be scaled easily. These criteria are met by the experimental fast flow chromatofocusing media and by the DEAE Sepharose fast flow used in this study.

Although the rhGH extract was selected here simply to demonstrate the use of Multibuffer-2 and fast flow media with a complex feedstock, we intend to further address parameters that can be modified to optimize the system for maximum resolution and recovery. We have not attempted here to resolve closely related protein species. Nevertheless, increased resolution could be achieved by slowing the flow rate for the pH gradient immediately in the range of the protein of interest (e.g., pH 6.5 to 5.5 for rhGH) (Giri, 1990). Such a modification does not obviate the advantage of chromatofocusing fast flow media for one step in a purification process because a high flow rate can be maintained for all other phases of column operation.

In practice, chromatofocusing could be employed at any stage in a purification process. Sample conditioning could be achieved by diafiltration or by placing chromatofocusing after a step that produces an eluent of low ionic strength. Indeed, after diafiltration, Multibuffer-5 was employed with PBE94 as a downstream step in CD4-IgG purification with 90% recovery.

In summary, we have developed a practical chromatofocusing system for the purification of therapeutic proteins that is suitable for scale up. We have identified a triethа-