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Development of chromatofocusing techniques employing mixed-mode column packings for protein separations

Hui Guo [a], Xiang Li [b], Douglas D. Frey [a,*]

[a] Department of Chemical, Biochemical and Environmental Engineering, University of Maryland Baltimore County, Baltimore, MD 21250, USA
[b] Department of Biological Sciences, University of Maryland Baltimore County, Baltimore, MD 21250, USA

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ABSTRACT

Recent studies reported in the literature using mixed-mode chromatography (MMC) column packings have shown that multiple modes of interactions between the column packing and proteins can be usefully exploited to yield excellent resolution as well as salt-tolerant adsorption of the target protein. In this study, a mixed-mode separation method using commercially available column packings was explored which combines the techniques of hydrophobic-interaction chromatography and chromatofocusing. Two different column packings, one based on mercapto-ethyl-pyridine (MEP) and the other based on hexylamine (HEA) were investigated with regard to their ability to separate proteins when using internally generated, retained pH gradients. The effects of added salt and urea on the behavior of the retained pH gradient and the protein separation achieved when using MMC column packings for chromatofocusing were also investigated. Numerical simulations using methods developed in previous work were shown to agree with experimental results when using reasonable physical parameters. These numerical simulations were also shown to be a useful qualitative method to select the compositions of the starting and elution buffers in order to achieve desired shapes for the pH and ionic strength gradients. The use of the method to fractionate blood serum was explored as a prototype example application.

1. Introduction

In recent years there has been an increasing interest in developing mixed-mode chromatography (MMC) methods for downstream processing in the biopharmaceutical industry. In particular, MMC is becoming a promising method to improve the selectivity achieved for protein separations [1,2]. The most commonly used MMC method for protein separations is the combination of hydrophobic-interaction chromatography (HIC) and ion-exchange chromatography (IEC) [3–7]. A particularly successful class of MMC, termed hydrophobic charge induction chromatography (HCIC), has been proposed by Burton and Harding [8–12]. In contrast to more common applications of MMC where multiple interaction modes simultaneously influence the adsorption of molecules, HCIC is based on the pH-dependent behavior of ligands that are more hydrophobic and uncharged at neutral or higher pH and ionize at lower pH so that during gradient elution the nature of the interactions between the protein and column packing varies significantly with time. More specifically, in HCIC proteins bind to the column packing ligands by hydrophobic interaction at the beginning of the process, and with a reduction of the fluid phase pH the bound proteins will be eluted by electrical charge repulsion. In addition to using a combination of HIC and IEC, other forms of MMC have also been developed, such as those that combine hydrophilic-interaction chromatography (HILIC) and IEC [13–16].

Mixed-mode chromatography has several significant advantages as compared to traditional single-mode chromatography when applied to the purification of proteins. Since proteins are amphiphilic molecules with both hydrophobic and hydrophilic surfaces, MMC can improve selectivity and also potentially achieve so-called "salt-independent" adsorption where proteins are able to adsorb onto the column packing at moderately high salt concentrations due to hydrophobic interactions, particularly if the multimodal components of the protein and column packing are complementary. Consequently, MMC can facilitate process step transitions such as performing ion-exchange chromatography directly on filtered cell extract without an intermediate desalting step [17]. Another advantage of MMC is that it may facilitate a reduction in the number of chromatographic steps by performing orthogonal chromatography techniques in a single column [6,7]. Reducing the number of chromatographic steps in this way is likely to increase yield and reduce the processing time so that the overall throughput is improved.

Despite the significant amount of past research regarding MMC for protein separation and purification described previously in the literature, no studies have been reported on the use in these

* Corresponding author. Tel.: +1 410 455 3418; fax: +1 410 455 6500.
E-mail address: dfrey1@umbc.edu (D.D. Frey).

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systems of retained pH gradients that are entirely generated internally, which is a technique generally termed "chromatofocusing." One recent study, however, separated proteins by using a mixed-mode column packing and an externally produced pH gradient that evidently was partly retained [18]. This study also demonstrated the ability of pH gradient elution to achieve better resolution as compared to the salt gradient elution for the case of separating α-lactalbumin, trypsin inhibitor, and β-lactoglobulin A using a mixed-mode cation exchanger.

Chromatofocusing is a variant of IEC where, in contrast to standard forms of IEC which employ an unretained salt gradient, a retained pH gradient is formed entirely inside the column by utilizing the buffering capacity of the column packing and the adsorption characteristics of the buffering species. The original version of this technique was developed by Sluyterman and coworkers who employed polyampholyte elution buffers similar to those used in isoelectric focusing [19,20]. The method has been investigated and further developed by a number of researchers over the last decade who have used simple mixtures of buffering species instead of polyampholyte buffers to form the pH gradient [21–31]. In this study, chromatofocusing will be extended to the use of mixed-mode column packings by employing these methods along with numerical simulations to aid in the selection of the buffer composition. In addition, it will be demonstrated in this study that additives such as urea or a neutral salt can be employed to usefully adjust the protein retention behavior.

Another goal of this work is to explore the use of chromatofocusing with a mixed-mode column packing for the fractionation of blood plasma. The blood plasma fractionation industry produces a number of commercial therapeutic proteins such as immunoglobulins and albumin, and it is by far the largest segment in global therapeutic protein manufacturing in terms of mass produced [32]. The Cohn process, which incorporates cold ethanol fractionation, is the oldest and most widely used method for blood plasma fractionation [33], but the method often exhibits poor yield and the albumin produced generally has relatively low purity [34]. For these reasons, chromatography in combination with ultrafiltration has been widely investigated since the 1980s as a means to improve the purity and yield in blood plasma fractionation. Among the possible alternatives, dye-ligand affinity chromatography [35] and immobilized metal chelate affinity chromatography [36] have shown considerable promise, although the higher cost of these methods has inhibited their widespread use so that there is a need to develop lower cost chromatographic methods for plasma fractionation.

2. Experimental

2.1. Materials

Myoglobin from equine skeletal muscle, cytochrome C from horse heart, lysozyme from chicken egg white, α chymotrypsinogen A from bovine pancreas, and bovine serum albumin were products M0630, C2506, L7651, C4879, A7638, respectively, obtained from Sigma–Aldrich (St. Louis, MO, USA). Rabbit blood serum (i.e., blood plasma with the clotting factors removed) was obtained from Covance Inc. (Princeton, NJ, USA) and stored at −20 °C until use.

Tris(hydroxymethyl)-aminomethane (Tris), 2-(N-morpholino)-ethanesulfonic acid (MES), 2-(cyclohexylamino)-ethanesulfonic acid (CHES), N-tris(hydroxymethyl)-methyl-3-amino-propanesulfonic acid (TAPS), N-tris(hydroxylmethyl)-methyl-2-aminoethanesulfonic acid (TES), 3-(N-morpholino)-propanesulfonic acid (MOPS), citric acid, urea, ethanol, PBS, NaOH, NaCl and HCl were also obtained from Sigma–Aldrich. Formic acid was obtained from J.T. Baker (Philipsburg, NJ, USA).

All buffer compositions are described in the figure captions corresponding to each experiment, and the buffer solutions were prepared using distilled water and were degassed by vacuum filtering using disposable filter units with nylon membranes having 0.2 μm pores (Part No. 0974024A, Thermo Fisher Scientific, Waltham, MA, USA). To produce a sample for injection, proteins were dissolved into a starting buffer and filtered with a nylon syringe filter having 0.2 μm pores (Part No. 431215, Corning Life Sciences, Lowell, MA, USA).

2.2. Columns

MEP HyperCel and HEA HyperCel particles (Pall Life Sciences, Port Washington, NY, USA) which were 90 μm in diameter were slurry packed into a 10-cm long glass Omnifit column (Diba Industries, Danbury, CT, USA) with 1.0 cm internal diameter and with one end fitting being adjustable in length. The column was packed using PBS buffer at a flow rate of 4 ml/min, and the packing process was terminated when the height of the bed became constant. The final height of the packed bed produced by this process varied from 3.8 to 6.6 cm. After packing, the column was washed with 20% (v/v) ethanol in deionized water for overnight at a flow rate of 0.1 ml/min.

2.3. Equipment

Experiments were performed using a LC Packings Ultimate HPLC instrument (now Thermo Scientific Dionex, Sunnyvale, CA, USA) and an Orion (now Thermo Scientific Orion, Beverly, MA, USA) model 520A pH meter. A Model FC49K 50 μl internal volume flow cell and a Model 450CD pH electrode (Sensorex, Garden Grove, CA, USA) were used to directly measure the pH of the column effluent. The same pH meter and electrode were used for measuring both the elution buffer pH and column effluent pH in order to enhance the accuracy of the pH measurements. All the chromatography experiments were controlled by Chromeleon software version 6.6 (Thermo Scientific Dionex).

2.4. Chromatofocusing experiments

To perform an experiment, the column was initially equilibrated with the starting buffer. The feed sample was then introduced into the column, and the column was subsequently eluted with a stepwise change to the elution buffer.

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All materials used for SDS-PAGE were obtained from Sigma–Aldrich. To prepare each 10 ml of 12% SDS-PAGE separation gel, 3.4 ml distilled water, 2.5 ml of 1.5 M Tris–HCl (pH 8.8), 0.05 ml of 20% (w/v) SDS, 4 ml of 30% acrylamide/bis-acrylamide (37.5:1), 0.05 ml of 10% ammonium persulfate and 0.01 ml N,N,N′,N′-tetramethylenediamine (TEMED) were mixed. To prepare 10 ml of stacking gel, 6 ml of Milli-Q water, 2.5 ml of 0.5 M Tris–HCl (pH 6.8), 1.33 ml of 30% acrylamide/bis-acrylamide (37.5:1), 0.05 ml of 10% ammonium persulfate and 0.01 ml TEMED were mixed. To prepare a 1.0 mm thick mini-gel, 4.8 ml of separation gel and 2 ml of stacking gel was used. The 10× stock solution of running buffer consisted of 15.0 g Tris, 72.0 g glycine, and 5.0 g SDS in 500 ml deionized water. The 2× stock solution of reducing sample buffer consisted of 1.0 ml of 0.5 M Tris–HCl (pH 6.8), 1.6 ml 10% (w/v) SDS, 2.0 ml glycerol, 0.08 ml 1.0% bromophenol blue, 0.4 ml β-mercaptoethanol, and 2.92 ml deionized water. The electrophoresis was performed at a voltage of 150 V for 1 h using