Journal of Chromatography A, 1233 (2012) 152–155

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Short communication

Improving selectivity in multimodal chromatography using controlled pH gradient elution

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ARTICLE INFO

Article history:
Received 22 November 2011
Received in revised form 23 January 2012
Accepted 25 January 2012
Available online 1 February 2012

Keywords:
Multimodal chromatography
pH gradient
Selectivity
Protein separations

ABSTRACT

Externally generated pH gradients are employed on a multimodal cation exchange chromatographic resin to improve the selectivity for a mixture of model proteins. By combining controlled pH gradients with the unique selectivities arising from the multiple interaction types exhibited by the multimodal resin, the separation of the protein mixture is significantly improved as compared to linear salt gradient operation. Several gradient conditions are explored and a shallow gradient from pH 3.8 to 5.5 is shown to be able to resolve the proteins. This work provides proof of concept for the use of pH gradients in multimodal chromatography and sets the stage for future applications.

1. Introduction

The increasing demands placed on downstream bioprocesses have catalyzed many recent advances in traditional separation processes as well as the emergence of entirely new approaches. These developments will be crucial for the development of efficient purification processes for complex biomolecules in the expanding portfolio of biopharmaceuticals. Charge based chromatographic separations continue to play a major role in downstream bioprocessing, with traditional ion exchange (IEX) processes often relying on an elution strategy where the net charge is decreased, either by increasing the ionic strength or by changing the pH. While many industrial separation processes employ step changes or gradients in salt concentration, superior chromatographic resolution has been obtained with pH-gradient elution strategies [1–4]. Further, pH-gradient elution eliminates the need for buffer exchange or dilution as is typically required when eluting with a high ionic strength solution.

Linear pH gradients have been generated through two different approaches depending on whether the pH gradient is generated internally or externally to the column. During chromatofocusing, the column is equilibrated with a buffer at an initial pH [5,6]. As an elution buffer at a different pH passes through the column, a pH gradient is developed internally during which the proteins desorb from the resin in the order determined by the relative isoelectric points of the proteins. While this technique has been used in a variety of applications [7,8], there are several drawbacks including the requirement of preparing different buffers for each pH range and the fact that the slope of the gradient is proportional to the width of the pH range [4]. Alternatively, the two buffer solutions can be mixed externally to introduce a pH gradient at the column inlet. While such externally controlled pH gradients are often employed for the IEX separation of protein isoforms and proteins with very small differences in isoelectric point (pI) [9–11], they have also been used for separating libraries of proteins containing diverse electrophoretic properties as well as crude protein mixtures [4,12]. A pH-conductivity hybrid gradient elution strategy has been employed for the IEX separation of isoforms of mAbs [13] and has also been demonstrated for process-scale mAb purification, resulting in high product throughput and process reproducibility with good purity and yield [14].

While the majority of pH gradient studies have been performed on IEX resins, there have also been some investigations using other stationary phases. The separation of aggregates from monoclonal antibody (mAb) cell culture has been demonstrated using pH-gradient protein A chromatography [15]. A two-step process consisting of pH gradient IEX chromatography followed by reversed phase chromatography has been used for the separation of complex mixtures of proteins [16] and peptides [17].

A major challenge associated with pH gradient elution is the difficulty of maintaining sufficient buffering capacity over a broad pH range. This challenge has recently been addressed by the

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doi:10.1016/j.chroma.2012.01.074

development of pISep-IEX chromatography for externally controlled pH gradients [4]. This technique employs low ionic strength buffers with overlapping buffering capacities to create ascending, descending, linear, nonlinear, multi-step, or multi-slope pH gradients. The effectiveness of this technique has been demonstrated on cation and anion exchange resins over a broad pH range [4,18,19].

Multimodal (or mixed mode) chromatographic resins can provide alternative and improved affinities and selectivities by offering a combination of interaction types within a single resin [20–24]. The binding selectivity of various biomolecules such as peptides [25], oligonucleotides [26], nucleic acids [27], and oligomer-like compounds [28] has been improved through the use of multimodal resins. The multiplicity of binding interactions between proteins and multimodal resins has been shown to provide stronger binding than with traditional stationary phases [29–31]. To date, there have been very few studies that employ pH-gradients with multimodal chromatography. The elution of mAbs from multimodal (Capto Adhere) and IEX (Capto Q) resins using a pH gradient showed that while the elution order was the same, the pH at which the mAbs eluted was significantly different in the two systems, indicating that the additional interactions were playing a role in the multimodal system [32].

In this investigation, externally generated pH gradients are employed in conjunction with multimodal cation exchange chromatography to carry out the separation of a protein mixture. Three proteins that are inseparable by a linear salt gradient elution strategy are analyzed using several pH gradient conditions and conditions are established that result in the separation of these proteins. This work demonstrates that the use of pH gradients in multimodal chromatographic systems may offer new opportunities for high resolution downstream bioprocessing applications.

2. Materials and methods

2.1. Materials

Capto MMC chromatographic media was obtained from GE Healthcare (Uppsala, Sweden) and packed into a Pharmacia Biotech glass column (5 mm × 50 mm). Hydrochloric acid, sodium chloride, acetic acid, sodium acetate, sodium phosphate (monobasic and dibasic), β-lactoglobulin A (bovine milk), α-lactalbumin (bovine milk), and trypsin inhibitor (soybean) were purchased from Sigma–Aldrich (St. Louis, MO). Sodium hydroxide was purchased from Thermo Fisher Scientific (Pittsburgh, PA). The pISep buffer concentrates and pISep pH-gradient software were donated by CryoBioPhysica, Inc. (Silver Spring, MD). The pISep buffers are comprised of polyionic organic buffering molecules that possess overlapping buffering capacities over a broad pH range [4]. Detailed buffer compositions are described elsewhere [33].

2.2. Linear gradient chromatography experiments

Analytical linear gradient experiments were performed under various conditions using an ÄKTA Explorer system controlled by a Unicorn 5.0.1 chromatography software manager. All experiments were carried out using a flow rate of 1 mL/min and a column volume of 1 mL. The column effluent was monitored using UV absorbance at 280 nm. The pH gradient buffers were prepared using the protocol described in the pISep pH-gradient software program (CryoBioPhysica Inc.). The pISep software program was also used to develop the ÄKTA Unicorn method for generating the linear pH gradients in the HPLC system. The exact buffer compositions for each set of gradient experiments and the gradient slopes are presented in the figure legends.

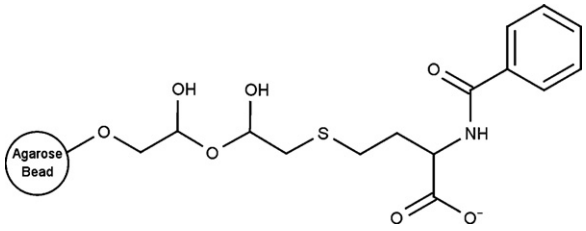

Fig. 1. Chemical structure of the multimodal cation exchange chromatographic resin (Capto MMC, GE Healthcare).

3. Results and discussion

A multimodal cation exchange chromatographic resin (Fig. 1) was used to evaluate protein binding behavior under linear pH gradient conditions. This resin possesses a combination of functional groups which allows it to bind through electrostatic, hydrophobic, and hydrogen bonding interactions. The model proteins α-lactalbumin, β-lactoglobulin A, and trypsin inhibitor with pIs of 4.5, 5.1 and 4.5, respectively, were used in these studies to evaluate the relative efficacy of linear salt and pH gradients for this mixture.

3.1. Salt gradient chromatographic retention behavior

Multimodal chromatographic resins have been shown to exhibit unique binding selectivities under linear salt gradient elution conditions [30,31]. The separation of the three component protein mixture was examined under linear salt gradient conditions. Fig. 2 shows the results for a gradient that transitioned from 0 to 1.5 M sodium chloride over 45 min. The single, large peak that eluted at 20 min (at 0.58 M sodium chloride) indicates that there was no resolution of the three proteins under these conditions. Control experiments were performed where each protein was injected separately to verify that each of the proteins was separately eluting at this time (data not shown).

Several other salt gradient conditions were examined in an attempt to achieve separation of the protein mixture. These included shallower gradients, narrower ranges of salts, and alternative pHs. Under all of these conditions, the results were similar to those shown in Fig. 2, with no resolution observed between the proteins on the multimodal resin using linear salt gradients.

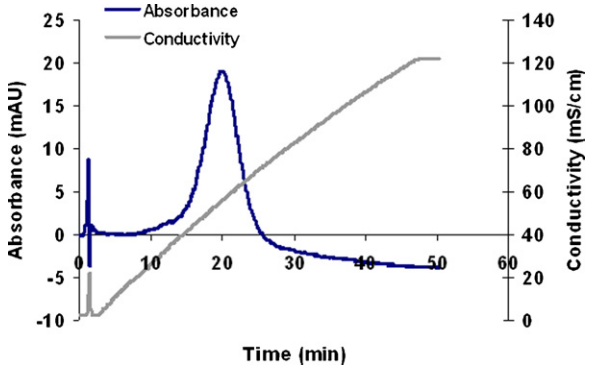

Fig. 2. Linear salt gradient chromatography results for α-lactalbumin, β-lactoglobulin A, and trypsin inhibitor on a multimodal resin. The linear salt gradient transitioned from buffer A (20 mM sodium acetate, pH 5) to buffer B (20 mM sodium acetate, 1.5 M sodium chloride, pH 5) over 45 min.

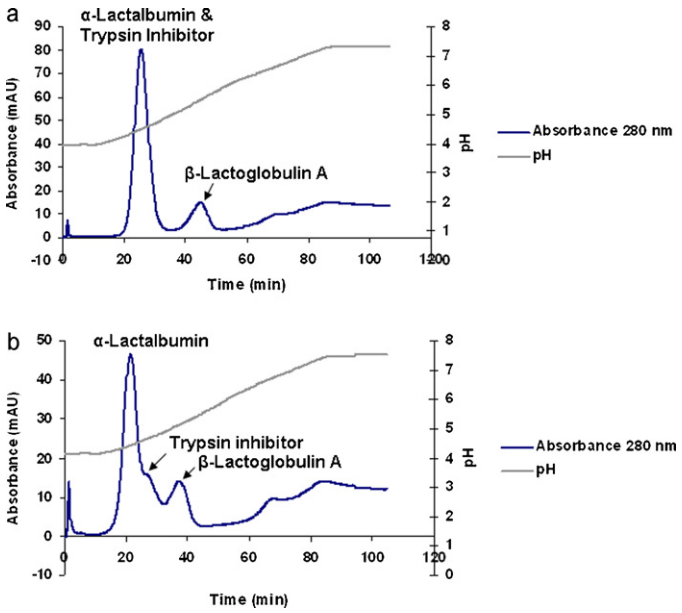

Fig. 3. Chromatography results for a mixture of α-lactalbumin, β-lactoglobulin A, and trypsin inhibitor on a multimodal resin using a pH gradient (pH 3.8–8.0, slope of 0.05 pH units/min) for elution in the absence (a) and presence (b) of 50 mM sodium chloride.

3.2. pH gradient chromatographic retention behavior

Externally generated pH gradients were also examined to see if resolution of these proteins could be achieved on the multimodal resin. Proteins were bound to the multimodal cation exchange resin under low pH conditions and then eluted using an increasing pH gradient. Different gradient slopes were examined, as well as the effect of background salt concentration.

One of the major benefits of the pISep-IEX chromatography system is the ability to generate pH gradients at low or high ionic strength over a large pH range. Fig. 3 shows the chromatography of the three component protein mixture using a linear pH gradient from pH 3.8 to 8.0 (0.05 pH units/min) in the absence (Fig. 3a) and presence (Fig. 3b) of 50 mM sodium chloride. Experiments were also performed with each protein individually to confirm the identity of the peaks. As can be seen in Fig. 3a, two distinct peaks were observed. The first peak contained α-lactalbumin and trypsin inhibitor and the second more retained peak consisted of β-lactoglobulin A. The pISep technology makes it possible to retain control over the formation of the pH gradient in the presence of different levels of sodium chloride ranging from 0 to 1.0 M [18]. Fig. 3b shows the results for the same gradient (pH 3.8–8.0) in the presence of 50 mM sodium chloride. A relatively low salt concentration was used in this experiment to weaken some of the electrostatic interactions while minimally affecting the hydrophobic interactions. As shown in the figure, while the separation of α-lactalbumin and trypsin inhibitor was slightly improved in the presence of the sodium chloride, the peaks were still not fully resolved. Furthermore, the presence of the sodium chloride reduced the retention of β-lactoglobulin A.

In order to improve the separation, the pH gradient was modified to target the range where the proteins were observed to elute from the multimodal resin. It has been shown previously that flattening the pH gradient leads to better resolution [19]. Accordingly, the slope of the gradient was reduced from 0.05 pH units/min to 0.01 pH units/minute and spanned from pH 3.8 to 5.5. Fig. 4 shows the results of this gradient for the three component protein mixture. As can be seen in the figure, while baseline separation was not quite achieved, three distinct protein elution peaks are

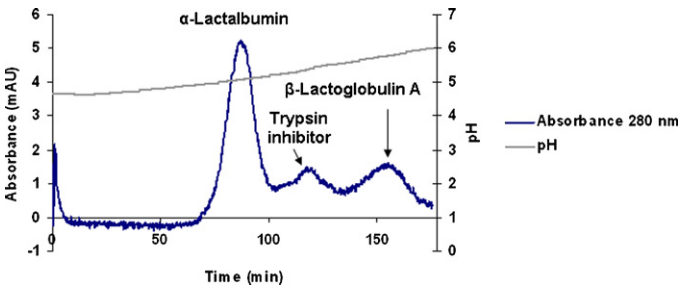

Fig. 4. Chromatography results for a mixture of α-lactalbumin, β-lactoglobulin A, and trypsin inhibitor on a multimodal resin using a pH gradient (pH 3.8–5.5, slope of 0.01 pH units/min) for elution.

clearly visible. As expected, while the shallower gradient reduced the sharpness of the peaks, better resolution of the proteins was indeed achieved. These results indicate that while these proteins could not be separated using salt gradients in the multimodal system, good separation could be achieved through the appropriate use of pH gradients. The combination of the unique selectivities afforded by multimodal chromatography with precise pH gradients offers a new tool in the arsenal of protein purification with potentially important implications for both analytical and preparative chromatographic separations.

4. Conclusions

A pISep externally controlled pH gradient was employed on a multimodal chromatographic resin to separate a protein mixture that was not able to be resolved using linear salt gradients. In addition to the inherent advantages of using pH gradients discussed in the introduction, the pISep technology made it possible to generate an externally controlled pH gradient over a wide pH range and in the presence of salt, greatly facilitating systematic optimization. This work demonstrates that by combining an externally controlled pH gradient with the unique selectivity provided by a multimodal chromatographic resin, it is possible to carry out separations that are not achievable through traditional methods. These initial results indicate that it may be possible to employ pH gradients to enhance the selectivity in systems where it is desirable to separate proteins from closely related variants, a key challenge in bioprocessing. Furthermore, they are consistent with the previously proposed model of pH-dependent protein binding to charged surfaces, where the titration of a protein's surface charges induced by a pH gradient causes the dominant binding patch(es) to shift from one region of the protein's surface to another [4]. This can also cause variations in the area available for hydrophobic binding, increasing the likelihood that closely related isoforms will differ in both electrostatic and van der Waals interactions as the gradient develops. The ability to tune selectivity and to elute proteins using pH gradients in multimodal chromatography without salt elution may have important implications for downstream processing of biopharmaceuticals. Future work will further examine the underlying phenomena involved in these selectivity changes and will apply these methods to more challenging bioseparation problems.

Acknowledgement

This work was partially supported by NSF Grant CBET 0933169.

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