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Theory and applications of a novel ion exchange chromatographic technology using controlled pH gradients for separating proteins on anionic and cationic stationary phases

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ABSTRACT

pISep is a major new advance in low ionic strength ion exchange chromatography. It enables the formation of externally controlled pH gradients over the very broad pH range from 2 to 12. The gradients can be generated on either cationic or anionic exchangers over arbitrary pH ranges wherein the stationary phases remain totally charged. Associated pISep software makes possible the calculation of either linear, nonlinear or combined, multi-step, multi-slope pH gradients. These highly reproducible pH gradients, while separating proteins and glycoproteins in the order of their electrophoretic pIs, provide superior chromatographic resolution compared to salt. This paper also presents a statistical mechanical model for protein binding to ion exchange stationary phases enhancing the electrostatic interaction theory for the general dependence of retention factor k, on both salt and pH simultaneously. It is shown that the retention factors computed from short time isocratic salt elution data of a model protein can be used to accurately predict its salt elution concentration in varying slope salt elution gradients formed at varying isocratic pH as well as the pH at which it will be eluted from an anionic exchange column by a pISep pH gradient in the absence of salt.

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1. Introduction

1.1. Background

The purification of proteins to a degree sufficient to allow molecular weight analysis by mass spectroscopy (MS) is of central importance to the emerging field of proteomics. Highly purified genetically engineered proteins are becoming essential for both the safety and the efficacy of modern biomedicine and agriculture. In addition, the ability to provide highly purified proteins for biomedical and agricultural research in general is of great importance in the effort to characterize the vast number of proteins of unknown function in a large number of important organisms. Optimum protein purity is vital to minimize side effects when expressed or purified proteins from natural sources are used in clinical or agricultural settings. Therefore, a method that enhances the selectivity and resolution of liquid chromatography (LC) is of great practical importance.

In general, proteins can be purified by one or more of the following strategies:

1. Separation based on sieving by size exclusion chromatography (SEC). Size differences are commonly exploited using SEC resins with variable pore sizes that allow proteins below a certain critical molecular weight to move through the gel at a rate proportional to their molecular weight. SEC is practical and widely used but has low selectivity and is generally confined to being an intermediate or a polishing step in a chain of purification procedures and is very often followed by a protein concentration procedure.
2. Hydrophobic interaction (HIC). HIC exploits the reversible interaction of a protein with a hydrophobic surface of a stationary phase in an eluent environment with high ionic strength. The protein is released from the column upon decreasing the ionic strength of the eluent. HIC is a preferred choice for fractionation after an ammonium precipitation or high salt ion exchange (IEX) chromatographic step.
3. Reversed phase chromatography (RPC). RPC is a very powerful separation method in which the proteins are bound to a hydrophobic surface in a polar solvent, and then eluted by a gradually increasing nonpolar eluent stream. It is highly selective and is a crucial purification step in most modern protocols.

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Its biggest disadvantages are the low binding capacity of the stationary phases and the tendency of globular proteins to denature during the procedure.

4. Affinity interactions (AC). AC relies on a highly specific affinity binding interaction between a protein and a ligand immobilized on the surface of a resin. The protein is released from the stationary phase upon increasing the ionic strength of the eluent, which reduces the strength of binding. This technique is very useful for first concentrating, then rapidly and very selectively removing a natural or expressed protein from a complex mixture of proteins. Its drawbacks are elution of nonspecifically bound proteins and ligand leaching in the fraction of the purified protein. The latter is especially undesirable in purification involving antigen–antibody interactions.
5. Ion exchange (IEX) chromatography. IEX chromatography is widely used technique that exploits the variations in net electrical charge of proteins as a function of pH. IEX is generally carried out by first adjusting the pH of an aqueous solution of proteins to a pH value such that all of the proteins of interest will have the same net charge (negative or positive), and then binding the proteins to an ion exchanger containing immobilized groups of the opposite charge. Next, the retained proteins are eluted from the exchanger one after the other by an electrolyte gradient with increasing ionic strength. Most often a gradient of NaCl at the chosen pH is the eluent. The selectivity of IEX arises from the differences in the free energy of binding of the proteins to a particular ion exchanger at a given pH and, as a rule, the tighter the binding of a protein to the ion exchanger the higher the salt concentration required for elution. The chief deficit of the method is that proteins with the same number of effective charges will be released from the ion exchanger close to each other. This significantly limits the selectivity of IEX when complex protein mixtures are separated.
6. In addition to these five liquid chromatographic techniques there is isoelectric focusing (IEF), a very powerful electrophoretic separation technique which involves the use of immobilized pH gradients. IEF is usually performed in either a slab gel or a capillary. The gel or capillary containing a complex mixture of ampholytes (polyionic organic electrolytes buffering either a short or a broad pH range) is placed in an electric field. The electric field causes the ampholyte variants to migrate to pH zones on the gel known as their isoelectric points (pIs) where they have a net charge of zero. This creates a steady state pH gradient in which each of the proteins applied to the gel will migrate to its electrophoretic pI and will thereby be separated from all other proteins of differing pI. While IEF is perhaps the most powerful of the known separation technologies, the difficulty of removing the separated proteins from the gel has confined the method to an analytical rather than a preparative separation technique.
7. In the late 1970s Sluyterman et al. [1–5] developed a pH gradient LC technique called chromatofocusing (CF). In this method, one equilibrates a specially designed weak anion exchanger such as PBE94, PBE118 or Mono P with an initial buffer at a pH high enough to allow the binding of the target proteins to the exchanger. In general, after application and binding of the proteins, followed by washing of the CF stationary phase with several column volumes of the initial buffer, an elution is performed with a second (final) buffer containing Polybuffer (a mixture of ampholytes) at a final pH lower than the pH of the initial buffer. As the final buffer descends through the column the various ampholyte components bind differentially to the stationary phase developing an internally retained pH gradient. This gradient dynamically evolves from the higher pH of the initial buffer to the lower pH of the final buffer as the final buffer moves down the column. During this process proteins detach sequentially from the stationary phase as their ionized side groups are neutralized and begin to move down the column with the bulk flow at a pH usually near the pH of their electro-neutrality measured by IEF.

CF has several serious shortcomings. First, for each pH range over which one might wish to create a gradient, it is necessary to prepare different initial and final buffer solutions. Second, for any given pH range, the slope of the gradient inside the column is proportional to the width of that pH range, thus precluding variations in the slope during the separation. Third, no single formulation of the ampholytes can maintain good buffering capacity over more than three pH units, requiring three different Polybuffer formulations (G. E. Healthcare) which cover the pH ranges 11–8, 9–6, and 7–4. Finally, ampholytes are expensive and some of them bind to purified proteins requiring their removal by SEC. It is therefore not practical to use CF in mid and large scale preparative protein purifications. Yet, despite these drawbacks, CF has sparked renewed interest as the first dimension LC technique of the Beckman-Coulter ProteomeLab™ PF 2D Protein Fractionation System, because uncontrolled retained pH gradients deliver significantly higher chromatographic resolution than salt gradients at isocratic pH when proteins from complex mixtures are separated.

In the intervening years since the initial work of Sluyterman et al., numerous attempts have been made to address CF's disadvantages [6–17]. Generally, those attempting to improve on CF have assumed that complex interactions between the buffers and the preferred stationary phase, a weak anionic exchanger, are the key to successful pH gradient formation, even though this inevitably results in an inability to accurately predict and control the development of the pH gradient. As a consequence, it has been widely accepted that controlled pH gradients over very wide pH ranges are not achievable. In a few instances the buffers forming the pH gradient have been mixed externally to the CF column [6,7,13,14]. However, these efforts have failed to achieve: (1) the formation of multi-step, multi-variable slope linear or non-linear pH gradients over a very wide pH range of up to 10 pH units under software-enabled algorithmic control, (2) a single low ionic strength buffering composition allowing the controllable formation of pH gradients on both AEX and CEX stationary phases with arbitrary starting point and length over the entire pH range or (3) the use of strong ion exchangers as the preferred stationary phase. A more recent effort [18], using a buffer chemistry very similar to pISep, but at a much higher ionic strength, was able to achieve uncontrolled, descending, wide ranging pH gradients but without attempting to develop software-enabled algorithmic control of the pH gradient formation. Because of the high ionic strength of the mobile phase in that study, the AEX stationary phase failed at pH 10 to strongly bind proteins with electrophoretic pIs in the neutral (pH 6–8) range causing the elution of all these proteins at around pH 9 independent of their pI.

As a consequence of the lack of progress in achieving accurately controlled pH gradients, chromatographers face a number of shortcomings of LC that remain unresolved and which formed the impetus for this study. The more obvious needs are for: (1) software-driven, multi-step ascending or descending pH gradients with changing slopes limited only by the accuracy of modern LC gradient pumps; (2) software-driven capability to vary the slope of a pH gradient arbitrarily throughout a LC separation independent of the initial and final pH and without changing the chemistry of the mobile phases in the source reservoirs with each slope change; (3) generation of controlled pH gradients on both anionic and cationic stationary phases using the same mobile phase; (4) software-driven control of pH gradients formed in the presence of isocratic additives such as nonionic detergents, organic solvents, salts, etc. as well as controllable development of simultaneous independent

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