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pIsep: Anion, Cation and Combined Ion Exchange Chromatography for Separation of Proteins and Charged Molecules by External pH Gradients

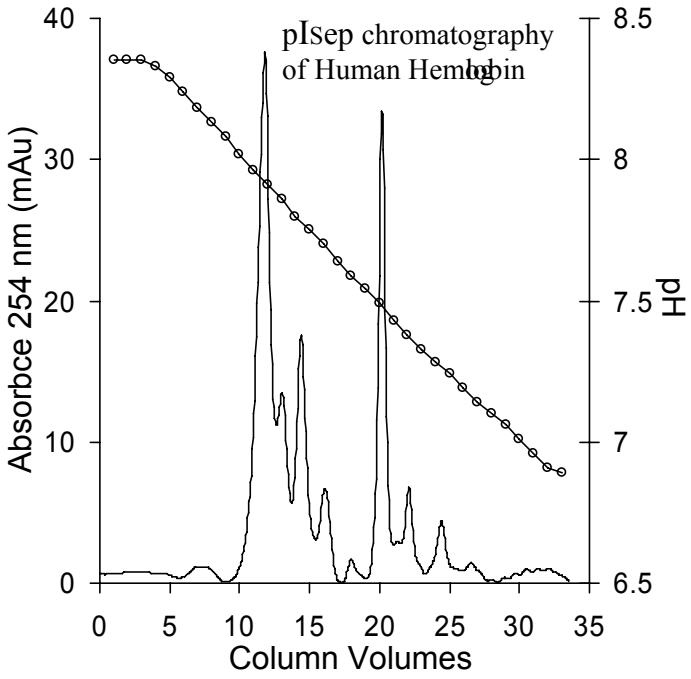
pIsep chromatography replaces traditional Polybuffer chromatofocusing, originally developed by Sluyterman (1, 2), to fractionate proteins in order of their apparent isoelectric point (**pI**) by a self-generated, retained (**internal**) pH gradient (3) on a weak anion exchange column. During chromatofocusing the pH gradient is automatically formed throughout the length of the separation column as the Polybuffers (amphoteric multi-buffering species) in an acidic elution buffer bind differentially to the functional groups of a weak anion exchanger which is specifically designed for Polybuffer chromatofocusing. Up until now, chromatofocusing has been the most selective, high-resolution, chromatographic technique available for separating proteins.

Columns and Polybuffers for chromatofocusing are supplied by GE Healthcare (formerly Amersham Biosciences). For protein separations over the pH range from pH 10.5 to 4, GE provides three different weak anion exchangers using three different Polybuffers, each covering a limited pH range of 3 pH units. For the pH range 10.5 to 8, Pharmalyte 8-10.5 is used with a weak anion exchanger, PBE 118. For the pH ranges 9 to 6 and 7 to 4, Polybuffer 96 and Polybuffer 74 are used with a weak anion exchanger PBE 94. For high resolution, analytical chromatofocusing, a high resolution, weak anion exchanger, Mono P, is available and can be used with the three types of Polybuffer.

pIsep is an ion exchange chromatography (IEX) using an **external** pH gradient created outside the column by a gradient-forming device. The technique is highly effective for fractionating polyampholyte biological molecules such as proteins and polysaccharides, based on differences in their net surface electrical charges. It requires no special chromatographic column. It enables user controlled formation of pH gradients over very wide pH range, both linear and non-linear, as well as stepwise and augments the current chromatographic techniques with a new ion exchange method called combined IEX (combined chromatofocusing, discussed below). **pIsep** can accurately separate proteins that differ by only a single amino acid as well as proteins with similar or nearly identical **pIs** including different isoforms of the same protein. Virtually any class of charged molecule can be separated. The fractionation produces a sharp peak of concentrated, highly purified product, often requiring no further purification or polishing. It is a stand-alone, IEX purification method that can be used in both column and batch separation processes. Alternatively, it can be easily incorporated as either an initial or an intermediate step in a number of existing separation protocols using chromatographic techniques. **pIsep** IEX completely replaces Polybuffer chromatofocusing and, as a far better alternative in most cases, can substitute salt elution IEX steps in already developed chromatographic protocols. **pIsep** buffers can be used to selectively elute antibodies or other proteins bound to an affinity column by a pH jump or a pH gradient instead of salt, thereby eliminating undesired, nonspecifically bound impurities from the product of interest. The low ionic strength **pIsep** buffers need not be removed from the separated protein fractions and, when used in IEX separations as a replacement of salt, the desalting steps will be eliminated. Proteins fractionated by **pIsep** can be further purified immediately by either reversed phase, ion exchange (after pH adjustment if necessary), gel filtration or affinity chromatography.

During **pIsep** separation the pH gradient is generated externally using either HPLC or FPLC dual pump systems or any other gradient-forming device, then applied to either an anion or a cation exchanger or a sequential arrangement of oppositely charged ion exchangers. The pH gradient can be started at any pH within the range pH 2.4 to pH 10.7 and can be expanded to the full range of 8.3 pH units. The buffering capacity of **pIsep** buffers remains essentially constant as the pH changes

throughout the entire pH range, allowing the development of gradients with varying slope or shape from alkaline to acidic or acidic to alkaline pH. Such control over the gradient formation permits a flexible optimization of the separation process that is free of the restrictions inherent in Polybuffer chromatofocusing using weak anion exchangers. **pIsep** allows the scale up of an analytical separation to a large preparative scale fractionation similar to the scale up of a conventional salt-elution, ion exchange, chromatographic separation. **pIsep** is relatively inexpensive and industrial production separations are economically feasible.



Example of external, descending pH gradient pIsep

Buffer A: pIsep acidic buffer pH 2.4

Buffer B: pIsep basic buffer pH 10.7

Sample: Freeze dried human hemoglobin from Sigma Cat # H7379, 50 µl (8mg/ml) pH 8.5. Sample was applied, and the column washed and equilibrated with starting buffer 67.4%B pH 8.4

pH gradient: slope 0.05 pH units/CV, from pH 8.4 to pH 6.9 in 30 CVs.

1. 67.4%B to 60.5%B in 10 CVs;

2. 60.5%B to 56%B in 7.6 CVs;

3. 56%B to 49.5%B in 12.4 CVs

4. at 49.5%B 3 CVs.

Column: Mono Q HR 5/5 - column volume 0.98 ml (Amarsham Pharmacia)

Flow rate: 1 ml/min

Detection: 254 nm

pIsep does not use the proprietary weak anion exchangers required for Polybuffer chromatofocusing, but utilizes the strong ion exchange resins already available in every chromatographic laboratory. Although the chromatographer may use ion exchangers with particle sizes of 20, 30, 60 µm or larger, resins with particle sizes of 15 µm, 10 µm or smaller in well-packed columns (plate number ≥ 20000 per meter) are preferable in order to take full advantage of the effectiveness and separation power of the method. Nevertheless, whatever the choice of resin, utilization of **pIsep** chromatography will always result in separation with better resolution then the IEX separation with salt elution on the same resin. **pIsep** resolves peaks differing in **pI** by 0.04 pH units or less. Since **pIsep** can separate proteins over the very wide pH range of 8.3 pH units, the benefits of that capability are best exploited using resins with broad working pH-range stability. Some good choices of strong ion exchangers are: 400VHP strong cation exchange and 300VHP575 Q anion exchange columns from Crace Vydac which are stabile from pH 0 to pH 14; Mono Q, Mono S, Source 15Q, Source 15S, Q Sepharose and SP Sepharose from GE Healthcare offer very good selectivity and stability from pH 2 to pH 12; Micra-Cold S1000, Micra-Silver S300 and Micra-Silver Q300 from Eichrom Technologies; Protein-Pak Q 8HR and Protein-Pak SP 8HR from Waters. Weak DEAE-based anion and weak carboxymethyl-based cation exchangers can also be used with this technique but they will not permit exploiting the full advantages of **pIsep**. In general these types of resins exhibit ion exchange capacity that varies markedly with pH and as a consequence the length as well as the shape and the slope of the externally created pH gradients will be altered.

pIsep offers three basic approaches for IEX chromatographic separation of charged molecules:

1. External Descending pH Gradient, pIsep (ExtGpIsep): For this anionic IEX technique, the sample molecules are bound to a strong anion exchanger at a pH higher than their **pI**, then eluted with a descending external pH gradient from alkaline towards acidic pH.

2. External Ascending pH Gradient, pIsep (ExtRGpIsep): For this reverse, or cationic IEX technique, sample molecules are bound to a strong cation exchanger at a pH lower than their **pI**, then eluted with an ascending external pH gradient from acidic towards alkaline pH.

3. Combined External pH Gradient, pIsep: a technique using a tandem of ion exchangers of opposite charge connected in series:

a. Combined External Descending pH Gradient, pIsep (CoExtGpIsep) - the tandem column consists of an anion exchanger followed by a cation exchanger or, alternatively,

b. Combined External Ascending pH Gradient, pIsep (CoExtRGpIsep): The tandem column consists of a cation exchanger followed by an anion exchanger.

Each of these tandem column techniques can be performed in two different ways:

In the first variant, the charged molecules (proteins) to be separated are applied to both ion exchangers arranged in series (tandem) at a chosen pH. Some of these molecules will then selectively bind to either the anion or the cation exchanger, and some will pass through both columns without binding. The behavior of each protein depends on its charge at that chosen pH. After isocratic washing and equilibration, the two exchangers are then separated from each other and individually eluted starting at the same chosen pH. The anion exchanger is eluted with a descending external pH gradient to fractionate negatively charged molecules and the cation exchanger with an ascending external pH gradient to fractionate positively charged molecules.

In the second variant, a sample is first applied to either an anion or a cation exchanger at a pH chosen to allow binding of the targeted molecules. After washing, an ion exchanger of the opposite kind is connected in series with the first. This tandem of exchangers is then washed and equilibrated at the same chosen pH. In the next phase, the bound molecules are fractionated and eluted from the first exchanger to be bound to the second exchanger by applying an appropriate ascending or descending external pH gradient. Finally, the first exchanger is removed and the bound target molecules are separated and eluted from the second ion exchanger with an appropriate ascending or descending external pH gradient.

We have demonstrated that two proteins with very close **pIs** that are difficult to separate by an external descending pH gradient on an anion exchanger can usually be very well fractionated by an external ascending pH gradient on a cation exchanger or vice versa. We have found that most of the proteins separated by **pIsep** exhibit different apparent **pIs** during anionic vs. cationic **pIsep** chromatography. This observation led to the development of sequential, combined **pIsep** IEX which offers a hitherto non-existent chromatographic technique for protein purification.

More recently, the capabilities of the pIsep chromatography have been greatly extended, making it possible for the chromatographer to form controlled pH gradients in the presence of 0 to 8 M urea and/or of 0 to 1M salt. This now enables the purification of proteins that are difficult to separate such as those trapped in inclusion bodies, membrane, hydrophobic or other proteins with low solubility.

No company other than CryoBioPhysica offers buffer solutions for cationic chromatofocusing. CryoBioPhysica also offers an exclusive software package allowing protein purifications in

presence or absence of non-ionic detergents by pIsep chromatography. This computer program will calculate the chromatographic parameters necessary to develop complex pH gradients containing multiple linear segments of user-defined length and slope, as well as nonlinear concave, convex and stepwise pH gradients. Together the pIsep buffers and the "pIsep pH Gradient Maker" software package provide the necessary flexibility for complete control over optimization of the separation process.

References

1. L.A. AE, Sluyterman, O. Elgersma, Chromatofocusing: isoelectric focusing on ion-exchange columns I. General principles, J Chromatogr. 150 (1978) 17-30.

2. L.A. AE, Sluyterman, O. Elgersma, Chromatofocusing: isoelectric focusing on ion-exchange columns II. Experimental verification, J. Chromatogr. 150 (1978) 31-44.

3. Chromatofocusing with Polybuffer and PBE. Handbook 18-1009-07, Amersham Biosciences AB (2001), 3-7.