Separation of Protein Standards on Agilent 3 µm Ion Exchange Columns by Cation Exchange Chromatography

Application Note

Biopharmaceuticals

Author

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Abstract

In addition to being a widely used technique for protein purification, protein separations by ion exchange chromatography provide valuable information about the charge variants present in the protein sample. We present a method of protein separation using an Agilent Bio IEX (WCX and SCX) and MAb, nonporous, 3 µm poly(styrene divinylbenzene) columns. Furthermore, protein separation on an Agilent Bio IEX and MAb is compared and the difference in elution between the two columns determined.

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Introduction

Ion exchange chromatography is the most frequently used method for the purification of charged biomolecules such as proteins, polypeptides, and nucleotides. The nondenaturing method, high resolving power, and extensive applicability makes ion exchange chromatography a very favorable technique.

Ion exchange chromatography allows the separation of molecules based on their charge. The technique requires charge-charge interactions between the proteins in the sample and the charge immobilized on the resin. Cation exchange chromatography works on the basis of positively charged molecules being attracted to a negatively charged solid support. Conversely, in anion exchange chromatography, negative charged molecules are attracted to a positive charged solid support.

Separation of proteins according to their net charge is dependent on the composition of the mobile phase. Various proteins can be separated by adjusting the pH and the ionic strength of the mobile phase. Varying the pH is a powerful way of influencing net charges of sample molecules and is therefore normally used to control selectivity. A change in buffer pH so that the protein has a net charge that is the same as the charge on the column will result in protein elution. Elution by altering the ionic strength of the mobile phase is the more common way of performing ion exchange HPLC. This will not influence selectivity but provides a means of desorbing the sample molecules in order of increasing charge, with the least charged eluting first.

Separation by ion exchange is dependent on the reversible adsorption of charged solute molecules to immobilized ion exchange groups. Optimized binding of charged molecules is achieved by an initial mobile phase solution of low conductivity. The strength of the interaction between the opposing charges of the ionic groups on the molecule and the functional ligand on the resin determines the salt concentration required for protein elution.

Materials and Methods

Agilent Bio Ion Exchange (IEX) columns are packed with polymeric, nonporous media and are designed to provide high resolution, high recovery, and high efficiency separations of biomolecules. Grafting the rigid PS/DVB particles with a hydrophilic, polymeric layer greatly minimizes nonspecific binding, while increasing efficiency and percentage recovery. Agilent Bio MAb columns are optimized specifically for antibody separations.

The Agilent Bio IEX family offers strong cation exchange (SCX), weak cation exchange (WCX), strong anion exchange (SAX), and weak anion exchange (WAX) phases. The Agilent Bio MAb family is a highly uniform, densely packed, weak cation exchange resin. Each is available in 1.7, 3, 5, and 10 µm particle sizes. The work presented here uses the 3 µm columns for high efficiency.

Conditions

Columns	Agilent Bio SCX, NP 3, 4.6 × 50 mm, SS Agilent Bio WCX, NP 3, 4.6 × 50 mm, SS Agilent Bio MAb, NP 3, 4.6 × 50 mm, SS
Mobile phase	A - 10 mM $NaH_2PO_4 \cdot 2H_2O$, pH 5.70 B - A + 1 M NaCl
Gradient	0 minutes - 100% A : 0% B 25 minutes - 0% A : 100% B
Flow rate	0.5 mL/min
Temperature	Ambient
Injection volume	2 µL
Sample	Cytochrome c, ribonuclease A, lysozyme and protein mix
Sample concentration	2 mg/mL
Detection	UV at 220 nm
Instrument	Agilent Infinity 1260 Bio-inert HPLC system

Each of the protein samples were made up to a final stock concentration of 2 mg/mL in buffer A. The protein mix comprised 2 mg/mL of each of the individual proteins. Each column was equilibrated with five column volumes of buffer A prior to and following a protein injection. A linear gradient of increasing ionic strength was subsequently used to elute the proteins from the columns.

Results and Discussion

Figures 1 to 4 and Table 1 illustrate that the Bio WCX, SCX, and MAb columns are all capable of producing protein separations. Protein elution was accompanied by an increase in ionic strength. The point of elution depends on the isoelectric point (pI) (the point at which the protein carries no net charge) of the individual proteins, whereby proteins with higher isoelectric points generally require increased ionic strength to be eluted from the column, while proteins with lower isoelectric points are eluted at lower salt concentrations.

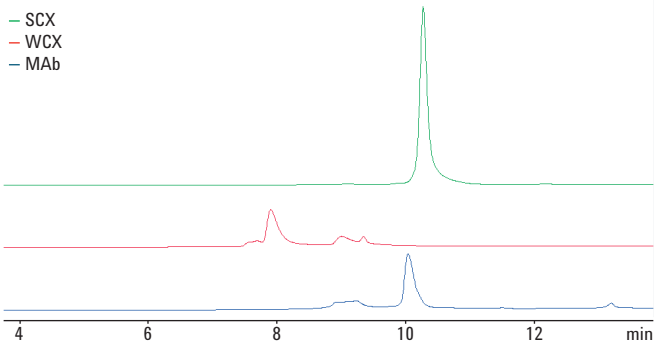

Figure 1. Elution of cytochrome c on Agilent Bio Ion Exchange columns.

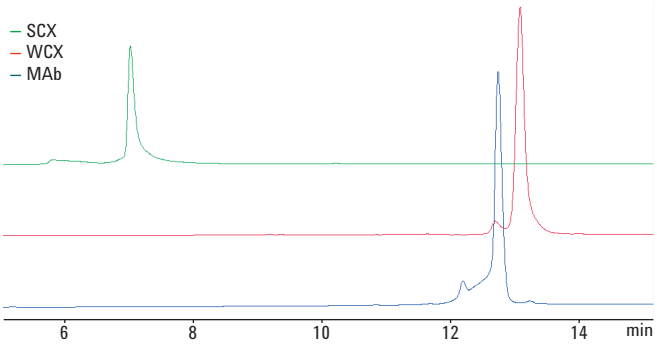

Figure 3. Elution of lysozyme on Agilent Bio Ion Exchange columns.

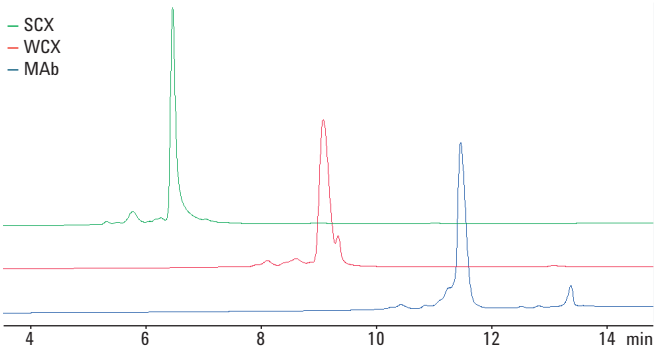

Figure 2. Elution of RNase A on Agilent Bio Ion Exchange columns.

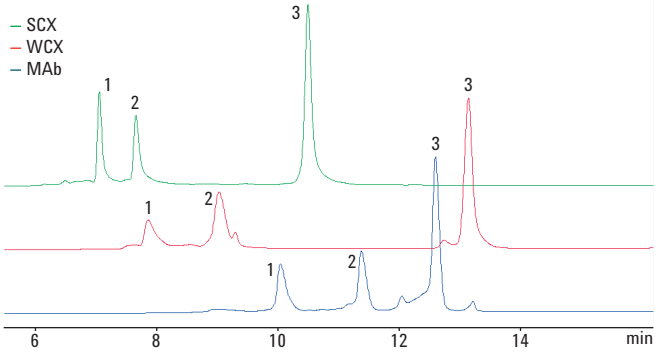

Figure 4. Overlaid chromatograms of elution of protein mix on Agilent Bio Ion Exchange columns.

Table 1. Illustration that Bio WCX, SCX and MAb Columns are Capable of Producing Protein Separations

Agilent column	Peak number	Peak name	RT [min]	Height [mAU]	Area [mAU*s]	Plates	Width [min]	Resolution
Bio WCX, NP, 3 µm	1	Cytochrome c	7.86	123.617	1833.392	7844	0.2089	-
	2	RNase A	9.03	240.980	3357.965	10800	0.2044	3.32
	3	Lysozyme	13.13	635.979	7274.018	44488	0.1466	13.73
Bio SCX, NP, 3 µm	1	RNase A	7.06	395.912	2616.430	39847	0.0832	-
	2	Cytochrome c	7.66	296.481	2777.710	28920	0.1060	1.08
	3	Lysozyme	10.49	762.518	7185.960	44828	0.1167	1.37
Bio MAb, NP, 3 µm	1	Cytochrome c	10.04	202.515	2369.341	21814	0.1600	-
	2	RNase A	11.37	256.270	2689.885	33314	0.1467	3.11
	3	Lysozyme	12.59	652.369	6615.773	56734	0.1244	5.28

Cytochrome c, RNase A and lysozyme have an isoelectric point of 10.5, 8.7, and 10.7, respectively. The accessibility of the ionic groups on each of the proteins will also affect the elution position of the protein.

The weak cation exchange column was the most efficient at protein separation, as signified by the more distinct peak shapes. The minor peaks that are evident in the chromatograms from the Bio WCX column are either not visible or less distinct in the chromatograms from the Bio SCX and MAb columns. For example, comparison of cytochrome c elution on each column (Figure 1) allows the differences in elution to be identified. Figure 1 (elution on Bio WCX) demonstrates a predominantly single peak with a small shoulder beforehand, followed by two other components. The same protein on the Bio SCX column illustrates a predominantly single peak with a small amount of material eluting earlier and an absence of later eluting protein. Protein elution on Bio MAb shows cytochrome c elution to be a predominant single peak with some early eluting protein followed by very small amounts of later eluting material.

The order of protein elution is also somewhat different. The Bio WCX and MAb columns showed the order of elution to be cytochrome c, RNase and then lysozyme. However, the Bio SCX column demonstrated the order to be RNase A, cytochrome c, and then lysozyme, in order of isoelectric points. Both the WCX and MAb columns are weak cation exchange columns, whereas the SCX column is a strong cation exchange column. The difference in the ionisable group on the resin determines the separation behaviour of the resin as the charge on the media will depend on the pH of the eluent.

The strong cation exchange resin contains a sulphonic acid (SO_3H) functional group and is highly ionisable in both acid and salt states. Varying the pH is a powerful way of influencing net charges of sample molecules and is therefore normally used to control selectivity. Consequently, the exchange capacity of strong acid resins is independent of solution pH.

In the weak cation exchange resin, the ionisable group is a carboxylic acid (COOH) and these resins have a much higher affinity for hydrogen ions than do strong acid resins. Thus, the degree of dissociation of a weak acid resin is strongly influenced by the solution pH.

Conclusions

The Agilent Bio WCX, SCX, and MAb columns demonstrate their ability to perform protein separations. There are clear differences in the separation of a protein test mixture, which is attributed to differences in ion exchange capacity and strength. Under the conditions evaluated here, the columns gave better separation of both positive and negative charge variants.

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