Improving Low Molecular Weight Separation Using BioSep™ GFC Columns

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To obtain the optimal resolution of low molecular weight peptides and proteins by Gel Filtration Chromatography (GFC), one must use unique mobile phase conditions compared to the near-physiological conditions used for protein separation. For peptides, organic solvents and ion-pairing reagent (TFA) are used to minimize interactions resulting in sharp peaks with distinct retention typically based on the molecular weight of the peptide on a BioSep™-SEC-S2000 GFC column.

Introduction

Probably the most common application for gel filtration chromatography is to look at the aggregation state of a protein under non-denaturing conditions. Protein dimer and aggregates are separated from a native protein by differences in sizes of protein complex's compared to a monomer protein; such retention differences somewhat correspond to differences in molecular weight between aggregates and the native protein. The mobile phase conditions for such separations are often close to physiological pH and osmolarity to best quantitate non-covalent aggregates; however, such conditions also have some secondary interactions between proteins and the gel filtration stationary phase resulting in reduced resolution and anomalous molecular weights.

While for protein separations secondary interactions may not adversely affect chromatography, for peptide separation such secondary interactions can result in anomalous peak retention and reduced peak efficiency. Such reduced performance can result in poor isolation and quantitation of peptides by gel filtration chromatography. For such separations a mobile phase using an organic solvent can greatly reduce hydrophobic secondary interactions resulting in improved peak shape and higher efficiency. The addition of an ion-pairing buffer (0.1 % TFA) can further reduce secondary interactions. Several examples of peptide separation on a BioSep-SEC-S2000 column are shown demonstrating the performance using such mobile phase condition.

Materials and Methods

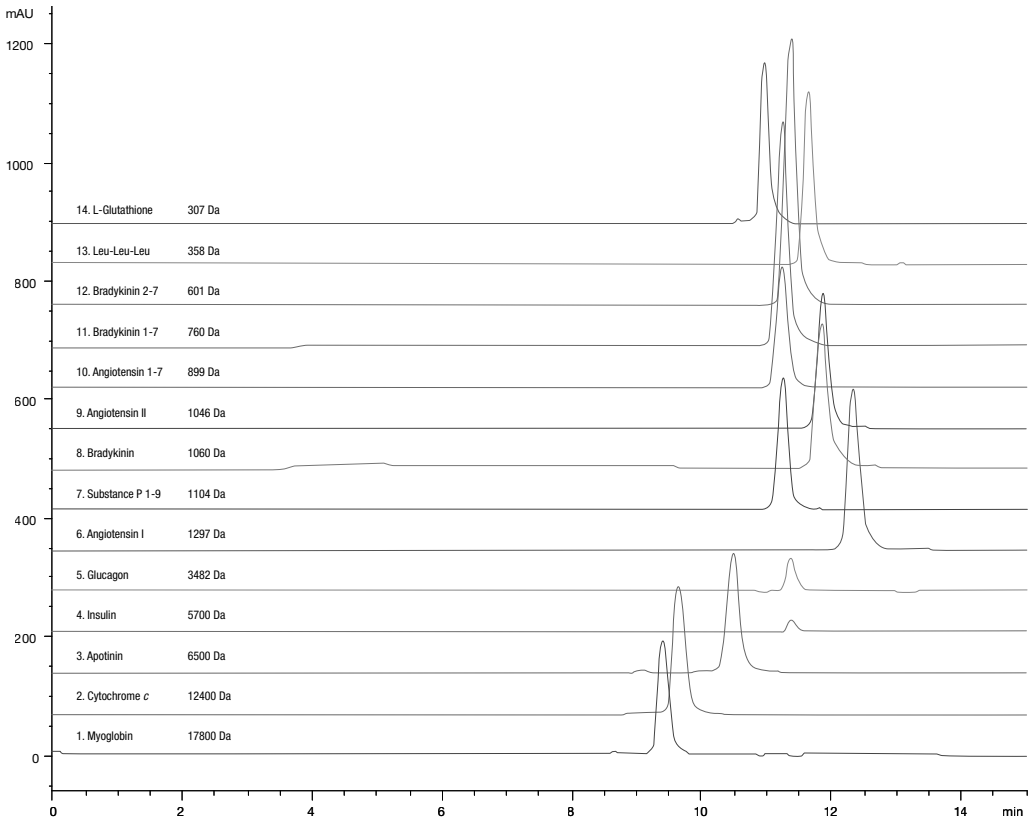
All reagents were obtained from Sigma (St. Louis, MO) and solvents were obtained from EMD (San Diego, CA). Chromatography was performed on an Agilent HP1100 HPLC system equipped with an autosampler and UV detector utilizing Agilent ChemStation™ software, and a BioSep-SEC-S2000 GFC column, 300 x 7.8 mm (Phenomenex, Torrance, CA, USA) for all protein and peptide separations. Mobile phase used was 50 mM sodium phosphate, pH 6.8/ 300 mM sodium chloride or 45 % acetonitrile with 0.1 % TFA pH~3. Flow rate for both separations was 1 mL/min. Protein elution was detected at 214 nm.

Results and Discussion

Peptide separation on gel filtration media typically approach the lower end of the resolution range of gel filtration chromatography and thus the smallest pore size media of a gel filtration product line is used. In the BioSep product line that media is the BioSep-SEC-S2000 column which is best suited for proteins below 250 kDa molecular weight. While native conditions using physiological buffers is ideal for aggregate assays, for small protein and peptide separations secondary interactions between the stationary phase and proteins of interest can result in mediocre peak shapes and low efficiencies. Secondary structures of small proteins and peptides can also have a major influence on retention resulting in anomalous retention of peptides compared to other peptides of equal molecular weight. An example of such a separation is shown in **Figure 1** where chromatograms of several different low molecular weight proteins and peptides are compared on a BioSep 2000 column. Extreme examples of anomalous molecular weights are shown; glutathione and leu-enkephalin elute earlier than oxytocin and bradykinin despite being significantly smaller peptides. Basic peptides can potentially have secondary interactions with silica columns leading to increased retention; other small peptides can be completely unfolded resulting in reduced retention. Minimizing both types of interactions is needed to improve accuracy of molecular weight determinations.

APPLICATIONS

Figure 1.
Peptides separated under native conditions. Note the broad peaks and anomalous retention of many peptides in comparison to their known molecular weight. Secondary structure and non-specific interactions can reduce the separation of low molecular weight peptides.



App ID 19234

Column: BioSep-SEC-S2000
Dimensions: 300 x 7.8 mm
Part No.: 00H-2145-K0
Mobile Phase: 300 mM Sodium chloride, pH 6.8 in 50 mM Sodium phosphate
Flow Rate: 1 mL/min
Temperature: Ambient
Detection: UV @ 214 nm
Sample: Low MW proteins and peptides

1. Myoglobin 17800 Da
2. Cytochrome c 12400 Da
3. Apotinin 6500 Da
4. Insulin 5700 Da
5. Glucagon 3482 Da
6. Angiotensin I 1297 Da
7. Substance P 1-9 1104 Da
8. Bradykinin 1060 Da
9. Angiotensin II 1046 Da
10. Angiotensin 1-7 899 Da
11. Bradykinin 1-7 760 Da
12. Bradykinin 2-7 601 Da
13. Leu-Leu-Leu 358 Da
14. L-Glutathione 307 Da

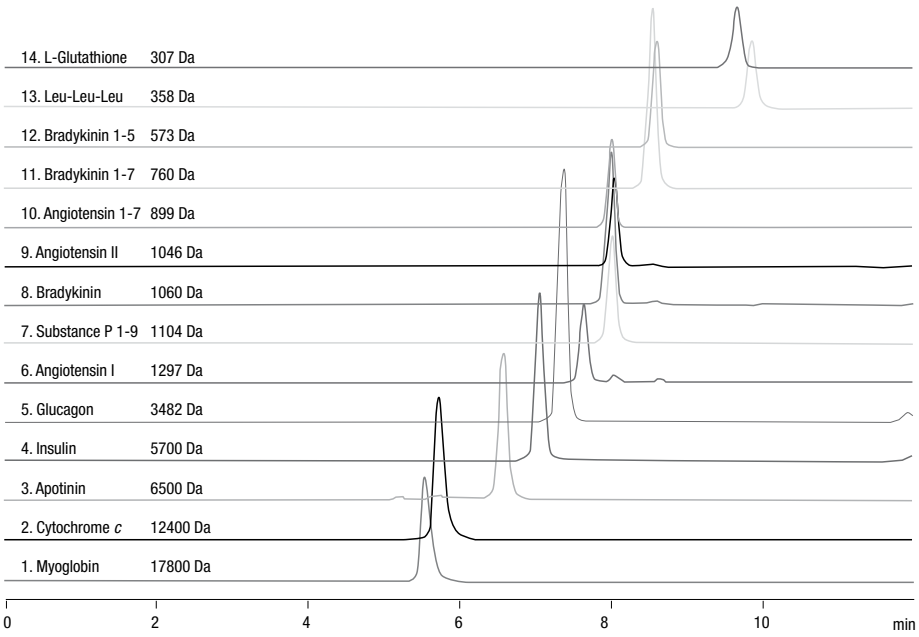
Organic mobile phase and ion-pairing buffer can be used to minimize secondary interactions and unfold small proteins and peptides. By minimizing such interactions peptides and small proteins will more closely elute to their expected molecular weight (based relative to other unfolded proteins). An example of using acetonitrile and TFA mobile phase to perform such a separation is shown in **Figure 2**. In this example peptides are run on the BioSep 2000 column using aqueous/organic buffer to minimize molecular weight anomalies between different peptides. Also note that most peptides elute much earlier compared to native separations; this is likely due to peptides being unfolded resulting in less permeation into the gel filtration media, leading to reduced retention. Finally, peak shape is greatly improved allowing for better resolution of closely eluting peptides.

Conclusions

The experiments shown in this note demonstrate the utility of using BioSep-SEC-S2000 with modified denaturing conditions (acetonitrile/TFA) for resolving low molecular weight proteins and peptides. These conditions allow for getting accurate gel filtration results for smaller proteins which can sometimes elute anomalously using native separation conditions. While not discussed in depth here, comparing peptide separated in native versus denaturing mobile phase conditions can elucidate some information on the nature of folding that may be occurring with specific peptides.

APPLICATIONS

Figure 2.
Separation of peptides on BioSep 2000 using TFA/organic buffer. Note the improved peak shape and better resolution between peptides of similar molecular weight. While some peptides behave anomalously, most peptides elute based closely to their molecular weight.



App ID 18945

Column: BioSep-SEC-S2000
Dimensions: 300 x 7.8 mm
Part No.: 00H-2145-K0
Mobile Phase: 45 % Acetonitrile, 0.1 % TFA
Flow Rate: 1 mL/min
Temperature: Ambient
Detection: UV @ 214 nm
Sample: Low MW proteins and peptides

1. Myoglobin 17800 Da
2. Cytochrome c 12400 Da
3. Apotinin 6500 Da
4. Insulin 5700 Da
5. Glucagon 3482 Da
6. Angiotensin I 1297 Da
7. Substance P 1-9 1104 Da
8. Bradykinin 1060 Da
9. Angiotensin II 1046 Da
10. Angiotensin 1-7 899 Da
11. Bradykinin 1-7 760 Da
12. Bradykinin 1-5 573 Da
13. Leu-Leu-Leu 358 Da
14. L-Glutathione 307 Da

BioSep™ Ordering Information

Stainless Steel Columns (mm):	Narrow Bore	Analytical		Preparative
Phases	300 x 4.6	300 x 7.8	600 x 7.8	300 x 21.2
BioSep-SEC-S2000	00H-2145-E0	00H-2145-K0	00K-2145-K0	00H-2145-P0
BioSep-SEC-S3000	00H-2146-E0	00H-2146-K0	00K-2146-K0	00H-2146-P0
BioSep-SEC-S4000	00H-2147-E0	00H-2147-K0	00K-2147-K0	00H-2147-P0

Stainless Steel Guard Columns (mm)	Narrow Bore	Express	Analytical
Phases	30 x 4.6	35 x 7.8	75 x 7.8
BioSep-SEC-S2000	03A-2145-E0	03Q-2145-K0	03C-2145-K0
BioSep-SEC-S3000	03A-2146-E0	03Q-2146-K0	03C-2146-K0
BioSep-SEC-S4000	03A-2147-E0	03Q-2147-K0	03C-2147-K0

guarantee

If BioSep analytical columns do not provide at least an equivalent separation as any other GFC column of similar porosity, type and dimensions, return the column with comparative data within 45 days for a FULL REFUND.

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