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Peptide Separations Using Size Exclusion Chromatography

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INTRODUCTION

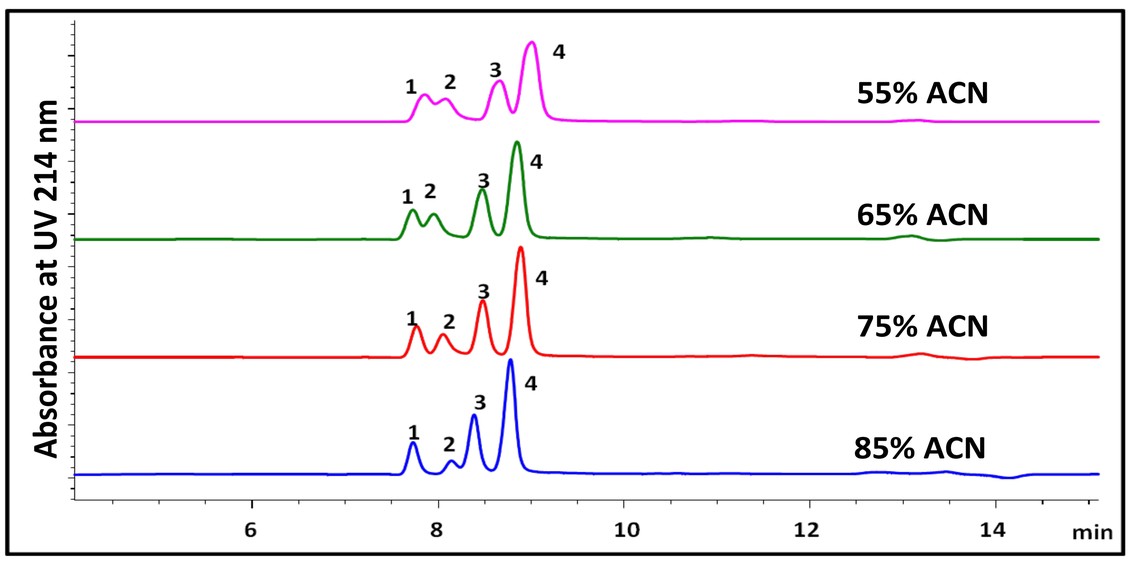
Size exclusion chromatography (SEC) has been applied successfully to separate different sizes of proteins under native conditions. Different pore sizes have been developed to accommodate different ranges of molecular weights of biological samples. In order to apply the size exclusion chromatography to peptides under 10,000 Da, a few limitations have to be overcome. Even very small peptides can exist in different conformations and exhibit secondary structures. Therefore, peptides tend to adsorb to column matrices by ionic and hydrophobic interactions. High salt concentrations, denaturing agents, and organic additives will minimize such interactions, thus enabling the separation of peptides according to their molecular weights. Sepax Zenix™ SEC columns are based on uniform, hydrophilic, and neutral nanometer thick films chemically bonded on high purity and mechanically stabilized silica. Zenix™ SEC-80 is specifically designed for small protein and peptide separations. Its phase is similar to the other Zenix™ SEC with the same particle size 3 µm, but different pore size at 80 Å. Here we present separation of four peptides bradykinin (1,060 Da), angiotensin I (1,297 Da), glucagon (3,483 Da) and insulin (5,778 Da) under different separation conditions. Separation of E.coli tryptic digest on Zenix™ SEC-80 is also investigated with different mobile phase conditions.

EXPERIMENTAL

HPLC System: Agilent 1200 HPLC with binary pump
SEC column: Zenix™ SEC-80 (3 µm, 80 Å, 7.8x300 mm)
Mobile Phases: different percentages of acetonitrile with 0.1% TFA and water, high salt and methanol additives.
Flow rate: 0.8 mL/min
Detection UV 214 nm
Column Temperature: 25 °C
Samples: Bradykinin acetate salt (MW 1,060 Da)
Angiotensin I Acetate (MW 1,297 Da)
Glucagon (MW 3,483 Da)
Insulin from porcine pancreas (MW 5,778 Da)
E. coli Lysate tryptic digest

All four peptide samples were purchased from Sigma-Aldrich. 5 mg/mL stock solutions were made with 50 mM acetic acid. For sample injections, stock solutions were further diluted with 50 mM acetic acid to desired concentration. Sequencing grade modified trypsin was purchased from Promega.

EFFECT OF ACETONITRILE MOBILE PHASE CONCENTRATION

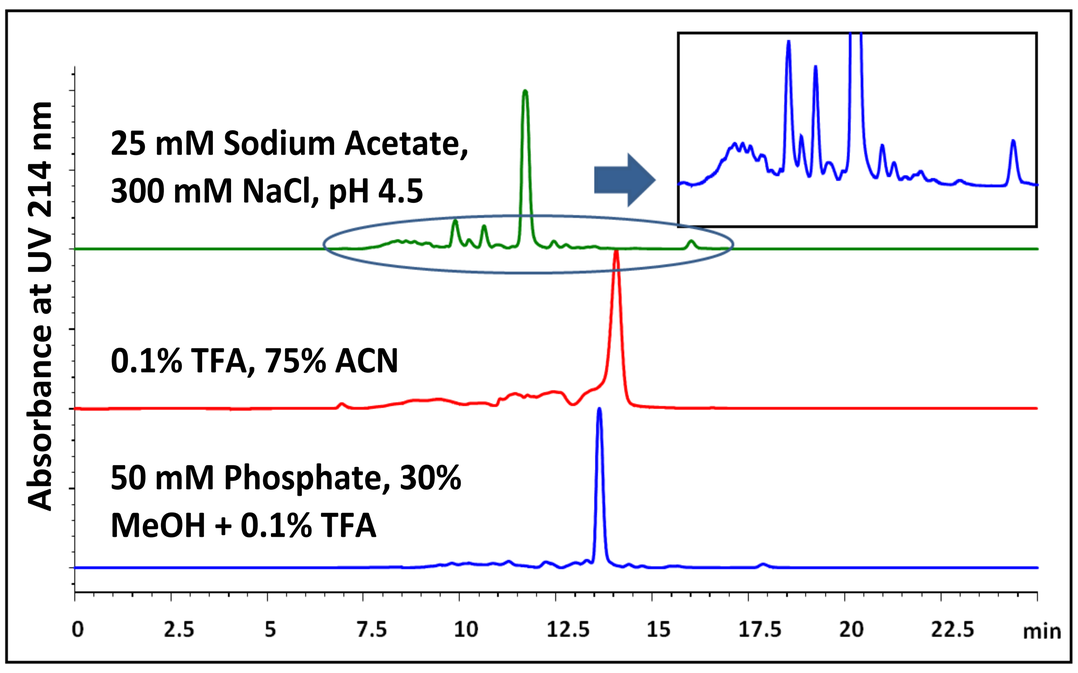


Effect of mobile phase acetonitrile concentration on the separation of the four peptide mixtures (Peak 1 Insulin, Peak 2 Glucagon, Peak 3 Angiotensin I, Peak 4 Bradykinin). The mobile phases contained 0.1% TFA with the indicated percentage of acetonitrile. The flow rate was 0.8 mL/min. 5 µL of the peptide mixture (0.5 mg/mL concentration for each peptide) was injected.

The table below summarizes the separation parameters for the four peptides on Zenix™ SEC-80, using the optimal acetonitrile concentration of 75%. The elution order of the four peptides was related to its molecular weight or size under the denaturing 0.1%TFA/75% acetonitrile. Angiotensin I and Bradykinin achieved baseline separation.

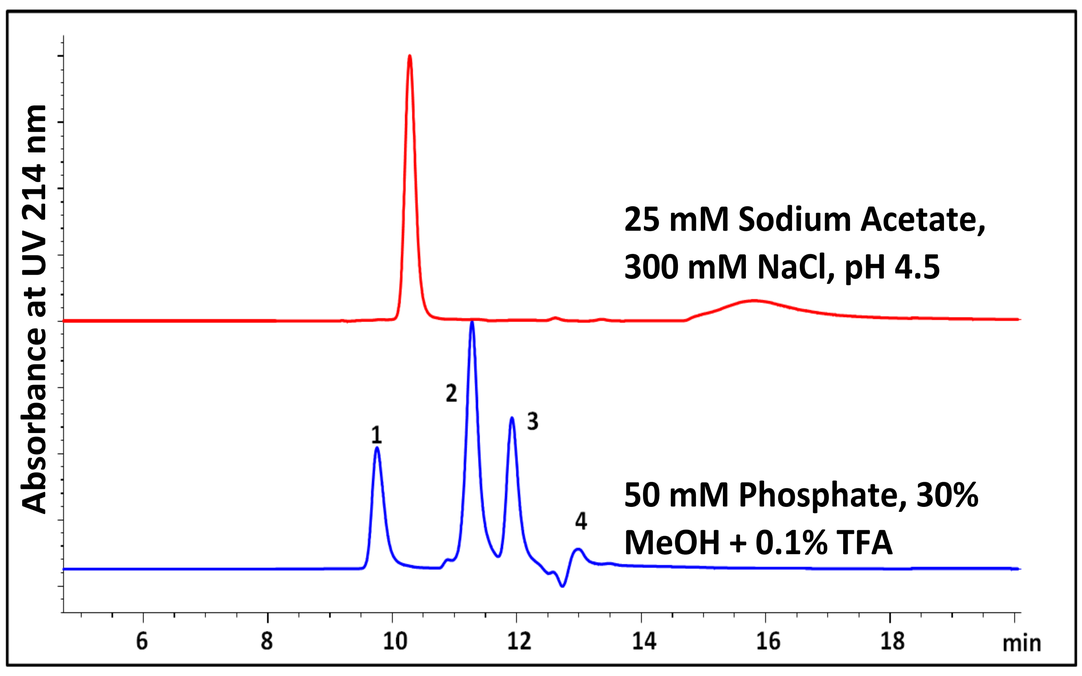
Peak	Protein	MW (Da)	Retention Time (min)	Resolution	Plate Count
1	Insulin	5777.60	7.749		16711
2	Glucagon	3482.75	8.034	1.07	12132
3	Angiotensin I	1296.48	8.457	1.58	19741
4	Bradykinin	1060.21	8.858	1.65	21060

SEPARATION OF E. COLI DIGESTS ON ZENIX™ SEC-80



For complex E. coli tryptic digest, a mobile phase of 25 mM sodium acetate/300 mM NaCl gave the best separation. The bottom chromatogram was run with 50 mM phosphate/30% MeOH/0.1% TFA. The middle one was run with 0.1% TFA/75% ACN. The top chromatogram represents the best degree of peptide separation and was run with 25 mM sodium acetate/300 mM NaCl, pH 4.5.

EFFECT OF SALT AND ORGANIC ADDITIVES ON PEPTIDE SEPARATION



Peak elution order: 1. Insulin, 2. Bradykinin, 3. Angiotensin I, 4. Glucagon. The flow rate was 0.8 mL/min. The top chromatogram was run with the mobile phase of 25 mM sodium acetate and 300 mM NaCl, pH 4.5. The bottom one was run with the mobile phase 50 mM Phosphate with 30% MeOH and 0.1% TFA.

CONCLUSION

Zenix™ SEC-80 successfully separated four peptides whose molecular weights range from 1 kD to 6 kD. Organic additives help disrupt the resin-peptide interaction. There is an optimum concentration of acetonitrile at 75% in aqueous 0.1% TFA as a mobile phase to give the best separation of the four peptides. Two 7.8x300 mm Zenix™ SEC-80 columns in tandem will improve separation with wider retention times between peptides. Rarely, a complex protein digest can be resolved in a single reversed phase LC run. With Zenix™ SEC-80, E. coli lysate can be pre-fractionated and the fractions can be further subjected to ion exchange or C18 reversed phase separation. SEC fractions can be directly applied to 2D LC/MS/MS as well for peptide mapping and identification purposes. Thus, sample complexity can be greatly reduced with Zenix™ SEC-80 pre-fractionation.

REFERENCES

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