Peptide Separations Using Size Exclusion Chromatography

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Peptide Separation

Abstract

Size exclusion chromatography (SEC) has been widely applied in protein separation based on their molecular sizes. Here we present a peptide mixture from 1kD to 6 kD separation on size exclusion chromatography column Zenix™-80. The effect of different mobile phases on the peptides' separation performances was also explored. In the separation of insulin, glucagon, angiotensin I and bradykinin, 0.1% TFA/75% acetonitrile/H_2O proved to be the optimum mobile phase. Peptide SEC can be applied to fractionate complex peptide mixtures as a first dimension chromatography in a multidimensional chromatography scheme. For complex *E. coli* tryptic digest, a mobile phase of 25 mM sodium acetate/300 mM NaCl gave the best separation.

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 weight 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.[1] Therefore, peptides tend to adsorb to column matrixes by ionic and hydrophobic interactions.[2] 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 3μm, but different pore size at 80 Å. In this application note, 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™-80 is also investigated with different mobile phase conditions.

Experimental

HPLC system:

Agilent 1200 HPLC with binary pump

SEC column and LC method:

Zenix™-80 (3 μM, 80 Å, 7.8x300 mm) was used for the peptide size exclusion separations. Mobile phases include different percentages of acetonitrile with 0.1% TFA and water, high salt and methanol additives. The flow rate was at 0.8 mL/min.

Chemicals and Reagents:

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

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.

E. coli lysate tryptic digestion:

E. coli lysate tryptic digestion was performed according to the procedure described previously.[3, 4] Briefly 0.5 mg dried lysate were reconstituted in 100 μl 6 M urea, 50 mM Tris-HCl, pH 8.0. A 0.2 M dithiothreitol (DTT) stock solution was added to obtain 10 mM concentration and the protein mixture was incubated at room temperature for 1 hour. A final concentration of 30 mM iodoacetamide (IAM) was reached by adding a stock solution of 0.2 M IAM. The alkylation was performed at room temperature for 1 hour. The final urea concentration was reduced to 0.6 M with the addition of 50 mM Tris-HCl, 1 mM $CaCl_2$, pH 7.6. The protein mixture was then digested with trypsin overnight at 37 ^{o}C with a trypsin: protein ratio of 1:50 (w/w). 1 μl of formic acid was added to stop the digestion. The digest was maintained at 4 ^{o}C in an auto sampler for SEC-HPLC runs or frozen at -20 ^{o}C for future analysis.

Results

Figure 1A showed the separation profile of four peptides with mobile phase 25 mM sodium acetate/300 mM NaCl, pH 4.5. Four peptides did not get separated with the addition of high salt. Based on the individual peptide injections (data not shown), the wide peak between 14.5 and 16.5 minutes in figure 1A was glucagon and insulin. Interaction between the peptides and the solid phase was the reason that glucagon and insulin were eluted later than angiotensin I and bradykinin (the main peak at 10.2 minutes). When the mobile phase was switched to 50 mM phosphate, 30% MeOH and 0.1% TFA, four peptides were separated, but not according to their molecular weight order. According to the molecular weights, the order of elution should be insulin, glucagon, angiotensin I and bradykinin.

Figure 1B showed the separation profile with the elution in the order of insulin, bradykinin, angiotensin I and glucagon. The combination of methanol and TFA was not strong enough to disrupt the interactions between the column matrix and peptides. Thus, it did not achieve separation of four peptides according to their molecular sizes.

When the mobile phase was switched to a TFA/acetonitrile system, all four peptides were eluted at earlier retention times, and separation resolutions were improved dramatically. The solid phase and peptide interaction was minimized with the addition of high concentration acetonitrile. As seen in Figure 2, the separation of the four peptides was closely associated with the percentage of acetonitrile in the mobile phases. To achieve the best separation of the peptide mixture, the optimal acetonitrile concentration was at 75% in aqueous 0.1% TFA. Table 1 summarized the separation parameters for four peptides on Zenix™-80. Angiotensin I and bradykinin achieved baseline separation. Furthermore, the elution order of the four peptides was related to their molecular weights or sizes under the denaturing 0.1% TFA/75% acetonitrile/H_2O.

Size exclusion chromatography can be used as a pre-fractionation first dimension separation for complex peptide mixtures. Figure 3 showed the separation profiles of *E. coli* digest under different mobile phase conditions. Under acidic salt condition with 25 mM sodium acetate/ 0.3 M NaCl, pH 4.5, the chromatogram exhibited a higher degree of peptide separation than the ones with the other two mobile phases - 0.1% TFA/75%acetonitrile/H_2O and 50 mM phosphate/ 30% MeOH/0.1% TFA.

Conclusion

Zenix™-80 successfully separated four peptides whose molecular weights range from 1 kD to 6 kD. Organic additives help disrupt the column-peptide interaction. There is an optimum concentration of acetonitrile at 75% in aqueous 0.1% TFA as mobile phase to give the best separation of the four peptides. Two 7.8x300 mm Zenix™-80 columns in tandem will improve separation with wider retention time between peptides.

Rarely, a complex protein digest can be resolved in one single reversed phase LC run. With Zenix™-80 SEC, 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 purpose. Thus sample complexity can be greatly reduced with Zenix™-80 SEC pre-fractionation.

For more information on Zenix™- SEC products, please visit our website: http://www.sepax-tech.com/Zenix.php or contact us at 1-877-SEPAX-US.

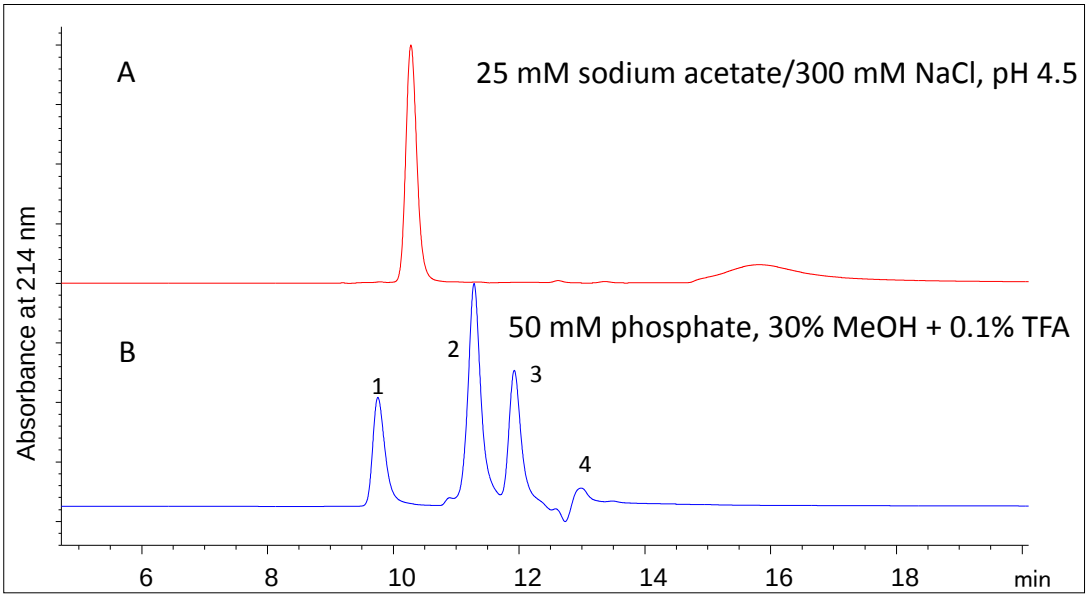

Figure 1. Peptide separation (bradykinin, angiotensin I, glucagon and insulin) on Zenix™-80 (7.8x300 mm). Panel A was with mobile phase 25 mM sodium acetate and 300 mM NaCl, pH 4.5. Panel B was with mobile phase 50 mM phosphate, 30% MeOH and 0.1% TFA. Peak elution order: 1. Insulin, 2. Bradykinin, 3. Angiotensin I, 4. Glucagon. The flow rate was 0.8 mL/min.

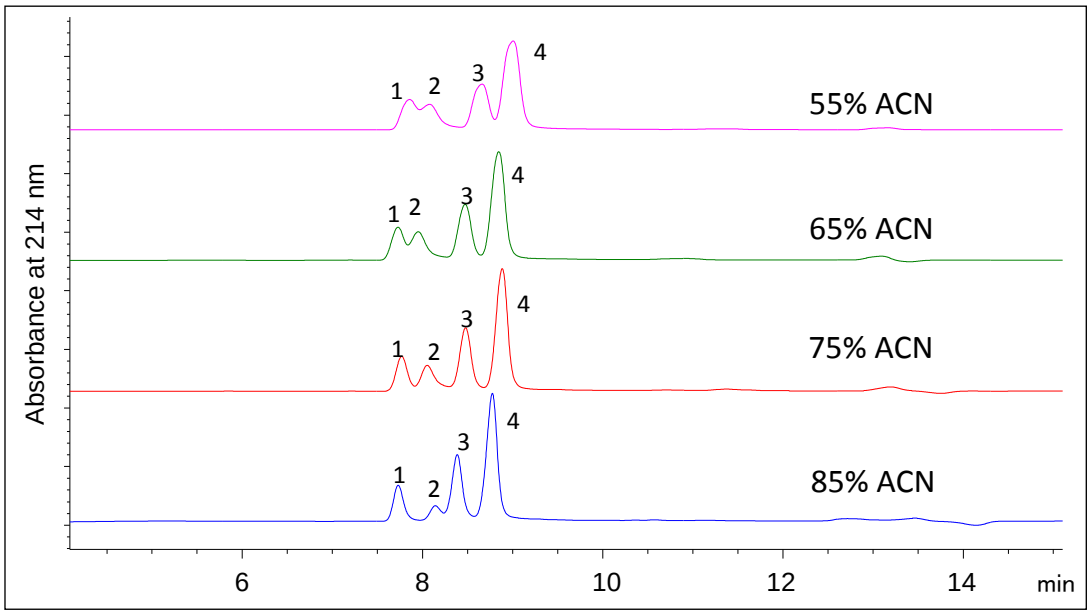

Figure 2. Effect of mobile phase acetonitrile concentration on the separation of the four peptide mixtures on Zenix™-80 (7.8x300 mm) (Peak 1, insulin, Peak 2, glucagon, Peak 3, angiotensin I, Peak 4, bradykinin). The mobile phases contained 0.1% TFA with the indicated percentage of acetonitrile. The flow rate was 0.8 mL/min. 5 µL of peptide mixture (0.5 mg/mL concentration for each peptide) was injected.

Table 1. Separation parameters for the four peptide mixture with mobile phase 75% Acetonitrile in 0.1% TFA with water.

Peak	Protein	MW (Da)	Retention time (min)	Resolution	Plate counts
1	Insulin (porcine)	5,778	7.75		16,711
2	Glucagon	3,483	8.03	1.07	12,132
3	Angiotensin I	1,297	8.46	1.58	19,741
4	Bradykinin	1,060	8.86	1.65	21,060

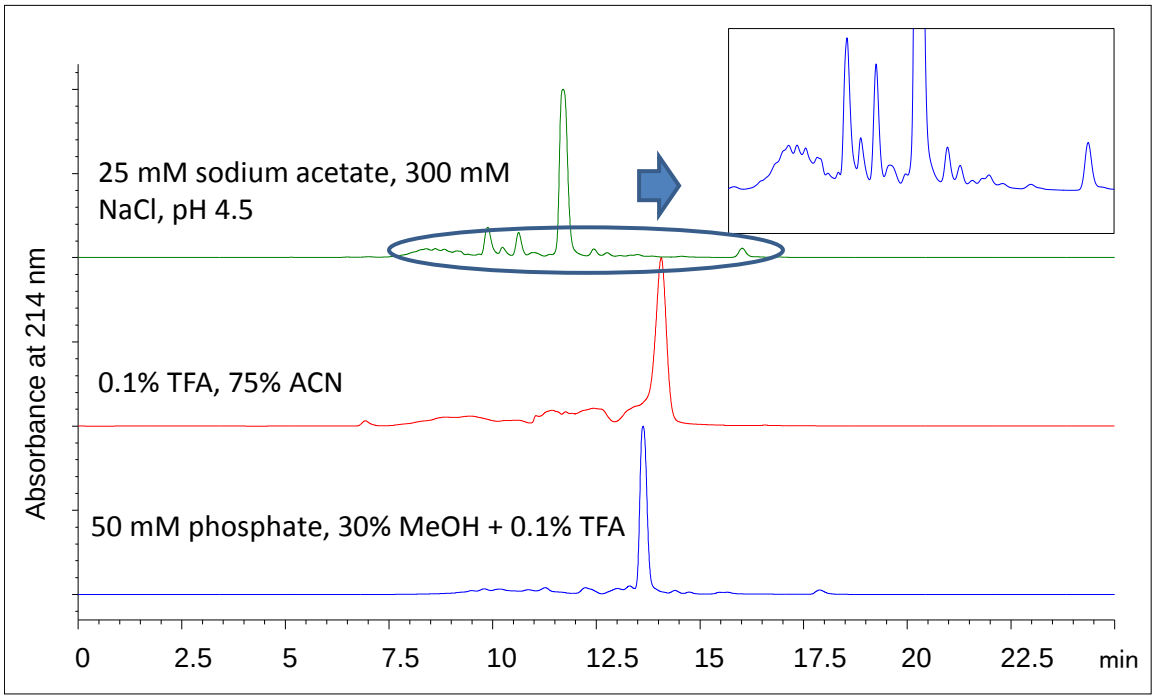

Figure 3. Separation of *E. coli* digests on Zenix™-80 (7.8x300mm) under indicated mobile phases. Bottom chromatogram was for the run with 50mM phosphate/30%MeOH/0.1% TFA. The middle one was for the run with 0.1% TFA/75% acetonitrile. The top chromatogram represented the run with 25 mM sodium acetate/300 mM NaCl, pH 4.5. The inset was for the zoom view for the part of the chromatogram circled.

Reference:

1. Irvine G. J. Biochem. Biophys. Methods, (2003) **56** 233-242.
2. Swergold G., and Rubin C. Anal. Biochem. (1983) **131**, 295-300
3. Tryptic digestion protocol: http://ipmb.sinica.edu.tw/proteomics/Documents/In-Sol-Digest.pdf
4. Kinter, M., and Sherman, N. E. 2000, Protein sequencing and identification using tandem mass spectrometry. JohnWiley & Sons, Inc. pp.161-163

Order information

Part Number	Particle Size	Pore Size	IDxLength
213080-2105[1]	3 µm	80 Å	2.1x50mm
213080-2130	3 µm	80 Å	2.1x300mm
213080-4605[1]	3 µm	80 Å	4.6x50mm
213080P-4605[1][2]	3 µm	80 Å	4.6x50mm
213080-4615	3 µm	80 Å	4.6x150mm
213080-4625	3 µm	80 Å	4.6x250mm
213080-4630	3 µm	80 Å	4.6x300mm
213080P-4630[2]	3 µm	80 Å	4.6x300mm
213080-7805[1]	3 µm	80 Å	7.8x50mm
213080-7815	3 µm	80 Å	7.8x150mm
213080-7820	3 µm	80 Å	7.8x200mm
213080-7830	3 µm	80 Å	7.8x300mm
213080-10005[1]	3 µm	80 Å	10.0x50mm
213080-1010	3 µm	80 Å	10.0x100mm
213080-10015	3 µm	80 Å	10.0x150mm
213080-10025	3 µm	80 Å	10.0x250mm
213080-10030	3 µm	80 Å	10.0x300mm
213080-21205[1]	3 µm	80 Å	21.2x50mm
213080-21225	3 µm	80 Å	21.2x250mm
213080-21230	3 µm	80 Å	21.2x300mm

[1] Guard column
[2] Column packed with PEEK tubing