Application Update 188

High-Resolution Cation-Exchange Alternative to Peptide Mapping for Protein ID and QA/QC

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Key Words
Ion Exchange, ProPac SCX-10 Column, MabPac SCX-10 Column, Fast Separation

Goal
To develop an efficient and high-resolution peptide mapping method in cation-exchange mode as an alternative to reversed-phase separation

Introduction
Peptide mapping is commonly used to demonstrate protein identity. In later phases of pharmaceutical development and in quality assurance/control (QA/QC), peptide mapping with UV detection of the protein drug serves as a primary protein QC method. Although a reversed-phase separation is the typical choice for separating peptides, high-resolution ion-exchange chromatography is an alternate method that provides additional information and a different selectivity. In proteomics applications, strong cation-exchange chromatography is routinely the choice for the first dimension separation of peptides.[1]

The Thermo Scientific ProPac SCX-10 and MabPac SCX-10 Analytical Columns are constructed by grafting cation-exchange groups onto a nonporous polymeric particle that has a hydrophilic coating. This construction results in high-efficiency peaks and high-resolution separations of protein variants.[2] The work shown here demonstrates that strong cation-exchange chromatography can provide good resolution for peptide mapping, and thereby serve as an alternate or supplementary method for separating peptides.

Equipment
- Thermo Scientific Dionex UltiMate 3000 ×2 Dual Biocompatible Analytical Liquid Chromatography (LC) System, including:
  - DGP-3600BM Biocompatible Dual-Gradient Micro Pump with SRD-3600 Integrated Solvent and Degasser Rack
  - WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection
  - TCC-3000SD Thermostatted Column Compartment
  - DAD-3000 Diode Array Detector
- Thermo Scientific Dionex Chromeleon Chromatography Data System software version 6.80, SR9 or higher
- Thermo Scientific Orion 2-Star Benchtop pH Meter

Reagents and Standards
- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (CH_3CN), HPLC grade (Fisher Scientific P/N AC610010040)
- Formic acid, analytical grade, SCRC, China
- Sodium perchlorate, analytical grade, SCRC, China
- Triethylamine, HPLC grade, CNW Technologies GmbH, Germany
- Trypsin from bovine pancreas (Sigma-Aldrich® P/N T1426)
- Myoglobin from equine heart (Sigma-Aldrich P/N M1882)
- DL-Dithiothreitol, ≥99% (Fluka P/N 43819, Sigma-Aldrich)
- Iodoacetamide, ≥99% (Sigma-Aldrich P/N I6125)

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Preparation of Solutions

Buffers for Tryptic Digestion

For detailed methods of preparing buffers for tryptic digestion, refer to Dionex (now part of Thermo Scientific) Application Update (AU) 183.[3]

Mobile Phases with Triethylamine Phosphate (TEAP)

Prepare a 20 mM triethylamine (TEA) solution, then adjust the pH of the solution to 2.0 or 3.9 using phosphoric acid. Add acetonitrile and sodium perchlorate to make mobile phase A or mobile phase B for the ion-exchange separation. For example, mix the pH 2 TEAP solution 1:1 with acetonitrile to prepare mobile phase A shown in Figure 1. To prepare mobile phase B shown in Figure 1, prepare 1 L of mobile phase A and dissolve 14.05 g of sodium perchlorate in that solution.

Sample Preparation

Reduce, alkylate, and dialyze myoglobin extensively against 50 mM sodium bicarbonate. Then digest the resulting reduced and alkylated myoglobin with trypsin overnight. For detailed procedures, refer to AU 183.

Results and Discussion

Use of a TEAP/sodium perchlorate mobile phase with manipulation of the pH and organic solvent modifier concentration allowed high-resolution separation of an equine heart myoglobin tryptic digest (Figure 1). This separation was easily accelerated without compromising resolution by simply increasing the flow rate. This method can also be applied to a synthetic peptide. Figure 2 shows similar high resolution and fast separation of a synthetic peptide and its byproducts. In contrast to the myoglobin tryptic digest separation, better separation was observed at pH 3.9 than at 2.0. This may be due to the basic nature of the synthetic peptide.

Separation on the cation-exchange column is primarily determined by analyte charge, but hydrophilic and hydrophobic interactions also play a role. Organic solvent modifiers such as acetonitrile improve separation by changing solubility, hydrophilic interaction, and possibly peptide conformation. Sodium perchlorate was used to elute peptides due to its better solubility in acetonitrile and its stronger elution power compared to the commonly used sodium chloride.

Column:	ProPac™ SCX-10, 10 µm (4 × 250 mm, P/N 075725)
Mobile Phase:	A: 20 mM TEAP, pH 2.0, 50% acetonitrile B: 100 mM sodium perchlorate in A
Gradient:	**1**. 0–40 min, 0–100% B, at 0.7 mL/min **2**. 0–28 min, 0–100% B, at 1.0 mL/min **3**. 0–20 min, 0–100% B, at 1.4 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV, 214 nm
Sample:	Myoglobin (from equine heart) tryptic digest
Sample Preparation:	Reduce, alkylate, dialyze, and digest with trypsin overnight

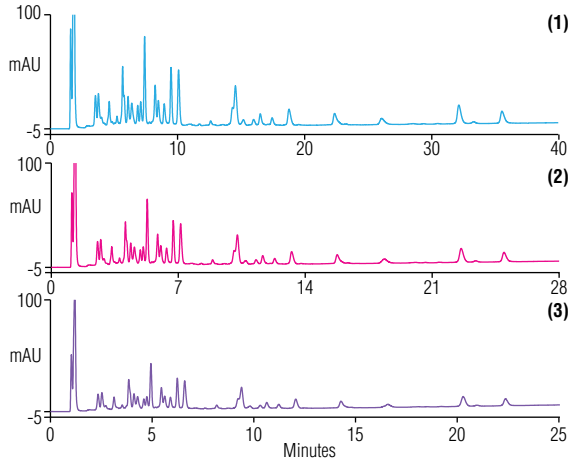

Figure 1. Chromatograms of a myoglobin tryptic digest with different flow rates.

Column:	ProPac SCX-10 10 µm (4 × 250 mm)
Mobile Phase:	**1**. A: 20 mM TEAP, pH 3.9, 50% acetonitrile B: 100 mM perchlorate in A **2**. A: 20 mM TEAP, pH 2.0, 50% acetonitrile B: 100 mM perchlorate in A
Gradient:	0–10 min, 0–50% B
Flow Rate:	1.4 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV, 214 nm
Sample:	A synthetic peptide and its byproducts The sequence of the peptide: (Ac-)YNIQKESTLPLVLRLRGG (–$CONH_2$) Calculated pI of the peptide: 11.4

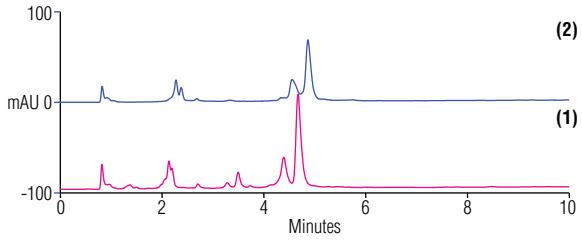

Figure 2. Chromatograms of a synthetic peptide and its byproducts at different mobile phase pH values.

Figure 3 shows that the ProPac SCX-10 column can provide high resolution comparable to the reversed-phase separations that use formic acid as a mobile phase modifier. This is in contrast to many ion-exchange columns that yield incomplete resolution and poor peak shape in peptide mapping. Figure 4 shows that a reversed-phase separation with trifluoroacetic acid (TFA) can be better than the cation-exchange separation.

Columns:	**1**. ProPac SCX-10, 10 µm (4 × 250 mm, P/N 075725) **2**. Acclaim™ PA2, 3 µm (3.0 × 150 mm, P/N 063705) **3**. Acclaim 300, C18, 3 µm (3.0 × 150 mm, P/N 063684)
Mobile Phase:	**1**. A: 20 mM TEAP, pH 2.0, 50% acetonitrile B: 100 mM sodium perchlorate in A **2**. A: 98% water, 2% acetonitrile, 0.1% formic acid B: 98% acetonitrile, 2%water, 0.08% formic acid **3**. Same as 2
Gradient:	**1**. 0–20 min, 0–100% B, at 1.4 mL/min **2**. 0–5 min, 0% B; 5–28 min, 0–100% B, at 0.6 mL/min **3**. 0–5 min, 0% B; 5–28 min, 0–100% B, at 0.5 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV, 214 nm
Sample:	Myoglobin (from equine heart) tryptic digest
Sample Preparation:	Reduce, alkylate, dialyze, and digest with trypsin overnight

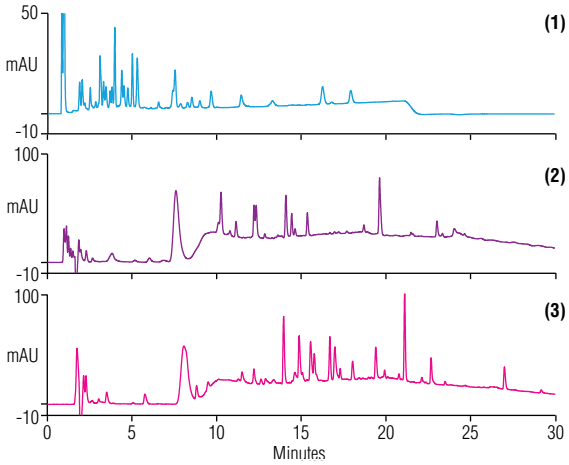

Figure 3. Chromatograms of a myoglobin tryptic digest separated by 1) a ProPac SCX-10 column, 2) a Thermo Scientific Acclaim PolarAdvantage II (PA2) column, and 3) an Acclaim 300 C18 column with formic acid as the mobile phase modifier of reversed-phase separation.

Columns:	**1**. ProPac SCX-10, 10 µm (4 × 250 mm, P/N 075725) **2**. Acclaim PA2, 3 µm (3.0 × 150 mm, P/N 063705) **3**. Acclaim 300, C18, 3 µm (3.0 × 150 mm, P/N 063684)
Mobile Phase:	**1**. A: 20 mM TEAP, pH 2.0, 50% acetonitrile B: 100 mM sodium perchlorate in A **2**. A: 98% water, 2% acetonitrile, 0.04% TFA B: 98% acetonitrile, 2%water, 0.03% TFA **3**. Same as 2
Gradient:	**1**. 0–20 min, 0–100% B, at 1.4 mL/min **2**. 0–5 min, 0% B; 5–28 min, 0–100% B, at 0.6 mL/min **3**. 0–5 min, 0% B; 5–28 min, 0–100% B, at 0.5 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV, 214 nm
Sample:	Myoglobin (from equine heart) tryptic digest
Sample Preparation:	Reduce, alkylate, dialyze, and digest with trypsin overnight

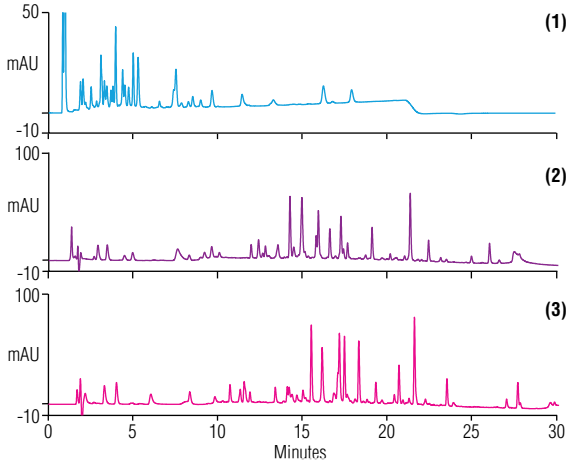

Figure 4. Chromatograms of a myoglobin tryptic digest separated by 1) a ProPac SCX-10 column, 2) an Acclaim PA2 column, and 3) an Acclaim C18 column with TFA as the mobile phase modifier of reversed-phase separation.

Table 1 summarizes peak capacity of an ion-exchange column, the Dionex ProPac SCX-10, and two reversed-phase columns, the Acclaim PA2 and Acclaim 300. Peak capacity(n_C) was calculated by the equation:

$$n_C = 1 + \frac{t_G}{W}$$

where t_G is the gradient time and W the peak width measured at 4σ (13.4% of the peak height). For detailed information, refer to Dionex (now part of Thermo Scientific) Technical Note (TN) 74.[4]

Table 1. Peak capacity comparison of three columns.

	ProPac SCX-10 (10 µm)	Acclaim PA2 (3 µm)	Acclaim 300 C18 (3 µm)
Formic acid as the mobile phase modifier in a reversed-phase separation	73	79	140
TFA as the mobile phase modifier in a reversed-phase separation	73	136	158

With the next-generation MabPac™ SCX-10 (3 µm) column, both separation efficiency and resolution can be further improved (Figure 5). Separation time can be easily reduced to <10 min by simply increasing the flow rate. Additionally, the peptides have better retention and are better resolved on the MabPac SCX-10 column compared to the ProPac SCX-10 column.

For the synthetic peptide and byproducts separation, the ProPac SCX-10 and MabPac SCX-10 (3 µm) columns provide similar resolution (Figure 6). The latter column performs better for resolving the main product (Peak 1) and a nearby byproduct (Peak 2).

Conclusion

This work shows that the ProPac SCX-10 column delivers high-resolution separations for peptide mapping and provides an alternate or supplementary method for reversed-phase peptide separation. The new MabPac SCX-10 (3 µm) column delivers faster high-resolution peptide mapping. Both columns can be used to separate a synthetic peptide and its byproducts, providing an alternative to reversed-phase separation.

References

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3. Dionex (now part of Thermo Scientific) Application Update 183: Separation of Peptides from Enzymatic Digestion of Different Acclaim Columns: A Comparative Study. Sunnyvale, CA, 2011. [Online] www.dionex.com/en-us/webdocs/111297-AU183-LC-Peptides-EnzyDigest-AcclaimCompar-02Nov2011-LPN2973.pdf (accessed June 18, 2012).
4. Dionex (now part of Thermo Scientific) Technical Note 74: High Peak Capacity Nano LC Peptide Separations Using Long Packed Columns. Sunnyvale, CA, 2009. [Online] www.dionex.com/en-us/webdocs/70394-TN74-HPLC-Peptides-LongColumns-29Jan09-LPN2111.pdf (accessed Aug 8, 2012).

Columns:	**1**. ProPac SCX-10, 10 µm (4 × 250 mm, P/N 075725) **2**. MabPac SCX-10, 3 µm (4 × 50 mm, P/N 077907)
Mobile Phase:	A: 20 mM TEAP, pH 2.0, 50% acetonitrile B: 100 mM sodium perchlorate in A
Gradient:	**1**. 0–20 min, 0–100% B, at 1.4 mL/min **2**. 0–15 min, 0–100% B; 15.5–25 min, 0% B, at 0.4 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV, 214 nm
Sample:	Myoglobin (from equine heart) tryptic digest
Sample Preparation:	Reduce, alkylate, dialyze, and digest with trypsin overnight

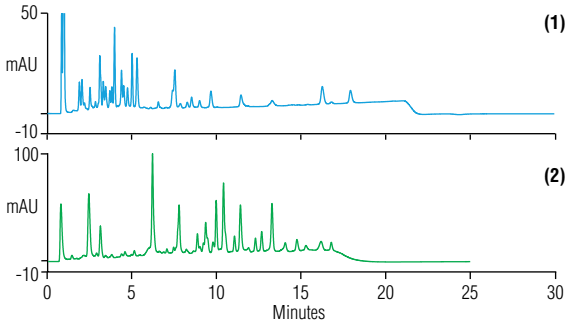

Figure 5. Chromatograms of a myoglobin tryptic digest separated by 1) a ProPac SCX-10 column (10 µm particle size) and 2) a MabPac SCX-10 column (3 µm particle size).

Columns:	**1**. ProPac SCX-10, 10 µm (4 × 250 mm, P/N 075725) **2**. MabPac SCX-10, 3 µm (4 × 50 mm, P/N 077907)
Mobile Phase:	A: 20 mM TEAP, pH 3.9, 50% acetonitrile B: 100 mM sodium perchlorate in A
Gradient:	**1**. 0–20 min, 0–100% B, at 1.4 mL/min **2**. 0–15 min, 0–100% B; 15.5–25 min, 0% B, at 0.4 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV, 214 nm
Sample:	A synthetic peptide and its byproducts The sequence of the peptide: (Ac-)YNIQKESTLPLVLRLRGG(–$CONH_2$) Calculated pI of the peptide: 11.4

Peak X is also shown in the blank, so it should not be considered a real peak.

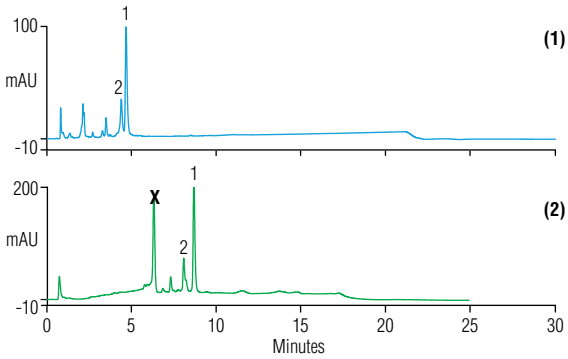

Figure 6. Chromatograms of a synthetic peptide and its byproduct using 1) a ProPac SCX-10 column (10 µm particle size) and 2) a MabPac SCX-10 column (3 µm particle size)

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