Strong Cation-Exchange and Reversed-Phase Purification of a Synthetic Peptide

Introduction

A reversed-phase column (218TP54) and a sample of a peptide (23-mer with no cysteine or tryptophan, but containing a methionine) were sent to Vydac by a synthetic peptide company for the purpose of improving the resolution. Similar separation problems had occurred on both the analytical column and a preparative C18 column.

During peptide synthesis, hydrophobic contaminants often accumulate from condensation of side chain protectants and scavenger reagents. These contaminants can complex with peptides and bind tightly to reversed-phase columns, causing problems with chromatography. Although most synthetic peptides are purified by reversed-phase alone, difficult purifications can be simplified by running ion exchange first with at least 25% acetonitrile (ACN) in the ion-exchange mobile phase. Including ACN in the mobile phase improves retention of charged peptides. Ionic interactions are stronger in solvent than in pure water. ACN also acts as a chaotrope. It breaks up hydrophobic complexes between peptides, protecting-group residues, and scavenger reaction products. The hydrophobic contaminants then elute early in the ion-exchange run, ahead of the charged peptides. If not separated by ion exchange, hydrophobic complexes can remain intact on reversed phase and appear at a different position than either the peptide or contaminant alone.

Advantages of this procedure

When reversed phase is preceded by ion exchange:

1. *The combined effect of the two chemistries produces better purification than either chemistry alone.* Ion exchange and reversed phase are orthogonal separation methods. The effects of the two methods are synergistic; final resolution is greater than with either method alone.
2. *The ion-exchange chromatogram can aid in locating the desired product.* Sometimes reversed-phase analysis of a crude synthetic peptide does not clearly indicate a main peak. When ion exchange is run first, the last major peak on the ion-exchange chromatogram usually contains the peptide of interest. Shorter peptides missing a charged amino acid will elute earlier. Shorter peptides with the correct number of charged amino acids will also elute last, but can be distinguished on the reversed-phase run.
3. *Hydrophobic reaction products are removed by the ion-exchange step.* This reduces the loading capacity required for the reversed-phase separation. In addition, fouling of the reversed-phase column that may require cleaning with IPA or stronger solvents is eliminated.
4. *Strongly acidic cleavage reagents are removed prior to reversed-phase.* HF and TFA reagents used in cleavage reactions can attack a silica-based reversed-phase column. An initial step of ion-exchange on a chemically resistant polymer-based column removes these reagents, thereby protecting the reversed-phase column.

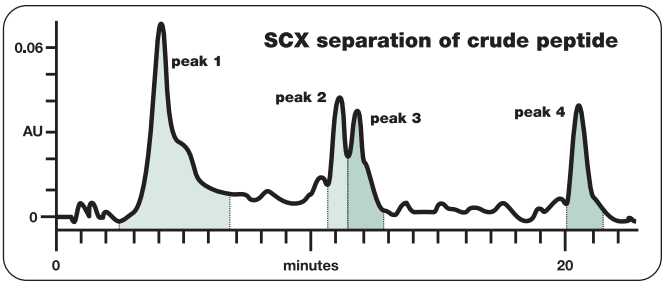

Figure 1. SCX separation of the crude peptide in 25% ACN. Column: Vydac 400VHP575 sulfonic acid polymer cation exchange, 5µm, 7.5mm ID x 50 mm L. Conditions: 1mL/min. 220nm. Buffer A= 0.1% TFA (w/v) in 25% ACN. Buffer B = A + 500 mM NaCl. Gradient = 0 to 60%B in 30 minutes.

Choosing the ion-exchange mode

If the sequence of the peptide is unknown, strong cation exchange (SCX) at pH 2 is the first choice. Most peptides are cationic at pH 2 (acidic amino acids are protonated), and this is the same pH as reversed-phase in 0.1% TFA. As long as the peptide has 2-3 positive charges (the amino terminal and one or two histidines, lysines or arginines) the peptide will bind to an SCX column in 25% ACN.

In the case of synthetic peptides, one normally *does* know the sequence and can choose anion exchange (Vydac 301VHP575 column) if the peptide is anionic, or run cation exchange at pH 4 if the net charge is still positive at that pH. Ion exchange at a pH where all charged amino acids are ionized gives the highest resolution of charge differences.

In the case described here, the sequence was not known by Vydac. So a Vydac 400VHP575 column ("SCX" cation exchange, 5µm, 7.5 mm ID x 50 mm L) was used.

Results

Figure 1 shows the ion-exchange chromatogram of the crude peptide. There are four peaks. The first is close to the injection. It elutes before the salt gradient reaches the column, due to the delay volume (4 mL) in the HPLC system. The molecules in Peak 1 are probably not cationic, but side-chain protecting groups and scavenger reaction products. Peak 4 has the most positive charges and is therefore predicted to contain the full-length peptide.

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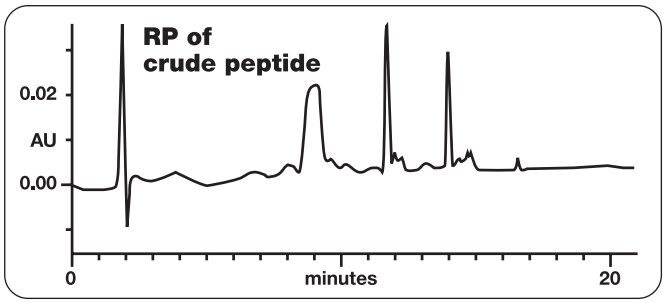

Figure 2. Reversed-phase separation of crude peptide. Column: Vydac 218TP54 C18, 5µm, 4.6mm ID x 250mm L. Conditions: 1.5 mL/min. 220nm. Gradient = 20-60% ACN over 30 min in 0.1% TFA (w/v).

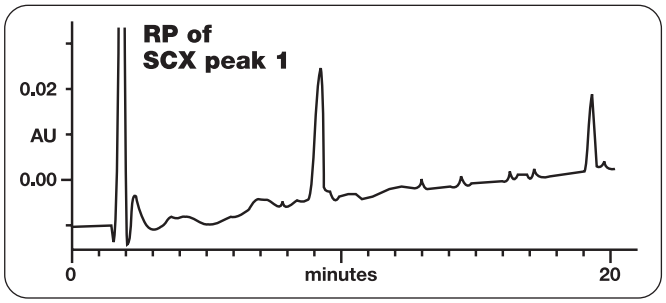

Figure 3. Reversed-phase separation of SCX Peak 1. Column and Conditions: As in Figure 2. There are two peaks. One elutes at the same time as the wide, asymmetric peak in Figure 2. The other, at 19 minutes, was not present in Figure 2. The appearance of this new peak at 19 minutes is a confirmation that ion exchange in solvent was needed. The molecules in this peak probably eluted as complexes in Figure 2. The complexes were broken up by the 25% ACN in the ion-exchange separation.

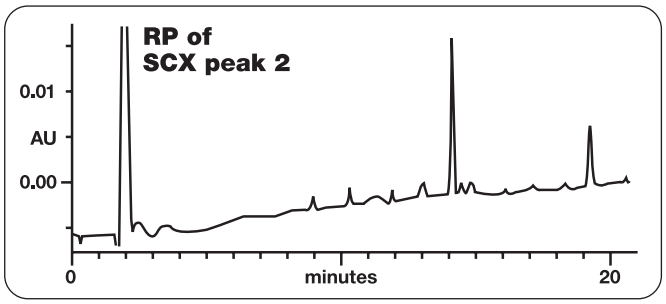

Figure 4. Reversed-phase separation of SCX Peak 2. Column and Conditions: As in Figure 2. There are two peaks; one at 14 minutes and one at 19 minutes. The 14-minute peak was the last main peak in Figure 2, but cannot be the full length peptide because it is missing at least one positive charge.

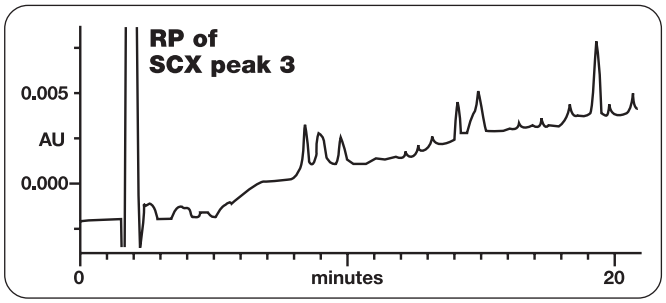

Figure 5. Reversed-phase separation of SCX Peak 3. Column and Conditions: As in Figure 2. There are a variety of peaks as well as the 19 minute peak. None of these have the same number of charges as the full length peptide. This is a good example of ion exchange simplifying the subsequent reversed-phase isolation. Unless the 19 minute peak is important, all this material is best removed by SCX prior to reversed-phase.

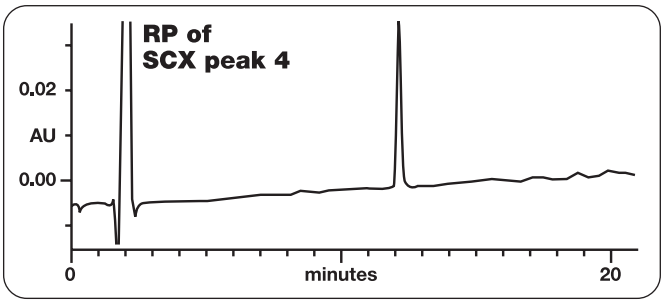

Figure 6. Reversed-phase separation of SCX Peak 4. Column and Conditions: As in Figure 2. This separation yields only one main peak which elutes at the same time as the 12-minute peak in Figure 2. There is no 19 minute peak. There are very few contaminant peaks. The 12-minute peak was predicted to be the full-length peptide, and this was confirmed. An initial SCX step would make large scale purification of this peptide very efficient because the preparative C18 load contains only this fraction. All the material that eluted in Figures 3, 4 and 5 is eliminated.

Figure 2 shows what a reversed-phase separation of the crude peptide looks like without first performing ion exchange. There are three peaks. The first is wide and asymmetric. This poorly defined peak at the beginning of a reversed-phase analysis suggests the presence of small molecules that can elute either alone or as complexes. *It is one sign indicating a need for ion-exchange in organic solvent prior to reversed-phase.* The lack of an obvious main peak is another sign that an ion-exchange step is needed.

Figures 3 through 6 show reversed-phase separations of peaks from the ion-exchange column. Each chromatogram is discussed in detail in the figure legend. The simple chromatogram of Figure 4, containing the desired product, shows the power of this procedure.

Conclusion

Ion exchange prior to reversed phase simplifies difficult peptide purifications, helps in identifying the desired peptide, and improves product purity. The procedure may also be useful for purifying peptides and proteins from other sources.

ORDERING INFORMATION:

Vydac Cat. #	Description
	Cation-exchange column:
400VHP575	Polymer-based Sulfonic Acid, 5µm, 7.5mmID x 50mmL
	Reversed-phase column:
218TP54	Silica-based C18, 5µm, 4.6mmID x 250mmL

Alternative (see page 1)

	Anion-exchange column:
301VHP575	Polymer-based DEAE, 5µm, 7.5mmID x 50mmL

Larger columns are also available.

To place an order contact, The Nest Group, Inc. toll-free at 800-347-6378.