Flash Peptide Purification and Method Development: Thymosin

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Chromatography Application Note AN125

Abstract

This application note continues the exploration of purification of peptides with flash chromatography. In our previous Application Note, AN124, we demonstrated that flash chromatography could purify small peptides. As flash chromatography has long been used for small molecules, purification of small peptides would be expected. In this application note, however, a larger peptide molecule is evaluated. Despite a molecular weight of 2950 Da, thymosin was purified with flash chromatography. Larger peptides contain more impurities, making them difficult to be cleaned with flash chromatography. Redi*Sep* Gold® reverse phase columns purified the peptide very well; selectivity differences between the columns were exploited to provide pure sample.

Background

Peptides, especially larger chain lengths, are traditionally purified on preparative HPLC systems. Synthesized peptides typically contain several impurities—most commonly deletions, which are chromatographically like the desired peptides. Until now, flash chromatography didn't have the resolution needed to resolve these compounds, especially as the peptides become larger and the proportion of impurities is increased. Single amino acid deletions are similar to the desired compounds. Thymosin (sequence H2N-SDAAVDTSSEITTKDLKEKKEVVEEAEN-OH) was chosen for these experiments because it is a reasonably large peptide.

Experimental and results

Method Development

Method development consists of two parts, determining the best column and determining the elution conditions. The pI of this peptide was estimated to be ~4.5, so the solvent system was buffered to pH 3.8 with 50 mMol ammonium formate to keep the peptide in a single ionization state. The organic solvent, methanol, contained no additives. Ammonium formate is volatile, and so may be used with a mass spectrometer; the fluid interface reduces the buffer concentration to a level compatible with the mass spectrometer. Method development was run on 15 g columns.

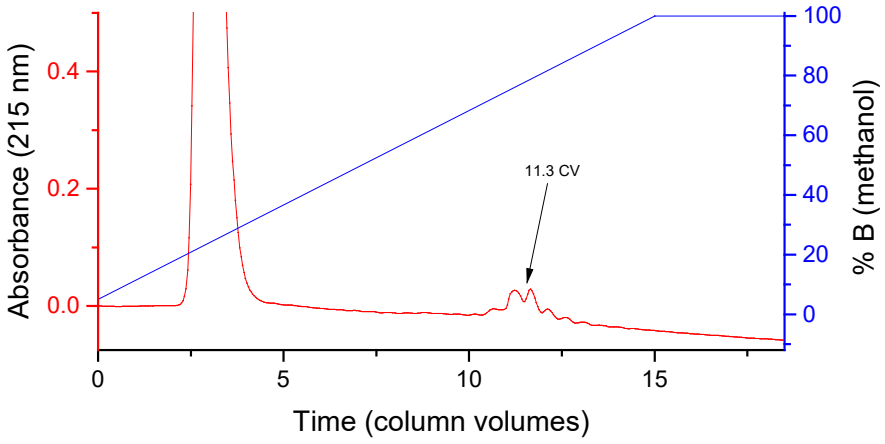

Figure 1—Scouting gradient of Thymosin on 15.5 g RediSep C18 columns (1 milligram of peptide).

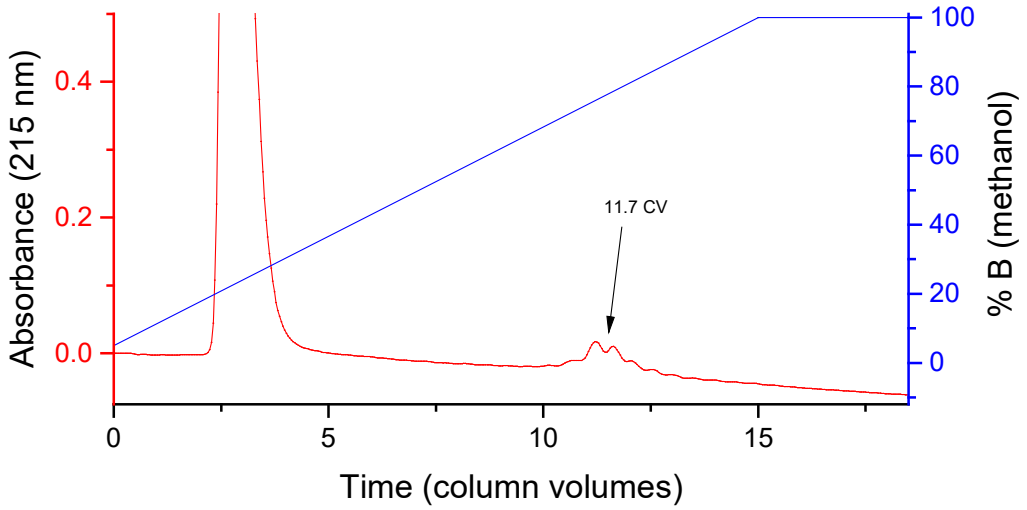

Figure 2—Thymosin purified on a 15.5 g RediSep Gold C18 flash column. 60 mg of peptide was recovered from 100 mg loaded.

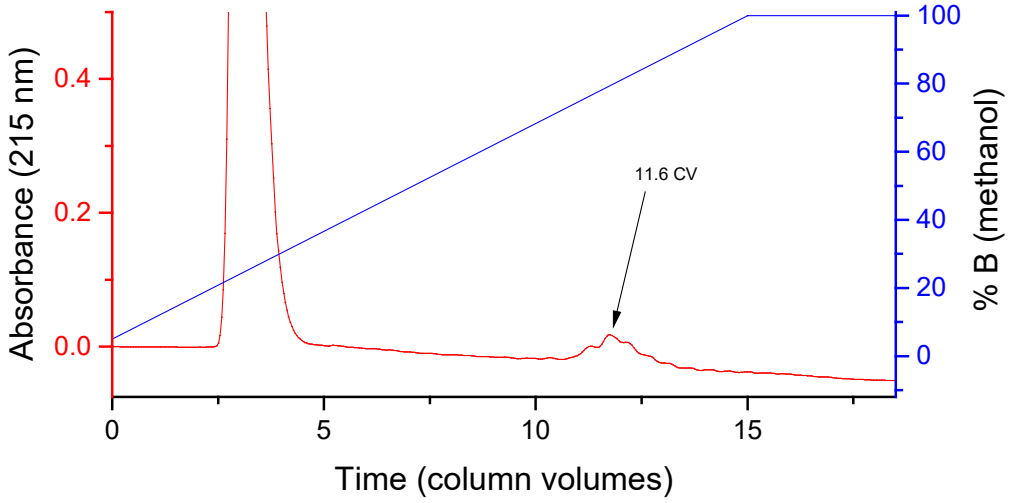

Figure 3—Scouting gradient of Thymosin on 15.5 g RediSep C8 column (1 milligram of peptide).

The runs all used the same scouting gradient, solvent, and sample. Only one milligram of sample was injected for each run. Although the peptide itself is soluble in water, there was non-water-soluble material in the peptide. Neat trifluoroacetic acid dissolved the sample very well, so the injection was 1 mg of sample dissolved in 0.2 mL TFA.

Although all columns showed similar retention, the C8 column showed a narrower peak with improved resolution from later eluting materials at ~11.6 column volumes (CV). The focused gradient was determined using the Flash Focus Gradient Generator as described in Chromatography Technical Note 65.

Purification

The solvents for the purification were the same as those used in the scouting gradients. The focused gradient ran from 36% to 56% methanol for all runs. Rather than a step, the gradient went to 100% methanol to reduce the tailing of the peptide peak and to wash out late-eluting compounds. Mass detection (PurIon™ L mass spectrometer with fluid interface) was used for these runs. The purification was run on 15 g columns. The C8 column showed narrower peaks and less tailing.

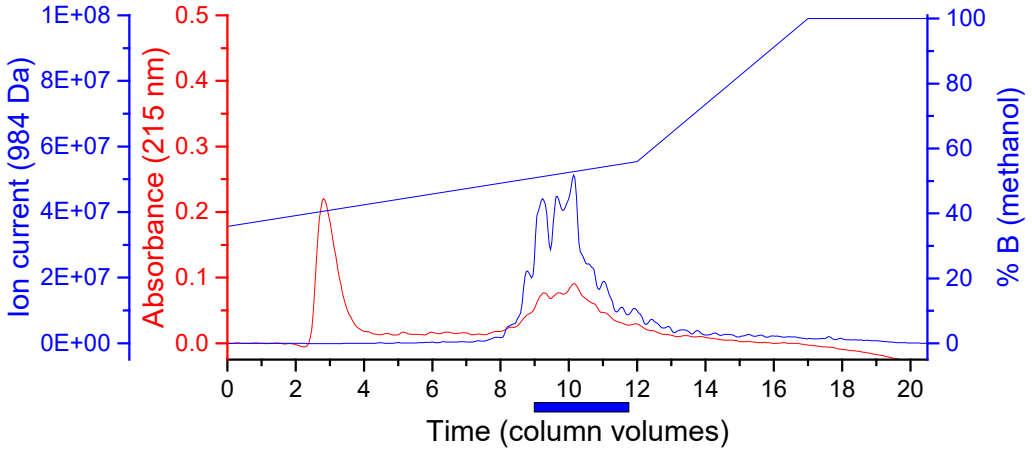

Figure 4—Purification of Thymosin on a 15,5 g RediSep Gold C18 reverse phase column. Of 100 mg loaded, 60 mg was recovered.

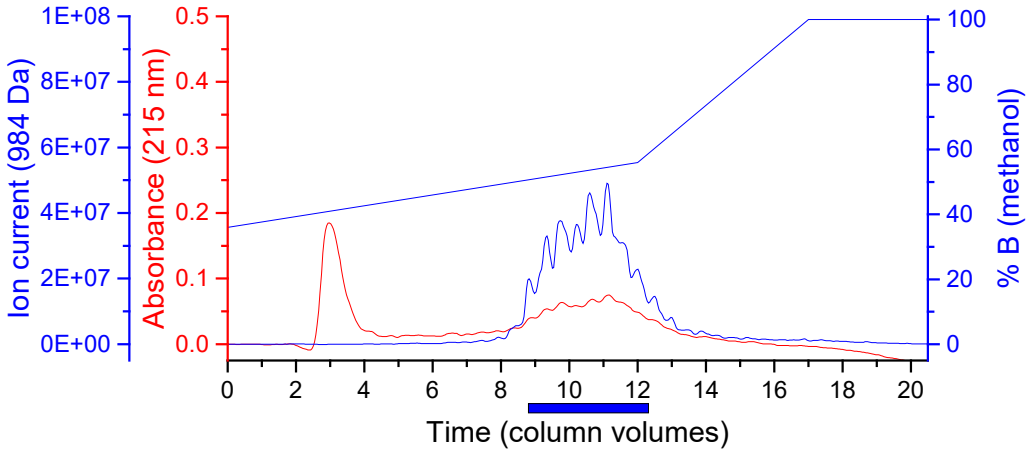

Figure 5—Purification of Thymosin on a 15,5 g RediSep Gold C18AQ reverse phase column. Of 100 mg loaded, 60 mg was recovered.

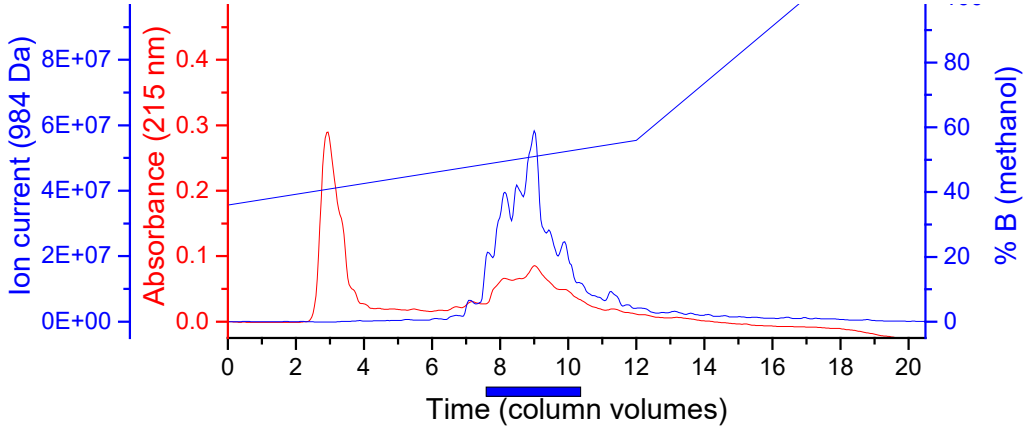

Figure 6—Purification of Thymosin on a 15,5 g RediSep Gold C8 reverse phase column. Of 100 mg loaded, 60 mg was recovered.

Analytical HPLC

Analytical HPLC was run on an Agilent 1290 with a Redi*Sep*® Prep 2 x 50 mm column in water/acetonitrile, both containing 0.1% TFA. The fractions were combined and analyzed prior to removal of methanol and freeze-drying. The differences between the flash runs is small, but the C8 run is slightly cleaner. In all cases, the peptide could be used without further purification.

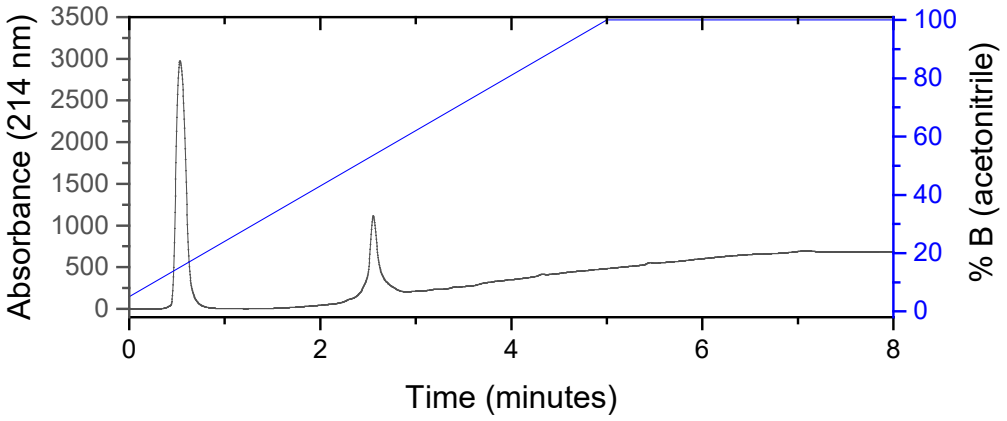

Figure 7—Thymosin before purification

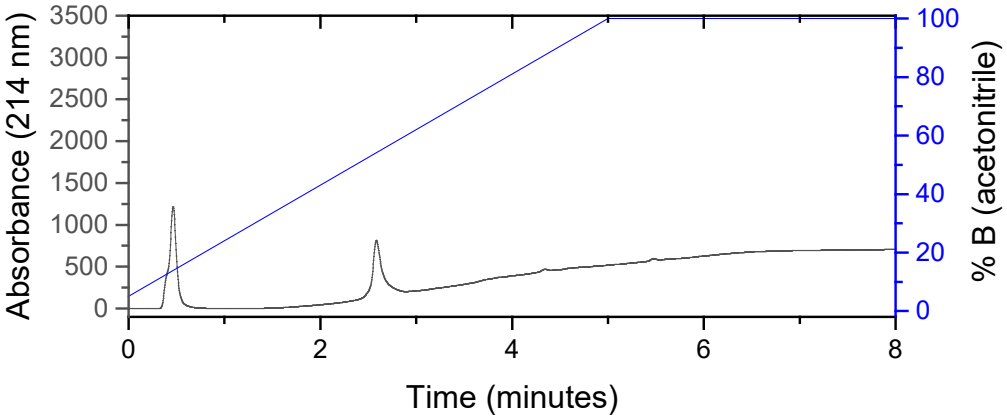

Figure 8—UHPLC run of crude thymosin after flash purification on RediSep Gold C18 column.

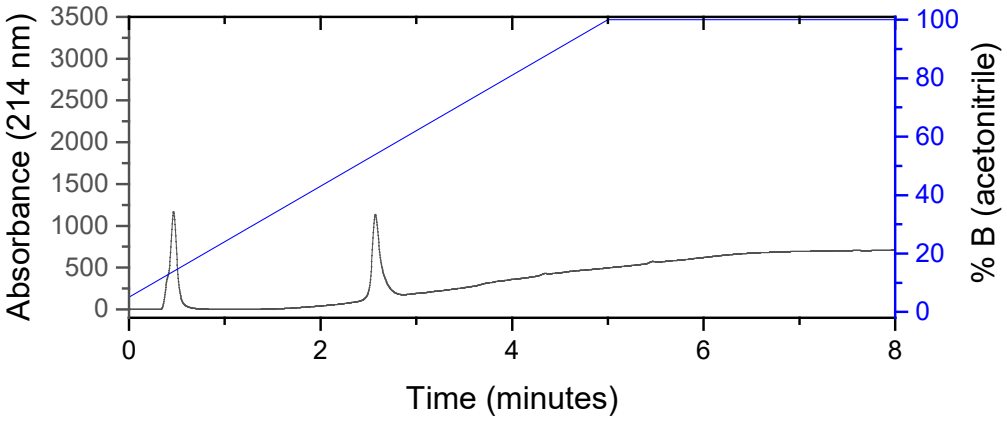

Figure 9—UHPLC run of crude thymosin after flash purification on RediSep Gold C18Aq column.

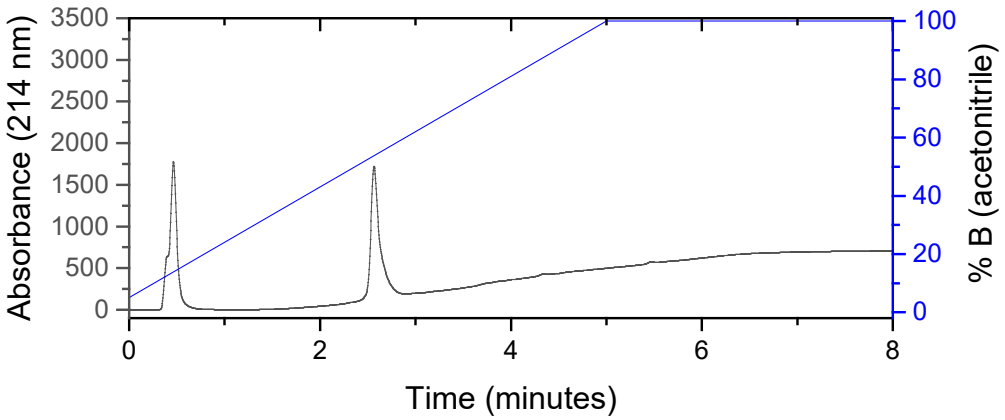

Figure 10—UHPLC run of crude thymosin after flash purification on RediSep Gold C8 column.

Conclusions

Flash chromatography works very well to purify peptides with a molecular weight of ~3000 Daltons. The use of the PurIon L mass spectrometer allowed confirmation of the eluting peak from impurities. The flash chromatography total run time was about 9 minutes, with the desired peptide eluting within 5 minutes, and so the purification time was very fast.

[1] *Silver, J. Overview of Analytical-to-Preparative Liquid Chromatography Method Development.* ACS Combinatorial Science ***2019***, 21 (9), *609–613.*

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