DIONEX

Application Note 230

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Purity Analysis of Synthetic Thymosin α1 by Reversed-Phase HPLC with an Acclaim 300 C18 column

INTRODUCTION

Reversed-phase high-performance liquid chromatography (HPLC) is an efficient separation mode extensively applied to the purification and analysis of proteins and peptides. The stationary phases typically used for these applications are C18, C8, and C4, with C18 being the phase of choice for peptide analysis.[1] The Acclaim® 300 C18 column was designed for protein, peptide, and other biological macromolecule separations. This column contains a resin consisting of 3 µm diameter silica particles with 300 Å diameter pores, bonded with a highly characterized C18 phase. The unique bonding chemistry, which includes exhaustive alkyl group bonding for a high-density, highly uniform phase coverage, and extensive endcapping on 3 µm silica particles, results in fast, high-resolution separations.[2]

In this Application Note (AN), we establish HPLC methods for analyzing thymosin α1 and its synthetic precursors using an Acclaim 300 C18 column. These methods feature high-resolution separations and efficient peaks. Thymosin α1 is a 28 amino acid hydrophilic peptide with a sequence of Ac-S-S-A-A-V-D-T-S-S-E-I-T-T-K-D-L-K-E-K-K-E-V-V-E-E-A-E-N-OH. Its molecular formula is $C_{129}H_{215}N_{33}O_{35}$ (molecular weight 3108.37 after acetylation), and its isoelectric point (pI) is 4.25 (before acetylation). Thymosin α1 is derived from the 113 amino acid polypeptide prothymosin alpha, which is produced by the thymus gland. Medicinally, thymosin α1 is used to treat Hepatitis B either alone or in combination with other drugs. Thymosin α1 is also used as a vaccine adjuvant to enhance a vaccine's effectiveness (for example, it has been used in the influenza vaccine), and is used to treat other diseases, including Hepatitis C and certain types of cancers.

EQUIPMENT

Dionex UltiMate® 3000 HPLC system consisting of:

- HPG 3400A pump / DGP3600A pump / DGP3600M pump*
- WPS-3000TSL autosampler
- TCC-3200 thermostatted column compartment
- VWD-3400 UV-vis detector

MSQ Plus™ Mass Spectrometer with electrospray ionization (ESI) source

Chromeleon® 6.80 SP5 Chromatography Data System

*Any of these pumps can be used, but for best performance, we recommend the HPG 3400A. The DPG3600M is optimized for working with smaller column diameters.

REAGENTS

Water Milli-Q® Gradient A10

Methanol (CH_3OH) (HPLC grade, Fisher Scientific)

Acetonitrile (CH_3CN) (HPLC grade, Fisher Scientific)

Formic acid (FA) (analytical grade, SCRC, China)

Ammonium acetate (NH_4Ac) (analytical grade, SCRC, China)

Trifluoroacetic acid (TFA) (HPLC grade, Sigma-Aldrich)

SAMPLES

Three samples were obtained from a biotechnology company: a purified thymosin α1 product, a raw (unpurified) product, and a mixture of six synthetic precursors of thymosin α1, p-5, p-10, p-15, p-20, p-25 and p-28, which contain 5, 10, 15, 20, 25, and 28 amino acids respectively. Precursor p-28 was thymosin α1 without acetylation. All samples were dissolved in water.

CONDITIONS

Chromatographic Conditions

Analytical Column:	Acclaim 300 C18, 3 µm, 4.6 × 150 mm, P/N 060266
	Acclaim 300 C18, 3 µm, 2.1 × 150 mm, P/N 060264, for MS
Column Temp.:	25 °C
Mobile Phase / Gradient:	See Figures
Flow Rate:	1.0 mL/min for the Acclaim 300 C18, 3 µm, 4.6 × 150 mm column
	0.2 mL/min for the Acclaim 300 C18, 3 µm, 2.1 × 150 mm column
Inj. Volume:	10 µL
Detection:	UV Absorbance at 214 nm

MSQ-Plus Conditions

Ionization Mode:	ESI
Operating Mode:	Positive Scan
Probe Temperature:	400 °C
Needle Voltage:	3500 V
Mass Range:	400 ~ 1200 amu
Scan Time:	0.5 sec
Cone Voltage:	50 V
Nebulizer Gas:	Nitrogen at 75 psi

RESULTS AND DISCUSSION

Method Development

The Acclaim 300 C18 column can be used successfully with the different types of eluents typically used for reversed-phase separation of peptides and proteins with UV or MS detection. To achieve efficient peaks and high-resolution peptide and protein separations, TFA is widely used. It is typically used at 0.1 or 0.05%. The Acclaim 300 C18 column's unique bonding chemistry delivers efficient peptide peaks with TFA levels as low as 0.04%, and sometimes 0.01%.[2] On the Acclaim 300 C18 column, other reagents can be substituted for TFA (e.g. formic acid and ammonium acetate) while maintaining good peak shape and chromatographic efficiency. We used these features to develop methods for the analysis of thymosin α1 and its synthetic precursors.

In early method development, it is useful to proportion the mobile phase from three different eluent bottles: water, acetonitrile, and 0.5% TFA (or another ion-pairing reagent). Figure 1 shows seven consecutive separations of thymosin α1 and five synthetic precursors of thymosin α1 developed and executed with proportioning from three eluent bottles. The separation uses 0.05% TFA and there is some positive baseline drift due to the increased refractive index of TFA in CH_3CN compared to water. Peptides are detected at 214 nm, the absorbance maximum of the peptide bond. Sometimes 280 nm is useful for peptide detection, but because thymosin α1 has no aromatic amino acids, it has no UV absorption at 280 nm.

After the appropriate chromatographic conditions have been determined, premixing mobile phase components and using a high-pressure gradient pump can deliver a better baseline and the best reproducibility for routine analysis. We used two eluent bottles with each bottle containing a mixture of mobile phase components (Figure 2). Bottle A contained 0.05% TFA and bottle B contained 50% CH_3CN and 0.04% TFA. Because the highest amount of organic solvent in the mobile phase used in our separation is 20%, we can make an eluent B

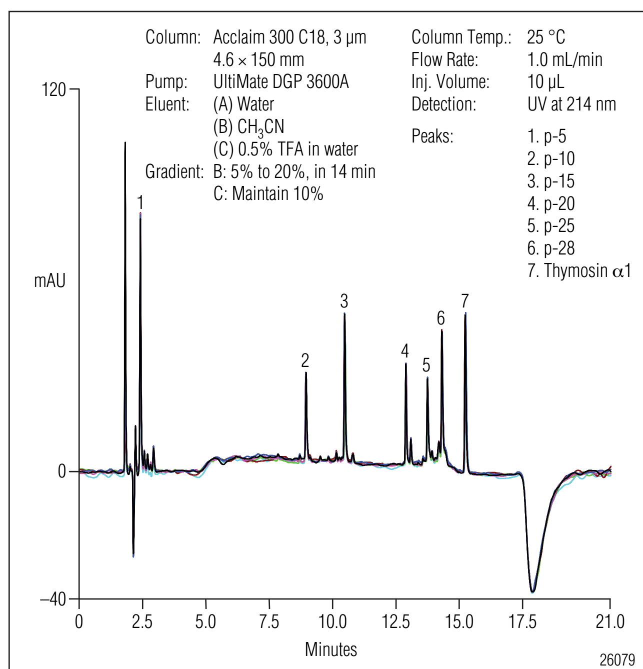

Figure 1. Overlay of chromatograms of seven consecutive injections of a mixture of thymosin α1 and its synthetic precursors.

containing 50% CH_3CN. Many samples will require more than 50% organic solvent to eluent more highly retained peptides and proteins. In those cases, 90% CH_3CN should be used. Using 50% organic solvent instead of 90% also allows a shallower solvent gradient to be used in a fixed time frame, which can deliver better and more reproducible separation. Additionally, by putting less TFA in bottle B compared to bottle A (0.04% vs. 0.05%), we were able to compensate for the change in refractive index as the percentage of organic solvent increases. An enlargement of the baseline (Figure 2B) shows only a small increase. Table 1 lists retention time RSD values of each analyte when the pump prepares the mobile phase from (a) three eluent bottles each containing a single mobile phase component, and (b) two eluent bottles containing premixed mobile phase components. Although the RSD values obtained by using three bottles are not as good as those obtained by using two bottles, they are acceptable for the method development phase of a project.

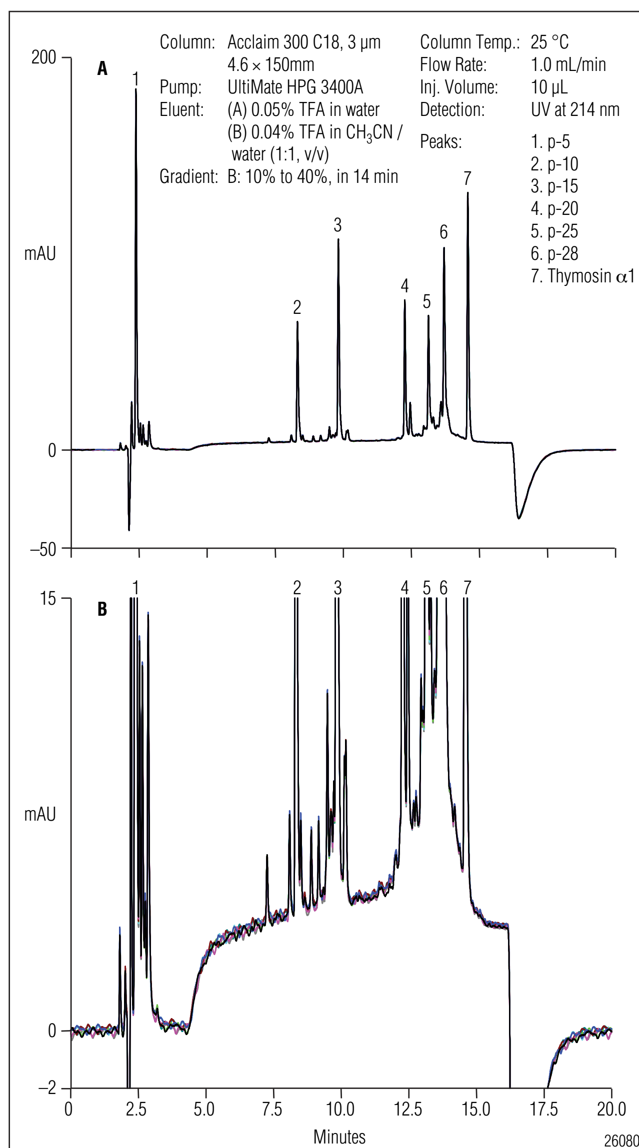

Figure 2. Overlay of (A) chromatograms and (B) enlarged chromatograms of eight consecutive injections of a mixture of thymosin α1 and its synthetic precursors.

Table 1. Retention Time Reproducibly Using Different Methods to Prepare the Mobile Phase

Peptide	RSD	
	Three-bottle mode	Two-bottle mode (premixing mobile phase components)
precursor p-5	0.104	0.032
precursor p-10	0.065	0.010
precursor p-15	0.132	0.016
precursor p-20	0.065	0.011
precursor p-25	0.063	0.017
precursor p-28	0.064	0.014
thymosin α1	0.086	0.016

MS Data for Peak Confirmation

Because TFA concentrations of 0.1% or higher have been shown to reduce analyte sensitivity when using electrospray ionization interfaces in LC-MS,[2] the 0.05% TFA was replaced with 0.1% FA. ESI interfaces work best at lower flow rates, therefore we replaced the 4.6 mm i.d. Acclaim 300 C18 column with a 2.1 mm i.d. column of the same length, reducing the flow rate from 1 to 0.2 mL/min. The pump prepared the mobile phase from three eluent bottles containing water, CH_3CN, and 1% FA in water. We used these three bottles for convenience. If we were seeking the best reproducibility, we would have used two bottles with one containing 0.1% FA and the other containing 50% CH_3CN and 0.1% FA. The molecular ions calculated from the observed mass to charge ratios matched the calculated values of the analyte molecular weights. For example, Figure 3 shows mass spectra of thymosin α1 and its synthetic precursors p-25 and p-28. The calculated values of molecular weights are the same as the theoretical values, 3108.3, 2794.9, and 3067.3, respectively.

Analysis of Unpurified Thymosin α1 Product

The analysis of unpurified thymosin α1 product was performed on the Acclaim 300 C18 (4.6 mm i.d. × 150 mm) column with UV detection and a mobile phase prepared from three eluent bottles containing water, CH_3CN, and 1% TFA or 100 mM ammonium acetate in water, respectively. It is difficult to separate thymosin α1 and the impurities in this preparation using TFA in the mobile phase (Figure 4A). When using ammonium acetate instead of TFA, the separation is significantly improved (Figure 4B). Seven impurity peaks were separated from thymosin α1. As ammonium acetate is MS compatible, we can use the MSQ for peak identification. Figure 5 shows the mass spectra of thymosin α1 and peak 3, an impurity. Assuming this impurity is a peptide, the MS data suggests it is Ac-S-S-A-A-V-D-T-S-S-E-I-T-T-K-D-L-K-E-K-K-E-V-V-E-E-A-OH, which is thymosin α1 with a loss of its two C-terminal amino acids. No synthetic precursors of thymosin α1 were found in the unpurified product.

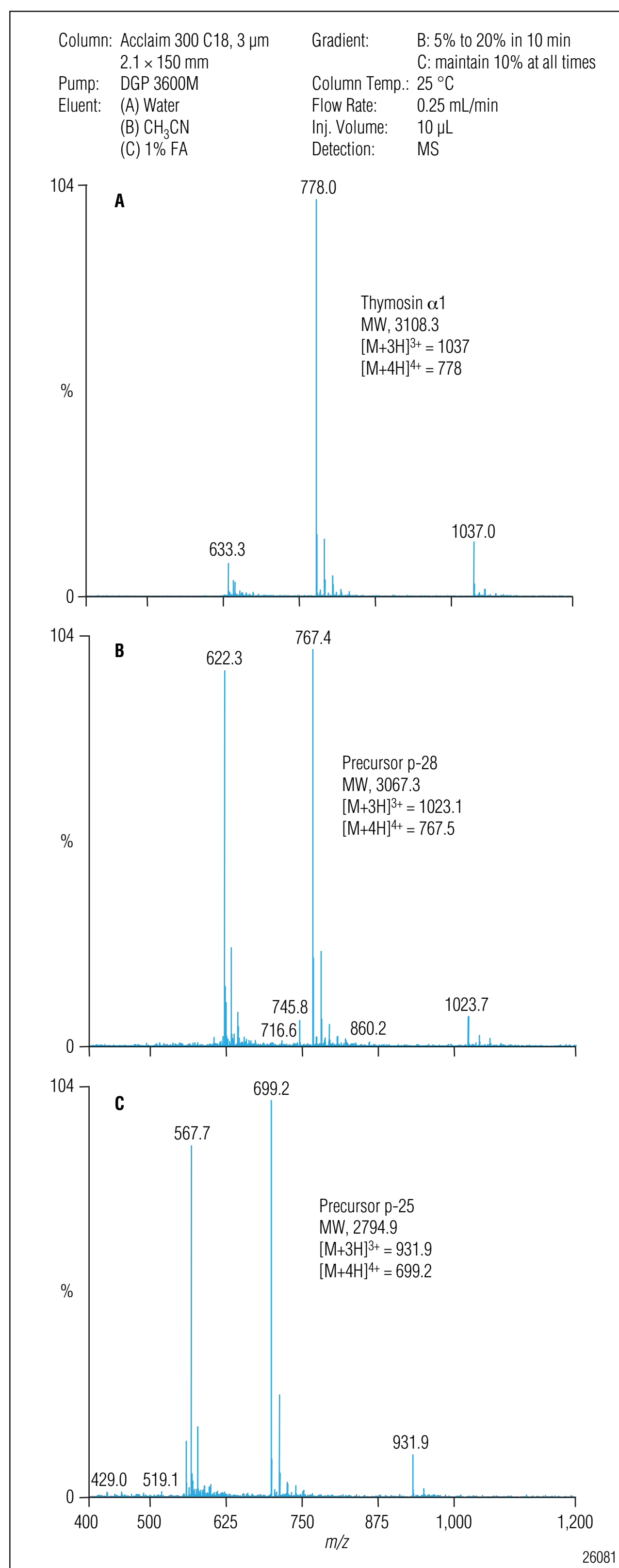

Figure 3. MS spectra of (A) thymosin α1, (B) precursor p-28, and (C) precursor p-25.

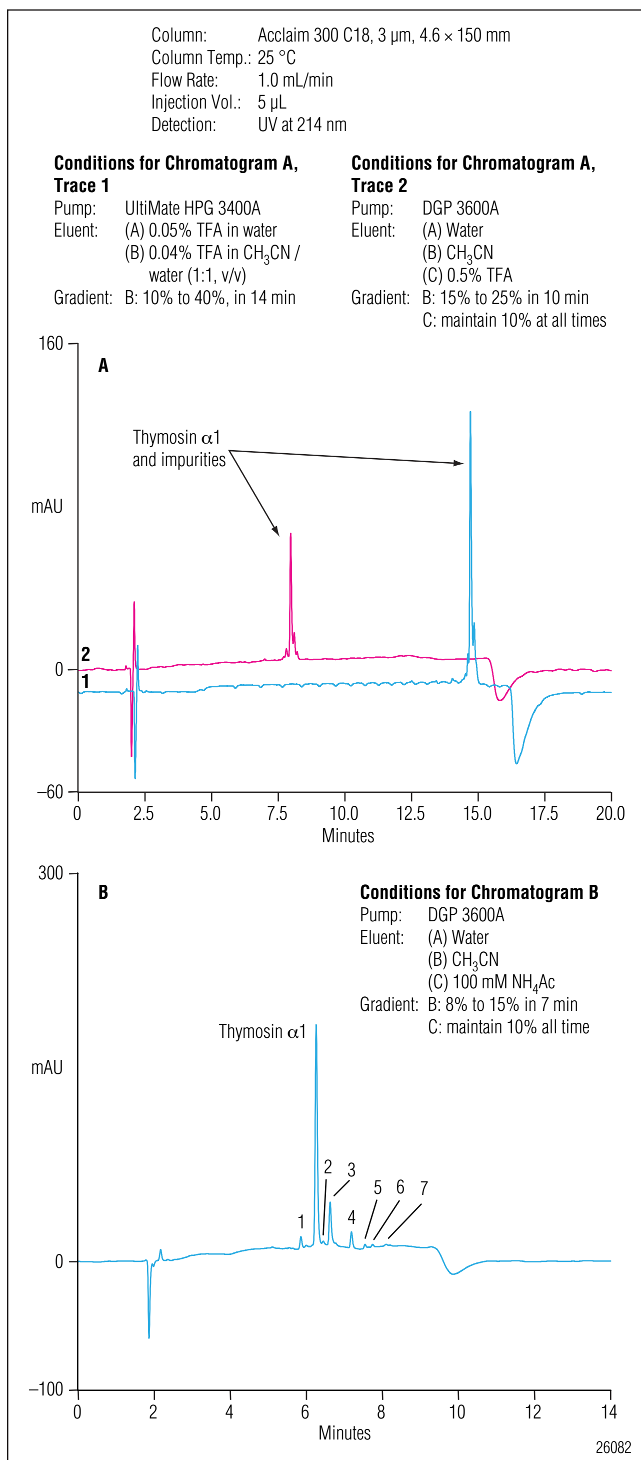

Figure 4. Chromatograms of the unpurified thymosin α1 product using (A) TFA and, (B) 100 mM NH_4Ac in the mobile phase.

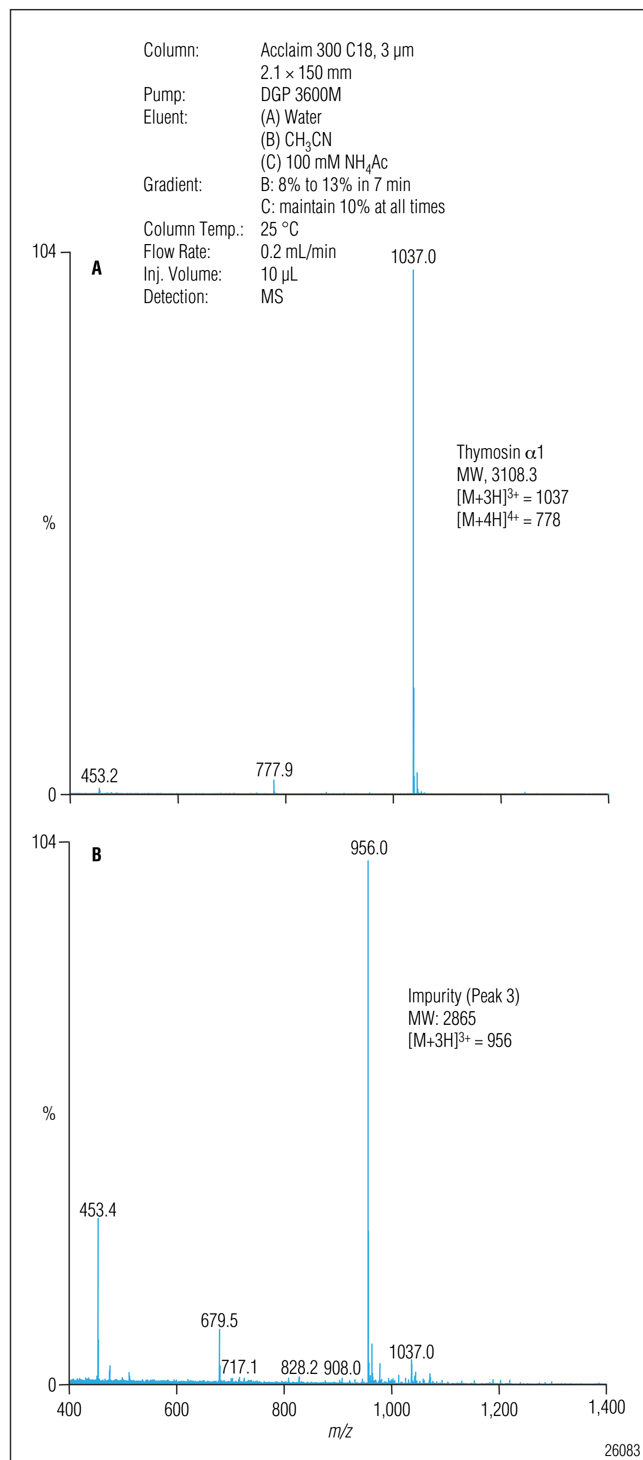

Figure 5. MS spectra of (A) thymosin α1 and (B) impurity (peak 3).

PRECAUTIONS

A smoother baseline can be delivered by filling the eluent bottles with solutions of the mobile phase components. To achieve better reliability with single mobile phase component solutions in eluent bottles A and B, a high-quality, high-pressure gradient HPLC pump (for example, UltiMate HPG 3400A system) is needed.

CH_3CN and TFA purities are critical for this application. Because TFA is light sensitive, eluents containing TFA greater than a week old may not be good, and should be discarded.

CONCLUSION

This application note describes an efficient LC method for analyzing peptides on the Acclaim 300 C18 column, using thymosin α1 and its synthetic precursors as an example. The Acclaim 300 C18 column delivers symmetrical efficient peptide peaks, and therefore high resolution with all the eluents typically used for reversed-phase analysis of peptides. Its bonding chemistry is compatible with MS detection. This makes the Acclaim 300 C18 column ideal for developing protein and peptide separations.

REFERENCES

1. Zhu X.N., Su Z.G. Application of Reversed-Phase Liquid Chromatography in Separation and Analysis of Proteins and Peptides (Review), *Chinese J. Anal. Chem.* **2004,** *32* (2), 248–254.
2. Dionex Corporation. *Acclaim 300 HPLC Columns*. 2003, LPN 1511 5M 2/03. Sunnyvale, CA.

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