Fast, efficient HPLC purification of peptides from solid-phase synthesis

Application Note

Marc Dauber, Jörg Hoheisel, Anette Jacob
Deutsches Krebsforschungszentrum, Funktionelle Genomanalyse, Heidelberg, Germany
Udo Huber, Edgar Nägele
Agilent Technologies, Waldbronn, Germany

Abstract

This Application Note demonstrates:

- Configuration of the Agilent 1200 Series Rapid Resolution LC (RRLC) analytical scale (AS) purification system.
- Use of the Agilent 1200 Series RRLC AS purification system with an optimized method for purification of peptides from solid-phase synthesis.

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Introduction

The concept of solid-phase synthesis of peptides, which was first introduced by Merrifield et al., simplifies the synthesis of peptides compared with their classical synthesis in solution.[1] In solid-phase synthesis, the first amino acid of the peptide is bound to a solid resin at the amino acid's C-terminus while the amino group – the N-terminus – is chemically blocked by a so-called protecting group. To enlarge the peptide sequence, the N-terminal protecting group is removed by treating the resin-bound amino acid or peptide with a reagent that cleaves off the N-terminal protecting group specifically. In the second step, the next N-terminally-protected and C-terminally-free amino acid is coupled to the resin-bound peptide with an activating reagent to form the new peptide bond.

The main advantage of this synthesis is the fast peptide chain elongation and the easy cleanup of the resin-bound peptide by a washing step to prepare for the next step. This procedure enlarges the peptide repetitively by one amino acid per cycle. It is possible to synthesize peptide sequences with up to 100 amino acid residues with this technique.

Peptides that are 10 to 30 amino acids in length represent binding sites, and are often used for biological applications like phosphorylation studies, epitope mapping, and drug development. These applications require many different highly purified peptides, but only small amounts of each are needed. Therefore, a rapid, analytical-scale LC purification system is ideal.

Experimental

Equipment

1200 Series RRLC AS purification system consisting of:

- Agilent 1200 Series binary pump SL with degasser
- Agilent 1200 Series high-performance autosampler with cooler
- Agilent 1200 Series thermostatted column compartment
- Agilent 1200 Series diode-array detector (DAD) SL, using a flow cell with 10-mm path length
- Agilent 1200 Series analytical scale fraction collector

General method parameters

- Column: Agilent ZORBAX Eclipse XDB-C18, 4.6 x 50 mm, 1.8 µm particle size
- Injection volume: 10 µL (mixture of a 20-mer, an acetylated 19-mer and an acetylated 17-mer, 2 % of a 0.4-µmol synthesis scale each)
- Sample temperature: 4 °C
- Column temperature: 45 °C
- DAD:
  217 ±4 nm, Ref. 600 ±4 nm
- Collection of fraction was triggered by UV signal. The system delay volume was determined by the procedure for delay volume calibration described in an Agilent Application Note.[2]

LC method for purification (10 min)

- Solvent A: 0.1 % aqueous trifluoroacetic acid (TFA_{aq}) (v/v).
  Solvent B: 0.085 % TFA/acetonitrile (v/v)
- Gradient:
  0 min, 15 %B, 1 mL/min
  3 min, 25 %B, 1 mL/min
  10 min, 35 %B, 1 mL/min
  11 min, 100 %B, 2 mL/min
  13 min, 100 %B, 2 mL/min
  13.5 min, 15 %B, 1 mL/min
  Stop time: 13.5 min
  Post time: 1 min

LC method for purification (4 min)

- Solvent A: 0.1 % TFA_{aq} (v/v).
  Solvent B: 0.085 % TFA/acetonitrile (v/v)
- Gradient:
  0 min, 15 %B, 2ml/min
  1 min, 25 %B, 2 mL/min
  4 min, 35 %B, 2 mL/min
  4.5 min, 100 %B, 2 mL/min
  7 min, 100 %B, 2 mL/min
  7.5 min, 15 %B, 2 mL/min
  Stop time: 7.5 min
  Post time: 2 min

Results and discussion

Peptides were synthesized in a 384-multiwell filter plate with an automatic peptide synthesizer (Intavis, Cologne) in a 0.4-µmol scale.[3] After synthesis, the peptides were cleaved from the resin. Due to coupling yields below 100 %, truncated sequences were generated, which required removal by HPLC.

In order to test the separating power of the HPLC purification, a crude product of a 20-mer peptide were spiked with the respective acetylated 17- and 19-mer. In figure 1A, the HPLC chromatogram and the MALDI-TOF MS spectrum of the spiked crude peptide is shown. The three compounds separated by HPLC were fractionated, evaporated and redissolved in 30 µL of 15 % acetonitrile/0.1 % TFA_{aq}.

MALDI-TOF MS and a second HPLC analysis of the three fractions demonstrated the purity and identity of the purified peptide and the respective truncated sequences (figure 1B, 1C, and 1D).

Figure 2 demonstrates that separation and therefore purification time can be further decreased from 10 to 4 minutes by optimizing the gradient. The identity and purity of the HPLC fractions of the crude product were confirmed via MALDI-TOF MS analysis.

With the gradient and the HPLC system used here, automatic high-throughput purification of peptides synthesized in multi-well-plates can be carried out, using automatic sample loading out of 384-micro-well-plates, as well as automated fraction collection of the product in another micro-well-plate. By applying alternating column regeneration, the time normally used for washing and equilibration (~ 3.5 minutes) can be used for another run, thereby dividing the overall purification time for high-throughput applications. The capacity of the column employed allows for the purification of a 0.1-µmol synthesis scale.

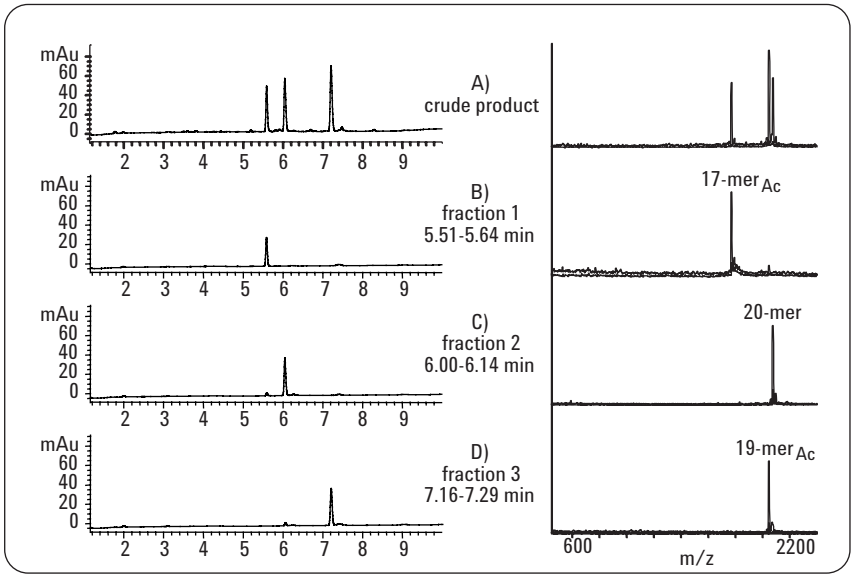

Figure 1
A) HPLC purification of a crude 20-mer peptide, additionally spiked with two acetylated truncated sequences (full-length product 20-mer: AEFGILNQRPTVAFGINTAG at 2075 Da, 19-mer: $_{Ac}$EFGILNQRPTVAFGINTAG at 2046 Da, and 17-mer: $_{Ac}$GILNQRPTVAFGINTAG at 1770 Da). B-D) Reanalysis of the fractions by HPLC (left side) and MALDI-TOF MS (right side) demonstrates the purity and identity.

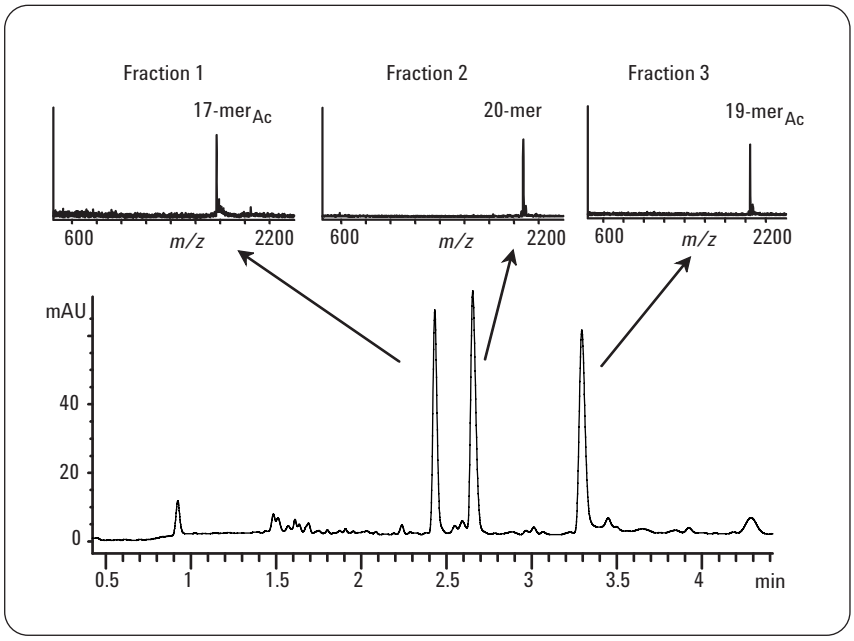

Figure 2
HPLC chromatogram of a crude 20-mer peptide additionally spiked with acetylated 19- and 17-mer peptides and MALDI-TOF MS spectra of the single fractions. Fraction 1 contains the acetylated 17-mer (1770 Da), fraction 2 the 20-mer full-length product (2075 Da), and fraction 3 the acetylated 19-mer (2046 Da).

Conclusion

This Application Note demonstrates the use of the Agilent 1200 Series Rapid Resolution LC analytical scale purification system for the purification of peptides synthesized on a solid phase. By this method, a 20-mer can be easily separated from the respective acetylated 19-mer, which differs by only one alanine. The desired peptide can be isolated from the crude mixture in high purity and

with short separation time by an automated purification process that is triggered by a UV signal. The purity of the isolated peptide can be confirmed by reanalysis of the fraction. While in the beginning, purification time for one run, including washing and equilibration of the column, was about 14 minutes per peptide, the method was further optimized to meet the requirements of high-throughput applications. By the additional use of alternating column regeneration, purification of a 0.1-µmol synthesis scale within 4 minutes per peptide can be established.

References

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Marc Dauber, *Jörg Hoheisel*, *Anette Jacob are Scientists at at Deutsches Krebsforschungszentrum*, *Funktionelle Genomanalyse*, *Heidelberg*, *Germany*
Udo Huber and Edgar Nägele are Application Chemists at Agilent Technologies, *Waldbronn*, *Germany.*

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Published June 15, 2010
Publication Number 5989-8306EN

Agilent Technologies