The new dimension of purity

Orthogonality

The purification of the hydrophilic peptide Histone H3 (1-20), synthesized via SPPS, is a formidable challenge due to co-eluting impurities. In this study, we show in collaboration with Bachem how to achieve high purities and lower solvent consumption by using the PEC technology and RP-HPLC in a simple orthogonal purification approach.

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1. Introduction

Peptide impurities can have a significant influence on the outcome of assays during drug research and development (e.g., false-positive results). Ultimately, such impurities must be removed entirely from active pharmaceutical ingredients to provide the highest quality material. Thus, high purity is crucial throughout the whole value chain of peptide drug development.
Purification of chemically synthesized peptides is mainly carried out by traditional chromatographic methods. Reversed-phase high-pressure liquid chromatography (RP-HPLC) is the most commonly used method here. Also, reversed-phase flash chromatography (flash) represents an established technique for research-grade peptide purifications and intermediate/clean-up purification steps of higher-grade material. Due to similar properties, however, co-eluting impurities may be overlooked with both these chromatographic techniques.
In contrast, chemo-selective separation principles drive Belyntic's catch-and-release technology Peptide Easy Clean (PEC). Capping during solid-phase peptide synthesis (SPPS) ensures that only the full-length peptide is accessible for modification with a traceless cleavable purification linker (PEC-Linker) at the end of SPPS. The target peptide can then be isolated from a complex mixture.[1]
In this case study, we demonstrate the orthogonality of PEC with RP-HPLC by purifying the polar peptide fragment Histone H3 (1-20).

2. Method

Synthesis. Bachem (UK) synthesized the peptide Histone H3 (1-20) in a 200 µmol scale on H-Leu-2-Cl-Trt resin (Table 1). The synthetic resin was split into two parts, of which 100 µmol were kindly provided for PEC purification. The second 100 µmol of crude product remained with Bachem UK for flash chromatography and additional RP-HPLC purification. Routine capping using acetic anhydride after each coupling step ensured the selective coupling of the PEC-Linker only on the full-length peptide.
The basic cleavable PEC-Linker BL (4 eq.) was employed and coupled using Oxyma (8.75 eq.) and DIPEA (8.5 eq.) in DMF for 2 h. TFA cleavage was performed for 2 h using Reagent K (10 mL cleavage cocktail per 100 µmol peptide).

Table 1: Peptide used in this study.

name	sequence
Histone H3 (1-20)	H-ARTKQ TARKS TGGKA PRKQL-OH

PEC-Purification. The crude material was first dissolved in 4.5 mL DMSO. Then, 500 µL citric acid/GdmCl buffer (0.1 M/7M; pH 4.5) was added. The dissolved peptide was added to a pre-conditioned suspension (3 mL) of activated filter material, and the immobilization was performed for 90 min before washing off impurities.
A 0.2 M solution of ethanolamine at pH 11.0 was added in six bead volumes for 60 min to release the peptide. After collecting the filtrate, an additional release-step was performed with the same portion of the alkaline solution for 30 min. The obtained mixture was lyophilized.

Flash purification. Flash purification was carried out using a Teledyne ISCO CombiFlash instrument on Daiso C18 20 µm media with a gradient of 10-100% B in 30 minutes (A: 0.1% heptafluorobutyric acid (HFBA) in H_2O, B: 0.1% HFBA in 60/40 MeCN/H_2O).

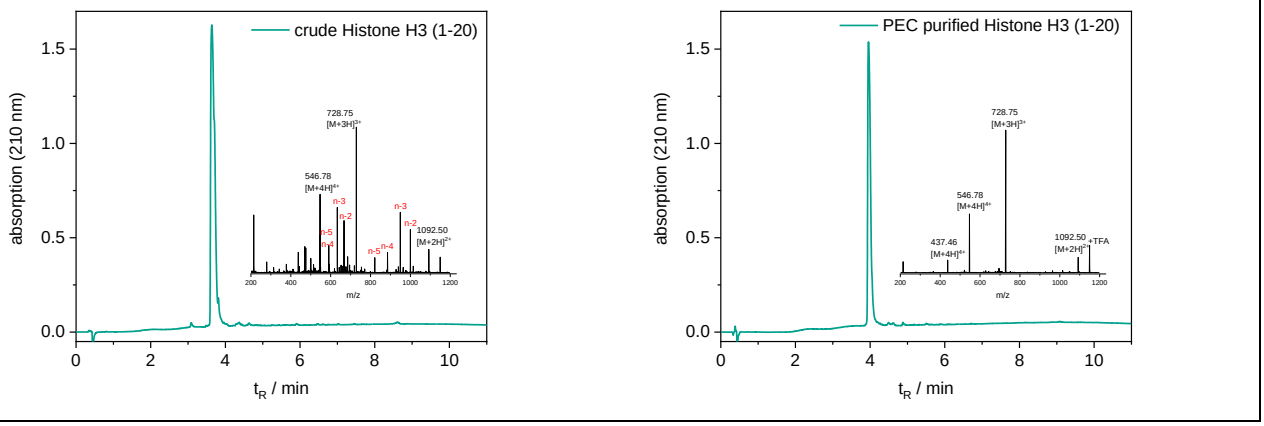
RP-HPLC purification. RP-HPLC purification was carried out using a Varian ProStar prep HPLC on Daiso C18 8 µm media with a gradient of 25-100% B in 105 minutes (A: 0.1% HFBA in H_2O, B: 0.1% HFBA in 60/40 2 MeCN/H_2O).

Analysis. UPLC-UV and mass spectra were recorded with an analytical Acquity H-Class UPLC-ESI-MS system from Waters on a C-18 column (1.7 µm, 2.1 x 500 mm). As the mobile phase, mixtures of H_2O (A) and MeCN (B) with 0.1% TFA were used.

3. Discussion

Purity. The UV-chromatogram of the crude Histone H3 (1-20) appears very clean at first glance (Figure 1, left). However, several capped deletion sequences assemble in the mass analysis of the corresponding peak (inset of Figure 1, left). Major impurities were Ala (A)-, Arg (R)- and Thr (T)-deletions. 0.1% HFBA for an analytical HPLC run, resolved the underlying peaks, and a crude purity of 29% was determined (chromatogram is not shown).

Figure 1: Chromatograms of crude and PEC purified Histone H3.



Due to the ability of HFBA to separate the target peptide from the truncated sequences, an HFBA-enhanced reversed-phase flash chromatography served as the first-dimension purification. The purity increased to 66% with the applied method (Table 2). On the contrary, a single run with PEC resulted in 86% purity.
Figure 1 (right) shows the chromatogram of the PEC purified peptide. A closer look into the mass spectra (insets in Figure 1, right) reveals that PEC removes the co-eluting impurities efficiently in a single step.

Table 2: Purities and solvent/total waste usage.

1st dimension purification	2nd dimension purification	final purity	MeCN used	total waste
PEC		86%	50 mL	200 mL
PEC	RP-HPLC	96%	1050 mL	3200 mL
Flash		66%	500 mL	1500 mL
Flash	RP-HPLC	85%	1500 mL	4500 mL

Orthogonality. Next, we performed an additional RP-HPLC purification with both the flash- and the PEC-purified peptides as the second dimension purification.
The purities were increased from 29% (crude) to 85% and 96%, respectively (Table 2), clearly demonstrating the superior purification efficiency of PEC in orthogonal combinations with chromatography for peptides that show co-eluting impurities in the crude mixture.

Solvent economy. MeCN consumption is a reasonable measure to evaluate the ecological benefit of this complementary approach. Due to PEC's catch-and-release principles, only a small amount of organic solvents incur. Only 50 mL of MeCN and 200 mL total waste accumulated during the process. In contrast, the flash purification required 500 mL MeCN and produced 1500 mL total solvent. When adding the additional RP-HPLC purification run, we calculate 1000 mL MeCN and 3000 mL total waste.

An orthogonal purification approach using PEC and RP-HPLC can, therefore, result in a total saving of costly solvent and harmful waste of overall 30%.

4. Results at a glance

- efficiently remove co-eluting truncations in a single step
- get highest purities for challenging peptides with a combination of PEC and RP-HPLC
- save up to 30% of total waste during in orthogonal purification processes

References

[1] Reimann O.; Seitz O.; Sarma D.; Zitterbart R. *J. Pep. Sci.* 2019; 25:e3136.

About Belyntic

Belyntic GmbH is a chemistry-for-healthcare enterprise focusing on the manufacturing of biopolymers, especially peptides. Belyntic offers the world's first broadly applicable peptide purification kits as well as development and implementation services of its proprietary Peptide Easy Clean (PEC) technology.

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