Difficult Peptides

Cracking tough nuts

Linear SPPS of long peptides can result in very complex and difficult-to-purify mixtures using liquid chromatography. Also, peptides with hydrophobic or aggregating properties hardly dissolve in media that is compatible with this method. The PEC purification technology can overcome these hurdles, as shown by the purification of very long PTH (1-84) and hydrophobic, strongly aggregating CMV (81-95) peptide.

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1. Introduction

Due to its broad applicability and good separation ability, preparative reversed-phase high-pressure liquid chromatography (RP-HPLC) is the most common method for peptide purification.
However, several well-known shortcomings make successful applications tedious: The first hurdle lies in finding a suitable, HPLC-compatible solvent that enables the dissolution of the peptide. Also, the injection volume restricts probe dissolution. The second challenge comes with hydrophobic peptides that cause problems through adherence to the C18-coating of the RP-HPLC column. Thirdly, solid-phase peptide synthesis (SPPS) of long peptides leads to the formation of many truncations, which may co-elute with the desired product.
We meet these challenges with the Peptide Easy Clean (PEC) technology. PEC tolerates the use of many different organic solvents in large volumes for dissolution and processing while overcoming solubility problems. Besides, the chemo-selective separation can extract even the smallest peaks from a large cohort of truncated sequences.
In this case study, we demonstrate these features on the example of two difficult peptides, real "tough nuts": the 84-mer peptide parathyroid hormone (PTH)[1] and the sequence 81-95 from human cytomegalovirus (CMV) lower matrix phosphoprotein[2]. The PTH (1-84) with a length of 84 amino acids reaches feasibility limits of SPPS, and the 15-mer CMV peptide is poorly soluble due to hydrogel formation.

2. Method

Synthesis. PTH (1-84) was synthesized by GPT in a 300 µmol scale on ChemMatrix resin. Double-couplings of amino acids (2 x 10 min. with a 5-fold excess of amino acids). CMV (81-95) was synthesized in 100 µmol scale by Belyntic on Rink Amide AM resin. Routine capping using acetic anhydride after each coupling step ensured the selective coupling of the PEC-Linker only on the full-length peptide. Table 1 lists the sequence for PTH (-84) and CMV (81-95).
For both peptides, the PEC-Linker RC+ (4 eq.) was coupled using Oxyma (6 eq.) and DIPEA (6 eq.) in DMF for 2 h. TFA cleavage was performed for 2 h using Reagent K (TFA/EDT/PhOH/PhSMe/H_2O 83.5:2.5:5:5:5, 10 mL cleavage cocktail per 100 µmol peptide).

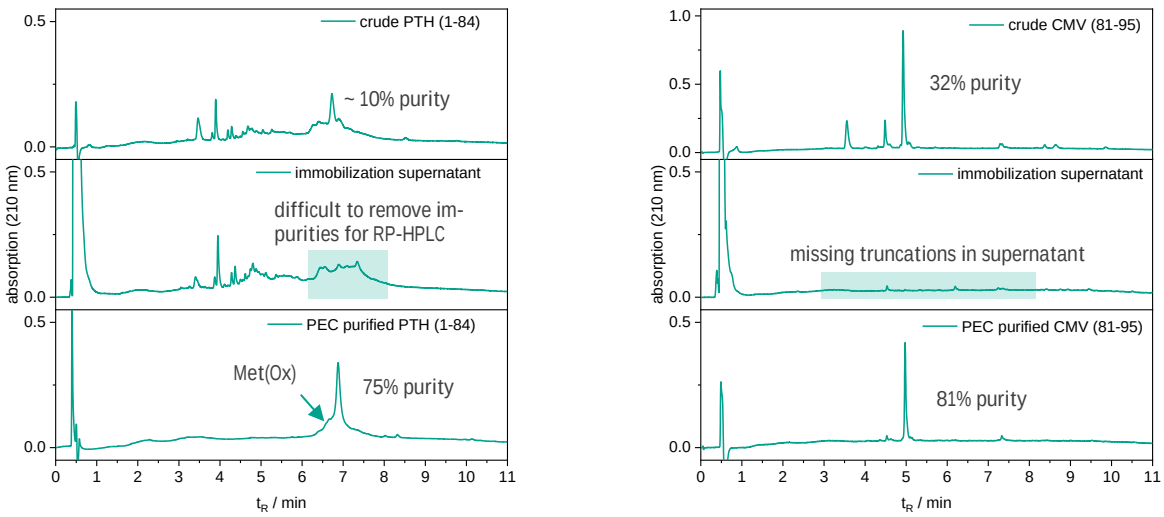
Table 1: Peptides used in this study

name	sequence
PTH (1-84)	H-SVSEI QLMHN LGKHL NSMER VEWLR KKLQD VHNFV ALGAP LAPRD AGSQR PRKKE DNVLV ESHEK SLGEA DKADV NVLTK AKSQ-NH_2
CMV (81-95)	H-YFTGS EVENV SVNVH-NH_2

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 3.5) was added. The dissolved peptide was added to 3 mL pre-conditioned suspension of activated filter material (150 µmol aldehyde content), and the immobilization was performed for 90 min before washing off impurities.
0.5 ml 2 w% L-Cys in a citric acid buffer (pH = 4.5) was then added to block unreacted aldehydes and displace imines (15 min incubation time).
Per peptide, 500 mg of PPh_3 in 9 mL MeCN/H_2O/AcOH (90:5:5) was added to reduce the linker (15 min reaction time). The activated filter material was washed to remove excess PPh_3. The acidic PEC-Linker cleavage was initiated by adding TFA/H_2O (2:3). After 60 min, 2 ml TFA was added, and the filtrate was collected in 50 ml tubes. Each sample was washed/eluted twice with 2 ml 95% aq. TFA. The peptide precipitated in chilled diethyl for direct use.

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 water (A) and MeCN (B) with 0.1% TFA were used.

Figure 1: Chromatograms of PTH (1-84) and CMV (81-95) before (crude) and after PEC.



3. Discussion

PTH (1-84). The synthesis of the 84-mer lead to a very complex crude mixture after SPPS due to the accumulation of truncated sequences (Fig. 1, left). The crude purity of this sample couldn't be determined by UV-analysis with UPLC-MS, but was around 10% by ESI-MS mass analysis. After the peptide immobilization on activated filter material via the linker molecule, the supernatant was analyzed and revealed the presence of compounds underneath the peptide peak. In RP-HPLC, those impurities might have been very difficult to remove (see supernatant analysis in Fig. 1, left center).

In contrast, PEC purification of PTH (1-84) masters target peptide isolation in a single step even from such complex mixtures that result from extensive linear SPPS. Impurities that would co-elute or closely elute with the product on RP-HPLC can efficiently be removed. This example also highlights possible applications of PEC as an additional purification dimension for difficult peptides and high purity missions.

CMV (81-95). The sequence 81-95 from (CMV) lower matrix phosphoprotein[2] can be very difficult to be handled and purified with chromatography due to lack of solubility: Dissolution in pure MeCN or mixtures of MeCN/water lead to hydrogel formation. Applying PEC, however, enabled to dissolve the peptide in DMSO successfully. Even after adding 10 vol.% citric acid buffer with 7 M GdmCl, the peptide stayed well dissolved.

The aggregating nature is manifested after immobilization because only a few peptide-related impurities are detectable in the supernatant (Fig. 1, right center). Usually, all impurities remain in the supernatant as in the example of PTH (1-84) on the left side. Due to the highly aggregating nature of CMV (81-95), truncated peptide sequences might adhere to the immobilized full-length peptide or filter material. Nonetheless, these aggregating impurities were quantitatively removed by the routine washing procedure, and the desired peptide was obtained with 81% purity.

4. Results at a glance

- purify very long peptides (>50 AAs) in a single step
- isolate peptides from complex mixtures and widen the scope of linear SPPS
- overcome limitations for the manufacturing of hydrophobic or aggregating peptides

References

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About Belyntic

Belyntic GmbH is a chemistry-for-healthcare enterprise focusing on the purification 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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