Advanced peptide modification

Disulfide formation

Disulfide formation remains one of the most attractive modification pathways to improve the properties of peptide drugs. The disulfide bridge provides exceptional chemical and conformational stability. However, manufacturing disulfide peptides is challenging, especially due to unwanted dimer and multimer formations in solution phase attempts. This case study shows how to create purified disulfide peptides in a combined approach using solid-phase modification with PEC.

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1. Introduction

Disulfide bonds are typically formed through the oxidation of free thiols or thiol-protected precursors after solid-phase peptide synthesis. Conventional oxidation in solution requires high dilution (typically at 10 – 100 µM) to avoid the aggregation and intermolecular side reactions.[1] Subsequent reversed-phase high-pressure liquid chromatography (RP-HPLC) removes the excess reagents and impurities. However, during purification with RP-HPLC, the solubility of the peptide can often be a challenging task.

Solid-phase disulfide formation provides a remedy because it allows washing off oxidizers and by-products. Moreover, pseudo-high-dilution through immobilization of the peptide on beads helps suppress intermolecular reactions.

The Peptide Easy Clean technology (PEC) is a new tool for peptide purification by chemo-selective isolation of the target peptide using a safety-release cleavable linker and immobilization on aldehyde-modified (activated) beads.[2] Beyond purification, handling the unprotected peptide on the activated beads enables late-stage solid-phase modifications such as disulfide formation.

In this case study, we demonstrate a disulfide modification protocol and showcase the benefits of this strategy to manufacture purified and modified Solnatide in a combined approach.

2. Method

Synthesis: Test peptides #1 & #2 (Table 1) were synthesized in 100 µmol scale on 2-chlorotritylchloride resin. Target peptide #3 (Solnatide) was synthesized on Fmoc-L-Cys(Trt)-TCP-Resin. Capping after each coupling step (2 M Ac_2O, 2 M pyridine in DMF 5 min) ensured the selective coupling of the PEC-Linker RC+ only on the full-length peptide in the last step. TFA cleavage was performed for 2 h using 10 mL TFA/H_2O/DTT/TIS (84:8:6:2).

Purification & disulfide formation (test peptide #1 & #2): The crude peptide (10 µmol) was first dissolved in 1 mL HFIP and 0.5 mL of sodium citrate/GdmCl buffer (0.1 M/7 M; pH 3.5) before adding to 0.26 g (4 eq) activated polymethacrylate beads (63 µM, ~150 µmol/g). The immobilization was performed for 180 min followed by washing away impurities and adding 1 mL of 0.5 wt% NH_2OMe to block unreacted aldehydes (15 min). The reduction was carried out by shaking the beads with 50 mg of DTT in 1 mL of DMF/ 5 w% aq. $NaHCO_3$ (1:1) for 60 min. Excess of DTT and DTT(ox) was removed through washing. Adding 6.8 mg (1.5 eq) of Rabenstein reagent in 1 mL of 0.1 M phosphate buffer (pH = 7.3) and agitation for 60 min promoted disulfide formation. By-products were subsequently removed through washing.

Purification & disulfide formation (target peptide #3): The crude peptide (10 µmol) was first dissolved in 1.5 mL HFIP and 0.75 mL of citric acid/GdmCl buffer (0.1 M/7 M; pH 3.5) before adding to 0.48 g (3 eq) activated polymethacrylate material (213 µM, ~62 µmol/g). The immobilization was performed for 240 min before washing away impurities and adding 3 mL of 0.5 wt% NH_2OMe to block unreacted aldehydes (15 min). The reduction was carried out by shaking the beads with 75 mg of DTT in 1500 µL of DMF/ 5 w% aq. $NaHCO_3$ (1:1) for 20 min. Excess of DTT and DTT(ox) was removed through washing. Rabenstein reagent promoted disulfide formation as described above. By-products were subsequently removed through washing.

Table 1: Peptides used in this study

ID	Peptide sequence	Cys SPPS protecting group
#1	H_2N-CYFQNCPRG-$CONH_2$	S-tBu
#2	H_2N-VRVPGCAHCADSLY-$CONH_2$	S-tBu
#3	H_2N-CGQRETPEGAEAKPWYC-COOH (Solnatide)	Trityl

Peptide release. The PEC-Linker cleavage and release of purified and modified peptide was initiated by adding 500 µL of TFA:H_2O (95:5) and shaking for 45 min. After 45 min, the sample was washed/eluted twice with 750 µL (500µL for #3) 95% aq. TFA. Ice-cold diethyl ether was used to precipitate the peptides before lyophilization.

Analysis: UPLC-UV chromatograms 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.

3. Results and Discussion

In this study, we highlight the strength of PEC to enable fast access to purified disulfide peptides using PEC-assisted solid-phase modification.

The modification procedure. We used Cys(StBu) building blocks for the test peptides #1 and #2 to suppress the reattachment of the protecting groups during TFA cleavage. StBu removal occurs during the PEC-Linker reduction step at neutral conditions with DTT. After that, the peptide stays attached to the filter material via the reduced yet stable PEC-Linker. Oxidation of Cys with Rabenstein reagent results in efficient disulfide formation (results in Table 2, DMSO and I_2 showed unwanted side reactions). The acidic treatment finally releases the modified peptide from the resin, confirming the compatibility of PEC with disulfide chemistry.

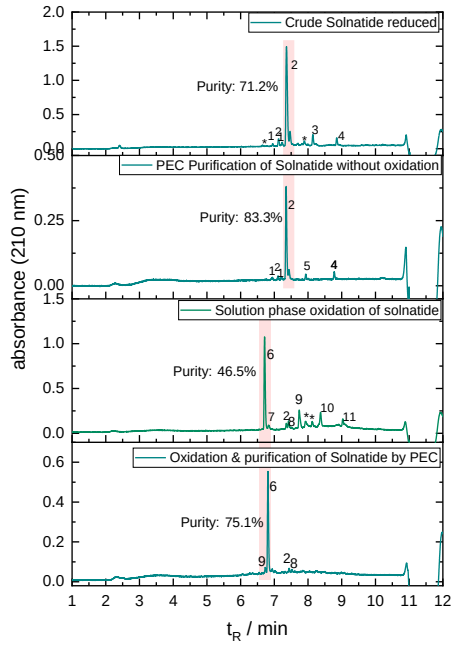
Table 2: Overview of peptides #1 - #3 before and after PEC

ID	$purity_{crude}$ / %	Disulfide / %	$purity_{PEC}$ / %	$yield_{PEC}$
#1	72[a]	100	~78	3.2 mg
#2	73	100	~72	5.2 mg
#3	71	96.8	~75	3.4 mg

[a] crude purity after PEC-Linker coupling

Rapid manufacturing of a COVID-19 drug target. Next, we prepared the target peptide Solnatide (#3), a cyclic 17 amino acid peptide with one disulfide bond. Solnatide is a clinical Phase IIb medicinal product, currently in evaluation for treating SARS-CoV-2-induced acute respiratory distress syndrome (ARDS) in COVID-19 patients (https://www.solnatide.eu/).

Figure 1: Crude and modified peptide #3 (Solnatide)



A detailed list of impurities in the Annex (Link). * Unknown impurity > 1%

We used trityl as an alternative Cys protecting group for the Solnatide peptide to minimize C-terminal ß-(1-piperidyl)alanine formation.[3] Yet, PEC purification (without modification) reveals that this SPPS-related impurity is still present (Compare peak 1 in Figure 1; 3.8%) in the precursor – next to SPPS-related tert-butylation on Cys (4, 5; in sum 10.1%).

For comparison, we performed solution-phase oxidation of PEC purified Solnatide. The impurities are mainly over-oxidation of Cys to sulfinic acid (7; 2.8%, 9; 12.0%), dimer formation (10; 9.5%), along with other unknown impurities (11; 11.0%).

In contrast, PEC-assisted solid-phase modification completely eradicates the dimer formation (10) due to the pseudo-high-dilution effect, and reduces the over-oxidation-related impurities significantly (7;<1.0%, 8; 2.4%, 9; 3.5%). The two impurities in the crude mixture with one tert-butylation of Cys (4 and 5) presumably changed into multiple minor impurities (<1%) by different side-reactions on the remaining non-butylated Cys-residue. We obtained the PEC-modified Solnatide product with 75% UV-purity compared to 47% for in-solution oxidation.

This example shows how adding a modification step to the PEC purification protocol helps rapidly synthesize purified cyclic disulfide peptides. Optimizing SPPS and oxidation procedures can provide higher-purity products.

4. Results at a Glance

- Benefit from a rapid approach to create purified and modified peptides in a combined approach
- Accelerate development of disulfide peptide drugs
- Access complex modification patterns through PEC-assisted solid-phase modification

5. References

[1] Chan, W. C. White, P. Fmoc Solid-Phase Peptide Synthesis: A Practical Approach 2000, p. 91-93

[2] Zitterbart, R. et al. Chemical Science 2021, 12, 2389-2396

[3] Felix, A. Methods of Organic Chemistry, 4th Edition Supplement 2004, p. 391-392

About Belyntic

Belyntic GmbH is a chemistry-for-healthcare enterprise focusing on the purification and modification of peptides. Belyntic offers the world's first broadly applicable peptide purification kits and custom solutions of its proprietary Peptide Easy Clean (PEC) technology.

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