Application Note 72:

Cleaving peptides in flow

Produced by Vapourtec

Abstract

In this application note, we demonstrate how safe, fast and reliably a peptide is cleaved from a resin in continuous flow. Different cleavage conditions were evaluated using Glucagon-like peptide-1 (GLP-1) as an example peptide. By using the Variable Bed Flow Reactor (VBFR), the packing density of the resin was controlled throughout the reaction, minimizing dilution and eliminating channeling of reagents. Temperature, residence time and cleavage cocktail were evaluated. The optimized protocol yielded GLP-1 with a crude purity of 82 % in only 30 minutes, compared to a traditional batch reaction taking between 2 to 4 hours.

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Background

Cleaving peptides is one key reaction in solid-phase peptide synthesis (SPPS) which often possess as many challenges as assembling the peptide itself.

Most peptides are naturally found in the amide state, as the C-terminal amide is more stable to degradation compared to C-terminal acids [1]. In SPPS, the type of linker attached to the resin defines the C-terminal of the peptide.

To break the linker that connects the peptide to the resin, as well as removing side protective groups, acidic conditions are required. Typical cleavage cocktails are composed of trifluoroacetic acid (TFA) and carbocations scavengers [2]. TFA is a corrosive and fuming chemical, harmful if inhaled and can cause severe chemical burns in contact with skin.

Good purity peptide is not solely based on the actual synthesis. Side reactions during cleavage can modify residues, generating additional impurities, as well as compromising the integrity of the peptide [3].

C-terminal N-alkylation of peptide amides can occur from the Rink amide linker [4]. Alkylation of Tryptophan, Tyrosine or Lysine, to name a few, can occur when TFA liberates t-Butyl or pentamethyl-3,4-dihydrochromene-6-sulfonyl (Pmc) protecting groups. In the absence of scavengers, these

cleaved protecting groups can re-attach back to the peptide.

The overall cleavage efficiency will impact the purity and yield of the peptide. Resin core, type of linker, and the composition of the cleavage cocktail can be easily controlled. Current batch cleavage processes do not allow for an accurate control of temperature nor mixing of reagents, making results less reproducible. Any change in temperature, stirring efficiency or reaction time, will lead to unreliable results. In addition to it, TFA needs to be manually handled in all the steps; from the cleavage cocktail preparation to the filtration step, increasing the risk of exposure.

Continuous flow can offer a safer and more reproducible alternative. It only requires the user to place the cleavage cocktail in a bottle and fit the pump connections. TFA will then be pumped through the resin beads in the reactor at a controlled temperature. The cleaved peptide would finally be collected and precipitated in a vial with cold ether.

This application note explores the effect of key parameters on peptide cleavage. Temperature, residence time and cleavage cocktail acid strength were evaluated against crude purity and yield.

GLP-1, synthesized on rink amide ChemMatrix resin was used as example peptide as it contains a variety of side protective groups prone to reattach. By using the VBFR, GLP-1 packing density was maintained through the synthesis and cleavage, yielding the best results.

To the best of our knowledge, this is the first publication covering cleaving peptides in continuous flow.

Setup

To evaluate different cleaving conditions, a Vapourtec R-Series system was set with a VBFR. An R2C+ pump module was used to pump cleavage reagents using bottled reagent. If an R2S pump module was to be used, it would need to be equipped with GREEN tubes.

The R4 reactor heater module independently controlled the reactors' temperature. Figure 1 shows the schematic of the flow set up for the cleavage step.

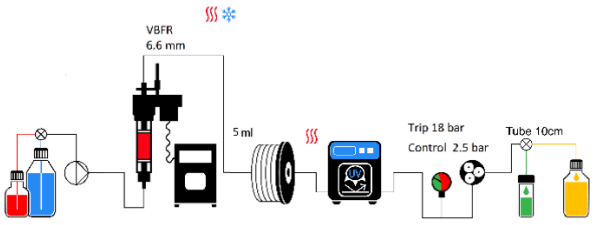

Figure 1 - Schematic of the R-Series used for this work

An SF-10 pump was configured as active BPR and connected post UV detector.

Reagents

GLP-1 was synthesized on ChemMatrix resin as Application Note 69 describes [5]. It was used for this work without further treatment. All reagents were purchased from commercial suppliers.

Sigma Aldrich - Dichloromethane (DCM), Trifluoroacetic acid (TFA) Reagentplus 99%, Acetonitrile (ACN) HPLC grade 99.9%, Water HPLC plus. *Fluorochem - Triisopropylsilane* (TIPS).

System Parameters

Reactor 1 - VBFR loaded with 200-500 mg of GLP-1 peptidyl resin.

Reactor 2 – Tubular reactor of different volumes 0-10 ml.

Cleavage cocktail: 15 ml containing 2.5 % Water, 2.5 % TIPS and TFA (at different concentrations 20-95 %), balanced with DCM.

Flow rate: 0.4 ml/min.

Temperature: Room temperature to 50 °C.

System solvent: DCM.

System pressure: 3 bar.

Final work up and analysis

The product was collected in a vial with cold ether. The precipitated peptide was centrifuged, and the supernatant discarded. Crude samples were dissolved in ACN:H2O 1:1, and analyzed via HPLC (Agilent 1200 equipped with a Eclipse XDB-C18 5 µm column at 40 °C, flow rate = 2 ml/min, the column was eluted using a linear gradient from 5 % to 60 % ACN 0.1 % TFA over 20 minutes). Mass analysis was carried out by ESI-MS (Advion expression CMS).

Results and Discussion

Work started by packing the peptidyl-resin in the VBFR and allowed to swell and stabilize in DCM at the desired temperature for 20 minutes.

If the cleavage reaction is programmed after the last deprotection of the synthesis, swelling is not necessary. 10 ml of DCM will be pumped before the cleavage cocktails to remove DMF from the reactor.

Effect of type of packed bed reactor

Before the launch of the VBFR, the only option to handle solid media, key for SPPS, was the use a fixed bed column reactor. The effect of type of reactor during the synthesis was discussed in Application note 69, and it is further explored in this section. In presence of TFA, polyethylene glycol resin swells more than in DCM, Figure 2 shows how significant this volume change can be at 50 °C. The VBFR increased its volume an extra 0.9 ml due to the swelling of the resin in presence of TFA.

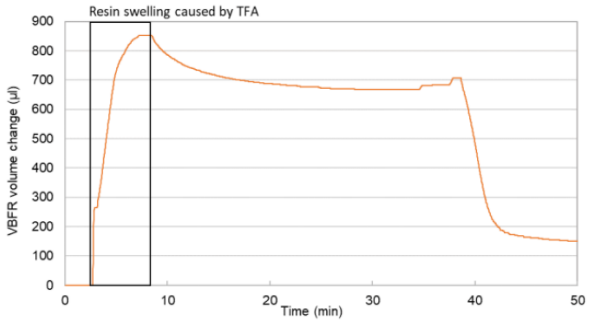

Figure 2 - VBFR volume change recorded during cleavage

To compare the impact of maintaining a constant packing density, an aliquot of the same batch of peptidyl resin was cleaved using a fixed bed column.

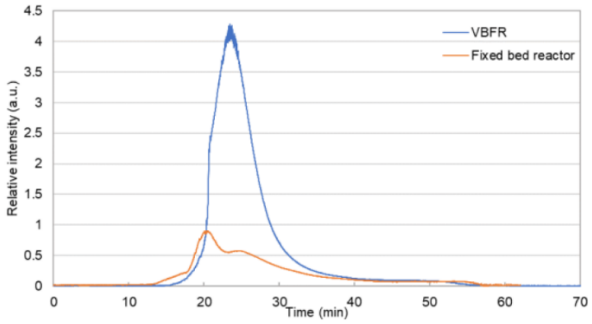

Figure 3 - UV spectral output (at 280 nm) of flow cleavages using the VBFR and a fixed bed reactor.

Figure 3 shows the UV spectral output of both cleavages carried out from the same peptidyl-resin batch. Despite having the same loading, the UV absorption recorded when using a fixed bed reactor is a fraction of what the VBFR achieved.

When using a fixed bed reactor, sufficient dead volume needs to be given to accommodate the swelling of the resin. This extra volume will dilute the cleavage cocktail before reaching the resin and, the lack of packing density will allow channeling of reagents through the path of least resistance.

With a fixed bed reactor, GLP-1 was afforded with a purity of 58.6 % and 61 % yield. These results were drastically improved when using the VBFR. With a controlled packing density, and with the same GLP-1 peptidyl-resin, GLP-1 was afforded with a purity of 76.9 % and 81 % yield.

Effect of residence time

To separate the effect of residence time on linker breakage and side protective group removal, flow rate was fixed to 0.4 ml/min. Different residence times were achieved by changing the tubular reactor's volume. With temperature fixed at 50 °C, 10, 20 and 30 minutes residence time were evaluated.

Figure 4 shows an overlap of the different UV signals of each reaction as the product reaches the UV detector.

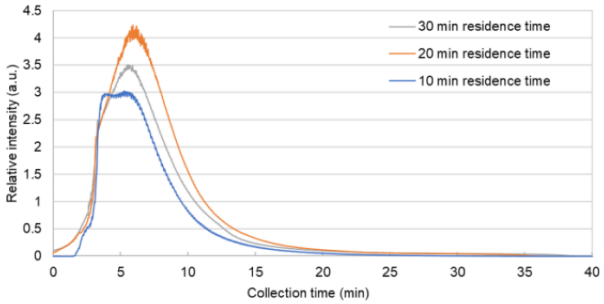

Figure 4 - UV spectral output (at 280 nm) of flow cleavages at different residence times

LC-MS was required to evaluate the purity profile of the cleaved peptides. Table 1 compiles all the cleavage results in both flow and batch.

Table 1: Flow cleavages differences over residence time and batch procedure

Rt	Flow Purity
10 min	53.2 % GLP-1 26 % GLP-1 adduct with side protective groups attached
20 min	77.7 %
30 min	77.7 %

10 minutes of residence time was insufficient to remove all the protective groups from GLP-1, as traces of uncleaved peptide were observed by LC-MS. The main impurities were GLP-1 with tBu and Pbf still attached to it.

20 and 30 minutes residence time yielded the same quality peptide indicating 20 minutes residence time was enough to fully cleave GLP-1.

Effect of temperatures

Different cleaving temperatures were evaluated with a residence time of 20 minutes. Figure 5 shows the effect of the UV absorption of the reaction mixture for the different reactions.

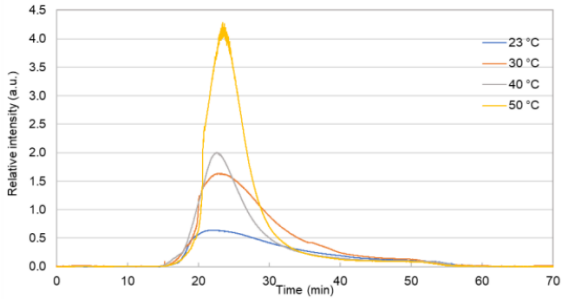

Figure 5 - UV spectral output (at 280 nm) of flow cleavages at different temperatures

Although the cleavages done at 23 °C to 40 °C yielded the desired peptide in high purity, the HPLC baseline was not as satisfactory as the sample cleaved at 50 °C (see S.I. Figure S2).

Table 2: GLP-1 purity and yield at different cleaving temperatures

Temperature	Flow purity (yield)
23 °C	63.5 % (29 %)
30 °C	57.9 % (103 %)
40 °C	62.5 % (60 %)
50 °C	77.7 % (68 %)

Effect of cleavage cocktail acid strength

Lowering the amount of TFA used at any given time could potentially reduce the consequence of a hazardous event (i.e. skin contact). For this reason, different cleavage cocktail acid strengths were evaluated. Reaction conditions were maintained to 50 °C, 20 minutes residence time, while keeping the same concentration of scavengers.

Figure 6 shows the effect of TFA concentration on the UV spectral output of the reaction. Table 3 compiles the obtained crude purity and yield.

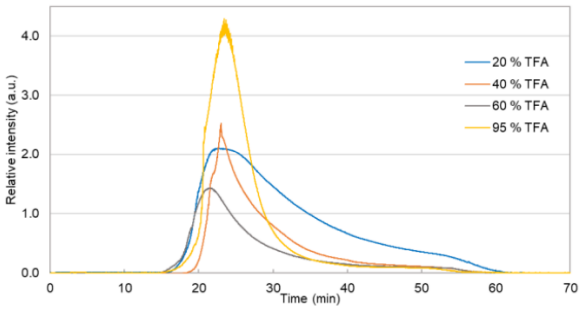

Figure 6 - UV spectral output (at 280 nm) of flow cleavages with different TFA concentrations

Only the cleavage done with 95 % TFA resulted in the complete extraction of GLP-1, indicating the concentration of TFA is critical for a complete cleavage. Reducing the concentration of TFA below 80 % did not only reduced the crude purity of GLP-1, but it took longer for the peptide to be fully cleaved off the resin.

Table 3: GLP-1 purity and yield with different cleaving cocktail acid strengths

TFA concentration	Flow purity (yield)
20 %	N/A
40 %	25.8 % (49 %)
60 %	59.3 % (67 %)
80 %	78.8 % (66 %)
95 %	82 % (81 %)

Figure 7 shows the HPLC traces of GLP-1 cleaved with 40 % and 95 % TFA.

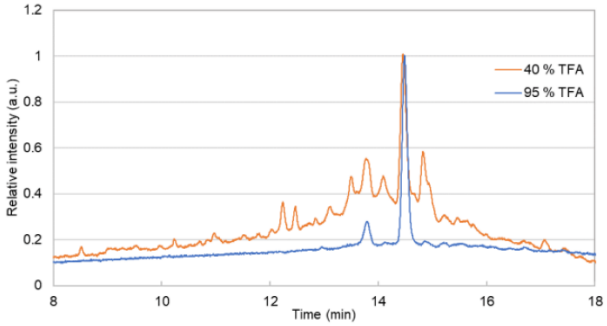

Figure 7 – HPLC of crude GLP-1 with different TFA concentrations

These results could also explain why a fixed bed reactor is not suitable for cleavage in continuous flow. The dead volume, filled with solvent, will dilute the cleavage cocktail before reaching the resin, leading to an incomplete cleavage.

GLP-1 combined synthesis and cleavage

The optimized conditions were implemented as last reaction step in the synthesis of GLP-1. A 10 ml DCM wash was programmed to ensure the complete removal of DMF prior cleaving the peptide. Figure 8 plots the VBFR volume change with UV data for the synthesis and cleavage of GLP-1, achieving a crude purity of 82 % and 81% yield.

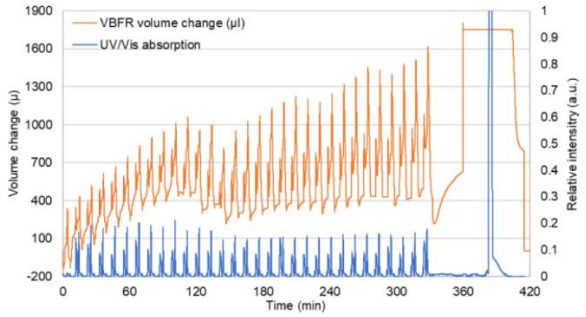

Figure 8 – Synthesis and cleavage in flow of GLP-1 peptide

Conclusions

This application note demonstrates the advantage of cleaving peptides in flow in a fast, reliable, and safe manner. By using the VBFR, the resin's packing density was maintained, avoiding dilution and channeling of reagents.

Residence time and TFA concentration were found to be critical for a complete cleavage of the peptide. These optimized conditions afforded GLP-1 with a crude purity of 82 % and 81 % yield in just 30 minutes.

Acknowledgment

Vapourtec would like to thank Novo Nordisk Copenhagen, DK, and specially Dr Carsten Jessen for the fruitful discussions and further LC-MS analysis.

References

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2. KING DS, FIELDS CG, FIELDS GB. A cleavage method which minimizes side reactions following Fmoc solid phase peptide synthesis. *Int J Pept Protein Res*. 1990;36(3):255-266.
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4. Stathopoulos P, Papas S, Tsikaris V. C-terminal N-alkylated peptide amides resulting from the linker decomposition of the Rink amide resin. A new cleavage mixture prevents their formation. *J Pept Sci*. 2006;12(3):227-232.
5. Vapourtec Ltd. Application Note 69 - *Automated CF-SPPS and Evaluation of GLP-1 Peptide*.; 2021.

Supporting Information

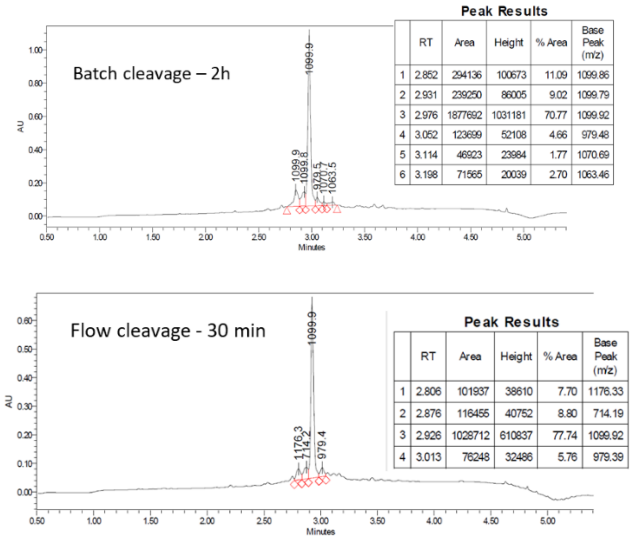

Figure S1 – LC-MS of GLP-1 cleaved in batch (top) and optimized flow conditions (bottom)

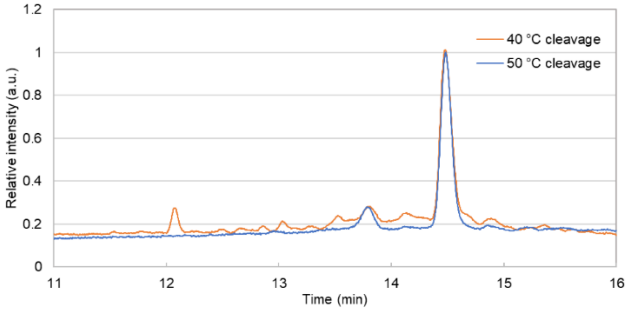

Figure S2 – HPLC of crude GLP-1 with different cleavage temperatures