Investigating the use of Mixed Stationary Phases for Peptide Purification via Reversed Phase Flash Chromatography

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Introduction

Peptide purification using standard reversed phase HPLC methods are hampered by low loading capacity, resulting in purifications that demand significant time investment. Recently, the use of reversed phase flash chromatography has increased in popularity for peptide purification due to the significant reduction of purification time, enabled by the increased loading levels of the larger stationary phase particles. Resolution, though, is somewhat diminished with the larger particle size, demanding creative techniques to retain a highly pure peptide product.

Herein, we present an investigation that uses multiple stationary phase materials, connected in-line for a single peptide purification with the goal of improving the overall peptide purity using reversed phase flash chromatography. We present chromatrographic results for peptide purifications that compare the purification efficiency of a single C4-functionalized and a C18-functionalized stationary phase, and followed by a comparison of the purification efficiency achieved by linking the C4- and C18-functionalized cartridges. For a more comprehensive evaluation, two peptides differing in amino acid length, crude purity and hydrophobic content will be compared.

Experimental protocol

Peptide Synthesis and Analysis

Peptides were synthesized with a Biotage® Initiator+ Alstra™ microwave assisted automated peptide synthesizer on Rink-amide Chemmatrix® resin using default methods, DIC, and Oxyma as coupling reagents, and Fmoc-protected amino acids.

Peptide cleavage occurred in a cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O for 3 hours at room temperature. The cleavage cocktail was evaporated using the Biotage® V-10 evaporation system and the resulting residue was washed with cold diethyl ether then lyophilized. Crude and purified peptides were analyzed with an Agilent 1260 Infinity series HPLC and AB Sciex MS equipped with Biotage® Resolux C18 column (2.1 x 150 mm)

Peptide Purification

Purification was performed on a Biotage® Isolera™ Dalton 2000 equipped with a 10 gram Biotage® SNAP Bio C18 cartridge, SNAP Bio C4 cartridge or both. Peptides were dissolved in a minimal volume of DMSO, injected onto a pre-equilibrated cartridge, and purified with optimized gradients. Fractions were collected by UV absorbance (200-280 nm PDA) and were monitored by mass spectrometry.

Results and discussion

The selectivity profiles of C4- and C18-functionalized media are certainly different, causing alterations in peptide and peptidic impurity retention. These differences can potentially be exploited to improve peptide purity with flash chromatography when used in tandem. To more thoroughly evaluate the utility of this approach, two peptides with grossly different physical properties and crude purities were chosen for this evaluation, Table 1.

Name	Amino acids	Molecular weight	Isoelectric Point (pI)	Average hydrophobicity and hydrophilicity (GRAVY)	Aliphatic index	Crude purity
18A	18	2201.51	4.78	-0.478	76.11	69%
HA2-TAT	33	3979.52	10.2	-1.045	65.15	

Table 1. *Properties of the peptides evaluated in this investigation.*

Purification of 18A using a single stationary phase

Equivalent quantities of 18A were purified using either a Biotage SNAP Bio C4 or a Biotage SNAP Bio C18 cartridge using a linear elution gradient from 20 to 70% MeCN over 10 column volumes, Figure 1.

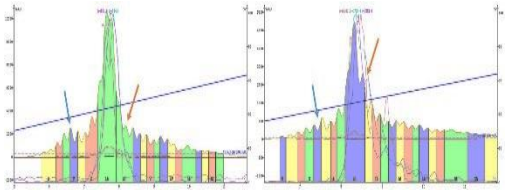

Figure 1. *Purification of peptide 18A using either a single C4-functionalized (left) or C18-functionalized (right) stationary phase. Subtle differences in the chromatographic behavior are indicated with arrows.*

As expected, 18A eluted from the C4-functionalized stationary phase slightly earlier than from the C18-functionalized media (7.5 vs 8.5 column volumes respectively). Importantly though, the unique chromatographic behavior expected for the different selectivity profiles is also observed. An impurity is clearly resolved, eluting earlier than the desired product using the C4 cartridge that is not identifiable with the C18 cartridge purification (blue arrows). Conversely, a late eluting impurity is resolved using the C18 cartridge but not when using the C4 cartridge (orange arrows).

Purification of 18A using mixed stationary phases

The 18A peptide was then purified using a mixed stationary phase configuration - where the C4 and C18 cartridges were connected in-line, using the same sample loading and elution gradient as for the single cartridge purifications Figure 2.

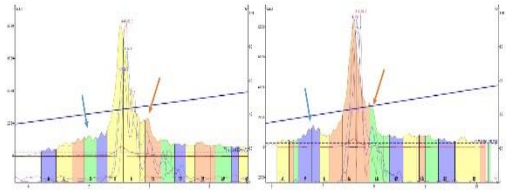

Figure 2. Purification of peptide 18A using two cartridges stacked in-line with either a C4 connected to a C18 cartridge (left) or a C18 connected to a C4 cartridge (right). Sample load and elution gradient were maintained and arrows indicate differences in chromatographic behavior.

The above mentioned selectivity differences become more apparent when the cartridges are connected in line. The leading impurity peak, well resolved only with the C4 cartridges, is only resolved when the C4 cartridge is the second cartridge in line (blue arrows). However, the trailing impurity is sufficiently resolved regardless of cartridge orientation. Taken together, these results suggest that resolution obtained for more hydrophilic impurities will only be maintained if the less retentive C4 cartridge is second in line.

Purification of HA2-TAT using a single stationary phase

Equivalent quantities of crude HA2-TAT were purified using either a Biotage SNAP Bio C4 or a SNAP Bio C18 cartridge and a linear gradient from 30 to 70% MeCN over 8 column volumes, Figure 3.

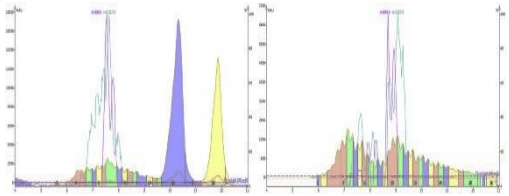

Figure 3. Purification of HA2-TAT peptide using either a single C4-functionalized (left) or C18-functionalized (right) stationary phase. HA2-TAT and all sample impurities elute significantly earlier using a C4-functionalized stationary phase than observed with the C18-functionalized stationary phase.

The differences in chromatographic behavior when comparing the two purifications are much more significant for HA2-TAT than observed for 18A. Purification using the C4 cartridge yielded a very broad, ill-defined chromatogram, suggesting that the both the peptide and the impurities are similarly hydrophilic and therefore poorly distinguished by the stationary phase. In contrast, two well-defined peaks are present in the C18 purified sample.

Purification of HA2-TAT using mixed stationary phases

The HA2-TAT peptide was then purified using a mixed stationary phase, Figure 4. The same sample loading and elution gradient was used to ensure consistency in the chromatographic analysis.

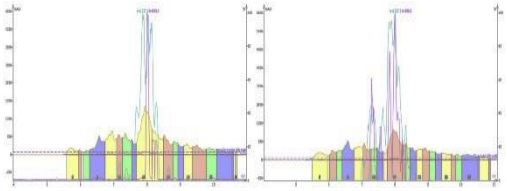

Figure 4. Purification of HA2-TAT using two cartridges stacked in-line with either a C4 connected to a C18 cartridge (left) or a C18 connected to a C4 cartridge (right).

The obvious differences in the chromatographic profiles observed when purifying HA2-TAT with a single cartridge are eliminated when using mixed stationary phases for purification, regardless of cartridge orientation. In this case, mixed stationary phase purification exhibits some characteristics of the single cartridge purifications, but neither chromatographic profile dominates the mixed stationary phase purification. Both chromatograms exhibit the same broad and mostly ill-defined profile that was observed with the C4 cartridge purification. Using mixed stationary phases for the purification though did increase the desired peptide retention somewhat and allowed for separation of the hydrophilic impurities. Importantly though, the increased selectivity contributed by the C18 cartridge sharpened the product peak, enabling quick identification of the fractions containing the desired product.

Conclusions

The compound selectivity differences dictated by C4- and C18-functionalized stationary phase is critical to the success or failure of any purification effort. One hydrophobic peptide and one hydrophilic peptide were purified using a C4 cartridge, a C18 cartridge or both cartridges connected in-line. Purifying the hydrophobic peptide was most improved when the C4 cartridge second in series with the C18 cartridge. However, purifying the hydrophilic peptide improved when both cartridges were use in-line, regardless of relative orientation. Herein we demonstrated that combining the selectivity differences in a single purification effort indeed improves the purification efficiency for two peptides with significantly different physical properties, reducing the overall time dedicated to peptide purification in general.

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