Strategies Toward Optimizing Automated On-Resin Disulfide Bond Formation in Disulfide Rich Peptides

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Introduction
Disulfide rich peptides exhibit exquisite stability due to the covalent stabilization of their secondary structure. After a particular sequence has been identified and mapped, these peptides are typically folded in solution under redox conditions, assuming that a single, thermodynamically stable conformation will be the predominant species. However, there have been several cases where multiple disulfide bonding patterns are observed upon folding completion in solution, demanding additional purification and significant characterization efforts before any biological assays can be performed.

A simplified on-resin synthesis strategy is attractive as the purification and characterization steps can be minimized, if not completely eliminated, thereby increasing the overall yield of the peptide with the proper disulfide bond pattern. Herein we describe a fully automated, optimized solid phase synthesis of Apamin, an 18 amino acid peptide which is conformationally constrained by two disulfide bonds. Using Branches™, the synthesis and on-resin disulfide bond formation was readily visualized and programmed, simplifying the total synthesis ensuring that the proper disulfide bond pattern was achieved.

Experimental protocol
Peptide Synthesis and Analysis
Peptides were synthesized on 0.1 or 0.5 mmol scale using a Biotage® Initiator+ Alstra™ automated peptide synthesizer on Rink amide ChemMatrix® resin using DIC, Oxyma, and Fmoc-protected amino acids. Disulfide bonds were selectively formed on resin in two steps using the Branches™ software feature. First, Mmt protecting groups were removed with 2% TFA in DCM with 5% TIPS reacting for 10 min and repeated 4 times. The free Cys thiol were then oxidized for 5 min at 50 °C with 1 equivalent N-chlorosuccinimide (NCS). Second, the Acm protecting groups were removed and disulfide bonds formed with I2 with a variety of conditions described herein.

Peptide cleavage was performed in a cocktail of 92.5% TFA, 2.5% EDT, 2.5% TIS and 2.5% H2O for 2 h at room temperature. The cleavage cocktail was rapidly evaporated using the Biotage® V-10 evaporation system and the resulting residue was washed with cold diethyl ether and dissolved in 0.1% TFA (aq) for HPLC analysis. Crude peptides were analyzed with an Agilent 1260 Infinity series HPLC and AB Sciex MS equipped with Restek Raptor ARC-18 column (2.1 x 150 mm).

Results and Discussion
Optimized Synthesis of Linear Apamin
H-CH3(CH2)6-Cys(Mmt)-OH
Linear Apamin was first synthesized using microwave assisted methods and evaluated for overall synthesis efficiency. The standard coupling reaction temperature was decreased from 75 °C to 50 °C for all Cys and his residues to prevent side chain racemization. Rather than utilize a double coupling strategy for residues 5 (Ala), 13 (Arg), 14 (Arg), and 16 (Gln), the coupling reaction was decreased to 50 °C and reaction time extended to 15 min. to increase coupling efficiency while decreasing reagent consumption. The linear peptide was synthesized in 53% crude purity, despite the presence of residual protecting groups Figure 1.

Strategic Pairing of Orthogonally Protected Cysteines
For automated synthesis of the fully oxidized peptide, pairs of methoxysulfonyl (Mmt)- and aceticamidomethyl (Acm)-protected cysteine residues were incorporated in place of standard trityl-protected cysteines used in the linear peptide synthesis. Using the unique software feature Branches™, each orthogonal deprotection and subsequent cysteine oxidation can be visualized, individually programmed and the synthesis order assigned, Figure 2.

Figure 1. Crude analytical HPLC of fully reduced, partially protected Apamin. The peptide was synthesized with 53% crude purity and confirmed by mass spectrometric analysis (inset).

Figure 2. A) ChemDraw® structural representation of Apamin and B) Branches™ representation of Apamin for a fully automated synthesis and subsequent on-resin disulfide bond formation where C represents Fmoc-Cys(Acm)-OH and C′ represents Fmoc-Cys(Mmt)-OH building blocks. Each numeric value indicates the order in which the synthesis was programmed to proceed.

Optimizing Concomitant Acetamidomethylated Removal and Cysteine Oxidation
While there are several reported strategies for Acm oxidative removal with concomitant cysteine oxidation, iodine (I2) was selected to avoid use of toxic heavy metal reagents. Conditions for Acm removal reported previously utilize 15 equivalents of I2, which was reacted for 60 min. at room temperature. These conditions were varied in order to determine conditions that require the least time and fewest equivalents of I2, while still maintaining quantitative conversion to the fully oxidized peptide product, Table 1. It is important to note that the first disulfide bond between Cys residues 1 and 11 was formed using optimized Mmt removal conditions and NCS-mediated cysteine oxidation3 to ensure regioselective disulfide bonds prior to removing the Acm protecting group. The 3-15 disulfide bond was selected for I2 mediated reaction optimization to avoid steric interference that may be caused by the remaining N-terminal Fmoc protecting group.

Table 1. Conditions evaluated for Acm removal optimization.

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<th>Time (min)</th>
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While this is certainly not an exhaustive evaluation of all possible conditions, all conditions evaluated yielded crude peptide samples containing fully oxidized peptide and no mass spectrometric evidence of residual Acm protected peptides, Figure 3. These results are likely due to the conformational rigidity and increased local concentration effects induced by formation of the first disulfide bond.

Figure 3. Crude analytical HPLC chromatograms and representative mass spec analysis (inset) for Apamin samples analyzed during the Acm removal optimization. Representative chromatograms for conditions 3 (red) and 6 (black) are overlaid. No significant differences are evident.

Determining Disulfide Bond Formation Order
With optimized conditions for Mmt removal, NCS oxidation and Acm removal with concomitant Cys oxidation in hand, the order in which the disulfide bonds are formed was studied. Apamin synthesized with the N-terminal disulfide bond formed first and the results were compared to Apamin synthesized with the N-terminal disulfide bond formed second, Figure 4. Interestingly, regardless of the order in which disulfide bonds were formed, there was no difference in efficiency observed.

Figure 4. Crude analytical HPLC chromatograms and representative mass spec analysis (inset) for Apamin samples synthesized with the N-terminal disulfide bond formed first (red) or second (black). Peak sharpness suggests only a single disulfide conformation present in the sample. There is no evidence of partially reduced Apamin by mass spectrometry.

This outcome is somewhat surprising given the evidence suggesting that a preference for disulfide bond formation order exists in disulfide rich peptides.12 Factors such as macrocycle ring size, neighbouring amino acids, protecting groups and conformational flexibility could all restrict formation of the second disulfide bond. For this specific peptide, the first disulfide bond likely directs a conformational resemblance to the second disulfide bond given the close proximity of the exocyclic cysteine residues.

Conclusions
Disulfide rich peptides have gained interest as therapeutic scaffolds given their exquisite stability and sequence plasticity. Given the breadth of targets, protocols that simplify not only the linear synthesis but also the disulfide bond formation are highly desirable. Herein we identified an optimized protocol for removing an Acam protecting group using the Biotage® Initiator+ Alstra™ and applied the protocol for a fully automated synthesis with on-resin disulfide bond formation, simplified with the Branches™ software feature. The results presented herein provide a route amenable to the synthesis of other disulfide rich peptides, greatly reducing the effort put toward synthesizing these complex molecules.