Large Scale Stepwise Synthesis of Ubiquitin

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Introduction

Ubiquitin is a central member within a group of structurally conserved proteins found throughout (ubiquitous) eukaryotic organisms. The ubiquitin family of proteins regulate multiple processes through covalent binding to a substrate protein in a process called ubiquitination.[1] Covalent attachment can occur through binding to a lysine residue via an isopeptide bond, a cysteine through a thioester bond, serine/threonine residues through an ester bond, or the N-terminus amino group. Ubiquitination can trigger different cellular processes for the substrate protein such as degradation, activity changes, effects on protein interactions, or altering cellular location. Synthesis of ubiquitin is known to be challenging due to its length (76 aa). The use of pseudoprolines and/or native chemical ligation have been previously employed to aid in the synthesis.[2,3] Here, we report a stepwise linear synthesis of ubiquitin without pseudoprolines or native chemical ligation by using a new microwave based SPPS process that we recently introduced.[4] Additionally, the synthesis of ubiquitin was scaled from 0.1 mmol – 0.5 mmol to demonstrate the scalability of this approach even for very long peptides.

Experimental

HE-SPPS Materials and Methods: All peptides were synthesized on the CEM Liberty Blue Automated Microwave Peptide Synthesizer using Fmoc-PAL-PEG-PS resin (0.20 mmol/g substitution). Post-deprotection washing with DMF was followed by coupling using the DIC/Oxyma/DIEA activation method.[5] The peptide resin was cleaved with TFA/TIS/H_2O/DODT (92.5/2.5/2.5/2.5) on the CEM Accent Microwave Cleavage System. The peptide was precipitated in cold ether and the crude material was analyzed without any purification.

Analysis: Crude peptides were analyzed on a Waters UPLC ACQUITY H-Class with 3100 Single Quad MS using acetonitrile/water with 0.1 % TFA as the solvent system on a C18 Column (1.7 µm, 2.1 x 100mm).

Results and Discussion

Ubiquitin Sequence:

MQIFVKTLTGKTITLEVEPSDTIENVKAKIQDKEGIPPDQQRLIFAGKQLEDG RTLSDYNIQ KESTLHLVLRLRGG (76-mer)

(A) Standard Scale Synthesis at 0.1 mmol:

Ubiquitin is a well-known sequence for its synthetic challenges that involve difficult deprotection and coupling steps as the peptide chain grows longer than 30 amino acids. Also, this sequence consists of four aspartic acid segments DY, DG, DQ and DT that have a tendency to form the aspartimide side product with DG being the most susceptible. Our initial study on this peptide using Fmoc-Asp(OtBu)-(Dmb)-Gly-OH for the DG segment and Fmoc-Asp(OtBu)-OH for all other Asp residues with 1 min/90 °C deprotection using 10% piperazine/0.1M HOBt revealed minor amounts of aspartimide formation. Using a modified deprotection method of 10 min/55 °C lowered the aspartimide formation to negligible levels, but this method resulted in longer cycle times. Interestingly, use of Fmoc-Asp(OMpe)-OH allowed the fast 1 min/90 °C Fmoc deprotection method to be carried out for the whole sequence without the formation of aspartimide side product as determined by UPLC-MS analysis. Ubiquitin was synthesized at 0.1 mmol scale without the use of (expensive) pseudoprolines on the Liberty Blue using the new CarboMAX™ coupling with DIC/Oxyma/DIEA (1:1:0.1).[5] Cycles involved a single 1 min/90 °C deprotection and a single 2 min/90 °C coupling for the first 30 amino acid residues, and a double 2 min/90 °C coupling for the rest of the sequence. His was coupled with a special 2 min/RT-4min/50 °C microwave method and all Arg residues were coupled with double 2 min/90 °C method using a 5-fold excess of reagents (Figure 1).

Ubiquitin (76-mer)
0.1 mmol scale
Total Synthesis Time: 9 hours 45 min
Total Wash Solvent Usage (DMF): 1.5 L
Total Chemical Waste: 2.1 L

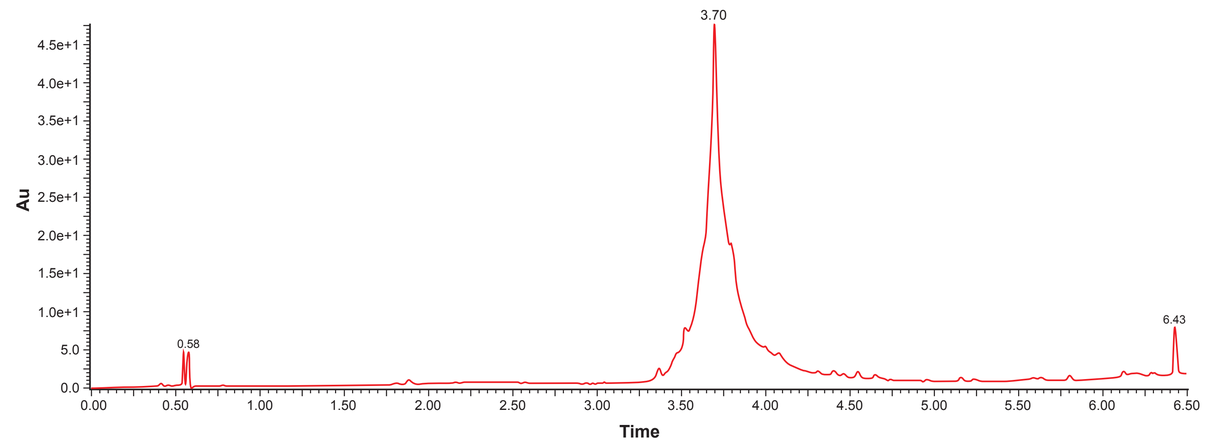

Figure 1. UPLC chromatogram of Ubiquitin synthesized at 0.1 mmol scale

(B) Large Scale Synthesis at 0.5 mmol:

Encouraged by the successful results at 0.1 mmol scale, we applied the new DIC/Oxyma/DIEA coupling procedure to the synthesis of Ubiquitin at 0.5 mmol scale. Cycles for 0.5 mmol scale involved a single 2 min/90 °C deprotection and a single 5 min/90 °C coupling for the first 30 amino acid residues, and a double 5 min/90 °C coupling for the rest of the sequence. His was coupled with a special 4 min/RT-8 min/50 °C microwave method and all Arg residues were coupled with double 5 min/90 °C method using a 5-fold excess of reagents (Figure 2).

Ubiquitin (76-mer)
0.5 mmol scale
Total Synthesis Time: 36 hours
Total Wash Solvent Usage (DMF): 7.8 L
Total Chemical Waste: 12 L

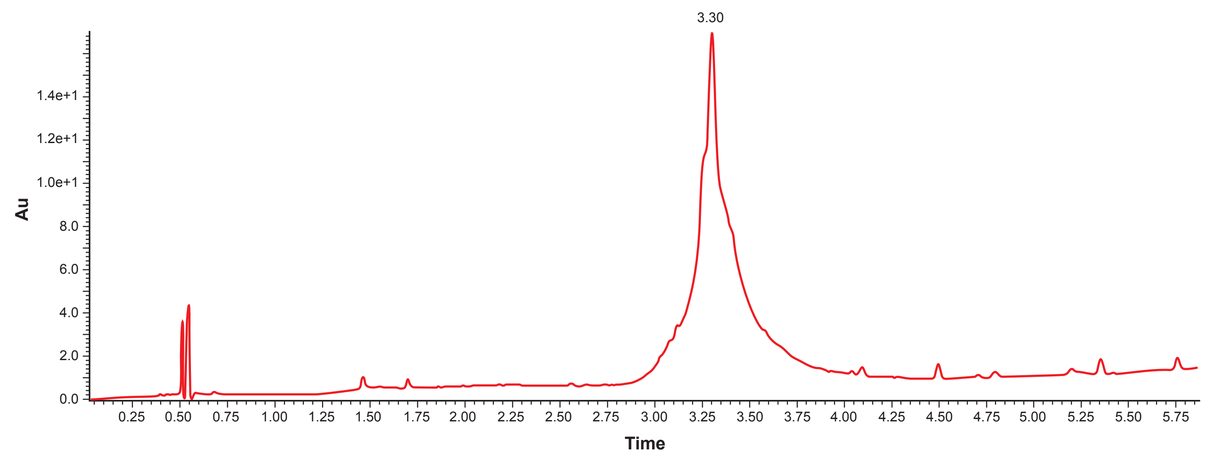

Figure 2. UPLC chromatogram of Ubiquitin synthesized at 0.5 mmol scale

Conclusion

The combination of new microwave based HE-SPPS chemistry[4] and ultrafast automation allowed high purity Ubiquitin synthesis on the Liberty Blue on a scale ranging from 0.1 to 0.5 mmol. Use of only 5 equiv. coupling reagents and optimized washing resulted in substantial savings in reagent cost and a 90% reduction in solvent usage and chemical waste.

References

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