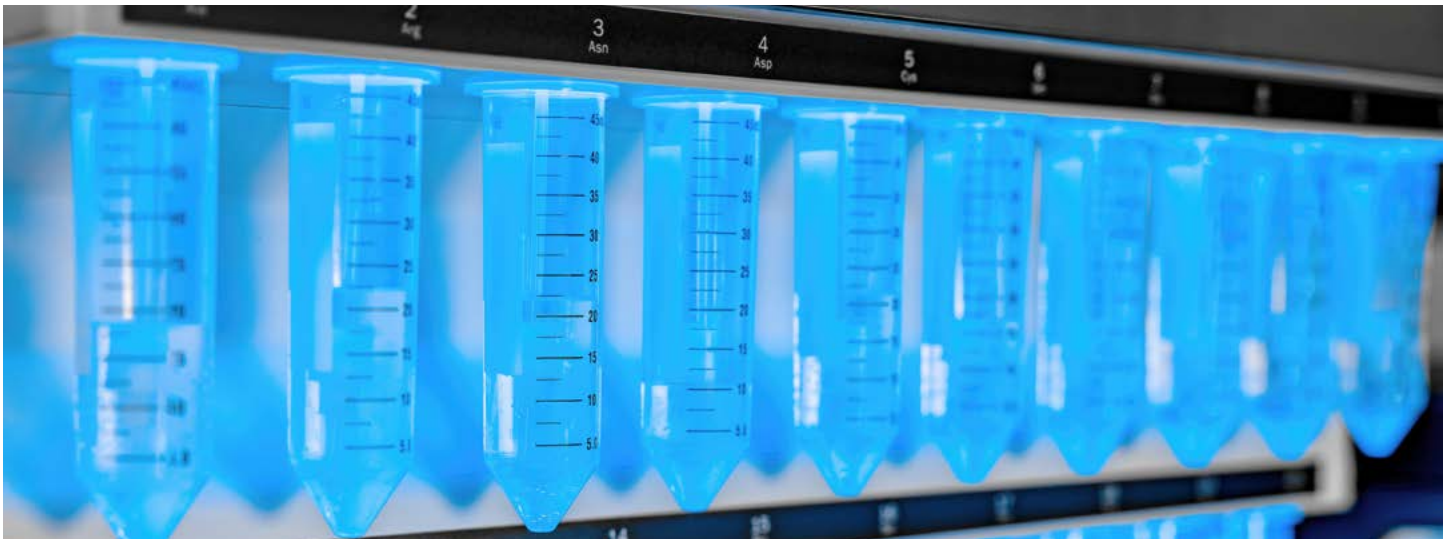
Automated Microwave-Enhanced Total Synthesis of Proteins



Summary

Proteins and long peptides can be synthesized rapidly and efficiently by microwave-enhanced SPPS on the Liberty Blue™ 2.0 and Liberty PRIME™ 2.0. The use of optimized microwave SPPS incorporating a new headspace flushing technology allows for higher purity synthesis of protein sequences. This technology was demonstrated on a series of biologically relevant proteins (ubiquitin, barstar, proinsulin, collagen, HIV protease, and MDM2) from 76-127 amino acids which were obtained in good purity through stepwise assembly without any ligation steps. High purity samples were isolated from the crude material by elevated temperature chromatography at 60 °C on the Prodigy™ Preparative HPLC Peptide Purification system.

Introduction

Proteins and long peptides are critical components of biological systems and comprise many important therapeutics, but their research is slowed by time-intensive expression or native chemical ligation production methods. Total synthesis by SPPS provides a direct synthetic route to target specific sequences and allows for rapid generation of analogs. However, long peptides and proteins can be challenging targets for SPPS, due to the iterative accumulation of impurities and tendency for aggregation to occur. Historically, SPPS was limited to making fragments for native chemical ligation and longer sequences have been of limited accessibility.[1] Recently, rapid flow-based methodology has shown the significant ability to assemble long sequences with extremely quick cycle times.[2] However, this process requires large excesses of amino acid (≥ 100 equivalents) and produces large amounts of waste.

Microwave heating is now widely used and has demonstrated the ability to overcome aggregation and drive the completion of difficult reactions on long peptide chains.[3, 4] Optimized carbodiimide-based coupling conditions with microwave heating (CarboMAX™) allow for minimal epimerization and higher purity synthesis than more aggressive activation based methods using onium salts with strong bases.[5] Additionally, the use of a one-pot coupling and deprotection process without any draining between the steps results in a faster and more efficient process.

In 2022, a new series of microwave peptide synthesizers (Liberty PRIME 2.0 and Liberty Blue 2.0) were released that utilize a new headspace flushing technology for ensuring cleaner surfaces of the reaction vessel during peptide synthesis. This headspace flushing technology is useful for preventing volatile reagents from re-condensing and contaminating subsequent reactions. The small purity increases to each reaction become significant gains in purity over the course of a long protein synthesis. The successful synthesis of several proteins including ubiquitin (76mer), proinsulin (86mer), barstar (89mer), a collagen-like sequence (99mer), HIV protease (99mer), and MDM2 (127mer) was achieved on the Liberty PRIME 2.0.

Cycles were optimized for protein synthesis with 10-20 equivalents of amino acid and a total average cycle time of around 7.5 minutes that enabled overnight synthesis (10–17 h per run) of the targeted proteins in high crude purity. The next day, the proteins were isolated and purified on the Prodigy system with elevated temperature chromatography at 60 °C. This streamlined production method is fast and uses reagents efficiently (**Table 1**).

Materials and Methods

Reagents

The following Fmoc amino acids were obtained from CEM Corporation (Matthews, NC) and contain the indicated side chain protecting groups: Ala, Asn(Trt), Arg(Pbf), Asp(OMpe), Asp(OtBu)-(Dmb)Gly, Cys(Trt), Gln(Trt), Glu(OtBu), Gly, His(Boc), Ile, Leu, Lys(Boc), Phe, Pro, Met, Ser(tBu), Thr(tBu), Trp(Boc), Tyr(tBu), and Val. Rink Amide ProTide™ LL resin (0.20 meq/g substitution) was also obtained from CEM Corporation. N,N'-Diisopropylcarbodiimide (DIC), pyrrolidine, trifluoroacetic acid (TFA), 3,6-dioxa-1,8-octanedithiol (DODT), and triisopropylsilane (TIS) were obtained from Sigma-Aldrich (St. Louis, MO). Dichloromethane (DCM), N,N-Dimethylformamide (DMF), anhydrous diethyl ether (Et2O), and acetic acid were obtained from VWR (West Chester, PA). LC-MS grade water (H2O) and LC-MS grade acetonitrile (MeCN) were obtained from Fisher Scientific (Waltham, MA).

Protein Synthesis

The proteins were synthesized on 0.05 or 0.1 mmol scale using the CEM Liberty PRIME 2.0 automated microwave peptide synthesizer and Rink Amide ProTide® resin LL. Deprotection was performed with pyrrolidine in DMF. Coupling reactions were performed with 10 or 20 equivalents of Fmoc-AA with DIC and Oxyma Pure in DMF (Modified CarboMAX).[5] Cleavage was performed at room temperature or 38 °C using TFA/H2O/TIS/DODT. Following cleavage, the proteins were precipitated with Et2O and lyophilized overnight.

Protein Purification

The proteins were purified by elevated temperature HPLC on a Prodigy system equipped with an Intrepid C18, 21.2 mm x 250 mm, 5 µm column. The crude proteins were dissolved in H2O and filtered before injection. Separations were performed at 40 or 60 °C with a gradient elution of 0.1% TFA in (i) H2O and (ii) MeCN.

Proteins Analysis

The proteins were analyzed on a ThermoFisher UPLC system with Q Exactive plus MS equipped with an Acquity UPLC BEH C8 column (1.7 mm x 100 mm). Peak analysis was achieved on Chromeleon software. Separations were performed with a gradient elution of 0.05% TFA in (i) H2O and (ii) MeCN.

Results

Table 1. Synthesis times and total waste generated for each protein.

Protein	Sequence length (AAs)	Total Synthesis Time (min)	Total Waste (mL)
Ubiquitin	76	9 h 44	1504
Proinsulin	86	11 h 15	1622
Barstar	89	11 h 26	1660
Collagen	99	12 h 30	1784
HIV Protease	99	12 h 48	1829
MDM2	127	16 h 26	2316

Table 2. Amino acid sequences for each protein. Bolded DG indicates that Asp(OtBu)-(DMB)Gly was used.

Protein	Sequence
Ubiquitin	MQIFVKTLTG KTITLEVEPS DTIENVKAKI QDKEGIPPDQ QRLIFAGKQL EDGRTLSDYN IQKESTLHLV LRLRGG
Proinsulin	FVNQHLCGSH LVEALYLVCG ERGFFYTPKT RREAEDLQVG QVELGGGPGA GSLQPLALEG SLQKRGIVEQ CCTSICSLYQ LENYCN
Barstar	KKAVINGEQI RSISDLHQTL KKELALPEYY GENLDALWDC LTGWVEYPLV LEWRQFEQSK QLTENGAESV LQVFREAKAE GCDITIILS
Collagen	GLPGAKGLAG APGAPGPDGK AGPPGPAGQD GRPGPPGPPG ARGQAGPPGF PGPKGAAGEP GKAGERGVPG PPGAVGPAGK DGEAGAQGPP GPAGPAGER
HIV Protease	PQVTLWQRPI VTIKIGGQLK EALLDTGADD TVLEEMSLPG KWKPKMIGGI GGFIKVRQYD QVSIEICGHK AIGTVLIGPT PVNIIGRNLL TQLGCTLNF
MDM2	MHHHHHHGSM CNTNMSVPTD GAVTTSQIPA SEQETLVRPK PLLLKLLKSV GAQKDTYTMK EVLFYLGQYI MTKRLYDEKQ QHIVYCSNDL LGDLFGVPSF SVKEHRKIYT MIYRNLVVVN QQESSDS

Conclusions

Microwave SPPS is a powerful and efficient method for the synthesis of large peptides and proteins. This was demonstrated by the synthesis of 6 different proteins ranging in lengths from 76 to 127 amino acids on the Liberty PRIME 2.0. Individual proteins were completed in 10–17 hours and the target proteins were quickly isolated using elevated temperature preparative HPLC with the Prodigy system. This methodology is fully automated, rapid, and offers a convenient alternative to native chemical ligation or protein expression methods. The Liberty PRIME 2.0 and Liberty Blue 2.0 systems are adaptable and can be used to install unnatural amino acids or make other nonconventional proteins. The assembly is fast, efficient, and produces low amounts of waste.

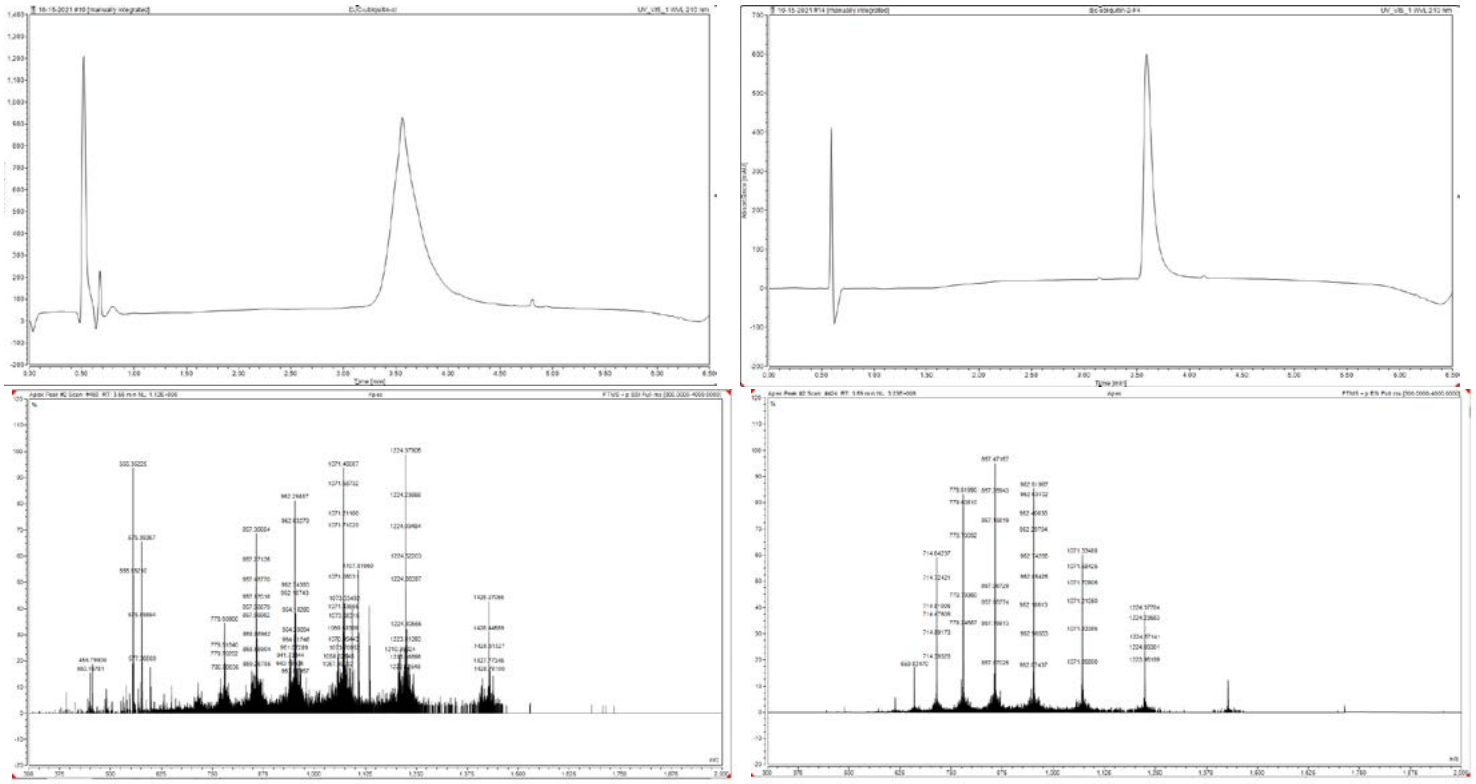


Figure 1. UPLC-MS analysis for crude (left panels) and purified (right panels) ubiquitin samples.

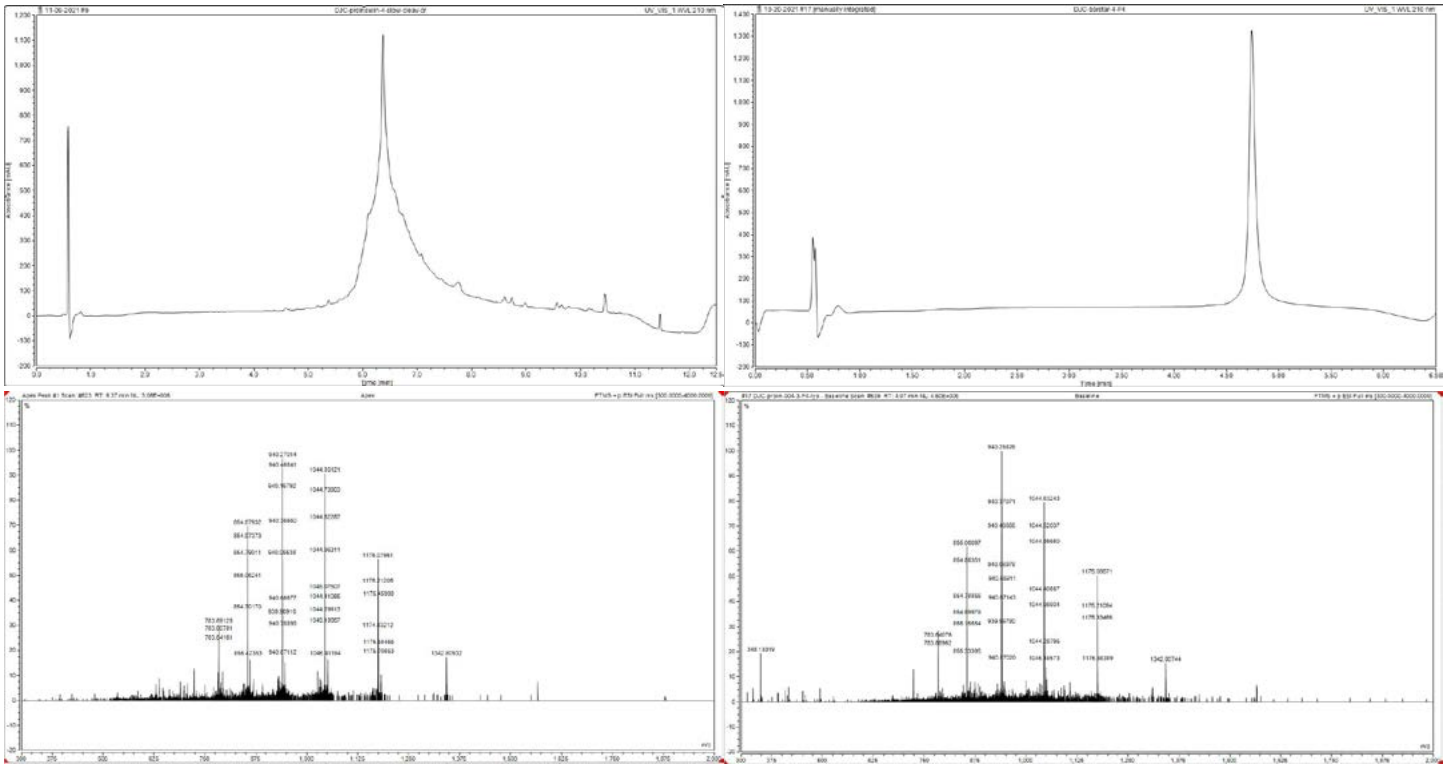


Figure 2. UPLC-MS analysis for crude (left panels) and purified (right panels) proinsulin samples.

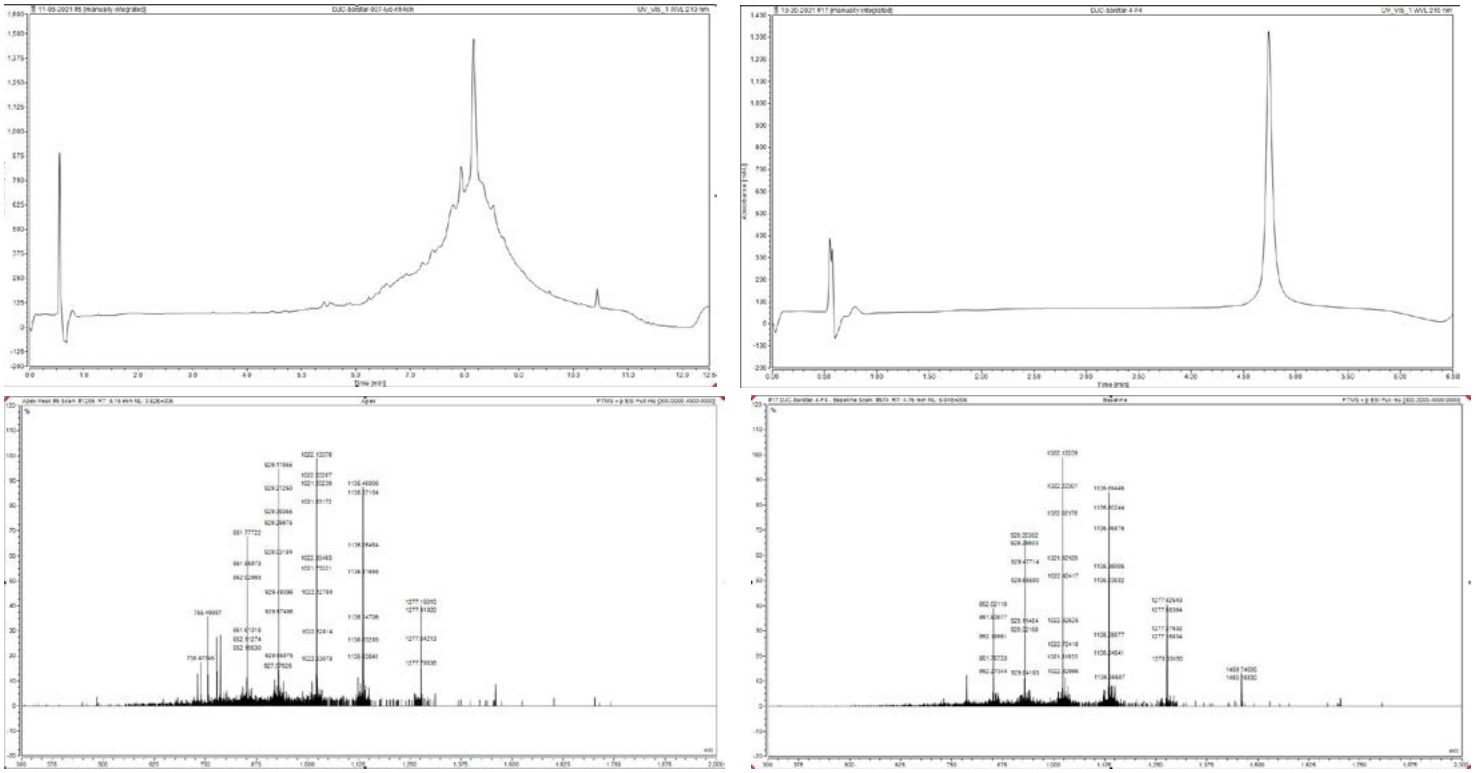


Figure 3. UPLC-MS analysis for crude (left panels) and purified (right panels) barstar samples.

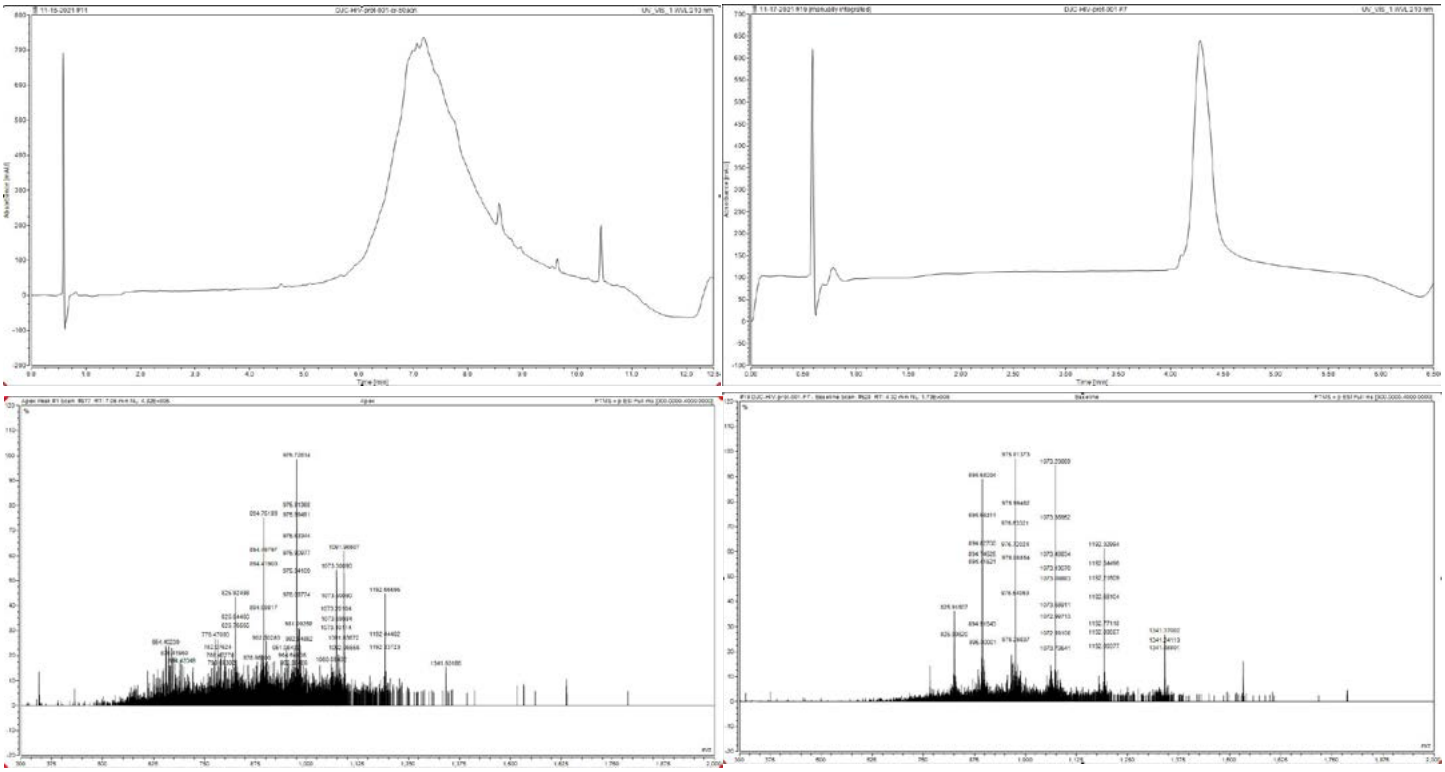


Figure 4. UPLC-MS analysis for crude (left panels) and purified (right panels) HIV protease.

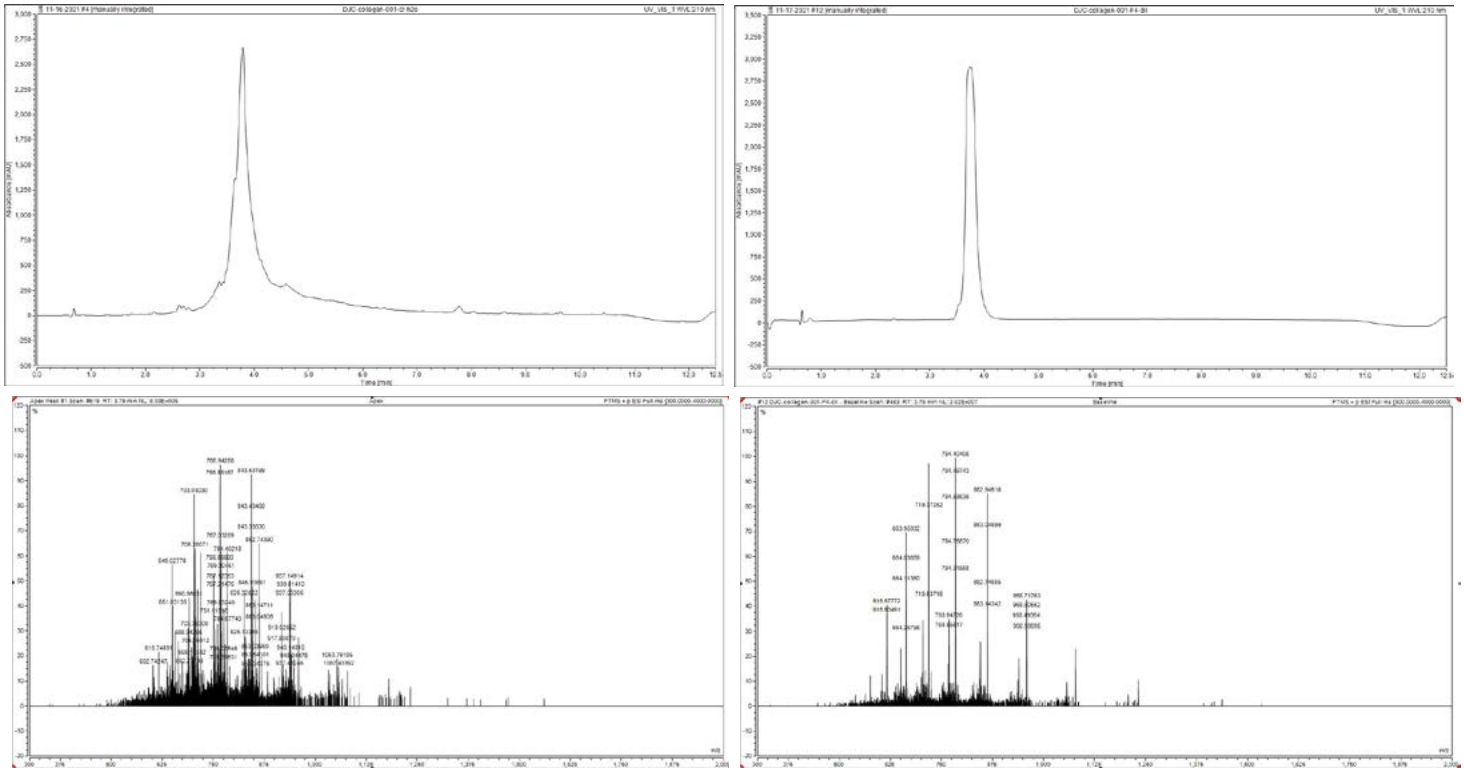


Figure 5. UPLC-MS analysis for crude (left panels) and purified (right panels) collagen samples.

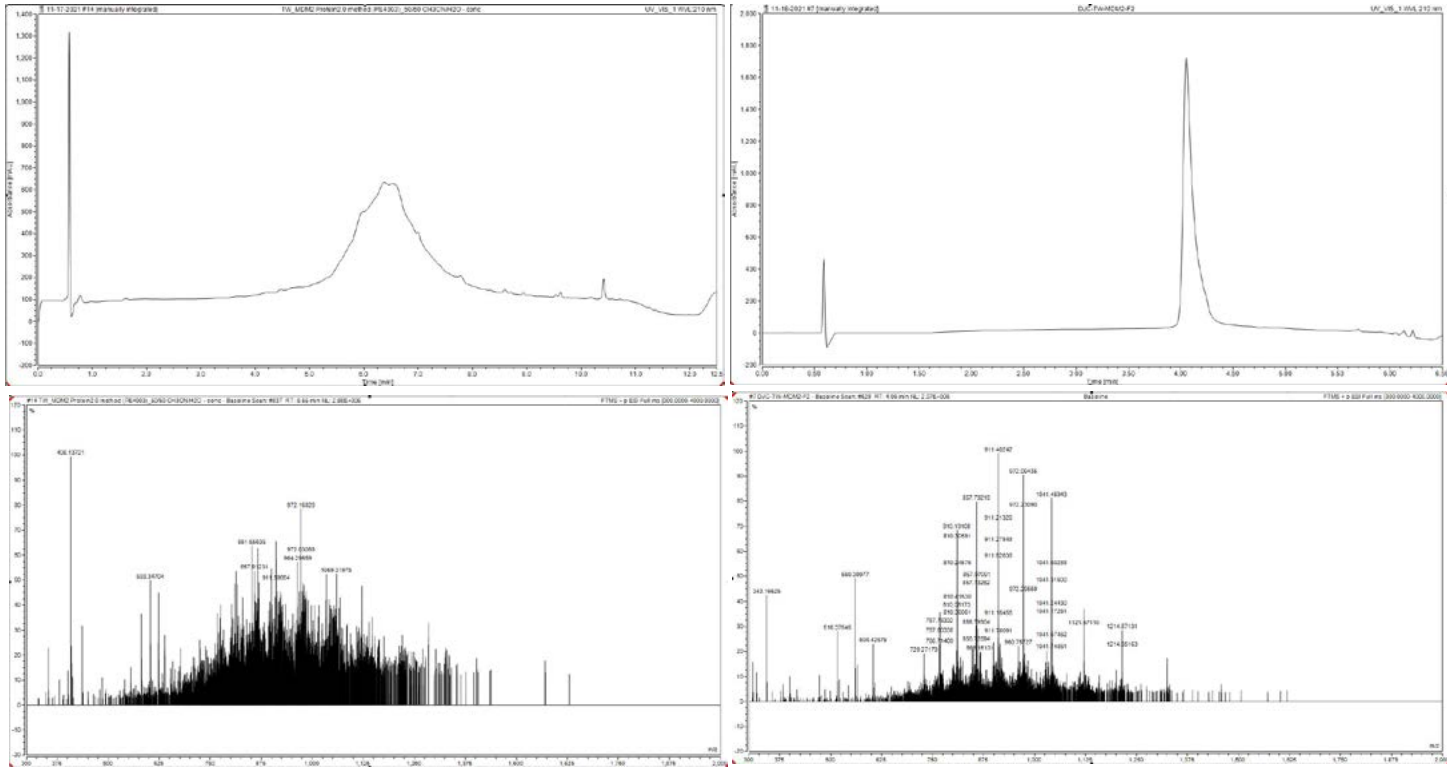


Figure 6. UPLC-MS analysis for crude (left panels) and purified (right panels) MDM2 samples.

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