Therapeutic peptides: Synthesis optimization using parallel automated SPPS

Andrew Kennedy¹, James Cain², Cyf Ramos-Colon², Daniel Martinez²

¹Gyros Protein Technologies, Sweden

²Gyros Protein Technologies, United States

<https://doi.org/10.17952/35EPS.2018.090>

Process development for the manufacture of therapeutic peptides remains a vital part of the commercial peptide production process. An optimized solid-phase synthesis protocol can be challenging to develop. Many of the recent advances in peptide therapeutics focus on peptides with greater structural complexity compared to naturally occurring peptides, allowing for the design of more physiologically stable products with increased target specificity and membrane permeability [1]. The advent of the neoantigen and personalized medicine sectors adds a further requirement for faster peptide synthesis protocols and reduced processing times, including adherence to regulatory guidelines and GxP requirements during manufacture.

Solid-phase peptide synthesis (SPPS) of linear peptides using heat (>50°C) and highly reactive coupling reagents have resulted in products with increased crude purities and yields when using significantly shorter coupling cycles compared to room-temperature methods[2]. Development of automated solid-phase (on-resin) protocols for cyclization, potentially using heat, may provide numerous advantages for the development of complex peptide products [3]. Parallel automated synthesis is a useful tool for quickly and efficiently synthesizing multiple peptide analogs simultaneously for structure-activity relationship (SAR) studies of synthetically challenging sequences.

Solid-support screening, reagent screening and temperature screening are demonstrated using an automated peptide synthesizer as part of the optimization process for difficult peptides such as Aib-Enkephalin (Aib-Enk) and Jung-Redemann (JR 10-mer). A number of automated solid-phase protocols are also shown to be more efficient replacements for traditionally manual or solution-phase manipulations, resulting in reduced processing times and high crude product purities and yields. We report the use of heat to increase the crude purity of Melanotan II peptide from 43% to 60% using solely on-resin synthetic methods.

Table 1: Synthesized peptide sequences.

Peptide	Sequence
Melanotan II (MT-II) Sequence	Ac-Nle-cyclo[Asp-His-D-Phe-Arg-Trp-Lys]-NH ₂
Jung-Redemann Sequence	H-Trp-Phe-Thr-Thr-Leu-Ile-Ser-Thr-Ile-Met-NH ₂
Aib-Enkephalin Sequence	H-Tyr-Aib-Aib-Phe-Leu-NH ₂

Methods

Protocol A: The MT-II linear peptides were synthesized at 100 μ mol scale using Rink Amide resin (loading 0.22 mmol/g). Deprotection was performed with 20% piperidine in DMF 2 x 2 min at 25°C. Couplings were performed at a final concentration of 100 mM AA (5 eq.), 100 mM COMU (5 eq.) and 200 mM DIPEA (10 eq.) for 30 min at 25°C. Alloc and Allyl side chain protection was used for Lys and Asp. Cyclization: Following Pd-mediated removal of the side chain protecting groups and washing, a solution of PyClock (5 eq) and DIEA (10 eq) in DMF was added to the resin. After cyclization, the resin was washed with DMF and DCM.

Protocol B: JR10 and Aib Enkephalin were synthesized using Rink Amide ChemMatrix Resin (0.47mmol/g substitution) at a 50 μ mol scale. Deprotection time was 2 minutes at 25°C, 60°C, or 90°C using 20% Piperidine in DMF. Amino acids were coupled using a six-fold excess and final concentration of 100mM for Amino Acids and Activators/Additive and 200mM for DIEA. Coupling time was 3 min at 25°C, 60°C, or 90°C. DMF washes followed both deprotect and coupling steps with 3 repetitions at 30 seconds each.

Cleavage: Final cleave used TFA:TIS:EDT:Water (95:1:2.5:2.5) for 2 hours at 25°C.

Analysis: MT-II was analyzed on a U3000 Thermo HPLC using a C18, 180 Å, 5 μ m, 250 x 4.6 mm column (Agilent Polaris), over 30 minutes with a flow rate of 1 mL/min, and using a gradient of 5-95% B, where Buffer A is 0.1% TFA in water, and Buffer B is 0.1% TFA in acetonitrile. Detection was at 214 nm. Mass analysis was performed on a Shimadzu LCMS-2020 Single-Quad mass spectrometer, equipped with a C18, 100 Å, 2.6 μ m,

50 x 2.1 mm column (Phenomenex Kinetex), over 7 min with a flow rate of 1 mL/min and using a gradient of 5-50% B where Buffer A is 0.1% formic acid in water and Buffer B is 0.1% formic acid in acetonitrile.

JR10 and Aib enkephalin prepared using Protocol A were analyzed using a Varian Microsorb MW 300-5 C18 50x4.6 mm column with a gradient of 5-95%B in 15 min using Water (0.1%TFA):ACN(0.1%TFA) at 1ml/min. A 1:10 dilution of a standard sample of 3 mg/ml was run on a Phenomenex Kinetex 2.6 um C18 100A 50x2.1 mm column for LCMS with a gradient of 5-95%B in 15 min for JR10 and 10-20%B in 9 min for Aib-Enkephalin using Water(0.1%FA):ACN(0.1%FA) at 1 ml/min.

Results

MTII

On resin cyclization is typically done using methods of 2-18 h, here we show that by increasing the temperature the cyclization of MTII is possible in 30 min with high crude purity. Increasing the temperature to 50°C resulted in a crude purity of 60.1% compared to 42.7% done at 25°C with a longer reaction time of 1h.

Table 2: Crude purity of cyclic MTII.

Condition	% Crude Purity
1 h @ 25°C	42.7
30 min @ 50°C	60.1

JR 10-mer and Aib-enkephalin

JR 10-mer and Aib-enk were synthesized using different coupling reagent combinations at three different temperatures (Table 3). Regardless of coupling reagent combination an increase in crude purity can be seen as the temperature is increased. COMU produced the best result on the synthesis of both JR-10mer (66.9%) and Aib-Enk (89.3%).

Table 3: Effect of coupling reagents and temperature on the crude purities of JR 10-mer and Aib-enk.

Reagent	JR 10-mer % Crude Purity			Aib-Enk % Crude Purity		
	25°C	60°C	90°C	25°C	60°C	90°C
HDMC/OxymaPure	20.8	52.9	59.8	29.2	54.2	71.3
HDMC	21.6	44.9	51.3	21.3	38.1	56.9
COMU	23.5	55.7	66.9	49.9	78.5	89.3

Figure 1: Crude purity profiles of Aib-Enk synthesized with COMU at A) 25°C and B) 90°C.

Conclusions

Difficult peptide sequences (JR 10-mer and Aib-Enk) and a cyclic peptide (MT-II) were successfully prepared in high purity with short synthesis times using automated peptide synthesizers. Independent induction heating on the Prelude X allowed multiple temperatures to be screened simultaneously on the synthesis of JR 10-mer and Aib-Enk. Purities of both difficult sequences improved with an increase in temperature up to 90°C. With MT-II, induction heating achieved a significant decrease of cyclization time without compromising peptide crude purity. Efficient cyclization can be obtained from 30 min cyclization reaction times. Finally, multi-variable conditions were effectively tested in parallel for the process optimization of JR 10-mer, Aib enkephalin, and MT-II syntheses.

References

- 1) S. H. Joo, "Cyclic peptides as therapeutic agents and biochemical tools," *Biomol. Ther.*, 20, 1,19-26, 2012.
- 2) T. Redemann and G. Jung, *Proc. 24th Eur. Pep. Symp.*, 1996, 749.
- 3) N. Tsomaia, "Peptide therapeutics: Targeting the undruggable space", 2015.