Conventional Heating Provides Higher Quality Peptides than Microwave Heating or Conventional Solid Phase Peptide Synthesis at Room Temperature

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ABSTRACT

Since Merrifield first reported solid phase peptide synthesis processes, large advances have been made in resins and protecting groups but progress has been slow in utilizing heating in peptide synthesis. Advancing from our earlier reports, we have found that a combination of a short reaction time at room temperature and conventional gradient heating produces peptides in higher yield and higher purity than standard protocols at room temperature or with microwave heating. We have compared room temperature synthesis along with synthesis utilizing gradient heating and published microwave synthesis of ACP (65-74), JR 10-mer, thymosin α1 amide, HIV tat (trans activator of transcription) and the ABRF 1992. These test peptides showed significant improvement in purity and yield with synthesis using gradient and delayed gradient methods. Also there was no significant racemization with amino acids such as Cys in these two conventional gradient heating methods.

METHODS AND RESULTS

ACP (65-74), JR 10-mer, thymosin α1 amide, HIV tat and the ABRF 1992 test peptides were prepared by standard solid phase peptide synthesis at room temperature and with rapid heating, gradient heating and delayed gradient heating from 24 °C to 75 °C.

Standard solid phase peptide synthesis (SPPS) performed at room temperature produced peptides with high yield and good purity with longer synthesis times. "Difficult sequences", highly hydrophobic peptides characterized by slow or difficult coupling or de-protection reactions, have significantly reduced yields of desired peptide and increased impurities. It has been shown that in difficult sequences, heating accelerates reactions and often produces higher purity and higher yield.

AAPPTec Focus XC 6RV

With conventional heating, coupling reactions were performed utilizing the delayed temperature gradient shown in Table 1. For the first two minutes the reaction was run at room temperature, from thereon the temperature was increased 10 -14 °C per minute. Within five minutes, the temperature reached 75 °C.

In addition to accelerating the chemical reactions involved, heating helped solvate the peptide-resin and expose reactive sites. Heating reduced the need for double couplings or extended coupling times.

Reaction Time (minutes)	Reaction Temperature (°C)
0-2	Room temperature
2-3	40
3-4	54
4-5	66
5-6	75
6-7	78

Table 1 – Temperature gradient applied to coupling reactions.

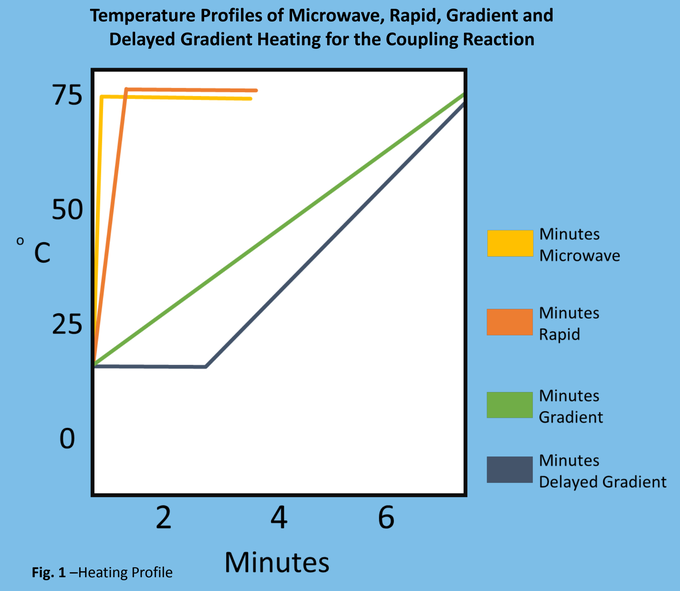

Fig. 1 –Heating Profile

GENERAL PROCEDURE

All peptides were synthesized on a Focus XC 2RV and/or a Focus XC 6RV from AAPPTec, LLC. Both instruments were equipped with heating and cooling capabilities. Each synthesis was performed on 100 mg of 0.5 mmol/g Rink amide resin (0.05 mmol scale). Fmoc-amino acids were freshly prepared as 0.2 M solutions in DMF. Diisopropylcarbodiimide (DIC) and OxymaPure were freshly prepared as 0.4 M solutions in DMF.

Fmoc de-protections were performed by adding 3 ml of 20% piperidine in DMF (v/v) containing 0.2 M OxymaPure to the resin. The OxymaPure was not added for room temperature reactions. The mixture was heated to 75 °C and mixed further for 1 minute. In difficult regions, the reaction time was extended to a total of 3 minutes. The liquid was drained and the resin was washed with DMF until the piperidine level was reduced to less than 0.02% piperidine (3 x 4 ml) which will react with 1% of activated amino acid available for the coupling. The wash procedure consisted of adding 4 ml of DMF to the resin and mixing at room temperature for 0.3 minute.
Standard couplings were performed by mixing 0.75 ml of the appropriate Fmoc protected amino acid solution (0.15 mmol, 3 equiv.), 0.375 ml of 0.4 M DIC solution (0.15 mmol, 3 equiv.) and 0.375 ml of
0.4 M OxymaPure solution (0.15 mmol, 3 equiv) in a pre-activation vessel for 30 seconds. Difficult couplings were performed using 5 equivalents of the appropriate Fmoc protected amino acid solution (1.25 ml,) activated with 0.625 ml of 0.4 M DIC solution (0.25 mmol, 5 equiv.) and 0.625 ml of 0.4 M OxymaPure solution (0.25 mmol, 5 equiv).

The solution of activated amino acid was added to the resin mixture and was mixed at room temperature for 2 minutes. The temperature was raised to 40 °C in one minute with mechanical mixing. The temperature was increased in 10-14 °C increments at 1 minute intervals until the final temperature of 75 °C was reached. The mixture was mixed at 75 °C -78 °C for 1 minute, then the resin was washed with DMF (3 x 4 ml). The same process was carried on through the entire synthesis.

The Fmoc protecting group was removed from the final amino acid in the peptide sequence following the deprotection procedure above. The resin was washed with ethanol (EtOH, 3 x 3 ml) and dried. The dried resin was treated with 3 ml of a cleavage cocktail containing trifluoroacetic acid (TFA), triisopropylsilane (TIS) and water (96:2:2 v/v/v). For peptides containing arginine residues, 0.5% thioanisole was added to the cleavage cocktail. The resin was mixed with the cleavage cocktail for 30 -90 minutes depending on the peptides. The mixture was filtered and the resin was rinsed with 1 ml of TFA. The combined filtrates were added to 5 ml of cold ether (0 °C). The precipitated crude peptide was centrifuged and the liquid decanted. The crude peptide was rinsed with cold ether (1 x 5 ml) and dried at room temperature.

The crude peptides were analyzed by HPLC (Figure 2) on a Spirit Peptide C-18 column (4.6 mm x 150 mm, 5 µm particle size). The samples were eluted with acetonitrile/water gradients. The HPLC analysis of the crude peptides is shown in Table 2. The identity of the crude peptide was confirmed by mass spectroscopy/HPLC.

Crude Peptide

Peptide	Purity (Room Temp)	Purity (Microwave)[f]	Purity (Gradient Heating)	Mass Spectrum
ACP(65-74)[a]	89.4%	93%	95.19%	$[M]^+$: 1062.22
JR 10-mer[b]	62.07%	67%	91.22%	$[M+Na]^+$: 1233.6
Thymosin α1[c]	68.87%	61%	77.62%	$[M]^+$: 3065.27
HIV tat[d]	78.91%		86.57%	$[M]^+$: 1620.986
ABRF 1992 Test Peptide[e]	93.07%	82%	96.13%	[M]+: 1626.808

Table 2 – Results of conventional heating with gradient to microwave heating and no heating. a Sequence: VQAAIDYING-NH2; b Sequence: WFTTLISTIM-NH2; c Sequence: SDAAVDTSSEITTKDLKEKKEVVEEAEN-NH2; d Sequence: GKKKRRQRRRPQ-NH2; e Sequence: GVRGDKGNPGWPGAPY-NH2; f JM Collins, KA Porter, SK Singh, GS Vanier, Org. Lett., 2014, 16, 940-3

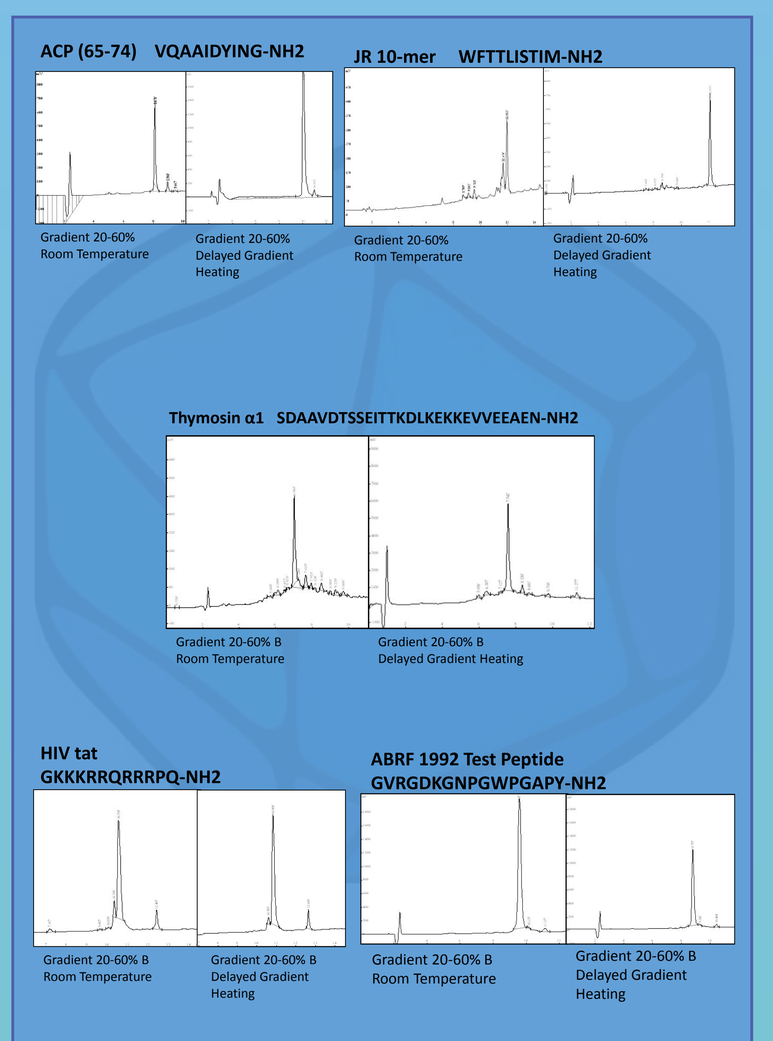

Fig. 2 – HPLC Profiles of ACP(65-74), JR 10-mer, Thymosin α1, HIV tat, ABRF 1992 Test Peptide Gradient: 5%B – 50%B, 20 min; A: H2O + 0.1% TFA; B: CH3CN + 0.1% TFA; Flow Rate: 1.0 ml/min; Detection: 220 nm; Column: C18 Peptide column from AAPPTec, 150 mm x 4.5 mm ID

CONCLUSIONS

We have examined solid phase peptide synthesis by rapid conventional heating, gradient conventional heating, delayed gradient conventional heating and microwave heating. Conventional heating with a gradient after 2-5 min reaction at room temperature provides much better peptide quality. Better control over the reaction time at room temperature provides better quality peptide because the majority of surface peptide resin is rapidly coupled at room temperature. Heating is only required for the pocket resin sites or aggregated peptides. Greater temperature control without overheating is resulting in higher quality peptides. The reactions go to completion, producing higher yields, and reduced impurities due to reduction of side reactions. Racemization of cysteine residues was reduced from 3.5% in microwave synthesis to less than 0.7% in delayed gradient temperature synthesis.

Peptide chains on the surface of the resin beads are exposed to the reaction mixture and react rapidly at room temperature or with mild heating. Diffusion and steric factors hinder the rate of reaction of chains within the pores of the resin and higher temperatures are required to accelerate the reactions. The increased temperature also increases the racemization rate of the amino acids. The rapid temperature increase incurred with microwave heating accelerates racemization of the amino acid as well as undesired reactions and the coupling reaction, allowing some racemized amino acid to be incorporated into the peptide resulting in diasteromeric impurities. With the slower temperature increase of gradient heating, the rapid reactions of the peptide chains on the resin surface are completed before the competing racemization reaction. Only when the few chains within the resin pores remain unreacted does the racemization of the amino acid become competitive. Hence, peptides synthesized with delayed gradient heating contain fewer diasteromeric impurities that peptides prepared with microwave heating.

Difficult sequences which in standard solid phase peptide synthesis require double coupling can be completed with a single coupling at high temperature. With gradient heating, cycle times average 10 to 15 minutes per cycle. Gradient heating greatly accelerated the synthesis of difficult peptides, increased the yield and slightly increased the purity of the crude peptides. One exception is the JR 10-mer, which went from 62% purity at room temperature to 91% purity when synthesized with gradient heating. We have observed that at higher temperatures, the synthesis time can be reduced, but the quality of the peptide is reduced as well. Conversely, at lower temperatures, the peptide quality is improved, but the synthesis time is longer. We have found that a 10 degree per minute gradient to 75°C to 78 °C with an initial 2-5 minute delay at room temperature generally produces an optimum compromise between peptide quality and synthesis speed.

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