◆◆ Scientific
◆◆ Research

Microwave Assisted Peptide Synthesis as a New Gold Standard in Solid Phase Peptide Synthesis: Phospholamban as an Example

Shadi Abu-Baker[1*], Philip Garber[1,2], Bryce Hina[1], Trevor Reed[1], Ghaffari Shahrokh[1], Mohannad Al-Saghir[1], Gary Lorigan[3]

[1]College of Arts and Sciences, Ohio University, Zanesville, USA
[2]Genesis Hospital, Ohio University, Zanesville, USA
[3]Department of Chemistry and Biochemistry, Miami University, Oxford, USA
Email: *abu@ohio.edu

ABSTRACT

In this study, we report the microwave assisted synthesis of Phospholamban protein (WT-PLB) as a new gold standard in solid phase peptide synthesis. Microwave energy offers benefits for both the coupling and deprotection reactions during peptide synthesis. The use of microwave energy for both the coupling and deprotection steps makes the microwave peptide synthesizers the most versatile and powerful systems available. It produces high yield and fast synthesis when compared to conventional peptide synthesizers.

KEYWORDS

1. Introduction

The use of microwave peptide synthesizers has several advantages over conventional peptide synthesizers. First, it allows the use of microwave energy for both the coupling and deprotection steps using fastest cycle times. Second, the peptide purity and the yield are comparable to conventional synthesis. Third, it significantly reduces purification time and waste. And finally, it allows the access to peptides impossible to synthesize under conventional conditions. In this study, we compared the synthesis of WT-Phospholamban (WT-PLB) using both the conventional and the microwave assisted solid-phase peptide synthesis. WT-PLB is a hydrophobic 52-amino acid transmembrane protein that is involved in regulating the contraction and relaxation of heart muscle [1-3]. Phosphorylation of PLB by cyclic AMP- and calmodulin-dependent kinases is believed to increase the rate of calcium re-uptake by the sacroplasmic reticulum and result in muscle relaxation [1-3]. The isolation and puri-

fication of large quantities of native PLB through molecular biology techniques have not yet been achieved due to difficulties encountered in the bacterial over expression of phospholambanc DNA [4,5]. Alternatively, WT-PLB has been prepared by chemical synthesis using standard solid-phase peptide synthesis and purification in organic solvents [6,7]. In addition, this approach gives the opportunity to synthesize site-specific isotopically labeled peptides and proteins [8-10]. The biochemical and biophysical comparison of synthetic PLB and native PLB revealed that they are both similar in size and functionally identical [6,7].

2. Materials and Methods

WT-PLB Synthesis and Purification

WT-PLB was synthesized using both the conventional solid-phase methods with an ABI 433A peptide synthesizer (Applied Biosystems, Foster city, CA) and with microwave assisted liberty peptide synthesizer (see **Figure 1**,

*Corresponding author.

Figure 1. New CEM liberty peptide synthesizer.

CEM liberty, Mathew, USA). During the conventional synthesis, we found that the coupling of Leu-7 to Thr-8 was difficult even after double coupling and extending the reaction time to six hours. However, this problem was solved by using the pseudoproline dipeptide of Fmoc-Leu-Thr ($\Psi^{Me,Me}$Pro)-OH from Novabiochem (San Diego, CA). The use of a pseudoproline dipeptide of Fmoc-Leu-Thr ($\Psi^{Me,Me}$Pro)-OH enhanced the yield (~25%, ~9 days of synthesis) after lyophylization. Using the similar protocol the microwave assisted solid-phase peptide synthesis an enhanced yield compared to of the conventional method (~35%, ~3 days of synthesis).This indicates that the microwave assisted method is preferable as it enhances the yield and reduce the synthesis time. To synthesize P-PLB, a pre-phosphorylated Fmoc-serine amino acid was used at amino acid position 16 instead of the regular Fmoc-serine used in the synthesis of PLB. The crude peptide was purified on an Amersham Pharmacia Biotech AKTA explorer 10S HPLC controlled by Unicorn (version 3) system software. The purified protein was lyophilized and characterized by matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry.

3. Results

3.1. Solid-Phase Peptide Synthesis of WT-PLB

The chemically synthesized form of the full length PLB (**Figure 2(A)**) and P-PLB (**Figure 2(B)**) was used for all of the solid-state NMR experiments. In general, solid-phase peptide synthesis (SPPS) starts with the C-terminal amino acid attached to a solid support (resin). Amino acids are then coupled one at a time till the N-terminus is reached. Each time an amino acid is added,

(A)H-Met1-Asp-Lys-Val-Gln-Tyr-Leu-Thr-Arg-Ser10-Ala-Ile-Arg-Arg-Ala-Ser-Thr-Ile-Glu-Met20-Pro-Gln-Gln-Ala-Arg-Gln-Asn-Leu-Gln-Asn30-Leu-Phe-Ile-Asn-Phe-Cys-Leu-Ile-Leu-Ile40-Cys-Leu-Leu-Leu-Ile-Cys-Ile-Ile-Val-Met50-Leu-Leu-OH

(B)H-Met1-Asp-Lys-Val-Gln-Tyr-Leu-Thr-Arg-Ser10-Ala-Ile-Arg-Arg-Ala-Ser(PO_3H_2)-Thr-Ile-Glu-Met20-Pro-Gln-Gln-Ala-Arg-Gln-Asn-Leu-Gln-Asn30-Leu-Phe-Ile-Asn-Phe-Cys-Leu-Ile-Leu-Ile40-Cys-Leu-Leu-Leu-Ile-Cys-Ile-Ile-Val-Met50-Leu-Leu-OH

Figure 2. **Primary sequence of (A) PLB; and (B) P-PLB. Sites of pseudoproline substitution are highlighted in red. P-Ser residue highlighted in blue was introduced using Fmoc-Ser (PO(OBzl)OH)-OH.**

the following three steps are repeated: First, deprotection of the N-terminal amino acid of the peptide bound to the resin (removal of the Fomc protecting group, see the aromatic part in **Figure 2**). This step is followed by activation and coupling of the next amino acid. And finally, the new N-terminal amino acid is deprotected [11].

To control the progress of the synthesis, the deprotection and coupling steps can be monitored using a UV detector. Several approaches including switching to different resins and activating reagents as well as using a pseudoproline dipeptide has been suggested to improve the yield of poor synthesis [12]. **Figure 2** shows the pseudoproline dipeptide Fmoc-Leu-Thr-(CMe,Mepro)-OH in red. In this dipeptide, the Thr residue has been reversibly protected as proline-like TFA-labile oxazolidine [12].

WT-PLB was synthesized using two peptide synthesizers using a procedure developed in the Lorigan's lab. Briefly, WT-PLB was synthesized using modified Fmoc-based solid-phase methods with an ABI 433A peptide synthesizer (Applied Biosystems, Foster city, CA) and the microwave assisted liberty peptide synthesizer (CEM liberty, Mathew, USA). WT-PLB is very hydrophobic; thus, the synthesis of this peptide is very challenging. Nevertheless, by using a combination of extended coupling and deprotection protocols with a single pseudoproline dipeptide substitution, we were able to obtain both purified PLB and P-PLB in a yield of ~25% in ~9 days using the conventional synthesis and 35% in ~3 days using the microwave assisted synthesis. Couplings were performed using 10-fold excess of Fmoc-amino acids activated with HBTU/DIPEA. The synthesizer was programmed to use conditional UV feedback monitoring; coupling and deprotection reactions are extended automatically, and a capping step introduced after the coupling step, based on the kinetic profile of the Fmoc deprotection reaction. The results of the the two methods of peptide synthesis is summarized in **Table 1**.

All peptides were cleaved from the resin by treatment with TFA/EDT/thioanisole/water (10:0.5:0.25:0.5) for 2.5 h, and isolated by centrifugation followed by precipi-

Table 1. Comparing the conventional and microwave assisted solid-phase peptide synthsis for PLB.

Method	Method
Convectional	Microwave assisted
~25% yield	~35% yield
~9 days	~3 days

tation with methyl *t-butyl* ether. PLB consists of a hydrophilic N-terminus (residues 1 - 20), a hinge region (residues 21 - 30) and a hydrophobic α-helical transmembrane tail (residues 31 - 52) [1]. From previous work by Lorigan and co-workers [13], it is known that the synthesis of the C-terminal transmembrane region of PLB is extremely difficult, particularly the region from Cys36 to Cys45. To overcome these difficulties, the Lorigan group developed a strategy involving extended double coupling together with capping and conditional repetition of the Fmoc deprotection reaction [13]. Using this approach, PLB (24 - 52) segment was obtained in a purified yield of 37% [13].

Initially, we attempted the synthesis of full length PLB with standard amino-acid building blocks using the protocols previously described [13]. A PEG-PS resin (0.22 mmol/g) was selected as the solid support to reduce steric crowding and aggregation during chain assembly. Using the conditional feedback monitoring, this synthesis was completed in 9 days, as compared to 10 days for the shorter PLB (residues 24 - 52) prepared on polystyrene resin [13]. And now using the microwave assisted method the synthesis is completed in about three days only.

UV monitoring of the Fmoc deprotection reactions indicated that the peptide assembly proceeded smoothly until Leu-7. However, following introduction of this residue, there was a marked decrease in the height of the Fmoc deprotection peak, indicating difficulties in the coupling of Leu-7 to Thr-8. Attempts to improve this coupling by double coupling or extending the reaction time to 6 hours had little effect. In view of the problems with the coupling of Leu-7 to Thr-8, the synthesis was repeated in exactly the same manner, except that Leu-7 and Thr-8 were introduced simultaneously using the pseudoproline dipeptide Fmoc-Leu-Thr (CMe, Mepro)-OH In the presence of this dipeptide, UV monitoring of the Fmoc deprotection reactions indicated that the peptide assembly proceeded reasonably smoothly until the end of this synthesis. Similar patterns were seen in both conventional and microwave assisted synthesis.

3.2. HPLC Purification of WT-PLB

Following global deprotection and cleavage of the peptide from the resin, PLB was purified by preparative reverse phase chromatography on a C4 column eluted with a gradient formed between 0.1% TFA in nanopure water (solvent A) and MeCN/isopropyl alcohol/water/TFA (38:57:5:0.1) (Solvent B). After lyophilization and using standard Fmoc-amino acid building blocks, the purified peptide was obtained in a yield of only 9% based on initial resin substitution. Conversely, with the dipeptide and using the conventional peptide synthesizer, the purified PLB was obtained in a yield of ~25%, nearly a 3-fold increase when compared to the synthesis using standard building blocks. And finally, with the dipeptide and using the microwave assisted peptide synthesizer, the purified PLB was obtained in a yield of ~35%, nearly 1.5 folds increase when compared to the conventional method.

3.3. Characterization of WT-PLB Using MALDI-TOF

When the dipeptide was used to synthesize WT-PLB, a correct mass of 6080 MU was obtained after the purification step. Conversely, when the dipeptide was not used, MALDI-TOF indicated the presence of an impurity with a mass of 5144 MU, which could be ascribed to Ac-PLB (residues 9 - 52).

4. Conclusion

The use of microwave peptide synthesizers has several advantages over conventional peptide synthesizers as it increases the peptide purity, yield and reduces the time of synthesis when compared to conventional synthesis. PLB was used as an example to emphasize the efficiency of the microwave assisted synthesis over the conventional synthesis and it showed better yields (1.5 fold increase) and faster synthesis(reduce the time from 9 to 3 days).

REFERENCES

[1] H. K. B. Simmerman and L. R. Jones, "Phospholamban: Protein Structure, Mechanism of Action, and Role in Cardiac Function," *Physiological Reviews*, Vol. 78, 1998, pp. 921-947.

[2] P. James, M. Inui, M. Tada, M. Chiesi and E. Carafoli, "Nature and Site of Phospholamban Regulation of the Calcium Pump of Sarcoplasmic Reticulum," *Nature*, Vol. 342, 1989, pp. 90-92. http://dx.doi.org/10.1038/342090a0

[3] M. A. Kirchberger, M. Tada and A. M. Katz, "Phospholamban a Regulatory Protein of the Cardiac Sarcoplasmic Reticulum," *Recent Advances in Studies on Cardiac Structure and Metabolism*, Vol. 5, 1975, pp. 103-115.

[4] J. Fuji, A. Ueno, K. Kitano, S. Tanaka, M. Kadoma and M. Tada, "Complete Complementary DNA-Derived Amino Acid Sequence of Canine Cardiac Phospholamban," *Journal of Clinical Investigation*, Vol. 79, 1987, pp. 301-304. http://dx.doi.org/10.1172/JCI112799

[5] Q. Yao, J. L. Bevan, R. F. Weaver and D. J. Bigelow, "Purification of Porcine Phospholamban Expressed in

Escherichia coli," *Protein Expression and Purification*, Vol. 8, 1996, pp. 463-468. http://dx.doi.org/10.1006/prep.1996.0125

[6]   J. G. Collins, E. G. Kranias, A. S. Reeves, L. M. Bilezikjian and A. Schwartz, "Isolation of Phospholamban and a Second Proteolipid Component from Canine Cardiac Sarcoplasmic Reticulum," *Biochemical and Biophysical Research Communications*, Vol. 99, 1981, pp. 796-803. http://dx.doi.org/10.1016/0006-291X(81)91235-3

[7]   E. J. Mayer, E. McKenna, V. M. Garsky, C. J. Burke, H. Mach, C. R. Middaugh, M. Sardana, J. S. Smith and R. G. Johnson Jr., "Biochemical and Biophysical Comparison of Native and Chemically Synthesized Phospholamban and a Monomeric Phospholamban," *The Journal of Biological Chemistry*, Vol. 271, 1996, pp. 1669-1677. http://dx.doi.org/10.1074/jbc.271.3.1669

[8]   D. J. Hirsh, J. Hammer, W. L. Maloy, J. Blazyk and J. Schaefer, "Secondary Structure and Location of a Magainin Analogue in Synthetic Phospholipid Bilayers," *Biochemistry*, Vol. 35, 1996, pp. 12733-12741. http://dx.doi.org/10.1021/bi961468a

[9]   A. Mascioni, C. Karim, J. Zamoon, D. D. Thomas and G.

Veglia, "Solid-state NMR and rigid body molecular dynamics to determine domain orientations of monomeric phospholamban," *Journal of the American Chemical Society*, Vol. 124, 2002, pp. 9392-9393. http://dx.doi.org/10.1021/ja026507m

[10] E. K. Tiburu, P. C. Dave, K. Damodaran and G. A. Lorigan, "Investigating Leucine Side-Chain Dynamics and Backbone Conformations of Phospholamban Incorporated in Phospholipid Bilayers Utilizing ^{2}H and ^{15}N Solid-State NMR Spectroscopy," *Biochemistry*, Vol. 43, 2004, pp. 13899-13909. http://dx.doi.org/10.1021/bi0490993

[11] http://brf.jcs.anu.edu.au/services/Peptidesynthesis/spps.html

[12] "Derivatives for Enhanced Peptide Synthesis," Novabiochem 2004/2005 Catalog, pp 185-188.

[13] E. K. Tiburu, P. C. Dave, J. F. Vanlerberghe, T. B. Cardon, R. E. Minto and G. A. Lorigan, "An Improved Synthetic and Purification Procedure for the Hydrophobic Segment of the Transmembrane Peptide Phospholamban," *Analytical Biochemistry*, Vol. 318, 2003, pp. 146-151. http://dx.doi.org/10.1016/S0003-2697(03)00141-6