Fast Conventional Synthesis of Human β-Amyloid (1-42) on the Symphony® and Prelude®

Protein Technologies, Inc.

Abstract

Human β-amyloid (1-42) peptide was synthesized on the Symphony® and Prelude® peptide synthesizers from Protein Technologies, Inc. Reaction and washing times were reduced, and the effect of different activators and resins on the purity of the crude peptides were compared. The synthesis time of the β-amyloid (1-42) peptide was reduced from 54 hours to 12 hours. The peptide was verified by mass spectrometry and successfully isolated.

> H$_2$N-DAEFRHDSGY EVHHQKLVFF
> AEDVGSNKGA IIGLMVGGVV IA-COOH

Figure 1: Sequence of β-amyloid.

Introduction

Human β-amyloid (1-42) peptide is a major component of the plaque deposits found in the brains of Alzheimer's disease patients [1]. Synthesis of the β-amyloid (1-42) peptide by conventional solid phase peptide synthesis has been reported to be difficult due to the high hydrophobicity of the C-terminal segment and subsequent on-resin aggregation [2]. The conservative approach to solid phase peptide synthesis typically uses coupling times from 20 min to one hour or more, deprotection times of 15 min with added repetitions, a minimum of 6 washes in between deprotection and coupling steps, as well as Kaiser tests to insure complete acylations. This increases the cycle time, and depending on the peptide length may extend the synthesis time to days. This approach has served many scientists well.

However, the increased demand for peptides has many researchers looking for different ways to do faster syntheses that may provide a faster turnout of peptides and produce them with a reasonable quality such that after purification the pure peptides are obtained. This has been a goal for many researchers and during the years, different ways of chemically speeding up the synthesis reactions have been proposed [3]. Miranda and Alewood indicated that the choice of activator agent plays an important role in the reduction of synthesis times because the rate of amino acid acylation either in solution or on a solid support is heavily dependent on the properties of the coupling reagents [4]. Earlier, kinetic studies by Hetnarski and Merrifield showed that diffusion was not a rate-limiting step for the coupling reaction [5]. The choice of deprotection reagent may also affect the synthesis time. While Atherton and Sheppard reported that piperidine removes Fmoc groups very quickly [6], Wade reported that DBU is an even more "rapid and efficient cleavage agent" for Fmoc removal [7]. The choice of resin materials and loadings is important as well for long or hindered peptide sequences [2b]. Consequently, the choice of the appropriate coupling and deprotectant reagents linked to the correct resin may provide the opportunity to reduce the total synthesis time of a peptide.

In a previous study at Protein Technologies Inc., the synthesis time of [65-74]ACP peptide was reduced from 8.4 hours to 1.9 hours by changing the chemistry [8]. In this study, the goal was to synthesize the β-amyloid (1-42) peptide using HCTU and HATU as activators with conservative and reduced synthesis times, and observe the effects on the purity of the crude peptide. Wang-ChemMatrix, HMPB-ChemMatrix, and Wang-PS-LL were compared as resins, and 2% DBU was added to the 20% piperidine deprotection solution, as it has been shown to be "beneficial in cases of slow, hindered Fmoc group removal in so-called 'difficult' syntheses" [9] and also because our internal studies showed that a mixture of the two bases allowed shorter deprotection times than either base alone (unpublished results).

Experimental

Solid-Phase Synthesis: The Symphony® and Prelude® peptide synthesizers from Protein Technologies, Inc. provided an excellent way to simultaneously synthesize this peptide using different variables. The scale of syntheses was 25 and 20 µmol on the Symphony® and Prelude®, respectively, using a 10-fold excess of Fmoc-amino acids with respect to the resins. Deprotection was performed using 2% DBU/20% Piperidine in DMF. Coupling was achieved using 1:1:2 amino acid/Activator/NMM in DMF. Capping was performed with 1:1:3 acetic anhydride/DIPEA/DMF. Cleavage was performed by treating the resin with 95/2/2/1 TFA/anisole/water/EDT for 2 hours. The side chain protecting groups utilized included tBu (t-butyl) for aspartic acid, glutamic acid, serine, and tyrosine; Trt (trityl) for asparagine, glutamine, and histidine; Pbf (2,2,4,67-pentamethyldihydrobenzofuran-5-sulfonyl) for arginine, and Boc (butyloxycarbonyl) for lysine. 1H-Benzotriazolium 1-[bis(dimethylamino) methylene]-5chloro-hexafluorophosphate (HCTU) was provided by Protein Technologies, Inc., (1-),3-oxide 2-(1H-7-Azabenzotriazol-1-yl)-1,1,3,3-tetramethyl uronium hexafluorophosphate (HATU) was purchased from Genscript, and 6-chloro-1-hydroxybenzotriazole (6-Cl-HOBt) was purchased from Lee BioSolutions, Inc. Fmoc-Ala-Wang-ChemMatrix resin (0.56 mmol/g) was purchased from and Fmoc-Ala-HMPB-ChemMatrix resin (0.54 mmol/g) was provided by Matrix Innovation. Fmoc-Ala-Wang-PS-LL (0.25 mmol/g) was purchased from Novabiochem.

Peptide Analysis: Crude peptides were precipitated with anhydrous ether, dissolved in HFIP (1,1,1,3,3,3-Hexafluoro 2-propanol) and analyzed using a Varian ProStar HPLC, equipped with a C8, 300 Å, 5 µm, 250 x 4.6 mm column (Varian Microsorb-MV), heated at 60°C, over 60 minutes with a flow of 1 mL/min and using a gradient of 5-95% B where Buffer A was 0.1% TFA in water and Buffer B was 0.1% TFA in acetonitrile. Detection was at 214 nm. Electrospray ionization and MALDI-TOF mass spectrometry (Voyager-DE™ Biospectrometry™ workstation from PerSeptive Biosystem) were utilized to verify the structure of the peptides.

Results

β-amyloid (1-42) was synthesized on Fmoc-Ala-Wang-ChemMatrix resin, Fmoc-Ala-HMPB-ChemMatrix resin, or Fmoc-Ala-Wang-PS-LL resin using a modified protocol from García-Martín [10] for the synthesis of β-amyloid (1-42) (Table 1, Protocol 1) or PTI's standard quality control protocol (Table 1, Protocol 2). Reaction and washing times were then minimized (Table 1, Protocol 3). The effects of the different activators and resins were compared (Figures 1-3).

Table 1: Reaction, washing, cycle and synthesis times for the different synthesis protocols.

	PROTOCOL 1	PROTOCOL 2	PROTOCOL 3
Deprotection Time:	1 x 15 min	2 x 2.5 min	2 x 1 min
Coupling Time:	1 x 35 min	2 x 10 min	1 x 5 min
Capping Time:	1 x 5 min	None	None
Washing Time:	6 x 30 sec	6 x 30 sec	2 x 30 sec
Cycle Time:	1.3 hours	44 min	18 min
Total Synthesis Time:	54 hours	30 hours	12 hours

For each resin, purer crude peptide was obtained at the shorter synthesis times (Figures 1-3). A possible explanation for this result could be that fewer side reactions are occurring at the reduced reaction times. Although the 54 hour syntheses were performed with capping, it was shown that the HPLC's of peptides synthesized with and without capping using Protocol 3 showed no differences (data not shown).

Peptides coupled with HCTU/6-Cl-HOBt came out only slightly less pure than those coupled with HATU (Figures 1 & 2), and in the case of the Wang-PS-LL resin were highly comparable (Figure 3).
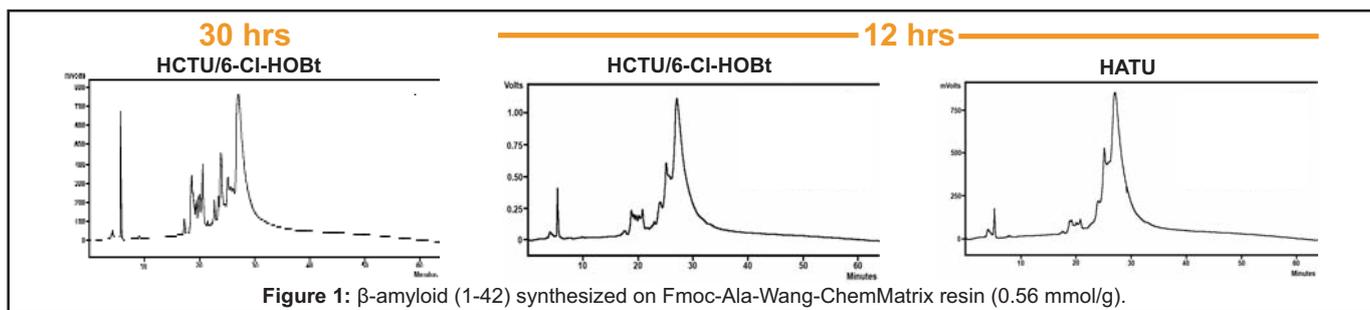
30 hrs ———— **12 hrs** ————

HCTU/6-Cl-HOBt   HCTU/6-Cl-HOBt   HATU

Figure 1: β-amyloid (1-42) synthesized on Fmoc-Ala-Wang-ChemMatrix resin (0.56 mmol/g).

54 hrs ———— **12 hrs** ————

HCTU   HCTU/6-Cl-HOBt   HATU
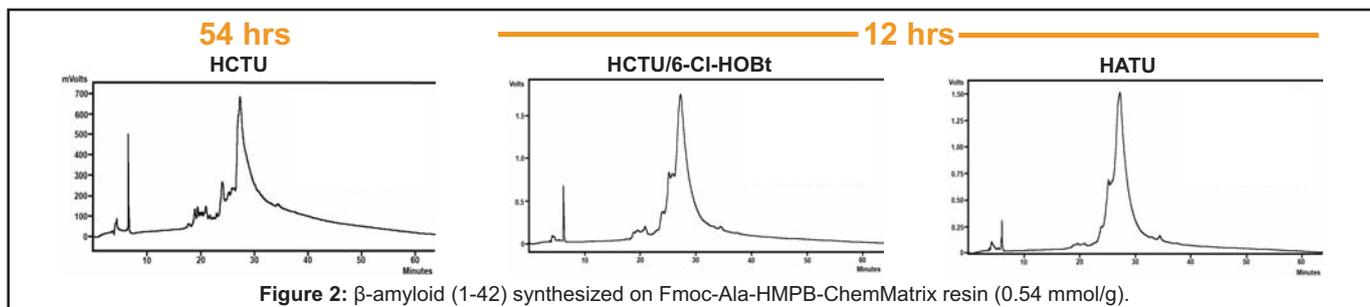
Figure 2: β-amyloid (1-42) synthesized on Fmoc-Ala-HMPB-ChemMatrix resin (0.54 mmol/g).

54 hrs ———— **12 hrs** ————
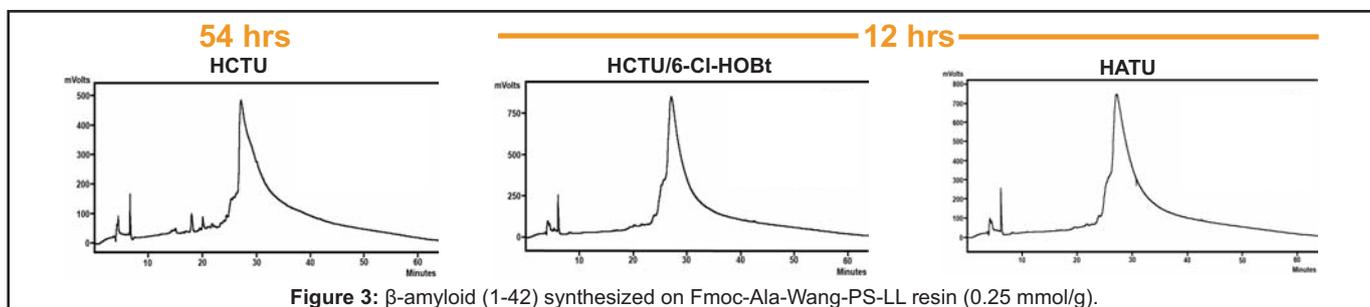
HCTU   HCTU/6-Cl-HOBt   HATU

Figure 3: β-amyloid (1-42) synthesized on Fmoc-Ala-Wang-PS-LL resin (0.25 mmol/g).

It was also found that the additive 6-Cl-HOBt provided no significant benefit (data not shown). HCTU may therefore be a good substitute for HATU when cost is an issue by providing peptides of comparable or only slightly lower purity than HATU with significant cost savings.

The HMPB linker in general produced a purer peptide than the Wang linker on ChemMatrix resin with the highest purity peptide obtained at 12 hours with HATU (Figures 1 & 2). Wang-PS-LL resin was able to produce a crude peptide of comparable purity at 12 hours with the less costly coupling reagent HCTU (Figure 3), although the HPLC product peak was slightly broadened. However, the higher purity with HCTU was due to the low substitution of the polystyrene resin rather than the material itself. When Fmoc-Ala-Wang-polystyrene resin with a loading of 0.56 mmol/g was used, the peptide purity was worse than the similarly substituted ChemMatrix resin (data not shown). The polystyrene resin produced a higher yield than ChemMatrix resins (69% vs. 25-27%, respectively).

The identity of each peptide was verified by either ESI or MALDI-TOF mass spectroscopy. Isolation of the major product peak by HPLC and subsequent analysis by MALDI-TOF mass spectroscopy (4510.86 m/z) identified it as the full-length β-amyloid peptide (Figure 4).

Conclusions

Human β-amyloid (1-42) peptide was successfully synthesized on the Symphony® and Prelude® peptide synthesizers in 12 hours. HCTU (with or without 6-Cl-HOBt) produced peptides of comparable or slightly lower purity compared to HATU, but with significant cost savings. HMPB-ChemMatrix resin with HATU produced the highest purity crude peptide, however, Wang-PS-LL resin with HCTU produced crude peptide of comparable purity at a lower cost, and polystyrene resin produced higher crude peptide yields than ChemMatrix resins.
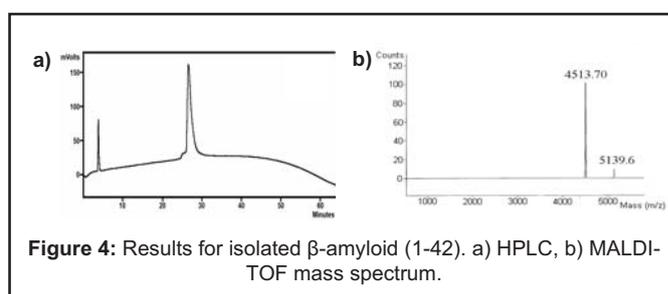

Figure 4: Results for isolated β-amyloid (1-42). a) HPLC, b) MALDI-TOF mass spectrum.

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