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Improved chemical synthesis of hydrophobic A β peptides using addition of C-terminal lysines later removed by carboxypeptidase B

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Abstract

Many amyloidogenic peptides are highly hydrophobic, introducing significant challenges to obtaining high quality peptides by chemical synthesis. For example, while good yield and purity can be obtained in the solid phase synthesis of the Alzheimer's plaque peptide A β ₄₀, addition of a C-terminal Ile-Ala sequence to generate the more toxic A β ₄₂ molecule creates a much more difficult synthesis resulting in low yields and purities. We describe here a new method that significantly improves the Fmoc solid phase synthesis of A β peptides. In our method, Lys residues are linked to the desired peptide's C-terminus through standard peptide bonds during the synthesis. These Lys residues are then removed post-purification using immobilized carboxypeptidase B. With this method we obtained both A β ₄₂ and A β ₄₆ of superior quality that, for A β ₄₂, rivals that obtained by recombinant expression. Intriguingly, the method appears to provide independent beneficial effects on both the total synthetic yield and on purification yield and final purity. Reversible Lys addition with carboxypeptidase B removal should be a generally useful method for making hydrophobic peptides that is applicable to any sequence not ending in Arg or Lys. As expected from the additional hydrophobicity of A β ₄₆, which is extended from the sequence A β ₄₂ by a C-terminal Thr-Val-Ile-Val sequence, this peptide makes typical amyloid at rates significantly faster than for A β ₄₂ or A β ₄₀. The enhanced amyloidogenicity of A β ₄₆ suggests that, even though it is present in relatively low amounts in the human brain, it could play a significant role in helping to initiate A β amyloid formation.

INDEXING – KEY WORDS AND PHRASES

Solid phase peptide synthesis; Hydrophobic; Lysine; Carboxypeptidase B; Immobilized enzyme; Amyloid beta; Yield; Purity; High performance liquid chromatography; Purification; Amyloid; β -sheet; Recombinant

INTRODUCTION

Solid-phase peptide synthesis of 'difficult sequence'-containing peptides is one of the most problematic fields in synthetic chemistry^{1–3}. These peptides often result in low yields and limited purities^{2,4–6}. Some examples are the amyloid forming peptides like amyloid- β

(A β)², α -synuclein^{7,8}, and acyl carrier protein (ACP)_{65–74}⁹, and some hydrophobic trans-membrane segments of proteins, like glycoporphin A, epidermal growth factor (EGFR) and M2 ion channel¹⁰. The hydrophobic stretches in these peptides appear to promote aggregation during synthesis and purification (see below). For example, the A β ₄₂ molecule associated with Alzheimer's disease (AD) contains a 13 amino acid segment at its C-terminus which is highly hydrophobic and has a high propensity to aggregate into β -sheet structures¹¹.

Various hypotheses invoking self-associated forms of A β peptides as playing a critical role in the etiology of Alzheimer's disease (AD) continue to inspire great interest and discussion^{12–14}, and a strong focus has been on understanding the biophysics and self-association products of the naturally occurring A β peptides responsible for oligomer and amyloid plaque formation in this disease¹⁵. This has placed a premium on the ability to obtain high quality A β peptides and their analogs for structural, self-assembly and toxicity studies. For example, longer versions of A β , like A β ₄₃¹⁶ and A β ₄₆¹⁷ are known to be generated in the brain, but have received little attention from biophysical and structural chemists, in part because of the perceived synthetic challenge in making these peptides and their analogs.

Aggregation during solid phase synthesis is attributed to intermolecular hydrophobic interactions between resin-bound peptide chains, resulting in formation of β -extended secondary structures during synthesis^{18–20}. This aggregation of the growing peptide chain on the resin can result in poor solvation and steric hindrance of the growing N-terminus leading to a decrease in the extents of addition and/or de-protection in each cycle and hence to incomplete repetitive coupling yields²¹. The β -aggregation of these hydrophobic, resin-bound peptides has been characterized by various techniques, including ssNMR¹⁹, FTIR²² and Raman spectroscopy²³. This aggregation is clearly a serious problem in solid-phase synthesis since amino acid deletion sequences of varying length and composition accumulate during the synthesis and are difficult to separate from the target peptide. The resulting decrease in the purity of the crude peptide, as well as the intrinsically poor solubility of these hydrophobic sequences, also often results in subsequent difficulties in purification⁶. These sometimes hidden impurities can have consequences for the properties of the synthetic product that are significantly amplified beyond what might be expected based solely on their intrinsic purity level. For example, it has been shown previously that even small amounts of certain impurities in synthetic A β peptide can radically reduce A β aggregation and neurotoxicity²⁴.

Several attempts have been made to improve the Fmoc solid phase synthesis of such peptides. Some of the approaches include the use of solvent additives to provide optimal coupling agents, like anisole to prevent aggregation^{25–28}, improved de-protection agents to aid in C-terminal synthesis²⁹, and the use of better coupling reagents, like HATU^{25–27} and other organic solvent additives which prevent aggregation during synthesis^{28,30,31}. Other techniques that have been explored include microwave assisted synthesis^{32,33} and the use of a more hydrophilic PEG based copolymer resin³⁴. Some approaches specifically target the peptide backbone. These include photo-triggered interruption of amide bond formation with ester bonds (o-acyl isopeptide)^{35–37}, introduction of pseudoproline, which acts as a β -sheet

breaker to prevent aggregation during coupling³⁸, and the use of other chemical moieties to act as backbone protecting intermediates^{4,39–41}. Most of these techniques are useful in producing higher yields of hydrophobic peptides but are also, to varying degrees, cumbersome and expensive to undertake on a routine basis.

Another approach is to alter or add side chains that might reduce aggregation on the resin and/or during purification, in such a way that the modification can be reversed after purification. For example, oxidation of Met35 was employed to suppress aggregation of resin bound A β ₄₂ during synthesis, followed by reduction after purification⁴². Reversible solubilization has also been accomplished by adding, during the synthesis, a cationic C-terminus separated from the peptide of interest by a labile non-peptide bond, which can either be removed to directly generate the peptide of interest^{43,44}, or can be incorporated into a fragment condensation approach to make longer polypeptides^{45–47}. Data on the efficiencies of cationic tail removal are generally not reported, however, making it difficult to assess the efficacy of this method. In some cases, the reversible cationic tail approach was determined to not be beneficial due to relatively low yields⁴⁸.

In this paper we introduce an alternative strategy for the reversible cationic tail approach in an effort to improve the synthesis of sequence variants of the Alzheimer's plaque peptide A β . The method involves direct addition of C-terminal Lys residues (instead of the previously used Arg residues) during solid phase synthesis through only normal peptide bonds (instead of the previously used non-peptidyl linkage), and subsequent removal of the Lys tail, after purification, using carboxypeptidase B (CPB) immobilized onto agarose beads. We show that the method provides superior yields and purities of the human brain peptides A β ₄₂ and A β ₄₆, doing so by making separate beneficial contributions to both total synthetic yield and to purification efficiency. We also provide preliminary amyloid formation data on the little investigated A β ₄₆ peptide that are consistent with a possible role in amyloid plaque formation and Alzheimer's disease etiology¹³.

MATERIALS AND METHODS

Materials and standard methods

Wild type A β ₄₂ and A β ₄₆ peptides without or with 2–6 C-terminal lysines (Table 1) were synthesized at the Small Scale Synthesis facility at the Keck Biotechnology Resource Laboratory of Yale University. Peptides were synthesized using Fmoc chemistry on a PTI Symphony peptide synthesizer. All amino acids were double coupled with a 4 fold amino acid excess using HBTU activator in 0.4M NMM (N-methylmorpholine) in DMF. The resins used were Life Technologies Peg-PS pre-loaded low load resins carrying the appropriate C-terminal amino acid. Recombinant A β ₄₂ was purchased from rPeptide.

Crude peptides were purified using a preparative reversed phase HPLC column (9.4 x 250 mm Agilent Zorbax SB-C3 (5 μ m particle size)) eluted with a 30–60% gradient of acetonitrile in water, with 0.05 % trifluoroacetic acid (TFA). Crude synthetic product (1–10 mg) was dissolved in 4 ml 50% aqueous formic acid with sonication for 2 min in a bath sonicator, followed by immediate injection onto the chromatography column to avoid formylation of the peptide. Purifications were carried out either at room temperature or at

65°C (Sidewinder LC column heater, Restek Corp.). The fractions obtained were checked for purity using an electrospray mass spectrometer (MS) and the pure fractions were pooled.

Peptide concentrations were determined by integration of A₂₁₄ peaks in analytical HPLC on an Agilent XDB-C18 4.6 x 50 mm column (1.8 µm particle size), applying standard curves of peak area vs. amount injected that were separately constructed for each chemically different peptide⁴⁹. Peptide concentrations in the stock solutions used to generate the standard curves were determined from the calculated UV extinction coefficient at 214 nm as described⁵⁰. Electron microscopy and Fourier transform infrared spectroscopy were performed as described previously⁵⁰.

Preparation of CPB-agarose

CPB, pre-treated with PMSF to remove any contaminating serine protease activity, was purchased from Worthington Biochemicals (Cat. No. LS001724 (Carboxypeptidase B, PMSF treated). Very High Density Glyoxal Beads (6BCL), containing 6% agarose beads conjugated with aldehyde groups reactive with protein primary amino groups, were obtained from Agarose Bead Technologies. Crosslinking of CPB to the glyoxal beads was performed according to the procedure previously described by Blanco et al.⁵¹. Approximately 10 mg CPB was dissolved in 10 ml of 0.1 M sodium bicarbonate buffer at pH 10.0. The ligand solution was then mixed with 2 ml Glyoxal Agarose Beads and stirred gently for 5–6 hrs at 4 °C until the enzyme activity measured in removed aliquots of beads remained constant, as monitored using the Hip-I-Arg assay. Then, 20 mg of sodium borohydride was added to the suspension and stirred for 30 min at room temperature. After reaction the CPB-agarose beads were loaded onto a column (one ml bed Bio-Rad Econo-column) and washed with 10 ml of pH 7.0 phosphate. The column was then further washed with Tris.HCl buffered saline (TBS), pH 9.0, followed by the same buffer containing 20% ethanol. The columns were stored at 4 °C.

Calculation of percent purity using LC-MS

Purity of Aβ preparations was assessed by electrospray mass spectrometry. Raw data of ion current vs. mass/z was transferred to Excel and plotted. Working in a mass/z region not occupied by significant peaks, a baseline was drawn through the mass/z noise. Purity was then calculated using the peak cluster with the highest charge state (for example, for Aβ₄₂ we used the +5 state) and the ion current values normalized to 100% for the highest peak (in all cases which corresponded to the desired peptide). All other peaks with a normalized amplitude of >1% were included in the analysis. All peaks were integrated and their areas combined and used as the denominator in the % purity calculation. Areas of small peaks (1–3%) with masses consistent with Aβ associated with one or two sodium counter-ions were added to the area of the major Aβ peak to obtain the total peak area associated with desired product.

Calculation of total synthetic yields

In order to determine an absolute yield that was not influenced by a peptide's relative efficiency of purification, we devised a method capable of accounting for all of the correct synthetic product generated. A small weighed amount of crude synthetic product was

solubilized in 50 % aqueous formic acid and immediately injected onto the preparative C3 HPLC column and subjected to purification at 65 °C in a gradient of acetonitrile in water with 0.05% TFA. Fractions were collected and analyzed individually by LC/MS on an analytical HPLC column at 25 °C. For those fractions exhibiting a peak at the expected retention time for the peptide of interest, the A₂₁₄ peak was integrated and the µg of total Aβ-like peptide calculated from a standard curve⁴⁹. From the mass spectrometry analysis of the peak we calculated the percent purity of the fraction. This percent purity was multiplied times the total µg of peptides in the peak to determine the µg of correct peptide. This analysis was conducted on all relevant fractions near the main elution position of the desired peptide. The plot of the content of desired peptide in each fraction gave a good overview of the efficiency of the purification, and the total µg of desired peptide in all the fractions gave the total synthetic yield.

Calculation of percent purity using 1H-NMR

In some cases purity was also assessed by integration of NMR spectra using the published procedure of Zagorski². Thus, proton-NMR spectra (900 MHz) of purified pools of Aβ₄₂ peptides are obtained in TFA-d solution (0.5ml). Peaks corresponding to the His-2H, aromatic and αH protons were integrated. Integrals were scaled to the three His-2H protons at 8.56 ppm and the total α-H protons relative to the His-2H protons were calculated. This value was related to the theoretical number of α-H protons to calculate purity².

Optimized method to remove C-terminal Lys residues

The standard protocol is summarized in Figure 1. The CPB-agarose column described above was equilibrated to room temperature and washed with at least five column volumes of TBS. An HPLC purification pool (adjusted to pH 7.5) containing purified Aβ-Lys tail peptide (typically 2–3 ml of ~ 1 mg/ml) was flowed over the column bed at approximately 0.2 ml/min, adjusting the flow rate with the column stopcock. The column was then washed with 2 ml TBS, pH 9.0. One ml fractions from both the peptide load and wash were collected. Fractions were analyzed by LC-MS to identify peptide-containing fractions and confirm complete removal of Lys residues. If Lys removal was found to be incomplete (in our experience using the conditions described here, this only happened with the K₆ tail peptide), the column flow-through was passed through the column again. Peptide containing fractions were pooled and the pool adjusted to pH 2.0, loaded onto the RP-HPLC column, and repurified using the standard gradient. Fractions were checked for purity, pooled, and lyophilized. After use, columns were washed with 20% ethanol in TBS and stored at 4 °C.

Aggregation kinetics of Aβ peptides

In determining spontaneous amyloid assembly kinetics it is very important that the starting monomer solution be freshly disaggregated to remove any trace of pre-existing amyloid that might influence assembly kinetics by seeding effects⁵². In the present study, peptides were disaggregated using the general approach of isolating fresh monomer by size exclusion chromatography (SEC) from peptide freshly dissolved in a denaturing solvent⁵³. SEC was carried out on a Superdex 200 10/300column (GE Health Sciences) equilibrated in PBS on an Agilent 1200 isocratic HPLC system with a flow rate of 0.5 ml/min and elution monitored at 215 nm. Purified, lyophilized peptides were dissolved in 6 M Gdn-HCl,

sonicated briefly (probe sonicator), injected into the SEC column, and the fractions collected. Separate peaks were observed eluting at the positions of oligomers and monomers. The monomer fractions were immediately analyzed for A β concentration⁴⁹, pooled, adjusted to 10 μ M by adding PBS, and incubated at 37°C. Samples were removed from reactions at various time-points and analyzed for the concentrations of monomeric A β left in the reaction as described previously⁴⁹.

RESULTS

General optimization for removal of lysines by immobilized CPB

Using Very High Density Glyoxal Beads we obtained 50–60% immobilization of CPB, in line with previous results reported for immobilization of carboxypeptidase A (CPA)⁵⁴. We chose to work with beads containing high densities of immobilized CPB to maximize efficiency of Lys removal and hence minimize the chance of losing product through aggregation after the Lys residues were removed. We found that some commercial preparations of CPB contained levels of contaminating enzymes like trypsin, chymotrypsin and CPA that, if not eliminated, resulted in decreased yields and introduction of new impurity fragments of the desired peptide. When these contaminating enzymes were present, we were not able to completely eliminate them using ion exchange chromatography (data not shown). Although trypsin and chymotrypsin levels could be eliminated by treatment of the immobilized enzyme with PMSF (data not shown), contaminating CPA cannot be inactivated in this way. We obtained better success using more highly purified CPB that was pre-treated by the manufacturer (Worthington) with PMSF. Immobilized CPB made with this product was free of measureable CPA levels and required no further PMSF treatment over multiple uses of the same column. We tested for low levels of contaminating enzyme that might introduce fragmentation during particularly long reaction times by running a 1.0 mg/ml solution of A β ₄₂-K₃ onto a column prepared with a 2X density of CPB per ml of resin, stopping flow for five mins, then resuming flow. Analysis of the product showed no contaminating peaks due to activity from contaminating enzymes.

Using the Hip-L-Arg substrate assay (Sigma-Aldrich), we characterized CPB-agarose activity over the pH range 5 – 10 and the temperature range 4 – 37 °C, and determined the best conditions to be pH9.0 and room temperature. We also used the Hip-L-Arg substrate to periodically check the activity of the column after storage and/or multiple uses. We found that the storage stability of covalently immobilized CPB was excellent, not exhibiting anydetectible loss after 30 days storage at 4°C. We found that CPB activity was unchanged when peptide substrates were dissolved in the HPLC elution buffer (40% acetonitrile v/v in H₂O), after adjusting pH to 9.0. This important result told us that we can remove Lys residues by directly treating the pH-adjusted HPLC pool from purification of the Lys-tail peptide. We found that when we used the same column to treat an A β ₄₂ peptide followed by an A β ₄₆ peptide, we did not detect any A β ₄₂ carryover into the A β ₄₆ product. This suggested that even though the peptides we processed were highly hydrophobic, it is possible to use the same column for treatment of different peptides. However, since the columns are inexpensive and simple to prepare, one could eliminate any concern about possible carryover by using a new column packing for each synthesis or each different

peptide. In principle, the HPLC purification of peptides after Lys-tail removal may not be required. However, it seemed possible that additional contaminants might be removed by doing this, and recoveries in the second HPLC purification were excellent (Table 2). Typically we obtained yields for the combined CPB-agarose and subsequent RP-HPLC purification steps of about 75% (Table 2).

Addition of lysines to the C-terminus of A β ₄₂ improves synthetic efficiency and RP-HPLC purification

Reversible incorporation of C-terminal lysines was first evaluated and optimized by working with various A β ₄₂ peptides synthesized using Fmoc chemistry (Table 1). First, we characterized the experience with wild type A β ₄₂ synthesized without C-terminal Lys residues. The RP-HPLC chromatogram of the initial purification of Fmoc-synthesized A β ₄₂ made without any C-terminal Lys residues (Fig. 2A) is typical for A β ₄₂ and its analogs synthesized using this method. The A₂₁₄-monitored chromatogram of the purification shows a broad profile with no obvious peak for the desired product. Although no single chromatographic fraction contained highly pure A β ₄₂, we collected the most enriched fraction for further analysis. As a general rule, we found that although analytical HPLC A₂₁₄ profiles of purified fractions often exhibit single peaks suggesting highly pure material, MS analysis of the peak shows that it can contain significant levels of impurities. For example, MS analysis of the analytical HPLC peak (LC-MS) from the synthetic A β ₄₂ purified at room temperature showed dominant mass/charge peaks at three charge states consistent with WT A β ₄₂ (Fig. 2A). However, closer examination of the MS spectrum reveals a large number of significant small peaks which taken together reduced the purity considerably. By integrating the m/z peaks of the +6 charge state cluster (Methods) we determined that the single most pure chromatographic fraction (grey bar) from this purification was only ~ 65% pure (Table 3). Many of the major impurity peaks correspond to A β deletion peptides, consistent with the expected outcome of peptide aggregation on the column (Introduction). The major impurity peaks correspond to losses of single Ala (M_{obs} 4441.7 Da), Gly (M_{obs} 4455.9 Da) or Ser (M_{obs} 4426.5 Da) residues from the wild type A β ₄₂ peptide (M_{obs} 4513.2 Da). All these impurities co-elute with the pure A β ₄₂ under our room temperature RP-HPLC conditions. Not only is the purity of the most pure fraction quite low, the amount of authentic wild type A β contained in this fraction represents a synthetic yield of only 2.7 % (Table 3). Presumably this yield would drop to even lower levels if further purification efforts were undertaken to get the purity to an acceptable level.

In some cases it is possible to improve the purification of synthetic peptides by operating the RP-HPLC column at higher temperatures^{55,56}. Therefore, we subjected the same crude A β ₄₂ peptide reaction product to RP-HPLC chromatography with the column equilibrated to 65°C. The chromatogram obtained (Fig. 2b) exhibits a dominant peak, which was confirmed to be A β ₄₂ by MS. The MS spectrum of the purification pool (Fig. 2B; Fig. 3A) exhibits higher purity (80.6 %) than the room temperature HPLC chromatography fraction (Table 3). We confirmed this purity level by determining purity using an alternative method, the proton NMR integration method of Zagorski². This method gave a purity of the 65 °C purified peptide of 82.3 % (Table 3), in very good agreement with the MS method. The amount of A β ₄₂ in the purified pool corresponds to a synthetic yield of 5.4 % (Table 3). The purity of ~

80 % may be sufficient for some studies, but it is not optimal and clearly not as high as the purities of 90–97 % for commercial recombinant A β ₄₂ we confirmed using the MS (Fig. 3C) and NMR methods (Table 3).

We next investigated the degree to which Lys-tails improved the overall quality of the synthetic product, and the percent yields, in A β ₄₂ syntheses. We observed a marked improvement in the chromatographic separations of both A β ₄₂-K₂ (Fig. 2C) and A β ₄₂-K₃ (Fig. 2D), each of which exhibited a major peak in the room temperature HPLC purification, similar to what is observed in the 65 °C purification of the A β ₄₂ product (Fig. 2B). Furthermore, the quality of the peptides purified at room temperature from the crude reaction mixtures (actually determined after Lys removal; Fig. 3B) was superior to synthetic A β ₄₂ purified either at room temperature or at 65 °C (Table 3).

Lysine removal using immobilized enzyme

Optimized CPB-agarose conditions (Methods) were used to remove the Lys tails from the A β ₄₂ peptides described above. After Lys removal and repurification by RP-HPLC, the purities of the final A β ₄₂ products were in the 90% range (Table 3). This purity level approaches that of recombinant A β ₄₂ (90% vs. 97% by MS, 87% vs. 90% by NMR; Table 3, Fig. 3C). Furthermore, the final percent yields of A β ₄₂ after Lys removal and repurification by RP-HPLC were in the 6–8 % range, significantly better than the yields obtained for A β ₄₂ synthesized without C-terminal Lys tails (Table 3). Considering purity as well as final yield, the approach of reversible addition of C-terminal Lys residues followed by room temperature HPLC purification and CPB removal is even superior to direct synthesis of A β ₄₂ alone followed by 65 °C chromatography (Table 3).

Addition of lysines to the C-terminus of A β ₄₆ peptides

Having validated the reversible Lys-tail method on A β ₄₂, we now turned the method to the peptide A β ₄₆, which is expected to be much more hydrophobic, aggregation prone, and difficult to synthesize and purify due to the added C-terminal Thr-Val-Ile-Val sequence (Table 1). In fact, we confirmed that conventional methods give very poor yields of this peptide, with poor final purities. The RP-HPLC chromatogram of the purification of the crude Fmoc synthesized A β ₄₆ at room temperature (Fig. 4A) exhibits a broad profile with a single fraction containing a small amount of partially pure peptide, with adjacent fractions containing peptide of much lower purity (Table 3). The LC-MS spectrum of the purified fraction (not shown) revealed various impurities which co-elute with the pure A β ₄₆ peptide. The yield (1%) and purity (69%) of A β ₄₆ obtained in this product pool were quite low (Table 3). Running the purification with a column temperature to 65°C gave a purified pool with only modest improvements in yield (2 %) and purity (75 %) (Table 3; Figs. 4B and 5A). Hence, as expected from its sequence, the A β ₄₆ peptide behaves more poorly than the A β ₄₂ peptide when directly synthesized by Fmoc chemistry.

We found, however, that addition of C-terminal lysines to the A β ₄₆ peptide afforded a marked improvement in product purity and yield of the Fmoc synthesized peptide. RP-HPLC chromatography of the crude synthetic product of both an A β ₄₆-K₃ (Fig. 4C) and A β ₄₆-K₆ (Fig. 4D) peptide exhibited dominant product peaks in the RP-HPLC purification,

in contrast with the broad, featureless profile of the peptide without added Lys residues (Fig. 4A,B). After removal of C-terminal Lys residues, the final yields of A β ₄₆ were in the range of 4.6 – 5.7 %, and the MS-determined purities were in the range of 88 – 91 % (Table 3). This is in contrast to yields in the 1 – 2 % range, and purities in the 69 – 75 % range, for A β ₄₆ synthesized without a Lys-tail, even using 65 °C HPLC.

Analysis of the source of the benefits of the Lys-tail method

Although the reversible addition of Lys residues was envisioned to mainly provide the benefit of improved chromatographic behavior of the initial synthetic product, we had the impression that there might also be a significant benefit in terms of the initial total synthetic yield. To put this impression on a firm basis, we quantified the contribution of the added Lys residues to both aspects of solid phase synthesis of A β ₄₂ and A β ₄₆ by carrying out small scale purifications at 65 °C and analyzing the chromatography fractions (Methods) (Figs. 6,7). The results clearly show that the addition of C-terminal Lys residues benefits the total synthetic yield and the purification yield separately, by substantial margins in the range of 50% each. For example, while the total amount of A β ₄₂ present in the crude reaction mixture represents about a 15% synthetic yield compared to theoretical, the total amount of A β ₄₂-KK corresponds to a 23% synthetic yield. This gives an improvement of about 52% in synthetic yield (Table 4). Similarly, the added lysine residues clearly aid purification. Thus, based on the total amount of A β ₄₂ generated in the synthesis, only 18.6% of that material is found in the most pure chromatographic fraction in the 65 °C “mini-purification” (Fig. 6A). In contrast, 31.5% of the total available A β ₄₂-KKK is found in the pool of the highest purity fractions of a similar purification (Fig. 6B). This corresponds to a benefit to the efficiency of the purification on the order of about 70% for the Lys tail method (Table 4). Together these two factors combine to produce a calculated 2.6-fold improvement in isolated yield of purified A β ₄₂, with an improvement in the purity of the pool from 79% to 93%.

A similar analysis shows similar benefits in the preparation of A β ₄₆ (Fig. 7; Table 4). We determined a benefit in synthetic yield of about 63%, and a benefit in purification yield of 42%, for a total 2.3-fold improvement in expected yield of purified peptide. The purity of the pool also increased, from 70% to 91%.

Aggregation of A β ₄₂ peptides synthesized by different methods

To assess the effects of the different purification levels of A β ₄₂ peptides on their ability to form amyloid fibrils, we determined the spontaneous aggregation kinetics with disaggregated samples (Methods) of various purified A β ₄₂ peptides at 37°C, pH 7.4. The reactions were carried out at a starting monomer concentration of 10 μ M and the kinetics of aggregation was determined by measuring soluble monomer levels at various time points using HPLC. We found that recombinant A β ₄₂ peptide obtained from rPeptide was the fastest aggregating peptide (Fig. 8A), exhibiting a time to 50% aggregation of 6 hrs (Table 3). We found that the Fmoc synthesized peptides described here aggregated at somewhat lower rates, with half-times of aggregation inversely proportional to their purity levels. Thus, A β ₄₂ obtained via A β ₄₂-K₃, at 90% purity, exhibited a half-time of aggregation of 9.5 hrs. A β ₄₂ synthesized directly and purified at 65 °C, which had a purity of 81 %, showed a half-time of aggregation of 17 hrs, and directly synthesized A β ₄₂ purified at room

temperature, with a 65 % purity, gave a half-time of aggregation of 26 hrs (Table 3). The source of this variation in $t_{1/2}$ values is not totally clear. Since the reactions were set up at 10 μM by assuming the entire $\text{A}\beta_{42}$ peak to be authentic $\text{A}\beta$, some of the drop in aggregation rate with decreasing purity levels could be due to a lower effective starting concentration of authentic $\text{A}\beta_{42}$. Thus, in another study, the $t_{1/2}$ for 10 μM $\text{A}\beta_{42}$ was found to be 6.5 hrs, while the $t_{1/2}$ for 5 μM $\text{A}\beta_{42}$ was found to be 25 hrs (S. Chemuru and R. Wetzel, Ms. in preparation). It is noted that the actual concentration of WT $\text{A}\beta_{42}$ in the sample purified at room temperature from conventionally synthesized product would have been only 6.7 μM in the Figure 8A comparison. If all the increase in aggregation time were due to reduced WT $\text{A}\beta_{42}$ concentration, it would imply that the contaminating $\text{A}\beta$ peptides are neutral in the aggregation process, neither contributing to it nor inhibiting it.

Another measure of the quality of a non-covalent aggregation product, such as the $\text{A}\beta_{42}$ fibrils produced here, is the concentration of the monomer at equilibrium, which reflects the thermodynamic stability of the amyloid fibril with respect to monomer⁵⁷. We monitored the $\text{A}\beta_{42}$ aggregation reactions until the amount of monomer in the supernatant, after centrifugation, did not change. We found that apparently the most stable amyloid is assembled from the recombinant material, giving a final monomer concentration of 0.07 μM (Table 3). In contrast, other $\text{A}\beta_{42}$ peptides gave final monomer concentrations proportional to the purity of the starting peptide (produced *via* $\text{A}\beta_{42}$ -K₃, 0.13 μM ; *via* 65 °C purification of directly synthesized $\text{A}\beta_{42}$, 0.16 μM ; *via* room temperature purification of directly synthesized $\text{A}\beta_{42}$, 0.24 μM). The elevated final concentrations of amyloid assembly reactions from impure $\text{A}\beta_{42}$ could be a reflection of less stable fibrils resulting from co-incorporation of $\text{A}\beta_{42}$ -related impurities, but more work would be required to show this. Alternatively, the higher values might be due to the presence in the monomer pool of $\text{A}\beta_{42}$ -related impurities that are incapable of assembling into fibrils. It is clear that a large portion of the $\text{A}\beta_{42}$ -related impurities, for example in the material that is only 65% pure, must be incorporated into some kind of sedimentable aggregate, otherwise the final monomer concentration would be much higher than 0.24 μM (Table 3). However, whether the aggregation by the $\text{A}\beta$ -related impurities is due to co-aggregation with WT $\text{A}\beta$ or is due to some independent, parallel aggregation process cannot be easily determined. As related below, EM images do suggest that the less pure starting preparations of $\text{A}\beta$ do generate some non-fibrillar material in the product. However, the composition of this material, and the composition of the fibrils, cannot be determined by EM.

We also analyzed the samples from the various aggregation reactions of synthetic and recombinant $\text{A}\beta_{42}$ peptides by negative-stain electron microscopy. As judged by examination and analysis of the entire EM grid for each sample (only representative images of which are shown here), fibrils formed from recombinant $\text{A}\beta_{42}$ (Fig. 9C) showed a more homogeneous morphology than fibrils formed from $\text{A}\beta_{42}$ that was Fmoc-synthesized without C-terminal Lys residues and purified by HPLC (Fig. 9A,B). These less homogeneous aggregates appear to have oligomers (white arrows) and/or curvilinear protofibrils (black arrows) associated with the mature fibrils in the EM images. These might represent the aggregates obtained from the significant amount of impurities present in these samples or the 'off-pathway' intermediates obtained by the incorporation of the impurities

into authentic A β ₄₂ aggregates. A β ₄₂ aggregates obtained from Lys-removal (Fig. 9D) do not exhibit such apparent dead-end intermediate products and have similar morphology to recombinant A β ₄₂ (Fig. 9C).

Amyloid formation by different A β variants

We compared the spontaneous aggregation of the two dominant A β species in the human brain, A β ₄₀ and A β ₄₂, with the behavior of the relatively rare variant, A β ₄₆, whose synthesis is described here. All peptides were highly pure chemically synthesized material. We found that at a 5 μ M starting concentration, A β ₄₆ monomers aggregate with a time to 50% aggregation of 9 hrs, compared with 36 hrs for A β ₄₂ and 140 hrs for A β ₄₀ (Fig. 6B). Thus, the additional hydrophobic residues at the C-terminus of A β ₄₆ significantly enhance spontaneous aggregation compared with shorter A β variants. Electron micrographs of the product fibrils (A β ₄₀, Fig. 9E; A β ₄₂, Fig. 9D; A β ₄₆, Fig. 9F) show them in each case to be uniform in morphology with no non-fibrillar aggregates present. FTIR spectra show the previously described doublet in the portion of the spectrum assigned to β -sheet for A β ₄₀ fibrils grown under quiescent conditions⁵⁸, while the A β ₄₂ and A β ₄₆ fibrils exhibit similar spectra with only a single absorption peak in this region (Fig. 10).

DISCUSSION

A major problem in AD research has been difficult to reproduce results on the *in vitro* aggregation of A β peptides. In one extreme recent case, the reported aggregation behaviors of A β peptides containing the Osaka E22 mutation⁵⁹ range from oligomer formation with no fibril formation, to unusually rapid fibril formation^{59–65}. There are a variety of possible explanations for these varied results, such as general chemical purity of the synthetic peptides, the presence of low but significant levels of backbone α -C-H epimerization during synthesis²⁴, the disaggregation protocol used, and the types of assays used to monitor aggregation kinetics and analyze aggregate morphology. Our lab has been systematically evaluating some of these variable procedures, and will be reporting on several in the near future. In this paper, we deal with the purity in synthetic A β and how to improve it.

Recognizing that the well-chronicled problems of working with A β peptides² are largely due to the extreme hydrophobicity of the C-terminal 60% of the peptide, Kim et al. devised a synthetic strategy in which methionine sulfoxide was inserted into the growing A β chain in place of Met₃₅ to provide improved solution properties⁴². After purification of the synthetic product, the methionine sulfoxide was reduced back to Met. Another way to improve the solution properties of hydrophobic peptides is to append cationic tails to their N- or C-termini⁶⁶. This general phenomenon has been exploited to devise a synthetic scheme in which T-Boc⁴³ or Fmoc⁴⁴ solid phase synthesis is initiated with a resin-bound oligo-Arg tail separated from the growing desired peptide sequence by a hydrolysable bond. In order to avoid the non-standard chemistry required by this method for both solid phase synthesis and product recovery, we evaluate here an alternative method for reversible addition of a cationic tail in which only peptide bonds are used in the chemical synthesis, and the cationic tail is removed enzymatically. This method was inspired by recognition of the potential effectiveness of highly specific removal of cationic amino acids by the C-

terminal endoproteinase carboxypeptidase B in a previously described enzyme model system for converting proinsulin to insulin⁶⁷ - a system that must be robust since it was ultimately developed for commercial production of human insulin⁶⁸.

In fact, we show here that an immobilized CPB column support is simple and inexpensive to prepare, is stable, and is simple to use in removing multiple Lys groups from the C-termini of A β peptides. CPB of sufficient purity is commercially available so that undesired further cleavage within the desired peptide product by contaminating proteases can be avoided. It is also worth noting that active, recombinant CPB has been described^{69,70} and would presumably be an even better source for minimizing the potential for side reactions during Lys removal. More work must be done to explore the full breadth of our new method. Some peptides may possess tertiary or self-assembled structure at the C-terminus which could impair CPB cleavage. However, in this regard the A β model system is a good test, since the highly hydrophobic, oligomerization-prone A β is processed cleanly in our tests. Perhaps the acetonitrile component of the cleavage buffer (i.e., the HPLC elution buffer) helps prevent the formation of inhibitory structures. Another potential limitation is that peptides ending in Lys or Arg cannot be prepared in this way. However, it should be possible to develop a simple, tandem carboxypeptidase column approach for such peptides, in which the desired C-terminal Lys or Arg is C-terminally masked in the synthesis with a neutral amino acid like alanine or valine, followed by the oligoLys tail. After removal of the Lys groups by immobilized CPB, the cleaved product could be run through another immobilized enzyme column containing a carboxypeptidase capable of removing the new C-terminal neutral amino acid, but not the desired Lys or Arg.

The experiments shown in Figures 6 and 7 and summarized in Table 4 show that our method provides benefits for both total synthetic yield and, separately, purification yield. The advantage given to purification yield is presumably due to the better solution behavior of the A β peptides with highly charged C-terminal Lys residues. This feature, which we expected and which in fact was the motivation for developing our method, does in fact narrow the elution profile of the desired product (Figs. 6, 7). Surprisingly, however, the addition of C-terminal Lys residues also clearly improves the total synthetic yield by more than 50% (Table 4). The basis of this effect is somewhat mysterious, since the Lys residues during SPPS are protected and therefore neutral and hydrophobic. We hypothesize that the important feature may be that Lys is not a β -branched amino acid. It is well-known that β -branched amino acids are particularly prone to form β -structure⁷¹ and hence β -aggregates. In fact, the very poor synthetic performance of A β ₄₆ may be due in large part not only to its general hydrophobicity, but also to the fact that seven out of the eight C-terminal residues of this peptide are β -branched. β -hairpin formation by the first formed C-terminal segment of a synthetic peptide might limit overall yield through steric interference by the resin itself on further additions. If so, then addition of a linker that does not contain β -branched residues (even though it may be highly hydrophobic) may be sufficient to limit the consequences of this interfering β -hairpin formation by better distancing it from the resin surface.

Since they were meant to explore the source of improved final, isolated yields, the studies summarized in Table 4 were conducted under ideal circumstances of 65 °C chromatography and without the final step of Lys removal. Excellent benefits of the method are also

observed, however, when comparing final, isolated yields after Lys removal, as shown in Table 3. These data show that isolated synthetic yields and purities of A β peptides are clearly improved by the reversible addition of Lys-tails during solid phase Fmoc synthesis. Restricted only to data from room temperature RP-HPLC chromatography, the yield of A β ₄₂ increased from 2.7% to 7.8%, and peptide purity increased from 65% to 90%, with the CPB-reversible addition of three C-terminal Lys residues during synthesis (Table 3). Likewise, HPLC purification of A β ₄₆ directly synthesized by Fmoc solid phase synthesis gave isolated yields of 1% (room temperature) and 2% (65 °C), and purities in the 69 – 75% range (Table 3). In contrast, synthesis of A β ₄₆ *via* the intermediate Lys-tail product gave isolated yields in the 4.7 – 5.5% range and purities in the 88 – 91 % range (Table 3). In contrast to the experience with directly synthesized peptides, using the CPB-reversible Lys-tail method we experienced no drop-off in isolated yield or purity in going from A β ₄₂ to A β ₄₆. This suggests that this method should be capable of making even longer A β peptides as well as other highly hydrophobic peptides. It is possible that the Lys tail method may also confer some advantages to the synthesis of peptides that do not appear particularly hydrophobic.

How important is the general purity of A β peptides to their aggregation behavior? We found that spontaneous amyloid formation kinetics trend with the purities of the various A β ₄₂ preparations, with the product of the synthesis *via* a Lys-tail aggregating considerably faster than the A β ₄₂ synthesized directly and purified at room temperature (Fig. 8A). Importantly, the best quality chemically synthesized A β ₄₂ peptide aggregates nearly as fast as recombinant A β ₄₂. The relative differences in aggregation rates we observed for our different A β ₄₂ preparations, while significant, should be considered relatively modest, quantitative differences that are not nearly as large as effects reported elsewhere²⁴. The mechanisms behind the differences we observed are far from clear. For example, the significant purity differences of the samples lead to corresponding concentration differences in bona fide A β ₄₂, which could lead to aggregation rate differences. However, the impurities are for the most part A β ₄₂ analogs many of which may well be able to participate in the amyloid formation reaction, perhaps to some extent negating the differences in concentration of the WT material. Sorting out these questions, and the related question of whether impurity A β peptides co-assemble with WT or independently aggregate, is well beyond the scope of this paper.

It is well known that multiple C-termini of A β peptides are generated in the AD brain, some of which owe to an apparent processive action of an endoproteinase function of the enzyme γ -secretase on cleavage of APP^{13,17}. One of the cleavages releases A β ₄₆. It has been speculated that some presenilin mutations may work by shifting the balance of A β peptide products to longer, more aggregation-prone versions of A β ¹³. It has also been reported that some γ -secretase inhibitors, in blocking the formation of A β _{40/42} peptides, result in the accumulation of intracellular A β ₄₆ peptide^{72,73}. It is therefore of considerable interest how the aggregation kinetics and morphologies of A β ₄₆ and other “minor” A β species might differ from those of A β ₄₂. A preliminary report on the aggregation of chemically synthesized A β ₄₆ focused on possible cross-seeding activity, but did not quantitatively compare individual aggregation tendencies or describe the quality of the synthetic peptides used⁷⁴. In the studies reported here, we focus on ensuring the purity of the A β ₄₆ used, and

in making direct comparisons of the aggregation kinetics and aggregate structure of A β ₄₆ compared with A β ₄₀ and A β ₄₂. We found that A β ₄₆ undergoes spontaneous amyloid formation significantly more rapidly than either A β ₄₂ or A β ₄₀ (Fig. 8b). The fibrils appeared similar to those of A β ₄₂ in both EM morphology (Fig. 9) and FTIR spectroscopy (Fig. 10). We did not explore the ability of A β ₄₆ to make oligomers. Our results are consistent with the possibility that A β ₄₆, in spite of being present in relatively low amounts in the brain, may have a disproportionate impact on amyloid burden or other aggregation-associated events.

The advent of efficient recombinant DNA methods for preparing useful amounts of high quality, chemically confirmed peptides and proteins^{75,76} introduced a major alternative to traditional methods of peptide synthesis for producing molecules for biophysical and biological studies as well as pharmaceutical applications. At the same time, chemical methods capable of producing high quality peptide material continue to have a major role to play. Although no longer unique in this regard, chemical methods can generally more readily be used to prepare polypeptides containing unnatural amino acids and specifically isotopically labeled amino acids. At least for chemically-oriented peptide labs, chemical synthesis can be a more straightforward, less labor intensive way to conduct a preliminary investigation on a new peptide sequence. Recently published results²⁴, however, suggested that for at least some classes of peptides, such as highly hydrophobic and amyloidogenic molecules, recombinant expression might possess some intrinsic advantage over chemical synthesis in terms of peptide quality and performance in replicating *in vivo* amyloid formation events. We describe here a general method for greatly improving the synthetic yield and quality of chemically synthesized, highly hydrophobic and amyloidogenic peptides. It should be possible to combine this method with other approaches for improving yields of hydrophobic peptides to obtain enhanced results. Using this method, we obtain purities and performance in *in vitro* amyloid growth assays that are similar to those from the recombinant version of the same molecule. We also show, however, that all chemical synthetic products are not equivalent, and that rigorous methods of synthesis, purification and analysis have important roles to play in generating quality molecules and quality data from those molecules.

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Figure 1.
Flow chart of the method.

Figure 2.

Addition of lysines to C-terminus of $A\beta_{42}$ improves reversed phase chromatography purification. RP-HPLC purification (see Methods) profiles of the various crude peptides synthesized using Fmoc chemistry. A) $A\beta_{42}$ at room temperature column temperature; B) $A\beta_{42}$ at column temperature of 65°C; C) $A\beta_{42}$ -K₂ purified at room temperature; and D) $A\beta_{42}$ -K₃ purified at room temperature. The shaded region represents the fractions which were of highest purity and the electrospray mass spectra of each pool is shown in the corresponding right panel.

Figure 3.

Electrospray mass spectrograms of +5 charge state of purified $A\beta_{42}$ peptides, as used in the calculation of purity (Methods). A) $A\beta_{42}$ synthesized using Fmoc chemistry and purified by 65°C RP-HPLC column; B) $A\beta_{42}$ obtained from $A\beta_{42}$ -K₂ as described; C) $A\beta_{42}$ obtained by recombinant methods (rPeptide).

Figure 4.

Addition of lysines to C-terminus of A β_{46} improves synthetic purity. RP-HPLC purification (see Methods) profiles of the various crude peptides synthesized using Fmoc chemistry. A) Crude A β_{46} purified at room temperature; B) Crude A β_{46} purified at column temperature of 65°C; C) A β_{46} -K₃ purified at room temperature column; and D) A β_{46} -K₆ purified at room temperature. The shaded region represents the fractions which were of highest purity and the electrospray mass spectra of each pool is shown in the corresponding right panel.

Figure 5.

Electrospray mass spectrograms of +6 charge state of pure Aβ₄₆ peptides, as used in the calculation of purity (Methods). A) Aβ₄₆ synthesized using Fmoc chemistry and purified by 65°C RP column; B) Aβ₄₆ obtained from Aβ₄₆-K₃ as described; C) Aβ₄₆ obtained from Aβ₄₆-K₆ as described.

Figure 6. Analysis of 65 °C purification of A β ₄₂ peptides. Several mg of crude deprotected reaction product chromatographed on the preparative column at 4 ml/min, collecting 0.8 ml/fraction. Total weight of correct peptide (Methods) in each fraction plotted as gray bar graph superimposed on the A₂₁₄ trace. Numbers inside the bars are the % purity in that fraction. Arrows indicate percent acetonitrile in the gradient. (A) 2.0 mg A β ₄₂ run on a 30–60% acetonitrile gradient (B) 1.4 mg A β ₄₂-KK run on a 20–50% acetonitrile gradient.

Figure 7.

Analysis of 65 °C purification of Aβ₄₆ peptides. Several mg of crude deprotected reaction product chromatographed on the preparative column at 4 ml/min with a linear gradient of 30 – 60 % acetonitrile, collecting 0.8 ml/fraction. Total weight of correct peptide (Methods) in each fraction plotted as gray bar graph superimposed on the A₂₁₄ trace. Numbers inside the bars are the % purity in that fraction. Arrows indicate percent acetonitrile in the gradient. (A) 2.7 mg Aβ₄₆; (B) 2.0 mg Aβ₄₆-KKK.

Figure 8.

Aggregation kinetics of Aβ derivatives. A. Comparison of Aβ₄₂ obtained by different methods. 10μM reaction of Aβ₄₂ peptide obtained from various sources: Aβ₄₂ from rPeptide (◆); from Aβ₄₂ - K₃ peptide after Lys removal (●); synthesized as Aβ₄₂ and purified at 65°C (▲) or room temperature (■). B. Comparison of 5 μM Aβ₄₆(◆; prepared from Aβ₄₆-K₆) with 5 μM Aβ₄₂(●; prepared from Aβ₄₂-K₃) and 5 μM Aβ₄₀ (○; purified at room temperature from crude Fmoc synthesis product).

Figure 9.

Electron microscopy analysis of various A β amyloid fibrils collected at the end of the aggregation reaction. Aggregates of A β_{42} (A) synthesized as A β_{42} and purified at room temperature; (B) synthesized as A β_{42} and purified at 65 °C; (C) synthesized by recombinant means; (D) synthesized as A β_{42} -K₃ and processed as described in this paper. Amyloid fibrils from (E) A β_{40} obtained purified from the Keck Biotechnology Center; (F) A β_{46} synthesized as A β_{46} -K₃ and processed as described here. Arrows indicate apparent fibrillization intermediates of oligomers (white arrows) and curvilinear protofibrils (black arrows).

Figure 10.

FTIR spectroscopy of various Aβ amyloid fibrils. Fibrils of Aβ₄₀, Aβ₄₂, and Aβ₄₆ were the same as those shown in Figure 7 panels D, E and F.

Table 1

Peptide sequences described in this work.

Peptide	Sequence
A β ₄₂	DAEFRHDSGY EVHHQKL VFF AEDVGSNKG A IIGLMVGGVV IA
A β ₄₂ K ₂	DAEFRHDSGY EVHHQKL VFF AEDVGSNKG A IIGLMVGGVV IAKK
A β ₄₂ K ₃	DAEFRHDSGY EVHHQKL VFF AEDVGSNKG A IIGLMVGGVV IAKKK
A β ₄₆	DAEFRHDSGY EVHHQKL VFF AEDVGSNKG A IIGLMVGGVV IATVIV
A β ₄₆ K ₃	DAEFRHDSGY EVHHQKL VFF AEDVGSNKG A IIGLMVGGVV IATVIVKKK
A β ₄₆ K ₆	DAEFRHDSGY EVHHQKL VFF AEDVGSNKG A IIGLMVGGVV IATVIVKKKK KK

Table 2

Recoveries of each step for A β ₄₂ preparation via A β ₄₂-K₃.

Step	Step recovery	Cumulative
Reverse phase HPLC (25 °C) purification from crude	10.3 %	10.3 %
Lysine removal by carboxypeptidase B column	79 %	8.1 %
Repeat reverse phase HPLC (25 °C) purification	96 %	7.8 %

Table 3

Data summary for Aβ syntheses and aggregation reactions.

Final Peptide	Fmoc Peptide	Column temp. °C	% purity (MS)	% purity (NMR)	Yield (overall)	Hours to 50% agg.	C _r (μM)
Aβ ₄₂	recomb.	N/A	96.9	90.0	N/A	6.0	0.07
Aβ ₄₂	Aβ ₄₂	25	64.9	n.d.	2.7	26	0.24
Aβ ₄₂	Aβ ₄₂	65	80.6	n.d.	5.4	17	0.16
Aβ ₄₂	Aβ ₄₂ K ₂	25	89.7	n.d.	6.2	n.d.	n.d.
Aβ ₄₂	Aβ ₄₂ K ₃	25	90.2	n.d.	7.8	9.5	0.13
Aβ ₄₆	Aβ ₄₆	25	69.4	n.d.	1.0	n.d.	n.d.
Aβ ₄₆	Aβ ₄₆	65	74.6	n.d.	2.0	n.d.	n.d.
Aβ ₄₆	Aβ ₄₆ K ₃	25	88.4	n.d.	5.6	n.d.	n.d.
Aβ ₄₆	Aβ ₄₆ K ₆	25	91.0	n.d.	4.7	n.d.	n.d.

n.d. = not determined.

Table 4

Relative contributions of approach to improved overall isolated yield of Aβ peptides.

Peptide	Total synthetic yield (%) ^a	% benefit to synthetic yield ^b	Purification efficiency (%) ^c	% benefit to efficiency ^d	Fold total improvement ^e	% Purity of pool
Aβ ₄₂	15.1	-	18.6	-	-	79
Aβ ₄₂ K ₂	23.0	52.3	31.5	69.4	2.6	93
Aβ ₄₆	11.5	-	18.2	-	-	70
Aβ ₄₆ K ₃	18.7	62.6	25.9	42.3	2.3	91

^aPercent theoretical yield based on determination of total synthetic yield of desired product (see Methods).

^bFor the same Aβ peptide target, total synthetic yield (before purification) for the Lys-tail approach compared to the standard approach.

^cThe percent of total desired peptide product (total synthetic yield) that is obtained by 65 °C chromatography in combined purification peak fractions of acceptable purity (see Supporting Information).

^dFor the same Aβ peptide target, purification efficiency for the Lys-tail approach compared to the standard approach.

^eExpected combined benefit of the Lys-tail method from improvements in synthetic yield and in purification. Expressed as fold improvement in isolated yield of peptides of acceptable purity.