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Novel Semi-synthetic Method for Generating Full Length β -Amyloid Peptides

Jessica J. Bockhorn¹, Kristi L. Lazar², Adam Gasser³, Laura M. Luther², Isam M. Qahwash⁴, Neeraj Chopra⁵, and Stephen C. Meredith^{1,5,*}

¹Department of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL 60637

²Department of Chemistry, The University of Chicago, Chicago, IL 60637

³Committee on Cancer Biology, The University of Chicago, Chicago, IL 60637

⁴Department of Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL 60637

⁵Department of Pathology, The University of Chicago, Chicago, IL 60637

Abstract

Bacterial expression of full length β -amyloid (A β) is problematic because of toxicity and poor solubility of the expressed protein, and a strong tendency of Met35 to become oxidized in inclusion bodies. We have developed a semi-synthetic method in which A β ₁₋₂₉ is expressed in bacteria as part of a fusion protein with a C-terminal intein and Chitin-Binding Domain (CBD). There is also a single residue, N-terminal Met extension. The protein, Met-A β ₁₋₂₉-Intein-CBD, is well expressed and highly water-soluble. After binding of the expressed protein to Chitin beads, treatment with sodium 2-mercapto-ethane sulfonate (MESNA) yields Met-A β ₁₋₂₉-MESNA, with a C-terminal thioester suitable for native chemical ligation. Met-A β ₁₋₂₉-MESNA is first subjected to CNBr cleavage, which removes the N-terminal Met residue, but leaves the thioester intact. We synthesized NH₂-A30C-A β ₃₀₋₄₀, which has an N-terminal Cys residue and is the partner for native chemical ligation with Met-A β ₁₋₂₉-MESNA. Native chemical ligation proceeds rapidly and efficiently (> 90% yield) to give A30C-A β ₁₋₄₀. The final step is selective desulfurization using Raney-Ni, which also proceeds rapidly and efficiently (> 90% yield) to give native sequence A β ₁₋₄₀. Overall, this system is highly efficient, and can yield $\approx$ 8-10 mg of pure A β ₁₋₄₀ from one liter of bacterial culture medium. This procedure is adaptable for producing other A β peptides. We have also expressed an A β construct bearing a point mutation associated with one type of familial Alzheimer's Disease, the Iowa mutation, i.e., Met-D23N-A β ₁₋₂₉-Intein-CBD. Since expression of the intein-containing fusion protein is robust in minimal media as well as standard enriched media, this procedure also can be readily modified for incorporating ¹⁵N or ¹³C labels for NMR. Future work will also include extending this system to longer A β peptides, such as A β ₁₋₄₂.

Introduction

Oscar Wilde's well-known aphorism, "Life imitates art far more than art imitates life", is a re-statement of an observation made by Ovid in Book 3 of *Metamorphoses*: "Nature in her genius had imitated art." Native chemical ligation, pioneered and artfully developed by Stephen Kent, to whom this issue of *Biopolymers: Peptide Science* is dedicated, was too

*Corresponding author: To whom correspondence should be addressed: Dept. of Pathology, University of Chicago, 5841 S. Maryland Ave., Chicago, IL 60637. Tel.: 773-702-1267; Fax: 773-834-5251; scmeredi@uchicago.edu..

good for nature not to imitate in self-splicing proteins. Native chemical ligation and the natural process in which inteins are spliced out of some proteins to join the exteins both have strikingly similar features. In both, an N-terminal Cys-containing peptide reacts spontaneously with a second peptide containing a C-terminal thioester, yielding a fusion peptide containing both fragments linked by a native, peptide bond. Both reactions occur rapidly and efficiently in ordinary aqueous media, on otherwise unmodified peptides, and with little in the way of sequence requirements. In this paper we use both techniques to develop a technique for making various β -amyloid (A β) peptides.

β -amyloid (A β) precursor protein undergoes proteolytic cleavage forming β -amyloid peptides including A β ₁₋₄₀ and A β ₁₋₄₂ among others. The deposition of these β -amyloid peptides into neuritic plaques and cerebral blood vessels are signatures of Alzheimer's disease [1,2]. A continuing goal for understanding this disease is the elucidation of the structure of A β soluble oligomers and fibrils, in which solution and solid-state NMR are invaluable. For these studies, it will be desirable to have methods for producing uniformly or specifically isotopically labeled A β peptides. Appropriately labeled A β can be generated through solid phase synthesis, but this method has several disadvantages, including the expense and laboriousness of synthesizing multiply labeled peptides, especially uniformly isotopically labeled A β peptides, such as are commonly used for two- and higher dimensional solution NMR analyses [3].

Bacterial expression of A β peptides can be problematical, however. Among the difficulties, the foremost is the strong tendency of Met35 of bacterially expressed A β peptides to become oxidized, probably in inclusion bodies. For this reason, several studies have resorted to using either A β containing methionine oxides, or A β peptides with conservative substitutions, such as Leu, for Met 35 [4,5]. In addition, the toxicity of recombinant A β peptides appears to limit bacterial expression [6]. Several procedures to express A β peptides in bacteria lead to accumulation of insoluble protein in inclusion bodies [7,8]. Another approach has been to render A β more soluble by expressing it as part of a fusion protein [9], but for structural studies, it would be preferable to avoid exogenous sequences that could modify the structure of A β in oligomers or fibrils.

For all of these reasons, we have developed a method, reported below, for semi-synthetic production of A β peptides, which combines bacterial expression and solid-phase peptide synthesis. As we will describe below, our method starts with bacterial expression of the N-terminal 29 residues of A β , A β ₁₋₂₉, which remains highly soluble in bacteria when it is part of an intein tagged system. In particular, as described below, we expressed the fusion protein, Met-A β ₁₋₂₉-Intein-CBD (CBD = Chitin-Binding Domain). This fusion protein is water-soluble and is purified using a Chitin column, from which Met-A β ₁₋₂₉ is cleaved using MESNA. This generates a C-terminal thioester, which is then used for native chemical ligation with a synthetic peptide containing the C-terminal sequence of A β . The use of the short synthetic peptide containing the problematic Met residue circumvents the tendency of bacterially-expressed Met35 towards oxidation. Met35, within a synthetic C-terminal peptide, can then be incorporated into A β peptides under the controlled conditions of native chemical ligation, yielding a peptide with a native (non-oxidized) Met at position 35. The ease of using native chemical ligation to generate a wide variety of fusion peptides and proteins has been amply demonstrated [10-13]. One goal of this procedure is to produce A β peptides with isotopically labeled amino acids for NMR. We will show that production of the fusion protein is efficient in both enriched and minimal media, and hence, can be adopted for incorporating labeled amino acids. In addition, this approach can be adapted for incorporating point mutations of A β through site directed mutagenesis. We demonstrate this point, below, by expressing the construct, Met-D23N-A β ₁₋₂₉-Intein-CBD, i.e., bearing the Iowa mutation, D23N, associated with familial Alzheimer's Disease and Cerebral Amyloid

Angiopathy. In principle, this approach can be extended to other point mutations of A β associated with familial Alzheimer's Disease [14-19]. The method described below is both versatile and straightforward, and yields large quantities of pure protein ($\approx$ 8-10 mg from one liter of bacterial culture) that are suitable for NMR and other biophysical studies.

Materials and Methods

Cloning

A β ₁₋₂₉ 5' GGT GGT CAT ATG GAT GCA GAA TTC CGA CAT GAC TCA GGA TAT GAA GTT CAT CAT CAA AAA TTG GTG TTC TTT GCA GAA GAT GTG GGT TCA AAC AAA GGT TGC GGA AGA GCA ACC ACC 3' was made and amplified by PCR using primers 5' CCA CCA GTA TAC CTA CGT C 3' (forward) and 5' GGT GGT TGC TCT TCC GC 3' (reverse), with a SapI site added to the 3' end and a NdeI site added to the 5' end. The PCR cycle included 95 °C for 20 minutes, 30 cycles of 95 °C for 30 seconds, 46 °C for 30 seconds and 72 °C for 1 minute and finally 72 °C for 5 minutes. PCR reaction utilized TAq polymerase (Roche). pTXB1 vector (New England Biolabs) and PCR product were digested with NdeI and SapI (New England Biolabs) and dephosphorylated using calf intestinal alkaline phosphatase (Invitrogen). The vector and insert were ligated using T4 DNA ligase (Fermentas). The ligation reaction was transformed into ER2566 cells and resulting colonies were screened on Luria broth (LB) plates containing ampicillin (amp) and using restriction digest. Sequencing verified that A β ₁₋₂₉ was correctly aligned into the pTXB1 vector.

For production of A β peptides bearing a point mutation associated with Familial Alzheimer's Disease, we performed site directed mutagenesis to obtain a construct with the Iowa mutation, D23N. This required altering a nucleotide in GAT (codon for D23) to AAT (for N). For this site directed mutagenesis, the following forward and reverse primers were used: 5' TCA AAA ATT GGT GTT CTT TGC AGA AAA TGT GGG TTC AAA CAA AGG 3', and 5' CCT TTG TTT GAA CCC ACA TTT TCT GCA AAG AAC ACC AAT TTT TGA 3', respectively (mutation site in bold and underlined). Site directed mutagenesis was performed using a Quickchange II XL Site-Directed Mutagenesis Kit (Stratagene). As described above, a miniprep of DNA for the pTXB1 vector containing A β ₁₋₂₉ was used as a template for synthesis of the mutated strand by PCR. The ligation reaction was transformed into XL10-Gold Ultracompetent cells (Stratagene) and resulting colonies were screened on (LB) plates containing amp. Amp-resistant colonies were then picked and cultured. Plasmid DNA was isolated by miniprep and then sequenced (using T7short and T7 terminator primers) to confirm the mutation, and to confirm that D23N-A β ₁₋₂₉ was correctly aligned into the pTXB1 vector.

Expression

Vector DNA was transformed into BL21DE3 or ER2566 cells and plated onto LB-amp plates. Individual colonies were inoculated in LB-amp and grown overnight at 37 °C. The overnight culture was used to inoculate a larger volume and when the OD 600 nm reached approximately 0.4-0.6, protein expression was induced with Isopropyl β -D-1-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Various growth conditions were tested prior to high yield expression. These conditions included 37 °C for 3 and 6 hours, 30 °C for 3 hours, and 20 °C overnight (approximately 18 hours); samples were examined for amount of expressed recombinant protein on Tris-glycine SDS-PAGE (12% acrylamide gels). Once it was determined that 20 °C overnight was optimal, induced cultures were then placed under those conditions. The culture was then harvested and frozen at -20 °C. To demonstrate the feasibility of using minimal media for incorporation of labeled amino acids in the future, bacteria originally grown in LB broth culture were

inoculated into M9 minimal medium, containing glucose as the only carbon source, and NH_4Cl as the only nitrogen source.

Purification

The initial purification scheme closely followed the recommendations from New England Biolabs. Briefly, bacteria from overnight culture were resuspended in lysis buffer (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.00) containing complete protease inhibitor (Roche), and sonicated on ice using a sonicator (Heat Systems-Ultrasonicator, Inc., model W140) for 5 minutes at output 8 with repeated 5 seconds on/5 seconds off. The lysate was then centrifuged at $12,000 \times g$ for 25 minutes at 4 °C. Chitin beads (New England Biolabs) were washed with buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.00), and the supernatant from the lysate was added to the beads. The lysate was incubated with the beads overnight at 4 °C. The intein beads were washed with buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 7.00), the supernatant was decanted, and then an equal volume of elution buffer (20 mM sodium phosphate, 150 mM sodium chloride, pH 7.00) also containing a final concentration of 70 mM 2-mercapto-ethane sulfonate (MESNA) was added to the bead slurry. In earlier experiments, thiophenol had been used rather than MESNA [20]. The beads were incubated in the elution buffer at room temperature for approximately 60-70 hours. The concentration of protein (mainly $\text{A}\beta_{1-29}$ peptides, as shown in Results) eluted from the Chitin beads was measured using a NanoDrop ND-1000 spectrophotometer, as per the manufacturer's instructions. This eluted protein was concentrated by lyophilization. For further analysis, the lyophilized protein was dissolved in 50:50 H_2O :acetonitrile (both containing 0.1% TFA) at 37 °C, by which procedure most of the precipitate was dissolved. The protein was then further analyzed by RP-HPLC, using a Zorbax C18 column, eluted with a 10:90 to 50:50 acetonitrile: H_2O (both 0.1% TFA, v:v) linear gradient. For further purification, a similar gradient was used on a preparative reverse phase HPLC using a C18 column (Zorbax). To verify that the expressed protein was $\text{A}\beta_{1-29}$, western blots using antibodies (1:500 dilution) to full-length β -amyloid (6E10, Santa Cruz) were performed.

CNBr cleavage

Cyanogen bromide cleavage followed the methods as in [21,22]. Briefly, lyophilized protein was dissolved in 40% formic acid, and CNBr was added to the solution at ≈ 1000 times the protein concentration. Care was taken that any use of cyanogen bromide was performed under the hood due to the toxicity of this compound. The solution was kept away from light and left incubating at room temperature for 72 hours or until most of the final product was present as assessed by MALDI-TOF mass spectrometry and analytical RP-HPLC. The product was then purified on HPLC using a Zorbax C18 column with 10:90 to 50:50 acetonitrile: H_2O (both 0.1% TFA, v:v) linear gradient. Peaks were checked using MALDI-TOF mass spectrometry. The peaks corresponding to $\text{A}\beta_{1-29}$, i.e., without the N-terminal methionine, were pooled and lyophilized.

Synthesis and purification of A30C- $\text{A}\beta_{30-40}$

$\text{NH}_2\text{-CIIGLMVGGVV-COOH}$ (i.e., $\text{A}\beta_{30-40}$ bearing an A30C point mutation) was synthesized, starting with Fmoc-Val Wang resin, and using standard Fastmoc chemistry on an ABI Model 433A peptide synthesizer. Fmoc-Met was from Midwest Biochemicals or Bachem, and was used without further modification. The peptide was cleaved from the resin using 94% TFA, 2.5% H_2O , 2.5% EDT and 1% TIS (Novabiochem). The peptide was purified by preparative reverse phase HPLC using a C8 column with a 20:80 to 60:40 acetonitrile: H_2O (both 0.1% TFA, v:v) linear gradient. Peaks were checked using by

MALDI-TOF mass spectrometry ($m/z = 1060.6$, expected = 1060.4). The peaks containing A30C-A β_{31-40} were pooled and lyophilized.

Synthesis and purification of A β_{1-29} -COSR

Native chemical ligation was also performed on synthetic peptides. A C-terminal thioester peptide of A β_{1-29} , NH₂-DAEFRHDSGYEVHHQKLVFFAEDVGSNKG-C(O)SR (A β_{1-29} -COSR) was synthesized using manual Boc synthesis with HBTU on a Boc-Leu-PAM resin to which S-Trityl- β -mercaptopropionic acid had been attached. For this synthesis, we used tBoc chemistry with “in situ neutralization” and 0.5 M HBTU in DMF, as described previously [23,24]. This results in a peptide consisting of A β_{1-29} , in which C-terminus is in a thioester bond to R = thiopropionyl-Leu. The peptide was cleaved from the resin using anhydrous HF with *p*-cresol (1:10, v:v). The peptide was purified by preparative reverse phase HPLC using either a Rainin C4 or C18 column heated at 60 °C with a 20:80 to 60:40 acetonitrile:H₂O (both 0.1% TFA, vol:vol) gradient over 50 minutes. The peaks were checked by ESI mass spectrometry ($m/z = 3520.69$, expected = 3520.65). The peaks containing A β_{1-29} -COSR were pooled and lyophilized.

Native chemical ligation

Native chemical ligation was performed essentially as described [12,13]. One mg of A β_{1-29} -COSR and 1.2 equivalents of Cys-A β_{31-40} were dissolved in 10 mM sodium phosphate, pH 7.00, containing 6M urea, 20 mM TCEP and 10 mM thiophenol. We initially performed the reaction using synthetic C-terminal thioester peptide of A β_{1-29} , (A β_{1-29} -COSR), made by the procedure described in the above paragraph. Yields using synthetic and bacterially expressed A β_{1-29} -COSR were essentially the same; yields reported in Table 1 are those for the bacterially expressed form of A β_{1-29} -COSR. The reaction was monitored on a HPLC using an analytical C4 Rainin column heated at 60 °C with a 20:80 to 60:40 acetonitrile:H₂O (both 0.1% TFA, v:v) gradient over 50 minutes. Peaks were checked by MALDI-TOF mass spectrometry ($m/z = 4330.5$, expected = 4329).

Selective desulfurization of A β_{1-40}

Selective desulfurization was performed essentially as described by Pentelute and Kent [25]. Briefly, Raney nickel was prepared by adding 25 mg NaBH₄ to 150mg Ni(OAc)₂(H₂O)₂ dissolved in 2ml of deionized H₂O. After 5 minutes, the slurry was filtered through a medium sintered glass funnel. 0.5 mg of peptide was dissolved in 2 ml of 35 mM TCEP, 6 M urea and 200 mM sodium phosphate, pH 6.80. The Raney nickel slurry was added to the peptide solution. The reaction was monitored by HPLC using a C18 column heated at 60 °C, and using a 20:80 to 60:40 acetonitrile:H₂O (both 0.1% TFA, v:v) gradient. The peaks were checked using a MALDI-TOF and ESI mass spectrometer. After 1 hour the reaction was complete.

RESULTS

Semi-synthetic strategy for generating A β_{1-40}

The experimental scheme of the semi-synthetic method is shown in Figure 1. By expressing only amino acids 1-29 of A β in bacteria, rather than full length A β , we circumvent two major problems. First, the toxicity that limits bacterial expression of the full length A β is largely avoided. Second, and more important is that Met35 of expressed A β_{1-40} is readily oxidized [4,5], so that expression of full length A β usually requires either a “conservative substitution” at this position (e.g., Leu for Met), or the use of methionine oxides in the peptide – neither of which is satisfactory for structural studies.

We demonstrate below that A β ₁₋₂₉ can be expressed in large quantities as a C-terminal thioester in an intein tag system [26-30], with an N-terminal extension of only one residue, Met, on the A β peptide. The fusion protein, Met-A β ₁₋₂₉-Intein-CBD contains a thioester between the intein and A β ₁₋₂₉. Thus, the protein is readily purified using a Chitin column, and Met-A β ₁₋₂₉ is released from the column after cleavage using MESNA. After removing the N-terminal Met residue using CNBr, the product is A β ₁₋₂₉, in a C-terminal thioester bond with MESNA. This product is suitable for native chemical ligation to A30C-A β ₃₀₋₄₀, or other peptides with an N-terminal Cys. A30C-A β ₃₀₋₄₀ was chosen because the Cys residue can be converted later to the Ala residue of the native sequence by selective desulfurization using Raney Ni. This procedure yields high quantities of native sequence A β ₁₋₄₀. The expression system is efficient for bacteria grown either in LB and minimal media, and thus should allow for facile labeling of peptides for NMR.

Cloning of A β ₁₋₂₉

DNA encoding A β ₁₋₂₉ was cloned into pTXB1 vector [31], which utilizes an intein tag system. This vector was chosen based on its ability to generate a C-terminal thioester suitable for native chemical ligation after cleaving the intein tag using MESNA. It also contains a C-terminal Chitin-Binding Domain, which facilitates purification. To allow expression of this protein in bacteria, we also added a methionine codon to the 3' end of the DNA segment encoding A β ₁₋₂₉. As we show below, it is possible to cleave this extra Met residue from A β ₁₋₂₉-MESNA using CNBr, since the MESNA thioester is stable to the acidic conditions under which CNBr cleavage is performed. DNA encoding A β ₁₋₂₉ was cloned using the NdeI and SapI sites. The vector alone shows a high rate of self-ligation, probably due to incomplete digestion by one of the restriction enzymes; for this reason, it was necessary to dephosphorylate the vector using calf intestinal alkaline phosphatase. Sequencing verified that Met-A β ₁₋₂₉ was correctly positioned into the vector.

Expression of Met-A β ₁₋₂₉-Intein-CBD

The induction conditions were optimized prior to large-scale expression. Various temperatures and times were used to determine the optimal induction condition for production of the Met-A β ₁₋₂₉ fusion protein. These included 37 °C for 3 and 6 hours, 30 °C for 3 hours and overnight at 15 °C or 20 °C. As shown in Figure 2, there was a prominent band with the appropriate molecular weight of 31 kD. Induction with 1 mM IPTG overnight at 15-20 °C gave the best expression of all sets of conditions tested. An examination of the centrifuged cell lysates by SDS-PAGE showed that the protein was abundant in the soluble fraction, and it was therefore not necessary to solubilize the protein out of inclusion bodies. From Coomassie blue staining of Tris-glycine SDS-polyacrylamide gels, we estimated that approximately 133 mg of Met-A β ₁₋₂₉ with the Chitin-Binding Domain was expressed in one liter of the cell lysate (Table 1). This is in contrast to previous reports in which the majority of the expressed recombinant protein was present in the insoluble fraction that needed to be solubilized with urea or guanidine hydrochloride before purification [7,8].

Purification of Met-A β ₁₋₂₉-Intein-CBD and Cleavage with MESNA

A typical purification of the expressed fusion protein is shown in Figure 3. Cleavage of the intein and elution with MESNA containing buffer yields a protein that is fairly pure ($\approx$ 90% pure by SDS-PAGE), and therefore still requires further purification by RP-HPLC. A typical HPLC trace of the purification (Figure 4) shows two peaks eluting at approximately 21 minutes. Mass spectrometric analysis showed that the first peak is Met-A β ₁₋₂₉ and the second peak is Met-A β ₁₋₂₉-MESNA. From these purifications, it was determined that the MESNA cleavage was approximately 50% efficient (Table 1). Although this procedure yielded sufficient protein for our purposes, it is possible that longer incubation times or a higher concentration of MESNA might have yielded more protein from the Chitin beads.

The identity of the isolated products as containing A β sequence was demonstrated by Western blotting, using an antibody to full-length β -amyloid peptide (Figure 5). The presence of two bands in the SDS-PAGE gels arises because of partial hydrolysis of the thioester of Met-A β_{1-29} -MESNA at the alkaline pH (8.80) under which samples are prepared for SDS-PAGE. The HPLC tracing shows that there is less hydrolysis of this thioester – approximately 35% – in the HPLC tracing than in the SDS-PAGE analysis. Thus, during the lysis, intein cleavage, and elution of Met-A β_{1-29} -MESNA from the Chitin beads, where the pH is maintained close to neutrality, thioester cleavage is not an insurmountable problem. It might be possible to decrease spontaneous hydrolysis of the thioester by maintaining the pH below 7 during intein cleavage and elution, but this lower pH would also inhibit intein cleavage by MESNA, and therefore would not result in a significantly higher yield of Met-A β_{1-29} -MESNA from the column. For these reasons, we concluded that pH 7 was optimal for intein cleavage and elution of Met-A β_{1-29} -MESNA from the column. The yield and purity of Met-A β_{1-29} -MESNA from purification on the intein beads is shown in Table 1.

CNBr cleavage of Met-A β_{1-29} -MESNA

CNBr cleavage was used to remove the N-terminal Met residue from Met-A β_{1-29} -MESNA. The reaction mixture was analyzed at various times to determine the point at which CNBr cleavage was complete. An analytical RP-HPLC tracing of the reaction mixture (Figure 6) shows a major peak with an elution time of approximately 22 minutes, and a shoulder that is present at time 48h, and remains apparent and of the same size at 72 h. MALDI-TOF mass spectrometry (Supplemental Figure 1) showed that the major peak is A β_{1-29} -MESNA (m/z = 3444.5, expected = 3442.72), while the shoulder is oxidized Met(O)-A β_{1-29} -MESNA (m/z = 3465.6, expected = 3466.76) and Met(O₂)-A β_{1-29} -MESNA (m/z = 3482.7, expected = 3482.76). MALDI-TOF mass spectrometry revealed Met(O)-A β_{1-29} , and small amounts of A β_{1-29} (m/z = 3320.7, expected = 3319.56), but no Met-A β_{1-29} or Met-A β_{1-29} -MESNA. Thus, the major impurities in the crude sample were due to Met oxidation, and not cleavage of the MESNA thioester, which appeared to be quite stable to these acidic conditions. From the HPLC tracing, the yield of A β_{1-29} -MESNA from CNBr cleavage of Met-A β_{1-29} -MESNA was estimated to be $\approx$ 80% (Table 1). Because of the stability of the MESNA thioester to acid, it might be possible to perform the reaction using 70-80% formic acid, and have the reaction proceed more quickly.

Native Chemical Ligation and Selective Desulfurization

As shown in Figure 7, native chemical ligation of A β_{1-29} -MESNA (or A β_{1-29} -thiophenolate) to A30C-A β_{30-40} proceeds extremely rapidly and efficiently. Analytical RP-HPLC shows a peak eluting at 25.87 minutes (Figure 7), which MALDI-TOF mass spectrometry confirmed was A30C-A β_{1-40} (m/z = 4367.5, expected = 4361.9). The elution time for this product was well separated from that for either A β_{1-29} -MESNA or A30C-A β_{30-40} (15.57 and 23.19 minutes, respectively).

Selective desulfurization of A30C-A β_{1-40} was performed using Raney Ni, as previously described (24), which results in conversion of the Cys residue into Ala, and yields the final product, A β_{1-40} . As shown in Figure 8, RP-HPLC chromatogram at time zero and one hour into the reaction shows a peak shift from 25.76 to 25.24 minutes, which MALDI-TOF mass spectrometry confirmed to be A β_{1-40} , i.e., with the correct Ala residue at position 30. The overall yield of A β_{1-40} was 1.43 mmol/L (5 mg/L), starting from 2.27 mmol/L (133 mg/L) of crude protein in one liter of culture broth, or 63.0 % of the starting material on a molar basis.

Cloning and Expression of Met-A β ₁₋₂₉-Intein-CBD in Minimal Medium

Since our goal is to use this procedure to produce isotopically labeled A β peptides for NMR studies, it was of interest to determine expression levels of the Met-A β ₁₋₂₉-Intein-CBD construct in minimal media, which contain no amino acids, as compared to the LB broth that is used routinely. As shown in Supplemental Figure 2A, expression of the Met-A β ₁₋₂₉-Intein-CBD fusion protein is slightly lower in minimal medium than LB broth, but the band is still visible, indicating robust expression in this medium.

Cloning and Expression of Met-D23N-A β ₁₋₂₉-Intein-CBD

To examine the feasibility of extending this procedure to point mutant forms of A β , and possibly other variants of A β peptides, we performed site directed mutagenesis to obtain a construct with the Iowa mutation, D23N [32-34], i.e., D23N-A β ₁₋₂₉ expressed as part of a Met-D23N-A β ₁₋₂₉-Intein-CBD fusion protein. This required altering a nucleotide in GAT, the codon for D23 of A β ₁₋₂₉, to AAT for N at the same position. Site directed mutagenesis was performed using a Quickchange II XL Site-Directed Mutagenesis Kit (Stratagene), which yielded colonies containing the desired substitution. Plasmid DNA was sequenced, and confirmed both the T to A mutation, and the correct alignment of D23N-A β ₁₋₂₉ in the pTXB1 vector. As shown in Supplemental Figure 2B, expression of the Met-D23N-A β ₁₋₂₉-Intein-CBD fusion protein is similar to that of the fusion protein containing the wild-type A β ₁₋₂₉.

DISCUSSION

In this paper, we present an efficient semi-synthetic method for producing native sequence A β ₁₋₄₀ that does not require any sequence substitutions, and does not yield undesired modifications in side chains, such as Met oxidation. This procedure centers on native chemical ligation of A β ₁₋₂₉-MESNA, containing MESNA as a C-terminal thioester, with A30C-A β ₃₀₋₄₀. After native chemical ligation, desulfurization using Raney Ni converts Cys to Ala, without modification of Met 35, yielding the native sequence of A β ₁₋₄₀. In addition, we demonstrated expression of a variant of the construct, Met-D23N-A β ₁₋₂₉-Intein-CBD, which will be used in the future for production of D23N-A β ₁₋₄₀, the Iowa mutant. Furthermore, we showed that expression of the peptide occurs in minimal medium, suggesting that it would be feasible to produce isotopically labeled A β peptides. Thus, the procedure is a versatile one, that we anticipate will be able to produce a variety of A β peptides, possibly also including A β peptides of other chain lengths.

The yield of Met-A β ₁₋₂₉-Intein-CBD expressed in BL21DE3 cells was approximately 133 mg of protein per liter of culture medium. Following cleavage and affinity purification, 10 mg of Met-A β ₁₋₂₉-MESNA was obtained, which represents a yield of $\approx$ 50% on a molar basis. The majority of the protein loss is not due to its insolubility, but rather, to the combination of incomplete cleavage of fusion protein on the Chitin beads, and sensitivity of the MESNA thioester to alkaline pH. These losses occur at tolerable levels at pH 7.00, and which MESNA cleavage of the intein and elution of Met-A β ₁₋₂₉-MESNA from the Chitin beads is performed. SDS-PAGE, which is performed at a higher pH (8.8) overestimates the extent of this loss; as stated above, analytical RP-HPLC gives a more accurate picture. The subsequent parts of this procedure – CNBr cleavage of the N-terminal Met residue, native chemical ligation of A β ₁₋₂₉-MESNA to Cys30Ala-A β ₃₀₋₄₀ and selective desulfurization to convert Cys30Ala-A β ₁₋₄₀ to native sequence A β ₁₋₄₀ – all occur with very high efficiency, generally > 90% yield at each step.

The advantage of this procedure over a purely synthetic method of making A β peptides is that it can readily be adapted for the production of isotopically labeled peptides for NMR

studies and other biophysical or biological techniques requiring isotopically labeled peptide. For example, efficient methods exist for producing selectively or uniformly labeled proteins, simply by incubating bacteria in ^{15}N - or ^{13}C -labeled sources (e.g., $^{15}\text{N-NH}_4^+$, and $^{13}\text{C-HCO}_3^-$ or ^{13}C -glucose, respectively; see references [35-40] as examples). These methods lead to high levels of labeled proteins, and do not in general impede bacterial growth or protein expression. In addition, this technique is readily adaptable for the production of point mutant forms of A β , as we have shown with the example of D23N-A β (1-40), the Iowa Mutant form of this peptide, associated with one form of Familial Alzheimer's Disease and Cerebral Amyloid Angiopathy [32-34]. The combination of bacterial expression with a short synthetic sequence allows for flexibility in the selection of C-termini for study, and avoids the problems, described above, inherent in expression of full length A β_{1-40} . This method also offers the additional advantage for NMR of simplifying the spectra whenever this is desired, e.g., of peptide uniformly labeled only in residues 1-29 rather than all 40 residues. The synthetic portion of A β_{1-40} can, of course, also be labeled by traditional synthetic methods. Perhaps most importantly, the semi-synthetic method offers the distinct advantage in far less expense of supplying simple labels, such as $^{15}\text{N-NH}_4^+$ or $^{13}\text{C-HCO}_3^-$ as compared with incorporating labeled amino acids into synthetic peptides.

Supplementary Material

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Acknowledgments

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Figure 1.

Scheme for semi-synthetic production of native sequence A β_{1-40} . DNA encoding A β_{1-29} with an N-terminal extension of a single amino acid, Met, is inserted into the pTXB1 vector for expression in *E. coli* (BL21DE3 cells). After induction with IPTG, the fusion protein is expressed at high levels. The fusion protein consists of Met-A β_{1-29} in a thioester bond to an intein segment, and a C-terminal Chitin-Binding Domain (CBD). This allows for affinity purification of the fusion protein using Chitin beads. Met-A β_{1-29} is cleaved from the Intein-CBD segment and eluted from the bead using MESNA in buffer, which yields Met-A β_{1-29} -MESNA, i.e., with MESNA in a thioester linkage to Met-A β_{1-29} . The next step is CNBr cleavage of the N-terminal Met residue; the thioester is stable to the acidic conditions under

which this is performed. The recombinant A β ₁₋₂₉-MESNA is then linked to A30C-A β ₃₀₋₄₀ by native chemical ligation. The final step is selective desulfurization, using Raney Ni, which yields full length, native sequence A β ₁₋₄₀.

Figure 2.

Expression of Met-A β ₁₋₂₉-Intein-CBD fusion protein under various growth conditions. The figure shows SDS-PAGE of expressed protein, with a prominent band at the appropriate molecular weight of 31 kD. Results are shown for the follow induction conditions: (B) 15 °C, 18 h; (C) 30 °C, 3 h; (D) 37 °C, 6 h; (E) 37 °C, 3 h. (A) shows molecular weight markers and (F) shows the uninduced control. The gel is 12% polyacrylamide and the gel buffer system is Tris-glycine SDS [41]. (F) shows an uninduced bacterial lysate and confirms that the expression of the fusion protein had been induced.

Figure 3.

Affinity purification and MESNA cleavage of Met-A β ₁₋₂₉-Intein-CBD fusion protein. Met-A β ₁₋₂₉-Intein-CBD was allowed to bind to Chitin beads, and the beads were washed with MESNA, as described in Methods. The figure shows 16.5% SDS-PAGE, electrophoresed using Tris-Tricine-SDS buffer [42]. (A) shows molecular weight markers. (B) shows the lysate of IPTG-induced bacterial cells, after centrifugation to remove insoluble material. A prominent band is present at the appropriate molecular weight of 31 kD. (C) shows protein remaining in the cell lysate supernatant after incubation with Chitin beads; little or none of the induced protein remains in the solution. (D) shows the eluent after treatment of the beads with MESNA. The main band has a molecular weight of 27 kD, consistent with released Intein-Chitin fusion protein. The other product of this reaction, as shown by HPLC and mass spectrometry, is Met-A β ₁₋₂₉-MESNA. This is not seen on the gel. (E) shows the same solution as (D), after purification of Met-A β ₁₋₂₉-MESNA by RP-HPLC, and lyophilization, resulting in a 50-fold concentration of this protein to allow for visualization by SDS-PAGE.

Figure 4.

RP-HPLC of products of MESNA cleavage of Met-A β ₁₋₂₉-Intein-CBD fusion protein. The largest peak was shown by mass spectrometry to be Met-A β ₁₋₂₉-MESNA. The other main peak in the profile is Met-A β ₁₋₂₉; this peak also contains a shoulder with methionine oxidation products. It is likely that some hydrolysis of the thioesters (with Intein-CBD and/or MESNA) occurs during the incubation of the beads with buffer at pH 7.00.

Figure 5.

Western blot of protein eluted from the Chitin column with MESNA-containing buffer. Lane 1 shows two bands, with molecular weights consistent with Met-A β ₁₋₂₉-MESNA and Met-A β ₁₋₂₉. The Western blot was developed using the 6E10 antibody from Santa Cruz Antibodies; this antibody recognizes full length A β , as well as A β ₁₋₂₉. The intensity of the bands is roughly equal. This is in contrast to the data shown in Figure 4, where the HPLC profile shows the A β ₁₋₂₉-MESNA peak to be larger than that for A β ₁₋₂₉, suggesting that additional lysis of the thioester occurs in the SDS-PAGE buffers at pH 8.80.

Figure 6.

RP-HPLC of products of CNBr cleavage of Met-Aβ₁₋₂₉-MESNA. The main peak, eluting at approximately 22 minutes, was shown by mass spectrometry to be Aβ₁₋₂₉-MESNA. The large shoulder contains other peptides, mainly ones with oxidized methionine. Little or no Met-Aβ₁₋₂₉ was present, and in general, the MESNA thioester was resistant to hydrolysis under these acidic conditions.

Figure 7. Native Chemical Ligation of $A\beta_{1-29}$ -MESNA or $A\beta_{1-29}$ -thiophenol (A) with Cys30Ala- $A\beta_{30-40}$ (B). The figure shows RP-HPLC tracings using gradients described in Methods. Native chemical ligation proceeds rapidly and can be performed with either $A\beta_{1-29}$ -MESNA from recombinant protein or $A\beta_{1-29}$ -thiophenol from synthetic peptide. The reaction product (C) was shown to be A30C- $A\beta_{1-40}$ by mass spectrometry.

Figure 8.

Selective desulfurization of A30C-A β_{1-40} . The figure shows analytical RP-HPLC as described in Methods. Two traces are shown, immediately after addition of Raney Ni (t = 0 h, solid line) and after incubation of the peptide with Raney Ni for one h (t = 1 h, dotted line). Mass spectrometry showed that A30C-A β_{1-40} had been converted to full length, native sequence A β_{1-40} .

Table 1
Expression and Purification of Semi-Synthetic A β ₁₋₄₀

Purification step	Yield (mg)	Yield (mmol)	Purity
Cell lysate	133 ^a	4.93	Not determined
MESNA elution	10 ^b	2.90	50% ^c
CNBr cleavage	9 ^c	2.61	90% ^c
Native Chemical Ligation and Desulfurization	8.5 ^c	2.47	> 90% ^c

^a Estimated from Coomassie blue stained SDS-PAGE gel against BSA standards.

^b Determined using Nanodrop.

^c Determined using HPLC.