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Novel Purification Process for Amyloid Beta Peptide(1-40)

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Received: 12 March 2020; Accepted: 8 April 2020; Published: 15 April 2020

Abstract: Amyloid beta peptide (Aβ)-related studies require an adequate supply of purified Aβ peptide. However, Aβ peptides are "difficult sequences" to synthesize chemically, and low yields are common due to aggregation during purification. Here, we demonstrate an easier synthesis, deprotection, reduction, cleavage, and purification process for Aβ(1-40) using standard 9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids and solid-phase peptide synthesis (SPPS) resin [HMBA (4-hydroxymethyl benzamide) resin] that provides higher yields of Aβ(1-40) than previous standard protocols. Furthermore, purification requires a similar amount of time as conventional purification processes, although the peptide must be cleaved from the resin immediately prior to purification. The method described herein is not limited to the production of Aβ(1-40), and can be used to synthesize other easily-oxidized and aggregating sequences. Our proposed methodology will contribute to various fields using "difficult sequence" peptides, such as pharmaceutical and materials science, as well as research for the diagnosis and treatment of protein/peptide misfolding diseases.

Keywords: peptide synthesis; reduction; purification; amyloid beta peptide; solid-phase synthesis; difficult sequence; aggregating peptide

1. Introduction

Protein misfolding diseases are increasingly common in aging populations and include Alzheimer's disease, Parkinson's disease, and Huntington's disease. These diseases involve the systematic or tissue-localized deposition of fibrillar, β-sheet-rich protein/peptide assemblies [1,2]. In Alzheimer's disease (AD) [3,4], amyloid beta peptide (Aβ) forms fibrillar aggregates known as amyloid fibrils [5,6], which are the principal component of extracellular deposits and are the likely causative agent of AD. Many scientists have investigated Aβ aggregation, but the mechanism by which Aβ aggregates in vivo, and how the generated aggregates affect disease development, remains unknown. Recent studies have implicated Aβ and its ability to self-assemble as key factors in the pathogenesis of AD. Aβ-related studies require an adequate supply of purified Aβ peptide. However, Aβ peptides are "difficult sequences" to synthesize chemically, and low yields are common due to aggregation during purification [7–10]. To address this, Mutter et al. developed pseudoproline building blocks that are dipeptide derivatives containing serine (Ser)/threonine (Thr)-derived oxazolidines or cysteine (Cys)-derived thiazolidine [11]. Sheppard et al. also reported a building block, 2-hydroxy-4-methoxybenzyl (Hmb), as a protecting group for the backbone amide nitrogen [12]; Martin et al. optimized and applied this method to Aβ(1-43) [13]; and very recently Kasim et al. used Hmb as a linker for preparation of Aβ(1-42) [14]. Sohma et al. developed a method involving the O–N intramolecular acyl migration reaction of O-acyl isopeptides. O-acyl isopeptides improve both peptide solubility in various media and the nature

of the difficult sequence during solid-phase peptide synthesis (SPPS) [15–17]. All these innovative studies provided efficient strategies towards the synthesis of amyloid beta peptide, but an easy, simple, low-cost, and versatile method is still needed.

Here, we demonstrate an easier synthesis, deprotection, reduction, cleavage, and purification process for Aβ(1-40) using standard 9-fluorenylmethyloxycarbonyl (Fmoc)-protected amino acids and SPPS resin that provides higher yields of Aβ(1-40) than previous standard protocols (Figure 1). This process is applicable not only to the Aβ(1-40) sequence but also to other easily oxidized, aggregating sequences. Our proposed methodology will contribute to various fields using "difficult sequence" peptides, such as pharmaceutical and materials science, as well as research for the diagnosis and treatment of protein/peptide misfolding diseases.

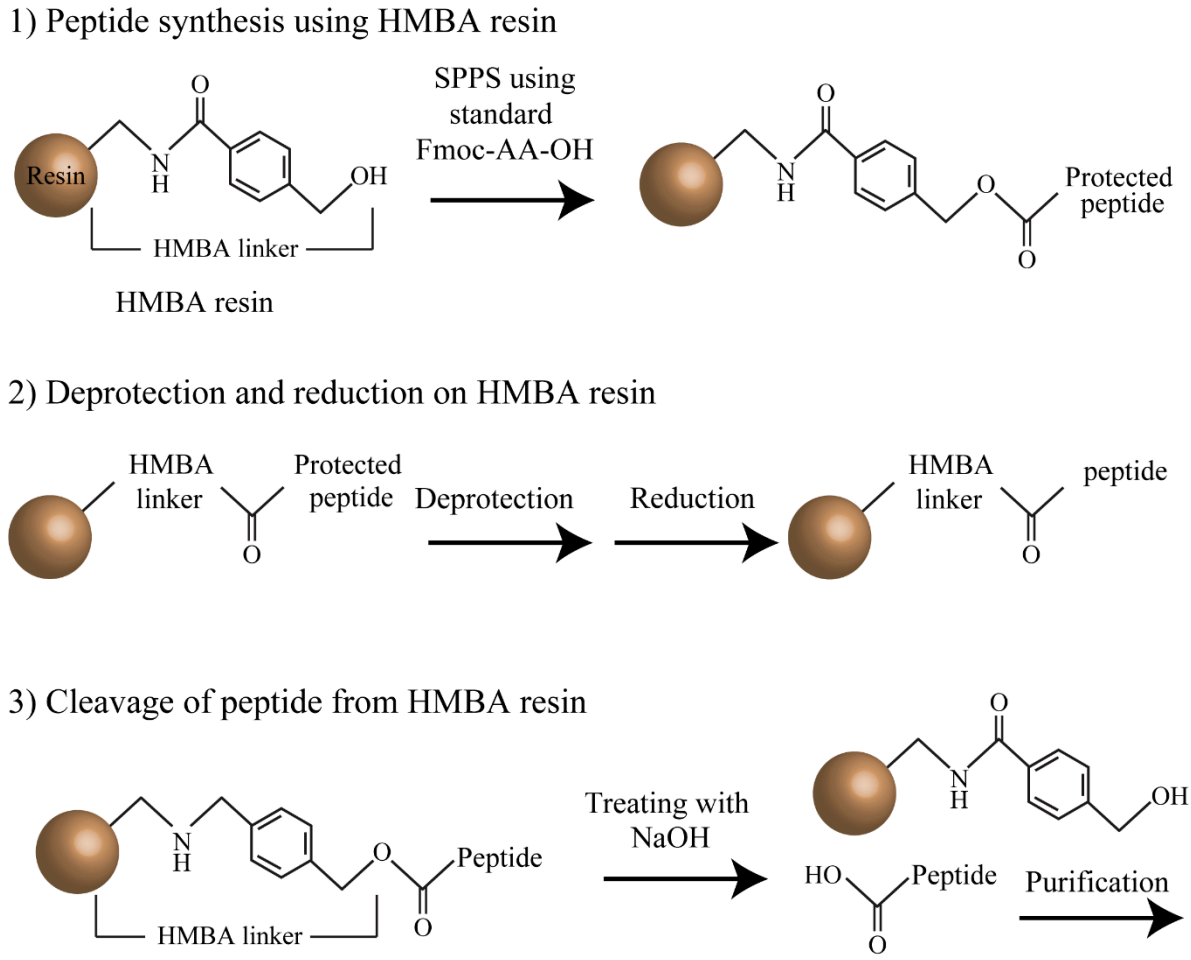

Figure 1. Scheme of the peptide synthesis, deprotection, reduction, cleavage, and purification process. **1**) After attaching the first amino acid to 4-hydroxymethyl benzamide (HMBA) resin by standard ester coupling, standard Fmoc peptide synthesis was conducted using the (HBTU)–1-hydroxy benzotriazole monohydrate (HOBt) method. **2**) Deprotection of all of the side-chain-protected peptide was conducted using the standard trifluoroacetic acid (TFA) method. Then some oxidized peptides were reduced using NH_4I and dimethyl sulfide (DMS). **3**) Cleavage of the peptide from HMBA resin using 500 mM NaOH and acetonitrile (1/1, v/v).

2. Results and Discussion

2.1. Fmoc Peptide Synthesis Using HMBA-Resin

We selected HMBA (4-hydroxymethyl benzamide) resin [18]. HMBA is a commonly used base-labile linker that allows the deprotection of the peptide with TFA without cleaving the peptide

from the resin. This allows the deprotected peptide to subsequently be cleaved quickly using a low concentration of aqueous base. The purity of the cleaved peptide is usually very high, and purification is frequently not required prior to characterization and use. Aβ(1-40) easily aggregates in acidic conditions but remains monomeric or oligomeric in basic conditions. A NaOH-based protocol for the preparation of Aβ(1-40) monomer was recently reported and has been widely adapted [19]. We thus used aqueous NaOH to both cleave Aβ(1-40) from the resin and keep it monomeric, allowing facile purification.

After attaching the first amino acid (valine) to HMBA resin by standard ester coupling, Fmoc peptide synthesis was conducted using the (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU)–1-hydroxy benzotriazole monohydrate (HOBt) method (10 eq., double coupling, see Section 3.2) (Figure 1). Although we adopted this method in order to synthesize the peptides certainly, we must improve the conditions such as equivalents in future studies. Following synthesis, the peptide was deprotected using the standard TFA method (see Section 3.3), then the peptide was cleaved with aqueous NaOH/acetonitrile (1/1, v/v). Acetonitrile is required to prevent nonspecific adsorption of Aβ(1-40) to the resin, thus providing higher yields (see Section 3.4). Peptide purity was checked by high performance liquid chromatography (HPLC) and matrix assisted laser desorption ionization (MALDI)—time of flight (TOF) mass spectrometry (MS) (see Section 3.6). The HPLC chart showed two main peaks, reduced Aβ (1-40) and oxidized Aβ (1-40), indicating that the methionine of Aβ(1-40) is partially oxidized to methionine sulfoxide during synthesis and deprotection (Figure 2a). The NaOH cleavage and monomerization method using HMBA resin was much easier than a conventional method using Wang resin (Figure S2), which needed a monomerization step using guanidine hydrochloride (GdnHCl) (see Section S1.3 in SI). The GdnHCl monomerization step takes 1 day, and GdnHCl can damage an HPLC column. Peptide reduction before cleavage is thus indispensable for obtaining higher yields of Aβ(1-40) in our method.

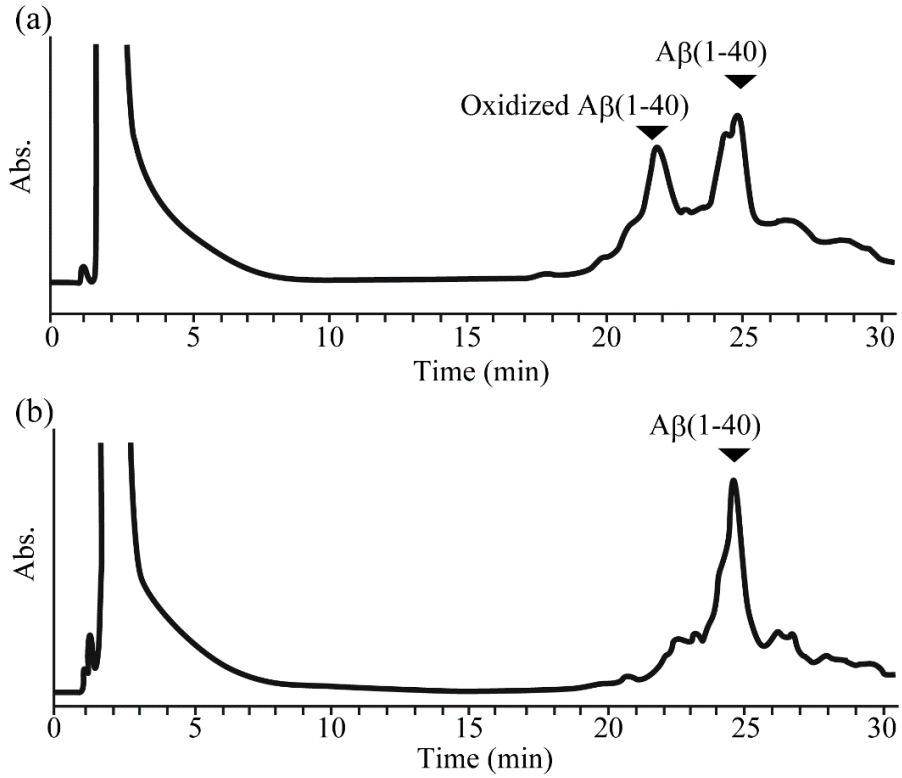

Figure 2. (**a**) HPLC chart of the cleaved Aβ(1-40) from HMBA resin without the reduction process. (**b**) HPLC chart of the cleaved Aβ (1-40) from HMBA resin with the reduction process. The peaks were characterized by MALDI-TOF MS: the oxidized Aβ(1-40), m/z 4346.9 ($(M+H)^+$ calcd. 4346.6); Aβ(1-40), m/z 4330.8 ($(M+H)^+$ calcd. 4330.6).

2.2. Reduction of Oxidized Aβ(1-40) and Aβ(17-40) on the Resin

We reduced methionine sulfoxide [20] after the deprotection step (Figure 1, see Section 3.5). NH_4I (30 eq.) and dimethyl sulfide (DMS, 30 eq.) in TFA/Milli-Q (7/3, v/v) were added to an ice-cooled column containing deprotected peptidyl resin. Keeping the column at 0 °C with ice, a half-volume of mercaptoacetic acid (TFA-Milli-Q/mercaptoacetic acid = 2/1, v/v) was added, which was indispensable for preventing from the generation of histidine iodide (Figure S1). After 30 min at 0 °C, the peptide was cleaved from the resin (see Section 3.4). The purity of the peptide was checked by HPLC and MS (see Section 3.6). The HPLC chart showed one main peak, corresponding to reduced Aβ(1-40), indicating that this reduction step was successful and efficiently provided a higher yield of the target peptide Aβ(1-40) (Figure 2b).

In addition, we also demonstrated the reduction of a sequence without histidine, Aβ(17-40) (Figure 3a, see Section 3.5). In this case, the addition of mercaptoacetic acid was not necessary. The reduction of Aβ(17-40) without this step was conducted. The HPLC chart (see Section 3.6) showed one main peak, corresponding to reduced Aβ(17-40), indicating that this reduction step was successful and efficiently provided a higher yield of the target peptide Aβ(17-40) (Figure 3b).

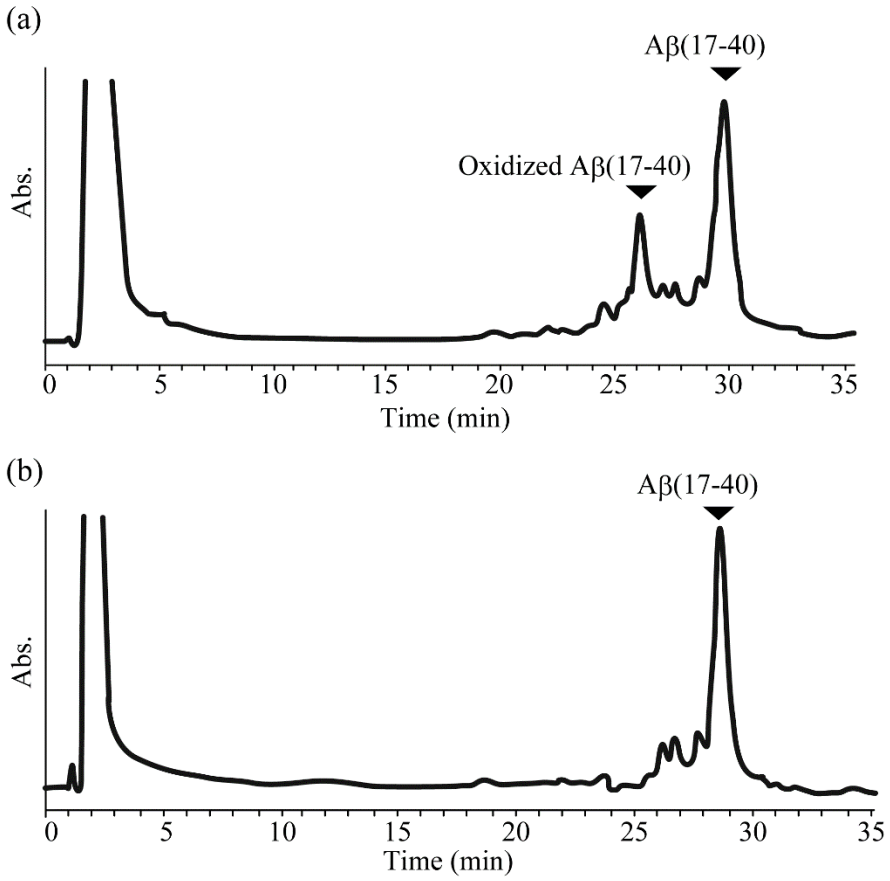

Figure 3. (**a**) HPLC chart of the cleaved Aβ(17-40) from HMBA resin without the reduction process. (**b**) HPLC chart of the cleaved Aβ(17-40) from HMBA resin with the reduction process not using mercaptoacetic acid. The peaks were characterized by MALDI-TOF MS: the oxidized Aβ(17-40), m/z 2409.9 ($(M+H)^+$ calcd. 2409.7); Aβ(17-40), m/z 2393.6 ($(M+H)^+$ calcd. 2393.7).

2.3. ThT Assay of Aβ (1-40) Cleaved from HMBA Resin

We checked if Aβ(1-40) aggregated after cleavage by fluorescence measurements using thioflavin T (ThT) (Figure 4) (see Section 3.7). ThT fluorescence originates only from binding to amyloid fibrils. It is widely assumed that the fluorescent increase of ThT is due to the selective immobilization of

a subset of ThT conformers [21]. The fluorescence intensity of ThT did not increase after cleavage, whereas the sample of dissolved Aβ(1-40) powder with 250 mM NaOH for monomerization showed higher fluorescence intensity of ThT than that after cleavage from HMBA resin with NaOH by our method. These results indicated that Aβ(1-40) was completely monomerized on the HMBA resin, and that Aβ(1-40) aggregation was inhibited by the basic conditions. This suggests that purification would be easier under basic rather than standard acidic cleavage conditions.

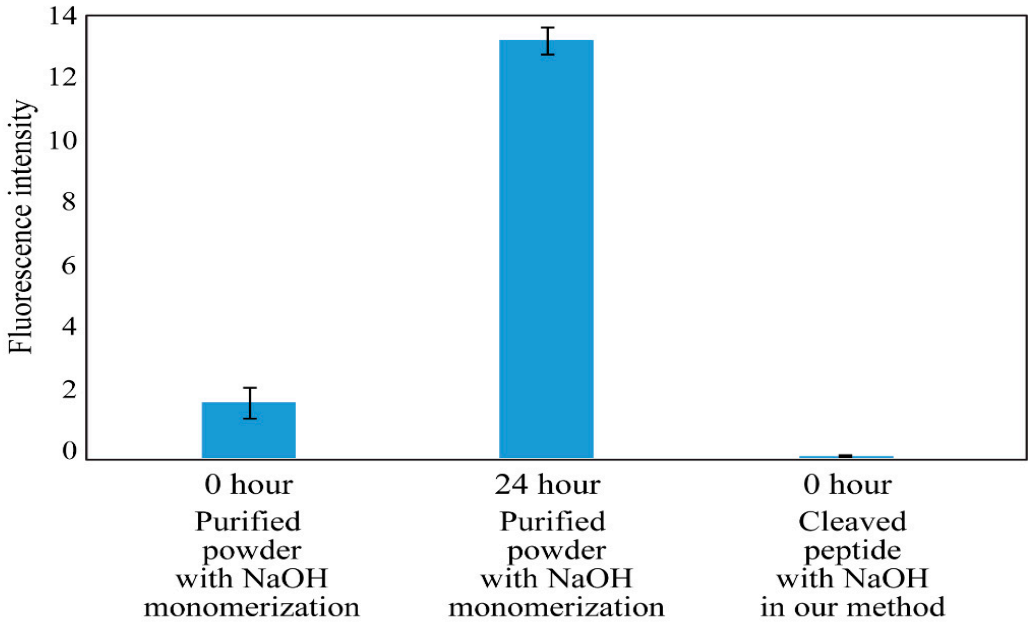

Figure 4. Thioflavin T (ThT) assay with Aβ (1-40) sample, 0 h or 24 h after preparation by dissolving the lyophilized powder in 250 mM NaOH for monomerization, and 0 h after cleavage from HMBA resin with NaOH by our method.

2.4. Optimization of the Purification Process of Aβ(1-40)

We then optimized the purification protocol to obtain higher yields and facilitate handling. Following peptide synthesis, an aliquot of the resin was deprotected, reduced, cleaved, and immediately purified (Figure 1). After deprotection and reduction, resin sufficient for one HPLC injection was cleaved in a 1.5 mL tube to prevent Aβ aggregation. Cleavage required 20 min, and thus the cleaved crude peptide solution was ready for injection on the HPLC (see Section 3.8) as soon as the previous injection of peptide had eluted from the C-18 column. The optimized batch purification process took a similar amount of time as the conventional purification process, although each injection required a separate cleavage step. In our method, the total yield was 3.6% despite a small scale in this time, and we could not purify the sample cleaved from the Wang resin because it was hard to separate the second peak completely. Additionally, the NaOH cleavage and monomerization method using HMBA resin was much easier than the conventional method using Wang resin, which needed a 1-day monomerization step using GdnHCl, which can damage a column. Thus, our methodology would offer easier handling and obtain higher yields of Aβ(1-40) than previous standard methodology.

3. Materials and Methods

3.1. Materials

All chemicals were used without further purification. The peptides were characterized by MALDI-TOF MS on an Autoflex III mass spectrometer (Bruker Daltonics, Billerica, MA, USA) using 3,5-dimethoxy-4-hydroxycinnamic acid as the matrix.

3.2. Fmoc Peptide Synthesis of Aβ(1-40) and Aβ(17-40) Using HMBA Resin

Aβ(1-40) and Aβ(17-40) peptides were synthesized using HMBA (4-hydroxymethyl benzamide)-PEG resin (HiPep Laboratories Co., Ltd., Kyoto, Japan) by Fmoc solid-phase synthesis [22] using the (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate) (HBTU)–1-hydroxy benzotriazole monohydrate (HOBt) method (10 eq., double coupling). The DIPCI (*N*,*N*′-Diisopropylcarbodiimide)—DMAP (*N*,*N*-dimethyl-4-aminopyridine) method was used for the first residue (valine). The side-chain-protecting groups used were t-butyloxy carbonyl (Boc) for Lys; 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for arginine (Arg); t-butyl (tBu) for serine (Ser), tyrosine (Tyr), aspartic acid (Asp), and glutamic acid (Glu); and trityl (Trt) for histidine (His), asparagine (Asn), and glutamine (Gln).

3.3. Deprotection of Aβ(1-40) and Aβ(17-40) Using HMBA Resin

The side-chain-protecting groups on the resins were removed by incubating the peptide-resin for 2 h in deprotection solution (trifluoroacetic acid (TFA)/triisopropylsilane/water (90/5/5, v/v)). The resin was washed 5 times with the deprotection solution and chloroform, and then the resins were dried completely in a desiccator. The peptide-resin was stored at 4 °C.

3.4. Cleavage of Aβ(1-40) and Aβ(17-40) from HMBA Resin

When the peptide-resin was dried, the resin was swelled by acetonitrile overnight. The swelled resin was treated with 500 mM NaOH and acetonitrile (1/1, v/v) for 20 min at room temperature.

3.5. Reduction of Oxidized Aβ(1-40) and Aβ(17-40) on the Resin

In the case of Aβ(1-40), the peptide-resin was incubated in TFA/Milli-Q (7/3, v/v) with DMS (30 eq.) and NH_4I (30 eq.) with ice cooling at 0 °C. Keeping the column at 0 °C with ice, a half-volume of mercaptoacetic acid (TFA-Milli-Q/mercaptoacetic acid = 2/1, v/v) was added to the reaction solution, and the resin was incubated for 30 min at 0 °C. After the reduction reaction, the resin was washed 10 times with TFA and Milli-Q.

In the case of Aβ(17-40), the peptide-resin was incubated in TFA/Milli-Q (7/3, v/v) with DMS (30 eq.) and NH_4I (30 eq.) with ice cooling at 0 °C. The resin was incubated for 30 min at 0 °C. After the reduction reaction, the resin was washed 10 times with TFA and Milli-Q.

3.6. Analysis of Aβ(1-40) and Aβ(17-40)

The HPLC was performed on the GL7410 pump and GL7450 detector system (GL Sciences Inc., Tokyo, Japan) using 220 nm absorbance. Aβ(1-40) was analyzed on a Shodex Asahipak ODS-50 column (4.5 × 150 mm, Showa Denko K.K., Tokyo, Japan) using an isocratic condition with 100% of A solvent over 5 min and then a linear gradient from 0% to 25% of B solvent (90% acetonitrile, 10% Milli-Q, and 0.1% NH_4OH) over 25 min at a flow rate of 1.0 mL/min. Aβ(17-40) was analyzed on a Shodex Asahipak ODS-50 column (4.5 × 150 mm, Showa Denko K.K) using an isocratic condition with 100% of A solvent over 5 min, and then a linear gradient from 0% to 30% of B solvent (90% acetonitrile, 10% Milli-Q and 0.1% NH_4OH) over 30 min at a flow rate of 1.0 mL/min.

3.7. Thioflavin T (ThT) Fluorescence Assay

Samples (20 μL) were diluted in phosphate buffer (final concentration: 50 mM sodium phosphate and 300 mM NaCl, pH 7.5) containing ThT (final concentration: 25 μM, total volume 100 μL), and fluorescence was measured (excitation at 450 nm, emission at 492 nm) using a fluorescence microplate reader (MTP-601, Corona Electric Co., Ltd., Hitachinaka, Japan) [8].

3.8. Purification of Aβ (1-40)

The HPLC was performed on the GL7410 pump and GL7450 detector system (GL Sciences) using 220 nm absorbance. After the reduction (see Section 3.5), the peptide was purified on a Shodex Asahipak ODS-50 column (10 × 250 mm for purification, Showa Denko K.K) using an isocratic condition with 100% of A solvent over 5 min, and then using a linear gradient from 0% to 30% of B solvent (90% acetonitrile, 10% Milli-Q and 0.1% NH_4OH) over 30 min at a flow rate of 3.0 mL/min.

4. Conclusions

We demonstrated easier synthesis, deprotection, reduction, cleavage, and purification processes for Aβ(1-40) using standard Fmoc-protected amino acids and a common SPPS resin, HMBA resin. In our process, we could use aqueous NaOH to both cleave Aβ(1-40) from the resin and keep it monomeric, allowing facile purification. Thus, our methodology provided higher yields of Aβ(1-40) than previous standard protocols. Additionally, the optimized purification process took a similar amount of time as the conventional purification process, although the peptide must be cleaved from the resin immediately prior to purification. The method described herein is not limited to the production of Aβ(1-40), and can be used to synthesize other easily-oxidized and aggregating sequences. Our proposed methodology will contribute to various fields using "difficult sequence" peptides, such as pharmaceutical and materials science, as well as research for the diagnosis and treatment of protein/peptide misfolding diseases.

Supplementary Materials: The following are available online at http://www.mdpi.com/2227-9717/8/4/464/s1, the additional materials and methods, Figure S1: HPLC chart of Aβ(1-40) cleaved from HMBA resin after the reduction process without mercaptoacetic acid, Figure S2: HPLC chart of Aβ(1-40) by conventional peptide synthesis using wang resin.

Author Contributions: Conceptualization, K.U. and Y.H.; methodology, K.I., S.-i.Y. and Y.H.; writing—original draft preparation, K.U. and S.-i.Y.; writing—review and editing, K.U., S.-i.Y. and Y.H. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by The Nipponham Foundation for the Future of Food (NFFF), and KU is grateful for KAKENHI Grant Number 19K05741 from the Japan Society for the Promotion of Science (JSPS).

Conflicts of Interest: The authors declare no conflict of interest.

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