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Synthesis of Aβ(1-42) and its derivatives with improved efficiency

MÁRTA ZARÁNDI,* KATALIN SOÓS, LÍVIA FÜLÖP, ZSOLT BOZSÓ, ZSOLT DATKI, GÁBOR K. TÓTH and BOTOND PENKE

Department of Medical Chemistry, University of Szeged, H-6720 Szeged, Dóm tér 8, Hungary

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Abstract: It has been proved that the principal component of senile plaques is aggregates of β-amyloid peptide (Aβ) in cases of one of the most common forms of age-related neurodegenerative disorders, Alzheimer's disease (AD). Although the synthetic methods for the synthesis of Aβ peptides have been developed since their first syntheses, Aβ[1-42] is still problematic to prepare. The highly hydrophobic composition of Aβ[1-42] results in aggregation between resin-bound peptide chains or intrachain aggregation which leads to a decrease in the rates of deprotection and repetitive incomplete coupling reactions during 9-flurenylmethoxycarbonyl (Fmoc) synthesis. In order to avoid aggregation and/or disrupt internal aggregation during stepwise Fmoc solid phase synthesis and to improve the quality of crude products, several attempts have been made. Since highly pure Aβ peptides in large quantities are used in biological experiments, we wanted to develop a method for a rational synthesis of human Aβ[1-42] with high purity and adequate yield.

This paper reports a convenient methodology with a novel solvent system for the synthesis of Aβ[1-42], its *N*-terminally truncated derivatives Aβ[4-42] and Aβ[5-42], and Aβ[1-42] labeled with 7-amino-4-methyl-3-coumarinylacetic acid (AMCA) at the *N*-terminus using Fmoc strategy. The use of 10% anisole in Dimethylformamide/Dichloromethane (DMF/DCM) can substantially improve the purity and yield of crude Aβ[1-42] and has been shown to be an optimal coupling condition for the synthesis of Aβ[1-42]. Anisole is a cheap and simple aid in the synthesis of 'difficult sequences' where other solvents are less successful in the prevention of aggregation during the synthesis.

Keywords: beta-amyloid; synthesis; difficult sequences; neurodegeneration; Alzheimer's disease; labeling of beta-amyloid

INTRODUCTION

Alzheimer's disease (AD) is a progressive age-related neurodegenerative disorder that is the most common form of dementia affecting mostly elderly people. The pathologic features of AD are the presence of amyloid deposits [1] and neurofibrillary tangles in various areas of the brain. The principal component of senile plaques is aggregates of ß-amyloid peptide (Aβ) which contains 39–43 amino acids. Aβ is produced from a large membrane-associated amyloid precursor protein (APP) and can be neurotoxic both *in vitro* [2,3] and *in vivo* [4,5].

Although the synthetic methods for the synthesis of Aβ peptides have been optimized and developed since their first syntheses, Aβ[1-42] is still problematic to prepare [6,7]. The highly hydrophobic composition of Aβ[1-42] results in aggregation between resin-bound peptide chains [6] or intrachain aggregation. This aggregation of the growing peptide chain on the resin can result in poor solvation and steric hindrance of the *N*-terminus leading to a decrease in the rates of deprotection [8] and repetitive incomplete coupling reactions. This is a serious problem in the solid-phase method since amino acid deleted sequences of varying length and composition accumulate during the synthesis are difficult to separate from the target peptide [8]. A decrease in the purity of the crude peptide and the poor solubility of Aβ[1-42] often results in subsequent difficulties in purification.

In order to avoid aggregation and/or disrupt internal aggregation during stepwise 9-flurenylmethoxycarbonyl (Fmoc) solid phase synthesis of long peptides and to improve the quality of crude products, several attempts have been made, such as the use of *N*-(2-hydroxy-4-methoxybenzyl) (Hmb) protected amino acids [9,10], Tmob [11] backbone protecting groups, incorporation of pseudoproline dipeptide building blocks [12–15], or different highly effective coupling reagents [16–18].

Improvement of solvation during the synthesis was achieved by the use of DMSO [19–22], *N*-methylpyrrolidine (NMP) [23], hexafluoro-2-propanol [24], solvent mixtures [25,26], and chaotropic reagents [8,27]. Incomplete removal of the Fmoc group during synthesis may also be a problem. Deprotection of Fmoc with DBU increased the availability of terminal amino groups for acylation, and improved crude peptide quality [28–31].

Since highly pure Aβ peptides in large quantities are used in biological experiments, we wanted to work out a method for a rational synthesis of human Aβ[1-42]

*Correspondence to: M. Zarándi, Department of Medical Chemistry, University of Szeged, H-6720 Szeged, Dóm tér 8, Hungary;
e-mail: zarandimarta@yahoo.com

that provides a product with high purity and sufficient yield. For this purpose, we used anisole, a special swelling solvent for solvation of the growing protected polypeptide chain in the polystyrene matrix.

MATERIALS AND METHODS

Synthesis

Aβ[1-42] was prepared by solid-phase methodology using Fmoc-chemistry. The amino acid derivatives, 1-hydroxybenzotriazole (HOBt), *N*-[(1*H*-benzotriazol-1-yl)(dimethyl-amino)methylene]-*N*-methylmethanaminum hexafluorophosphate *N*-oxide (HBTU) and dithiothreitol (DTT) were obtained from Orpegen (Heidelberg, Germany). Dichloromethane (DCM), dimethylformamide (DMF), methanol (MeOH), trifluoroacetic acid (TFA), acetonitrile of HPLC grade, and anisole were purchased from E. Merck (Darmstadt, Germany). *N*,*N*′-dicyclohexylcarbodiimide (DCC) was bought from Fluka (Buchs, Switzerland). The α-amino function was protected with Fmoc group, and the reactive side-chain functional groups were protected as follows: N^G-2,2,4,6,7-pentamethyl-dihydrobenzofuran-5-sulfonyl (Pbf) for Arg; *tert*-butyl for Asp, Glu, Ser, Thr, and Tyr; N^{im}-trityl (Trt) for His; and Boc for Lys. The side chains of Asn and Gln were unprotected. All peptides were constructed with a free carboxylic group at the *C*-terminus on Wang resin (0.41 mmol/g, Calbiochem-Novabiochem GmbH, Bad Soden, Germany). The synthesis scale was 0.25 mmol. Before starting the synthesis, the resin was swollen in DCM for 30 min. For the coupling reactions, a 3-fold excess of Fmoc-amino acid was dissolved immediately prior to use in 1–2 ml of DMF, and this solution was diluted to 10 ml with DCM containing 10% (v/v) anisole. DCC and HOBt were used as activating agents. After a coupling time of 2 h, the completeness of acylation was monitored at each stage by ninhydrin test. In cases where incomplete coupling was found, the coupling procedure was repeated with the use of HBTU or acetylation was carried out with acetic anhydride (30% v/v) in DCM/anisole (9 : 1) before removal of the Fmoc protecting group. Fmoc deprotection was achieved by treatment with 20% piperidine (v/v) in DMF containing 10% (v/v) anisole for 5 + 15 min, followed by wash with DMF and MeOH twice, with DCM twice, and a last time with DCM which contained 10% (v/v) anisole. After completion of the synthesis and removal of the *N*-α-Fmoc protecting group from Asp^1, the resin was washed several times with DMF and methanol and dried.

Similar syntheses were carried out for the production of Aβ[4-42] and Aβ[5-42] peptides.

Cleavage and Purification

Final deprotection as well as the cleavage of the peptide from the resin was performed with the mixture of TFA/DTT/H_2O (90 : 5 : 5 v/v) at 20 °C. After 4 h, the cleaved resin was removed by filtration and washed twice with TFA. The combined TFA filtrate was diluted with 0.1% TFA in acetonitrile to set the final acetonitrile concentration to 30% (v/v). The final volume was ~1500 cm^3. The diluted TFA solution was immediately loaded into a preparative HPLC column since dissolving and purification of the aggregated peptide was hard when the cleaved peptide was lyophilized prior to purification. Thus, the peptide was purified without prior lyophilization. Purification was carried out with a Simadzu SCL-8 preparative HPLC system (SHIMADZU Co. Kyoto, Japan) equipped with a PrepPak^R Cartridge 47 × 300 mm (Column No. M23582) Bakbond WP C4 15 μ Packing (Waters Division of Millipore). The following solvent system was used for the elution in a linear gradient mode, at a flow rate of 80 ml/min: (i) 0.1% aqueous TFA (v/v) and (ii) 0.1% TFA (v/v) in acetonitrile: distilled water (80 : 20). The eluent was monitored at 220 nm. The fractions were lyophilized and checked after lyophilization by analytical HPLC, and those containing Aβ[1-42] with a purity exceeding 95% were pooled and lyophilized.

Analysis

The HPLC analyses of crude and purified peptides were carried out on a Hewlett-Packard (Palo Alto, CA) model 1090 liquid chromatograph using a Phenomenex (Jupiter) C4 reverse-phase column (250 × 4.6 mm, 300 Å, 5 μm) with the solvent system described above. The column was eluted with a gradient from 30% to 70% B and 30% to 90% B in case of the crude (Figure 2) and purified peptides (Figure 3), respectively, at a flow rate of 0.2 ml/min. The peaks were detected at 220 nm.

The structure of the synthesized peptide was verified by amino acid analysis and electrospray mass spectrometry (ES-MS). Amino acid analysis of peptides was carried out on HP Amino Quant amino acid analyzer after hydrolysis of the samples (6 M HCl, 110 °C, 48 h in sealed tubes *in vacuum*). Amino acid analyses demonstrated the expected amino acid composition (data are not shown). Mass spectra were taken on a FinniganMat TSQ 7000 mass spectrometer in ESI-MS mode (Figure 2) and gave the expected molecular masses (Calculated: 4513.15, measured: 4512.0).

Fluorescent Labeling

Labeling was performed on the side-chain protected Aβ[1-42] attached to resin (0.035 mmol) applying six-fold excess of 7-amino-4-methyl-3-coumarinylacetic acid (AMCA) and *O*-(7-Azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluoro-phosphate (HATU) (GL Biochem Ltd. Sanghai, China) and 12-fold excess of *N*,*N*-diisopropylethylamine (DIPEA) in DMF for 20 h. The peptide was cleaved from the resin with a cleavage cocktail which consisted of 81% TFA, 5% phenol, 5% thioanisole, 3% H_2O, 2.5% DTT, 2% DMS, and 1.5% NH_4I [32] for 3 h. The crude peptide was precipitated in ice-cold diethyl-ether, washed with diethyl-ether and methanol, and dried. The crude peptide (60 mg) was dissolved in hexafluoroisopropanol (HFIP), diluted with d.water, and purified on a RP-HPLC column (Phenomenex Jupiter C4 column, 10 μ, the same solvent system as described above, and gradient: 30%–90%) yielding 13.6 mg of pure product. M_w (measured by ESI-MS): 4728.7 ($[M - H_5]^{5+}$ = 946.7 $[M - H_4]^{4+}$ = 1183.3 $[M - H_3]^{3+}$ = 1577.2), mass average; theoretical value: 4729.3.

MTT Assay for Neurotoxicity

Differentiated human neuroblastoma cells (SH-SY5Y) and spectrophotometric measurement of formazan, the reduced product of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [33] were used for determination of the

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neurotoxic activity of synthetic Aβ[1-42] and its *N*-terminally truncated derivatives Aβ[4-42] and Aβ[5-42] (Figure 4). Experiments were done in triplicate with seven measurements within each trial ($n = 21$).

RESULTS AND DISCUSSION

The goal of this investigation was to work out a rational synthesis of human Aβ[1-42] and related sequences by using the solid phase method. Our approaches focused on Fmoc chemistry with DCC and HOBt coupling reagents. To minimize aggregation of the growing peptide chain in the resin beads during the assembly of the peptide, a commercially available Wang resin with 0.41 mol/g loading was chosen. Fmoc-amino acids were dissolved immediately prior to use in DMF, and this solution was diluted with DCM containing 10% (v/v) anisole. Resin swelling in anisole/DMF/DCM solvent system was comparable with those obtained in DCM. Increasing the anisole concentration to approximately 20–30% did not appear to improve either the swelling of the resin or the rate of acylation although strict comparative studies were not performed. Higher than 10% of anisole decreased the solubility of certain Fmoc-protected amino acids. Acylations were more extensive with the use of anisole/DMF/DCM solvent system than without anisole. Only a few coupling steps had to be repeated. Figure 1 shows the sequences of Aβ and its fragments synthesized and residues which were double and triple coupled in the sequence of Aβ[1-42]. Recouplings were carried out with HBTU. Aβ[4-42] [34] and Aβ[5-42] which are *N*-terminally truncated derivatives of Aβ[1-42] [35] were synthesized with the same method. The synthesized peptides were cleaved from the resin with the mixture of TFA/DTT/H_2O (90 : 5 : 5 v/v) at 20 °C for 4 h. The analytical HPLC of the crude Aβ[1-42] without any signs of oxidized product is shown in Figure 2. The improved purity of the crude peptides is most probably due to the disruption of aggregation of peptide chains during the synthesis with anisole. Side chain protecting groups are also solvated by aromatic interactions with anisole. In order to prevent aggregation, the diluted solutions of the crude products were immediately purified without prior lyophilizations. After RP-HPLC purification, the purity of the peptides exceeded 95% (Figure 3). The yielding materials were white fluffy powders after lyophilization which could be stored in a freezer at −20 °C for months without the loss of their neurotoxic effects. The neurotoxicities of the stored Aβ[1-42], Aβ[4-42], and Aβ[5-42] were investigated by the MTT test [33] (Figure 4). The neurotoxicity of Aβ[4-42] was similar to that of Aβ[1-42], but Aβ[5-42] was less neurotoxic than Aß peptide with full length. Aβ[1-42] if aggregated is extremely insoluble in water which leads to difficulties in its purification, analysis, and use in biological and biophysical tests. It is very important to avoid the partial oxidization of Met[35]. The formation of Met-sulfoxide could be prevented by the use of cleaving-cocktails containing NH_4I and DMS [32] or tetrabutylammonium iodide (TBAI)/DMS [36], or the oxidized peptide can be reduced with TFMSA/DMS/TFA (1 : 3 : 6) [37] at 0 °C for 3 h.

(A) DAEF**RH**DSGY**E**V**HH**QKLVFFAEDVGSNKGAIIGL**M**VGGVVIA
(B) F**RH**DSGY**E**V**HH**QKLVFFAEDVGSNKGAIIGL**M**VGGVVIA
(C) **RH**DSGY**E**V**HH**QKLVFFAEDVGSNKGAIIGL**M**VGGVVIA

Figure 1 The sequences of (A) Aβ[1-42], (B) Aβ[4-42], and (C) Aβ[5-42] showing the double coupled (bold) and triple coupled (bold and underlined) residues.

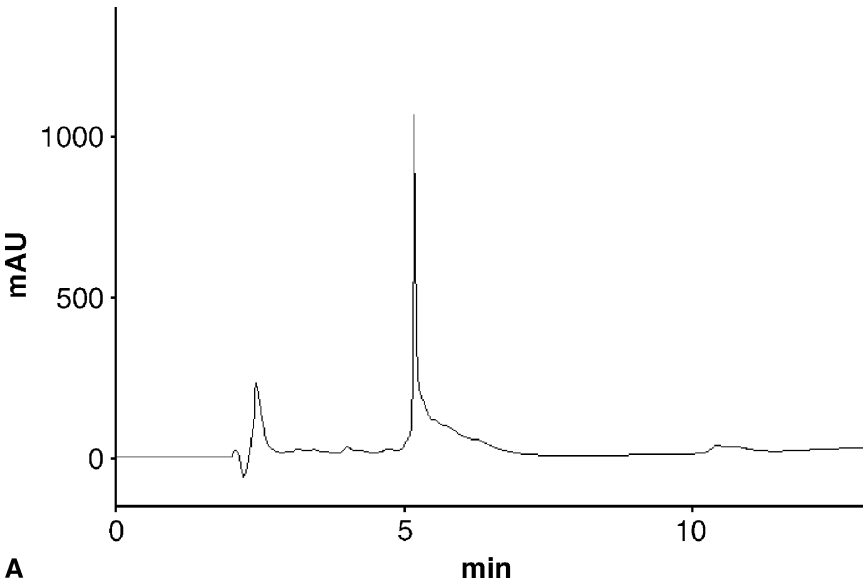

Figure 2 (A) Analytical RP-HPLC profile (conditions described in materials and methods) and (B) electrospray mass spectrum of crude Aβ[1-42] synthesized by Fmoc chemistry with the use of 10% anisole in the solvents.

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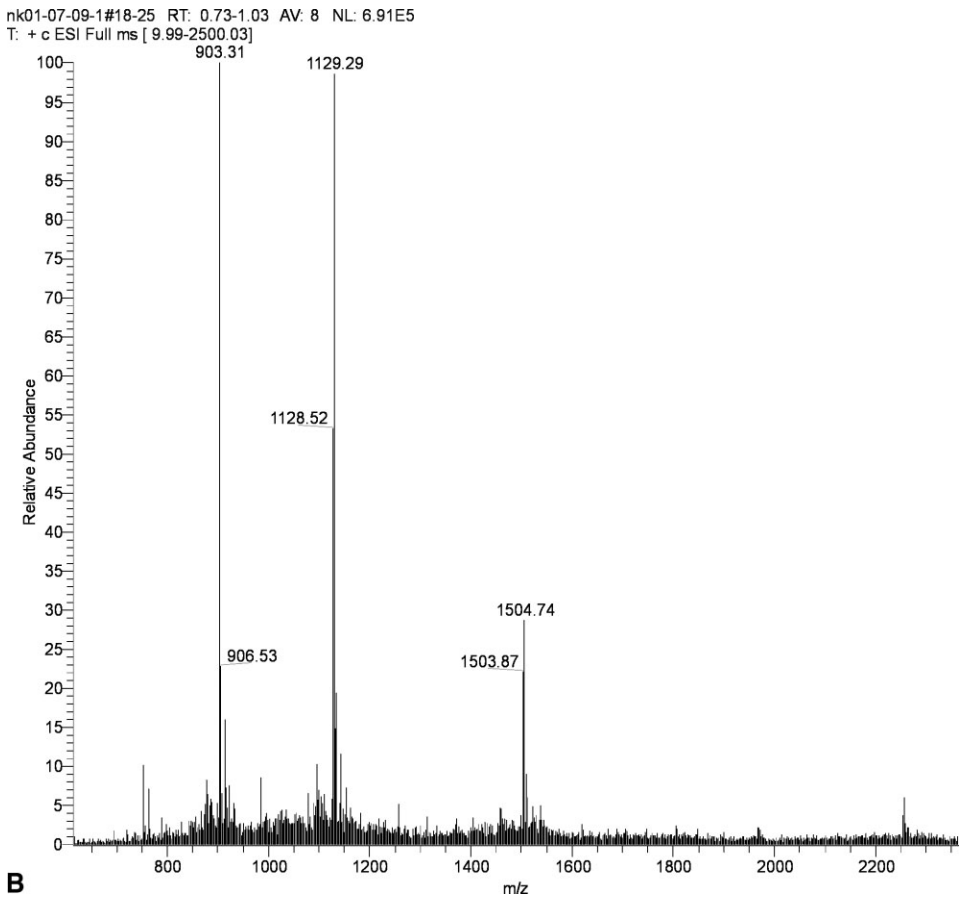

Figure 2 (*Continued*).

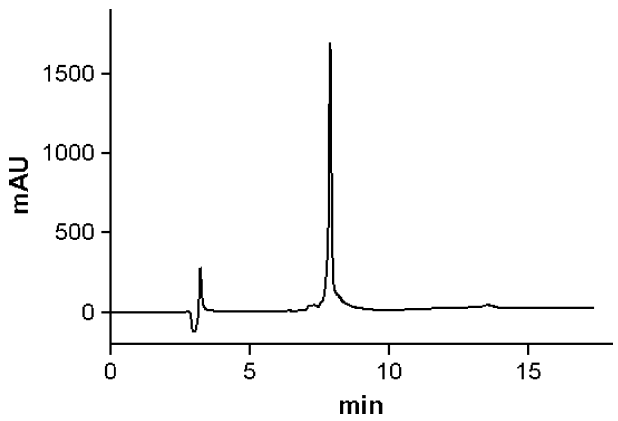

Figure 3 Analytical RP-HPLC chromatogram of purified Aβ[1-42] (conditions described in materials and methods).

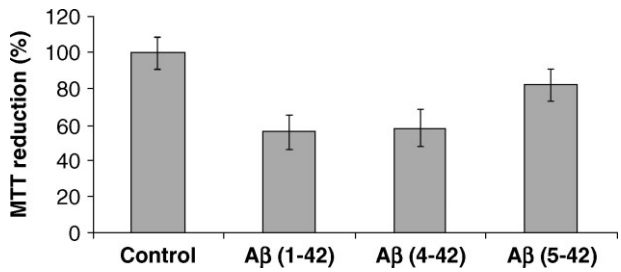

Figure 4 Neurotoxic activity of synthesized Aβ[1-42], Aβ[4-42], and Aβ[5-42] in MTT test on differentiated human neuroblastoma cells (SH-SY5Y) ($n = 21$, ANOVA Post Hoc Test, Bonferroni). Cell viabilities are expressed as percentages of untreated control.

We also aimed to prepare a fluorescently labeled derivative of Aβ[1-42]. The labeling of Aβ[1-42] was carried out in the solid phase at the *N*-terminus. Our fluorophore of choice was AMCA which can be easily activated and coupled to the *N*-terminus of the peptide. AMCA is much cheaper than the popular post-synthetic fluorescent labeling reagents; in addition, it remains chemically stable even under longer storage and possesses an intensive blue fluorescence ($\lambda_{ex} = 349$ nm, $\lambda_{em} = 448$ nm) [38]. Since the crude material was almost insoluble in most of the common solvents and contained a huge amount of scavengers which could not be washed out with diethyl-ether, the precipitate was washed three times with methanol without a considerable loss of the peptide. The peptide was dissolved in HFIP prior to purification and incubated overnight at room temperature, whereby its solubility improved, making the purification with HPLC possible.

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CONCLUSIONS

This paper reports a convenient methodology with a novel solvent system for the synthesis of Aβ[1-42] and related peptides. The crude peptides can be prepared in sufficient quantity and acceptable purity. Aβ[1-42] was also labeled with AMCA in the solid phase at the *N*-terminus.

In conclusion, the use of 10% anisole in DMF/DCM can substantially improve the purity and yield of crude Aβ[1-42], Aβ[4-42], and Aβ[5-42] and has been shown to be an optimal coupling condition for the syntheses of these peptides. Anisole in the solvent system seems to be a cheap and simple aid in the synthesis of "difficult sequences" where other solvents are less successful in the prevention of aggregation during the synthesis.

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