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Synthesis, Aggregation, and Neurotoxicity of the Alzheimer's Aβ1-42 Amyloid Peptide and Its Isoaspartyl Isomers

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Abstract

Amyloid Aβ1-42 peptide (Aβ1-42) and its isomers with an isoaspartyl residue at position 7 or 23 [Aβ1-42(isoAsp7) and Aβ1-42(isoAsp23)] were synthesized in high purity by the Fmoc-solid phase technique, followed by HPLC on a silica-based reversed-phase column under the basic conditions. Importantly, Aβ1-42(isoAsp23) aggregated more strongly than native Aβ1-42 and showed significant neurotoxicity, while the aggregation ability and neurotoxicity of Aβ1-42(isoAsp7) was weak. This suggests that the isomerization of the aspartyl residues plays an important role in fibril formation in Alzheimer's disease.

Keywords: aggregation; Alzheimer's disease; β-amyloid peptide; solid phase synthesis

Alzheimer's disease (AD) is characterized by the accumulation of insoluble fibrillar aggregates of β-amyloid peptides and the death of neurons in the brain region involved in learning and memory. β-Amyloid peptides produced from the amyloid precursor protein by enzymatic reactions mainly consist of two types of peptides, Aβ1-40 and Aβ1-42 [1]. Under pathological conditions, production of Aβ1-42 is significantly enhanced, suggesting that Aβ1-42 plays a significant role in AD [2,3]. Isoaspartyl residues of Aβ1-42 have also been identified in the cerebrum of AD patients [2], and investigation of the aggregation ability of these variants is needed.

To date, many studies on Aβ1-42 aggregation have been carried out [4-8]. However, most results remain controversial partly because of the low purity of the synthetic Aβ1-42 peptides employed. The synthesis of Aβ1-42 is difficult since it contains 14 hydrophobic and/or bulky amino acid residues in the C-terminus, and it easily aggregates even under acidic and neutral conditions, making it difficult to purify by normal reversed-phase HPLC using trifluoroacetic acid (TFA) in acetonitrile [7]. This paper describes the first practical synthesis and purification of Aβ1-42 and its isomers with an isoaspartyl residue at position 7 or 23 [Aβ1-42(isoAsp7) and Aβ1-42(isoAsp23)], together with their aggregation ability and neurotoxicity.

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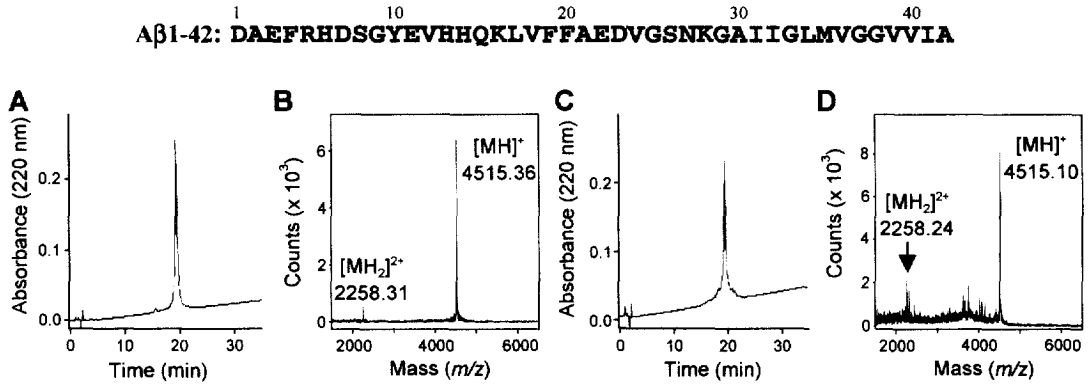

Figure 1. Reversed-phase HPLC chromatograms and MALDI-TOF-MS data of synthetic Aβ1-42 (A and B) and Bachem's one (C and D). The analytical conditions of HPLC are as follows: Develosil UG-3 (ODS), 35 min-linear gradient of 15-36% CH_3CN containing 0.1% NH_4OH, 0.2 mL/min, UV 220 nm. MALDI-TOF-MS measurements were performed on Voyager DE™ STR at the acceleration voltage of 20 kV. Aβ1-42 dissolved in 0.1% TFA aqueous solution (50 pmol/μL) was mixed with saturated α-cyano-4-hydroxy cinnamic acid in 30% CH_3CN containing 0.1% TFA in the ratio of 1:1. One microliter of the resultant solution was subjected to measurement. Angiotensin I and ACTH (7-38) were used as external references.

Aβ1-42, Aβ1-42(isoAsp7), and Aβ1-42(isoAsp23) were synthesized in a stepwise fashion using HATU [9,10] as a coupling reagent by the method reported previously with slight modifications [11-13]. Fmoc-Asp(OH)-O*t*Bu was used for the isoaspartic acid containing peptides. *N*-Terminus Fmoc group was deblocked with 20% piperidine in dimethylformamide (DMF) for 5 min, and the coupling reaction was carried out using each Fmoc amino acid (4 eq), HATU (4 eq), and *N,N*-diisopropylethylamine (8 eq) in DMF for 60 min. After each condensation step, the unreacted amino terminus was blocked with 5% acetic anhydride-pyridine in DMF for 5 min. After completion of the chain elongation, peptide-resin was treated with a cocktail containing TFA, phenol, triisopropylsilane, and water (8.8:0.5:0.2:0.5) for final deprotection and cleavage from the resin. Since Aβ1-42 peptides aggregate easily under acidic conditions, the crude peptides were each dissolved in 0.1% NH_4OH and purified by the reversed-phase HPLC on Develosil UG-5 (ODS) under the basic conditions. The overall yields of Aβ1-42, Aβ1-42(isoAsp7), and Aβ1-42(isoAsp23) were 10%, 15%, and 13%, respectively.

The purity of these synthetic peptides was checked by reversed phase HPLC, MALDI-TOF-MS, and amino acid composition analyses. We compared the purity of our synthetic Aβ1-42 with that of commercially available Aβ1-42 (Bachem, Swit.). As shown in Figure 1, our synthetic peptide gave a single and sharp peak on the reversed-phase HPLC while Bachem's peptide contained some impurities. The MALDI-TOF-MS analyses clearly showed the difference between the two peptides; significant impurities were detected in the Bachem's peptide. The amino acid composition of our synthetic Aβ1-42 coincided well with that of the theoretical value, but Bachem's peptide did not. Similar results were obtained with Aβ1-42(isoAsp7) and Aβ1-42(isoAsp23) (data not shown). Furthermore, the sequence analyses of these peptides gave satisfactory data (data not shown) [14]. These results indicate that the Aβ1-42 peptides were synthesized in high purity and serve as useful peptides for studies on the molecular mechanism of aggregation and neurotoxicity of the amyloid peptides.

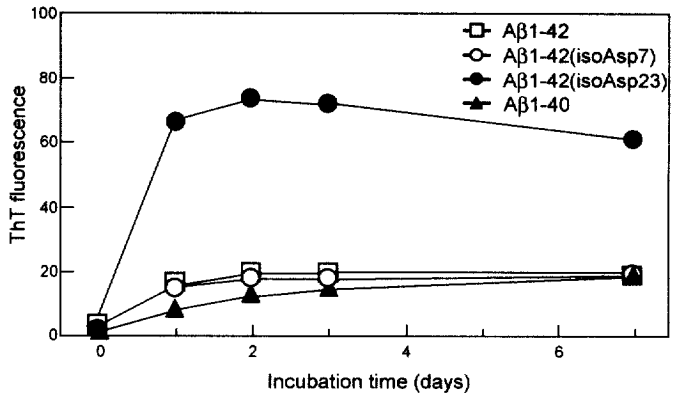

Figure 2. Aggregation of the synthetic Aβ1-42 peptides. Each Aβ peptide was dissolved in 10 mM NaOH and passed through a 0.22 μm pore filter. The resultant peptide solutions were neutralized with equal volumes of 10 mM HCl and diluted with PBS to 200 μM. Each solution was incubated at 37 °C for 1-7 days. Aggregation was monitored by fluorescence emission of thioflavin T (ThT) by the method reported previously [5]. Ten microliters of each peptide solution was added to 990 μL of 1 μM ThT in PBS. Fluorescence was measured at 450 nm excitation and 482 nm emission by Shimadzu Spectrofluorophotometer RF-5000.

The aggregation ability of synthetic Aβ1-42 peptides was measured by the thioflavin T (ThT) method as described in Figure 2. Each Aβ1-42 peptide was dissolved in phosphate buffered saline (PBS) at 200 μM. After incubation for various time intervals at 37 °C, an aliquot of each peptide solution was mixed with ThT which associated rapidly with the aggregated fibrils. Fibril formation can thus be measured by the fluorescence intensity. Importantly, Aβ1-42(isoAsp23) showed high levels of fluorescence during 1-7 days' incubation, while Aβ1-42 and Aβ1-42(isoAsp7) showed weak fluorescence. This indicates that isomerized Aβ1-42 at Asp23 rather than native Aβ1-42 may play an important role in fibril formation in AD, although there are no reports at present on the identificaiton of Aβ1-42(isoAsp23) in AD patients. As a control experiment, the aggregation ability of Aβ1-40 was lower than the Aβ1-42 peptides as expected.

The fibril formation of Aβ1-42 and Aβ1-42(isoAsp23) was clearly detected by the electron microscope. Incubation for 7 days at 37 °C of Aβ1-42 and Aβ1-42(isoAsp23) resulted in the significant fibril formation (Figure 3A and C). However, there was no fibril formation detected in the case of Aβ1-42(isoAsp7) (Figure 3B). These results indicate that isomerization of each Asp residue greatly alters the aggregation ability of the Aβ1-42 peptides.

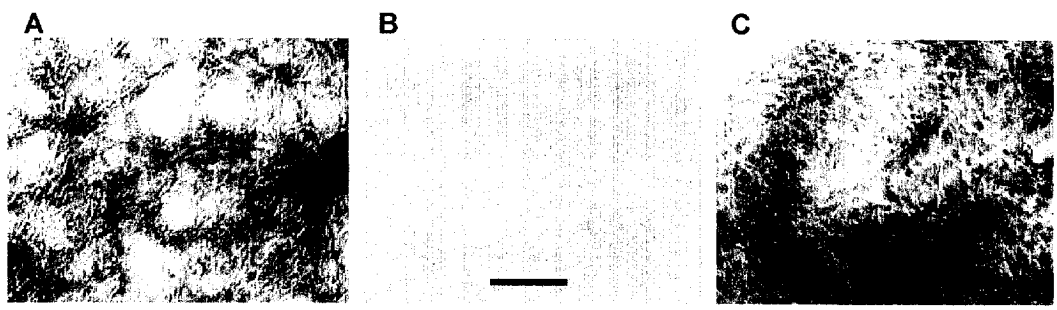

Figure 3. Electron micrographs of negative-stained preparations of the Aβ1-42 peptides. The peptides incubated at 200 μM for 7 days at 37 °C were adsorbed onto 200-mesh collodion-coated copper grids and negatively stained with 2% uranyl acetate. Fibrils formed by Aβ1-42 exhibited a morphology similar to that of typical amyloid (A). No fibril formation was observed in Aβ1-42(isoAsp7) (B). Fibrils formed by Aβ1-42(isoAsp23) showed an enhanced meshwork which is composed of typical fibril of amyloid (C). Scale bar; 100 nm.

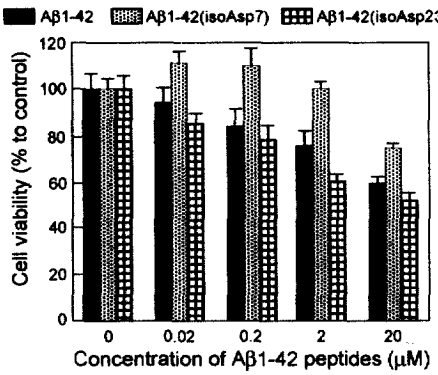

Figure 4. Neurotoxicity against rat embryonic cortical neurons of the synthetic Aβ1-42 peptides. Wistar rat brains of embryonic day 17.5 were extracted and the cerebral cortices were dissected. The dissociated cells were plated at an initial density of 4 x 10^3 cells/well onto the plastic 96-well plates pretreated with 0.5 mg/mL poly-L-ornithine, and cultured in the medium composed of 5% fetal calf serum and 95% DMEM containing 30 nM selenite, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate. The synthetic Aβ1-42 peptides were preincubated in PBS at 200 μM for 7 days at 37 °C. On day 4, the medium was changed to the serum-free culture medium containing various concentrations of the aged Aβ1-42 peptides. The serum-free culture medium contained 5 μg/mL human transferrin, 5 μg/mL bovine insulin, and 20 nM progestrone. On day 6, cell viability was measured by MTT reduction assay [15]. Results are expressed as a percentage of the control values. Each point represents the mean ± SEM of triplicate wells.

Since the aggregation of β-amyloid peptides is thought to be involved in the death of neurons [8], we investigated the neurotoxicity of the synthetic Aβ1-42 peptides against rat embryonic cortical neurons. Various concentrations of the aged Aβ1-42 peptides were administrated to cell culture. As shown in Figure 4, both Aβ1-42(isoAsp23) and native Aβ1-42 showed significant cytotoxicity while Aβ1-42(isoAsp7) showed the reduced activity, suggesting that fibril formation is necessary for the neuronal cytotoxicity.

In summary, we have synthesized the Aβ1-42 peptide along with its aspartyl isomers in high purity for the first time. This study has also shown that isomerization of the aspartyl residues of Aβ1-42 drastically changes the ability of fibril formation. Identification of the Aβ1-42(isoAsp23) in AD patients is currently in progress. These Aβ1-42 peptides afford unique opportunities for studies on the aggregation mechanism of the amyloid peptides, especially by NMR study.

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