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Short Communication

Synthesis of of β-Amyloid Precursor Peptide and Presenilin Segments

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Abstract: It seems likely that the β-amyloid precursor protein (APP) and the presenilins (PS-1/2) play important roles in the development of Alzheimer's disease (AD). Attempts to mimic the biochemical actions of these proteins are often made by the application of fragments of these proteins. However, the synthesis of these segments by conventional methods of peptide synthesis is problematic. We have synthesized several *C*-terminal fragments of APP and PS-1/2 by solid-phase synthesis through combination of automatic and manual methods of synthesis. This permits solution of the 'difficult sequences' in the solid-phase synthesis of these peptides. Some details of the syntheses of nine segments are presented in this paper. Copyright © 2001 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: Alzheimer's disease; β-amyloid precursor protein; presenilins; segments; solid-phase peptide synthesis

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the presence of neurofibrillary tangles and senile plaques [1]. The main constituent of the plaques is a 40–43-residue long peptide called β-amyloid (βA), produced by degenerative proteolytic processing from a transmembrane protein β-amyloid precursor protein (APP) [2]. The presenilins, PS-1 and PS-2, are integral 7–9 transmembrane spanning proteins. Familial AD seems to be caused not only by mutation in APP, but also by mutation in the presenilins [3,4]. Nishimoto *et al.* introduced a novel concept [5] concerning the possibility that AD could reflect a disorder of the APP-G_o signalling system, caused by structural alteration in APP. They suggested that APP is a putative, receptor protein-coupled G_o protein, and that abnormal APP-G_o signalling is involved in the AD process. For their studies, several APP-derived segment peptides were synthesized. However, as far as we are aware, no details of the syntheses are available.

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The APP and presenilin-derived peptides include several sequences that have been characterized in the literature as 'difficult sequences' during single solid-phase synthesis. Their presence leads to low yields and numerous side-products. To overcome these problems, we have applied a combination of manual and regular automatic peptide syntheses for nine *C*-terminal APP and presenilins segments. The *C*-terminally amidated sequences of the APP and presenilin-derived peptides are presented in Table 1, where the 'difficult sequences', the parts of the peptides that were synthesized manually, are highlighted. Not surprisingly, these sequences mainly consist of hydrophobic and sterically hindered side-chain-containing amino acids, such as **I L M N T V Q**, in different variations. It is somewhat surprising that **E** and **R** can also be found in these sequences. This hydrophobicity can be one of the explanations of 'difficult sequences'. For the very hydrophobic amino acids, double couplings on the synthesizer were generally applied with a Boc strategy. In the first approach, we utilized *t*-Boc strategy to prepare *C*-terminally amidated peptides **1**, **3**, **4** and **5**, an Applied Biosystems Model 431A

Table 1 Sequences and Molecular Weights of the Synthetic Segments of APP and Presenilins

Peptides	Sequences	Calculated MS	Obtained MS
1. APP(657-676)amide	HHG**VVEVD**AAVT**PEER**HLSK amide	2209.5	2209.3
2. APP(657-695)amide	HHG**VVEVD**AAVT**PEER**HLSK**MQQN**GYENPTKFFEQ**MQN** amide	4586.1	4587.9
3. APP(639-648)-(657-676)amide	**TIVITLVML**HHG**VVEVD**AAVT**PEER**HLSK amide	4586.1	3293.9
4. PS-1/2(429-467)amide	KKAT**PALP**ISITFG**LVF**YFFATDY**LVQP**FMDQLAFHQFYI amide	4554.5	4555.2
5. APP(639-648)amide	**TVIVITLYML** amide	1100.5	1100.5
6. PS-1(400-413)amide (mutant)	ASGDV**NTTI**AYFVA amide	1514.6	1513.0
7. PS-1(400-413)amide (wild)	ASGDV**NTTI**ACFVA amide	1453.6	1453.1
8. PS-2(129-143)amide (mutant)	PSVGQ**RLLN**SVLITL amide	1608.9	16101
9. PS-2(129-143)amide (wild)	PSVGQ**RLLN**SVLNL amide	1623.9	1623.2

'Difficult sequences' are printed in bold.

synthesizer, with stepwise coupling on a 0.1 mmol scale on a MBHA resin (1.1 mmol/g), using DCC/HOB*t* coupling with a tenfold excess of amino acids and coupling reagents. Our syntheses were unsuccessful.

In the second approach, the peptides were assembled on the same scale (0.1 mmol) on a MBHA resin. For the 'difficult sequences', the peptide-resin was taken from the synthesizer and the synthesis was continued manually, using the standard Kaiser test in each step. After successful assembly of the 'difficult sequences', the peptide resin was returned to the automatic synthesizer. We used a manual synthesis for the 'difficult sequences' in **1**, **3**, **4** and **5.** In this way, **1** and **3** were successfully prepared in the second run. The decapeptide **5** (very hydrophobic) could not be prepared in the synthesizer or manually (two attempts). We had special difficulties with **–PEER-**, **-NTTI-**, **-RLLN-**, and **-VVEVD-**, and especially with **ER** and **PE** couplings of our sequences. For these 'difficult sequences', we sometimes had to apply longer deprotection times and multiple (two, three, or even four) couplings. This is one of the keys to prepare these sequences successfully.

The other approach was with a solid-phase method, using a Rink-amide resin and the Fmoc strategy. For to all syntheses 0.1 mmol (0.62 mmol/g) scale Rink-amide resin was used. It is important that Fmoc(Trt)Gln and Fmoc(Trt)Asn were coupled by TBTU. We successfully synthesized *C*-terminally amidated peptides **2**, **6**, **7**, **8** and **9** on a Rink-amide resin with the Fmoc strategy, using double couplings for the very hydrophobic amino acids (**L**, **I** and **V**). We also used an Applied Biosystem Model 433A automatic synthesizer. In this way, no difficulties arose during these syntheses.

Deprotections and purifications were similar to those used for **1** or **2**. Peptide **4** was successfully purified only on an RPP1 analytical column (Rapp Polymers GmbH, Tübingen, Germany). The purities of the peptides were also demonstrated by means of analytical RP-HPLC and proved to be 97–99%. The correct molecular masses were measured with a Plasma Desorption Mass Spectrometer (PD-MS) and with FAB or Electrospray Mass spectrometers (ES-MS); the calculated and obtained masses can be seen in Table 1. Amino acid analyses were also carried in the regular ways, using scavengers in several cases.

EXPERIMENTAL

C-Terminal APP(657-676) amide

Synthesis of this *C*-terminally amidated peptide was first attempted by the SPS method with Boc strategy in stepwise manner, on an Applied Biosystem Model 431 A automatic synthesizer. The synthesis was unsuccessful. We next began on the synthesizer, but the two 'difficult sequences' tetrapeptides were performed manually, with the qualitative Kaiser test for control. The trifunctional amino acids were: Boc(Tos)Arg, Boc(OcHx)Asp, Boc-(OcHx)Glu, Boc(Dnp)His, Boc(2-Cl-Z)Lys, Boc(Bzl)-Ser, Boc(Bzl)Thr and Boc(2-Br-Z)Tyr. Starting with 0.1 mmol scale of *p*-methylbenzylhydrylamine (MBHA) resin, using *N*,*N*-dicyclohexylcarbodiimide

(DCC)/1-hydroxybenztriazole (HOB*t*) activation, the Boc amino acids were coupled as hydroxybenztriazole ester to the resin. Boc groups were removed with 50% (v/v) trifluoroacetic acid (TFA) in dichloromethane (DCM). The dinitrophenyl (Dnp) group was removed by using 20% (v/v) thiophenol/DMF solution for 1 h at room temperature. Removal of the other side-chain protecting groups and cleavage of the peptide from the resin was performed by treating the peptide-resin with liquid hydrogen fluoride at 0°C for 1 h. The scavengers used were 5% *p*-cresol and 5% *p*-thiocresol. The crude peptide was isolated with 0.1% TFA (scavengers were washed out with diethyl ether) and freeze-dried. The crude *C*-terminally amidated peptide was purified by RP-HPLC on a YMC 2.4 × 25 cm semipreparative C_{18} column with a gradient of A (water-0.1% TFA) against B (MeCN-0.1% TFA), and the main peak was collected and freeze-dried. The purity of the peptide was about 99%, as demonstrated by the means of RP-HPLC. Amino acid analyses include: Asx(1) 1.08; Glx(3) 3.13; Ser(1) 0.9; His(3) 2.97; Gly(1) 1.03; Thr(1) 1.19; Ala(2) 2.14; Leu(1) 1.06; Arg(1) 1.3; Lys(1) 1.04; Val(4) 1.16.; and Pro(1) 0.95.

C-Terminal APP(657-695) amide

We also used a solid-phase method, but with Fmoc strategy and 0.1 mmol scale Rink-amide resin to synthesize the *C*-terminally amidated peptide **2**. All the work was performed on the Applied Biosystem Model 433 A automatic synthesizer The trifunctional Fmoc-amino acids were Fmoc(OBu^t)Asp, Fmoc(Trt)Gln, Fmoc(Trt)His, Fmoc(Boc)Lys, Fmoc-(Trt)Ser, Fmoc(Bu^t)Thr and Fmoc(Bu^t)Tyr. Rink-amide resin (0.1 mmol), so called 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) and N-methylmorpholine (NMM) in NMP or in DMF were applied during all couplings, with double couplings for 'difficult sequences'. Fmoc-groups were removed with 20% (v/v) piperidine/DMF solution. At the end of the full synthesis, the *N*-terminal Fmoc groups were also removed on the synthesizer. The other protecting groups and the peptide were removed with 10 mL TFA, using a scavenger mixture of 0.75 g phenol, 0.25 mL ethanedithiol (EDT), and 0.5 mL thioanisole, for 3 h, at room temperature. The reaction mixture was filtered into cold ethyl ether, where the crude peptide precipitated. The precipitated peptide was centrifuged from the ethyl ether, washed five times with fresh ethyl ether, and separated by centrifugation. The dried crude peptide was dissolved in water and freeze-dried. The crude *C*-terminally amidated peptide was purified similarly as it was for **1**.

The purity of this peptide was also 99%, as demonstrated by analytical RP-HPLC. Amino acid analyses include: Asx(2) 1.98; Glx(3) 2.95; Ser(1) 0.87, His(1) 1.01; Gly(1) 1.02, Thr(2) 1.97; Ala(4) 3.96; Tyr(3) 2.96; Val(2) 1.96; Met(1) 0.93; Ile(3) 3.11; Phe(6) 6.06; Leu(5) 5.34; Lys(2) 2.02; and Pro(3) 3.00.

CONCLUSIONS

Naturally, the 'difficult sequences' we have found during our synthetic work with Boc strategy on automatic synthesizer are not the only ones. Our aim was merely to discuss these sequences. These results lead us to recommend mainly Fmoc chemistry, using Rink-amide resins and automatic synthesizers, with double coupling for 'difficult sequences'.

We consider that either of the two methods discussed can be good solutions for the synthesis of fragments of APP and presenilins containing 'difficult sequences', but care is necessary.

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