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Solid-phase synthesis and purification of β-amyloid (1-42)

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We report here the study of some new approaches to the solid-phase peptide synthesis, purification and analytical control of β-amyloid (1-42), a protein implicated in Alzheimer's disease.

Deposition and aggregation of β-amyloid (βA4) in brain plaque is one manifestation of Alzheimer's disease.[1] β-Amyloid is a 39/43 amino acid residue peptide (Figure 1) which is proteolitically derived from the much larger β-amyloid precursor protein APP.[2,3]

In a number of biological experiments concerned with the study of Alzheimer's disease some synthetic βA4 peptides were applied.[1,4,5] Some more or less successful attempts at the synthesis and purification of β-amyloid have already been reported.[3,6,7] However, the problem has not been satisfactorily solved until now. Firstly, intermolecular aggregation of the growing peptide chains in organic solvent accompanies the synthesis of the very hydrophobic C-terminal fragment, causing incomplete aminoacylation and accumulation of failed peptides.[8] Secondly, the extremely low solubility of βA4 and its tendency to aggregate in aqueous solutions dramatically complicate post-synthetic analysis and purification.[9]

We report here the search for the optimisation of solid-phase peptide synthesis (SPPS) conditions for the most hydrophobic βA4 (1-42) peptide and some approaches to its analysis and purification.

One of the methods used to overcome the difficulties encountered in the formation of hindered structures in the SPPS of β-amyloid involves the use of amide backbone protection using the *N*-(2-hydroxy-4-methoxybenzyl) group, described by T. Johnson.[10]

We tried to solve the problem of internal aggregation of the peptide chain within the peptide-resin matrix by choosing some different solvent mixtures to improve the possibility of good swelling of the peptide-resin. In addition, it was interesting to compare the efficiency of the two alternative approaches: stepwise SPPS and fragment-coupling strategy. All syntheses were carried out by a 9-fluorenylmethoxycarbonyl (Fmoc)-technique on polystyrene polymer (Wang resin) or on polyamide polymer (Pepsyn KA) with automatic peptide synthesizers Applied Biosystems 431A and Milligen Biosearch 9050 in continuous flow conditions, respectively. The side-chain function of Asp, Glu, Ser and Tyr were protected with *tert*-butyl (Bu^t) groups; of Asn, Gln and His with trityl groups; of Lys with a *tert*-butyloxycarbonyl (Boc) group; and Arg with a 2,2,5,7,8-pentamethylchroman-6-sulfonyl group. As a coupling agent in stepwise SPPS (benzotriazol-1-yl-oxy)tris(dimethylamino)phosphonium hexafluorophosphate/1-hydroxybenzotriazole (HOBT)[11] in the presence of 1.8 equiv. *N,N*-diisopropylethylamine was used. All couplings were repeated twice using a five-fold excesses of acylating agents without end-capping. A comparison of the swelling of Wang resin-bonded tetra- and hexapeptides (H-Val-Val-Ile-Ala-P and H-Gly-Gly-Val-Val-Ile-Ala-P) in the following solvents suitable for SPPS: dichloromethane (DCM), *N,N*-dimethylformamide (DMF), 1-methyl-2-pyrrolidone (NMP) and its mixtures: DCM/trifluorethanol (TFE) (1 : 1), DMF/NMP, dimethyl sulfoxide (DMSO)/NMP (1 : 9) and DCM/DMF/NMP (1 : 1 : 1) showed that the most promising was a mixture of DMF/NMP (3 : 1 or 1 : 1). Therefore, all SPPS operations (deprotection, coupling and washing) were performed in this mixture. The synthetic product was cleaved from the resin and deprotected using reagent K [82.5% trifluoracetic acid (TFA), 5% phenol, 5% thioanisole, 5% water, 2.5% 1,2-ethanedithiole] for 3–5 h.

We varied some parameters and evaluated their effects on the quality of the target product using electrospray ionization mass spectrometry (ESI-MS) and high-performance liquid chromatography (HPLC). The content of the target product and quantity and composition of by-products were found to be independent of the nature of resin and Fmoc-cleavage agents used. [In the first 14 cycles for α-amino group

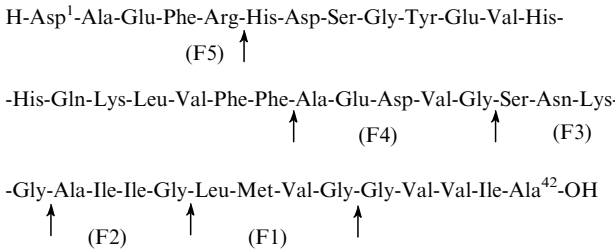

Figure 1 Amino acid sequence of βA4 (1-42).

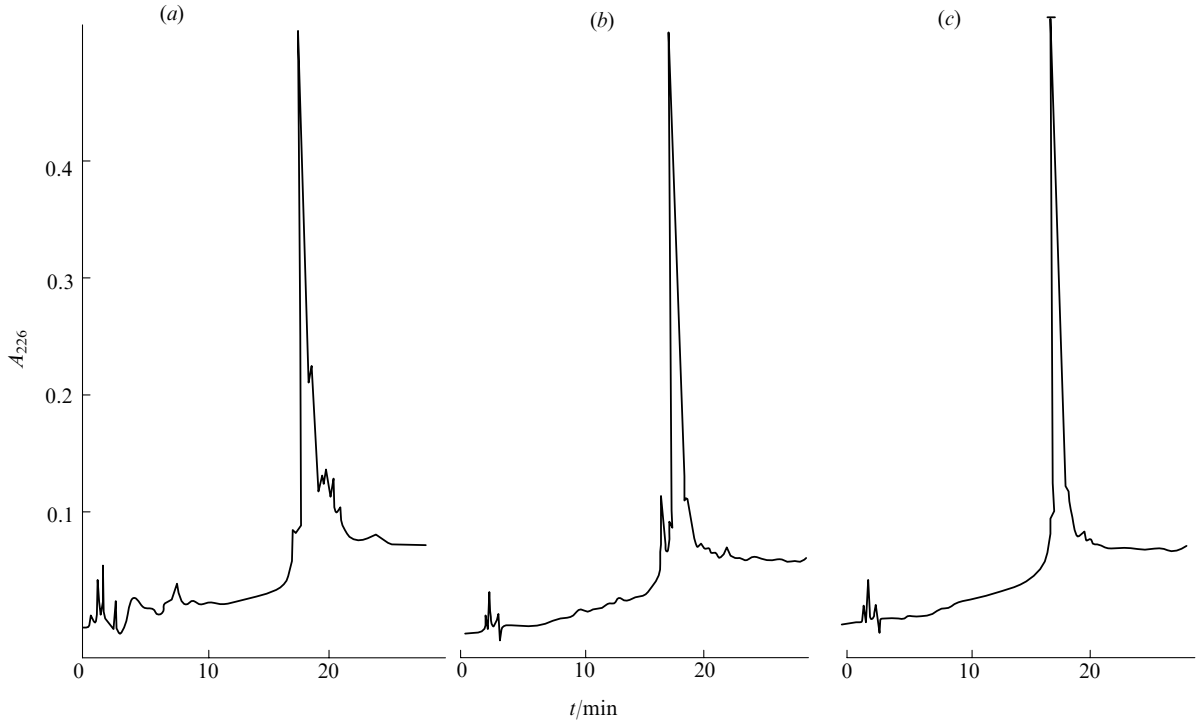

Figure 2 HPLC of final crude products from (*a*) stepwise; (*b*) fragment synthesis and (*c*) after Sephadex G 50. Conditions: Hamilton PRP 1 (4.6×250 mm) column. Eluent A: 0.05 M Na_2HPO_4; B: 80% CH_3CN + 20% A (v/v). Gradient 10–50% B in 30 min. Flow rate 1 ml min^{-1}. Detection at λ 226 nm, 0.5 AUFS.

deprotection either standard 20% piperidine (Pip) in DMF/NMP or 2% 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) and 2% Pip in DMF/NMP solution was used (sometimes DBU was suitable for Fmoc-group cleavage in some 'difficult sequence' syntheses[12])]. We expected that the level of resin substitution should affect the quality of the target product. Crude peptide which had been synthesized on high capacity polymer (0.45 mmol g^{-1}) contained many more failed peptides than those on a polymer of substitution level *ca.* 0.2 mmol g^{-1} (ESI-MS). The main results are shown in Table 1. The best results were achieved when the synthesis was performed on 0.22 mmol g^{-1} substituted polymer in a DMF/NMP (1 : 1) mixture (Table 1).

In the fragment SPPS only the C-terminal, the most difficult part of the βA4 sequence, was separated on tetra- and pentapeptide blocks (Figure 1). Moreover, *N*-terminal pentapeptide containing an unprotected arginine residue was also attached as a fragment. All the other amino acids were coupled in the same manner as in the stepwise version. In choosing the fragment with unprotected arginine, we were guided by the results of our own developments which showed that coupling of similar fragments in solution proceeded in high yields and without racemization.[13] In choosing the peptide block size we followed the data on the βA4 solid-phase fragment synthesis[6] in which the coupling yields of octapeptide or longer fragments were relatively low (70–90%) or unsatisfactory. Fragments Fmoc-Leu-Met-Val-Gly-OH (F1), Fmoc-Ala-Ile-Ile-Gly-OH (F2), Fmoc-Ser(Bu^t)-Asn-Lys(Boc)-Gly-OH (F3), Fmoc-Ala-Glu(OBu^t)-Asp(OBu^t)-Val-Gly-OH (F4) and Fmoc-Asp(OBu^t)-Ala-Glu(OBu^t)-Phe-Arg-OH (F5) were prepared by classical methods in solution and were purified by crystallization, absorption chromatography or RP-HPLC. Their homogenity was confirmed by HPLC, mass-spectrometry† and ^{1}H NMR spectroscopy (^{1}H NMR data are given in Table 2). Fmoc-Gly-Val-Val-Ile-Ala-P with alanine content 0.2 mmol g^{-1} was used as a starting material for the fragment synthesis. Fragments were condensed by *N*,*N*-diisopropylcarbodiimide and HOBT in a DMF/NMP (1 : 1) mixture. *N*-Terminal pentapeptide was coupled in the same way as shown above but in the presence of 1 equiv. pyridine hydrobromide, necessary for destruction of an intermolecular salt between the carboxyl and guanidine group derived from arginine.[13] The yields of fragment condensation were sufficiently high (>95%) according to Kaiser's test.[14] Figure 2 shows an analytical HPLC of crude products from stepwise and fragment synthesis. As one would expect, convergent solid-phase synthesis offered us the opportunity to produce more homogeneous product.

Analytical control of homogenity and purification of βA4 peptides is a very difficult problem because the crude product is extremely poorly soluble and inclined to aggregation.[9,15] Assuming the pI of βA4 to be 5–6, we worked with it under either acidic or basic conditions, *e.g.* in water–organic mixtures. The crude product of solid-phase synthesis was insoluble in an aqueous (10–30%) solution of acetic (AcOH) and formic acids, sodium dodecylsulfate, 8 M urea and other solvents commonly used for gel chromatography. Although the peptide is soluble in concentrated (>70%) formic acid, AcOH and TFA, these were impossible to use because of rapid aggregation. The same pattern was observed with an acetic acid–methanol mixture. The solubility of the crude product in 0.1% NH_4OH was sufficiently high (*ca.* 10–15 mg ml^{-1}) and the solution (a very important point) was stable over time. The crude product was successfully purified up to 90–95% in 0.1% aqueous ammonia using Sephadex G-50 (2.6×80 cm column) as a sorbent (Table 1). The scale of preparative separation was ~150 mg. Recovery yields of βA4 peptide were *ca.* 90%. It has been reported that HPLC of βA4 and related peptides was

† ESI-MS: F1 (Calc. 640.8) 641.5 $(M+H)^+$; F2 (Calc. 594.7) 595.4 $(M+H)^+$; F3 (Calc. 782.9) 783.7 $(M+H)^+$, 821.7 $(M+K)^+$; F4 (Calc. 823.9) 824.7 $(M+H)^+$, 846.6 $(M+Na)^+$; F5 (Calc. 971.1) 971.8 $(M+H)^+$.

‡ Amino acid analysis: D/N 4.07 (4), E/Q 4.03 (4), S 2.00 (2), G 6.00 (6), A 3.95 (4), V 6.01 (6), M 1.03 (1), I 2.94 (3), L 1.95 (2), Y 1.13 (1), F 2.74 (3), H 3.00 (3), K 2.30 (2), R 0.94 (1).

Table 1 Summary of some characteristics of βA4 and impotant synthetic data.

	Resin, capacity/ mmol g^{-1}	Solvent	HPLC (%) of product		ESI-MS[d], M(Da)± 0.01% M_{calc} = 4514.1
			crude	purified	
1	Pepsyn KA (PA)[b] 0.20	DMF/NMP (4 : 1)	77.8	92.4	4514.5 (βA4); impurities: 4571.7 (+Bu^t), 4429.4 (ΔSer), 4313.4 (ΔAspSer)
2	Wang-resin (PS)[c] 0.20	DMF/NMP (3 : 1)	74.3	91.0	4514.8 (βA4); impurities: 4571.4 (+Bu^t), 4426.1 (ΔSer), 4312.4 (ΔAspSer)
3	Wang-resin (PS) 0.22	DMF/NMP (1 : 1)	74.5	94.9	4515.2 (βA4); impurities: 4311.5 (ΔAspSer)
4	Wang-resin (PS) 0.45	DMF/NMP (1 : 1)	71.1	92.0	4514.0 (βA4) impurities: 4571.5 (+Bu^t), 4357.7 (ΔArg) or (ΔValGly), 4427.0 (ΔSer), 4312.1 (ΔAspSer)
5[a]	Wang-resin (PS) 0.20	DMF/NMP (1 : 1)	83.8	95.0	4515.3 (βA4) impurities: 4571.8 (+Bu^t)

[a] Fragment condensation. [b] PA: polydimethylacrylamide-type resin. [c] PS: polystyrene-type resin. [d] Mass spectra were recorded on a Finnigan MAT TSQ 700 quadrupole mass spectrometer with electrospray API ion source (Finnigan MAT). A shorter version of the principal peptide is marked by the relevant deletion notation (Δ).

Table 2 Chemical shifts δ (ppm) for amide and aliphatic protons of the constituent amino acid residues in peptide fragments.[a]

Peptide/residue	δ (ppm)					
	NH	$C_\alpha H$	$C_\beta H$	$C_\gamma H$	$C_\delta H$	Remarks
Fmoc-L-M-V-G-OH						
Leu1	7.540	4.057	1.425, 1.476	1.620	0.880, 0.840	
Met2	8.070	4.400	1.810, 1.910	2.430, 2.380		2.000(CH_3)
Val3	7.650	4.183	1.954	0.820, 0.850		
Gly4	8.280	3.800, 3.710				
Fmoc-A-I-I-G-OH						
Ala1	7.550	4.106	1.190			
Ile2	7.810	4.200	1.721	1.420, 1.050		
Ile3	7.710	4.210	1.720	1.430, 1.050		
Gly4	8.204	3.760, 3.680				
Fmoc-S(Bu^t)-N- -K(Boc)-G-OH						
Ser1	7.390	4.118	3.490, 3.427			1.358(Bu^t)
Asn2	8.140	4.547	2.560, 2.420			6.990, 7.430(NH_2)
Lys3	7.920	4.167	1.360, 1.480	1.690	1.360, 1.305	2.850(C_ε) 1.112(Boc)
Gly4	8.220	3.730, 3.670				
Fmoc-A-E(OBu^t)- -D(OBu^t)-V-G-OH						
Ala1	7.535	4.080	1.200			
Glu2	8.000	4.291	1.850, 1.720	2.210		1.348(OBu^t)
Asp3	8.280	4.610	2.690, 2.450			1.345(OBu^t)
Val4	7.590	4.180	1.970	0.840, 0.81		
Gly5	8.260	3.770, 3.700				
Fmoc-D(OBu^t)-A- -E(OBu^t)-F-R-OH						
Asp1	7.680	4.366	2.640, 2.440			1.366(OBu^t)
Ala2	7.994	4.230	1.138			
Glu3	7.675	4.195	1.648, 1.807	2.110		1.358(OBu^t)
Phe4	7.946	4.506	3.043, 2.790			
Arg5	7.670	3.911	1.610	1.478	3.050	

[a] [$^2H_6$]DMSO, 500 MHz, 300 K, $C \approx 3$ mg ml^{-1}.

either unsatisfactory, uninformative or generally impossible.[15] We checked as an aqueous phase (buffer A) for HPLC, either acidic [0.1% TFA, 0.05 M KH_2PO_4 (pH 3.0)] or basic [0.05 M K_2HPO_4 (pH 7.0, 7.5, 8.0) or 0.1% ammonia] solutions. As the organic phase (buffer B) a mixture 70–80% of acetonitrile and 20–30% buffer A was chosen. In our experiments the choice of sorbents was limited to Diasorb C16, Vydac C18, Ultrasphere ODS, Lichrosorb RP2 and Hamilton PRP1. We obtained satisfactory results only using a combination of Lichrosorb RP2 with 0.1% TFA and PRP1 under basic conditions (see above and Figure 2). These sorbents and conditions can be recommended for the preparative purification of βA4 (1-42) and related peptides up to >95% purity if necessary.

Thus, the performance of SPPS in a DMF/NMP (1:1) mixture on a support of substitution degree *ca.* 0.2 mmol g^{-1}, followed by purification of the crude product on Sephadex G50 in aqueous ammonia, allowed us to obtain molar quantities of βA4 (1-42) in high yields (45–50%) and purity (90–95%). The structure of the target peptide was confirmed by amino acid analysis,‡ ESI-MS (Figure 2) and Edman degradation.

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