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A Synthetic Substrate Assay for the Gamma-secretase of the β-A4 Amyloid of Alzheimer's Disease

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Abstract: γ-secretase, the endoprotease which releases the C-terminus of βA4 amyloid peptide, cleaves within the hydrophobic transmembrane domain of the amyloid precursor protein. In order to obtain a substrate for γ-secretase, a dodecapeptide which spans the cleavage site was synthesized, labelled with 125-iodine and conjugated to an agarose gel. A radiometric solid-phase assay was developed using this immobilized substrate. Peptide products were separated by reverse-phase HPLC and TLC to allow characterization of the cleavage site(s).

Keywords: βA4 amyloid peptide; radiometric assay; transmembrane peptide; reverse-phase TLC

Abbreviations

Ac-12, Ac-Tyr-Gly-Val-Val-Ile-Ala-Thr-Val-Ile-Val-Ile-Thr; PyBOP, benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate; DIEA, diisopropylethylamine; DMAP, 4-dimethylaminopyridine; EDC, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide.

INTRODUCTION

The main constituent of the plaques and congophilic angiopathy, which are characteristic of Alzheimer's disease (AD), is βA4 amyloid, a 4 kDa peptide [1, 2], derived by proteolytic cleavage from a larger transmembrane amyloid protein precursor (APP) [3]. The βA4 domain is located at the junction of the extracellular and transmembrane domain. This accounts for its hydrophobic character and partially explains its aggregating properties. Soluble forms of APP are produced by cleavage of the extracellular domain near the membrane. A major constitutive secretion pathway which results from the action of a unidentified protease termed α-secretase cleaves APP within the βA4 peptide, at position 15 or 16 (Figure 1) [4]. A minor secretion pathway due to the action of β-secretase cleaves at the N-terminus of βA4 [5, 6]. α- and β-secretases do not appear to be very specific since multiple minor cleavage products have been found [7]. The possibility exists that α- and β-secretase activities are identical and that a single membrane-bound protease could cleave at a site determined largely by its distance from the membrane.

Another quite distinct protease activity termed γ-secretase cleaves at the C-terminus of βA4 domain to liberate the fragments which eventually polymerize into amyloid filaments [7–9]. The γ-secretase cleavage is particularly puzzling since it takes place within the transmembrane domain of APP. Moreover, several C-termini have been reported for βA4 and its counterpart 'p3' [10], the 3 kDa fragment which results from α-secretase activity. The forms of βA4 which are secreted in the cerebrospinal fluid and in cell culture

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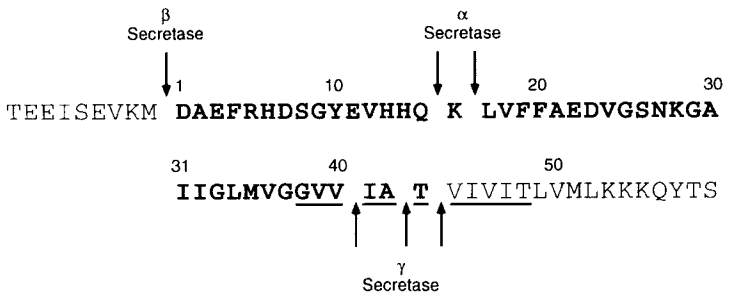

Figure 1 βA4 amyloid precursor protein secretase cleavage sites. The sequence of the βA4 amyloid peptide is shown in bold letters. The peptide selected as γ-secretase substrate is underlined.

media end at Val^{40} [8]. In contrast, the main components of the amyloid plaque cores and perivascular deposits end at Ala^{42}, with a minor component ending at Thr^{43} [3, 11–13]. The presence of the residues $(Ile\text{-}Ala)^{41-42}$ appears to be a major factor determining aggregation and possible cytotoxicity. Yet another form of βA4 which ends with Ala^{39} has also been described as a component of cerebrovascular amyloid [14]. Thus, the release of the βA4 C-terminus may involve several proteases or a single protease of broad specificity. Consequently, the study of the γ-secretase mechanism is of prime importance for the understanding and the control of amyloid formation in Alzheimer's disease. To characterize and purify the γ-secretase(s) we have prepared a synthetic substrate and developed a convenient assay which can distinguish between the several possible cleavage sites.

MATERIALS and METHODS

Fmoc-amino acid derivatives and BOP reagent were from Auspep, North Melbourne, Australia, the 'Wang resin' was from Novabiochem, Bubendorf, Switzerland. Other peptide synthesis solvents and reagents were from Pharmacia-LKB, Uppsala, Sweden, or from Applied Biosystems, Burwood, Australia. Pepsin (porcine, 1:10 000) and α-chymotrypsin (bovine, type I-S) were from Sigma (St Louis, Mo, USA). Papain was from Boehringer (Mannheim, Germany).

Peptide Synthesis

Peptides were synthesized manually on a Wang resin according to Fmoc protocol [15]. The sidechains of Tyr and Thr were protected as *t*-butyl ethers. The first amino acid was incorporated by esterification using BOP [16] and DMAP, in the presence of DIEA. Fmoc deprotection was done by 50% piperidine in *N,N*-dimethylformamide (DMF), (two steps; 5 and 15 min). Piperidine was completely removed by washing with DMF (3 × 1 min), with methanol (3 × 1 min) and again with DMF (3 × 1 min). Couplings were performed in DMF, using four equivalents of Fmoc amino acid and BOP or PyBOP [17] as compared to the peptidyl-resin, and eight equivalents of DIEA. Completeness of the coupling was checked by the ninhydrin test. When recoupling was necessary, it was done by using half the amount of reagents. If the ninhydrin test was still positive, the unreacted sites were blocked by acetylation, using 10% acetic anhydride in dichloromethane containing 5% DIEA, for 30 min. For final cleavage and deprotection, the resin was carefully dried, then suspended in 95% trifluoroacetic acid (TFA) in water and gently shaken for 2 h. The dodecapeptide Ac-12 required a longer deprotection time (4–18 h) to optimize peptide recovery. The resin was filtered off and the peptide was recovered by precipitation with diethyl-ether. The precipitate was dissolved in 10–50% acetic acid, depending upon peptide solubility, and lyophilized. The peptides were analysed by reverse-phase (high-performance liquid chromatography (HPLC), on a C-18 column (as described below), and characterized by amino acid analysis, after 24 h hydrolysis by HCl (6N), at 110°C. Amino acid composition of Ac-YGVVIA was: Y 0.95(1); G 1.09(1); V 1.90(2); I 1.01(1); A 1.05(1), as calculated from the mean of all residues. Ac-12 was particularly insoluble and could be dissolved only in TFA and formic acid solutions. It was analysed and purified by HPLC on a C-8 Aquapore column from which it eluted at about 50% acetonitrile in 0.1% TFA (Figure 2). Amino acid analysis of the crude product, after 48 h hydrolysis, was as follows: Y 1.19(1); T 1.57(2); G 1.26(1); V 3.63(4); I 2.68(3); A 1.39(1) (Thr was corrected by 5% to account for losses due to hydrolysis).

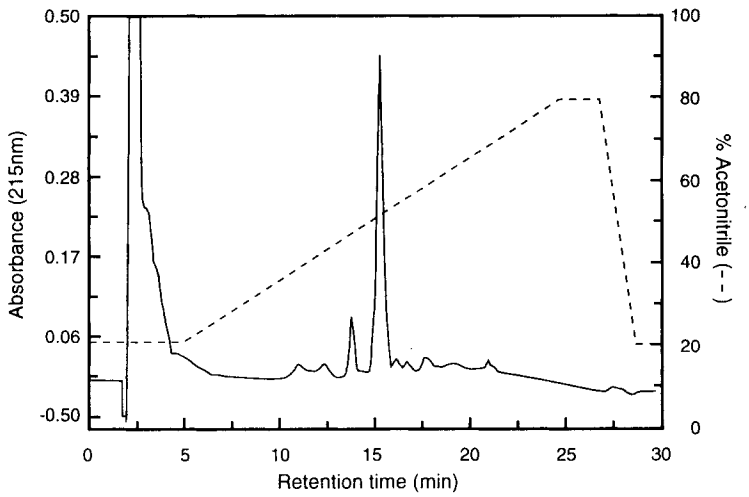

Figure 2 HPLC analysis of crude peptide Ac-12. The peptide was loaded onto an Aquapore-300 C-8 cartridge (Brownlee, 100 × 4.6 mm) and eluted with a linear gradient of acetonitrile in 0.1% TFA, at a flow rate of 1 ml/min.

Peptide Iodination

Peptide aliquots were purified by HPLC and dried prior to iodination. A 1 mg/ml solution in TFA was prepared from which 10 µl (10 µg) was transferred to an Eppendorf tube and dried in a Speed-Vac concentrator (Sorvall, Du Pont, Northside, Australia) before resuspension in 50 µl (0.1 M) phosphate buffer, pH 7.4, 0.5 mCi ^{125}I was added, followed with 10 µl of a 2.6 mg/ml chloramine-T solution in water [18]. Iodination was allowed to proceed for no more than 45 s in order to avoid diiodotyrosine formation, and was stopped by adding 50 µl of a 10 mg/ml solution of sodium metabisulphite in water. Peptide tracers were purified by extraction on C-18 Sep-pak cartridges (Millipore, Bedford, MA), eluting with 40–75% acetonitrile in 1% TFA water, depending on peptide solubility.

Conjugation to Affi-Gel 102

0.2 mCi of ^{125}-I labelled Ac-12 peptide was conjugated to 10 ml Affi-Gel 102 (Bio-Rad, Richmond, CA) suspended in 10 ml water, in presence of an excess of EDC reagent (Sigma, St Louis, MO), for 48 h, at 20°C. 5 mg of reagent was used to initiate the reaction, then another 5 mg was added after 2 h, and an additional 5 mg after 24 h. The gel was extensively washed with 0.1% Triton-100, then with 0.1 M Tris-HCl, pH 7.4, and stored at 4°C in the latter buffer.

Assay for Proteolytic Activity

Aliquots of 50 µl (about 100 000 c.p.m.) of peptide–gel were distributed in 1.5 ml microfuge tubes (Eppendorf, Germany), washed with 1 ml assay buffer (Tris-HCl 0.1 M, pH 7.4), pelleted by centrifugation, and washed another three times or until no counts were found in the wash buffer. The gel aliquots were then suspended in 200 µl assay buffer and 200 µl of diluted enzyme solution were added. The incubation was allowed to proceed for 30–60 min at 37°C with rotary shaking. The total radioactivity in each incubation was counted, then the tubes were centrifuged and a 200 µl aliquot of supernatant was counted. Ratios of released versus total counts were calculated, and a non-specific value corresponding to a mean of incubations where assay buffer, but no enzyme, was present was subtracted. All incubations were in duplicate.

Reverse-phase HPLC

Peptides were separated on a Brownlee Spheri-5 RP-18 cartridge (100 × 4.6 mm) equipped with a New Guard RP-18 cartridge (15 × 3.2 mm), Applied Biosystems, Burwood, Australia). After 5 min equilibration in 0.1% TFA, a linear gradient of 0–60% acetonitrile in 0.1% TFA was developed over 30 min, at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected and counted for radioactivity.

Products were identified by comparing their retention time with peptide standards.

Reverse-phase TLC

Aliquots of 1–2 µl of iodinated peptides (200–1000 c.p.m.) were loaded onto RP-8 plates (Merck, Darmstadt, Germany) which were developed with 38% isopropanol in 1% TFA. The plates were dried, sealed in a plastic bag, and exposed to a BAS-IIIS imaging plate, following by image analysis using a BAS-1000 FUJIX phosphorimager (Fuji, Japan). Exposure of 30 min was enough to detect 500–1000 c.p.m. Overnight exposures allowed good detection in the range of 200 c.p.m.

RESULTS

Peptide Synthesis

Peptides were synthesized according to the Fmoc methodology, using BOP or PyBOP as activation agents. Couplings were found to be slow. Two analogues were synthesized in parallel, Ac-YGVVIAT-VIVIT and Ac-YGVVIATVIIIT (the latter carries a mutation Ile/Val which was found to be associated with AD) [19] and the results of the ninhydrin tests were compared (Table I). Couplings started to become difficult at residue number 5, for both analogues. Interestingly, after residue 7, coupling difficulty seemed to alternate between the two analogues, and the difficult points occurred every three residues, which could correspond to a α-helix turn. Our interpretation is that the growing chains adopted different helix structures, but this is only speculation since we do not possess any structural data to confirm it.

Table I Coupling Efficiency Assessed by Ninhydrin Test

Native peptide	Coupled residue	I^{46}-mutant analogue
–	I	–
–	V/I	–
–	I	–
–	V	–
+/–	T	++/+
+/–	A	–
+/+	I	++/++
+/–	V	–
–	V	–
++/–	G	+/–
+/–	Y	–
–	ac	+/–

Ninhydrin test was assessed as follows: – , colourless beads and yellow solution; +, beads slightly blue, solution trace of blue; ++, beads moderately blue, solution slightly blue; +++, beads dark blue, solution moderately blue.

Table II Digestion of Ac-12–Substrate Gel by Various Proteases

Protease	Incubation time (h)		
	1	2.5	18
Pepsin			
20 µg/ml	13.16[a]	15.71	36.85
4 µg/ml	4.80		
Papain			
1.5 µg/ml	62.80	71.90	79.40
0.15 µg/ml	22.75		
Chymotrypsin			
20 µg/ml	37.15	46.26	77.50
2 µg/ml	11.79		
0.4 µg/ml	5.38		

[a] Results are expressed as the percentage of radioactivity released from the gel in the supernatant.

Iodination and Conjugation to Affi-Gel 102

To set up a solid-phase radiometric assay, peptide Ac-12 was iodinated and conjugated to Affi-Gel 102. Coupling yield of ^{125}I-Ac-12 to the gel in the presence of EDC was 18–20%, while an unrelated peptide, angiotensin I, used as a comparison, was coupled in the same conditions with 66% efficiency. The low yield of incorporation of Ac-12 tracer might be due to the poor solubility and aggregation properties of Ac-12 as well as to the presence of Thr, a residue difficult to activate, at the coupling site. Meanwhile, the preparation of different batches of substrate gave very similar yields, and batch to batch reproducibility of the protease assay was satisfactory.

Test of Ac-12-gel as a Substrate for Proteases

The peptide–Affi-Gel conjugate was tested as a substrate for various proteases known to cleave hydrophobic sequences, including chymotrypsin, papain and pepsin. Results (Table II) show that these proteases were able to release most of the radioactivity from the gel, confirming accessibility of the peptide to the proteases. The release of radioactivity was dependent upon time and enzyme concentra-

tion, confirming that release was due to proteolysis. Non-specific release of radioactivity from the gel was about 0.3% in 2 h incubations and less than 1% for overnight incubations. Duplicate experiments sometimes showed a standard error as high as 4 or 5% for 1 h or shorter incubations, but this was reduced to less than 0.5% after overnight incubations.

Separation of Peptide Products by Reverse-phase HPLC and TLC

Measuring the amount of radioactivity release from the gel did not permit cleavage site characterization. Thus a method was needed to separate the various peptide products. The N-terminal di-, tri-, tetra-, penta-, hexa- and heptapeptides were synthesized, iodinated and separated by reverse-phase HPLC on a C-18 column, eluting with a gradient of acetonitrile in 0.1% TFA. Eluted fractions were collected and counted for radioactivity. Table III shows HPLC retention times of the peptide fragments. Peptides larger than the heptapeptide (βA4 38–43) were not studied because of aggregation and insolubility considerations, and also because the six fragments studied were sufficient to characterize γ-secretase activities which are thought to cleave at positions 39, 40, 42 and 43.

Another method to separate the labelled peptides was also prepared. The peptides were separated by TLC on a C-8 reverse-phase TLC plate (Figure 3) and the radioactivity was detected by phosphorimage analysis of a screen exposed to the plate.

Stability of Ac-YGVVIA Tracer

The hexapeptide tracer was found to be quite unstable, as compared with the other tracers. Several iodinations were performed and every time the tracer decomposed quickly. To study the stability of the tracer, aliquots were incubated in buffers of various pHs and analysed by HPLC (Figure 4). After overnight storage at 4°C, results showed good stability of the tracer peptide at pH 3.2 as well as at pH 7.4. In contrast, at pH 5.2 and pH 6.2 a new peak appeared on the HPLC profile, which co-migrated with Ac-YG. In addition, the peak which corresponded to the hexapeptide was broader than usual, possibly due to aggregation. Indeed, βA4 peptides have been found to aggregate at pH 5.5, a pH at which α-helices are destabilized and β-pleated sheet formation is favoured [20, 21].

DISCUSSION

The design of synthetic substrates for βA4 γ-secretases is impaired by insolubility in aqueous buffers. Indeed, βA4 C-terminus is part of a transmembrane domain and therefore lipophilic. Ishiura *et al.* [22] prepared aminomethylcoumaride peptide derivatives and found that they could not use peptides larger than a dipeptide because of insolubility. Since several forms of βA4 have been described, varying at their C-termini (Val^{39}, Val^{40} and Ala^{42}, with sometimes presence of the Thr^{43}) (Figure 1) we aimed for an assay that would include these various cleavage sites. Furthermore, genetic mutations associated with hereditary AD occur at position 42 [23] or at position 46 [19, 24, 25] (numbering is from βA4 N-terminal Asp as residue 1), it seemed interesting to study if these interfered with the release of βA4 C-terminus. The chosen peptide sequence overlapped the several potential cleavage sites as well as the mutation sites. Because the peptide was very insoluble in all aqueous buffers and in most organic solvents, except for formic acid and TFA, a solid-phase radiometric assay was devised [26, 27]. The 'reporter' group, the labelled tyrosine, was attached to the N-terminus since it was intended to try alternative reporter groups with a same peptide. Indeed, to avoid the use of radioactivity, and its inconvenience, including the decay of ^{125}I and the

Table III HPLC Retention of Peptide Fragments on a C-18 Reverse-phase Column

Peptide	fragment of Ac-Y G V V I A T V I V I T	HPLC RT (min) Native	Iodinated
Ac-YG	———	13.3	18.5
Ac-YGV	————	17.4	21.0
Ac-YGVV	—————	18.7	22.5
Ac-YGVVI	——————	21.8	24.8
Ac-YGVVIA	———————	18.5	24.3
Ac-YGVVIAT	————————	20.0	24.0

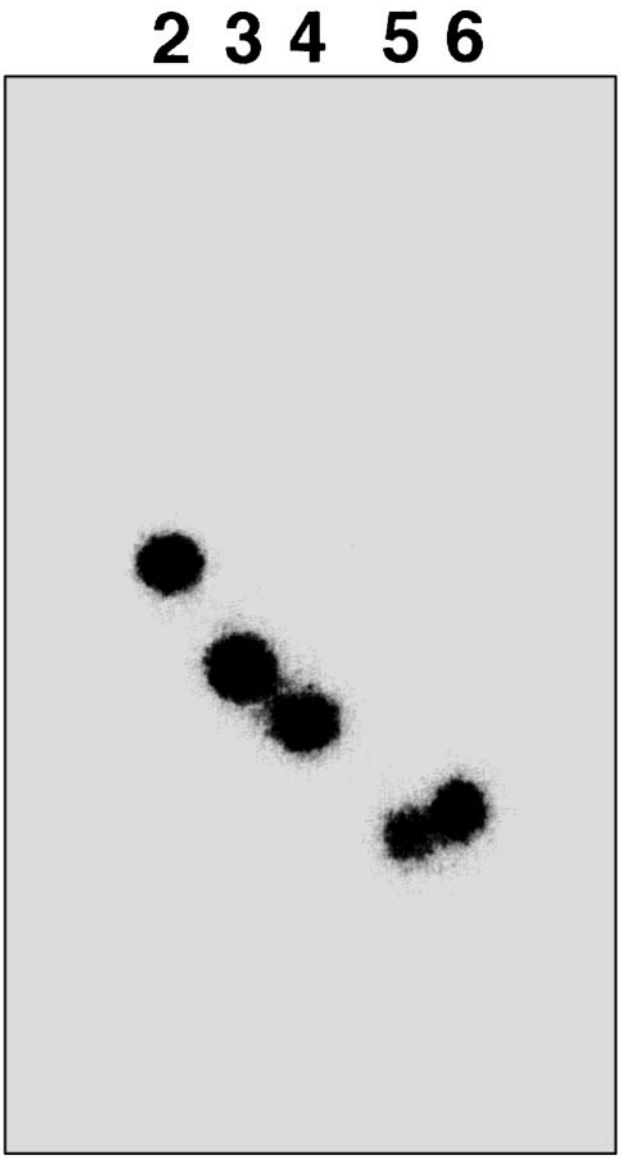

Figure 3 Separation of five standard peptides by TLC. 1000 c.p.m. aliquots of ^{125}I-labelled peptides were loaded onto a C-8 reverse-phase TLC plate developed with 38% isopropanol in 1% TFA. The plate was then analysed by phosphorimaging: lane 2, Ac-YG; lane 3, Ac-YGV; lane 4, Ac-YGVV; lane 5, Ac-YGVVI; lane 6, Ac-YGVVIA.

need to prepare new batches of substrate frequently, N-terminal dansylation was attempted, using dansyl chloride. The dansylation proved to be very slow and incomplete, so this approach was not pursued further.

The radiometric assay was easy to set up, reproducible and the problem of peptide insolubility

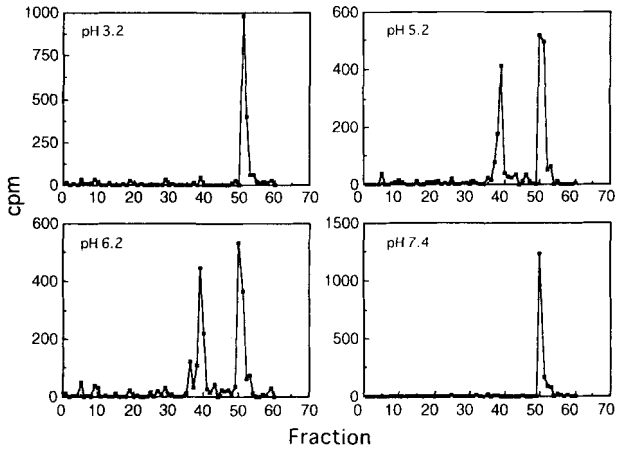

Figure 4 Stability of Ac-YGVVIA tracer at four different pHs. HPLC analysis of ^{125}I-labelled peptide after overnight incubation in buffers of different pHs. Samples were analysed on a C-18 reverse-phase column developed with a gradient of acetonitrile in 0.1% TFA, at a flow rate of 1 ml/min. 0.5 ml fractions were collected and counted for radioactivity. A standard solution of fresh tracer eluted as a single sharp peak, in fraction 50.

was circumvented since it consisted of a solid-phase assay and only tracer amounts of peptide were used. In addition, the assay requires little handling of samples (only pipetting and microcentrifugation), which is an advantage when biological and biohazardous samples are to be tested. Because of its simplicity, many samples can be processed simultaneously, which is convenient when purification fractions have to be assayed. To achieve optimum sensitivity, a longer spacer arm could have been used between the peptide and the gel (Affi-Gel 102 possesses only a 6-carbon spacer).

Identification of the cleavage products can be obtained by using reverse-phase chromatography techniques. Both HPLC (on a C-18 column) and TLC (on a C-8 plate) separated five or six peptide standards. The advantage of the HPLC is that supernatants from enzymatic incubations can be directly applied onto the column, but the disadvantages are that only one sample can be applied at a time and that crude samples may contaminate the column. TLC separation allows analysis of several samples simultaneously and direct comparison with standards, but the disadvantages are that the samples have to be concentrated before application to the plate, and it is less precise and reproducible. Both chromatographic methods are currently used in our laboratory to analyse digests of the substrate by

various proteases and brain samples. Application of this assay to search for γ-secretase from brain homogenates and subfractions is in progress.

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