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In vitro Generation of Amyloid βA4 Peptide from Amyloid Protein Precursor Through Nonspecific Proteolysis

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Abstract: Amyloid βA4 peptide, the principal constituents of the senile plaques in Alzheimer's disease (AD) originates from proteolysis of a larger protein precursor (APP). Several lines of evidence suggest that this peptide may be generated from aggregated precursor through nonspecific proteolysis. In this work, we used a sensitive *in vitro* method of detection to investigate the role of nonspecific proteases in the processing of a 100-amino acid C-terminal fragment (C100) inclusive of PA4 and cytoplasmic domain of APP. We demonstrate first that C100 forms high molecular weight aggregates *in vitro* as determined by size exclusion chromatography. Digestion of aggregated C100 with the nonspecific enzyme, proteinase K resulted in cleavage at the amyloidogenic y-secretase sites. This occurred at Ala 42-Val 43 generating βA4 12-42 and βA4 16-42 amyloid peptides. The enzyme cleaved most of the peptide bonds of the cytoplasmic domain and the upstream of βA4 domain of the substrate. The result suggests that both the N- and C-terminus βA4 can be generated by nonspecific proteases, acting on a aggregated substrate and support the notion that the βA4 can be formed in organelles containing proteases capable of cleaving most peptide bonds.

Key words: Amyloid βA4 peptide; Amyloid protein precursor; Nonspecific proteolysis

Introduction

The principal component of the extracellular senile plaque found in AD brain is βA4 amyloid. It is a 39-43 residue peptide and derived from the proteolytic processing of a larger precursor termed as amyloid precursor protein (APP) (Kang *et al.,* 1987). In the amyloidogenic pathway which occurs in the lysosomal-endosomal system, APP is first cleaved at the N-terminus by the Pesecretase yielding a potentially amyloidogenic C-terminal fragment of APP containing the intact βA4 and cytoplasmic domain (Estus *et al*., 1992) which will hereafter be referred to as C100 (Maruyama *et al*., 1990). C100 is further cleaved within its transmembrane domain by a y-secretase activity, thereby releasing βA4 peptide (Koo and Squazzo, 1994). The enzyme or enzyme catalyzing y-secretase cleavage have not yet been identified.

To identify the true y-secretase we are currently evaluating the proposed proteases implicated as candidates for y-secretase. In the previous paper, we have described the role of cathepsin D, an aspartic protease in the processing of APP (Sadik *et al*., 1999). An accumulation of evidence has implicated the nonspecific proteases as the y-secretase involved in the generation of βA4. First, a significant heterogeneity at the C-terminus of βA4 has been observed (Haass and Selkoe, 1993) which indicates a certain degree of nonspecificity of the y-secretase. Second, this enzyme is localized to an endosomal-lysosomal compartment (Haass *et al*., 1992a, b) which contain a variety of proteases with various substrate specificities and seems plausible to suppose that the substrate is processed in an environment of enzymes capable of cleaving most of the peptide bonds. Moreover this enzyme has previously been supposed to be capable of generating βA4-like peptides only from the aggregated substrate as βA4 domains of several C100 molecules may interact and generate a protease-resistant cores (Tjernberg *et al*., 1997; Dyrks *et al*., 1988), However, in these studies, βA4 peptide was not characterized at the amino acid sequence level (Dyrks *et al*., 1988) nor by the very specific antibodies that can determine the size of βA4 or can discriminate the different βA4's (Tjernberg *et al*., 1997). Therefore the role of nonspecific proteases in the generation of βA4 or the related peptides could not be comprehensively understood.

In this study, we investigated the generation of βA4 from the nonspecific proteolysis of a 100-amino acid C-terminal fragment (C100) containing βA4 and cytoplasmic domain of APP. This was conducted by developing suitable *in vitro* conditions. Improvements in the method of detection allowed us to characterise βA4 generated from the proteolysis and provide important clues regarding the role of nonspecific proteases in APP processing.

Materials and Methods

Materials: Proteinase K was from Sigma Chemical Company, USA. Monoclonal antibody specific for βA4 1.10 of βA4 was prepared as described (Shinoda *et al*., 1990). Unless indicated otherwise, all reagents used in this study were of analytical grade and obtained from Wako Pure Chemical Industries, Japan. The C100 protein was synthesized and purified as described (Sadik *et al*.,1999). This recombinant C100 (Fig.1) contained extra five residues at the C-terminal which were tagged to contribute to the expression and solubility of the protein. In brief, C100 was purified by a combination of urea solublization, size exclusion chromatography (SEC) and reversed phase-high performance liquid chromotography (RPHPLC) followed by lyophilization.

Size exclusion chromatography of C100: Size exclusion chromatography were performed using TSK G3000sw column (7.8 × 300 mm, TOSOH) in a Shimadzu HPLC chromatography system. A 1.0 ml aliquot was prepared by dissolving 85 μg of C100 in 0.2 ml of 8 M urea and diluted to 1.0 ml with 62.5 mM Tris-HCl buffer, pH 7.4 to adjust finally to 50 mM Tris-Hcl buffer containing 1.6 M urea, pH 7.4. The buffer used for SEC was identical to the solubliziog condition of C100. The whole aliquots were injected into the column which was equillibrated with the buffer and chromatography was performed with flow rate of 0.2 ml/min by monitoring absorbance at 280 nm. The standards used to calibrate the column and their masses are alcohol dehydrogenase (150,000), bovine serum albumin (68,000), ova albumin (43,000) and cytochrome C (12,000).

Digestion of C100 with proteinase K: Aggregated C100 (50 μg) collected after SEC was allowed to digest with proteinase K at an enzyme-substrate ratio of 1:50 and incubated at 37°C for 24 h, Due to the appearence of a considerable amount of precipitate during digestion the reaction mixture was centrifuged at 15,000 rpm, 4°C for 15 min. This separated supernatant from the precipitate. The clear supernatant was immediately subjected to RP-HPLC separation on an ODS column (4.6 × 150 mm) at a constant flow rate of 1 ml/min followed by elution with 60 min linear gradient of CH_30N (560%) in 0,1% TFA and the absorbance was monitored at 215 ram, The precipitate was analysed by SDS-PAGE followed by Coomassie brilliant blue staining (CBB) and western blotting, mass spectrometer and amino acid sequence analyser.

Amino acid sequence and mass spectral analyses: Peptides from digestion of C100 with proteinase K were subjected to Edman degradation using an Applied Biosystems 473A protein sequencer. Mass spectra was measured using a matrix assisted laser desorption/ionization time-of flight mass spectrometer (MALDI-TQF-M5), Finnigan MAT VISION 2000 (Sadik *et al*., 1999).

Results

The C-terminal fragment of APP that span the βA4 and cytoplasmic domain has a tendency to self-aggregate (Dyrks *et al*., 1988). Previously it was shown that under urea denaturing condition, C100 was inaccessible to proteolytic digestion (Sadik *et al*., 1999). The reason for the inaccessibility of C100 to proteolytic disestion was clearly clarified by the present data. Figure 2 shows the SEC elution profile of C100 prepared from 8 M urea. Compared to molecular size marker, 95% of C100 was eluted from the column with apparent molecular weight in the range of 150 kDa representing high molecular weight aggregates and the rest of 5% was eluted in the range of 12 kDa representing monomer.

To determine the nonspecific proteolysis, aggregated C100 fraction was allowed to digest for 24 h with proteinase K, an enzyme capable of cleaving most of the peptide bonds. After stopping the reaction, the digests were analyzed by conventional methods of peptide mapping. The digest products were separated by reversed phase-high performance liquid chromatography (RP-HPLC). As shown in Fig. 3 the digest was separated in seven major peaks and the amino acid sequences of each of the fraction are presented (Inset, Fig. 3). The amino acid sequence indicated that all the fraction except fraction 7 contained single polypeptide, while fraction 7 contained two peptides. The fraction was composed of tripeptide, tetrapeptide or pentapeptide. According to the location of the cleavage sites, proteinase K was found to cleave most of the peptide bonds of the cytoplasmic domain and the upstream of βA4 domain of C100. The complete sequences of βA4 was not detected by this method of analysis.

During the digestion period, a considerable amount of precipitate was formed in the reaction mixture and it was analysed by SDS-PAGE followed by CBB staining and western blotting. The precipitate was also analysed by mass spectroscopy and amino acid sequence analyser, CBB staining result (Fig. 4A) showed that the fragment migrated as a single species with an apparent molecular masses of 3 kDa. However in addition to this band, the high molecular weight aggregates were observed at the top of the separating gel interface, might be due to the spontaneous aggregation of the generated fragments. The Western blot of the precipitate (data not shown) revealed that the fragments were not immunoreactive against monoclonal anti βA4 antibody specific

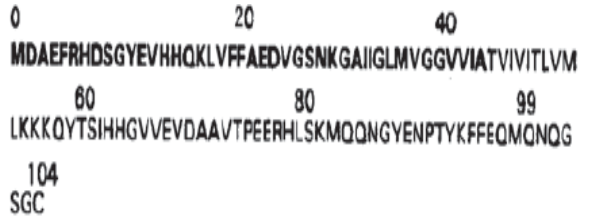

Fig. 1: Amino acid sequence of the C100 construct. The construct includes the initiation methionine residue and the C-terminal tagged residues. The sequences of the βA4 amyloid peptide is shown in bold letters

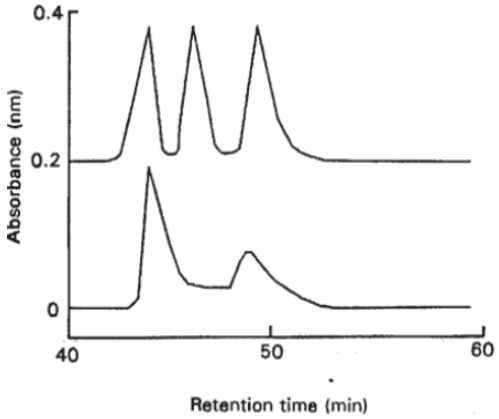

Fig. 2: Size exclusion chromatography of C100: C100 (85 μg) in 50 nnM Tris-HCI buffer (TB) containing 1.6 M urea, pH 7.4 was injected into a TB-urea equillibrated, precalibrated TSK G 3000sw column. The elution was monitored at 214 nm absorbance. Continuous line: elution profile of C100, Broken line: elution profile of molecular weight markers; from left to right, alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (33 kDa) and oytochrome C (12 kDa). The results are typical of three experiments

for βA4 1-10 of βA4. Both mass spectrometry (Fig. 4B) and direct amino terminal sequence analyses (Table 1) showed that the major species were βA4 12-42 and βA4 16-42. Thus the generation of C-terminal βA4 peptides were confirmed from the nonspecific proteolysis of C 100. However, due to strong hydrophobicity and aggregating tendency these βA4 species could not be purified by the conventional RP-HPLC system (Shinoda *et al*., 1996).

Discussion

In this study, we were primarily interested in examining the generation of βA4 peptide from the nonspecific proteolysis of the aggregated amyloid protein precursor. An appropriate protein substrate consisting of the carboxyl-terminal 100 amino acids of APP (C100), starting at Met0 of the βA4 domain was used that can aggregate (Dyrks *et al*., 1988) and generate a βA4-like peptide. We provide substantial evidence that the C-terminal βA4 can be generated through nonspecific proteolytic activity of proteinase K.

C100 synthesized *in vitto* was reported to be able to form self-aggregates (Dyrks *et al*., 1988). Aggregation has been observed even when the protein was inserted in cell membranes (Tjernberg *et al*., 1997). Moreover the transfection of C-terminal fragment of APP into COS cells caused visible

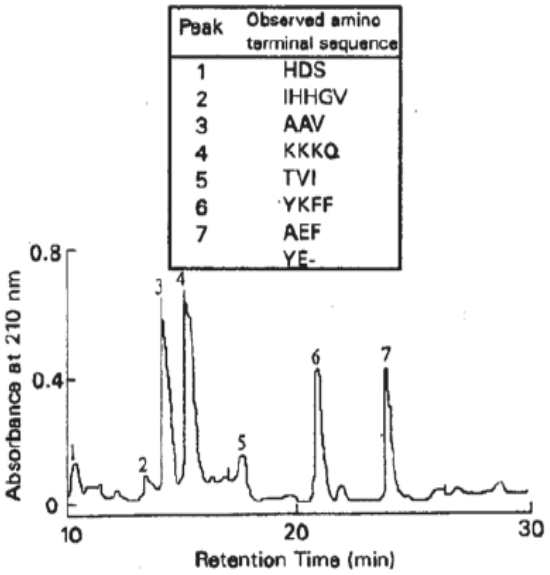

Fig. 3: FIP-HPLC profile of the digestion mixtures of aggregated C100 with proteinase K. A 50 μg sample of aggregated C100, dissolved in 50 mM Tris-HCl buffer containing 1.6 M urea, pH 7.4 was digested with proteinase K at an enzyme to substrate weight ratio of 1:50, 37°C for 24 h. After digestion, peptides were isolated and analysed by amino terminal sequence analyser as described in section 2. Inset, amino terminal sequences of major digestion products isolated by RP-HPLC

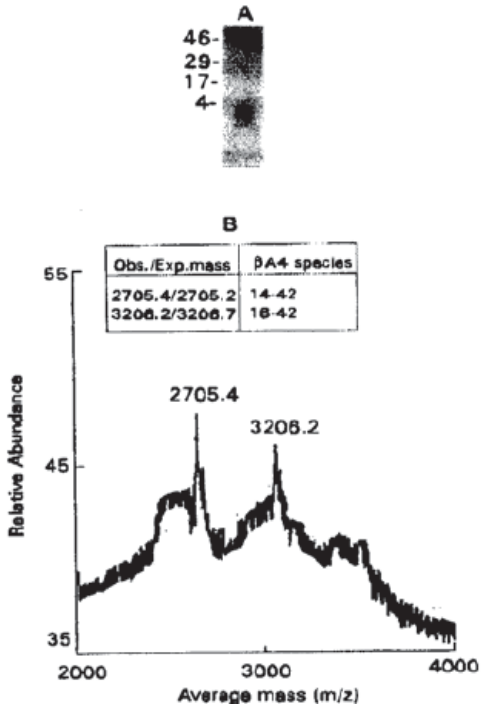

Fig. 4 (A-B): Analysis of the precipitate generated from digestion of aggregated C100 with proteinase K: Digestion of C100 with proteinase K and separation of the precipitate were carried out according to the text. (A) 20% Trisglycine SDS-PAGE of the precipitate stained with Coomassie brilliant blue. The positions of the molecular mass standards (kDa) are shown in the left. (B) MALDI-TOF-MS spectrum of the precipitate, Inset, expected and observed molecular masses of βA4 species with their position in the amino acid sequence

aggregates in the cells (Maruyama *et al.*, 1990). However, many other reports (Fukuchi *et al.*, 1992; Yoshikawa *et al.*, 1992) failed to observe any aggregational product of C-terminal APP fragment. From our experiment we demonstrate that C100 formed high molecular weight aggregates *in vitro,* indicating that C100 has a strong tendency to self-aggregate, The possible explanation is that C100 forms aggregates through interaction between βA4 domains, since βA4 readily forms fibril.

The generation of βA4 through nonspecific proteolysis was investigated in greater detail using recombinant protein substrate, purified protease and improved method of analysis. We have presented evidence that aggregated C100 on digestion with proteinase K, the bacterial enzyme capable of cleaving all peptide bond, yielded βA4 12-42 and βA4 16-42 (Table 1). The cytoplasmic domain and the upstream of βA4 domain of the substrate was almost completely degraded by the enzyme with formation of very short peptides like tripeptide, tetrapeptide or pentapetide. Proteinase K was choosen to mimic the environment of lysosome and endosome, which involve a multitude of endoproteases and exoproteases with various substrate specificities. The generation of such trunoated βA4 peptides is biologically relevant as the corresponding fragments have been found from the secretory processing in rat hippocampal neurons overexpressing human APP695 (Simons *et al.*, 1996). The result strongly suggests that βA4 variants can be formed in organelles such as endosomes and lysosomes, containing varieties of proteases covering a broad spectrum of substrate specificities. Apparently, nonspecific protease activity cleaving any peptide bond can serve as y-secretase, if the substrate has the aggregated state. The fact that an intact fragment corresponding to C100 lacking the βA4 domain has not yet been detected in cells may also be en argument against the idea that y-secretase is a specific enzyme (Citron *et al.*, 1996). The formation of βA4 from the aggregated substrate is also relevant to the findings *in vivo,* Podlinsy *et al.* (1995) demonstrated the secretion of βA4 as soluble oligomers in the culture media of APP transfected cells. The oligomers were SDS-stable after separation, thus speculating that the oligomers were formed prior to y-secretase cleavage.

Table 1: Amino terminal sequence and mass spectral analysis of the precipitate obtained from proteinase K digestion of aggregated C 100

Observed amino terminal sequence	Observed mass	Expected mass	Deduced sequence	C100 residue no.
(1°)	2705.4	2705.2	VHOXLVFFAEDVGSNKGAIIGILIVIVGGVVIA	12-42
(2°)	3206.2	3206.7	KLVFFAEDVGSINIKGAIIGLMVGGVVIA	16-42

In conclusion we propose that both N- and C-terminus of βA4 can b e generated by nonspecific proteases acting on a aggregated substrate *in vitro.* If such mechanism for βA4 generation is confirmed *in vivo,* an agent capable of antagonizing C100 aggregation would prevent the generation of βA4, thereby preventing amyloidogenesis in Alzheimer's disease.

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