Cathepsin D is involved in the clearance of Alzheimer's β-amyloid protein

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Abstract The cerebral deposition of 39–42 residue amyloid β-protein (Aβ) is a histopathological characteristic of Alzheimer's disease. The present study is aimed at finding proteinases responsible for the intracellular clearance of Aβ. The Aβ-degrading proteinase was purified from rat brain. Amino-terminal sequence analysis indicated the Aβ-degrading proteinase was cathepsin D. Purified cathepsin D hydrolyzed Aβ between Phe^{19} and Phe^{20}. Cathepsin D is likely to be involved in the intracellular clearance of aggregatable Aβ, since Aβ fragments with Phe^{20} at the amino-terminus have been reported to be secreted from several lines of cultured cells.

Key words: Alzheimer's disease; β-Amyloid; Amyloidosis; Cathepsin D; Rat brain; Aspartic proteinase

1. Introduction

One of the hallmarks of Alzheimer's disease is the cerebral deposition of 39–42 residue amyloid β-peptide (Aβ). Aβ is produced by proteolytic processing from β-amyloid precursor protein (βAPP), a 110–130 kDa type I integral membrane glycoprotein. Aβ is secreted from most cells including neurons and the overproduction of Aβ is likely to lead to the formation of neuritic plaques and vascular deposits in the brain microvessels (see [1] for review). Therefore, it is critically important to maintain the normal balance between production and clearance of Aβ to prevent the cerebral deposition of Aβ.

The proteolytic processing of βAPP has been studied using cultured cells and three endoproteinases designated α-, β-, and γ-secretases have been proposed to be engaged in the processing. The α-secretase has been shown to be present at or near the cell surface [2] and cleaves βAPP within the Aβ region (at Lys^{16}-Leu^{17} of Aβ) to produce a large secreted derivative (APPs) and a 9 kDa membrane-associated fragment, neither of which can produce amyloid because they do not contain the entire Aβ [3,4]. The β-secretase cleaves βAPP at the NH_2-terminus of Aβ, producing a 10 kDa COOH-terminal fragment, which is further cleaved at the COOH-terminus of Aβ by γ-secretase. It has been described that Aβ formation occurs at least in part in the endosomes/lysosomes [5,6] after cell surface βAPP has been reinternalized via clathrin-coated pits [7–9].

In addition to the cleavage after Lys^{16} of Aβ region, other cleavages are also suggested to be important to eliminate Aβ generation. For instance, Busciglio et al. reported that CHO cells secreted Aβ fragments with a heterogeneous NH_2-terminal sequence, reflecting cleavages after Lys^{16}, Phe^{19}, and Val^{24} [10] and Haass et al. described that kidney 293 cells secreted Aβ-related peptides, reflecting cleavages after Lys^{16}, Leu^{17} and Phe^{19} [11]. It is interesting that both cell lines produce Aβ-related peptides with Phe^{20} at the NH_2-terminus. However, proteinase(s) that cleave after Phe^{19} of the Aβ region have not been identified.

The present work was aimed at finding proteinases responsible for the degradation of Aβ in the brain and it was found that cathepsin D (EC 3.4.23.5) was the major proteinase that hydrolyzed Aβ in the middle of the sequence to eliminate the amyloidogenic peptide. The involvement of cathepsin D in the elimination of Aβ has been suggested by the observations that the proteinase specifically cleaves full-length Aβ between Phe^{19} and Phe^{20}.

2. Materials and methods

2.1. Materials

Synthetic $Aβ_{1-42}$ and $Aβ_{1-28}$ were purchased from American Peptide Co. (Sunnyvale, CA, USA). The purity of all synthetic peptides was above 98% on RP-HPLC. Pepstatin A, leupeptin, and E-64 were purchased from Sigma (St. Louis, MO, USA). Pepstatin A was coupled with Affi-Gel 10 (BioRad) by incubating the mixture of 0.05 mmol of pepstatin A, 0.05 mmol of diisopropylcarbodiimide, 0.06 mmol of hydroxybenzotriazole, and aminoethyl-Affi-Gel 10 (packed volume; 2 ml) in 2.5 ml of dimethylformamide at room temperature for 15 h. After coupling gels were successively washed with dimethylformamide, 1 M acetic acid, and 0.5 M borate buffer, pH 9.5.

2.2. Digestion of Aβ by the brain extract

Rat brain (2 g, stored at −20°C) was minced in 20 ml of Tris-buffered saline (TBS; 0.15 M NaCl, 10 mM Tris-HCl, pH 7.5), and homogenized for 30 s with a Polytron homogenizer at 4°C. The brain extract was obtained as the supernatant after the homogenate was centrifuged at $10^5 \times g$ for 60 min. To estimate the pH optimum of Aβ hydrolysis, $Aβ_{1-28}$ (1 nmol) was incubated with the rat brain extract (5 μl) in 20 μl of 0.1 M acetate buffer (pH 3.5 and 4.5) or phosphate buffer (pH 5.5–7.5), at 37°C for 30 min. After incubation the reaction was stopped by adding 0.1 ml of 0.05% trifluoroacetic acid (TFA) followed by heating at 100°C for 5 min. The reaction mixture was filtered through a membrane filter (0.22 μm; Amicon) and then the filtrate was chromatographed on an Inertsil 300C8 column (4.6×100 mm; GL Science Inc., Japan). The peptides were eluted at a rate of 1 ml/min with a 20-min linear gradient of 0–40% acetonitrile in 0.05% TFA and monitored by absorbance at 215 nm.

2.3. Purification of Aβ-degrading proteinase

Rat brain (30 g) was homogenized in 300 ml of TBS containing 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 μM leupeptin, and 5 mM EDTA, and centrifuged at $2 \times 10^4 \times g$ for 30 min. After the supernatant fraction was mixed with one-tenth volume of 1 M acetate buffer, pH 4.5, precipitates were removed by centrifugation at $10^5 \times g$ for 60 min. The supernatant fraction (290 ml) containing

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Abbreviations: Aβ, amyloid β-peptide; βAPP, β-amyloid precursor protein; FAD, familial Alzheimer's disease; HCHWA, hereditary cerebral hemorrhage with amyloidosis; RP-HPLC, reverse phase high performance liquid chromatography; TBS, Tris-buffered saline; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis

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108 units of Aβ-degrading proteinase was loaded on a column (1 ml) of pepstatin A-Affi-Gel 10 equilibrated with 0.1 M acetate buffer, pH 4.5. After the column was washed with 10 ml of the column buffer, Aβ-degrading proteinase was eluted with 0.5 M borate buffer, pH 9.6, and 0.5-ml fractions were collected, monitored by absorbance at 280 nm, and proteinase activity assayed using $Aβ_{1-28}$ as substrate. The proteinase preparation purified by affinity chromatography was loaded on a column (16×600 mm) of Superdex 75 pg column (Pharmacia Biotech) equilibrated with TBS. The column was eluted with TBS at a flow rate of 0.5 ml/min and 1-ml fractions were collected, monitored by absorbance at 215 nm, and Aβ-degrading activity assayed. The obtained enzyme preparation contained 34.4 units of Aβ-degrading activity (specific activity: 141 units/mg protein). One unit of Aβ-degrading activity was defined as the amount of proteinase required to hydrolyze 1 μmol of $Aβ_{1-28}$ after Phe^{19} in 0.1 M acetate buffer, pH 4.5, at 37°C in 1 h. SDS-PAGE was performed using 10% acrylamide gel with Tris-glycine buffer system [12] and gels were stained with Coomassie brilliant blue R-250.

2.4. Hydrolysis of Aβ by cathepsin D

$Aβ_{1-42}$ (1 nmol) was incubated with 1.2 μg of purified brain cathepsin D in 40 μl of 0.1 M acetate buffer, pH 4.5, at 37°C for 4 h. After incubation the reaction mixture was chromatographed on an Inertsil 300C8 column (4.6×100 mm) with a solvent system containing 0.05% TFA. The peptides were eluted at a rate of 1 ml/min with a 30-min linear gradient of 0–60% acetonitrile containing 0.05% TFA and fractions with UV absorbance at 215 nm were collected for peptide sequencing.

2.5. Analysis of protein sequence

Samples were spotted on polybrene-coated glass fiber disks and sequenced on an Applied Biosystems model 491 protein sequencing system. Data were analyzed using a model 610A Data Analysis Program.

3. Results and discussion

To examine the effects of pH on the degradation of Aβ, the rat brain extract was incubated with $Aβ_{1-28}$ at 37°C for 30 min, and subsequently the degradation products were analyzed on RP-HPLC (Fig. 1A). Since Aβ was effectively hydrolyzed at pH 3.5–4.5, the following experiments were performed at pH 4.5. To characterize the proteinase(s) that hydrolyze Aβ, the brain extract was incubated with $Aβ_{1-28}$ in the presence of specific proteinase inhibitors and the incubation mixture was analyzed on RP-HPLC (Fig. 1B). Neither E-64 (cysteine proteinase inhibitor) nor leupeptin (serine and cysteine proteinase inhibitor) inhibited the hydrolysis of $Aβ_{1-28}$ by the brain extract, whereas pepstatin A (aspartic proteinase inhibitor) completely inhibited the proteolysis. Neither EDTA (5 mM) nor PMSF (1 mM) inhibited the hydrolysis of Aβ (data not shown). The results suggest that the major proteinase involved in the hydrolysis of Aβ is aspartic proteinase.

The Aβ-degrading aspartic proteinase was purified by affinity chromatography using pepstatin A as an absorbent immobilized on Affi-Gel 10 (see Section 2). After affinity chromatography, gel filtration was performed to remove a ~70 kDa protein which had no Aβ-degrading activity (Fig. 2A). By these two steps of chromatography the Aβ-degrading proteinase was purified approximately 3000-fold with 32% recovery. The enzyme preparation after gel filtration gave a single band on SDS-PAGE at ~43 kDa (Fig. 2B). The NH_2-terminal sequence of the purified Aβ-degrading enzyme was determined to be EPVSELLKNYLDAQY, which is consistent with the protein sequence deduced from the nucleotide sequence of rat cathepsin D cDNA cloned from a pituitary cDNA library [13]. The molecular weight and the partial protein sequence indicated that the Aβ-degrading proteinase was cathepsin D. Cataldo et al. demonstrated the presence of cathepsin D and its mRNA in human brain neurons by immunohistochemical studies and by in situ hybridization [14], sug-

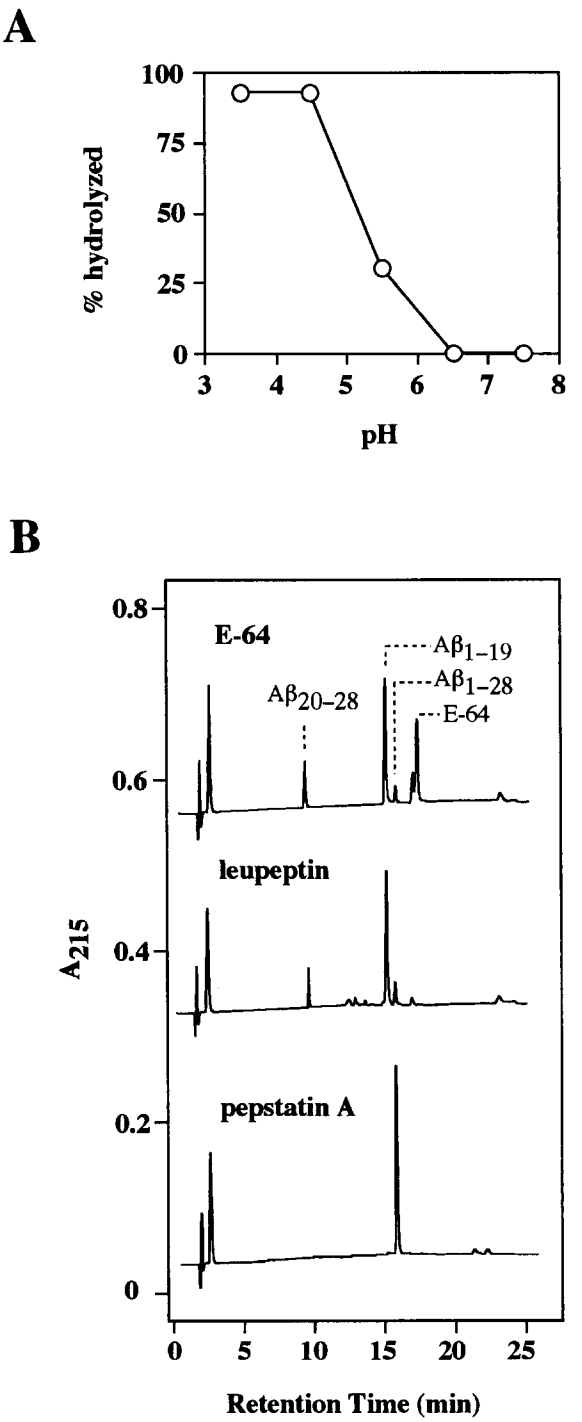

Fig. 1. Hydrolysis of Aβ in the middle of the sequence with rat brain extract. A: Effects of pH on the hydrolysis of $Aβ_{1-28}$. $Aβ_{1-28}$ was incubated at 37°C for 30 min with the brain extract. B: Inhibition of Aβ-degrading proteinase in the brain extract. $Aβ_{1-28}$ was incubated at 37°C with the brain extract in 0.1 M acetate buffer, pH 4.5, containing E-64 (3200 μM), leupeptin (1200 μM), or pepstatin A (1.5 μM). After 30-min incubation products were analyzed on RP-HPLC.

gesting brain neurons produce the same cathepsin D as peripheral tissues.

To show cathepsin D is able to degrade aggregatable full-length Aβ, $Aβ_{1-42}$ was incubated with the proteinase purified from rat brain. Fig. 3 shows that cathepsin D hydrolyzes $Aβ_{1-42}$ after Phe^{19} and Leu^{34}, producing $Aβ_{1-19}$, $Aβ_{20-34}$, and $Aβ_{35-42}$. About 80% of $Aβ_{1-42}$ was hydrolyzed in 4 h. The specific hydrolysis of Aβ between Phe^{19} and Phe^{20} by cathepsin D suggests that this endoproteinase contributes to the generation of Aβ-related peptides with Phe^{20} at the NH_2-terminus which have recently been described to be secreted from several lines of cultured cells [10,11].

The present results give direct evidence that cathepsin D is a major proteinase in the brain that is able to degrade full-length Aβ in the middle of the sequence. Since cathepsin D localizes in lysosomes and endosomes [14], where Aβ is generated by proteolytic processing [5,6], it is possible that cathepsin D eliminates Aβ at the site of generation. It has been described that cathepsin D was capable of producing the cleavages resulting in Aβ generation, especially when a synthetic peptide with Swedish-type mutation was used as substrate [15]. In conclusion, I would like to propose that cathepsin D is able to work as an Aβ-producing enzyme especially for Swedish-type mutants and also as an Aβ-eliminating enzyme by cleaving at Phe^{19}-Phe^{20} of the Aβ region and that the balance between the two cleavages affects the secretion of the amyloidogenic peptide.

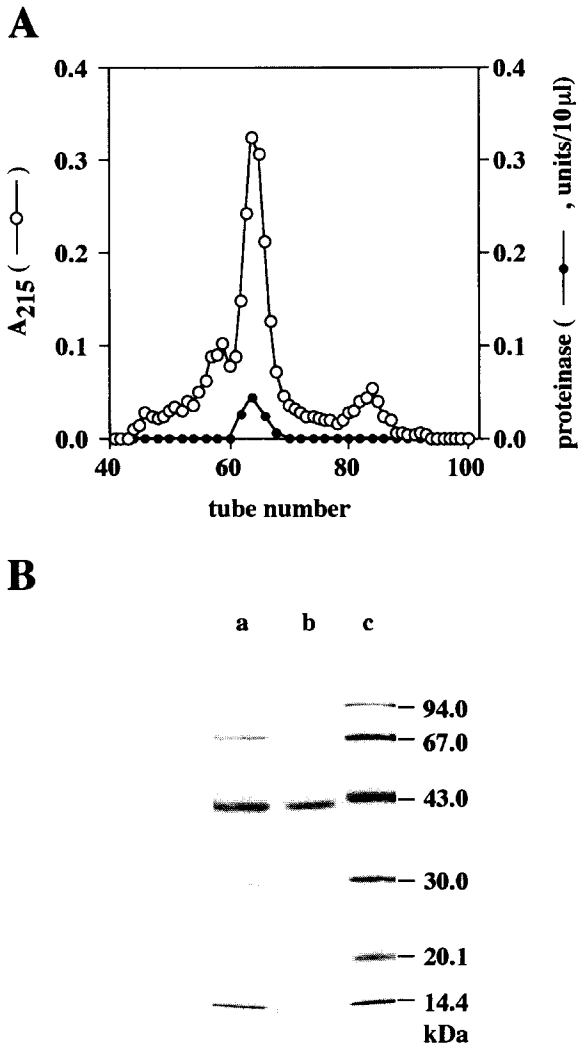

Fig. 2. Purification of Aβ-degrading proteinase. A: Gel filtration of affinity-purified Aβ-degrading enzyme. The proteinase preparation purified by affinity chromatography was loaded on a column (16×600 mm) of Superdex 75 pg column equilibrated with TBS. The column was eluted with TBS at a flow rate of 0.5 ml/min and 1 ml fractions were collected, monitored by absorbance at 215 nm, and proteinase activity assayed every second fraction. B: SDS-PAGE of the purified enzyme. Lane a: 2 μg of the enzyme preparation after affinity chromatography; lane b: 2 μg of the enzyme preparation after gel filtration; lane c: molecular weight standard.

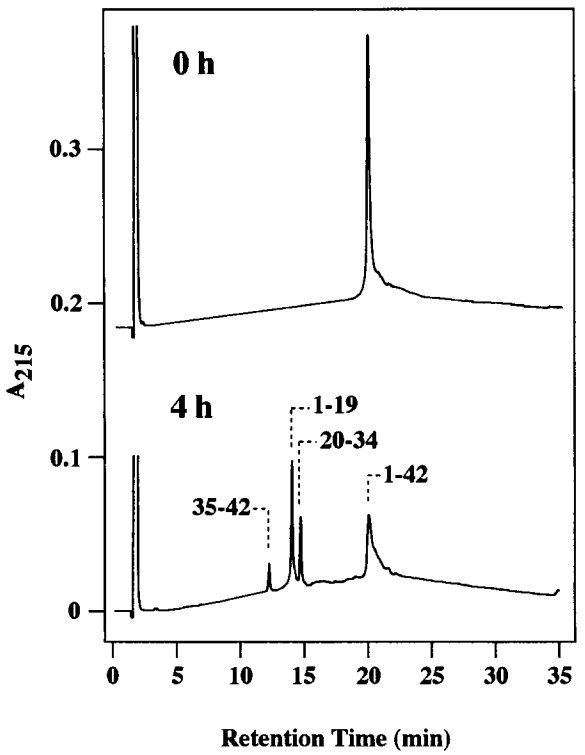

Fig. 3. Cleavage of full-length Aβ by cathepsin D. $Aβ_{1-42}$ (1 nmol) was incubated with 1.2 μg of purified brain cathepsin D in 40 μl of 0.1 M acetate buffer, pH 4.5, at 37°C for 4 h. After incubation the peptides were separated on an RP-HPLC and peaks with UV absorbance at 215 nm were collected for peptide sequencing.

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