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Two-way cleavage of β-amyloid protein precursor by multicatalytic proteinase

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The β-amyloid protein (β-AP) derived from a β-amyloid protein precursor (APP) is a hallmark of Alzheimer's disease. The abundant generation of β-AP suggests the abnormal processing of APP, but the molecular mechanism remains unclear. The main APP-processing enzyme was purified from the rat brain and identified to be a macropain-like multicatalytic proteinase. The purified enzyme cleaved the Gln^{15}–Lys^{16} bond of β-AP, but altered to cleave at the N-terminus of β-AP to release the extracellular domain of β-AP in the presence of Ca^{2+}. These findings suggest that the functional change in this multicatalytic proteinase may result in abnormal processing of APP.

β-Amyloid protein precursor; Multicatalytic proteinase; Macropain; Rat brain; Calcium ion

1. INTRODUCTION

Alzheimer's disease (AD) is pathologically characterized by extracellular deposits of β-amyloid protein (β-AP) in senile plaques and cerebral vessels [1]. β-AP is a small peptide fragment of 39 to 42 amino acids derived from a larger amyloid protein precursor (APP) [2–4]. There are at least three different isoforms of APP with 695, 751 and 770 amino acids, generated by alternative splicing of mRNA [4–7]. β-AP is encoded as an internal peptide at position 597–638 of APP695 [4]. The normal processing occurs within the β-AP sequence preventing the generation of β-AP [8,9]. Therefore, the abundant generation of β-AP in the Alzheimer brain suggests the abnormal processing of APP, but the molecular mechanisms of normal and abnormal processing of APP remain unclear.

To investigate the APP-processing enzyme and the β-AP-releasing enzyme in the rat brain, we used peptide AP33 (SEVKMDAEFGHDSGFEVRHQKLVFFAEDVGSNK), synthesised according to the sequence (residues 592–624) of rat APP695 [10]. The peptide consists of the extracellular domain of β-AP, i.e. from Asp^{1} to Lys^{28}, and five amino acids upstream from the N terminus of β-AP, i.e. Ser^{-5} to Met^{-1}. The enzyme activity was determined by the AP33 fragmentation assay.

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2. MATERIALS AND METHODS

2.1. *Assay for APP-processing enzyme and β-AP-releasing enzyme*

Peptide AP33 (SEVKMDAEFGHDSGFEVRHQKLVFFAEDVGSNK) was synthesized by the solid-phase method using a Beckman 990 peptide synthesizer. The peptide chain was elongated on a chloromethylated polystyrene resin using Nα-Boc derivatives of appropriate amino acids. The peptide resin was treated with anhydrous hydrogen fluoride to cleave the peptide from the resin and to remove all protecting groups. The crude peptide was purified by reverse-phase HPLC on a YMC ODS column (30 × 250 mm). AP33 (1 μg) was incubated with the sample in 40 μl of assay buffer (50 mM HEPES, pH 7.0) for 1 h at 37°C. The enzyme reaction was terminated by the addition of 40 μl of 2% trifluoroacetic acid (TFA). The reaction mixture (40 μl) was chromatographed by reverse-phase HPLC on a Bio-Rad RP-304 column (4.6 × 250 mm) using buffer A (0.1% TFA) and buffer B (0.07% TFA–95% acetonitrile). The chromatographic run was performed at a flow rate of 1 ml/min using a 20-ml linear gradient from 0% to 40% buffer B and a 2-ml linear gradient to 100% buffer B. One unit of APP-processing activity was defined as the amount of enzyme that catalyses AP33 as substrate to form one pmol of the peptide fragment corresponding to residues 16–28 of β-AP in 1 h at 37°C.

2.2. *Identification of the cleavage site of AP33 by the extract of rat brain*

About 70 frozen rat brains were homogenized in 60 ml of 50 mM HEPES, 1 mM EDTA, pH 7.5. Insoluble material was removed by centrifugation (40,000 rpm × 30 min). The supernatant (35 ml) was chromatographed at a flow rate of 300 ml/h on two Sephadex G-150 columns (11.2 × 20 cm) connected in series equilibrated with 20 mM HEPES, pH 7.0. Fractions (45 ml) were collected. AP33 (50 μg) was incubated for 3 h at 37°C with 0.5 ml of high-molecular-mass fraction (M_r>100 kDa) in the presence of 0.3 mM $CaCl_2$. The resulting fragments were purified by reverse-phase HPLC on an RP-304 column and analyzed for the N-terminal amino acid sequence on a protein sequencer (Applied Biosystems, model 477A) and a phenylthiohydantoin (PTH) amino acid analyzer (model 120A), while the amino acid compositions were determined on an amino acid analyzer (Hitachi, model 835).

2.3. *Purification and characterization of APP-processing enzyme*

The enzyme-rich fractions (Nos. 36–41) of Sephadex G-150 chromatography were dialyzed against 8 liters of 20 mM Tris-HCl, pH 7.5 (buffer C). The dialyzed enzyme solution was applied onto a benzamidine-Sepharose 6B column (1.6 × 25 cm) and eluted at a flow rate of 2.0 ml/min with a gradient of 0–1 M NaCl in 240 ml of buffer C. Fractions (6 ml) were collected. The active fractions (Nos. 19–26) were diluted twofold with buffer C. The enzyme solution was applied onto a TSK DEAE-5PW column (7.5 × 70 mm) and eluted at a flow rate of 1.0 ml/min with a gradient of 0–1 M NaCl in 60 ml of buffer C. The active fraction was rechromatographed on the same column. Fractions (0.5 ml) were collected. The active fractions (Nos. 66–68) were pooled and stored at −70°C. The purified enzyme was analyzed on a 4–30% polyacrylamide gradient gel without SDS and on a 10–20% polyacrylamide gel with SDS according to the method of Laemmli [11]. The gel was stained with Coomassie brilliant blue. The purified enzyme was chromatographed on a TSK G3000SW$_{XL}$ column (6 × 300 mm) equilibrated with 20 mM phosphate buffer (0.1 M NaCl, pH 7.0). The purified enzyme was chromatographed on an RP-304 column at a flow rate of 1.0 ml/min with a gradient of 0–95% acetonitrile using buffer A and buffer B, and the purified subunits were analyzed for the N-terminal amino acid sequence.

3. RESULTS AND DISCUSSION

We first examined the cleavage sites of AP33 in an extract of rat brain. The brain homogenate of 6-week-old rats was fractionated by gel filtration on two Sephadex G-150 colums (Fig. 1A). Most of the AP33 fragmentation activity was eluted in the high-molecular-mass fraction (M_r>100 kDa). To identify the cleavage sites, AP33 was incubated with the high-molecular-mass fraction, and the resulting fragments were purified by reverse-phase HPLC (Fig. 1B). The sequencing of these fragments revealed that the cleavage sites were at the Arg^{13}–His^{14}, Gln^{15}–Lys^{16}, Val^{18}–Phe^{19} and Phe^{19}–Phe^{20} bonds of the β-AP sequence. The main cleavage site was at the Gln^{15}–Lys^{16} bond. This finding is consistent with the known APP processing site, i.e. Gln^{15}–Lys^{16} or Lys^{16}–Leu^{17} bond, which was confirmed by direct protein structural analysis of the secreted fragment of native human APP in transfected cells [9]. There were some fragments cleaved at a middle site between Arg^{13} and Phe^{20} of the β-AP sequence, but no fragment due to cleavage at the site of the Met^{-1}–Asp^{1} bond. This result suggests the presence of APP-processing enzyme(s), but no active β-AP-releasing enzyme in the rat brain.

We purified the APP-processing enzyme, which cleaved β-AP at the site of the Gln^{15}–Lys^{16} bond, from about 130 rat brains. The active fractions from two Sephadex G-150 columns were fractionated on a benzamidine-Sepharose 6B column (Fig. 2A). The main active fraction was further purified by anion-exchange chromatography on a TSK DEAE-5PW column and rechromatographed on the same column (Fig. 2B). The enzyme was eluted as single symmetrical peak.

The purity of the finally purified enzyme was examined by native PAGE. As is evident from Fig. 2C, the enzyme was apparently purified to homogeneity. The enzyme was eluted as single peak coincident with the APP-processing activity by gel-filtration chromatography on a TSK G3000SW$_{XL}$ column. The molecular mass of the native enzyme was estimated at about 600 kDa by native PAGE and by gel filtration. The native

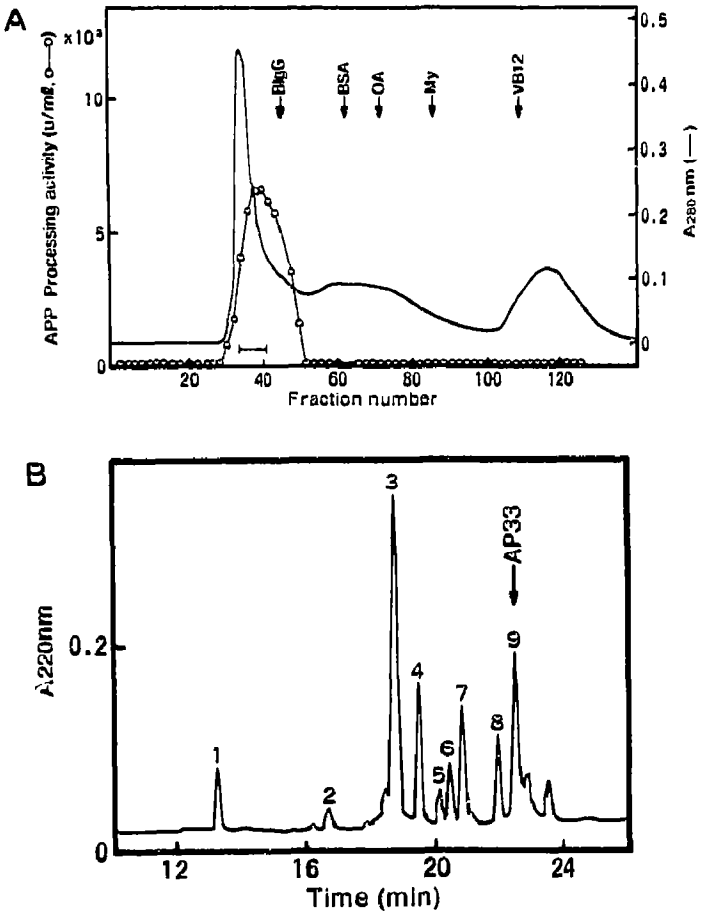

Fig. 1. Separation of APP-processing enzyme from the extract of rat brain by gel filtration on Sephadex G-150 (A). Separation and identification of the fragments of AP33 cleaved by rat brain extract (B). AP33 was incubated with the partially purified active fractions (M_r>100 kDa) on Sephadex G-150. The resulting fragments were separated by reverse-phase HPLC and analyzed for the amino acid sequence. The sequences are shown using the one-letter code. X represents an undetermined residue.

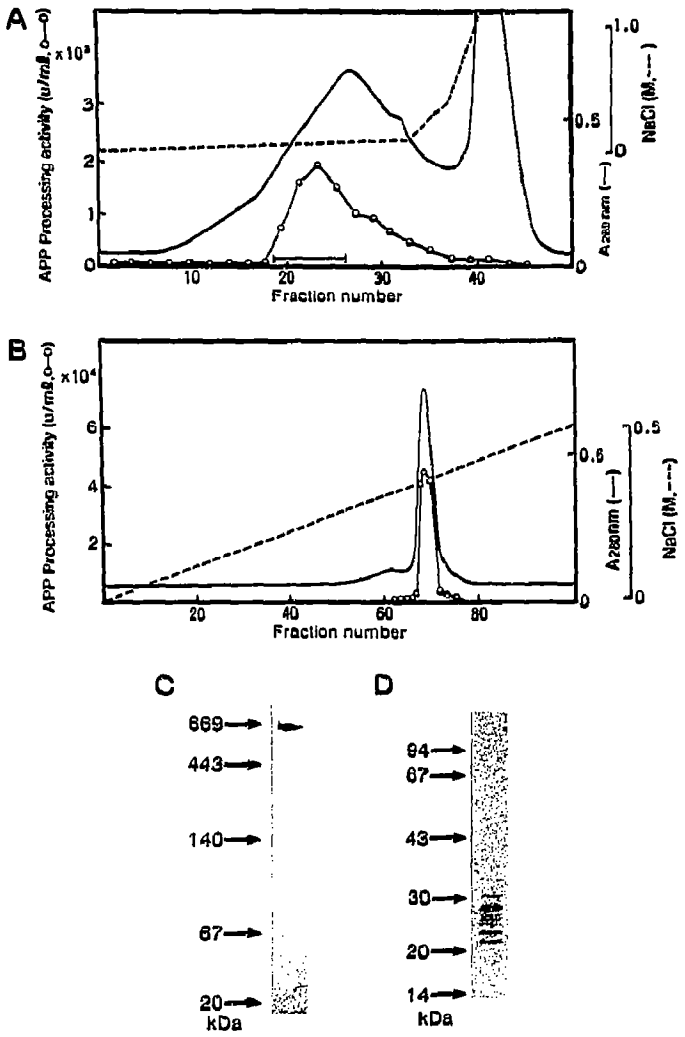

Fig. 2. Separation of APP-processing enzyme by benzamidine–Sepharose affinity chromatography (A) and by DEAE-5PW rechromatography (B). Native (C) and SDS-PAGE (D) of the purified APP-processing enzyme.

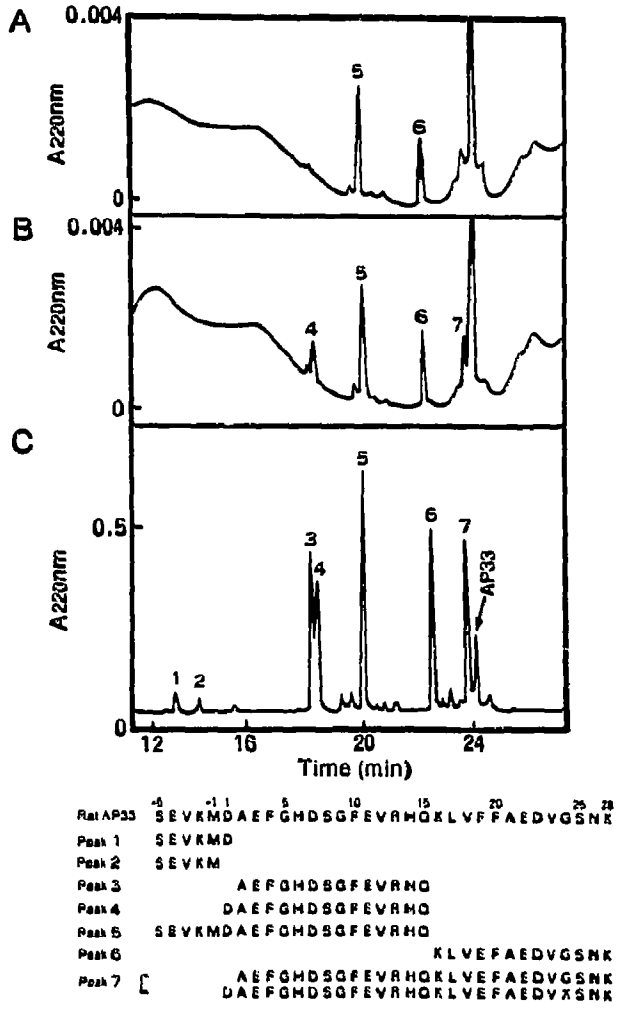

Fig. 3. Effects of Ca^{2+} on APP-processing activity. AP33 (1 μg) was incubated with 2 μl (0.26 μg) of the purified enzyme in 40 μl of 0.1 M phosphate buffer, pH 7.0 in the absence (A) or presence (B) of 25 mM Ca^{2+} for 1 h at 37°C. AP33 (50 μg) was incubated with 1.3 μg of the purified enzyme in 200 μl of assay buffer containing 0.6 mM Ca^{2+} for 3 h at 37°C (C). The resulting peptide fragments were purified and analyzed for the amino acid sequence.

enzyme was dissociated into about ten different subunits in the molecular mass range of 22 kDa–32 kDa by SDS-PAGE (Fig. 2D). Thirteen subunits of the enzyme were partially separated by reverse-phase HPLC on an RP-304 column (data not shown). The N-terminal sequence of one of the subunits (25 kDa) was identified as X-Gln-Asn-Pro-Met-X-Thr-Gly-Thr-Ser, which is identical to that of the β-subunit of human macropain [12]. Based on these results, we concluded that the APP-processing enzyme was a macropain-like proteinase [13].

When AP33 (1 μg) was incubated with this purified enzyme (0.26 μg) for 1 h, 37°C and pH 2–10, the maximum APP-processing activity was generated at pH 6.5–8. The APP-processing activity was inhibited at a final concentration of 100 μg/ml by leupeptin, chymostatin and antipain, but not by E-64, phosphoramidon or pepstatin (data not shown). Analysis of the substrate specificity using methylcoumarinamide-labeled synthetic peptides revealed that the enzyme cleaved the C-terminal bonds of Tyr, Trp and Arg residues (data not shown). These findings suggest that the APP-processing enzyme is a multicatalytic neutral proteinase. Our results show that this multicatalytic proteinase correctly cleaves the Gln^{15}–Lys^{16} bond of β-AP, but does not further degrade β-AP (Fig. 3A), and thus this is a candidate for the authentic APP-processing enzyme.

To examine the effects of metal ions on the APP-processing activity, we compared the fragmentation of AP33 (1 μg) by the purified enzyme (0.26 μg) in the presence of 25 mM Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} for 1 h incubation. Zn^{2+} inhibited the enzyme. Ca^{2+} altered the AP33 fragmentation profile by the enzyme, producing

new peptide fragments (Fig. 3B). We examined the fragmentation of AP33 (50 μg) by the purified enzyme (1.3 μg) in the presence of 0.6 mM Ca^{2+} for 3 h incubation to confirm the cleavage site (Fig. 3C). The sequences of the fragments showed that the APP-processing enzyme cleaved the peptide only at the Gln^{15}–Lys^{16} bond, but also cleaved the peptide at the Met^{-1}–Asp^{1} and Asp^{1}–Ala^{2} bonds, the N terminus of β-AP, in the presence of Ca^{2+}. Much to our surprise, we found that the purified enzyme generated the extracellular fragment of β-AP, i.e. peak 7 in Fig. 3C. This finding suggests that the APP-processing enzyme changes to a β-AP-releasing enzyme.

We have thus identified a macropain-like multicatalytic proteinase (also known as proteasome [14] and ingensin [15]) as a candidate for the APP-processing enzyme which cleaves at the Gln^{15}–Lys^{16} bond of β-AP. It is very important to note that the purified APP-processing enzyme also cleaved the Met^{-1}–Asp^{1} and Asp^{1}–Ala^{2} bonds of β-AP to release the extracellular domain of β-AP in the presence of Ca^{2+}. These findings lead to the new hypothesis that the generation of β-AP in AD may be due to the functional change in the multicatalytic proteinase. We have not confirmed whether the multicatalytic proteinase acts on the intact APP. However, the 11.8 kDa and 11.4 kDa C-terminal APP derivatives containing the entire β-AP at or near their N-terminus are shown to be present in human cerebral cortex [16]. These derivatives could be cleaved by the multicatalytic proteinase. Excessive rises in intracellular calcium have been implicated in AD [17,18]. The abnormalities of cell membrane phospholipid metabolism have also been shown in AD brain [19]. The accumulation of glycerophosphocholine suggests the increased activity of the deacylation pathway of phosphatidylcholine, which results in the increase of fatty acid. Fatty acids are known to enhance the chymotrypsin-like activity of the multicatalytic proteinase [15]. Taken together with our findings, there is a possibility that an abnormality of the system regulating the multicatalytic proteinase may be involved in AD.

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