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Putative N-terminal splitting enzyme of amyloid A4 peptides is the multicatalytic proteinase, ingensin, which is widely distributed in mammalian cells

Shoichi Ishiura, Toshifumi Tsukahara, Takeshi Tabira and Hideo Sugita

National Institute of Neuroscience, NCNP, Kodaira, Tokyo 187, Japan

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The main characteristic changes observed in Alzheimer's disease (AD) are the presence of neurofibrillary tangles and the deposition of amyloid A4 peptides. The most abundant amyloid A4 peptide species in AD (which we tentatively named A4′) is composed of 39 amino acids, which is devoid of the 3 N-terminal amino acids, Asp-Ala-Glu, of the originally reported A4 peptide. We synthesized a model peptide substrate, Suc-Ala-Glu-methylcoumarinamide (MCA), to identify the proteinase that splits the A4′ peptide. DEAE-cellulose column chromatography of rat liver and porcine brain extracts showed that only one peak material digested the synthetic substrate at pH 8. The results for the final preparation indicate that the Suc-Ala-Glu-MCA-degrading enzyme is a high-molecular-mass proteinase, with a molecular mass of above 500 000, and is composed of several low-molecular-mass subunits. These results suggest that a non-lysosomal multicatalytic proteinase (we named this enzyme ingensin (ingens = large in Latin), Ishiura, S. et al. (1985) FEBS Lett. 189, 119–123) catalyzes the above reaction. Antiserum against the purified multicatalytic proteinase, ingensin, crossreacted with the purified Suc-Ala-Glu-MCA-degrading proteinase. It is likely that ingensin shows a similar action toward amyloid precursor protein (APP) in vivo.

Multicatalytic proteinase; Amyloid A4; Proteolysis; Alzheimer's disease, A4 peptide; Amyloid precursor protein; (Rat liver)

1. INTRODUCTION

Alzheimer's disease (AD) is characterized by the deposition of amyloid in the brain, especially in senile plaques and neurofibrillary tangles [1–3]. Amyloid is thought to arise from abnormal cleavage of various proteins into self-aggregating fragments. The major component of AD amyloid is a 4.2 kDa polypeptide, referred to as the A4 or β-protein [4], corresponding to a membrane-spanning domain of a putative amyloid precursor protein (APP) whose amino acid sequence was deduced from the cDNA sequence.

The APP gene produces at least 3 mRNAs, APP695, APP751 and APP770, the last two containing a sequence highly homologous to that of Kunitz-type proteinase inhibitors [5–7]. All these mRNAs encode the A4 peptide. A4 at positions 597–638 of the initially identified APP695 is a hydrophobic protein and the C-terminal half of the A4 is buried in the membrane (see fig.1). The major peptide species in the amyloid plaque core in AD is peptide A4′, which corresponds to Phe_{600}–Ala_{638} (which comprises 64% of the total A4-related polypeptide) and an A4 peptide (12%) [8]. A4′ is found in larger amounts in neurofibrillary tangles than A4. A4′ is also a major material deposited in the brain of Down syndrome cases [9].

Correspondence address: S. Ishiura, National Institute of Neuroscience, NCNP, Kodaira, Tokyo 187, Japan

On the basis of this structure, it has been suggested that the N-terminal portion of the A4 peptide is first cleaved off from the APP protein, and then the C-terminal end of A4 is cut off by another proteinase at the membrane to release the A4 or A4′ peptide. To determine the initial cleaving enzyme for A4 production, we synthesized a fluorogenic peptide substrate with a cleavage point. Since the amount of A4′ in AD is greater than that of A4, i.e. a Glu-Phe bond is more easily cleaved in vivo than a Lys-Met bond (see fig.1), N-terminus-blocked Ala-Glu-methylcoumarinamide (MCA) was selected to demonstrate the usefulness of synthetic substrates for characterization of the A4′-producing enzyme. In addition, we investigated the presence of Met-endopeptidase to identify another A4-splitting activity.

2. MATERIALS AND METHODS

2.1. *Materials*

The peptide substrates, Suc-Ala-Glu-MCA and Suc-Leu-Met-MCA, were synthesized by Peptide Res. Inc., Osaka. Suc-Leu-Leu-Val-Tyr-MCA and microbial protease inhibitors were purchased from the same company. Aprotinin was obtained from Sigma.

The multicatalytic proteinase, ingensin, was purified from rat liver according to the method previously reported [10–14]. Antibodies against the enzyme were raised in rabbits. Characterization of the enzyme and the antibody was reported elsewhere [15].

2.2. *Assay method*

Standard assay mixtures comprised 50 mM Tris-HCl buffer, pH

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Table 1

Suc-Ala-Glu-MCA-degrading activity in the various organs of rat

Organ	Liberated AMC (nmol/mg per h)		
	Suc-Ala-Glu-MCA	Z-Phe-Arg-MCA	Suc-Leu-Leu-Val-Tyr-MCA
Brain	0.061	4 40	0.56
Tongue	0.061	1.44	0.44
Spinal cord	0.056	6.37	0 33
Skeletal muscle	0 028	2.03	0 52
Cardiac muscle	0.038	3.21	0 39
Lung	0.094	4.27	0.56
Liver	0.294	41.3	1 37
Kidney	0.327	41.7	3.65
Small intestine	0.320	3.67	18.6
Testis	0.168	4.25	1.94
Serum	0.010	0.08	0.01

7.0, containing 0.1 mM substrate and proteinase, in a total volume of 0.1 ml. Incubations were performed for 30 min at 37°C. The cleavage product, aminomethylcoumarin (AMC), was analyzed, after stopping the reaction with 5% SDS, with a Hitachi F-3000 fluorescence spectrophotometer. Methionine endopeptidase activity was determined at pH 8.0 with Suc-Leu-Met-MCA as a substrate. The analogue was first synthesized for cathepsins B&L [16], but a preliminary experiment showed that lysosomal cysteine proteinases do not digest it at slightly alkaline pH in the absence of an SH compound (data not shown).

2.3. *Extraction of tissue*

All operations were performed at 0–4°C. Fresh rat liver tissue (120 g) was suspended in 5 vols of 5 mM phosphate buffer, pH 7.0 (KPB), and then lysed with a Waring blender. The homogenate was centrifuged at 10000 × *g* for 10 min and then ammonium sulfate was added (final, 30%) to remove mitochondrial contamination. From the clear supernatant, proteins were precipitated by the addition of ammonium sulfate to 65% saturation. The resultant precipitate, which contained more than 95% of the cytosolic proteins, was dissolved in KPB and then dialyzed overnight against the same solution. Further purification was achieved at neutral pH by chromatographies on DEAE-cellulose (0–0.5 M NaCl gradient) and hydroxyapatite (5–300 mM phosphate gradient), HPLC gel filtration (TSK G3000 SW) and finally chromatography on heparin-Sepharose (0–1 M NaCl gradient). Fractions containing Suc-Ala-Glu-MCA-degrading activity were identified on the basis of cleavage of Suc-Leu-Leu-Val-Tyr-MCA as well as Western blot analysis.

2.4. *Gel electrophoresis and Western blotting*

Polypeptides were resolved in 12% polyacrylamide mini-slab gels and stained with Coomassie blue. For Western blotting, polypeptides were electrotransferred to nitrocellulose membranes at 150 mA for 1 h and then the membranes were blocked with 5% non-fat milk. Incubation with primary antisera was performed at room temperature for 1 h and detection was performed with an ABC kit [17].

3. RESULTS

3.1. *Distribution of the Suc-Ala-Glu-MCA-degrading enzyme in rat tissues*

Suc-Ala-Glu-MCA was used for investigation of the distribution of the putative A4-splitting enzyme in rat tissues. The activities of thiol cathepsins (substrate: Z-Phe-Arg-MCA) and the cytosolic multicatalytic proteinase, ingensin (substrate: Suc-Leu-Leu-Val-Tyr-MCA), were also checked for comparison. The results are summarized in table 1. Of the tissues tested, kidney, small intestine and liver showed the highest activity, followed by testis and lung. The hydrolytic activities toward Suc-Ala-Glu-MCA and Suc-Leu-Met-MCA at alkaline pH were roughly parallel in the rat tissues studied (data not shown). A similar tendency was observed in the case of thiol cathepsins B&L, but very high hydrolyzing activity toward Z-Phe-Arg-MCA at acidic pH was found in liver and kidney, but not in small intestine. On the other hand, hydrolyzing activity toward Suc-Leu-Leu-Val-Tyr-MCA, which is the specific substrate of the multicatalytic proteinase or ATP-dependent protease, is high in small intestine, followed by kidney and liver. A preliminary experiment showed that the high activity in the small intestine was due to chymase present in mast cells of the small intestine. More than 95% of each activity was recovered in the supernatant from the whole homogenate on homogenization and sonication. Thus, Suc-Ala-Glu-MCA-degrading activity was shown to be widely distributed in rat tissues. In addition, Suc-Ala-Glu-MCA-degrading activity was detected in a small amount in the serum (table 1).

Recent results of Weidemann et al. [18] showed that APP expressed in the cell surface (130 kDa) was quickly secreted in a C-terminal truncated form (112 kDa), suggesting that some serum enzyme cleaves the extracellular domain of APP to release the N-terminal portion of the precursor. The residual low-molecular-mass membrane-associated form of APP should contribute to the A4 amyloid formation. Although the Ala-Glu sequence is located in the extracellular domain in putative membrane-associated forms, we decided to purify the Suc-Ala-Glu-MCA-degrading enzyme from rat liver.

3.2. *DEAE-cellulose column chromatography*

Fig.1 shows the elution profile of the rat liver extract together with that of porcine brain on a DEAE-cellulose column. All of the activity was bound to the resin and was eluted at 0.25 M NaCl as a single peak.

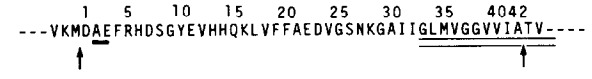

Fig.1. Amino acid sequence of the amyloid A4 peptide. The amino acid sequence of the A4 polypeptide, which is composed of 42 amino acids, is denoted by arrows. The N-terminal Asp of the A4 polypeptide is Asp597 of the predicted APP695. The double underline indicates a probable membrane-spanning sequence of the A4 polypeptide reported by Kang et al. [4]. The most abundant species, A4′, begins at Phe600 of APP. The model peptide sequence used in the search for the A4′-splitting enzyme is indicated by a thick underline.

The results indicate that the activity found in the crude extract was due to a single enzyme. Subsequent chromatographies confirmed this. We assayed these fractions using several fluorogenic substrates, such as Z-Phe-Arg-MCA, Suc-Leu-Leu-Val-Tyr-MCA, Suc-Leu-Met-MCA, Suc-Gly-Pro-Leu-Gly-Pro-MCA, Arg-MCA and Leu-MCA. We found that the elution profiles of Suc-Leu-Leu-Val-Tyr-MCA (pH 9) and Suc-Leu-Met-MCA (pH 8) coincided with those of neutral Suc-Ala-Glu-MCA in rat liver (fig.2a) and porcine brain (fig.2b).

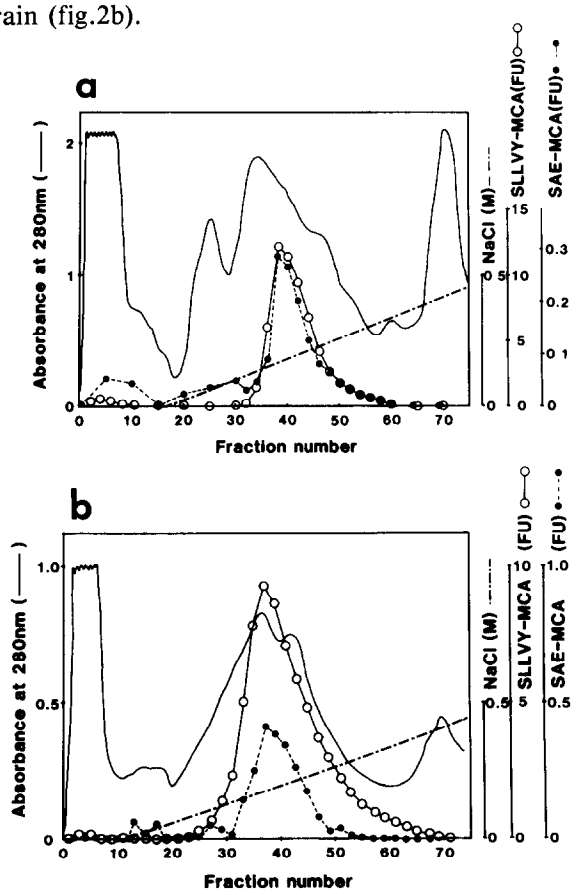

Fig.2. DEAE-cellulose column chromatography of tissue extracts. 80 g of rat liver (a) or porcine brain (b) was homogenized with 5 mM phosphate buffer, pH 7, and the supernatant was applied to a 200 ml DEAE-cellulose column. Proteins were eluted with a linear NaCl gradient (0–0.5 M, total, 1600 ml) and 20-ml fractions were collected. Suc-Ala-Glu-MCA (•) and Suc-Leu-Leu-Val-Tyr-MCA (○) degrading activities were determined as described under section 2. FU, fluorescence unit which liberates nanomoles of AMC in our standard assay system with 20 l of enzyme sample.

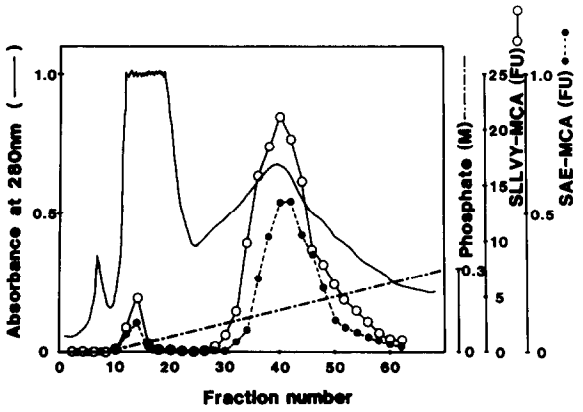

Fig.3. Hydroxyapatite column chromatography of the Suc-Ala-Glu-MCA-degrading enzyme. The active fractions of the rat liver extract from a DEAE-column were dialyzed and then applied to a 20-ml hydroxyapatite column. A linear phosphate gradient (5–300 mM, total, 400 ml) was employed and 5 ml fractions were collected.

3.3. *Hydroxyapatite chromatography and gel filtration*

The active fractions from rat liver were collected and applied to a hydroxyapatite column (20 ml) equilibrated with 5 mM phosphate buffer, pH 7. A linear gradient (5–300 mM) was applied and the fractions collected (6 ml) were assayed for enzyme activity. Two peaks of activity were detected (fig.3) and these

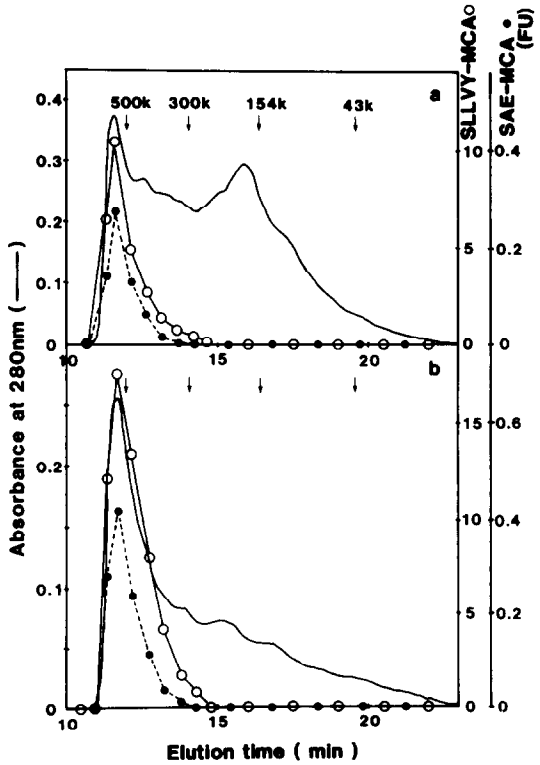

Fig.4. HPLC gel filtration. The two active peak materials were independently concentrated with ammonium sulfate and then applied to a HPLC gel filtration system (TSK G3000SW, 7.5 × 600 mm). The enzyme was eluted at 1 ml/min with 0.1 M phosphate buffer, pH 7.0. (a) Hydroxyapatite F.12-15 (corresponds to ingensin A [12]). (b) Hydroxyapatite F.35-45 (corresponds to ingensin B [12]).

peak materials degraded Suc-Leu-Leu-Val-Tyr-MCA, which corresponded to the previously reported two isoenzymes of the multicatalytic proteinase, ingensins a and b. Subsequent chromatography of these active fractions on an HPLC column (TSK G3000SW, 7.5 × 600 mm) indicated that both enzymes had a molecular mass of above 500000 (fig.4). They still gave single peaks. The results exclude the possibility that other proteinases such as chymase are copurified with this enzyme on DEAE-cellulose and hydroxyapatite.

3.4. *Identification of the Suc-Ala-Glu-MCA-degrading enzyme as multicatalytic proteinase, ingensin*

The purified enzyme hydrolyzed not only Suc-Ala-Glu-MCA but also other substrates (table 2). It cleaved Suc-Leu-Leu-Val-Tyr-MCA and Boc-Leu-Arg-Arg-MCA most rapidly. From the substrate specificity and subunit composition (fig.5), it is likely that this enzyme is closely related to the multicatalytic proteinase, ingensin [19].

To determine whether or not the purified enzyme was identical to the multicatalytic proteinase, Western blot analysis was performed (fig.5). Anti-rat liver ingensin antibody crossreacted with the Suc-Ala-Glu-MCA-degrading enzyme. These findings indicate that the cytosolic multicatalytic proteinase is one of the candidates for the amyloid A4 splitting enzyme.

Table 2

Synthetic substrate hydrolysis by the purified proteinase

X-hydrophobic-MCA	Activity(%)	X-Arg(Lys)-MCA	Activity(%)
Suc-Leu-Leu-Val-Tyr-MCA	100	Boc-Leu-Arg-Arg-MCA	100
Suc-Ser-Ser-Leu-Tyr-MCA	20	Boc-Leu-Lys-Arg-MCA	79
Suc-Ala-Ala-Phe-MCA	49	Boc-Leu-Gly-Arg-MCA	34
Ala-Ala-Phe-MCA	78	Boc-Gln-Arg-Arg-MCA	35
Suc-Ala-Ala-Ala-MCA	0	Boc-Gln-Ala-Arg-MCA	15
Suc-Ala-Pro-Ala-MCA	1	Boc-Gln-Gly-Arg-MCA	12
Suc-Leu-Met-MCA	5	Boc-Gly-Arg-Arg-MCA	0
Suc-Ile-Ala-MCA	1	Boc-Phe-Ser-Arg-MCA	28
Suc-Ala-Glu-MCA	1	Boc-Val-Leu-Lys-MCA	9
Glt-Phe-MCA	1	Boc-Glu-Lys-Lys-MCA	0

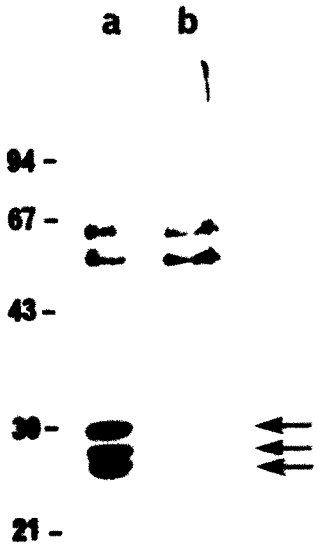

Fig.5. Western blot analysis of the Suc-Ala-Glu-MCA degrading enzyme. Purified enzyme (5 μg) was subjected to 12% SDS polyacrylamide gel and then transferred to a nitrocellulose sheet. Lane a, anti-rat liver ingensin antiserum; lane b, preimmune serum. Immunoreactive components of molecular masses of 25–30 kDa are present in lane a, but not in lane b. Non-specific 62 kDa and 55 kDa bands are observed in both lanes.

4. DISCUSSION

We do not known what the APP or A4 do. The results of immunocytochemical localization suggest that APP is located in neurons and skeletal muscle [20]. This and the proteoglycan nature of APP [21] suggested that APP is involved in cell-cell interaction. The recent finding that one of the predicted precursors, APP770, codes the Kunitz-type protease inhibitor also led to the proposal of the importance of a proteinase in amyloid formation. But no correlation of the expression of the inhibitor-containing APP with A4 deposition in AD was observed [22] and the exact role of the protease inhibitor remains unknown. In addition, the fact that in vitro-expressed APP lost its N-terminal extracellular sequence after 1–2 h, which resulted in a membrane-spanning, A4-containing peptide (17–18 kDa), seemed to indicate a role of the protease inhibitor domain in A4 formation [18]. This selective proteolytic release of the extracellular domain from an integrated membrane protein was also observed in several secretion processes, such as in the case of the IgA receptor [23]. It is important to determine whether or not the remaining peptide has the same N-terminus as the A4 peptide. But this is unlikely because A4 begins from position 597–695 of APP. The report of Weidemann suggests that APP was initially cleaved at the extracellular domain just after membrane insertion, resulting in a 17–18 kDa fragment. Then, the A4-splitting enzyme cuts the latter and produces the A4 peptide afterwards.

Our finding that a specific intracellular proteinase cleaves Suc-Ala-Glu-MCA indicates clearly that the A4-cleaving enzyme is derived from cellular parts after damage to the membrane. The tendency that an in vitro-expressed fragment beginning with the A4 sequence does aggregate also suggests the involvement of the intracellular proteinase in A4 formation as a consequence of primary or secondary membrane damage.

Two interesting results were obtained in this experiment. One is that the multicatalytic proteinase cleaved both Met-X and Ala-Glu-X bonds. This easily explains the creation of several A4 species with different N-termini. The other is the possible involvement of the multicatalytic proteinase in amyloid formation. Our re-

cent finding that this enzyme is the same as ATP-dependent proteinase [15,24] clearly suggests the importance of this proteinase in cellular processes. Once abnormal proteins are synthesized, the proteins are degraded in an ATP-dependent manner. Hershko and Ciechanover demonstrated that ubiquitin is first attached to abnormal proteins [25]. This process requires ATP. The ubiquitinated substrate is subsequently degraded by the proteinase, also in an ATP-dependent manner. Independently, it was demonstrated that a peptide hydrolysis in K562 cells is stimulated by ATP [24]. This is due to a multicatalytic proteinase, ingensin, which is stabilized at 37°C by ATP. If this enzyme recognizes abnormal proteins from the conjugated ubiquitin or through an unknown mechanism, it is proper to postulate that ingensin digests abnormal APP. The frequent observation of ubiquitinated proteins in paired helical filaments in AD suggests the above possibility [27]. We are now trying to purify APP to determine whether the purified multicatalytic proteinase releases A4 peptide or induces aggregation.

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