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Identification of a putative amyloid A4-generating enzyme as a prolyl endopeptidase

Shoichi Ishiura, Toshifumi Tsukahara, Takeshi Tabira, Teruo Shimizu, Kiichi Arahata and Hideo Sugita

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

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The A4 amyloid peptide is deposited in Alzheimer's disease inside neurons as neurofibrillary tangles or extracellularly as vascular amyloid. The A4 peptide is cleaved off by an unidentified proteinase from a larger precursor protein (APP), which resembles a cell surface receptor. The proteinase, which cleaves off the membrane-spanning domain of APP, may be important in amyloid formation. To evaluate this, a model peptide substrate, succinyl- isoleucyl-alanine-methylcoumarinamide, which is homologous to the C-terminal portion of A4 peptide, was synthesized to screen the putative A4-generating proteinase. On chromatographic purification, it was found that two proteinases are involved in the hydrolysis of the peptide, the major one being identified as a prolyl endopeptidase. This evidence may facilitate elucidation of the mechanism of amyloid deposition in Alzheimer's disease.

Amyloid; A4 peptide; Prolyl endopeptidase; Alzheimer's disease; (Rat brain)

1. INTRODUCTION

In Alzheimer's disease (AD), the amyloid A4 peptide is thought to arise from its precursor protein (APP) with deposition in brain tissue [1–3]. We therefore used rat brain to purify the A4-generating proteinase. The A4 peptide is the small C-terminal portion of APP, and includes part of the putative membrane-spanning region and part of the adjacent extracellular domain. A4 isolated from both amyloid plaque cores and neurofibrillary tangles shows N-terminal heterogeneity, indicating the involvement of several proteinases in its production. Considering its self-aggregating property, liberation of the A4 peptide from APP should occur after splitting of the N-terminal extracellular domain, excretion of the latter into the serum [4,5] and the following essential cleavage of the transmembrane domain. Initial cleavage of the transmembrane domain cannot occur in vivo. The fact that an APP lacking only the C-terminal 55–60 amino acids has never been found in serum confirms the above assumption.

To screen the putative A4-generating proteinase, we synthesized a model peptide homologous to the C-terminal portion of A4 peptide. Purification from rat brain revealed that the peptide was efficiently cleaved by an endogenous proteinase, identified as a prolyl endopeptidase.

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

2. MATERIALS AND METHODS

2.1. *Materials*

The model peptide, succinyl-isoleucyl-alanine-methylcoumarinamide (SIA-MCA) was synthesized by Peptide Inst. Co., Minoh, Osaka, Japan. The other peptides were also obtained from the same company. The specific inhibitor of prolyl endopeptidase, Z-thioprothiazolidine, was kindly provided by Dr Tadashi Yoshimoto, Nagasaki University.

2.2. *Assay method*

Our standard assay conditions were as follows: enzyme samples were mixed with a peptide substrate (final concentration: 0.1 mM), in a total volume of 0.1 ml, in 50 mM Tris-HCl, pH 7.0, containing 10 mM 2-mercaptoethanol. After incubation at 37°C for 30 min, the reaction was terminated by the addition of 0.1 ml of 10% sodium dodecyl sulfate and 1.3 ml of 0.2 M Tris-HCl, pH 9.0 [6,7]. The aminomethylcoumarin liberated was determined with a Hitachi fluorescent spectrophotometer F-3000 (ex. 380 nm; em. 460 nm).

3. RESULTS AND DISCUSSION

Fig.1 shows the purification of the A4-generating enzyme. DEAE-cellulose chromatography revealed the presence of two enzymes responsible for the degradation of a model peptide, succinyl-isoleucyl-alanine-methylcoumarin-amide (Suc-Ile-Ala-MCA or SIA-MCA). The major one, eluted at 0.10 M NaCl, was concentrated and applied to the next column. We also purified the minor one, eluted at 0.18 M NaCl, to homogeneity. The latter enzyme had different characteristics from the major A4-generating one [8].

The major active fraction was further purified on hydroxyapatite, gel filtration, heparin-Sepharose and

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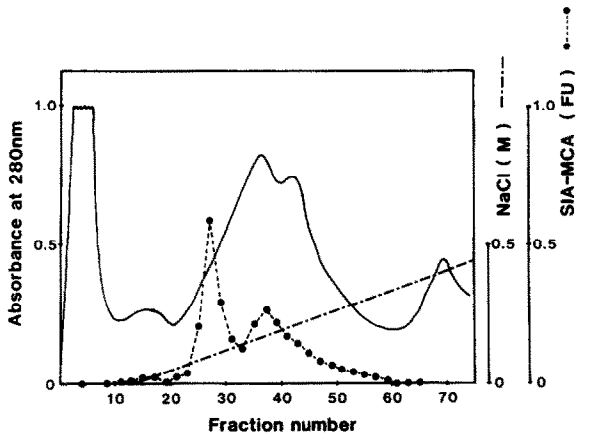

Fig.1. DEAE-cellulose chromatography profile of the succinyl-isoleucyl-alanine-methylcoumarinamide (SIA-MCA) degrading proteinase. Rat brain (23.5 g) was homogenized with 5 vols of 5 mM phosphate/10 mM 2-mercaptoethanol, pH 7.0 (buffer A), in a glass homogenizer, and then the homogenate was centrifuged at 10000 × *g* for 10 min. The clear supernatant was directly applied to a 200 ml DEAE-cellulose column equilibrated with buffer A. After washing, the column was eluted with a linear NaCl gradient (0–0.5 M; total, 1600 ml) at 4°C. 20 ml fractions were collected and assayed for SIA-MCA degrading activity. (——) Protein absorbance at 280 nm; (●---●) enzymatic activity, measured as the liberation of aminomethylcoumarin (FU, fluorescence unit) after 30 min. 1 FU approximately corresponds to 1 nmol AMC/h under our standard assay conditions.

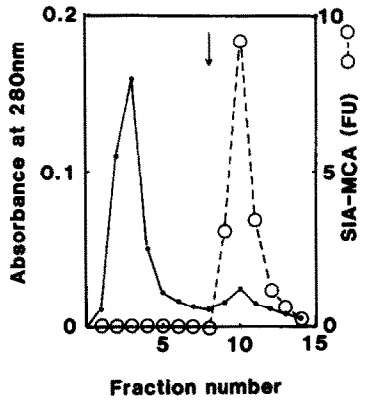

Fig.2. Final benzamidine-Sepharose column chromatography of putative A4-generating proteinase. The active fractions (fractions 26–30 in fig.1) were dialyzed against buffer A overnight and then applied to a 20 ml hydroxyapatite column equilibrated with the same buffer. The enzyme was adsorbed to the column and eluted with a linear phosphate gradient (0–300 mM; total, 400 ml). The peak of SIA-MCA degrading activity was eluted immediately after the application of the gradient, which separated a contaminating minor activity (see fig.1, fractions 35–45) from the major peak. The activity peak fractions were concentrated and applied to an analytical HPLC gel filtration column (TSKG3000 SW, 0.75 × 60 cm). The SIA-MCA hydrolytic activity was eluted as a single symmetrical peak corresponding to a molecular mass of 110000. Fractions containing the active enzyme were collected, again dialyzed against buffer A and then directly applied to a heparin-Sepharose column. Half the contaminating proteins were effectively removed by passage through this column. The SIA-MCA degrading activity was recovered in the flow-through fraction. Affinity isolation of the SIA-MCA degrading enzyme on *p*-aminobenzamidine-Sepharose was conducted at room temperature. The filtered enzyme samples were collected and applied to a 1 ml inhibitor column equilibrated with buffer A. After all the samples had been passed through the column, the unadsorbed protein was removed by washing with 10 bed vols of buffer A, and then the column was eluted with 5 bed vols of buffer A containing 0.1 M NaCl (arrow). The eluted sample was collected on ice. The eluate could be stored for a week at 4°C without significant loss of activity. (——) Protein absorbance at 280 nm; (○) enzyme activity.

benzamidine-Sepharose columns (fig.2). SIA-MCA degrading activity was adsorbed to *p*-aminobenzamidine-Sepharose and eluted at 0.1 N NaCl, with an almost 100% activity recovery. The protein with SIA-MCA hydrolyzing activity eluted from the column gave a single band under nondenaturing conditions. On HPLC gel filtration on TSKG3000 SW, the native molecular mass was estimated to be 110000. When electrophoresed on polyacrylamide in the presence of SDS under reducing conditions, one band appeared. It corresponded to 72000 as judged on comparison with molecular markers.

The rates of hydrolysis of the methylcoumarinamides of 8 synthetic peptides are shown in table 1. Surprisingly, the purified enzyme most efficiently cleaved Suc-Gly-Pro-Leu-Gly-Pro-MCA (SGPLGP-MCA), the substrate of prolyl endopeptidase (PEPase) [9,10], in the presence of 2-mercaptoethanol. We reassayed every chromatographic fraction for PEPase activity and found that Suc-Gly-Pro-Leu-Gly-Pro-MCA degrading activity coincided with Suc-Ile-Ala-MCA degrading activity, except for the minor peak of SIA-MCA hydrolyzing activity observed on DEAE-cellulose chromatography (see fig.1). No other PEPase activity was observed in subsequent chromatographies. The other substrates, such as Suc-Ala-Ala-Ala-MCA and Suc-Ala-Pro-Ala-MCA, were moderately digested by the purified enzyme, but the rates of hydrolysis were below 1/30. These characteristics are similar to those of PEPase from *Flavobacterium* [11,12] and bovine brain [13]. The rate of hydrolysis was almost zero when a trypsin substrate, which had an arginyl or lysyl residue as the residue immediately amino-terminal to the scissible bond (at P1), was used for the assay. In addition, Phe at the P1 site was not accessible to the enzyme.

The rate of hydrolysis of the substrate for PEPase was enhanced in the presence of an SH-compound. In the case of SGPLGP-MCA, the hydrolysis was increased 40-fold. The results indicate that the enzyme has an essential SH group at or near the active site, although it is not a cysteine proteinase because a specific cysteine proteinase inhibitor, E-64 [14,15], did not inhibit the peptide hydrolysis at all. The SIA-MCA and SGPLGP-MCA degrading activities of the purified sample were both strongly inhibited on the addition of the PEPase-specific inhibitor, Z-thiopro-thiazolidine [16] (see also table 2). About 70% of both activities was inhibited by 100 nM of the inhibitor. The results strongly suggest that the SIA-MCA degrading proteinase is a tissue prolyl endopeptidase.

Table 1

Substrate specificity of the purified enzyme

Substrate	Activity (nmol/h)		Activation (-fold)
	− 2ME	+ 2ME	
Suc-Ile-Ala-MCA	0.062	0.923	14.9
Suc-Gly-Pro-Leu-Gly-Pro-MCA	8.46	339	40.1
Suc-Ala-Ala-Ala-MCA	0.227	7.15	31.5
Suc-Ala-Pro-Ala-MCA	0.203	10.2	50.2
Suc-Ala-Ala-Pro-Phe-MCA	0.053	0.179	3.4
Suc-Ala-Ala-Phe-MCA	0	0	–
Ala-Ala-Phe-MCA	0	0	–
Glt-Phe-MCA	0	0	–

0.12 μg of the enzyme was incubated with 50 mM Tris-HCl, pH 7.0, and 0.1 mM of each substrate for 30 min in the presence or absence of 2-mercaptoethanol (2ME). The activity is expressed as nmol aminomethylcoumarin liberated/h. Suc, succinyl; Glt, glutaryl

Table 2

Effects of proteinase inhibitors on the PEPase activity of the purified enzyme

Proteinase inhibitor	Concentration	Relative activity (%)	
		SGPLGP-MCA	SIA-MCA
None	–	100	100
Kunitz BPTI	0.01 mg/ml	74	94
	0.1 mg/ml	25	30
Soybean trypsin inhibitor	0.01 mg/ml	100	100
	0.1 mg/ml	100	100
Aprotinin	0.01 mg/ml	91	40
	0.1 mg/ml	78	38
Bovine serum albumin	0.01 mg/ml	107	100
	0.1 mg/ml	91	100
Z-thiopro-thiazolidine	0.1 nM	100	99
	1 nM	92	90
	10 nM	59	61
	100 nM	24	17

The purified enzyme was incubated with 0.1 mM SGPLGP-MCA or SIA-MCA, as described in the legend to table 1, in the presence of various types of proteinase inhibitor. Bovine serum albumin was used as a control

The gene encoding the APP produces at least 3 mRNAs [17–19]. Two of them, APP750 and APP771, contain an insert, which is highly homologous to the Kunitz-type serine proteinase inhibitor (KPI). If the inhibitor domain functions as a proteinase inhibitor in vivo, it could either promote amyloidogenesis by inhibiting the A4-degrading proteinase or inhibit amyloidogenesis by inhibiting the A4-generating proteinase. To investigate the specificity of the purified PEPase, various proteinase inhibitors were examined (table 2). Kunitz-type bovine pancreas trypsin inhibitor (BPTI) and aprotinin inhibited the rat brain enzyme, whereas soybean Bowman-Birk trypsin inhibitor and the control albumin did not. The concentration of the inhibitor required for inhibition was slightly higher than estimated.

The activity of PEPase in the brain of 3 AD patients (356 ± 125 nmol/h/mg) was not statistically different from that in 7 age-matched controls (350 ± 75 nmol/h/mg). This rules out the possibility of the overproduction of PEPase in AD brain. Other factors, such as the different expression of the 3 mRNA species of APP or the presence of other proteinase inhibitors in the amyloid deposit, may contribute to the formation of the A4 peptide. In this connection, it would also be interesting to investigate the effect of α_1-antichymotrypsin [20], which is deposited in amyloid plaques, on the A4-generating proteinase.

The intracellular localization of the putative A4-generating proteinase, PEPase, leaves the question of the mechanism underlying A4 formation in AD open. Since the C-terminus portion of the A4 protein is thought to be located inside the plasma membrane, the A4-generating enzyme must cleave this part of the APP after binding to the membrane. The data so far obtained do not support the association of the PEPase with the membrane. However, the possible association of the enzyme in Alzheimer's brain remains to be confirmed. As a working hypothesis, we propose that the PEPase may directly act as an amyloid A4-generating proteinase in AD.

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