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A new brain metalloendopeptidase which degrades the Alzheimer ß-amyloid 1-40 peptide producing soluble fragments without neurotoxic effects

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Abstract

A new metalloendopeptidase was purified to apparent homogeneity from a homogenate of normal human brain using successive steps of chromatography on DEAE-Trisacryl, hydroxylapatite and Sephacryl S-200. The purified enzyme cleaved the Gly^{33}-Leu^{34} bond of the 25-35 neurotoxic sequence of the Alzheimer ß-amyloid 1-40 peptide producing soluble fragments without neurotoxic effects. This enzyme activity was only inhibited by divalent cation chelators such as EDTA, EGTA and *o*-phenanthroline (1 mM) and was insensitive to phosphoramidon and captopril (1 µM concentration), specific inhibitors of neutral endopeptidase (EC 3.4.24.11) and angiotensin-converting enzyme (EC 3.4.15.1), respectively. The high affinity of this human brain endopeptidase for ß-amyloid 1-40 peptide (Km = 5 µM) suggests that it may play a physiological role in the degradation of this substance produced by normal cellular metabolism. It may also be hypothesized that the abnormal accumulation of the amyloid ß-protein in Alzheimer's disease may be initiated by a defect or an inactivation of this enzyme.

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Key words
- Brain metalloendopeptidase
- Alzheimer ß-amyloid 1-40 peptide
- Alzheimer's disease

The major component of the amyloid deposits in Alzheimer's disease is an extremely insoluble 39- to 42-amino acid peptide with a strong tendency to aggregate, known as amyloid ß-protein (AßP). It has been shown that the 25-35 sequence of this peptide (...SNKGAIIGLM...) is the main factor responsible for its neurotoxic effects (1,2). AßP is part of the hydrophobic domain of the Alzheimer ß-amyloid precursor protein (APP), an integral transmembrane protein which may be processed via two pathways: i) cleavage of APP within the AßP sequence by an enzyme not yet characterized called "secretase", producing soluble fragments, and ii) cleavage of APP by lysosomal enzymes resulting in intact AßP that can precipitate to form amyloid plaques.

It was demonstrated that AßP is also produced by normal cellular metabolism, but the proteases involved in its physiological degradation are not known (3-8).

In the present study, a new metalloendopeptidase which hydrolyzes AßP producing

soluble fragments by a major cleavage of the Gly[33]-Leu[34] bond of its 25-35 neurotoxic sequence was purified and characterized from the soluble fraction of normal human brain.

The brains obtained from three normal subjects (6 to 8 h after death in traffic accidents) aged 20 to 30 and kept at -80ºC were homogenized in 4 volumes (w/v) of 50 mM Tris-HCl, pH 7.5, and centrifuged at 25,000 *g* for 60 min at 4ºC. The pellet was discarded and the supernatant was exhaustively dialyzed against the same buffer used for brain homogenization. A volume of 1 liter of the dialyzed supernatant was applied to a DEAE-Trisacryl column (2.6 x 50 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, at a flow rate of 90 ml/h. After sample application, the column was developed with steps of 50 mM Tris-HCl, pH 7.5, containing NaCl at the following concentrations: 50 mM, 100 mM, 150 mM, 300 mM and 1000 mM. The fractions eluted with 50 mM NaCl containing the enzyme activity were pooled and concentrated to 10 ml in a dialysis tube under reduced pressure. This sample was exhaustively dialyzed against 10 mM potassium phosphate, pH 7.5, and applied to a hydroxylapatite column (2.5 x 10 cm) equilibrated against the same buffer. The column was eluted with a 10-500 mM linear potassium phosphate gradient, pH 7.5, at a flow rate of 30 ml/h. Enzymatically active fractions were pooled, concentrated and exhaustively dialyzed against 50 mM Tris-HCl, pH 7.5. Finally, this sample was further fractionated on a Sephacryl S-200 column (2 x 160 cm) equilibrated against the same dialysis buffer and developed at a flow rate of 60 ml/h. Fractions containing enzyme activity were concentrated and stored at 4ºC. Under such conditions, the purified enzyme was stable for over 3 months. A summary of the purification procedure is presented in Table 1.

Table 1 - Purification of human brain metalloendopeptidase.

The enzyme activity was evaluated in 20-µl aliquots from each purification step; [dArg[8]]kermit was used as substrate. Activities are reported as nmol peptide cleaved at the Ser-Phe bond per hour. Specific activity is reported as nmol substrate cleaved per hour per mg protein.

Step	Total protein (mg)	Total activity (nmol/h)	Specific activity (nmol h^{-1} mg^{-1})	Purification (times)	Yield (%)
Homogenate	11300	63906	5.6	1	100
Supernatant	6690	51426	7.7	1.4	80
DEAE-Trisacryl	237	30855	13	23.2	48
Hydroxylapatite	8	18485	2310	412.5	29
Sephacryl S-200	0.1	10057	10057	1796	16

Enzyme activity was monitored using as substrate the diaminobenzylthiocyanate (DABTC) derivative of [dArg[8]]kermit, a derivate of kermit (Asp-Val-Asp-Glu-Arg-Asp-Val-Arg-Gly-Phe-Ala-Ser-Phe-Leu-NH_2) that undergoes a single cleavage at the Ser-Phe bond, as described previously (9). This assay was chosen because it was used previously with success to monitor the purification of another metalloendopeptidase (peptide hormone inactivating enzyme, PHIE) from the skin granular gland secretions of *Xenopus laevis* which also hydrolyzed AßP with high affinity (9). Protein content was evaluated by the method of Bradford (10).

The purity of the enzyme was demonstrated by polyacrylamide gel electrophoresis: i) under nondenaturing conditions showing a single protein band coinciding with the enzyme activity, and ii) under denaturing conditions (heating at 100ºC in the presence of 1% SDS and 10% ß-mercaptoethanol) also showing a single protein band with an apparent molecular weight of 200 kDa (data not shown).

The effects of various classical protease inhibitors on the enzyme activity were tested (data not shown). The serine- (PMSF, STI, TPCK and aprotinin), cystine- (PCMPS, PCMB and NEM), and carboxyl- (pepstatin and GEMSA) protease inhibitors, at 1 mM concentration, did not inhibit the enzyme activity. However, metalloprotease inhibitors such as EDTA, EGTA and *o*-phenan-

throline, at 1 mM concentration, totally inhibited the enzyme activity, suggesting a metalloendopeptidase nature of the enzyme. Furthermore, phosphoramidon and captopril, specific inhibitors of neutral endopeptidase (EC 3.4.24.11) and angiotensin-converting enzyme (EC 3.4.15.1), respectively, did not inhibit the enzyme at micromolar concentrations.

The metalloendopeptidase performed a major cleavage of the Gly^{33}-Leu^{34} bond of the 25-35 neurotoxic sequence of AßP producing soluble fragments without neurotoxic effects (Figure 1). The Km value for the enzyme-catalyzed hydrolysis of AßP was 5 µM (data not shown). This result shows the high affinity of the metalloendopeptidase for AßP.

During the last three years, several lines of evidence have suggested that AßP plays a key role in the process of plaque formation in Alzheimer's disease brain tissue (1,2,10). Although the enzymes involved in the major steps of AßP processing have not been identified, the protease activities that mediate these cleavage events are called ß- and γ-secretase (11,12). Furthermore, another protease activity called α-secretase is not involved in the formation of AßP and cleaves within the AßA domains of APP between Lys^{612} and Leu^{613} producing soluble peptides. The true α-secretase has yet to be identified. APP, which escapes α-secretase cleavage, is endocytosed and degraded in the endosomes and lysosomes by ß- and γ-secretases producing AßP (11-13).

It was demonstrated that AßP is also produced by normal cellular metabolism (3-8). However, the proteases involved in the physiological degradation of this peptide are not known. It is clear that these enzymes are very important to avoid the accumulation of AßP in normal human brain. In the present study, we purified a new metalloendopeptidase from normal human brain that degrades AßP within its neurotoxic sequence, producing soluble fragments without neurotoxic effects. The high affinity of this endopeptidase by the AßP (Km = 5 µM) suggests that it may play a physiological role in the degradation of this substance produced by normal cellular metabolism. It may also be hypothesized that the abnormal accumulation of AßP in Alzheimer's disease may be initiated by a defect or an inactivation of this enzyme.

Further studies will be necessary to elucidate the primary structure of this enzyme and to better understand its possible physiological role in AßP degradation.

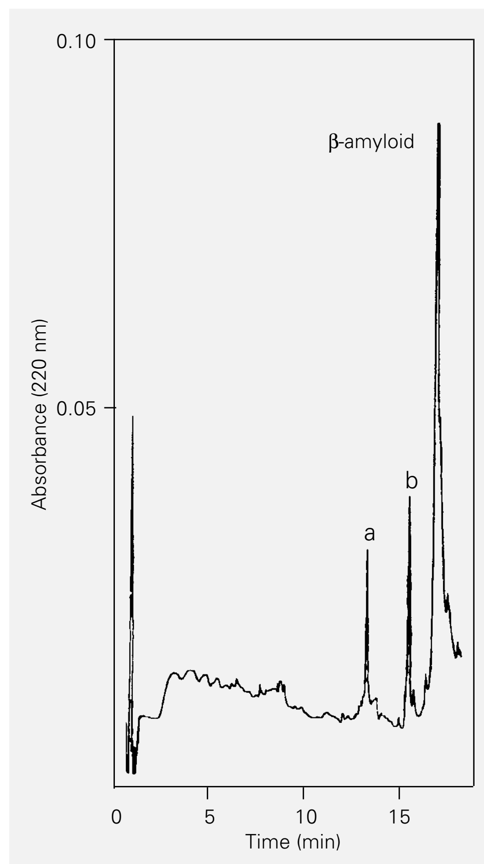

Figure 1 - HPLC elution profile of products of ß-amyloid 1-40 peptide generated after incubation with human brain metalloendopeptidase. The substrate (20 nmol) was incubated with 5 µl of the purified enzyme in a final volume of 50 µl 50 mM Tris-HCl buffer, pH 7.5, for 30 min at 37°C. After centrifugation at 10,000 *g* for 10 min, the supernatant fraction was injected into an HPLC column (Nucleosil 5 µm C_{18} 145 x 4.5 mm) eluted with a 0-50 gradient of acetonitrile containing 0.05% TFA, over a period of 20 min at a flow rate of 1 ml/min. Fragments were identified by amino acid composition: a and b are the 1-33 and 34-40 fragments of ß-amyloid 1-40, respectively.

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