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PURIFICATION AND CHARACTERIZATION OF A NOVEL METALLOPROTEASE FROM HUMAN BRAIN WITH THE ABILITY TO CLEAVE SUBSTRATES DERIVED FROM THE N-TERMINUS OF ß-AMYLOID PROTEIN

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The main component of amyloid plaques in Alzheimer's disease (AD) is the ß-amyloid peptide (ß/A4), derived from ß-amyloid precursor proteins (ß-APPs). In order to identify proteases possibly involved in the cleavage at the N-terminal site of ß/A4 a chromogenic peptide corresponding to this region of ß-APP was used. Here the purification and characterization of a new human brain protease with the ability to cleave the ß-APP peptide as well as ß-APP in vitro are described. The enzyme has a molecular mass of 100 kDa and belongs likely to the class of metalloproteases. It should further be named "MP100". The enzyme has a very broad substrate specificity in vitro.

Senile plaques and neurofibrillary tangles are the main histopathological alterations found in AD brains. The major component of the extracellular senile plaque is the ß-amyloid peptide (ß/A4) (8,15), which is derived from ß-amyloid precursor proteins (ß-APPs). ß-APPs are cleaved between amino acid 15 and 17 of the ß/A4 sequence by the still unknown sequence unspecific "α-secretase" (7,14,15). Therefore, ß/A4 amyloid cannot be generated by this pathway and alternative ways must exist to generate ß/A4. It was shown that cultured cells produce ß/A4 peptides during normal metabolism (9,22). Therefore, it can be expected that the enzymes involved in the generation of ß/A4 are active under normal physiological conditions.

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The abbreviations used are: MP100, purified human brain metalloprotease, ß-APP, ß-amyloid precursor protein; ß/A4, ß-amyloid peptide; AD, Alzheimer's disease; pNA, p-nitro-anilide; AMC, 7-amido-4-methylcoumarin; SDS, sodium dodecyl sulfate; EGTA, (Ethylenebis(oxyethylenenitrilo))tetraacetic acid; DFP, Diisopropyl fluorophosphat; PMSF, Phenylmethanesulfonyl fluoride; pCMB, 4-(chloromercury) benzenesulfonic acid.

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The purpose of this study was to characterize and purify proteases that may cleave ß-APP at the N-terminus of ß/A4. Several candidate enzymes with such a specificity have been described until now (1,10,11,16,17,18,19). The approach to identify corresponding proteases was based upon a chromogenic ß-APP peptide substrate derived from ten amino acids upstream of the N-terminal aspartic acid residue of ß/A4.

RESULTS AND DISCUSSION

Protease purification and biochemical properties. Using the procedure described under "Methods" a human brain metalloprotease called MP100 was purified in a 7.6% yield from a non-detergent brain extract (Fig. 1). Purification resulted in a 638-fold increase in the specific activity (Table 1). The purified protein revealed under reducing conditions a molecular mass of 98 ± 2 kDa in SDS-polyacrylamide gel electrophoresis (Fig. 2, lane 2). Under native conditions in a gel filtration (Superose 12) the molecular mass was calculated to be 100 ± 3 kDa as compared with standard proteins. Therefore the purified enzyme in its active form is assumed to be a monomer. The failure of MP100 to react positively in a glycan detection assay indicates that it is not a glycoprotein (data not shown).

Table 2 shows the amino acid composition of MP100. Only six methionine residues are present in the enzyme. The number of aspartic and glutamic acids plus the corresponding amids is more than twice the value for arginine and lysine residues. Due to the isoelectric point of the protein, determined to be between 4.7 and 4.9 by isoelectric focusing, it is likely that most of the acidic residues are amidated.

Catalytic properties. The pH-optimum of the protease was determined to be at pH 6.7 using the substrate Z-Val-Lys-Met-AMC. In Table 3 the influence of various inhibitors and modulators on MP100 is shown. Several metal ion chelators such as EDTA, EGTA and phenanthrolin strongly inhibit the enzyme activity indicating that the purified protease belongs to the class of metalloproteases. The serine and cysteine protease inhibitors DFP, PMSF and E64 did not inhibit the enzyme. However a SH-residue seems to be necessary for catalytic action: dialysis of the purified enzyme against a buffer lacking dithiothreitol resulted in a total loss of protease activity that could be recovered by readdition of dithiothreitol. pCMB, which reacts covalently with SH residues, as well as divalent metal ions are also able to block the activity of MP100.

Substrate specificity. An important characteristic of proteases is their specificity for different substrates. The substrate specificity of the purified MP100 was evaluated using a set of very different chromogenic amino acid- and peptide-AMC and -pNA substrates. The result was very striking: besides the synthetic ß-APP substrate used for protease purification, KTEEISEVKM-pNA, and a truncated peptide of the same ß-APP sequence Z-Val-Lys-Met-AMC, the protease was also able to cleave peptide-AMC substrates that are very different from these (Table 4). The best substrates according to the kcat/Km values are the amino acid-AMCs with the basic amino acids arginine and lysine. But the enzyme has also methionine and leucine aminopeptidase activity with lower kcat/Km values. For analyzing endoprotease activity several peptides specific for different proteases were tested. Besides the prolyl

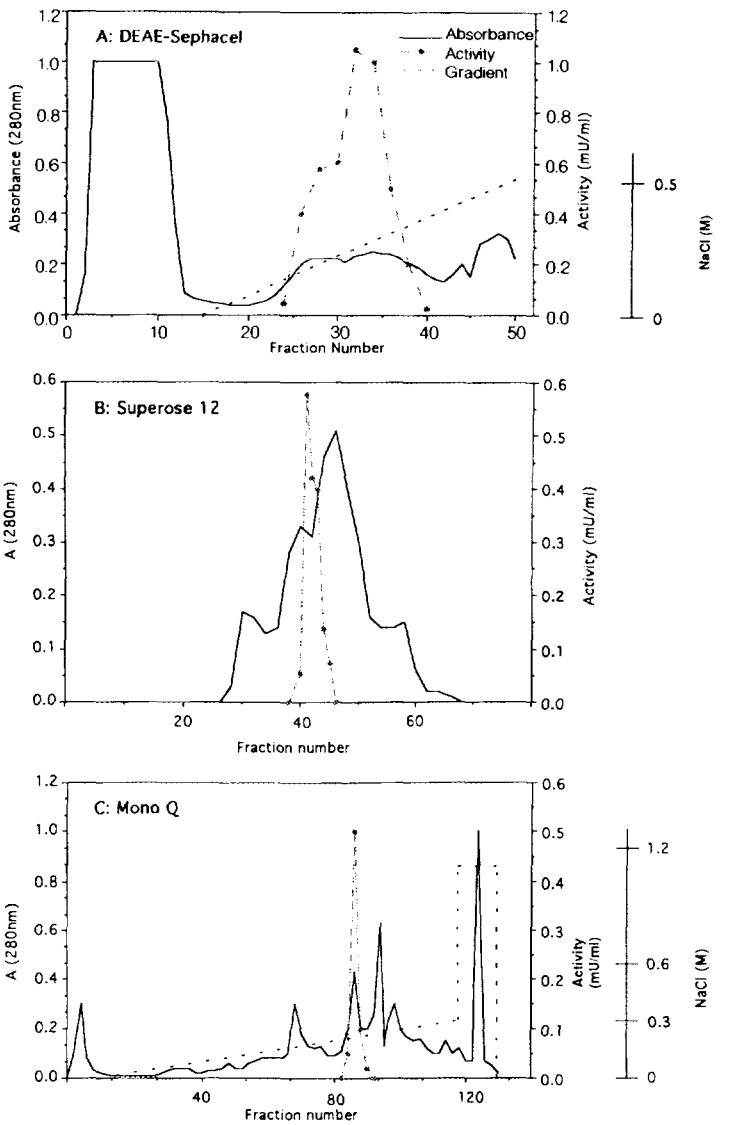

Figure 1. *Elution profiles of purification of human MP100.*

A:DEAE-Sephacel chromatography. Human brain supernatant was applied to a DEAE-Sephacel column (20 x 200 mm) equilibrated with buffer A and after column-washing 10 ml fractions were eluted with a 500 ml gradient of 0 - 0.5 M NaCl at 1 ml/min. Subsequently protease activity was determined.

B: Superose 12 chromatography. Active fractions from the DEAE-Sephacel column were pooled, concentrated by Centricon 30 to 2 ml and 250 µl were applied per run to a Superose 12 column (10 x 300 mm) equilibrated with buffer A. The flow rate of the column was 0.5 ml/min. Fractions of 0.5 ml were collected and analyzed.

C: Mono Q chromatography. Pooled active fractions from the Superose 12 column were applied to a Mono Q column (5 x 50 mm) equilibrated with buffer A. Proteins were eluted with a 55 ml linear gradient of 0 to 0.3 M sodium chloride at a flow rate of 1 ml/min. Fractions of 0.5 ml were collected, assayed for protease activity and analyzed on SDS-polyacrylamide gel electrophoresis for homogeneity.

Table 1
Purification table of human brain MP100

Step	Volume (ml)	Total activity (mU)	Total protein (mg)	Specific activity (mU/mg)	Purification factor	Recovery (%)
Supernatant	61	36.9	610	0.06	1	100
DEAE-Sepharose	36	18	4.8	0.26	4.4	49
Superose 12	5	11	0.68	16.3	272	30
Mono Q	4	2.8	0.075	38.3	638	7.6

endoprotease substrate Suc-Gly-Pro-AMC all other substrates tested were cleaved by MP100. The best endoprotease substrates tested according to kcat/Km were a shortened substrate for the N-terminal site of ß/A4, and Z-Phe-Arg-AMC, a cathepsin B substrate. In addition, the purified MP100 also cleaves a chymotrypsin substrate (Glutaryl-Phe-AMC), an elastase substrate (Suc-Ala-Ala-Ala-AMC), a calpain substrate (Suc-Leu-Tyr-AMC) and two other substrates (Z-Gly-Ala-Met-AMC and Boc-Glu-Lys-Lys-AMC) with a lower specificity. Two α-secretase-site derived substrates could not be cleaved by MP100.

Degradation of ß-APP by MP100. To investigate whether the enzymatic activity of MP100 could be relevant in the amyloidogenic pathway of ß-APP processing, the degradation of ß-APP in a human brain extract by purified MP100 was analyzed. The ß-APP bands of 105, 110, and 120 kDa recognized by the antibody 22C11 clearly disappeared after only 1h incubation with MP100 and new immunoreactive bands of 70, 75, 82, and 97 kDa could be

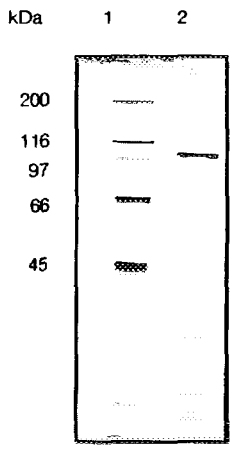

Figure 2. SDS-polyacrylamide gel electrophoresis of the purified human brain MP100. 5 µg of the purified active MP100 was applied to 10% polyacrylamide gel electrophoresis and silver stained (lane 2). Lane 1 shows molecular mass markers: Myosin (200 kDa), ß-galactosidase (116 kDa), phosphorylase B (97.4 kDa), serum albumin (66.2 kDa) and ovalbumin (45 kDa). Molecular mass of MP100 was determined after scanning with a video densitometer model 620 (BioRad) with the corresponding software.

Table 2

Amino acid composition of the purified MP100

Amino acid	Number of residues	Percentage of total number of residues (%)
Alanine	75.7	8.36
Arginine	39.4	4.35
Aspartic acid/Asparagine	98.9	10.94
1/2-Cystine	ND[a]	ND[a]
Glutamic acid/Glutamine	112.9	12.48
Glycine	64.8	7.16
Histidine	18.5	2.04
Isoleucine	43.2	4.77
Leucine	90.1	9.96
Lysine	58.3	6.44
Methionine	6.1	0.66
Phenylalanine	40.7	4.49
Proline	48.0	5.30
Serine	57.8	6.38
Threonine	50.8	5.61
Tryptophane	ND[a]	ND[a]
Tyrosine	30.7	3.39
Valine	68.7	7.59
Total	904.6	100

ND[a] not determined.

The purified protease was hydrolyzed at 110°C for 24 h in 6 N HCl. The values for serine and threonine were corrected by an experience factor originating from previous determinations. Amino acid residues were calculated based on a molecular protein mass of 100 kDa.

identified (Fig. 3A). Since 22C11 binds to the N-terminal region of ß-APP, these degradation products obviously originated from cleavage in the C-terminal region. In fact, immunostaining of full-length ß-APPs with an anti-C-terminal antibody completely disappeared within 3h of incubation with no new high molecular mass proteins appearing (data not shown). On the other hand, a protein fragment of 19 kDa which was recognized by an antibody against the N-terminal domain of ß/A4 accumulated during MP100 incubation (Fig. 3B). The size of this amyloidogenic fragment suggests a cleavage at or close to the N-terminus of the ß/A4 domain. Although other proteases are present in the brain extract the observed degradation of ß-APP seems to be exclusively due to MP100 as no degradation occurred in the absence of the enzyme. Furthermore, the degradation catalyzed by MP100 was virtually completely inhibited by 10 mM EDTA, which also inhibited MP100 in the peptide substrate assay (see above).

Conclusion. The proteolytic processing of ß-APP may result in the generation of the ß/A4 peptide which is subsequently deposited in extracellular senile plaques in AD brains. Until

Table 3
Effect of various inhibitors on MP100 activity

Effector	Concentration (mM)	residual activity (%)
DFP	2	100
PMSF	1	100
Pepstatin	1	100
E64	0.1	100
Leupeptin	0.1	100
p-CMB	0.5	0
	0.05	28
EDTA	10	10
	1	13
	0.1	18
EGTA	1	4
	0.1	22
Phenanthroline	1	4
Phosphoramidone	0.5	100
$ZnCl_2$	0.5	0
$MnCl_2$	0.5	3
$CaCl_2$	0.5	18
$CoCl_2$	0.5	0
$MgCl_2$	0.5	100

now it is not yet known where the proteolytic steps leading to ß/A4 generation occur and which enzymes are involved. However, cleavage at the N-terminus of ß/A4 is a crucial step in the generation of the amyloid peptide and therefore several attempts have been made to identify an N-terminal splitting enzyme (1,10,11,16,17,18). In the present study an as yet unknown metalloprotease was purified from human brain and characterized with the ability to cleave chromogenic substrates derived from the N-terminal cleavage site of ß/A4 as well as native ß-APP in a human brain extract. Therefore MP100 might be a candidate protease involved in ß-APP processing or degradation. Additional investigations are necessary to determine whether ß-APP is a specific substrate for MP100 and to identify further ß-APP degradation products.

METHODS

Purification of MP100, molecular mass determination and proteinchemical methods. Protein purification was started with 15 g of human brain tissue stored at -70°C; postmortem delay before freezing the tissue was at most 10 h. All steps were carried out at 4°C. The thawed tissue was homogenized 20 times in 75 ml of buffer A (50 mM sodium phosphate pH 7 containing 1 mM dithiothreitol) using a glass homogenizer with a teflon pistil. Then the homogenate was centrifuged at 20,000 g for 30 min and the supernatant was applied to a

Table 4

Kinetic constants of different substrates of MP100

Substrate	Km (M) * 10^7	kcat (s^{-1})	kcat/Km ($M^{-1}s^{-1}$) 10^{-3}
Arg-AMC	1.2 ± 0.16	2.7 ± 0.1	22400 ± 3080
Lys-AMC	6.1 ± 0.07	0.8 ± 0.02	1340 ± 140
Met-AMC	23.0 ± 2.0	1.8 ± 0.06	697 ± 65
Leu-AMC	19.2 ± 2.0	0.14 ± 0.001	72.9 ± 5
Glu-AMC	not	hydrolyzed	
Z-Val-Lys-Met-AMC	5.38 ± 1.0	0.062 ± 0.014	115 ± 30
Z-Gly-Ala-Met-AMC	833 ± 120	0.02 ± 0.005	0.24 ± 0.11
Boc-Glu-Lys-Lys-AMC	425 ± 23	0.20 ± 0.005	4.7 ± 1.0
Z-Phe-Arg-AMC	11.6 ± 2.0	0.23 ± 0.013	206 ± 13
Suc-Leu-Tyr-AMC	108 ± 16	0.006 ± 0.002	0.52 ± 0.17
Glutaryl-Phe-AMC	20.7 ± 4.8	0.023 ± 0.002	11.1 ± 2.7
Suc-Ala-Ala-AMC	260 ± 50	0.009 ± 0.003	0.34 ± 0.15
Suc-Gly-Pro-AMC	not	hydrolyzed	
KTEEISEVKM-pNA	2340 ± 125	8.5 ± 0.2	36.3 ± 2.2
DAEFGHDSGFEVRHQ-pNA	not	hydrolyzed	
DAEFGHDSGFEVRHQK-pNA	not	hydrolyzed	

DEAE-Sephacel column (20 x 200 mm) equilibrated with buffer A. After washing the column with 200 ml of buffer A to remove unbound proteins elution was performed with a 500 ml linear gradient from 0 to 0.5 M sodium chloride in buffer A. Fractions were collected, concentrated and loaded on a Superose 12 HR column (10 x 300 mm) equilibrated with buffer A. Active fractions were subsequently applied to a Mono Q column (5 x 50 mm) equilibrated with buffer A. Proteins were eluted with a 55 ml linear gradient (0 to 0.3 M sodium chloride in buffer A). Fractions of 0.5 ml were collected and analyzed for purity by SDS-polyacrylamide gel electrophoresis. Molecular mass determination of MP100 was carried out under nondenaturing conditions by gel filtration on a Superose 12 column (10 x 300 mm). The molecular mass was calculated according to (2). Polyacrylamide gel electrophoresis was done according to Laemmli (12) using a Mini-Protean II Electrophoresis Cell (Bio Rad), and proteins were visualized by silver staining (4). For western blotting the proteins were electrophoretically transferred from gels to nitrocellulose membranes (BioRad), which were blocked with 5 % milk in Tris buffered saline containing 0.05 % Tween 20 and incubated with the primary antibody overnight. As secondary antibody alkaline phosphatase conjugated anti rabbit-immunoglobulin (Dakopatts, Denmark) was used. Detection was performed using an alkaline phosphatase substrate kit (BioRad).
For isoelectric focusing on Immobiline Dry plates pH 4-7 (Pharmacia) 5 µg of the human brain metalloprotease were applied.
The amino acid composition of MP100 was determined after hydrolysis for 24 h at 110°C in 6 N HCL with an amino acid analyzer (Liquimat III, Kontron) (23).
Protein concentrations were determined by the method of Bradford (6) using a commercial assay (BioRad) with bovine serum albumin as standard.

Assay of protease activity, determination of substrate specificity, pH dependence and inhibitor profile. A standard protease assay was performed by reading the absorbance of pNA released from the substrate KTEEISEVKM-pNA at 405 nm. The reaction mixture

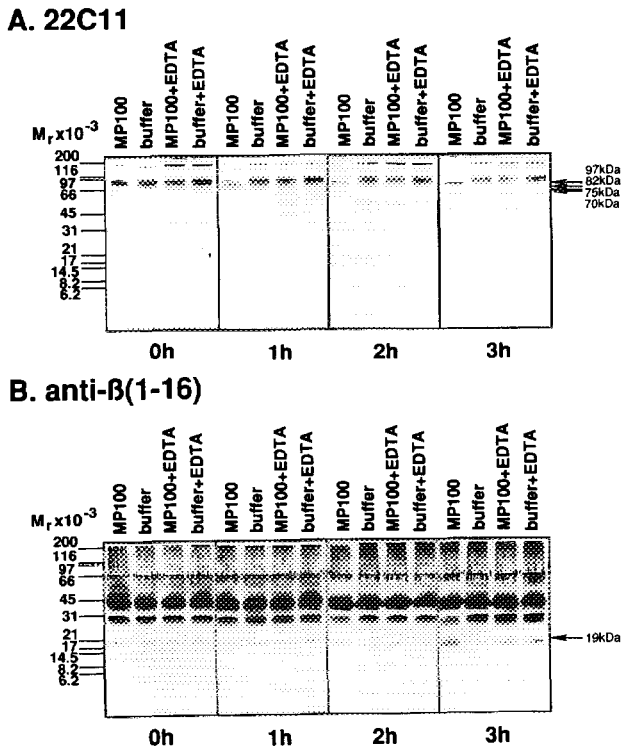

Figure 3. ß-APP degradation by MP100. Solubilized human brain proteins were incubated for 0, 1, 2, or 3h with or without MP100 in the absence or presence of EDTA, separated in a Tris-Tricine electrophoresis system, and transferrred to nitrocellulose membranes. The membranes were probed with antibody 22C11 (**A**) or anti-ß(1-16) (**B**).

consisted of 20 µl of substrate (0.4 mM), 40 µl of 50 mM Hepes buffer pH 7 containing 2 mM dithiothreitol (buffer B) and 20 µl of enzyme solution. Measurements were done on an ELISA reader (UV max, Molecular devices) at 30°C for 15 min. One unit of protease activity is defined as the amount of protease that liberates 1 µmol/min of pNA at 30°C. Kinetic measurements with the AMC-substrates were done on a fluorimeter (LS 50, Perkin Ellmer) at an excitation wavelength of 383 nm and an emission wavelength of 455 nm at 30°C. The reaction mixture consisted of 1 ml buffer B, 1 ml of different substrate concentrations and 20 µl of MP100 (4-20 ng/ml). The kinetic constants kcat, Km and kcat/Km were calculated using a Lineweaver/Burk plot.

The pH dependence of the purified protease was determined with Z-Val-Lys-Met-AMC (Peninsula Laboratories, USA) at a concentration of 10 µM which corresponds to approximately 20 x Km. The influence of various inhibitors was tested by the following procedure: 40 ng of MP100 were preincubated for 10 min with the inhibitors or other modulators in 50 mM Hepes buffer pH 7, 2 mM dithiothreitol, before the reaction was started by adding the substrate Z-Val-Lys-Met-AMC at a concentration of 10 µM. All measurements were done in triplicates.

ß-APP degradation assay. Proteins of human brain homogenate in buffer A (see above) were solubilized by addition of CHAPS (final concentration 2%). After 30 min on ice with occasional mixing, the solubilisate was cleared by a short centrifugation (ca. 1 min) in a desktop microcentrifuge. 15 µl of the supernatant (120 µg protein) were combined with 20 µl

incubation buffer (40 mM Hepes pH 7.0, 2 mM dithiothreitol) and 7.5 µl purified MP100 in buffer A, and the mixture was incubated at 37°C. In control assays MP100 was replaced by buffer A, or incubation buffer containing additionally 20 mM EDTA, or both. After 0, 1, 2, or 3h, the incubation was stopped by addition of 10 µl 10xSDS-sample buffer and heating the sample at 95°C for 5 min. The samples (11 µl per lane) were subjected to electrophoresis in 10% polyacrylamide gels using the Tris-Tricine buffer system (21) and transferred to nitrocellulose as described. Western blot analysis was done with antibody 22C11 (Boehringer Mannheim) binding to the N-terminal part of ß-APP, or anti-ß(1-16) binding to the N-terminal part of the ß/A4 domain (13).

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