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Cathepsins S, B and L with aminopeptidases display β-secretase activity associated with the pathogenesis of Alzheimer's disease

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Abstract

β-site APP-cleaving enzyme (BACE1) cleaves the wild type (WT) β-site very slowly (k_{cat}/K_m: 46.6 M^{-1} s^{-1}). Therefore we searched for additional β-secretases and identified three cathepsins that split the WT β-site much faster. Human cathepsin S cleaves the WT β-site (k_{cat}/K_m: 54 700 M^{-1} s^{-1}) 1170-fold faster than BACE1 and cathepsins B and L are 440- and 74-fold faster than BACE1, respectively. These cathepsins split two bonds flanking the WT β-site (K-MD-A), where the K-M bond (85%) is cleaved more efficiently than the D-A bond (15%). Cleavage at the major K-M bond yields Aβ (amyloid β-peptide) extended by N-terminal Met that should be removed to generate Aβ initiated by Asp1. The activity of cytosol and microsomal aminopeptidases on relevant peptides revealed rapid removal of N-terminal Met but not N-terminal Asp. Brain aminopeptidases showed similar specificity. Thus, aminopeptidases would convert Aβ extended by Met into regular Aβ (Asp1) found in amyloid plaques. Earlier studies indicate that Aβ is likely produced in the endosome and lysosome system where cathepsins S, B and L are localized and cysteine cathepsin inhibitors reduce the level of Aβ in cells and animals. Taken together, cathepsins S, B and L deserve further evaluation as therapeutic targets to develop disease modifying drugs to treat Alzheimer's disease.

Keywords: BACE1; cleavage of β-site sequence; cleavage points; k_{cat}; K_m; new β-secretases; WT β-site sequence.

Introduction

Many studies indicate that the amyloid β peptide (Aβ), deposited in the brain as amyloid plaques, plays a major role in the pathogenesis of Alzheimer's disease (AD) (Selkoe, 2001). The Aβ peptide is generated from the amyloid precursor protein (APP) by the consecutive action of two proteases: β-secretase and then γ-secretase. Major efforts have been directed to identify the secretases because inhibitors that block their activity reduce the formation of Aβ and thus would slow down, stop or reverse the progression of AD. The most successful clinical application of this approach are inhibitors of the HIV protease to treat AIDS and inhibitors of the angiotensin-converting enzyme to treat hypertension and congestive heart failure. Approximately 10 years ago, the β-site APP-cleaving enzyme (BACE1) was identified as β-secretase (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999; Lin et al., 2000) and the enzyme has been intensively studied (Wolfe, 2006; Cole and Vassar, 2008). Potent BACE1 inhibitors are available (Abbenante et al., 2000; Turner et al., 2001) but progress in drug development to treat AD is rather slow (Cole and Vassar, 2008). Therefore, alternative approaches have been evaluated (Hook et al., 2008b). It was shown that cathepsin B can function as β-secretase and that cysteine protease inhibitors improve memory and reduce amyloid plaques in the brain (Hook et al., 2005, 2008a). The glutaminyl cylase mediates the formation of pyroglutamated truncated Aβ ($pGlu_3$, $Aβ_{3–42}$) that facilitates Aβ aggregation, and inhibitors of glutaminyl cyclase reduce plaque burden (Schilling et al., 2006, 2008).

Compelling evidence suggest that BACE1 is the genuine β-secretase. Five research groups identified BACE1 as the β-secretase by using different strategies: cloning expression (Vassar et al., 1999), expressed sequence tag (EST) (Hussain et al., 1999; Lin et al., 2000), analysis of *Caenorhabditis elegans* genome and human EST (Yan et al., 1999), and affinity chromatography (Sinha et al., 1999). All research teams assumed that the β-secretase should be an aspartic protease with optimal activity at acidic pH, cleaving the β-site of the Swedish (SW) mutant sequence faster than the β-site of the wild type (WT) sequence. Accordingly, in all cases β-secretase activity was monitored by peptide substrates containing the β-site SW sequence and for affinity purification, the immobilized ligand was also derived from the β-site SW sequence. The presumed properties of the β-secretase were mainly based on the activity of brain extracts on SW and WT peptides. We have shown that the specificity of cathepsin D toward SW and WT peptides is similar to that of BACE1 and that the apparent β-secretase activity in brain extracts is mainly due to cathepsin D, not BACE1 (Schechter and Ziv, 2008). Additional problems with BACE1 are outlined below.

BACE1 cleaves the mutated SW β-site sequence of APP present in two Swedish families much better than the WT sequence present in the worldwide population. BACE1 cleaves the WT β-site very slowly (k_{cat}/K_m: approx. 50 M^{-1} s^{-1}, Lin et al., 2000; Shi et al., 2001; Schechter and Ziv, 2008)

whereas proteases acting on relevant substrates are much more efficient, k_{cat}/K_m: 10 000–1 000 000 M^{-1} s^{-1} (Schechter, 1970; Dunn and Hung, 2000). BACE1 is a membrane bound enzyme and it was argued that enzymes anchored in the cell membrane could have reduced activity when freed in solution. However, application of the active site mapping procedure (Schechter and Berger, 1967) to evaluate the specificity of BACE1 have led to the synthesis of good peptide substrates that differ from the WT β-site sequence (Turner et al., 2001; Tomasselli et al., 2003; Shi et al., 2005). For example, a peptide that extensively differs from the WT β-site octapeptide sequence (P4-P4′) in seven out of eight amino acid residues, is cleaved by BACE1 with k_{cat}/K_m of 342 000 M^{-1} s^{-1} (Turner et al., 2001). These findings suggest that BACE1 acts on yet unknown substrate(s) and it is unlikely that the WT APP is the physiological substrate (Schechter and Ziv, 2008). Knockout of BACE1 in mice markedly reduces Aβ formation (Cai et al., 2001; Lou et al., 2001; Roberds et al., 2001). Yet, it was shown that knockout of other genes in rodents, and the corresponding genetic defects in human, might reveal different phenotypes. Nine different tumor suppressor genes associated with human tumors were mutated in mouse and rat but only two mutated genes overlapped with the human phenotype (Jacks, 1996). Considering these issues, we searched for other β-secretase candidate(s). We found cathepsin D and evaluated properties of cathepsin D related to BACE1 that were not examined before. It was found that human BACE1 and cathepsin D display the same specificity; they have similar kinetic constants (k_{cat}, K_m, k_{cat}/K_m) for cleaving β-sites of relevant peptides, cleaving SW better than the WT sequence. G-100 chromatography of brain extract reveal that β-secretase activity is eluted at the position of cathepsin D (42 kDa) and not at that of BACE1 (72 kDa). Pepstatin A at 20 nM completely inhibits the cleavage of SW peptides by both cathepsin D and brain extracts, whereas BACE1 activity on the same peptides is not inhibited even by 1000-fold higher concentration of pepstatin A (20 μM). Quantitative Western blots reveal that in human brain, cathepsin D is approximately 280-fold more abundant than BACE1. Therefore, it is conceivable that β-secretase activity observed in brain extracts is mainly due to cathepsin D and not BACE1 (Schechter and Ziv, 2008). Nevertheless, because both BACE1 and cathepsin D show poor activity towards the WT β-site sequence we continued the search for additional β-secretase candidate(s). Here we report that cathepsins S, B, and L cleave the WT β-site sequence much better than BACE1 and cathepsin D.

Results

Search of β-secretase activity in the kidney reveals cathepsin S

The 25V fraction of bovine brain homogenate was chromatographed on a G-100 column and aliquots of the eluate were reacted with the P17 WT peptide. A complex pattern of cleavage products was observed that was resolved as work progressed (see below). Therefore, we analyzed bovine kidney because the APP is expressed ubiquitously (Neve et al., 1988) and many cell types, including kidney cells, produce Aβ (Vassar et al., 1999).

The 25V fraction of bovine kidney was chromatographed on a G-100 column and β-secretase activity was monitored by mixing aliquots of the eluate with the WT peptide substrates P4 or P17 (see Figure 1 for sequences of the peptide substrates and cleavage products). Peptide degradation was observed in two fractions (Figure 2). A fraction of high molecular mass (≥70 kDa, between the void volume and BSA) yielded multiple degradation products of varying size likely due to a mixture of proteases, and it was not further investigated. The other fraction of low molecular mass (approx. 22 kDa, near the myoglobin size marker) showed a distinct peak of activity and a simple pattern of cleavage products, indicating for activity by a single protease. The cleavage products of P4 and P17, observed by paper electrophoresis, were consistent with cleavage at the β-site of these peptide substrates. It was previously shown that BACE1 and cathepsin D cleave the P4 WT peptide at the M-D β-site to yield the P6 C-terminal peptide [subsequently it was found that the peptide product is P6+M and not P6 that have similar electrophoretic mobility (see Figure 6)], and the P9 N-terminal peptide (migrates like P4 on paper electrophoresis, Schechter and Ziv, 2008), as observed in Figure 2A. It was also shown (Schechter and Ziv, 2008) that cleavage at the M-D β-site of P17 yields the N [subsequently it was found that the peptide is N-M and not N that has similar electrophoretic mobility (see Figure 5)] and C terminal peptide products with electrophoretic mobility like that observed (Figure 2B). Therefore, the active fractions (Figure 2, fractions 32–36) were pooled, concentrated and loaded on CM52 cation exchanger. The β-secretase activity was found in the effluent. It was concentrated and loaded on a gel made in neutral buffer. After electrophoresis the gel was sliced into

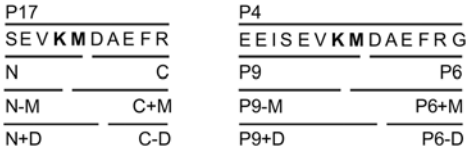

Figure 1 Sequences of WT peptide substrates and of cleavage products.

Substrates (P17, P4) match with the amino acid sequence flanking the β-site of the WT APP, except for the carboxyl-terminal residue of the P4 peptide where glycine is replacing histidine present in APP. Cleavage products are designated with reference to cleavage of the M-D bond of the WT β-site sequence. Accordingly, cleavage of P17 (10-mer) can yield the N (N-terminal, 5-mer) and C (C-terminal, 5-mer) peptides (cleavage at the β-site M-D bond), the N-M (N minus Met residue) and C+M (C plus Met residue) peptides (K-M bond split), the N+D (N plus Asp) and C-D (C minus Asp) peptides (D-A bond split). Similarly, cleavage of P4 (14-mer) can yield the P9 (8-mer) and P6 (6-mer) peptides (cleavage at the β-site M-D bond), the P9-M (P9 minus Met) and P6+M (P6 plus Met) peptides (K-M bond split), the P9+D (P9 plus Asp) and P6-D (P6 minus Asp) peptides (D-A bond split).

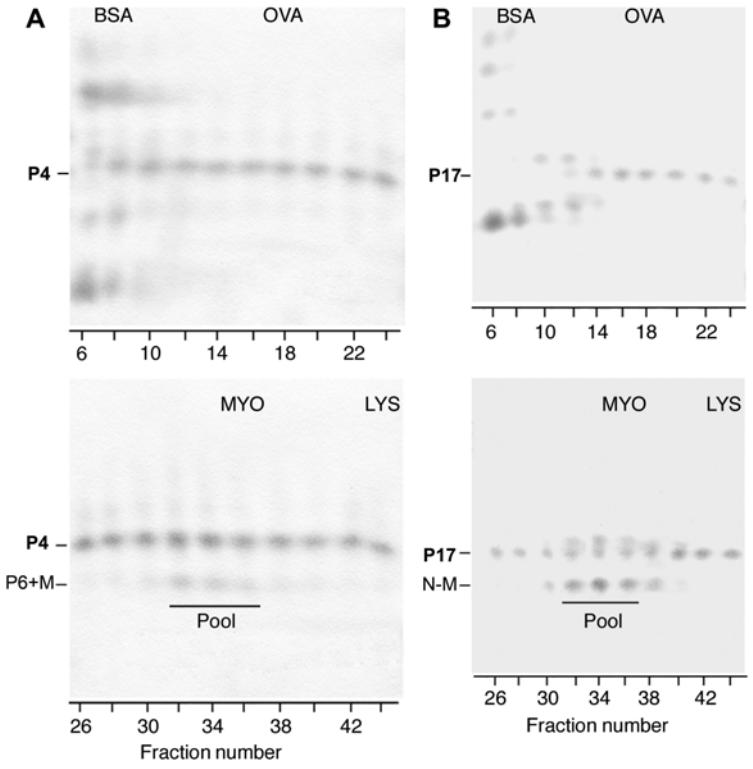

Figure 2 β-Secretase activity of the 25V fraction of bovine kidney prepared in the pH 7.3 buffer and subjected to chromatography on Sephadex G-100 at pH 7.3.

β-Secretase activity was tested by using P4 (A) and P17 (B) peptide substrates. Active fractions (32–36) were pooled for further purification. Components of the reaction mixture were resolved by paper electrophoresis in the pH 6 buffer. Bovine serum albumin (BSA), ovalbumin (OVA), myoglobin (MYO) and lysozyme (LYS) indicate the position of protein markers eluted from the G-100 column. Electrophoretic mobility of the peptide substrates (bold P4 and P17) and of resolved major cleavage products are shown on the left.

strips and strong β-secretase activity was found in eluates of strips one to three that were pooled (Figure 3A), concentrated and loaded on a gel made in acidic buffer. The gel was sliced and strong β-secretase activity was detected in eluates of strips six and seven that were pooled (Figure 3B). Trichloroacetic acid was added, the protein pellet was dissolved in loading buffer and it was resolved by SDS-PAGE. The stained gel showed three bands, two of which had molecular masses of approximately 25 kDa and 29 kDa (Figure 3C). Because G-100 chromatography reveals β-secretase activity with a molecular mass of approximately 22 kDa, the 25 kDa and 29 kDa bands were cut from the gel and digested by trypsin. Tandem mass spectrometry revealed that the 25 kDa band contained tryptic peptides derived from bovine cathepsin S.

Cleavage of WT peptides by cathepsins S, L and B

Some peptide substrates and cleavage products are not resolved by paper electrophoresis. Therefore, in order to determine the site of substrate cleavage by cathepsin S, the enzyme digest was further analyzed by HPLC and mass spectroscopy. HPLC of digests of the P17 and P4 peptides by cathepsin S revealed four new peaks (Figure 4B and H). The HPLC patterns of P17 and P4 digests by cathepsin S differ from those of the P17 and P4 digests by cathepsin D or BACE1 (Schechter and Ziv, 2008). The latter enzymes yield other HPLC peaks of peptide products formed by cleavage at the M-D bond of the β-site sequence of the P17 or P4 substrates (Figure 1).

Mass spectroscopy analyses of HPLC peaks of P17 digest by cathepsin S (Figure 4B) were as follows. The two major peaks correspond to cleavage products at the K-M bond of P17 to yield the N-M peptide (461 Da) and the C+M peptide (767 Da). The two minor peaks correspond to cleavage products at the D-A bond of P17 to yield the N+D peptide (707 Da) and C-D peptide (521 Da). Thus, cathepsin S does not split at the β-site M-D bond of the P17 WT substrate, but it cleaves the WT sequence at two bonds flanking the WT β-site: K-MD-A (hyphens indicate the bonds cleaved, Figure 1). From areas of the peaks (N-M plus C+M compared to N+D plus C-D, six measurements) we estimate 85% cleavage at the K-M bond and 15% cleavage at the D-A bond of P17. The resolved peaks (Figure 4B) were concentrated approximately 25-fold and subjected to paper electrophoresis (Figure 5). All cleavage products migrated similarly to the appropriate peptide markers in complete agreement with their mass as determined by mass spectrometry.

Analyses of the P4 peptide digested by cathepsin S were similar to those obtained with P17. HPLC (Figure 4H) and mass spectrometry of resolved peaks showed: (1) two major

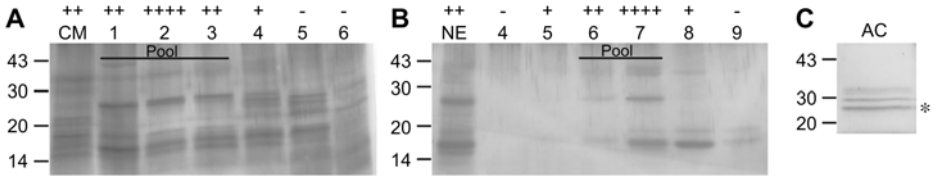

Figure 3 Purification of β-secretase activity from bovine kidney by polyacrylamide gel electrophoresis.
(A) Neutral gel analysis. Effluent of the CM52 column with β-secretase activity was concentrated and subjected to electrophoresis on a neutral polyacrylamide slab gel. After the run, the gel was sliced into twelve 3 mm wide strips that were soaked in elution solution. Aliquots of the eluate were analyzed for proteins and β-secretase activity. SDS-PAGE developed with silver stain demonstrated proteins in the CM52 effluent (CM) loaded on the neutral gel and in eluates from strips 1–6 (counting from top of the neutral gel). The β-secretase activity indicated on top of the gel was tested by using the P17 peptide substrate and paper electrophoresis. The extent of P17 degradation was qualitatively graded between – (P17 not degraded) to ++++ (P17 completely degraded). Strong β-secretase activity was found in eluates from strips 1–3 that were further analyzed. (B) Acidic gel analysis. Active fractions of the neutral gel (strips 1–3) were pooled, concentrated (NE), resolved on an acidic gel and analyzed as above. Strong β-secretase activity was found in eluates of strips six and seven. (C) Active fractions of the acidic gel (AC, stripes six and seven) were pooled, concentrated and resolved by SDS-PAGE developed with Coomassie Blue. Asterisk indicates the 25 kDa band that contained tryptic peptides of bovine cathepsin S. Numbers indicate the molecular mass (kDa) of protein size markers.

peaks corresponding to cleavage products of P4 at the K-M bond to yield the P9-M peptide (832 Da) and the P6+M peptide (824 Da) and (2) two minor peaks corresponding to cleavage of P4 at the D-A bond to yield the P9+D peptide (1078 Da) and P6-D peptide (578 Da). Here again, cathepsin S cleaves the P4 WT peptide at two bonds flanking the WT β-site: K-MD-A, as observed for the P17 peptide. From peak areas (see above) we estimate that cleavage of P4 occurs mainly at the K-M bond (approx. 90%) and less at the D-A bond (approx. 10%), similar to the finding with P17. Cleavage products resolved by HPLC subjected to paper electrophoresis migrated similarly to the expected peptide markers (Figure 6), in complete agreement with their mass determined by mass spectrometry.

The use of peptide markers, paper electrophoresis and HPLC clearly identified the substrate and the cleavage products. Peptides (for example, P4 and P9-M) that were not resolved by one procedure (paper electrophoresis, Figure 6)

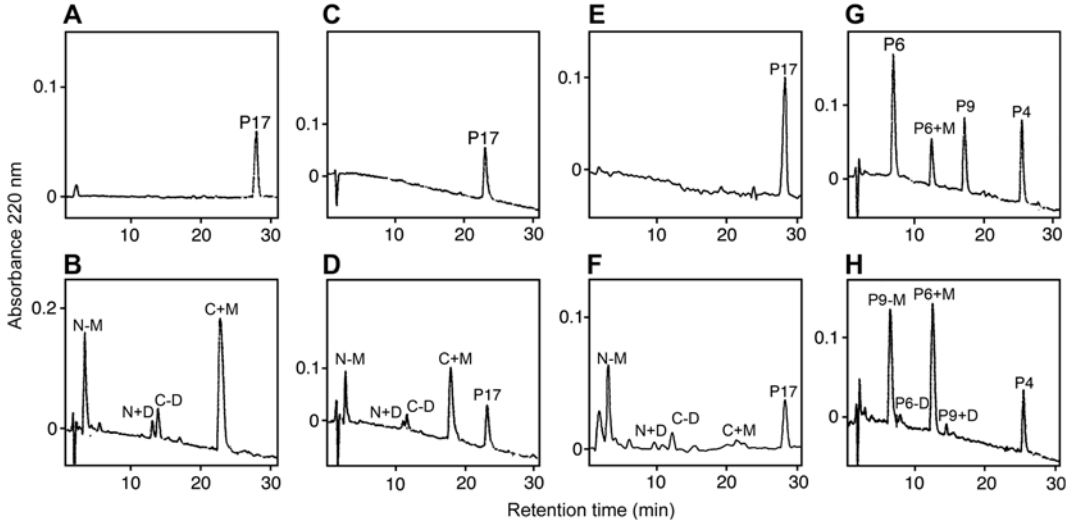

Figure 4 HPLC profiles showing hydrolysis of the P17 and P4 peptide substrates by human cathepsins S, L and B.
(A) Control of P17 (1210 Da). (B) P17 with cathepsin S. Cleavage yields two major peaks of N-M (molecular mass: 461 Da) and C+M (767 Da), and two minor peaks of N+D (707 Da) and C-D (521 Da). Molecular mass (Da) of the peptides was determined by mass spectrometry of the resolved HPLC peaks. (C) Control of P17. (D) P17 with cathepsin L. HPLC profile is similar to that observed in panel (B). Molecular mass values of peptides in resolved peaks are identical to those obtained in B. (E) Control of P17. (F) P17 with cathepsin B. HPLC profile is similar to that observed in panels (B) and (D) except for markedly reduced C+M peak. Molecular mass values of peptides in resolved peaks are identical to those obtained in panel (B). (G) Control of the P4 peptide substrate (1638 Da) and of three markers of possible cleavage products. (H) P4 with cathepsin S. Cleavage yields two major peaks of P9-M (832 Da) and P6+M (824 Da), and two minor peaks of P6-D (578 Da) and P9+D (1078 Da). Peptides were eluted from HPLC columns by 0.1% TFA in acetonitrile gradients of 5–20% in (A, B, E, F); 5–25% in (C, D) and 10–25% in (G, H). Similar HPLC profiles and identical molecular mass values of resolved peaks were obtained by analyses of the P17 and P4 WT peptide substrates digested by bovine cathepsins S, L and B.

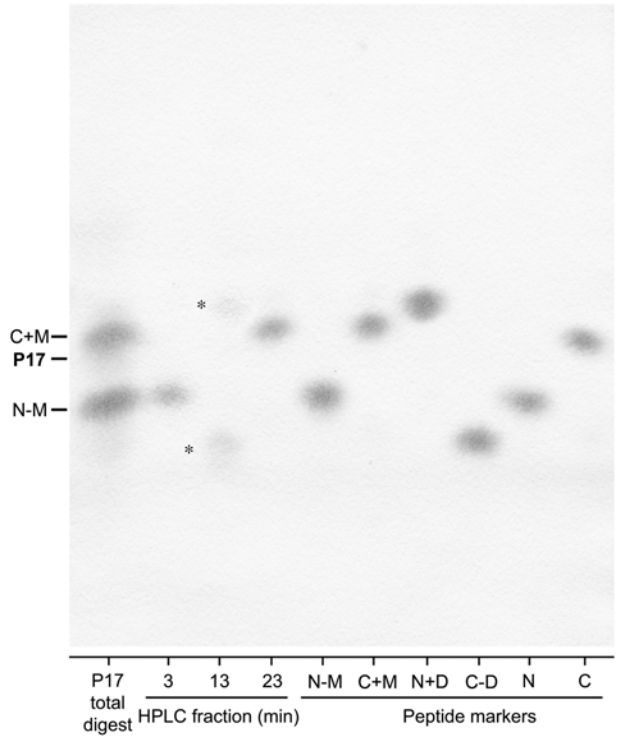

Figure 5 Characterization of the P17 cleavage products produced by cathepsin S.
P17 digested by human cathepsin S: total digest and fractions of the total digest eluted from the HPLC column after 3 min, 13 min and 23 min. Asterisks (13 min sample) indicate positions of the two minor cleavage products (N+D top, C-D bottom) that are resolved by HPLC. The positions of peptide markers are shown (amino acid sequences of the markers are in Figure 1). Samples were resolved by paper electrophoresis in the pH 6 buffer. Indicated on the left are positions of the P17 peptide substrate (bold) and of the two major cleavage products.

were clearly resolved by the other (HPLC, Figure 4H). Peptide identification was further ascertained by mass spectrometry. These analyses performed on peptide digests of cathepsin S as well as of cathepsins L and B (see below) enabled unambiguous identification of the bonds cleaved by the enzymes.

In addition to cathepsin S, we studied cathepsins B and L because high levels of these proteases were found in neurons and amyloid plaques in the brain of Alzheimer's patients by enzyme cytochemistry and immunohistochemistry (Cataldo and Nixon, 1990; Cataldo et al., 1991). Experiments conducted with cathepsin L and the P17 peptide show cleavage products similar to those observed with cathespsin S. Digests of P17 by both enzymes have similar HPLC (compare Figure 4B and D) and paper electrophoresis (Figure 7A) patterns. Mass spectrometry of HPLC peaks of P17 digest by cathepsin L had molecular mass values identical to those obtained from HPLC of P17 digest by cathepsin S. Thus, cathepsin L splits the WT P17 peptide at two bonds flanking the WT β-site (K-MD-A), where the K-M bond (approx. 85%) is cleaved more frequently than the D-A bond (approx. 15%), as found for cathepsin S.

Analyses of the P17 peptide digest by cathepsin B reveals minor cleavage at the D-A bond, as seen from two small HPLC peaks of N+D and C-D (Figure 4F) and confirmed by mass spectrometry and paper electrophoresis. Cleavage at the K-M bond is indicated by identifying the peptide pair, N-M and C+M. However, the amount of the N-M peptide is significantly higher than that of the C+M peptide as seen by HPLC (Figure 4F) and paper electrophoresis (Figure 7A). Conceivably this is due to the dual activity of cathepsin B, as endopeptidase (cleaving the K-M and D-A bonds) and as dipeptidyl carboxypeptidase (Aronson and Barrett, 1978). The latter activity would degrade the C+M peptide by stepwise removal from its C-terminus of the PheArg, AlaGlu and MetAsp dipeptides (Figure 1). Indeed, the P17 digest by cathepsin B reveals that the additional peptide spots comigrate with these dipeptide markers (Figure 7A). Thus, the three cathepsins (S, L and B) exhibit similar specificity: a major cleavage at the K-M bond (approx. 85%) and a minor cleavage at the D-A bond (approx. 15%) flanking the WT β-site (K-MD-A).

Cleavage products of P17 digest by cathepsin B is different from that obtained by cathepsins S and L (Figure 7A) due to the dipeptidyl carboxypeptidase activity of cathepsin B. The cathepsin B cleavage pattern of P17 was observed in the 25V fraction of bovine brain chromatographed on a G-100 column that eluted at the molecular weight region of

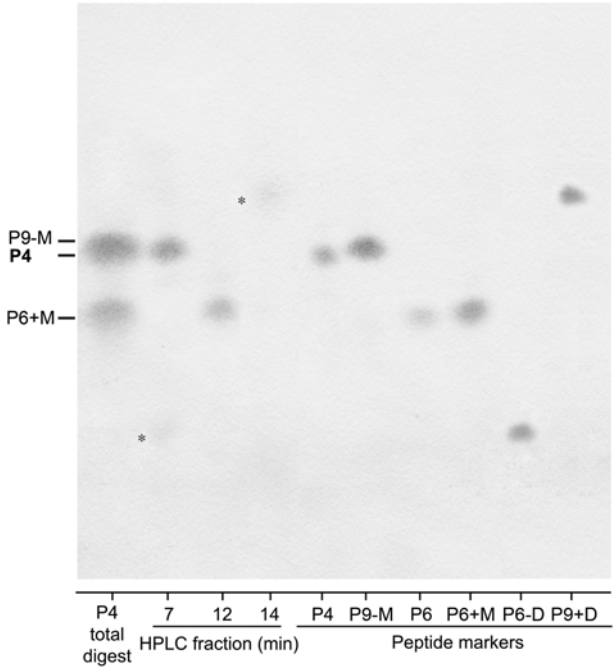

Figure 6 Characterization of the P4 cleavage products produced by cathepsin S.
P4-digested by human cathepsin S: total digest and fractions of the total digest eluted from the HPLC column after 7 min, 12 min and 14 min. Asterisks indicate positions of the two minor cleavage products (P6-D 7 min, P9+D 14 min). Samples were resolved by paper electrophoresis in the pH 6 buffer. Indicated on the left are positions of the P4 peptide substrate (bold) and of the two major cleavage products.

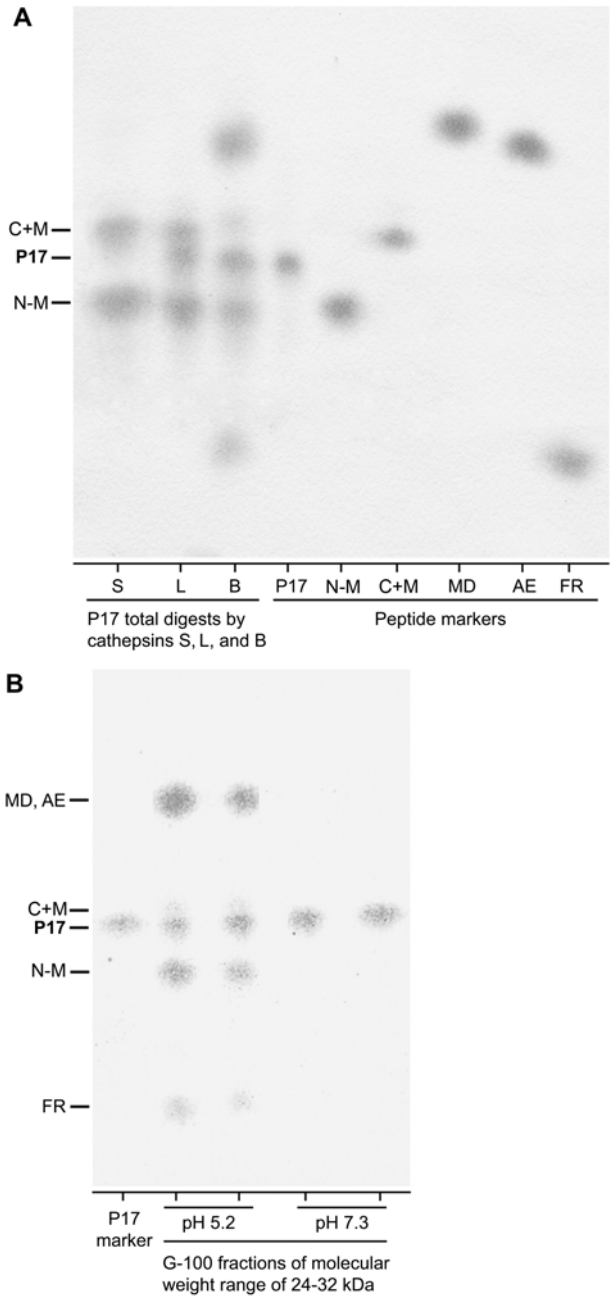

Figure 7 Characterization of the P17 cleavage products produced by cathepsin B and by the 25V fraction of bovine brain subjected to chromatography on a Sephadex G-100 column.
(A) Total digest of P17 by human cathepsin B, compared with the P17 total digests by cathepsins S and L. Peptide markers are P17, N-M and C+M peptides, the dipeptides MetAsp (MD), AlaGlu (AE) and PheArg (FR). Samples were resolved by paper electrophoresis in the pH 6 buffer. Indicated on the left are positions of the P17 peptide substrate (bold) and of the two major cleavage products. Similar patterns of cleavage product were obtained by paper electrophoresis analyses of P17 digested by bovine cathepsins S, L and B. (B) 25V fractions of bovine brain were prepared as described for bovine kidney. The 25V brain fraction prepared in the pH 5.2 or pH 7.3 buffers were chromatographed on G100 equilibrated at pH 5.2 or pH 7.3, respectively, and reacted with the P17 peptide. Reaction of G-100 fractions eluted in the range of 24–32 kDa (mass of cathepsin B is 28 kDa) are shown. Note that fractions from the acidic G100 column (pH 5.2) cleave P17 to peptide products as observed for P17 cleavage by cathepsin B (see A), whereas fractions from the neutral column (pH 7.3) do not degrade P17. Indicated on the left are positions of the P17 peptide substrate (bold), N-M and C+M peptides, the dipeptides MetAsp (MD), AlaGlu (AE) and PheArg (FR).

24–32 kDa (cathepsin B molecular mass is 28 kDa). Notably, P17 degradation was observed in the brain 25V fraction prepared in the pH 5.2 buffer and chromatographed on G-100 equilibrated with the pH 5.2 buffer but not in 25V fraction prepared and chromatographed at pH 7.3 (Figure 7B). This finding is consistent with cathepsin B activity in the brain, because cathepsin B is stable at acidic pH but it is unstable at neutral pH, whereas cathepsin S is stable at both acidic and neutral pH (Bromme et al., 1993).

Kinetic constants for cleaving the WT peptides by cathepsins S, L and B

To evaluate the relationship between the various cathepsins and BACE1 in quantitative terms, we determined kinetic parameters of these enzymes towards the P17 and P4 WT peptide substrates (Table 1) by using paper electrophoresis. The extent of hydrolysis of P17 was determined from the N-M peptide spot because it originates from the major cleavage site of P17 (approx. 85% cleavage of the K-M bond) and it is well separated from other components of the reaction mixture (Figures 2 and 5). Extent of hydrolysis of P4 by cathepsin S was determined from the P6+M peptide because it originates from the major cleavage site of P4 (approx. 90% cleavage at the K-M bond) and it is well separated from the P4 substrate and other cleavage products (Figures 2 and 6). Kinetic constants (k_{cat}, K_m, k_{cat}/K_m) were derived from Lineweaver-Burk plots for cathepsins S and L.

Cathepsin B has endopeptidase activity that cleaves the same bonds as cathepsins L and S. However, cathepsin B has additional dipeptidyl carboxypeptidase activity that rapidly degrades the C+M cleavage product (90% in 1 h, data not shown), it slowly degrades the N-M cleavage product (5% in 1 h, data not shown) likely because N-M (tetrapeptide) is smaller than C+M (hexapeptide) (Schechter and Berger, 1967) and it probably degrades the C-terminus of the P17 substrate. Therefore, in this case the P17 hydrolysis data are problematic to construct Lineweaver-Burk plots to evaluate the k_{cat} and K_m constants. Instead, we estimated only the k_{cat}/K_m value for P17 cleavage by cathepsin B. For this, low P17 concentrations (0.4–1 mM) were used and the released N-M peptide was determined. Apparent reaction rates were first order with respect to the P17 concentration, i.e., enzyme saturation was low. These values were used to calculate k_{cat}/K_m, which represents the rate of hydrolysis at infinite dilution of the substrate (Schechter, 1970).

The activity of bovine cathepsins S, L and B towards the P17 and P4 peptides is similar to that observed for the corresponding human enzymes, as observed in the HPLC profiles (Figure 4), paper electrophoresis (Figure 7A) and kinetic constants for cleaving the peptides (Table 1).

Table 1 Kinetic parameters for cleavage of the P17 and P4 WT peptide substrates by cathepsins S, L, B and D and by BACE1.

Enzyme[a]	Substrate[b]	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m M^{-1} s^{-1}	Ratio[c] Cat./BACE1
Cat.S-bov	P17 (10)	34.2±4.7	1.8±0.36	19 200±2200	
Cat.S-bov	P4 (14)	52.4±5.1	2.4±0.43	21 600±1100	
Cat.L-bov	P17 (10)	13.8±0.8	3.7±0.15	3720±310	
Cat.B-bov	P17 (10)			16 220±1500	
Cat.S-hum	P17 (10)	148±34	2.7±0.61	54 700±4800	1170
Cat.S-hum	P4 (14)	137±40	3.1±0.70	44 800±9500	570
Cat.L-hum	P17 (10)	9.7±1.6	2.8±0.52	3460±580	74
Cat.B-hum	P17 (10)			20 470±1900	440
Cat.D-hum	P17 (10)			29.5	0.6
Cat.D-hum	P4 (14)			134	1.7
BACE1	P17 (10)			46.6±6.7	
BACE1	P4 (14)			78.5±16.5	

[a]Kinetic constants of cathepsins S, L and B determined in the present study are presented as means±SD values from four experiments. Data of cathepsin D and BACE1 are from Schechter and Ziv (2008). The k_{cat}/K_m values for the cleavage of P17 and P4 by BACE1 are average of values from two laboratories (Schechter and Ziv, 2008). Cat, cathepsin.
[b]Substrate designation (P17 or P4) and the number of amino acid residues in the substrate (in brackets).
[c]Ratio of k_{cat}/K_m for cleavage of the same WT peptide substrate by human cathepsin (S, L, B or D) compared to human BACE1.

Human cathepsin S rapidly hydrolyzes the P17 (10-mer) and P4 (14-mer) WT peptides with k_{cat}/K_m values of 54 700 and 44 800 M^{-1} s^{-1}, respectively. Human cathepsin B rapidly hydrolyzes the P17 peptide but less rapidly than cathepsin S (k_{cat}/K_m: 20 470 M^{-1} s^{-1}). The human cathepsin L hydrolyzes the P17 peptide significantly slower (k_{cat}/K_m: 3460 M^{-1} s^{-1}) than the human cathepsins S and B (Table 1). Human BACE1 cleaves the P17 and P4 WT peptides very slowly with k_{cat}/K_m values of 46.6 and 78.5 M^{-1} s^{-1}, respectively. Human cathepsin D has comparably poor activity toward these peptides (Schechter and Ziv, 2008). Thus, cathepsins S, L and B cleave the WT peptides much faster than BACE1 and cathepsin D. Cathepsin S cleaves the P17 and P4 peptides 1170- and 570-fold faster than BACE1, respectively. Cathepsins B and L cleave the P17 peptide 440- and 74-fold faster than BACE1, respectively (Table 1).

BACE1 and cathepsin D split the P17 and P4 peptides at the WT β-site (M-D bond, Schechter and Ziv, 2008), whereas cathepsins S, B and L cleave at two bonds flanking the WT β-site (K-MD-A). Cleavage at the major K-M bond (85%) would yield a modified Aβ extended at the N-terminus by one Met residue. Therefore, we studied aminopeptidases to evaluate if they would rapidly remove the N-terminal Met and slowly remove N-terminal Asp, to favor the formation of authentic Aβ initiated with Asp1.

Aminopeptidases cleave N-terminal Met faster than N-terminal Asp

The C+M and P6+M cleavage products have N-terminal MetAsp…, and sequential degradation by aminopeptidase would expose new N-termini: AspAla…, AlaGlu…, GluPhe…, etc. (Figure 1). Aminopeptidase readily cleaves free dipeptides, whereas carboxypeptidase and endopeptidases weakly hydrolyze free dipeptides (Schechter and Berger, 1966b, 1967). Therefore, we used four dipeptides corresponding to the above N-termini (MetAsp, AspAla, AlaGlu, GluAla instead of GluPhe) as substrates for the cytosol and microsomal aminopeptidases.

Cleavage of the dipeptides was measured at two enzyme concentrations (2 μg/ml and 20 μg/ml) after 1 h and 10 h incubation. Hydrolysis of dipeptides by 2 μg/ml enzyme after 1 h and 10 h is shown (Figure 8). Both enzymes rapidly cleave MetAsp: 10–25% after 1 h and approximately 90% after 10 h. The AlaGlu dipeptide is cleaved somewhat slower than MetAsp, and GluAla is cleaved similar to AlaGlu. On the other hand, the AspAla dipeptide is a poor substrate. Hydrolysis of AspAla was not detected after 1 h and 10 h incubation with cytosol aminopeptidase. With microsomal aminopeptidase, the AspAla was slightly hydrolyzed after 1 h (approx. 3%). After 10 h, hydrolysis increased but it was significantly less than that observed with the MetAsp, AlaGlu and GluAla dipeptides (Figure 8).

Table 2 gives the information that can be extracted from Figure 8 (2 μg/ml enzyme) and from enzyme concentration at 20 μg/ml, regarding the effect of the N-terminal residue on susceptibility to hydrolysis by the two aminopeptidases. The Asp residue at the N-terminus is fairly resistant to hydrolysis. The cytosol aminopeptidase cleaves the N-terminal Met, Ala and Glu residues more efficiently: 92-, 35- and 44-fold faster than Asp. The microsomal enzyme cleaves N-terminal Met, Ala and Glu residues 21-, 8.1- and 6.6-fold

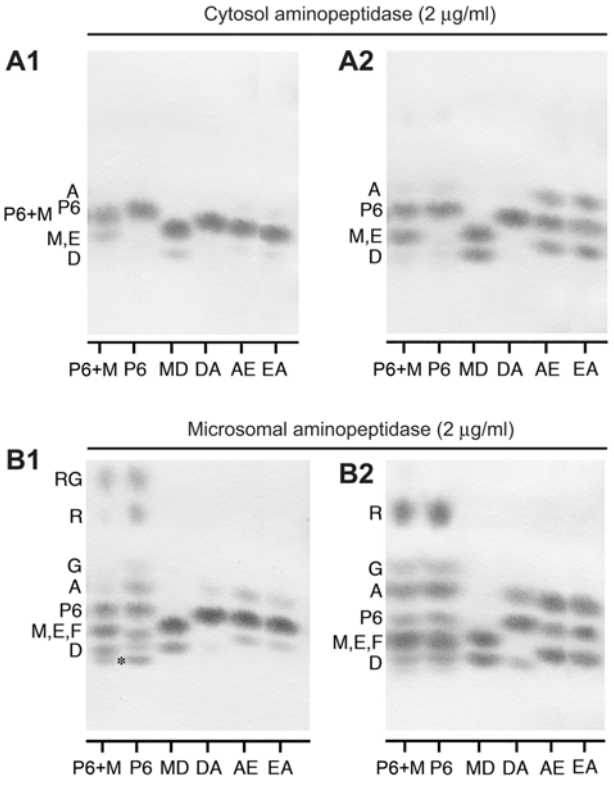

Figure 8 Cytosol and microsomal aminopeptidases cleave N-terminal Met faster than N-terminal Asp.
Cytosol (A1, A2) and microsomal (B1, B2) aminopeptidases (2 μg/ml) were reacted with the P6+M and P6 peptides and with the dipeptides MetAsp (MD), AspAla (DA), AlaGlu (AE) and GluAla (EA) for 1 h (A1, B1) and 10 h (A2, B2). The positions of amino acid markers Arg (R), Gly (G), Ala (A), Met (M), Glu (E), Phe (F) and Asp (D), in addition to ArgGly (RG), P6+M and P6 are shown on the left. Asterisk in B1 indicates a peptide likely formed by endopeptidase activity contaminating the microsomal aminopeptidase. Samples were resolved by paper electrophoresis in 0.5 M formic acid.

Table 2 Aminopeptidases cleave N-terminal Met faster than N-terminal Asp.

Peptide pair	Pep1/Pep2 ratio of rate of cleavage by aminopeptidase[a]	
	Cytosol	Microsomal
P6+M (Met)/P6 (Asp)[b]	40±4	n.d.
MetAsp/AspAla	92±8	21±4
AlaGlu/AspAla	35±5	8.1±1.9
GluAla/AspAla	44±4	6.6±1.4

[a]Pep1/Pep2 ratio of rate of cleavage rate is average of two experiments.
[b]Cleavage of N-terminal Met of P6+M compared to cleavage of N-terminal Asp of P6 by cytosol aminopeptidase. Ratio for cleavage of the same peptides by microsomal aminopeptidase could not be determined (n.d.) due to possible contamination by endopeptidase (see Figure 8 and text).

faster than Asp (Table 2, Figure 8). Both Asp and Glu are negatively charged. However, the N-terminal Glu is susceptible to hydrolysis whereas N-terminal Asp is weakly hydrolyzed (Figure 8, Table 2).

Rapid cleavage of N-terminal Met compared to Asp was also observed with longer peptides derived from the N-terminus of Aβ, that is, the P6+M (7-mer) and P6 (6-mer) peptides. Cytosol aminopeptidase (2 μg/ml) cleave the N-terminal Met of P6+M to yield Met and P6 (approx. 15% after 1 h, 100% after 10 h), and the P6 product is not further hydrolyzed. In agreement, the P6 substrate with N-terminal Asp is not cleaved after 1 h and weakly (approx. 3%) after 10 h (Figure 8). Based on these findings and data of cytosol aminopeptidase at 20 μg/ml (data not shown), we estimate that the N-terminal Met residue of P6+M is cleaved approximately 40-fold faster than the N-terminal Asp residue of P6 (Figure 8, Table 2).

The microsomal aminopeptidase cleaves the P6+M and P6 peptides faster than the cytosol enzyme (Figure 8). Inspection of the cleavage products indicates that this enzyme contains an additional endopeptidase activity that complicates interpretation of the data. After 1 h incubation the P6+M and P6 peptides reveal a spot with slow mobility, which disappears after 10 h (Figure 8B). The same spot is also observed when the 25V fraction of the brain extract was reacted with P6+M and P6 (Figure 9). It is likely that this spot is a peptide generated by endopeptidase activity and it serves as substrate to aminopeptidase. Therefore, at this stage, we did not evaluate the relative rates of N-terminal Met or Asp release from P6+M or P6 by the microsomal enzyme.

It was now of interest to evaluate aminopeptidase activity in the brain. The 25V and S100 fractions of bovine brain extract were chromatographed on G-100 columns and aliquots of the eluate were reacted with the peptides. The results showed reaction patterns similar to those observed with the purified aminopeptidases. The MetAsp dipeptide was degraded. The AlaGlu and GluAla dipeptides were degraded slower or at comparable rate to MetAsp, whereas the AspAla dipeptide was not degraded. The N-terminal Met residue of P6+M was cleaved whereas the N-terminal Asp residue of P6 was not (Figure 9).

The 25V fraction reveals two additional peptides from P6+M and P6: one with slow mobility and one moving rapidly corresponding to mobility of the ArgGly dipeptide (Figure 9). This is probably due to endopeptidase activity. We speculate that it cleaves the F-R bond to yield the MetAspAlaGluPhe pentapeptide from P6+M or the AspAlaGluPhe tetrapeptide from P6 plus the dipeptide ArgGly (Figure 1). The ArgGly dipeptide is then cleaved into arginine and glycine (Figure 8B). The MetAspAlaGluPhe is rapidly cleaved into Met plus AspAlaGluPhe. We propose that the peptide indicated by an asterisk (Figures 8 and 9) is the presumed AspAlaGluPhe tetrapeptide. Indeed, its electrophoretic mobility at 0.5 M formic acid corresponds to the expected mobility for this acidic tetrapeptide (Schechter and Berger, 1966a).

The aminopeptidase activity overlaps and flanks the void volume of the G-100 column (≥150 000 kDa) and it is

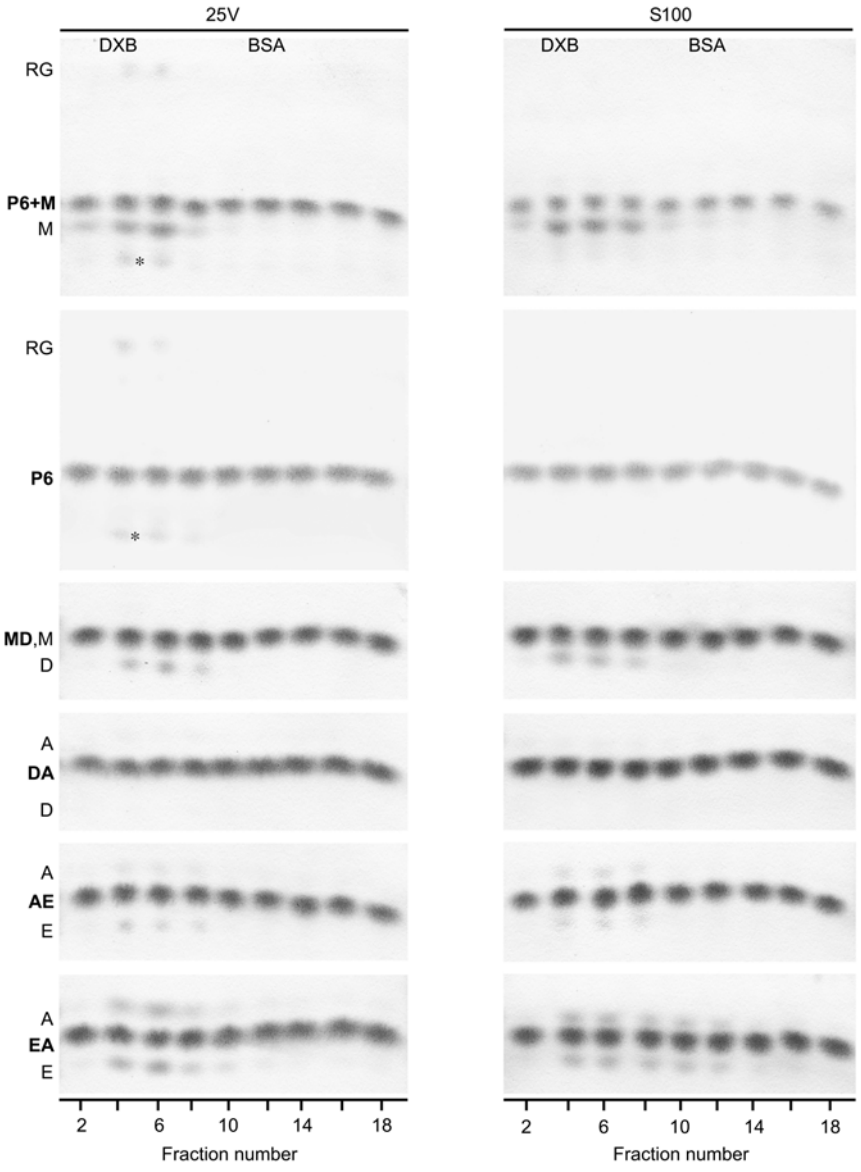

Figure 9 Aminopeptidase activity in the brain cleaves N-terminal Met faster than N-terminal Asp.
The 25V (left column) and S100 (right column) fractions of bovine brain were loaded on a Sephadex G-100 column. Aminopeptidase activity was tested by mixing aliquots of the eluate with the P6+M and P6 peptides and with several dipeptides. Positions of the peptide substrates (bold), of the cleavage products ArgGly (RG) and of amino acids (single letter code) are shown on the left. Asterisk indicates a peptide probably formed by endopeptidase activity of the 25V fraction eluted near the void volume. The positions of two size markers are shown: dextran blue (DXB, indicates the void volume ≥150 kDa) and BSA (67 kDa). Samples were resolved by paper electrophoresis in 0.5 M formic acid.

slower than the BSA marker (67 kDa, Figure 9). This position conforms with the size of aminopeptidases that form large aggregates that range in mass from 100 to 400 kDa (Taylor, 1993). In addition, the active fractions cleave the AlaAla dipeptide into alanine, the AlaAla-NH_2 is split into alanine plus Ala-NH_2, and N-acetyl-AlaAla is not cleaved, as expected for an aminopeptidase (data not shown). Additional aminopeptidase activity was not detected in G-100 fractions down to the position of the 14 kDa lysozyme marker (data not shown).

Cathepsin B is approximately 75-fold more abundant than BACE1 in human brain

Because cathepsins S, B and L cleave the WT β-site sequence, it was of interest to estimate the amount of these enzymes in human brain. Quantitative Western blots were performed by using increasing amounts of human cathepsins as calibration standard (1–20 ng) and increasing amounts of total protein extracts of cortex and hippocampus obtained from human brain (2–8 μg) as described (Schechter and Ziv, 2008).

Antibodies to cathepsin B detect cathepsin B, but not BACE1 or cathepsins S, L and D. Scanning of the blots shows linear dependence of densitometry units to the amount of cathepsin B or brain protein loaded on the gel. From these data we estimate that the amount of cathepsin B (ng enzyme/μg brain protein) is 1.6±0.3 ng/μg in the cortex and 1.4±0.3 ng/μg in the hippocampus of human brain (average values from two experiments). Others reported 0.02 ng BACE1/μg protein in human cortex (Yang et al., 2003). Thus, in human brain cathepsin B is approximately 75-fold (1.5/0.02) more abundant than BACE1.

We failed to detect cathepsin L in brain extracts because antibodies to cathepsin L (three commercial preparations) did not detect the control of purified cathepsin L loaded on the gel. However, immunohistochemistry reveals that cathepsin L is abundant in the neurons of normal human brain and in the neurons and amyloid plaques of Alzheimer's patients, similar to cathepsin B (Cataldo and Nixon, 1990; Cataldo et al., 1991).

Antibodies to cathepsin S specifically detect cathepsin S (1–20 ng) but not BACE1 and not cathepsins B, L and D. However, specific immunoreactive bands were not observed in the brain extracts, likely due to low concentration of the enzyme in normal human brain. It was previously shown that cathepsin S is weakly detected in a small minority of normal human brain, but it is upregulated and expressed in the brain of Alzheimer's patients (Lemere et al., 1995; Munger et al., 1995).

Discussion

Because BACE1 has very low activity toward the WT β-site (k_{cat}/K_m: approx. 50 M^{-1} s^{-1}) we searched for other β-secretase candidates and identified three cathepsins that split the WT β-site sequence better than BACE1. Human cathepsin S cleaves the WT β-site (k_{cat}/K_m: 54 700 M^{-1} s^{-1}) 1170-fold better than BACE1, whereas cathepsin B (k_{cat}/K_m: 20 470 M^{-1} s^{-1}) and cathepsin L (k_{cat}/K_m: 3460 M^{-1} s^{-1}) are 440- and 74-fold better than BACE1, respectively (Table 1). Western blots of brain extracts reveal that cathepsin B is 75-fold more abundant than BACE1. We failed to detect cathepsin L by Western blotting but earlier immunohistochemical studies showed that cathepsin L levels in the brain are similar to that of cathepsin B (Cataldo et al., 1991) and that cathepsins B, L and D are the most abundant lysosomal proteases (Turk et al., 2000). The expression of cathepsin S in normal brain is very low but it is induced in the brain of AD patients (Lemere et al., 1995).

The three cathepsins split the WT sequence at two bonds flanking the WT β-site (K-MD-A) where the K-M bond (85%) is cleaved more frequently than the D-A bond (15%). Cathepsins S, B and L are cysteine proteases of the papain family (Rawlings and Barrett, 1993) in which specificity is determined by a hydrophobic subsite S2, that is, cleavage occurs next to P2 (P2P1-P1′, hyphen indicates the bond cleaved) where P2 is a hydrophobic amino acid residue (Schechter and Berger, 1968; Schechter and Ziv, 2006). In agreement, the two susceptible bonds in the APP WT β-site sequence are preceded by a hydrophobic P2: Val (VK-M) or Met (MD-A). Cleavage of the K-M bond in the WT β-site sequence was previously reported. Cells transfected by cathepsin S cleave at the K-M bond to generate Aβ peptide extended at the N-terminus by one Met residue (Munger et al., 1995). Peptides spanning the WT β-site sequence are cleaved at the K-M bond by cathepsin B (Bohme et al., 2008) and cathepsin L (Sahasrabudhe et al., 1993). Neuroblastoma cells transfected by human WT or SW APP secrete into the culture medium Aβ peptides generated by cleavage at the D-A bond (Wang et al., 1996).

The interest in AD has led to the study of many enzymes as β-secretase candidates (Hussain et al., 1999). To our knowledge, except for BACE1 and recently cathepsin B, these enzymes have not been further investigated. This report deals with properties of cathepsins S, B and L in relation to Aβ-peptide formation that were not known before, these include: kinetic constants, two cleavage points at the WT β-site sequence and the role of aminopeptidases to produce the Aβ-peptide. Thus, it was reported that transfection of cells with cathepsin S increases the secretion of modified Aβ (N-terminal Met) into the culture medium, suggesting that cathepsin S could be relevant to the pathogenesis of AD (Munger et al., 1995). However, kinetic parametrs for cleavage at the WT β-site sequence were not determined, two cleavage points at the WT β-site sequence were not identified, and the need for aminopeptidase to generate the authentic Aβ (N-terminal Asp) was not shown. One research group identified cathepsin B as the β-secretase (Hook et al., 2005) and another group evaluated properties of the enzyme in this context (Bohme et al., 2008). The published data on cathepsin B differ on points of cleavage in the WT β-site sequence, that is, cleavage at the M-D bond (Hook et al., 2008a), mainly at the K-M bond, or at multiple bonds (Bohme et al., 2008). The k_{cat}/K_m values for cleavage of the WT β-site peptide substrates differ extensively, that is, 317 000 M^{-1} s^{-1} (Hook et al., 2008a) or 458 M^{-1} s^{-1} (Bohme et al., 2008). It is likely that the above discrepancies are due to the use of peptide substrates bearing markedly hydrophobic groups (methylcoumarinamide; 5-[(2-amidoethyl) amino] naphthalene-1-sulfonyl acid; 4-(4-dimethylaminophenyl-azo)benzoyl) in close proximity to the β-site sequence, that probably affects the rate of hydrolysis and point of cleavage. We have used the P17 peptide substrate that spans a larger portion of the WT β-site sequence, without added hydrophobic groups. This WT β-site peptide is cleaved by cathepsin B with k_{cat}/K_m of 20 470 M^{-1} s^{-1} and analyses of the cleavage products enabled unambiguous determination of the bonds split, that is, 85% at the K-M bond, 15% at the D-A bond and none at the M-D bond. Cathepsin L was not implicated in AD.

Cleavage at the major K-M bond yields modified Aβ extended at the N-terminus by one Met residue that should be removed to generate the authentic Aβ initiated by Asp1. To address this issue, we examined the activity of cytosol and microsomal aminopeptidases on peptides corresponding

to Aβ extended with N-terminal Met, the regular Aβ with N-terminal Asp and dipeptides corresponding to N-termini of intermediate cleavage products generated by sequential degradation of Aβ by aminopeptidases. It was found that the enzymes rapidly remove the N-terminal Met but not the N-terminal Asp, which is cleaved 21- to 92-fold slower than Met (Figure 8, Table 2). Aminopeptidase activity in the brain reveals similar specificity, that is, N-terminal Met is readily hydrolyzed whereas N-terminal Asp is weakly hydrolyzed (Figure 9). The major cathepsin cleavage product of Aβ extended by N-terminal Met has not been detected in the brain of AD patients (Munger et al., 1995). This is probably due to the specificity of aminopeptidases that rapidly convert the modified Aβ (N-terminal Met) into regular Aβ peptide (N-terminal Asp). Poor susceptibility of Asp to hydrolysis by aminopeptidases would prolong the *in vivo* lifetime of regular Aβ that aggregates to form amyloid plaques.

Brain aminopeptidases were classified to a major fraction (>80%) of Ala-aminopeptidase that cleaves neutral and basic amino acids but not N-terminal acidic residues and a minor fraction of Asp-aminopeptidase that cleaves N-terminal acidic amino acids (Asp, Glu) (Iribar et al., 1995). It was reported that the brain Asp-aminopeptidase is selectively decreased in aged brain (Iribar et al., 1995), a process that might reduce Aβ catabolism, prolong Aβ retention in the aging brain, and thus favor its deposition as plaques (Saido, 1998). Trimming by exopeptidases is a common process in the brain involving neuropeptide hormone maturation (Hook et al., 1994). In other tissues the precursors of several cathepsins (D, B, L, H) are processed by endopeptidases and are then trimmed by aminopeptidases to produce the mature active protease (Erickson, 1989).

Further studies in cells and animals are needed to establish that cathepsins S, B and L are relevant *in vivo* to the formation of the Aβ peptide and to the pathogenesis of AD. However, studies on the *in vivo* activity of these enzymes in relation to AD have been published. Summarized below is information on the colocalization of cathepsins and site of Aβ formation, the effect of cysteine protease inhibitors on the level of Aβ in cells and in AD animal models, and knockout of cathepsin B in APP transgenic mice.

The localization of APP processing is controversial. However, many studies implicate the endosome and lysosome compartment as the major site of Aβ formation. APP contains the re-internalization signal and APP molecules from the cell surface are targeted intracellularly to the endosome and lysosome vesicles in which APP amyloidogenic fragments have been found (Golde et al., 1992; Haass et al., 1992). This acidic compartment contains cathepsins that potentially could process the amyloidogenic fragments to form the Aβ peptide. In the past 20 years it was realized that proteolytic enzymes are key players in a wide range of biological processes in health and disease (Turk et al., 2000; Hooper, 2002). Cathepsins are involved in bone remodeling and antigen presentation (Chapman et al., 1997), tumor growth and metastasis (Mohamed and Sloane, 2006), thyroglobulin processing (Friedrichs et al., 2003) and other processes. The cathepsins are mainly localized in the lysosome and endosome vesicles but they were also found *in vivo* in the extracellular space, on the cell membrane, in the cytosol and inside nuclei (Turk et al., 2000; Tedelind et al., 2010). The cysteine cathepsins B and L and the aspartic cathepsin D are ubiquitous and they are the most abundant lysosomal proteases (Turk et al., 2000). Cathepsins S and K exhibit restricted and regulated tissue expression (Chapman et al., 1997). The presenilin complex was initially found in the endoplasmic reticulum and Golgi. However, it was later shown that Presenilin-1, Nicastrin, APP and γ-secretase activity colocalize in the lysosomal membrane (Pasternak et al., 2003).

High levels of cathepsins B and L were found in neurons and amyloid plaques in the brain of AD patients (Cataldo et al., 1991). Studies on brains from early stages of Down syndrome and of AD patients reveal the appearance of activated or enlarged endosomes containing soluble Aβ prior to amyloid deposition (Cataldo et al., 2004). Cathepsin S is weakly detected in normal human brain, but in AD brain the enzyme is readily detected (Lemere et al., 1995).

Knockout of the cathepsin B gene reduces the levels of Aβ and of the C-terminal β-secretase fragment (CTFβ) in the brain of mice expressing the human WT APP (Hook et al., 2009). The cysteine protease inhibitor E64d and the related inhibitor CAO74Me (preferentially inhibits cathepsin B) infused into the brain of a mouse AD model reduced amyloid plaques, decreased Aβ and CTFβ levels in the brain and improved memory deficit of the animals (Hook et al., 2008a). Transfection of human kidney cells by cathepsin S increased the secretion of Aβ and E-64d reduced Aβ secretion by the cathepsin S transfected cells (Munger et al., 1995).

Because the specificity of cathepsins S, B and L is determined mainly by a hydrophobic S2, peptide with penultimate hydrophobic P2 are inhibitors due to the interaction with the subsite S2 (Schechter and Berger, 1968; Schechter, 2005). It was reported that N-blocked di- and tri- peptide aldehydes with penultimate hydrophobic P2 inhibit the secretion of Aβ from cells transfected with APP in a dose dependent manner (Sinha and Lieberburg, 1999). The tripeptide aldehyde Z.ValLeuLeu-CHO inhibited the secretion of Aβ and analysis of the cellular intermediates of APP degradation products indicated inhibition of the β-secretase (Abbenante et al., 2000). Infusion of Ac.LeuValLys-CHO into the brain of guinea pigs decreased Aβ in the brain (Hook et al., 2007).

In summary, cathepsins S, B and L with aminopeptidases display β-secretase activity. These cathepsins cleave the WT β-site sequence much better than BACE1; cathepsins B and L are more abundant than BACE1; the expression of cathepsin S is induced in the brain of AD patients; these cathepsins are localized in the endosome and lyzosome system where Aβ formation likely occurs; cysteine protease inhibitors can reduce the production of Aβ in cells and animals, in addition to improving memory deficit of the AD animal model. Taken together, these findings indicate that cathepsins S, B and L are promising therapeutic targets to develop inhibitors serving as disease modifying drugs that would slow down, prevent or reverse the progress of Alzheimer's disease.

This paper supports and extends earlier reports suggesting that aminopeptidases and cathepsin S might play a role in the pathogenesis of AD, that cathepsin B can function as β-secretase and it does not imply that BACE1 is not involved in the processing of APP *in vivo*. Processing of the WT APP present in the worldwide population could be due to the joint action of BACE1, cathepsins S, B and L, and aminopeptidases. Further research would clarify the relative contribution of each enzyme at early and late stages of development of the disease.

Many studies demonstrate that the presenilin complex is associated with γ-secretase activity. However, the role of the presenilin complex in APP cleavage is not conclusive: direct action as protease or indirect action as regulator of γ-secretase. Therefore, we searched and identified additional γ-secretase candidates. Early studies show that the new enzymes cleave the γ, ζ and ε bonds in CTFβ probably faster than the presenilin complex. They are susceptible to inhibition and modulation by a subset of non-steroidal anti-inflammatory drugs more than the presenilin complex and they are not associated with Notch. We suggest that enzymatic cleavage of CTFβ is initiated at the ε-site located close to the membrane cytosol interface, followed by sequential exo/endo peptidase activity through the ζ-site until the γ-site, to form the neurotoxic Aβ-peptide.

Materials and methods

Purification of a putative β-secretase from bovine kidney

Bovine kidney (2 h after sacrifice) was homogenized in 0.25 M sucrose, 1 mM DTT and buffer (SDB) (1 g tissue per 3 ml SDB solution). Buffer solution was 20 mM Tris HCl pH 7.3 or 20 mM Na-acetate pH 5.2. The homogenate was spun (3000 *g*, 10 min) to sediment nuclei and unbroken tissue. The supernatant was spun (25 000 *g*, 30 min). The pellet was suspended in SDB to yield the 25V fraction (vesicles sedimented at 25 000 *g*) enriched with endosome and lysosome (Schechter and Ziv, 2008). The supernatant was spun again (100 000 *g*, 1 h) to yield the S100 fraction. The 25V and S100 fractions were kept in liquid nitrogen until used. Triton X-100 (1.5 mg detergent/1 mg protein) was added to the 25V fraction in order to disrupt the 25V vesicles. After 20 min on ice the solution was spun (8000 *g*, 10 min). The clear supernatant was loaded on a Sephadex G-100 column (40 mM Na-acetate pH 5.2, 1 mM DTT or 40 mM Tris HCl pH 7.3, 1 mM DTT). Aliquots of the eluate were analyzed for β-secretase activity by paper electrophoresis (see below) and proteins by SDS-PAGE. Active fractions were pooled, concentrated by vivaspin concentrator (Sartorius, Binbrook Hill, UK) and loaded on CM52 cation exchanger equilibrated with 40 mM Na-acetate pH 5.4, 1 mM DTT, 0.05% Triton X-100. The β-secretase activity was found in the effluent that was collected, concentrated and further purified by electrophoresis in neutral (pH 7.2) and acidic (pH 5.4) polyacrylamide gels (Maizel, 1971). These gels provide resolution based on the distinct pI of the protein, causing different electrophoretic mobility at different pH values. Furthermore, β-secretase activity was recovered from the gels in good yields (≥75% of activity loaded on the gel). Active fractions from the acidic gel were resolved on 8–14% polyacrylamide gradient SDS-PAGE as described (Schechter and Ziv, 2008). The gel was stained with Coomassie Blue, protein bands were excised and digested by trypsin. Tryptic peptides were separated by reverse phase HPLC (Model SP8800, Spectra Physics, San Jose, CA, USA). Eluted peptides were directed online into a nanospray injector and peptides were characterized by Q-STAR TOF tandem mass spectrometer (MDS-Sciex, Foster City, CA, USA).

Peptide substrates and cleavage products

Two WT peptide substrates were used to assay β-secretase activity: SEVKMDAEFR (P17, 10-mer) and EEISEVKMDAEFRG (P4, 14-mer). Peptides produced by enzymatic cleavage of the WT P17 and P4 substrates at the β-site (M-D bond) and at flanking sites (K-M and D-A bonds) are shown in Figure 1. The WT P17 and P4 peptide substrates were prepared in the department of Organic Chemistry (Schechter and Ziv, 2008). All other peptides (cleavage products) were synthesized by GL Biochem (Shanghai, China). The dipeptides MetAsp, AspAla, AlaGlu and GluAla were purchased from Bachem (Bubenorf, Switzerland).

β-Secretase activity at different purification stages

This was assayed in 10 μl reaction mixture containing: 2–5 μl of test sample, 2 mM peptide substrate, 25 mM Na-acetate buffer pH 5.2, 10 mM mercaptoethanol. After incubation (4–6 h at 37°C) samples (3–6 μl) were applied on a Whatman number three paper and components of the reaction mixture were resolved by paper electrophoresis run in the pH 6 buffer (2.9 mM citric acid/140 mM Na_2HPO_4) or in 0.5 M formic acid, and peptide spots were developed by ninhydrin, essentially as described (Schechter and Berger, 1966a). Enzyme digests of all peptides were analyzed by reverse phase HPLC performed with a RP18 5 μ column (Merck, Darmstadt, Germany) developed with acetonitrile gradient in 0.1% trifluoroacetic acid (TFA) as indicated in the Figure legends. Elution was monitored at 220 nm. Peptides in HPLC peaks were characterized by paper electrophoresis compared to synthetic peptide markers and by MALDI-TOF mass spectrometry (reflex 3; Bruker, Bremen, Germany).

Determination of the kinetic constants k_{cat}, K_m and k_{cat}/K_m

Human cathepsin S (recombinant, 24.5 kDa), cathepsin L (liver, 29 kDa) and cathepsin B (liver, 27.5 kDa) in addition to bovine cathepsin S (spleen, 24 kDa), cathepsin L (kidney, 31 kDa) and cathepsin B (spleen, 28 kDa) were purchased from Calbiochem (Darmstadt, Germany). Enzyme concentration and molecular mass were based on values provided by the manufacturer. Cathepsin activity was determined in a reaction mixture (20–40 μl) containing 1.8 mM DTT, 1.8 mM EDTA and 50 μg/ml BSA at pH 6 for cathepsin S (12 mM citric acid/43 mM Na_2HPO_4), at pH 5.5 for cathepsin L (14 mM citric acid/39 mM Na_2HPO_4) and at pH 4.5 for cathepsin B (18 mM citric acid/31 mM Na_2HPO_4). Duplicate aliquots (6–15 μl) were removed from the reaction mixture after 6–20 min incubation and from substrate hydrolyzed to completion (excess enzyme and prolonged incubation). Components of the reaction mixture were resolved by paper electrophoresis in the pH 6 buffer. The paper sheet was developed by ninhydrin (Figure 2), the colored spots were cut out, dipped in elution solution, centrifuged and the color of the clear supernatant was quantified by measuring absorbance at 570 nm (Schechter and Berger, 1966a). Extent of substrate hydrolysis was determined from the color of cleavage products in the test sample and the color of the same cleavage products obtained

from substrate hydrolyzed to completion, as described (Schechter and Ziv, 2008). Kinetic constants of cathepsins S and L were derived from Lineweaver-Burk plots of P17 and P4 degradation at nine different concentrations (0.52–2.4 mM). For cathepsin B only the k_{cat}/K_m constant was determined (Results section).

Aminopeptidase activity assays

Cytosol leucine aminopeptidase (L5658, Sigma, St. Louis, MO, USA) and microsomal leucine aminopeptidase (L5006, Sigma) were used. Enzyme concentration was based on values provided by the manufacturer. The cytosol aminopeptidase reaction mixture contained enzyme, peptide (3 mM), 50 mM Tris HCl pH 8, 5 mM $MgSO_4$ and 100 μg/ml BSA. The microsomal aminopeptidase reaction mixture contained enzyme, peptide (3 mM), 25 mM Tris HCl pH 7.2, 2.5 mM $MgSO_4$ and 100 μg/ml BSA. The 25V and S100 fractions of bovine brain were chromatographed on a G100 column equilibrated with 40 mM Tris HCl pH 7.3 and 1 mM DTT. Aliquots from the effluent were reacted with peptide (3 mM) in presence of 5 mM $MgSO_4$. Reaction mixtures were resolved by paper electrophoresis in 0.5 M formic acid.

Western blotting

Total protein extracts of human brain cerebral cortex and hippocampus (Clontech, Palo Alto, CA, USA), goat antibodies to the enzymes (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and horse radish peroxidase anti-goat antibodies (Jackson Immune Research Laboratories, West Grove, PA, USA) were purchased from commercial sources. Protein extracts and enzymes were resolved by SDS-PAGE, electroblotted onto PVDF membranes and the relevant enzymes were immunodetected as described (Schechter and Ziv, 2008).

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