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# Isolation and characterization of mammalian D-aspartyl endopeptidase

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**Summary.** The accumulation of D-isomers of aspartic acid (D-Asp) in proteins during aging has been implicated in the pathogenesis of Alzheimer's disease (AD), cataracts and arteriosclerosis. Here, we identified a specific lactacystin-sensitive endopeptidase that cleaves the D-Asp-containing protein and named it D-aspartyl endopeptidase (DAEP). DAEP has a multi-complex structure (MW: 600 kDa) and is localized in the inner mitochondrial membrane. However, DAEP activity was not detected in *E. coli*, *S. cerevisiae*, and *C. elegans*. A specific inhibitor for DAEP, i-DAEP: (benzoyl-L-Arg-L-His-[D-Asp]-$CH_2Cl$; MW: 563.01), was newly synthesized and inhibited DAEP activity ($IC_{50}$, 3 μM), a factor of ten greater than lactacystin on DAEP. On the other hand, i-DAEP did not inhibit either the 20S or 26S proteasome. And we identified succinate dehydrogenase and glutamate dehydrogenase 1 as components of DAEP by affinity label using biotinylated i-DAEP. In the long life span of mammals, DAEP may serve as a scavenger against accumulation of racemized proteins in aging. Insights into DAEP will provide the foundation for developing treatments of diseases, such as AD, in which accumulation of D-Asp-containing proteins are implicated.

**Keywords:** D-Aspartate – Protease – Mitochondria – Alzheimer's disease – Amyloid

## Introduction

Recent reports indicate that L-aspartyl (Asp) or L-asparaginyl (Asn) residues in many proteins isomerize, deamidate or racemize by spontaneous non-enzymatic reactions to produce isoaspartate (isoAsp) or D-Asp residues (Geiger and Clarke, 1987; Muraoka et al., 1987). These isoAsp-containing proteins are reverted to their original form and function by protein L-isoaspartyl methyltransferase (PIMT, McFadden and Clarke, 1982; Aswad, 1984). However, D-Asp-containing protein accumulates and may cause the following diseases: amyloid β protein (Aβ) and tau protein in Alzheimer's disease (AD, Shapira et al., 1988; Tomiyama et al., 1994; Iwatsubo et al., 1996; Kenessey et al., 1995), αA-crystallin in cataracts (Fujii et al., 1994), elastin in arteriosclerosis and lung diseases (Powell et al., 1992; Shapiro et al., 1991), myelin basic protein in multiple sclerosis (Fisher et al., 1986), collagen in metabolic bone diseases (Paget's disease, osteoporosis, etc, Cloos and Fledelius, 2000), and in prion disease (Weber et al., 1998). D-Asp containing Aβ (D-Aβ) synthesized in vitro forms an abnormally folded β-sheet and fibril, and is cytotoxic (Tomiyama et al., 1994; Mori et al., 1994; Shimizu et al., 2000). Racemization is known to be caused in part by reactive oxygen species (ROS), which are abundant in mitochondria. Since the amount of D-Asp-containing protein in a cell is very small, and free D-Asp has been detected in mammalian tissues, such as brain and particularly AD brain, at high concentrations that increase with age (Man et al., 1983; Fisher et al., 1991), we hypothesized that an endopeptidase hydrolyzing D-Asp-containing proteins would be present, though reports on the hydrolysis of D-Asp containing proteins in mammalian systems are limited (Watanabe et al., 1996; Lee et al., 2002). Therefore, we sought to identify an unknown proteolytic system specific for the D-Asp-containing protein, namely D-aspartyl endopeptidase (DAEP), which would control levels of the noxious racemized proteins present. Characterization of DAEP and its anti-aging function would prove useful in the treatment and prevention of diseases related to the D-Asp-containing protein. We therefore searched for DAEP in rabbit and mouse tissue and in *E. coli*, *S. cerevisiae*, and *C. elegans*.

## Materials and methods

### *Materials*

The following species (strains) were used in this study: rabbit (JW), mouse (DDY), *C. elegans* (N2), *S. cerevisiae* (TM101), and *E. coli* (OP50, XL2-Blue, and BL21(DE3) pLysS). Peptidyl MCAs except Suc-[D-Asp]-MCA were purchased from Peptide Institute, Inc. (Osaka, Japan). Other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO), Nakarai Tesque, Inc. (Kyoto, Japan), and Kanto Chemical Co. Inc. (Tokyo, Japan).

### *Proteolytic assay*

The DAEP activities were determined with the synthesized substrate succinyl-D-aspartic acid α-(4-methyl-coumaryl-7-amides) (Suc-[D-Asp]-MCA). Suc-[D-Asp]-MCA is hydrolyzed and the fluorescence of liberated aminomethylcoumarin is measured at $l_{ex} = 380$ and $l_{em} = 460$ nm. This substrate (1 mM in dimethyl sulfoxide, DMSO) was added to the assay buffer (10 mM Tris–HCl (pH 8.5), 200 mM NaCl, 3 mM $MnCl_2$) with the enzyme at a final concentration of 100 μM, and the assay mixture was incubated at 37 °C. Fluorescence was measured every two minutes with a Wallac ARVOsx multilabel counter (PerkinElmer Life & Analytical Sciences, Boston, MA). Other peptidyl MCAs shown in Table 2 were used to determine the substrate specificity of DAEP. In searching for an inhibitor of DAEP, various components were added to the assay buffer at appropriate concentrations, and the activity was measured in the same condition as Suc-[D-Asp]-MCA. The protein concentration of various samples was determined with a DC protein assay kit (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard. Purification and proteolytic assays for mouse 26S and yeast 20S were performed according to the methods described in (Seeger et al., 1996; Kanayama et al., 1992).

### *Distribution of DAEP activity in rabbit tissues*

All subsequent operations were performed at 4 °C. Various rabbit tissues (∼1 g) were homogenized in a Potter-Elvehjem homogenizer (Asahi Techno Glass, Tokyo, Japan) in homogenization buffer (20 mM Tris–HCl (pH 8.5), 250 mM sucrose, 0.5 mM EDTA) and were centrifuged at $100 \times g$ for 10 min to remove debris. These supernatants were used in the DAEP assay.

### *Subcellular fractionation of rabbit liver*

Rabbit liver was homogenized in a Potter-Elvehjem homogenizer in homogenization buffer (10 volumes) and centrifuged at $100 \times g$ for 5 min. The supernatant was centrifuged, first at $600 \times g$ for 10 min (precipitate = nuclei), then at $9000 \times g$ for 10 min (precipitate = mitochondria), and finally at $100{,}000 \times g$ for 60 min (precipitate = microsomes). The supernatant was designated as cytosol.

### *Subfractionation of rabbit liver mitochondria*

The mitochondrial fraction of rabbit liver was suspended in 1.5 ml of homogenization buffer and was disrupted by French press (model 5615, Ohtake, Tokyo, Japan). The homogenate (1 ml) was layered on top of a centrifugal tube (SW 28, Beckman, Palo Alto, CA) filled with a stepwise gradient in increments of 5 ml of 20, 25, 30, 35, 40, 50 and 60% sucrose in buffer (20 mM Tris–HCl (pH 8.5), 1 mM EDTA) and was centrifuged for $100{,}000 \times g$ for 18 h. After centrifugation, the layer was collected in 1 ml fractions and applied to the DAEP assay and Amplex™ Red monoamine oxidase assay kit (Molecular Probe, Inc., Eugene, OR).

### *Purification of DAEP from mouse liver*

All subsequent operations were carried out at 4 °C and column works in the purification step were performed by ÄKTAFPLC™ system (Amersham Biosciences, Piscataway, NJ). The mitochondrial fraction was separated from mouse liver (∼100 g) and suspended in osmotic shock buffer (20 mM Tris–HCl (pH 8.5), 1 mM EDTA, 1 mM dithiothreitol (DTT), 14 μM E-64, 21 μM leupeptin, 15 μM pepstatin A, 36 μM bestatin, 0.8 μM aprotinin, and 1.4 mM AEBSF) for 60 min. After osmotic shock treatment, the fraction was mixed with an equal volume of 1 M LiCl solution, sonicated and centrifuged at $100{,}000 \times g$ for 60 min. The precipitate (= mitochondrial membrane fraction) was suspended in extracting buffer (20 mM Tris–HCl (pH 8.5), 1 mM EDTA, 1% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate), 20% glycerol, 0.5 M $NaSO_4$, 1 mM DTT, 14 μM E-64, 21 μM leupeptin, 15 μM pepstatin A, 36 μM bestatin, 0.8 μM aprotinin, and 1.4 mM AEBSF) and stirred on a tube rotator for 12 h. To extract DAEP from the mitochondrial membrane, CHAPS was included in all buffers in the following purification steps. The CHAPS extract after centrifugation at $100{,}000 \times g$ for 60 min was loaded onto a centrifugal filter device: MacroSep® 100 (molecular cut-off limit: 100 kDa, Pall, East Hills, NY) to concentrate the extract and to remove small molecules included in the extract. The concentrated extract was diluted by a factor of 50 in buffer A (20 mM Tris–HCl (pH 8.5), 1 mM EDTA, 0.25% CHAPS, 20% glycerol, 1 mM DTT, 14 μM E-64, 21 μM leupeptin, 15 μM pepstatin A, 36 μM bestatin, 0.8 μM aprotinin, and 1.4 mM AEBSF), loaded onto a DEAE Affi-Gel® blue column (Econo-Pac® DEAE Blue Cartridge 5 ml × 2, Bio-Rad, Richmond, CA) and finally eluted with a linear gradient of 0–0.6 M NaCl in buffer A. The active fractions detected by the DAEP assay were collected and diluted by a factor of three in buffer A, and then loaded onto RESOURCE™ Q column (6 ml, Amersham Biosciences, Piscataway, NJ). In this column chromatography step, the fractions were eluted with a linear gradient of 0–0.6 M NaCl in buffer A. The active fractions were dialyzed in to 10 mM potassium phosphate in buffer B (1 mM EDTA, 0.25% CHAPS, 20% glycerol, 1 mM DTT, pH 8.0) and placed on a column of hydroxyapatite: Bio-Scale CHT2-I (Bio-Rad, Richmond, CA), and bounded protein was subsequently eluted with a linear gradient of 10–600 mM potassium phosphate in buffer B. Active fractions were collected and concentrated by MacroSep® 100, and then loaded onto a gel filtration column: Superose® 6 HR 10/30 (Amersham Biosciences, Piscataway, NJ) and finally eluted with buffer C (20 mM Tris–HCl (pH 8.5), 1 mM EDTA, 150 mM NaCl, 0.25% CHAPS, 20% glycerol, 1 mM DTT). Using these purification steps, DAEP was finally purified by a factor of 271 compared to the original mitochondrial fraction, and the purified sample was used in the following experiment.

### *Analysis of pH dependence of rabbit DAEP*

The pH dependence of enzyme activity of the purified rabbit DAEP (2 μg) was examined using 10 mM Tris–acetate buffer in the pH range of 5.0–9.0, and 10 mM glycine–NaOH buffer in the range of 9.0 and 11.0 at 37 °C.

### *Analysis of the synthesizd Aβ peptides hydrolyzed by rabbit DAEP*

2 mg/ml of the synthesized Aβ peptides consisting of 10 amino acid residues, D-$A\beta^{1-10}$ ([D-Asp]AEFRH[D-Asp]SGY) or L-$A\beta^{1-10}$ ([L-Asp]-AEFRH[L-Asp]SGY), in DMSO (1 μl) were mixed with the DAEP assay buffer including 2 μg of purified rabbit DAEP (10 μl) and incubated for 21 h at 37 °C. After the reaction was terminated by heating to 100 °C, the reaction mixtures were independently applied to a high-performance liquid chromatography (HPLC) reverse phase column (RP) (TSKgel ODS-80$T_M$; TOSOH, Tokyo, Japan), and eluted with a linear gradient of 0–90% acetonitrile in 0.1% trifluoroacetic acid. Amino acid sequences of the five peaks indicated by arrows in Fig. 2 were determined with a model 477A-120A amino acid sequencer (Applied Biosystems, Foster City, CA).

### *Identification of the DAEP component by MALDI-TOF MS*

Purified mouse DAEP (10 μg) was incubated with 100 μM i-DAEP or DMSO (final concentration: 1%) for 1 h at room temperature, and 0.1,

1, 10, 100 μM synthesized biotinylated i-DAEP (biotinyl-(ε-aminocaproic acid)-L-Arg-L-His-[D-Asp]-$CH_2Cl$ (MW: 798.35)) was subsequently added and incubated for 1 h at room temperature. After confirmation of the inactivation of DAEP in the reaction mixtures (35% activity remained at 10 μM), samples were then subjected to SDS-PAGE, and the gels (10%) were electroblotted onto polyvinylidene difluoride (PVDF) membranes (Immobilon™-P, Millipore, Bedford, MA). Western blots were probed with streptavidin-horseradish peroxidase conjugate (Amersham Biosciences, Co., Piscataway, NJ) used at a 1:1000 dilution and were detected by chemiluminescence (ECL™, Amersham Biosciences, Piscataway, NJ). The detected protein bands on PVDF membrane were sliced to a minimum, and were digested on the membrane by sequencing grade modified trypsin (Promega, Madison, WI), followed by peptide mass fingerprinting (PMF) and post-source decay (PSD) incorporated with matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS, Voyager DE-STR, Applied Biosystems, Foster City, CA, Iwamatsu and Yoshida-Kubomura, 1996). To identify other DAEP components, the affinity-labeled DAEP sample with biotinylated i-DAEP was centrifuged and concentrated by streptavidin Sepharose® beads (Amersham Biosciences, Piscataway, NJ), and analyzed as described above.

## Results

We developed an assay system for DAEP activity using the synthetic D-Asp containing peptide, Suc-[D-Asp]-MCA as a substrate (Ishiura et al., 1997). Liver was shown to have the highest DAEP activity among rabbit tissues (Fig. 1A), and DAEP activity was not detected in *E. coli*, *S. cerevisiae*, and *C. elegans* (Kinouchi et al., 2004). The mitochondrial fraction exhibited the highest specific activity of DAEP (Fig. 1B), and thus was used as the starting material. Subfractionation of the mitochondria by sucrose density gradient centrifugation indicated that

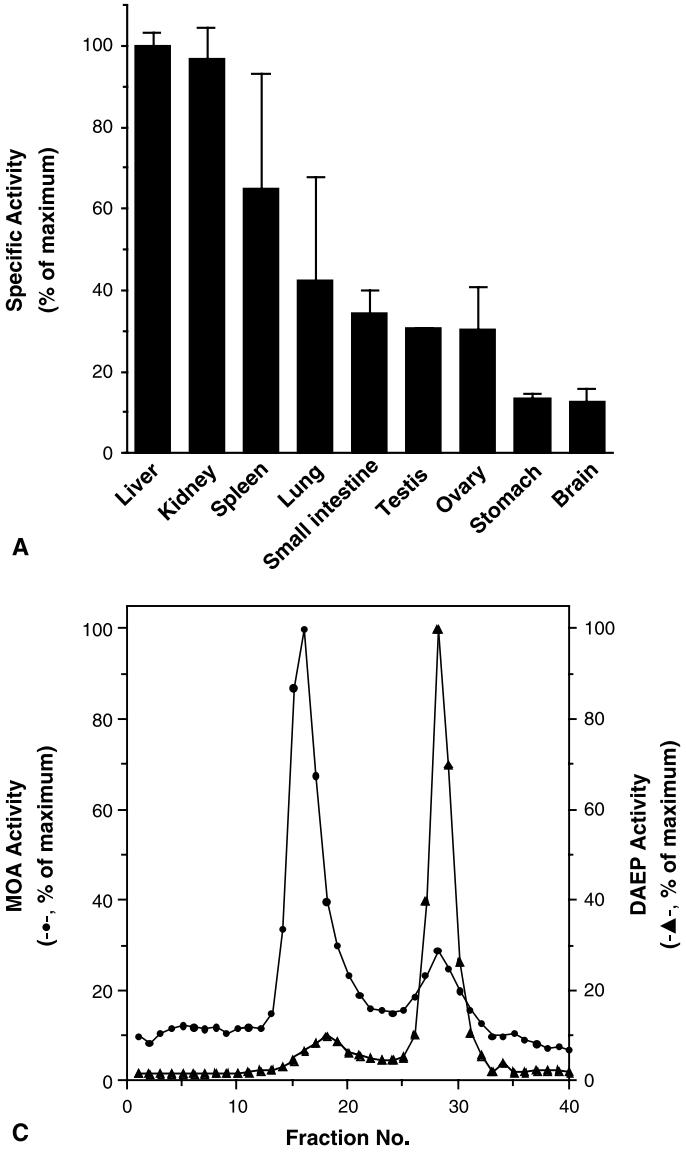


**Fig. 1.** Distribution and localization of DAEP. **A** DAEP activity in various rabbit tissues. Y-axis indicates specific activity of DAEP (% of maximum). $n = 4$ (one male and three females). **B** Subcellular localization of DAEP in rabbit liver. X and Y-axes indicate protein contents (% of total protein) and specific activity of DAEP (% of maximum) in each fraction of the organelle, respectively. **C** Localization of DAEP in mitochondria. Right and left Y-axes indicate DAEP (–▲–) and MAO (monoamine oxidase, –●–) activities in each fraction, respectively. Fraction number indicated on X-axis scale. Sucrose density in each fraction increases in a stepwise gradient every 5 ml from 20, 25, 30, 35, 40, 50 and 60%

**Table 1.** Summary of purification of DAEP

| Step | Total activity (Units) | Protein (mg) | Specific activity (Units/mg) | Purification factor | Yield (%) |
|---|---|---|---|---|---|
| Mitochondrial fraction | 4800 | 5850 | 0.821 | 1 | 100 |
| CHAPS extract | 1840 | 885 | 2.08 | 2.53 | 38.3 |
| MacroSep® (100 K) | 1280 | 258 | 4.96 | 6.05 | 26.7 |
| DEAE Affi-Gel® Blue | 790 | 44.5 | 17.8 | 21.6 | 16.5 |
| RESOURCE™ Q | 371 | 8.45 | 43.9 | 53.5 | 7.73 |
| Bio-Scale CHT2-I | 132 | 1.44 | 91.7 | 112 | 2.75 |
| Superose® 6 HR 10/30 | 98 | 0.44 | 223 | 271 | 2.04 |

**Table 2.** Substrate specificity of DAEP

| Substrates | Proteinase/Peptidase | Relative activity (%) |
|---|---|---|
| Suc-[D-Asp]-MCA | DAEP | 100 |
| Ac-Tyr-Val-Ala-Asp-MCA | Caspase-1 | 1.42 |
| Ac-Asp-Glu-Val-Asp-MCA | Caspase-3/7/8 | 0.11 |
| Suc-Gly-Pro-Leu-Gly-Pro-MCA | Collagenase-like peptidase, Prolyl endopeptidase | 0.03 |
| Z-Arg-Arg-MCA | Cathepsin B | <0.01 |
| Z-Phe-Arg-MCA | Cathepsin B/L, Plasma kalikrein, Arg-gingipain | <0.01 |
| Leu-MCA | Aminopeptidase | 0.52 |
| Suc-Leu-Leu-Val-Tyr-MCA | Proteasome, Chymotrypsin, Calpain | 0.02 |
| Z-Leu-Leu-Glu-MCA | Proteasome | <0.01 |
| Ala-Ala-Phe-MCA | Tripeptidyl peptidase II | <0.01 |
| Suc-Ala-[D-Ser]-MCA | | <0.01 |
| Suc-[D-Ser]-MCA | | 0.45 |

Structures of synthetic peptidyl MCAs and specific proteinase/peptidase degrading them22 are indicated. DAEP activity for the substrates is evaluated with regard to 100% of Suc-[D-Asp]-MCA hydrolyzed by DAEP. *Suc* Succinyl, *Ac* Acetyl, *Z* Benzyloxycarbonyl

DAEP was bound to the inner mitochondrial membrane (Fig. 1C). Therefore, DAEP was purified from the mitochondrial fraction of mouse liver by the addition of the detergent, CHAPS, and was purified by a factor of 271 (Table 1). The molecular weight of the purified mouse DAEP was determined by gel filtration to be 600 kDa. The properties of purified rabbit DAEP were as follows: $K_m$ was 0.12 mM, optimal pH was 8.5 (at 37 °C), and the specific activity was increased by a factor of two following addition of 3 mM divalent cations ($Mg^{2+}$, $Ca^{2+}$, $Sr^{2+}$, $Ba^{2+}$, $Mn^{2+}$), but was strongly inhibited by $Zn^{2+}$. In contrast to proteasomes, which are activated with 0.03% sodium dodecyl sulfate (SDS), DAEP was inhibited by treatment with 0.01% SDS. The ATP-dependent increase in DAEP activity was not detectable. DAEP differed from well-characterized peptidases in its substrate specificity (Table 2). Although D-Ser-containing protein also accumulates with age in human tissue, DAEP could not hydrolyze D-Ser-containing peptide. To examine whether DAEP can degrade the long D-Asp-containing peptide, we used peptides with 10 amino acid residues derived from $A\beta^{1-10}$. Following incubation of D-$A\beta^{1-10}$ peptide ([D-Asp]AEFRH[D-Asp]SGY) with purified DAEP, the peptide was degraded at D-$Asp^7$ and two peptides were produced: [D-Asp]AEFRHX and SGY (Fig. 2A). X was not identified in this study. On the other hand, L-$A\beta^{1-10}$ peptide could not be degraded by DAEP (Fig. 2B). These results indicate that DAEP recognizes and degrades specifically the internal D-Asp residue. Only lactacystin, a specific inhibitor of proteasomes (Omura et al., 1991), effectively inhibited DAEP activity ($IC_{50}$, 150 μM), but the other proteasome inhibitors, epoxomicin and ALLN (*N*-acetyl-L-leucyl-L-leucyl-norleucinal), did not inhibit DAEP activity (Table 3). In order to clarify the enzymatic properties of DAEP and the difference between DAEP and proteasomes, the specific inhibitor, i-DAEP (benzoyl-L-Arg-L-His-[D-Asp]-$CH_2Cl$; MW: 563.01), was newly designed to covalently bind to the active site of DAEP. It demonstrated a high inhibitory activity; $IC_{50}$ was 3 μM, a factor of ten greater than lactacystin on DAEP (Table 3). On the other hand, i-DAEP did not inhibit either the 20S or 26S proteasome. To identify the active site residues of

DAEP, purified mouse DAEP was affinity-labeled with biotinylated i-DAEP: biotinyl-(ε-aminocaproic acid)-L-Arg-L-His-[D-Asp]-$CH_2Cl$ (MW: 798.35, Fig. 3). The biotinylated i-DAEP inhibited DAEP with an inhibitory activity identical to that of free i-DAEP. Western blotting analysis with a streptavidin-peroxidase probe revealed protein bands, with a relative mobility of 30, 45, 55, 60, and 75 kDa, as indicated in Fig. 3. These bands were analyzed by peptide mass fingerprinting (PMF) and post-source decay (PSD) incorporated with matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS). Individual proteins were attributed to the bands as follows: 30 kDa was succinate dehydrogenase subunit B (iron sulfur (Ip)), 55 kDa was glutamate dehydrogenase (GDH1, EC 1.4.1.3), 60 kDa was succinate dehydrogenase subunit A (flavoprotein (Fp)), and 75 kDa was glucose regulated protein 75 (GRP 75), and their mobility was consistent with their theoretical molecular weight. The 45-kDa protein was derived from an ATPase $F_1$ β subunit.

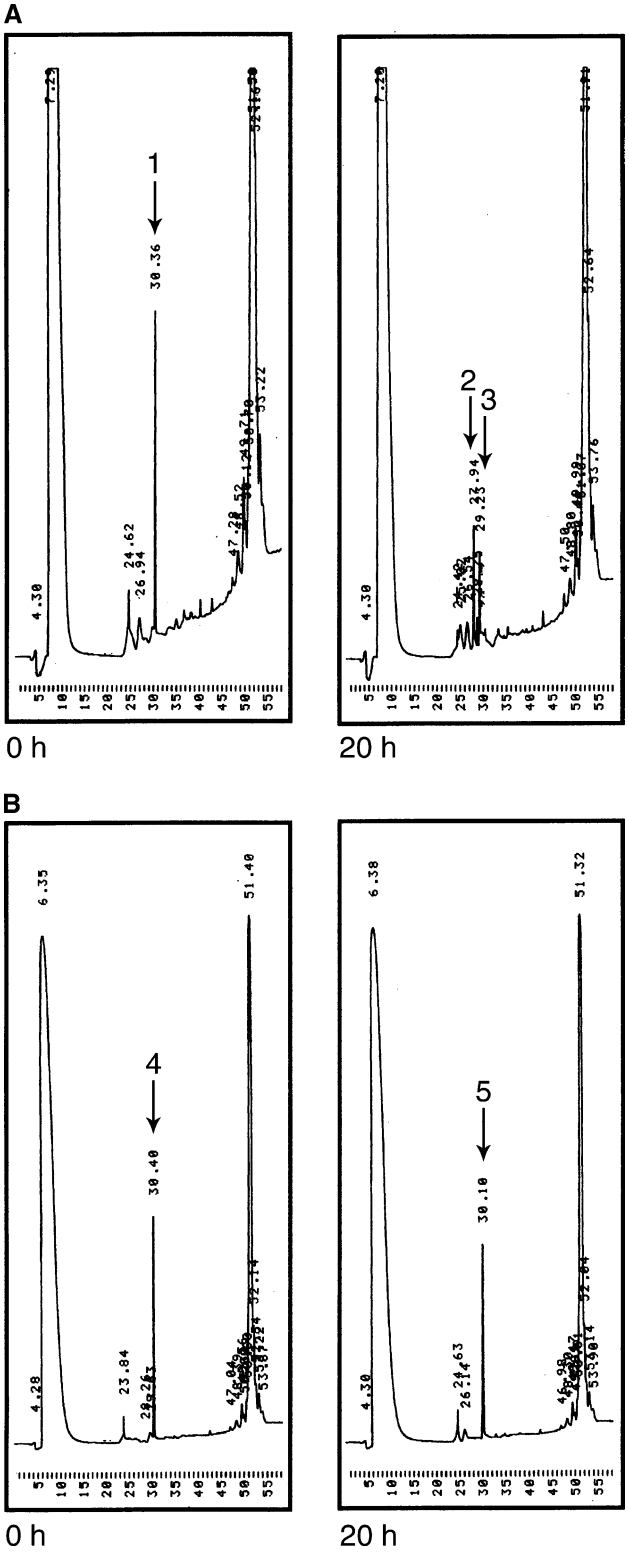


**Fig. 2.** Analysis of synthesized Aβ peptides hydrolyzed by DAEP. **A** Chromatograms of RP-HPLC in which purified rabbit DAEP and D-$A\beta^{1-10}$ peptide have been mixed and incubated for 21 h at 37 °C. Analysis of peak (1), (2), and (3) by the amino acid sequencer resulted in peak (1) [D-Asp]AEFRH[D-Asp]SGY, peak (2) SGY, and peak (3) [D-Asp]AEFRHX. **B** Chromatograms of RP-HPLC in which the purified DAEP and L-$A\beta^{1-10}$ peptide have been mixed and incubated for 21 h at 37 °C. Analysis of peaks (4) and (5) by the amino acid sequencer resulted in the same product: ([L-Asp]AEFRH[L-Asp]SGY)

## Discussion

DAEP activity was localized in both the mammalian mitochondria and nucleus. In preliminary experiments, nuclear DAEP was also detected in the internal space of the nucleus. Nuclear DAEP was inhibited in the presence of i-DAEP, lactacystin and $Zn^{2+}$, but in contrast to mitochondrial DAEP, the inhibitory sensitivities were different: $IC_{50}$ of i-DAEP was 5 μM and for lactacystin was 100 μM. A dipeptidase, located in the brush border membrane of pig kidney cortex, can hydrolyze the D-amino acid containing peptides such as Gly-[D-Asp] (Watanabe et al., 1996); however, that enzyme is a homodimer of a 48-kDa protein subunit, has a very broad spectrum of substrate specificity, and also hydrolyzes L-amino acid containing peptides. Considering these enzymatic properties, it is reasonable to suppose that this dipeptidase is different from DAEP. As kidney has high DAEP activity compared with other tissues, it may be that DAEP recognizes and degrades L-Asp-containing peptide/protein at the initial degrading stage to produce short peptides for further hydrolysis by the dipeptidase and/or other pepti-

**Table 3.** Sensitivity of DAEP to various inhibitors

| Inhibitor | Concentration | Proteinase peptidase | Relative inhibition (%) |
|---|---|---|---|
| i-DAEP | 3 μM | DAEP | 50 |
| Lactacystin | 0.15 mM | Proteasome | 50 |
| Epoxomicin | 0.1 mM | Proteasome | <0.1 |
| ALLN | 0.1 mM | Calpain, Proteasome | <0.1 |
| TPCK | 10 μM | Serine Proteases of Chymotrypsin family | <0.1 |
| TLCK | 0.1 mM | Serine Proteases of Trypsin family | <0.1 |
| PMSF | 1 mM | Serine Proteases | 0.5 |
| DFP | 0.5 mM | Serine Proteases | 15.5 |
| Aprotinin | 0.1 mM | Kalikrein, Trypsin, Chymotrypsin, Plasmin | 13.8 |
| E-64 | 0.1 mM | Thiol Proteases | <0.1 |
| Pepstatin A | 10 μM | Aspartic Proteases | 5.3 |
| Phosphoramidon | 10 μM | Metalloproteinases | 9.9 |
| o-Phenanthroline | 0.1 mM | Metalloproteinases | 8.3 |
| Leupeptin | 30 μM | Serine Proteases, Cysteine Proteases (except for Chymotrypsin) | 8.5 |
| Chymostatin | 5 μM | Chymotrypsin, Chymase, Papain, Calpain, Cathepsin A/B/D/G | <0.1 |
| AAF–$CH_2Cl$ | 1 mM | Giant Protease, Chymotrypsin, Chymase | <0.1 |
| EDTA | 1 mM | Metalloproteinases | <0.1 |

An appropriate concentration of each inhibitor was added to the DAEP assay buffer and activity was measured using Suc-[D-Asp]-MCA. DAEP activity for inhibitors is indicated as a percentage of the control. *TPCK N-p*-tosyl-L-phenylalanine chloromethyl ketone, *TLCK N-p*-tosyl-L-lysine chloromethyl ketone; *PMSF* phenylmethylsulfonyl fluoride; *DFP* diisopropyl fluorophosphate; *E-64 trans*-epoxysuccinyl-L-leucylamido(4-guanidino)butane; *AAF-$CH_2Cl$* L-alanyl-L-alanyl-L-phenylalanyl-chloromethane

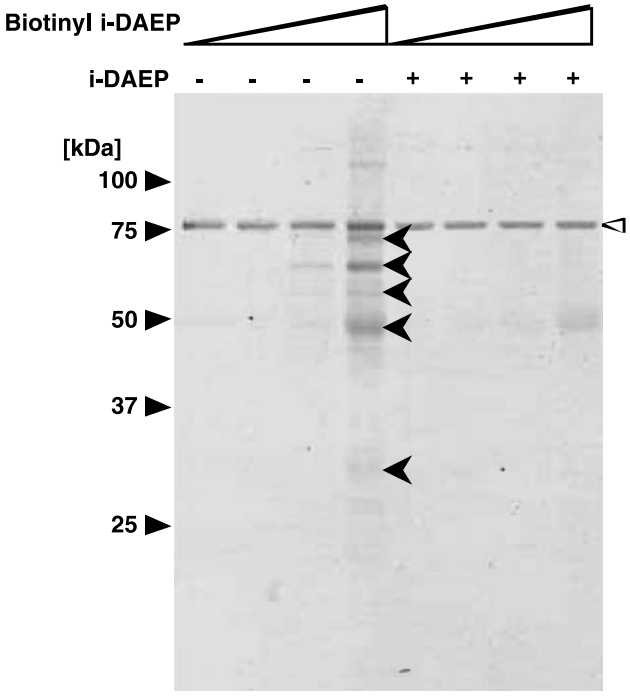


**Fig. 3.** Identification of DAEP by affinity labeling with biotinylated i-DAEP. Identification of the active site of DAEP by affinity labeling with biotinylated i-DAEP. Purified mouse DAEP was incubated with or without 100 μM i-DAEP and 0.1, 1, 10, 100 μM synthesized biotinylated i-DAEP was subsequently added. The DAEP samples affinity-labeled by biotinylated i-DAEP were subjected to SDS-PAGE, electroblotted onto PVDF membrane, probed with streptavidin-horseradish peroxidase conjugate and finally detected by ECL™. Several protein bands, with relative mobility of 30, 45, 55, 60 and 75 k, were detected and are indicated by arrowheads. The white arrowhead at 78 k indicates non-specific band

dases. Another hydrolyzing activity of the small synthetic D-Asp-containing Aβ peptide is found in the brain cortex (Lee et al., 2002). Although the enzyme preparation is a crude extract from the total homogenate of brain using Triton X-100, DAEP may also be present in brain tissue. The possibility of a coupled reaction of DAEP with other peptidases can not be excluded. The existence of DAEP in mitochondria might be explained by ROS, because they damage and modify various molecules in mitochondria. In fact, D-Asp is detected in rat liver mitochondria (Nagata et al., 2001). Therefore, in the long life-span of mammals, DAEP may serve as a scavenger against accumulation of racemized proteins in both the mitochondria and nucleus. Insights into the biochemical function and regulation of DAEP will provide the foundation for developing treatments of diseases, such as AD, in which damaged proteins are implicated.

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