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ORIGINAL ARTICLE

Screening system for D-Asp-containing proteins using D-aspartyl endopeptidase and two-dimensional gel electrophoresis

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Abstract D-Asp-containing proteins have been implicated in many aging-related diseases. To clarify the role of D-Asp-containing proteins in such diseases, we developed a screening system for these proteins using a D-aspartyl endopeptidase that specifically cleaves the proteins at the C-terminus. The digested proteins were detected by means of two-dimensional gel electrophoresis and identified using nano-liquid chromatography/tandem mass spectrometry. We were able to detect myelin basic protein, a known D-Asp-containing protein, in the brain tissues of mice; this indicates that our system is effective for screening D-Asp-containing proteins.

Keywords D-Asp · D-Aspartyl endopeptidase · Two-dimensional gel electrophoresis · Racemized protein

Introduction

It was long believed that only L-enantiomers of amino acids occur in nature, and D-amino acids were regarded as unnatural isomers or laboratory artifacts (Corrigan 1969). However, owing to progress in analytical methods, various D-amino acids have been found, both in free form and as components of proteins, in both vertebrates and invertebrates (D'Aniello et al. 1995; Okuma et al. 1995). Biologically uncommon D-aspartyl residues have been reported in proteins in teeth (Helfman and Bada 1976), the eye lens (Masters et al. 1977), the aorta (Powell et al. 1992), skin elastin (Fujii et al. 2002), and the brain (Man et al. 1983) of elderly humans. Aspartic acid is the most easily racemizable amino acid, and D-Asp may be formed by racemization in metabolically inactive tissues during the chronological aging process. Accumulations of D-Asp-containing proteins are thought to cause many diseases that involve abnormal proteins, such as amyloid β protein and tau protein in Alzheimer's disease (Iwatsubo et al. 1996; Kenessey et al. 1995; Shapira, et al. 1988; Tomiyama et al. 1994); αA-crystallin in cataracts (Fujii et al. 1994); elastin in arteriosclerosis and lung diseases (Powell et al. 1992; Shapiro et al. 1991); and myelin basic protein in multiple sclerosis (Fisher et al. 1986).

Our understanding of the relationship between racemized proteins and aging-related diseases would be increased if there were a screening system for novel D-Asp-containing proteins. Until now, however, there has been no effective method for detecting D-Asp-containing proteins. Because there are thousands of proteins in the brain alone, any screening method would have to be highly selective. In this study, we developed a system for selective screening of D-Asp-containing proteins using D-aspartyl endopeptidase (DAEP), an enzyme that recognizes D-Asp residues in proteins. DAEP is a novel mammalian endopeptidase that specifically cleaves D-Asp-containing proteins at the C-terminus. DAEP has a multicomplex structure (MW, 600 kDa) and is localized in the inner mitochondrial membrane of mice and rabbits, but not *Escherichia coli*, *Saccharomyces cerevisiae*, or *Caenorhabditis elegans*. A peptidyl-D-amino acid hydrolase that is an endopeptidase at D-Asp residue of small peptides was discovered in *Loligo vulgaris* Lam (D'Aniello and Strazzullo 1984). DAEP is

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believed to control levels of noxious racemized proteins (Kinouchi et al. 2004). We used a two-dimensional gel electrophoresis (2-DE) differential system, a high-resolution technique for protein separation and for visualizing differences in protein expression, to detect the products of protein cleaved by DAEP, and the protein was identified by means of nano-liquid chromatography/tandem mass spectrometry (nano-LC/MS/MS).

Materials and methods

R1 mice (male, 20 weeks old), which are often used as control mice of the senescence-accelerated mouse, a murine model of accelerated senescence, were purchased from Japan SLC (Shizuoka, Japan). Diethyl ether, urea, dithiothreitol (DTT), thiourea, sodium dodecyl sulfate (SDS), ammonium bicarbonate, ammonium peroxodisulfate, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), glycerol, acetonitrile, methanol, acetic acid, trifluoroacetic acid (TFA), acrylamide, *N,N′*-bisacrylamide, bromophenol blue, glycine, tris(hydroxymethyl)aminomethane (Tris), 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonic acid (CHAPS), and ethylenediamine-*N,N,N′,N′*-tetraacetic acid (EDTA) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Mass spectrometry-grade trypsin was purchased from Promega (Madison, USA). Immobiline dry strips, linear pH range 3.0–10.0, were purchased from GE Healthcare UK Ltd (Buckinghamshire, England). LC-MS-grade acetonitrile was purchased from Merck (Darmstadt, Germany). LC-MS-grade formic acid was purchased from Fluka (Bucks, Switzerland). Protease inhibitor cocktail was purchased from Sigma (St Louis, USA). Succinyl-D-aspartic acid α-(4-methyl-coumaryl-7-amide) (Suc-[D-Asp]-MCA) was purchased from PEPTIDE INSTITUTE, Inc. (Osaka, Japan). DAEP was purified from rabbit liver according to the methods described in a previous report (Kinouchi et al. 2004).

Sample preparation

Mice were anesthetized with diethyl ether and sacrificed by exsanguination, in accordance with the guidelines of the ethical committee of Musashino University. Brain samples were excised and then immediately frozen at −80°C until use. Frozen samples were thawed, weighed, and homogenized on ice in five volumes of lysis buffer (30 mM Tris/HCl (pH 8.5) containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% protease inhibitor cocktail, and 0.2 mM EDTA). The suspensions were sonicated and then centrifuged at 12,000*g* for 15 min at 4°C. The supernatants were further centrifuged at 100,000*g* for 60 min at 4°C. The supernatants were dialyzed against 10 mM Tris/HCl (pH 8.5) containing protease inhibitor cocktail and EDTA. The resultant samples were then stored at −80°C until use. Protein concentrations were measured by Bradford's method (Bradford 1976).

Two-dimensional gel electrophoresis

Samples containing about 1 mg total protein were digested by 5 μL DAEP solution at 37°C overnight. The control sample was treated in the same way with inactivated DAEP by heat treatment in boiling water for 10 min. Samples were applied to immobilized pH 3–10 strips and isoelectrically focused with 10,000 V in an IPGphor isoelectric focus system (GE Healthcare UK Ltd) according to the manufacturer's instructions. The two-dimensional electrophoresis (NIHON EIDO Co., Tokyo, Japan) was performed on 12.5% homogeneous polyacrylamide gels at 40 mA per gel. Gels were then stained with coomassie brilliant blue (CBB) for visualization of the in-gel proteins, and then scanned with an image analyzer LAS-3000 (Fujifilm, Photo Film Co., Tokyo, Japan).

In-gel digestion

Selected proteins were excised from the gels and digested as follows: gel plugs containing proteins were washed with 150 μL of 50 mM NH_4HCO_3:methanol (1:1) for 20 min at 37°C and then with 150 μL of 100% acetonitrile for 10 min to remove stain. The gels were then dried by vacuum centrifugation. The gels were rehydrated in 30–50 μL of 20 mM NH_4HCO_3 containing 2.5 mg/mL trypsin and incubated at 37°C for 16 h. The supernatant was collected, and digested peptides were extracted by sonication with 50 μL of 0.1% TFA/50% ACN for 5 min, and then with 70 μL 0.1% TFA/50% ACN for 5 min and 50 μL 100% ACN for 15 min. The extract was collected and then dried by vacuum centrifugation.

Nano-LC/MS/MS system

Digested samples were resuspended in 15 μL of water. An UltiMate nanoflow HPLC system (Nippon Dionex K.K., Osaka, Japan) was run in the trapping mode with a C18 PepMap100 analytical column (150 × 0.075 mm, 3 μm particles) and a C18 PepMap100 enrichment column (5 × 0.3 mm, 5 μm particles). The mobile phase consisted of solvent A (water:ACN = 98:2 (v/v) with 0.1% formic acid) and solvent B (water:ACN = 20:80 (v/v) with 0.1% formic acid). After the peptides had been trapped with

100% solvent A for 5 min, the column was developed with a biphasic linear gradient of solvent B from 0 to 50% in solvent A (Masuda et al. 2004). The flow rate was 30 μL/min for trapping and 270 nL/min for separation. MS analysis was conducted on an Esquire HCT Plus ion-trap MS (Bruker Daltonics, Germany) equipped with an orthogonal nanoelectrospray source with MS/MS acquisition. The source was operated at 2,300 V with a PicoTip emitter from New Objective (Woburn, USA).

Data searches of the peptide masses were conducted with the Mascot search engine (http://www.matrixscience.com/) and the SwissProt database for tryptic peptides. A peptide mass tolerance of ±0.5 Da was used, and one missed cleavage was allowed.

Result and discussion

Because there are thousands of proteins in a mouse brain, finding a novel D-Asp-containing protein among the protein mixture requires a highly selective screening system. We combined two methods for our screening system: (1) selective recognition by DAEP, an endopeptidase that is specific for D-Asp-containing proteins, and (2) a 2-DE differential system (Fig. 1). 2-DE followed by mass spectrometry detection is the method most widely used for proteome analysis. When D-Asp-containing protein was digested by DAEP, the molecular mass and p*I* of the resulting peptides change. The changes in molecular mass and the p*I* were easily detected by means of the 2-DE differential system when samples treated with inactivated DAEP were subjected to electrophoresis as a control experiment (Fig. 1b, c). Because heat treatment of DAEP in the boiling water for 10 min completely inactivated the DAEP activity (data not shown), the heat-treated DAEP was used as a control. We confirmed that the DAEP solution and the heat-treated DAEP solution did not give any visible spots when subjected to 2-DE (data not shown). In our previous study using a D-Asp-containing peptide, we found that DAEP specifically degraded the internal D-Asp residue of the peptide, whereas the L-Asp-substituted peptide was not affected by DAEP (Kinouchi et al. 2004). Although DAEP activity is detected in rat brain, the activity is very low compared with that in other tissues (Kinouchi et al. 2004). Therefore, the intrinsic DAEP activity in the brain can be considered to be negligible. Because many well-known protease inhibitors (except for lactacystin) do not inhibit DAEP activity, a protease inhibitor cocktail was added to the reaction mixture to inhibit protein digestion by other proteases during sample preparation. In this system, the DAEP solution was used in the penultimate step, that is, before final purification, because DAEP activity in the penultimate step is much more stable than the final purification state.

The DAEP activity was determined with a synthesized substrate, Suc-[D-Asp]-MCA, and the fluorescence intensity of liberated aminomethylcoumarin was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm. The substrate was added to the assay buffer (10 mM Tris–HCl (pH 8.5), 200 mM NaCl, and 3 mM $MnCl_2$), and the activity was 1.1 nmol min^{-1} mg^{-1}.

Figure 2 shows the expression pattern in the cerebrum of a male mouse at 20 weeks old. Protein spot positions and intensities in the gel patterns of DAEP-treated and control samples were compared. By analyzing the protein spot expression on the gels, we identified one protein spot that was not observed on the control gel (Fig. 2). This pattern difference was reproducible for samples from different mice. This spot, which appeared upon DAEP treatment, was identified as myelin basic protein (score, 302) by means of nano-LC/MS/MS and a subsequent Mascot search. Although there seem to be other differences

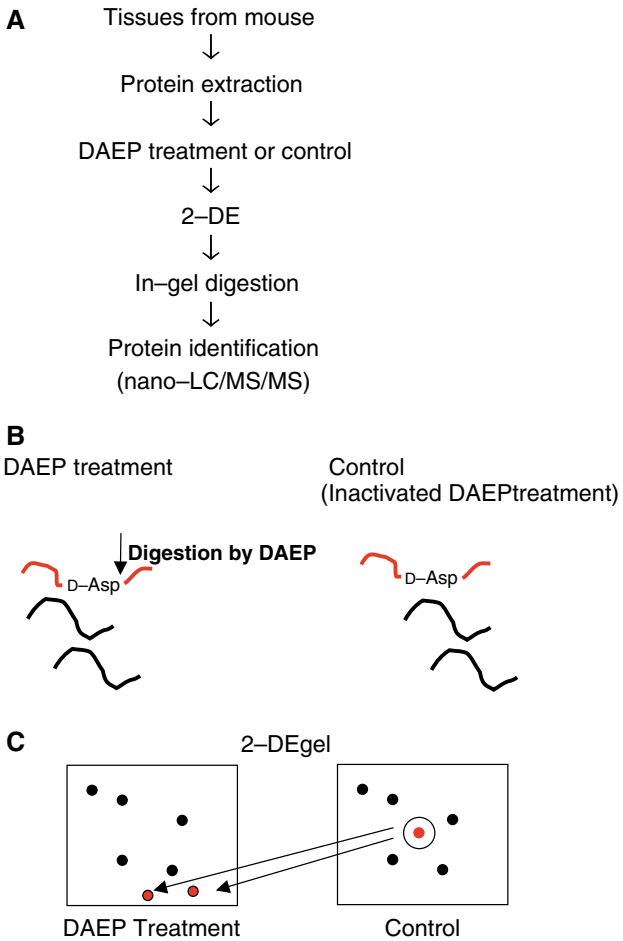

Fig. 1 Schematic of the screening system for D-Asp-containing proteins. **a** Flow chart of the procedure. **b** Schematic of specific digestion of D-Asp-containing protein by DAEP. The *red lines* signify D-Asp-containing protein. The *black lines* signify proteins that do not contain D-Asp. **c** Schematic of the 2-DE differential system

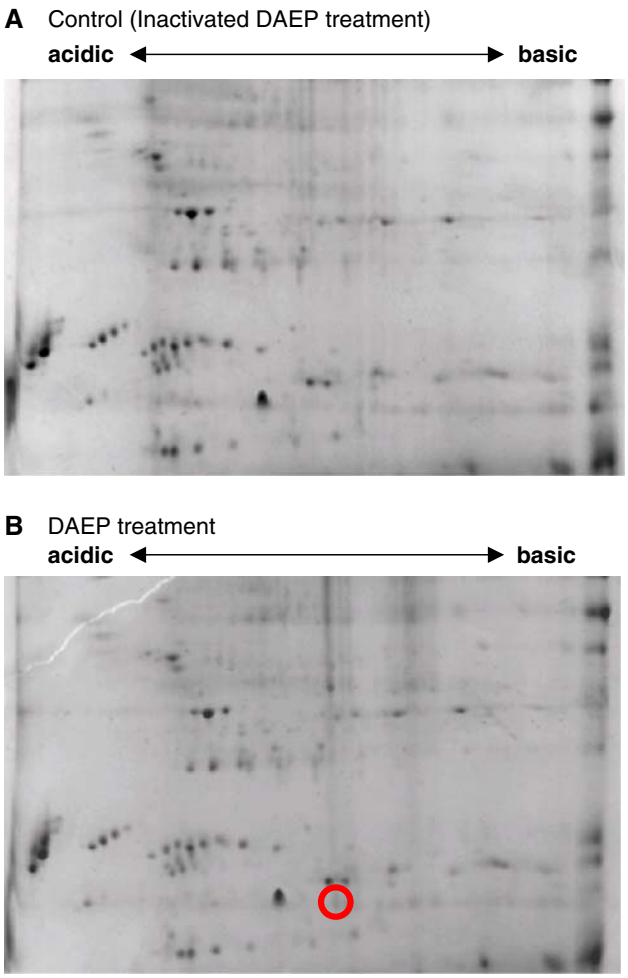

Fig. 2 2-DE differential pattern of a mouse cerebrum (R1, 20 weeks old, male). The gel patterns for the sample treated with DAEP (**b**) and control sample (treated with inactivated DAEP) (**a**) are compared. The *red circle* indicates the new spot observed after DAEP treatment; the spot was identified as myelin basic protein

in spot pattern between two gels, these differences were not reproducible using the same samples. Myelin basic proteins are reported to contain D-aspartic acid in some animals, and the ratio of D-aspartic acid to L-aspartic acid was about 15% in bovine myelin basic protein (Fisher et al. 1986). Our results indicate that mouse myelin basic protein containing D-Asp residues was digested by DAEP, and the digested peptide appeared as a new spot on the gels.

In conclusion, we developed a system for screening D-Asp-containing proteins by using DAEP and a 2-DE differential system. In brain tissues from mice, we showed that this system could screen D-Asp-containing protein, a myelin basic protein. In this report, we could screen only one protein. More D-Asp-containing proteins probably could be discovered by improving this system much more, including the yield of the protein extraction from 2DE gel and reproducibility of 2DE. Because D-Asp-containing proteins have been implicated in many aging-related diseases, we expect that this method will be helpful for clarifying the mechanism of many of these diseases.

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References

Bradford MA (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Corrigan JJ (1969) D-Amino acids in animals. Science 164:142–149

D'Aniello A, Nardi G, De Santis A, Vetere A, Di Cosmo A, Marchelli R, Dossena A, Fisher G (1995) Free L-amino acids and D-aspartate contents in the nerve system of cephalopods. Comp Biochem Physiol 112B:661–666

D'Aniello A, Strazzullo L (1984) Peptidyl-D-amino acid hydrolase from *Loligo vulgaris* Lam. J Biol Chem 259:4237–4243

Fisher GH, Garcia NM, Payan IL, Cadilla-Perezrios R, Sheremata WA, Man EH (1986) D-Aspartic acid in purified myelin and myelin basic protein. Biochem Biophys Res Commun 135:683–687

Fujii N, Ishibashi Y, Satoh K, Fujino M, Harada K (1994) Simultaneous racemization and isomerization at specific aspartic acid residues in alpha B-crystallin from the aged human lens. Biochim Biophys Acta 1204:157–163

Fujii N, Tajima S, Tanaka N, Fujimoto N, Takata T, Shimo-Oka T (2002) The presence of D-β-aspartic acid-containing peptides in elastic fibers of sun-damaged skin: a potent marker for ultraviolet-induced skin aging. Biochem Biophy Res Commun 294:1047–1051

Helfman PM, Bada JL (1976) Aspartic acid racemisation in dentine as a measure of ageing. Nature 262:279–281

Iwatsubo T, Saido TC, Mann DM, Lee VM, Trojanowski JQ (1996) Full-length amyloid-beta (1-42(43)) and amino-terminally modified and truncated amyloid-beta 42(43) deposit in diffuse plaques. Am J Pathol 149:1823–1830

Kenessey A, Yen SH, Liu WK, Yang XR, Dunlop DS (1995) Detection of D-aspartate in tau proteins associated with Alzheimer paired helical filaments. Brain Res 675:183–189

Kinouchi T, Ishiura S, Mabuchi Y, Urakami-Manaka Y, Nishio H, Nishiuchi Y, Tsunemi M, Takada K, Watanabe M, Ikeda M, Matsui H, Tomioka S, Kawahara H, Hamamoto T, Suzuki K, Kagawa Y (2004) Mammalian D-aspartyl endopeptidase: a scavenger for noxious racemized proteins in aging. Biochem Biophys Res Commun 314:730–736

Man EH, Sandhouse M, Burg J, Fisher GH (1983) Accumulation of D-aspartic acid with age in human brain. Science 220:1407–1408

Masters PM, Bada JL, Zigler JS Jr (1977) Aspartic acid racemisation in the human lens during ageing and in cataract formation. Nature 268:71–73

Masuda M, Toriumi C, Santa T, Imai K (2004) Fluorogenic derivatization reagents suitable for isolation and identification of cysteine-containing proteins utilizing high-performance liquid chromatography-tandem mass spectrometry. Anal Chem 76:728–735

Okuma E, Fujita E, Amano H, Noda H, Abe H (1995) Distribution of free D-amino acids in the tissues of crustaceans. Fish Sci 61:157–160

Powell JT, Vine N, Crossman M (1992) On the accumulation of D-aspartate in elastin and other proteins of the ageing aorta. Atherosclerosis 97:201–208

Shapira R, Austin GE, Mirra SS (1988) Neuritic plaque amyloid in Alzheimer's disease is highly racemized. J Neurochem 50:69–74

Shapiro SD, Endicott SK, Province MA, Pierce JA, Campbell EJ (1991) Marked longevity of human lung parenchymal elastic fibers deduced from prevalence of D-aspartate and nuclear weapons-related radiocarbon. J Clin Invest 87:1828–1834

Tomiyama T, Asano S, Furiya Y, Shirasawa T, Endo N, Mori H (1994) Racemization of Asp23 residue affects the aggregation properties of Alzheimer amyloid beta protein analogues. J Biol Chem 269:10205–10208