Journal of Chromatography B, 879 (2011) 3349–3352

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Short communication

Substrate stereoselectivity of mammalian D-aspartyl endopeptidase☆

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ARTICLE INFO

Article history:
Received 21 February 2011
Accepted 24 August 2011
Available online 30 August 2011

Keywords:
Peptidase
Racemization
D-Aspartate
D-Amino acid
Aging
Protein-misfolding disease

ABSTRACT

The formation and accumulation of D-aspartate residue (D-Asp) in proteins caused by oxidative stress leads to dysfunction and/or denaturation of proteins, and is consequently responsible for aging-related misfolding diseases such as cataracts, prion disease, and Alzheimer's disease. We sought to identify that an unknown protease selectively degrades the noxious D-Asp-containing protein, namely D-aspartyl endopeptidase (DAEP), and finally purified it from the inner mitochondrial membrane of mouse liver. In order to analyze the substrate stereoselectivity of DAEP, we synthesized a peptide corresponding to 55–65 (Thr-Val-Leu-Asp-Ser-Gly-Ile-Ser-Glu-Val-Arg) of human αA-crystallin and its corresponding diastereoisomers in which L-α-Asp was replaced with L-β-, D-α- or D-β-Asp residue at position 58. Following incubation of that peptide with purified DAEP, it was only degraded at D-α-Asp^{58}, independent of ATP or NAD. This result indicates that DAEP stereoselectively recognizes and degrades its substrate at the internal D-α-Asp residue. DAEP therefore seems to physiologically serve as the quality control system against the noxious D-Asp-containing protein in the long life span of mammals.

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1. Introduction

The D-isomer of aspartyl residue (D-Asp), identified in human proteins, would be responsible for age-related protein-misfolding diseases (i.e., cataract [1,2], prion disease [3] and Alzheimer's disease [4–6]). It is strongly suggested that the formation of D-Asp in proteins is nonenzymatically caused by oxidative stress and triggers the conformational change of proteins (Fig. 1). In fact, D-Asp-containing β-amyloid protein (Aβ) synthesized *in vitro* forms a β-sheet structure and fibril [7,8]. Since these D-Asp-containing proteins have never been found in the extracellular region, we hypothesized that an unknown proteolytic system would selectively eliminate the noxious D-Asp-containing protein, which is spontaneously generated in a cell. In order to identify such a proteolytic enzyme, we developed an assay system using the synthetic D-Asp-linking fluorescent substance, succinyl-D-aspartic acid α-(4-methyl-coumaryl-7-amide) (Suc-[D-Asp]-MCA), as a substrate mimic. We identified a multi-complex protease (MW: 600 kDa) from the inner mitochondrial membrane of mouse liver [9]. This enzyme could degrade the D-Asp-containing peptide: [D-Asp]^{1}-Ala-Glu-Phe-Arg-His-[D-Asp]^{7}-Ser-Gly-Tyr, corresponding to the amino acid sequence 1–10 of Aβ, at the internal D-Asp^{7} residue. Consequently, we named it D-aspartyl endopeptidase (DAEP) [9]. On the other hand, DAEP degraded neither Suc-L-Asp-MCA nor other synthetic peptidyl MCAs, which are used for proteolysis assays of well-characterized proteases, i.e., Suc-L-Leu-L-Leu-L-Val-L-Tyr-MCA for proteasome. Therefore, it was shown that DAEP has high substrate specificity.

In addition to detection of D-Asp in aged proteins, it was reported that the spontaneous isomerization of Asp residue also occurs in proteins. Consequently, four different isomers of Asp residue (L-α-, D-α-, L-β- and D-β-Asp) can be produced in various proteins (Fig. 1) [2,10–13]. As described above, DAEP can degrade D-Asp-containing substrate, but stereoselectivity of DAEP for other Asp-isomers remains unidentified. In the expectation of gaining a better understanding of the substrate specificity of DAEP from its structural features, we addressed the analysis of a higher order structure of DAEP by atomic force microscopy (AFM), which has the advantage of being able to observe fragile multi-complex molecules. As a result, an AFM image indicated that DAEP forms a ring-like structure with an average diameter of ~40 nm, that is a common structural feature of the AAA+ protease family, which is a class of ATP-dependent proteases such as proteasome, FtsH and Lon, functioning in protein quality control [14]. However, it is not clear whether DAEP degrades the substrate in an ATP-dependent manner, as well as AAA+ protease. We therefore investigated the substrate stereoselectivity of DAEP with or without ATP.

☆ This paper is part of the special issue "Analysis and Biological Relevance of D-Amino Acids and Related Compounds", Kenji Hamase (Guest Editor).

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1570-0232/$ – see front matter © 2011 Published by Elsevier B.V.
doi:10.1016/j.jchromb.2011.08.031

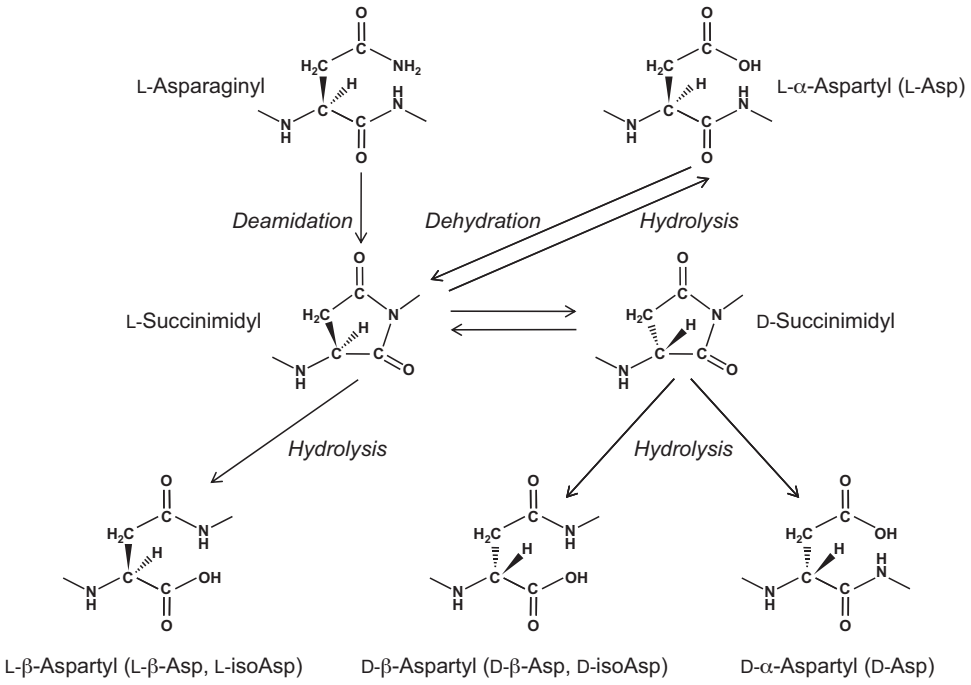

Fig. 1. Formation pathway of the altered Asp residues in protein. Spontaneous nonenzymatic dehydration of Asp and/or deamidation of Asn result in an unstable succinimidyl intermediate. The racemized succinimide is more likely to undergo spontaneous hydrolysis, and leads to the generation of four different isomers of the Asp residue (L-α-, D-α-, L-β- and D-β-Asp) in protein.

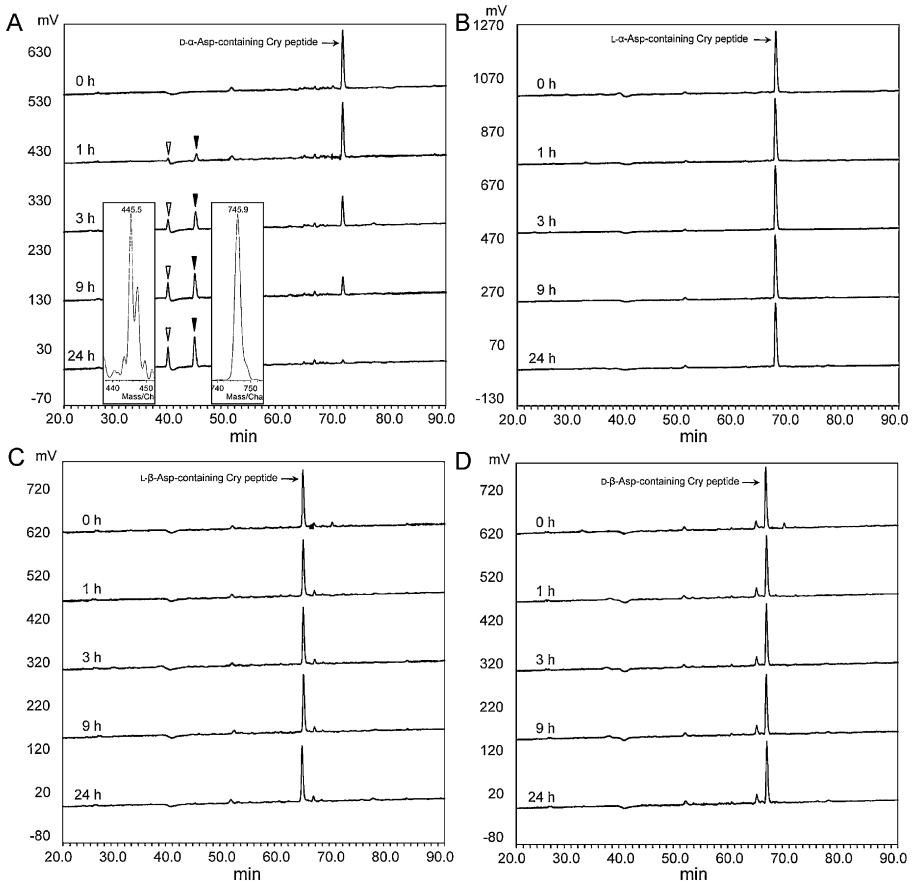

Fig. 2. RP-HPLC analysis of the altered Asp-containing Cry peptide hydrolyzed by DAEP. (A) Chromatograms of the reaction mixture of D-α-Asp-containing Cry peptide incubated with DAEP for 0, 1, 3, 9 and 24 h at 37 °C. Mass spectrometry analysis of the peaks, indicated by white and black arrowheads, reveals that the molecular masses correspond to those of Cry peptide fragments: Thr-Val-Leu-Asp (theoretical mass: 446.5, measured mass: 445.5 described in the left MS spectrum) and Ser-Gly-Ile-Ser-Glu-Val-Arg (theoretical mass: 746.8, measured mass: 745.9 described in the right MS spectrum), respectively. (B) Chromatograms of the reaction mixture of L-α-Asp-containing Cry peptide incubated with DAEP. (C) Chromatograms of the reaction mixture of L-β-Asp-containing Cry peptide incubated with DAEP. (D) Chromatograms of the reaction mixture of D-β-Asp-containing Cry peptide incubated with DAEP. In chromatograms (B)–(D), new fragments, which would be degraded by DAEP, were not detectable.

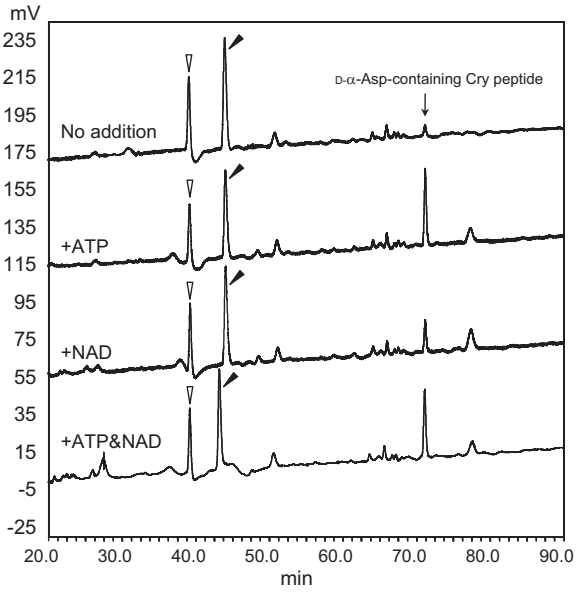

Fig. 3. Effect of ATP and/or NAD on the hydrolysis of substrates by DAEP. Chromatograms of the reaction mixture of D-α-Asp-containing Cry peptide incubated with DAEP for 24 h at 37 °C in the presence or absence of ATP and/or NAD. Mass spectrometry analysis of the peaks, indicated by white and black arrowheads, revealed that the molecular masses correspond to those of the Cry peptide fragments Thr-Val-Leu-Asp and Ser-Gly-Ile-Ser-Glu-Val-Arg, respectively.

2. Materials and methods

2.1. Materials and chemicals

The purification of DAEP from mouse (DDY) liver was previously described [9]. Purification factor of the purified DAEP used in this study was above 200. A peptide: Thr^{55}-Val-Leu-Asp-Ser-Gly-Ile-Ser-Glu-Val-Arg^{65}, corresponding to the amino acid sequence of human αA-crystallin (Cry peptide), and its corresponding diastereoisomers in which L-α-Asp was replaced with L-β-, D-α- or D-β-Asp residue at position 58 were synthesized by Fmoc (9-fluorenylmethoxycarbonyl) solid phase chemistry in dimethylformamide [15]. Other chemicals were purchased from Sigma–Aldrich (MO, USA), Nakalai Tesque, Inc. (Kyoto, Japan), and Kanto Chemical Co. Inc. (Tokyo, Japan).

2.2. Analysis of the altered Asp-containing Cry peptide isomers hydrolyzed by DAEP

10 μg of each Cry peptide containing normal (L-α-Asp) or abnormal isomers (L-β-, D-α- and D-β-Asp) was mixed and incubated with the DAEP assay buffer (10 mM Tris–HCl (pH 8.5), 200 mM NaCl, 3 mM $MnCl_2$) including 0.1 μg of purified mouse DAEP with or without ATP (3 mM) and/or nicotinamide adenine dinucleotide (NAD, 84 μM) (total volume: 200 μl) at 37 °C. Each reaction mixture following 0-, 1-, 3-, 9- and 24 h incubation was independently applied to reversed-phase HPLC (flow rate: 0.5 ml/min, detection wavelength: 215 nm, a reversed-phase column: CAPCELL PAK C18 (UG80), 5 μm of particle size, 3.0 mm i.d. × 250 mm, Shiseido, Tokyo, Japan), and eluted with a linear gradient of 0–40% acetonitrile in 0.1% TFA/120 min. The molecular masses of digested peptides (peaks indicated by arrowheads in Figs. 2 and 3) were determined using MALDI-TOF mass spectrometers, KOMPACT MALDI IV and AXIMA-TOF^2 (Shimadzu Biotech, Kyoto, Japan). The peptide solution and the matrix solution (5 mg/ml α-cyano-4-hydroxycinamic acid and 0.1% trifluoroacetic acid in 50% acetonitrile) were mixed and applied onto a sample plate and air-dried. The instruments were operated at 20 keV acceleration voltage. All MS data were acquired and analyzed with MALDI-MS software (Shimadzu Biotech, Kyoto, Japan).

3. Results and discussion

3.1. Substrate stereoselectivity of DAEP

In order to investigate the stereoselectivity of DAEP for four different isomers of Asp residue: L-α-, D-α-, L-β- and D-β-Asp, in a substrate, we used Cry peptide, Thr^{55}-Val-Leu-Asp-Ser-Gly-Ile-Ser-Glu-Val-Arg^{65}, corresponding to the amino acid sequence of human αA-crystallin as a model substrate. The reason why we chose that peptide for this assay was that the four isomers at the Asp^{58} residue have actually been found in αA-crystallin purified from aged human lens [13] and can be a substrate of DAEP *in vivo*. Each altered Asp-containing Cry peptide was individually mixed with purified mouse DAEP, and incubated for 24 h at 37 °C. Following incubation of D-α-Asp^{58}-contanining Cry peptide with DAEP, the peptide was degraded at D-α-Asp^{58} and new two fragments were produced as incubating time advanced: Thr-Val-Leu-Asp^{58} and Ser-Gly-Ile-Ser-Glu-Val-Arg (Fig. 2A). However, L-α-, L-β- and D-β-Asp^{58}-contanining Cry peptides could not be degraded by DAEP even after 24 h incubation (Fig. 2B–D). These results indicate that DAEP recognizes the internal D-α-Asp residue of the substrate in a stereoselective manner and specifically degrades it.

Although it is still unclear whether DAEP selectively degrades D-α-Asp-containing protein *in vivo*, the report that free D-aspartic acid accumulates in human brain with age would support that a physiological substrate of DAEP is D-α-Asp-containing protein [16] since free D-aspartic acid, in part, is considered as the degradation product of D-Asp-containing protein by DAEP. This would be consistent with our previous study in which, in the liver of mice, DAEP activity was 2.5-fold higher in middle-aged mice (69-weeks-old) than in young mice (12-weeks-old) [17].

3.2. ATP-dependency of DAEP activity

Some unique features of DAEP, namely its molecular weight (600 kDa) and the ring-like complex structure, suggest that DAEP might be a new member of the AAA+ protease family [14]. Thus, we examined the effect of ATP on the hydrolysis of substrates by DAEP (Fig. 3). As D-α-Asp^{58}-contanining Cry peptide was mixed and incubated with purified mouse DAEP in the presence of ATP (3 mM), 29% of the Cry peptide remained undegraded after 24 h incubation. In contrast, no more than 3.7% of the Cry peptide remained in the absence of ATP. NAD, which is well known as a coenzyme for stereoselective enzymes such as (*R*)- and (*S*)-hydroxypropylthioethanesulfonate dehydrogenases [18], was also rather inhibitory to DAEP activity. On the other hand, L-α-, L-β- and D-β-Asp^{58}-contanining Cry peptides could not be degraded by DAEP even in the presence of ATP and/or NAD (data not shown). These results strongly suggest that neither ATP nor NAD play an important role in DAEP activity and stereoselectivity. It is under investigation whether DAEP can degrade D-Asp-containing protein *in vivo*, which is much larger than Cry peptide used in this study, in the presence or absence of ATP.

DAEP exhibits strict substrate stereoselectivity and therefore seems to physiologically serve as the quality control system against the noxious D-Asp-containing protein in the long life span of mammals. To investigate the coordination of DAEP with other quality-control proteases will provide a basis for developing treatments for protein-misfolding diseases caused by isomerization of Asp residue in protein.

Acknowledgement

This work was supported in part by Grants-in-Aid for Scientific Research (No. 20590278, to T.K.) from the Japan Society for the Promotion of Science.

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