PR2 Project Research on the Elucidation of Generating Mechanism of Damaged Protein Induced by Aging and Irradiation

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Objectives and Allotted Research Subjects:

The aim of this project research is to elucidate the correlation between the change of the protein structure induced by various post-translational modifications with UV irradiation, gamma-irradiation, aging and protein function. We also investigate the repair mechanism for the damaged protein by irradiation. In this year, the 7 research subjects were carried out. The allotted research subjects (ARS) are as follows;

ARS-1: Consideration for the distribution of D-aspartyl endopeptidases activity in various living things (T. Kinouchi and N. Fujii)

ARS-2: Damage to biological molecules induced by ionizing irradiation and biological defense mechanisms against ionizing radiation III (T. Saito and N. Fujii)

ARS-3: Separation condition of the Prion Peptide (106-126) after treatment of protein L-isoaspartyl methyltransferase (PIMT) (Y. Sadakane and N. Fujii)

ARS-4: Analysis of hearing impairments in mice exposed to environmental stress (N. Ohgami and N. Fujii)

ARS-5: Identification of contiguous β-aspratyl residues in peptide using MS (N. Fujii, S. Kishimoto and N. Fujii)

ARS-6: The stability of D-β-Asp:kinetics of the competitive reactions of isomerization and peptide bond cleavage (K. Aki, E. Okamura and N.Fujii)

ARS-7: Change of protein function by the replacement of a single aspartyl isomer in a protein (N. Fujii, H. Sakaue, T. Kinouchi, N. Fujii and T. Takata).

Main Results and Contents of This Project

ARS-1: Kinouchi and Fujii have been researching an evolutional origin of D-Aspartyl endopeptidase (DAEP), which stereoselectively degrades D-aspartate-containing protein. As a result of searching for a distribution of DAEP in various living things, the activity could be detected only in the animal reproductive tissue. We therefore supposed that the original and primitive physiological functions of DAEP might be associated with fertilization and/or oocyte maturation.

ARS-2: Saito *et al.* determined the 1% survival rate of the E. coli clone to be 271 Gy and generated an E. coli cell population with approximately 3-fold resistance to gamma rays, compared with wild-type E. coli, by a directed evolution experiment.

ARS-3: Sadakane *et al.* determined the HPLC condition for separation of the prion peptides (106-126) containing isomerized Asp residues generated after treatment of a repair enzyme for aged protein, PIMT.

ARS-4: Ohgami *et al.* suggested that mice exposed to elements including barium and manganese via drinking water accumulates in inner ears resulting in hearing loss with neurodegeneration of auditory neurons. Further study is needed to investigate aggregation of a specific protein in the auditory neurons in inner ears.

ARS-5: Fujii *et al.* identified the contiguous β-aspratyl residues in a peptide by the analysis of the 2nd generation product ion spectrum of ion trap MS.

ARS-6: Aki *et al.* determined the rate constants of two competitive reactions, isomerization and peptide bond cleavage at Asp residue in αA-crystallin fragment containing L-α- and D- β-Asp-58 isomers. The results clearly indicate that D- β-Asp is stable in competitive reaction pathway of isomerization and peptide bond cleavage at Asp residue.

ARS-7: Fujii *et al.* synthesized the Asp isomer-containing RNase A using expressed protein ligation (EPL). The RNase A variants with different Asp isomers decreased the enzyme activity and solubility.

PR2-1 Consideration for the Distribution of D-Aspartyl Endopeptidases Activity in Various Living Things

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INTRODUCTION: D-Aspartyl endopeptidase (DAEP), which was discovered in the liver of mammals, stereoselectively recognizes and degrades D-isomer of aspartate (D-Asp) residue in peptides/proteins [1]. D-Asp residue is often detected in low-turnover proteins, e.g., crystallin, prion protein and β-amyloid protein, and these harmful proteins are mostly causing age-related diseases e.g., cataract, prion disease and Alzheimer's disease, respectively. Therefore, it seemed that DAEP would physiologically serves as a kind of quality-control system against the abnormally aggregated D-Asp-containing protein. However, in searching for a tissue-distribution of DAEP in various living things besides mammalians, the high activity of DAEP could not be specifically detected in their livers. For example, in African clawed frog (*Xenopus laevis*), high DAEP activity was detected in its testes, ovaries and unfertilized eggs. As a result of searching the distribution of DAEP in other aquatic animals; Japanese green sea urchin, *Hemicentrotus pulcherrimus*, and the ascidian, *Ciona intestinalis*, high DAEP activity was shown in their reproductive organs as a common feature. On the other hand, the existence and distribution of DAEP in plants are still ambiguous. Because, although we tried to detect the DAEP activity in some plants; radish, tomato and broccoli, it was difficult to distinguish the fluorescence liberated in the measurement of DAEP activity from the auto-fluorescence of pigments in those plant samples. In order to make clear the distribution of DAEP in plants, we searched the DAEP activity in the common duckweed (*Lemna minor*) grown under the low-light condition. Because the common duckweed grows and loses the green pigment even under the relatively dark condition.

EXPERIMENTS: Plant Materials and Growth Conditions> Common duckweed (*Lemna minor*) was kindly gifted by Dr. Masaru Kobayashi, Kyoto University, and was cultivated at 23°C in hydroponic media containing major nutrients (1 mM $Ca(NO_3)_2$, 0.5 mM KH_2PO_4, 0.5 mM K_2SO_4, 1 mM $MgSO_4$, and 1.5 mM NH_4NO_3) and micronutrients (75 μM EDTA-Fe, 46 μM H_3BO_3, 9 μM $MnSO_4$, 0.8 μM $ZnSO_4$, 0.3 μM $CuSO_4$, and 0.8 μM Na_2MoO_4) under a 16-h low-light/8-h dark cycle in a 60%-humidified growth chamber.

Extraction of Crude Enzyme> Vegetatively propagated common duckweeds were harvested and immediately homogenized by Polytron® PT2100 agitator in ice-cold sodium phosphate buffer (pH 7.5). The suspension was filtered through a double layer of cheesecloth and centrifuged at 600 xg for 10 min at 4°C. Since the supernatant was crude enzyme mixtures that might include not only DAEP but also other proteases, the appropriate dose of protease inhibitor cocktail for plant cell extracts (purchased from Sigma-Aldrich, Inc) was added to it. This final supernatant was applied to the measurement of DAEP activity.

Measurement of DAEP activity> We developed an assay system for DAEP activity using the synthetic D-Asp containing substrate, Succinyl-D-Aspartic acid α-(4-methyl-coumaryl-7-amide) (Suc-D-Asp-MCA) [1,2]. Supernatant of the above samples was mixed and incubated with 0.1 mM Suc-D-Asp-MCA and the assay buffer (10 mM Tris-HCl (pH 8.5), 200 mM NaCl, 3 mM $MgCl_2$) at 25°C. The fluorescence of aminomethylcoumarin liberated from Suc-D-Asp-MCA by DAEP was measured at *l*ex = 380 nm and *l*em = 460 nm.

RESULTS & DISCUSSION: As had been expected, the extract from the common duckweed grown under the low-light condition did not include a high amount of spontaneous pigments. However, the DAEP activity was not detectable. It was suggested that plants do not have any DAEP activity, or have quite low amounts. On the other hand, in animals, the DAEP activity was distributed in the reproductive organ, regardless of any species of animal. We therefore supposed that DAEP might be developed and associated with fertilization and/or oocyte maturation in the evolution of animals.

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PR2-2 Damage to Biological Molecules Induced by Ionizing Irradiation and Biological Defense Mechanisms against Ionizing Radiation III

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INTRODUCTION: There are various environments in nature, and organisms have evolved diversely to adapt to most of them. Some organisms can survive in environments that are considered extremely severe by our common sense. Elucidation of the adaptive mechanisms of organisms to severe environments will provide meaningful information regarding the evolution and diversity of whole organisms. Of these organisms, some bacteria have extremely high resistance to ionizing radiation [1]. The extreme resistance mechanisms of these bacteria are an interesting research subject to consider when studying adaptation to the environment. In order to elucidate radioresistance mechanisms, it is important to investigate the molecular mechanisms of the biological defense of organisms with radioresistant activity against external stress and the damage of biological molecules due to the ionizing radiation. Nevertheless, the study of radioresistant organisms in nature is expected to face several challenges, as knowledge on their genetic and biochemical properties is poor. In this study, we attempted to generate radioresistant *Escherichia coli* by a directed evolution experiment in which the following operations were repeated: gamma-ray irradiation of *E. coli*, whose genetic and biochemical properties are well known, growth of surviving cells, and irradiation of the grown cells.

EXPERIMENTS: Evaluation of the sensitivity of *E. coli* to gamma rays: *E. coli K*-12 cells were grown to the early log phase in LB medium at 37°C at 200 rpm. One milliliter of culture was centrifuged at 4000 ! *g* at 20°C for 10 min. The supernatant was discarded and the pellet was suspended in 1 mL of PBS(–). The cell suspension was irradiated with gamma rays at a dose rate of 25 Gy/min at room temperature. Gamma irradiation was carried out at the Co-60 Gamma-ray Irradiation Facility of the Kyoto University Research Reactor Institute. The gamma-irradiated cell suspension was diluted appropriately with PBS(–), plated on LB agar, and incubated at 37°C for 12 hr. Colonies were counted, colony forming units (CFUs) were determined, and survival rates were calculated.

Selection with gamma rays: *E. coli K*-12 clone cells obtained by single colony pick-up were grown to the early log phase in LB medium at 37°C at 200 rpm. The cell suspension was prepared as described above. The cell suspension was irradiated with 1% survival dose of gamma rays at a dose rate of 25 Gy/min at room temperature. One milliliter of gamma-irradiated cell suspension was inoculated in 100 mL LB medium and grown to the early stationary phase at 37°C at 200 rpm. Glycerol stock was prepared and stored at –80°C. This protocol was repeated with cells from the glycerol stock grown to the early log phase.

RESULTS: In order to evaluate the sensitivity of *E. coli* to gamma rays, *E. coli* clone cells were irradiated with gamma rays, and CFUs of the irradiated cells were determined by the colony forming method. Fig. 1 shows the relationship between the absorbed dose of gamma rays and the survival rate of *E. coli*. The 1% survival rate of this clone was determined to be 271 Gy.

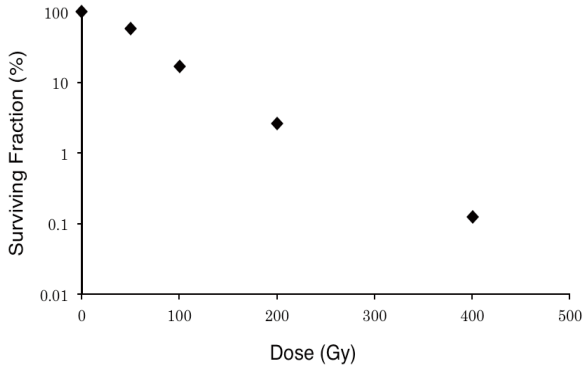

Fig. 1. Relationship between the absorbed dose of gamma rays and the survival rate of *E. coli*.

In order to generate radioresistant *E. coli* by directed evolution using gamma rays as the selection pressure, *E. coli* were irradiated with 1% survival dose of gamma rays, surviving cells were grown, the 1% survival dose of the grown cell population to the same radiation was evaluated, and the cell population was irradiated again with 1% survival dose. By repeating this operation eight times, an *E. coli* cell population with approximately 3-fold resistance to gamma rays compared with *E. coli* without selection, was obtained. Further studies, namely the generation of higher gamma-ray-resistant *E. coli* by employing a higher number of repetitions of the selection cycle and analysis of the genetic and biochemical properties of the resulting *E. coli* strain, will be required to reveal the biological defense mechanisms in response to ionizing radiation.

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PR2-3 Separation Condition of the Prion Peptide (106-126) after Treatment of Protein L-Isoaspartyl Methyltransferase (PIMT)

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INTRODUCTION: In previous study, we prepared PIMT by *E. coli* expression system, and determined the HPLC condition for separation of the peptides containing isomerized Asp residues generated after treatment of repairing enzyme, PIMT. Using model peptide T10 (HFSPEDLTVK of crystalline), we succeeded in finding the separation condition for the mixture of six T10 peptides bearing L-Asp, L-isoAsp, D-Asp, D-isoAsp, methyl ester Asp and succinimide. The mixture was separated by reversed-phased HPLC with 15 % acetonitrile with pH 3.0 of 15 mM phosphate buffer. The stereoconversion of aspartyl (Asp) residue arise through intramolecular rearrangement, such as *via* a succinimide intermediate. L-isoaspartyl methyltransferase (PIMT) catalyzes repair of L-isoAsp peptide bonds in aged proteins by transferring a methyl group from S-adenosylmethionine to a α-carboxyl group of L-isoAsp residue (Fig.1). In this study, we determined the separation condition for prion peptide (106-126) containing isomerized Asp residues generated after treatment of repairing enzyme, PIMT because the deamidation of Asn residue in prion peptide has been reported to affect its channel activity in planar lipid bilayer.

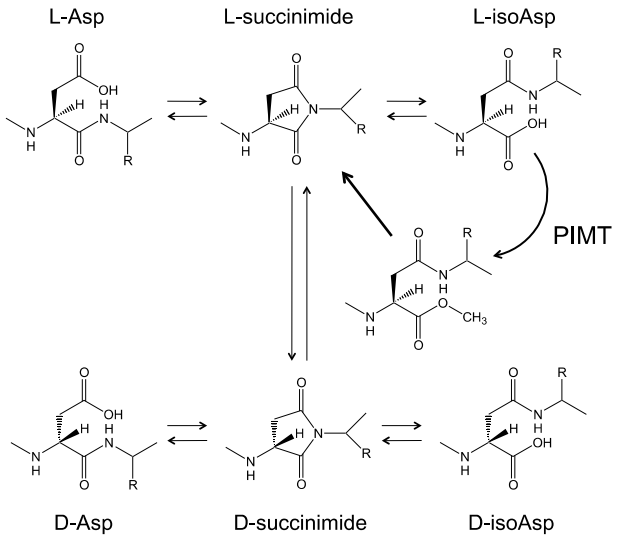

Fig. 1 The β–linkage isomerization and stereoinversion to D-form of aspartyl residue and PIMT repair system.

EXPERIMENTS: The recombinant PIMT protein was prepared by His-tag conjugated *E. coli* expression system, and the four isomerized prion peptides (KTNM-KHMAGAAA AGAVVGGLG) were synthesized by using Fmoc amino acids. The prion peptide bearing L-isoAsp residue was treated by PIMT for 60 min at 37 °C, and the reaction was stopped by addition of HCl for generating the peptides bearing methyl ester and succinimide. The reacted sample was analyzed by reversed-phase HPLC with acetonitrile/H_2O containing 15 mM phosphate buffer, detected by absorption at 215 nm.

RESULTS: PIMT transfers the methyl group of S-adenosylmethionine onto a α-carboxyl group of L-isoAsp residue of prion peptide, then resulted in generating the peptide bearing methyl ester. The methyl ester undergoes spontaneously and rapidly demethylation (15 min, half-time) to generate succinimide intermediate. The intermediate was hydrolyzed (1 hr, half-time) to produce the prion peptide beating L-Asp or L-isoAsp residue. To determine the separate condition the isomerized peptides, we prepared the mixture of the prion peptides bearing L-Asp, L-isoAsp, D-Asp and D-isoAsp, and PIMT-reacted solution, which included the peptide bearing methyl ester and succinimide. (Fig. 2). We became able to determine easily the kinetics of cyclization and de-cyclization of succinimide intermediate.

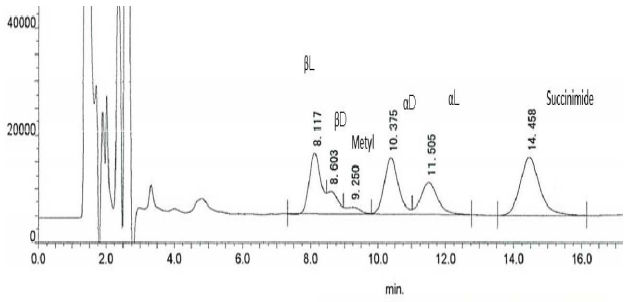

Fig. 2 Separation of prion peptides containing various isomerized Asp residue by standard HPLC. The mixture of six peptides were separated by reversed-phase HPLC with ODS column using the eluent 20 % acetonitrile with pH 3.0 of 15 mM phosphate buffer and 100 mM NaCl. βL: the peptide containing L-isoAsp residue, β D: D-isoAsp residue, αD: D-Asp residue, methyl: methyl ester, αL: L-Asp residue.

PR2-4 Analysis of Hearing Impairments in Mice Exposed to Environmental Stress

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INTRODUCTION: Exposure to elements in occupational and daily environments is known to cause health risks in humans [1,2]. However, there is very limited information that the ingestion of elements from food and water can be a risk to our health. In a previous study, it has been suggested that we are exposed to barium via drinking water and foods [1]. Also, exposure to barium has been shown to affect blood pressure [2], and direct injection of barium to inner ears has been shown to affect physiological functions in inner ears [3]. However, it remains unknown whether ingestion of barium via drinking water affects hearing levels in experimental animals. This study aimed at analyzing whether ingestion of barium affects hearing levels and morphology of inner ears in mice.

EXPERIMENTS: Oral exposure to barium chloride ($BaCl_2$) to wild-type ICR mice for 2 weeks via drinking water was performed. We measured barium levels in various tissues with inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500cx). We also performed pathological analyses of inner ears as described previously [4]. After oral exposure to $BaCl_2$ via drinking water, we performed auditory brain stem responses (ABR) to measure hearing levels in mice [4]. This study was approved by the Institutional Animal Care and Use Committee in Chubu University (approval number: 2510053). This study also followed the Japanese Government Regulations for Animal Experiments. We further performed an epidemiological analysis to determine a correlation between hearing loss and barium levels in biological samples in humans. This study was performed after an approval of Nagoya University International Bioethics Committee following the regulations of the Japanese government (approval number 2013-0070).

RESULTS: Mice orally exposed to barium showed hearing loss, especially at higher frequency in mice. Mice orally exposed to barium showed neurodegeneration in inner and outer hair cells, stria vascularis and spiral ganglion neurons in the organ of Cotri. Correspondingly, mice orally exposed to barium showed higher levels of barium in inner ears than those in control [5]. In a previous study, exposure to manganese has been shown to cause aggregation of proteins resulting in neurodegeneration [6]. Therefore, we further determine hearing loss in mice orally exposed to manganese. After exposure, the exposed group showed hearing loss and neurodegeneration of auditory neurons [7].

Conclusions: This study suggests that mice exposed to elements including barium and manganese via drinking water accumulates in inner ears resulting in hearing loss with neurodegeneration of auditory neurons. In our epidemiological study, barium levels in toenails and hair were significantly associated with hearing loss in humans [8]. Thus, it is possible that oral exposure to barium causes hearing loss in humans. On the other hand, our previous study showed that exposure to noise stress caused increased levels of oxidative stress and positive signals stained by anti-D-beta-Asp antibody in inner ears [9]. Therefore, we will pursue a research to elucidate a mechanism of degeneration of auditory neurons, especially to investigate whether exposure to noise or elements causes a protein aggregation in inner ears.

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PR2-5 Identification of Contiguous β-aspratyl Residues in Peptide Using MS

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INTRODUCTION: We developed a method for four Asp isomers (Lα-, Lβ-, Dα-, Dβ-Asp) at individual sites in proteins by liquid chromatography - tandem mass spectrometry (LC-MS/MS) system because the four Asp isomers containing peptides were eluted at the different time on the chromatogram [1]. However, to identify the four Asp isomers on the chromatogram, it is necessary to synthesize reference peptides containing the for different Asp isomers as standards. This is time-consuming. Last year, we reported a method for identifying the β-Asp containing peptides by detecting the specific 2nd generation product ions using an ion trap mass spectrometer (MS). In this study, the contiguous Asp residues in peptide couldn't be ever identified an isomer, but it is possible to identify the β-Asp residue by an advanced method using MS.

EXPERIMENTS: The peptides containing Asp isomers were made using a fmoc solid-phase chemistry. The synthetic peptides were human αA-crystallin sequence from residues 89 to 99 peptides (αAT11 peptide) containing Lα- or Lβ-Asp isomers; VQ(Lα-D)(Lα-D)FVEIHGK (αAT11 α-α), VQ(Lα-D)(Lβ-D)FVEIHGK (αAT11 α-β), VQ(Lβ-D)(Lα-D)FVEIHGK (αAT11 β-α), VQ(Lβ-D)(Lβ-D)FVEIHGK (αAT11 β-β).

MS was performed on an ion trap system (LCQ Fleet, Thermo). The 1st and 2nd generation precursor ion of αAT11 peptides were selected the $[M+2H]^{2+}$ ion at 643.8 m/z and the y_8 fragment ion at 944.4 m/z or the y_9 fragment ion at 1059.5 m/z.

RESULTS: In order to analyze the fourth Asp residues from N-terminal side in peptide, the 1st and 2nd generation precursor ion of αAT11 peptides were selected the $[M+2H]^{2+}$ ion at 643.8 m/z and the y_8 fragment ion at 944.4 m/z. Fig. 1 shows the 2nd generation product ion spectrum of αAT11 peptide containing four types of Asp isomers. The specific fragment ions of 871.4 m/z (y_8-73) and 853.4 m/z (y_8-91) were observed in the spectrum of αAT11 α-β and αAT11 β-β. The y_8-91 and y_8-73 were observed in the 2nd generation product ion spectrum of fourth β-Asp residues from N-terminal side containing αAT11 peptides (Fig. 1b, d), while these product ions were not observed for fourth α-Asp residues from N-terminal side containing αAT11 peptides (Fig.1a, c).

In order to analyze the third Asp residues from N-terminal side, the 1st and 2nd generation precursor ion of αAT11 peptides were selected the $[M+2H]^{2+}$ ion at 643.8 m/z and the y_9 fragment ion at 1059.5 m/z. Fig. 2 shows the 2nd generation product ion spectrum of αAT11 peptide containing four types of Asp isomers. The specific fragment ions of 986.4 m/z (y_9-73) and 968.4 m/z (y_9-91) were observed in the spectrum of αAT11 β-α and αAT11 β-β. The y_9-91 and y_9-73 were observed in the 2nd generation product ion spectrum of third β-Asp residues from N-terminal side containing αAT11 peptides (Fig 2c, d), while these product ions were not observed for third α-Asp residues from N-terminal side containing αAT11 peptides (Fig. 2a, b).

These results indicated that to select the 2nd generation precursor ion of the peptides distinguish the individual contiguous β-aspratyl residues in peptide.

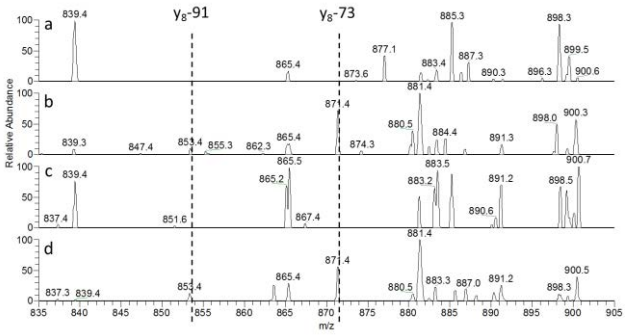

Fig. 1 Second generation product ion spectrums of four types of isomeric Asp residues in αAT11 synthetic peptides; a) αAT11 α-α, b) αAT11 α-β, c) αAT11 β-α and d) αAT11 β-β.

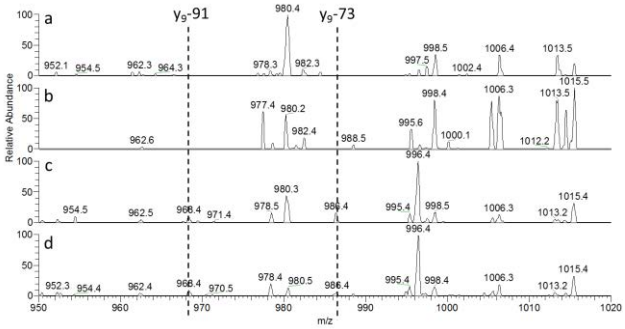

Fig. 2 Second generation product ion spectrums of four types of isomeric Asp residues in αAT11 synthetic peptides; a) αAT11 α-α, b) αAT11 α-β, c) αAT11 β-α and d) αAT11 β-β.

CONCLUSION: Identification of contiguous β-aspratyl residues in peptide has been establish by the analysis of the 2nd generation product ion spectrum of ion trap MS us.

REFERENCE:

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PR2-6 The Stability of D-β-Asp :kinetics of the Competitive Reactions of Isomerization and Peptide Bond Cleavage

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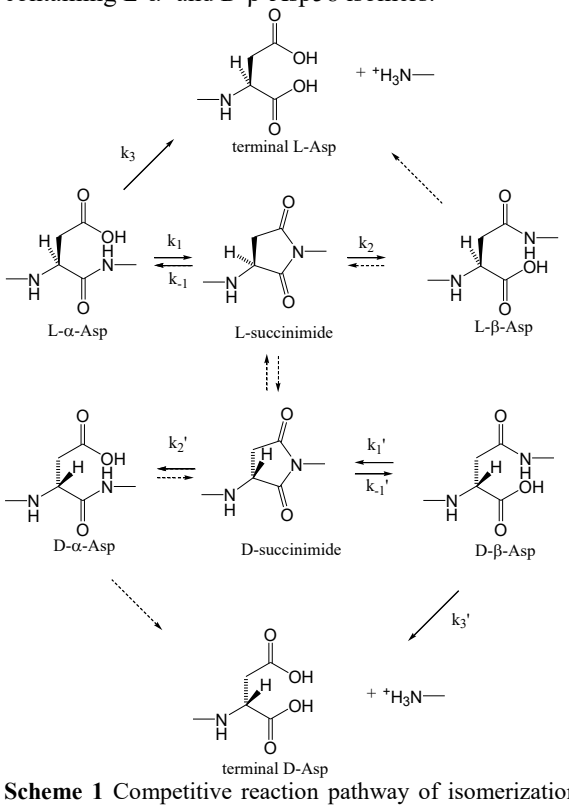
INTRODUCTION: It is well known that L-amino acids are main constituents of peptides and proteins in a living body. D-β-aspartyl (Asp) residue has been found in a living body such as aged lens crystalline [1]. Natural L-α-Asp isomerize to the uncommon D-β-Asp form via a succinimide intermediate. At Asp residue, isomerization and peptide bond cleavage compete with each other as shown Scheme 1. No information has been provided about these competitive processes because each reaction has been studied separately. In this study, the two competitive reactions, isomerization and peptide bond cleavage at Asp residue, were simultaneously observed and compared in αA-crystallin fragment, $S^{51}LFRTVLD^{58}SG^{60}$ containing L-α- and D-β-Asp58 isomers.



Scheme 1 Competitive reaction pathway of isomerization and peptide bond cleavage next to Asp.

EXPERIMENTS: Isomers of αA-crystallin fragment $S^{51}LFRTVLD^{58}SG^{60}$ composed of L-α- and D-β-Asp residues were synthesized by Fmoc solid-phase chemistry using an automated solid-phase peptide synthesizer (Shimadzu PSSM-8). The peptides were dissolved in 50 mM acetate buffer (pH 3.6 – 4.5) and incubated at 60 °C. Each solution was sampled typically after 0–120 h, and subject to RP-HPLC. The loss of reactants and the increase of products were quantified by using HPLC elution profiles.

RESULTS: In Figures 1A and 1B, the increase in the products of isomerization and peptide bond cleavage is quantified as a function of time, together with the decrease of the respective reactants, L-α- and D-β-Asp58. To quantify how each competitive reaction proceeds in L-α- and D-β- isomers, we have determined the rate constants ($k_{\pm1}$ ($k_{\pm1}$') for succimide formation, k_2 (k_2') for isomerization, k_3 (k_3') for peptide bond creavage) of isomerization and bond cleavage reactions at L-α- and D-β-Asp in accordance with the first-ordered reaction model as illustrated in Scheme 1. In Table 1, the obtained rate constants of isomerization and peptide bond cleavage of L-α- and D-β-Asp58 in an αA-crystallin fragment are summarized. The stability of D-β-Asp is because of the suppression of peptide bond cleavage and the enhanced reverse reaction from D-Suc to D-β-Asp after D-β-Asp is converted into D-Suc. Such specific reactivity at D-β-Asp residue is probably the reason for the gradual accumulation of abnormal D-β-Asp in a native αA-cystallin [2].

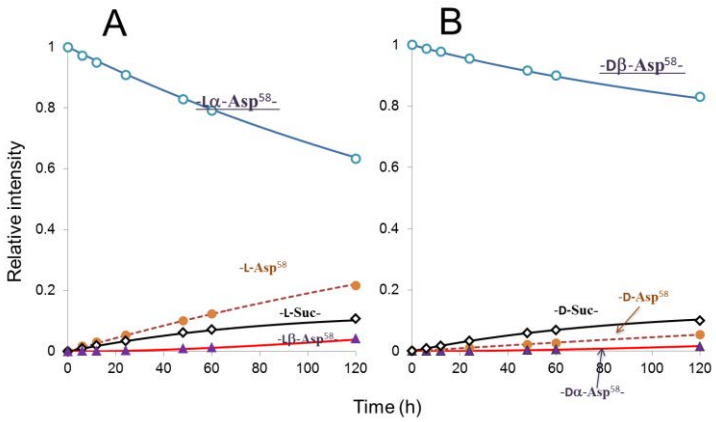


Figure 1 Change in the HPLC peak intensities accompanied by isomerization and peptide bond cleavage at (A) L-α- and (B) D-β-Asp58 at pH 4.0.

Table 1 Rate constants of isomerization and peptide bond cleavage.

	L-α-Asp58					D-β-Asp58				
pH	k_1	k_{-1}	k_2	k_3	pH	k_1',	k_{-1}',	k_2',	k_3',
3.6	0.7±0.1	n.d.	n.d.	3.6±0.1	3.6	0.8±0.1	n.d.	n.d.	1.2±0.1
4.0	1.8±0.1	2.9±0.9	5.5±0.2	2.3±0.1	4.0	1.6	7.9	2.1	0.5
4.5	1.5±0.1	6.6±1.1	11.0±0.2	1.1±0.1	4.5	1.1±0.1	13.5±1.4	3.8±0.1	0.14±0.01

In 10^{-3} h^{-1} unit.

Acknowledgment: The authors are grateful to Prof. N. Fujii of Research Reactor Institute, Kyoto University.

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PR2-7

Change of Protein Function by the Replacement of a Single Aspartyl Isomer in a Protein

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INTRODUCTION: Protein composed of exclusively L-amino acids. However, recent studies have demonstrated the presence of many D-aspartyl (Asp) -containing proteins in various aged tissues such as eye lens, brain, and skin. Lα-Asp residues in proteins can be non-enzymatically isomerized to abnormal Lβ-, Dα- and Dβ-Asp isomers under physiological conditions via formation of a succinimidyl intermediate over time. Isomerization of Asp is considered to be a trigger of protein denaturation because it induces elongation of the main chain and a different orientation of the side chain within the protein structure. However, previous studies have found no direct evidence of the effects of Asp isomers on protein function. Therefore, the production of Asp isomer-containing proteins is required to verify the effects of Asp isomerization. Here, we describe the process of obtaining an Asp isomer-containing protein using expressed protein ligation (EPL).

EXPERIMENTS: As a model protein, bovine pancreatic ribonuclease A (RNase A, 124 amino acid residues, EC 3.1.27.5), which catalyzes the cleavage of phosphodiester bonds in RNA, was used. In this study, Lα-Asp at position 121 in RNase A was replaced with Lβ-, Dα- and Dβ-Asp because aspartic acid at position 121 is located near the active site and related to RNA cleavage. We synthesize the recombinant RNase A (1-109) with intein at C-terminus by recombinant DNA technology and RNase A (110-124) containing Lα-, Lβ-, Dα- and Dβ-Asp at position 121 by chemical synthesis. The two segments of RNase A were combined by EPL to create the RNase A which was replaced with Lβ-, Dα- and Dβ-Asp at the position 121. The catalytic activity of RNase A toward 2',3'-cCMP was measured by monitoring the increase in absorbance at 284 nm.

RESULTS: It is the first time to create a protein containing four different Asp isomers[1]. The short segment of RNase A (110-124) was connected to the large segment of RNase A (1-109). The ligation efficiency did not differ among the different types of Asp isomer. Figure 1 shows the time course of the activity of the RNase A variants with different Asp isomers. The catalytic activity of Lα-RNase A was at the same level as commercially available RNase A (Sigma-Aldrich, St. Louis, MO, USA). On the other hand, replacement of the Lα-Asp isomer at position 121 with Lβ-, Dα- and Dβ-Asp decreased the RNase activity.

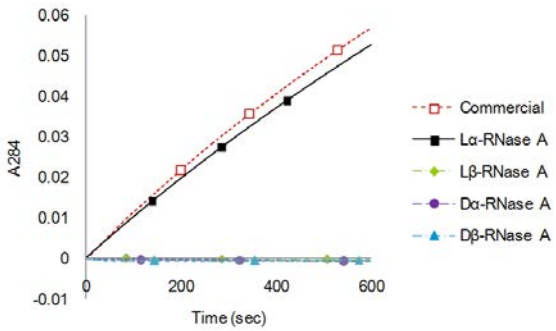

Figure 1. Comparison of catalytic activity among four RNase A proteins containing different Asp isomers.

The solubility of RNase A differed depending on the type of replacement Asp isomer. RNase A containing Lα-Asp and Dα-Asp was soluble in water at a final concentration of 1 mg/mL. By contrast, RNase A containing Lβ-Asp and Dβ-Asp showed very low solubility in water.

DISCUSSION

This study clearly indicated that the introduction of Asp isomers into a protein decreases protein function and leads to insolubilization. Our previous study demonstrated that the isomerization of one Asp residue in a partial peptide of αB-crystallin induced changes in secondary structure, hydrophobicity, and chaperone activity[2]. Molecular dynamics simulation also revealed that inversion of L-leucine to D-leucine at position 2 in the tetrapeptide Leu-Leu-Gly-Asp induced a change in three-dimensional structure[3]. These structural changes may be a trigger of protein dysfunction and insolubilization. Exploring the role of D-amino acids in proteins in an L-amino acid world opens up a new field of protein science and will be useful to understand the mechanism underlying the onset of age-related disease.

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