

PR4 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. This research program has started in 2013. In this year, the 6 research subjects were carried out. The allotted research subjects (ARS) are as follows;

ARS-1: Detection of D-aspartyl endopeptidases activity in the Ascidian (*Ciona intestinalis*). (T. Kinouchi and N. Fujii)

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

ARS-3: Separation condition of the peptides containing isomerized Asp residues after treatment of protein L-isoaspartyl methyltransferase (PIMT) (Y. Sadakane and N. Fujii)

ARS-4: Analysis of environmental stress-related hearing loss in mice and humans (N. Ohgami and N. Fujii)

ARS-5: Identification of biologically uncommon β -aspartyl residues in proteins using LC-MS/MS (N. Fujii, S. Kishimoto and N. Fujii)

ARS-6: D- β -Asp containing peptides are resistant to peptide bond cleavage compared with L- α -Asp containing peptides (K. Aki, E. Okamura)

ARS-7: One-shot LC-MS/MS analysis of deamidation of rat lens α - and β -crystallins induced by γ -irradiation (N. Fujii, I. Kim, T. Saito and T. Kanamoto).

Main Results and Contents of This Project

ARS-1: Kinouchi *et al.* detected D-Aspartyl endopeptidase (DAEP) activity in an experimental aquatic animal, the ascidian (*Ciona intestinalis*). The high DAEP activ-

ity in the ascidian was distributed in its reproductive tissues, but was quite low or not detectable in other organs. Since this activity was strongly inhibited by a synthesized DAEP inhibitor, it was suggested that the ascidian DAEP belongs to the same taxonomic family of the mammalian DAEP.

ARS-2: Saito *et al.* have irradiated rat lens tissue with 5 Gy γ -rays and determined the specific sites of the resulting oxidized amino acid residues (Cys 19, Cys 42, Trp 43, Met 44, Trp 69, Met 70, Met 102, Trp 157, Met 160, and Met 171) in γ E and/or γ F-crystallin.

ARS-3: Sadakane *et al.* prepared a repair enzyme for aged proteins, protein L-isoaspartyl methyltransferase (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. We became able to determine easily the kinetics of cyclization and de-cyclization of succinimide intermediate.

ARS-4: Ohgami *et al.* suggested that barium levels in biological samples could be a new risk factor for hearing loss, especially at high frequencies of 8 and 12 kHz, in humans. We are investigating whether barium-mediated hearing loss involves aggregation of a specific protein in the auditory neurons in inner ears.

ARS-5: Fujii *et al.* established a new method to identify the β -Asp containing peptides by the analysis of the 2nd generation product ion spectrum of liquid chromatography - tandem mass spectrometry (LC-MS/MS).

ARS-6: Aki *et al.* demonstrated that D- β -Asp in α -crystallin mimic peptide was less active to peptide bond cleavage than L- α -Asp by using real-time NMR. The trans conformer and low pKa of D- β -Asp side chain are thought to be the reason of less reactivity to peptide bond cleavage.

ARS-7: Fujii *et al.* determined the deamidation sites in crystallins after exposure to 5, 50, and 500 Gy of irradiated rat lenses by one-shot LC-MS/MS. The deamidation sites in rat irradiated crystallins resemble those reported in crystallins from human age-related cataracts. Thus, this study on deamidation of crystallins induced by ionizing irradiation may provide useful information relevant to the formation of human age-related cataracts.

PR4-1 Detection and Tissue Distribution of D-Aspartyl Endopeptidases Activity in the Ascidian (*Ciona intestinalis*)

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INTRODUCTION: We are researching a unique protease: D-Aspartyl Endopeptidase (DAEP), which we first discovered in the liver of mammals [1]. In these high body-temperature and long life span animals, DAEP seems to physiologically serve as a kind of quality-control system against the abnormal protein which contains D-isomer of Aspartate (D-Asp). It is strongly suggested that formation of D-Asp residue in proteins would eventually trigger the protein misfolding. Actually, D-Asp is detected in misfolded proteins of the age-related diseases (i.e., cataract, prion disease and Alzheimer's disease), and the formation of D-Asp in proteins is potentially noxious for the normal protein turnover in our cells and tissues. However, in searching a distribution of DAEP in various living things, except for mammals and birds, the activity could not be detected in livers, but rather in the reproductive tissue. For example, the tissue distribution of DAEP activity in the African clawed frog, *Xenopus laevis*, was remarkable in its testis and unfertilized eggs but was imperceptible in its liver. The trend mentioned above has been also observed in other non-vertebrates. As a result of searching for DAEP activity in Japanese green sea urchin, *Hemicentrotus pulcherrimus*, its high DAEP activity was also detectable in their testes, ovaries and unfertilized eggs. We therefore supposed that the original and primitive physiological functions of DAEP might be associated with fertilization and/or oocyte maturation and started examining the distribution of DAEP in an experimental aquatic animal, the ascidian, *Ciona intestinalis*.

EXPERIMENTS: Material> Wild and mature ascidians, *C. intestinalis* (were kindly gifted by Dr. Reiko Yoshida and Dr. Yutaka Satou, Kyoto University) were collected and then maintained in indoor tanks of artificial seawater at 18°C at the Maizuru Fisheries Research Station of Kyoto University.

Sample preparation> All subsequent operations were performed at 4°C. Surgically removed organs of the ascidian: heart, ovary, testis, stomach, intestine and genital duct were individually cut into fine pieces, homogenized by a small homogenizing tool, BioMasher[®] II, in 3 volumes of homogenization buffer (20 mM Tris-HCl (pH 8.5), 150 mM NaCl, 1 mM EDTA), and then centrifuged

at 100 × g for 5 min. The supernatant was centrifuged at 15,000 × g for 10min. The precipitate was suspended in 200 µl of solubilization buffer (20 mM Tris-HCl (pH 8.5), 150 mM NaCl, 0.25% CHAPS, 1 mM EDTA) and was sonicated for 30 sec by TOMY Ultrasonic Disruptor UD-200 (at output level 4 and 50% duty cycle). Sonicated samples were centrifuged at 15,000 × g for 10 min, and the supernatants were applied to the DAEP activity assay

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]. 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 MgCl₂) at 25°C. The fluorescence of aminomethylcoumarin liberated from Suc-D-Asp-MCA by DAEP was measured at λ_{ex} = 380 nm and λ_{em} = 460 nm.

RESULTS & DISCUSSION: The high DAEP activity in the ascidian was distributed in its ovary and testis, but was quite low or not detectable in other organs (Table). The stomach also seemed to have low DAEP activity. Since contents in the stomach consisted of the fed and undigested diatoms (*Chaetoceros gracilis*) which includes a large quantity of reddish-brown pigment, it was assumed that the observed data was not a result of the DAEP activity in the ascidian stomach, but caused by auto-fluorescence of the diatomaceous pigment. The DAEP activities in the ascidian ovary and testis were strongly inhibited by the addition of Zn²⁺ or a synthesized DAEP inhibitor (Benzyl-L-Arg-L-His-D-Asp-CH₂Cl) [1]-[2]. Considering this result with previous studies regarding the sensitivity of the mammalian DAEP against the inhibitor, it was suggested that the ascidian DAEP belongs to the same taxonomic family of the mammalian DAEP.

Table. Relative activity of DAEP in organs of the ascidian. Meaning of symbols in the table, -: not detectable, +: very low, +++: high, ++++: very high

Organs	Heart	Ovary	Testis	Stomach	Intestine	Genital duct
DAEP activity	-	+++	++++	+	-	-

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PR4-2 Damage to Biological Molecules Induced by Ionizing Irradiation and Biological Defense Mechanisms against Ionizing Radiation II

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INTRODUCTION:

Some organisms have biological defense mechanisms against ionizing radiation [1]. Investigating the details of damage induced by ionizing radiation to biological molecules such as protein, DNA, and lipids is essential to elucidate the defense mechanisms of these organisms. However, there have been very few reports on protein damage induced by ionizing radiation *in vivo*. Ionizing radiation decomposes water and generates free radicals and reactive oxygen species such as $\cdot\text{OH}$, O_2^- , and H_2O_2 , which can oxidize biological molecules [2]. Crystalline lens is excellent tissue for investigation of protein oxidation induced by external stresses, because its protein composition is simple. In this study, we irradiated rat lens tissue with γ -rays and analyzed the resulting oxidation of amino acid residues of γ -crystallin, which is considered to play an important role in maintaining the transparency and refractive index of the lens [3], by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

EXPERIMENTS:

γ -Irradiation to rat lenses:

Lenses isolated from male Wistar rats, 4-weeks old, in 50 mM sodium phosphate buffer (pH 7.4) and 150 mM NaCl were irradiated with γ -rays at a dose of 5 Gy and a dose rate of 2.8 Gy/h. γ -Irradiation was carried out at the Co-60 Gamma-ray Irradiation Facility of the Kyoto University Research Reactor Institute.

Extraction and enzymatic digestion of proteins from rat lenses:

γ -Irradiated rat lenses were homogenized by ultrasonication in 50 mM sodium phosphate buffer (pH 7.4), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mM EDTA. The samples were fractionated into water-soluble (WS) and water-insoluble (WI) fractions by centrifugation at $12,000 \times g$ for 20 min at 4°C. The WI proteins were dissolved in 8 M urea, 50 mM sodium phosphate buffer (pH 7.4), and 150 mM NaCl; the urea concentration was then diluted to less than 1 M in the same buffer (pH 7.4) and 150 mM NaCl. The

WS and WI proteins were digested with trypsin for 17 h at 37°C at an enzyme-to-substrate ratio of 1:50 (w/w).

Liquid chromatography-mass spectrometry analysis of tryptic peptides:

Peptides resulting from trypsin digestion were separated by nano-flow HPLC. MS analysis was carried out by alternating between full MS and MS/MS scans. MS/MS scans were performed using the collision-induced dissociation mode with dynamic exclusion function.

RESULTS:

In order to identify γ -irradiation-induced oxidation sites of amino acid residues in γ -crystallin, WS and WI proteins from γ -irradiated and non-irradiated rat lenses were digested with trypsin, and the resulting peptides were analyzed by LC-MS. The mass of the peptides were measured and analyzed by MS/MS, and then all peptides were identified using the Proteome Discover 1.0 software attached to the LC-MS system. In general, Met, Trp, and Cys are the amino acid residues susceptible to oxidation in proteins, and these residues can be converted into methionine sulfone (+16 mass as +10), methionine sulfoxide (+32 mass as +20), hydroxytryptophan (+16 mass as +10), and cysteine sulfenic acid (+16 mass as +10) or sulfinic acid (+32 mass as +20). γ -Irradiation at 5 Gy resulted in oxidation of Trp 69, Met 70, and Met 102 in γ E and/or γ F-crystallin in the WS fraction, and oxidation of Cys 19, Cys 42, Trp 43, Met 44, Trp 69, Met 70, Met 102, Trp 157, Met 160, and Met 171 in γ E and/or γ F-crystallin in the WI fraction. The oxidation of amino acid residues can cause structural and functional changes of proteins. The study on details of ionizing radiation-induced damage of biological molecules will provide useful information for investigations of the biological effects of ionizing radiation and the corresponding biological defense mechanisms.

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PR4-3 Separation Condition of the Peptides Containing Isomerized Asp Residues after Treatment of Protein L-Isoaspartyl Methyltransferase (PIMT)

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INTRODUCTION: The stereoconversion of aspartyl (Asp) residue arise through intramolecular rearrangement, such as *via* a succinimide intermediate. The native L-Asp residue in the protein was converted to the L-succinimide intermediate, and it is quickly hydrolyzed and produced the mixture containing L-Asp and L-isoAsp residue in a ratio of approximately 1:3. 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 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.

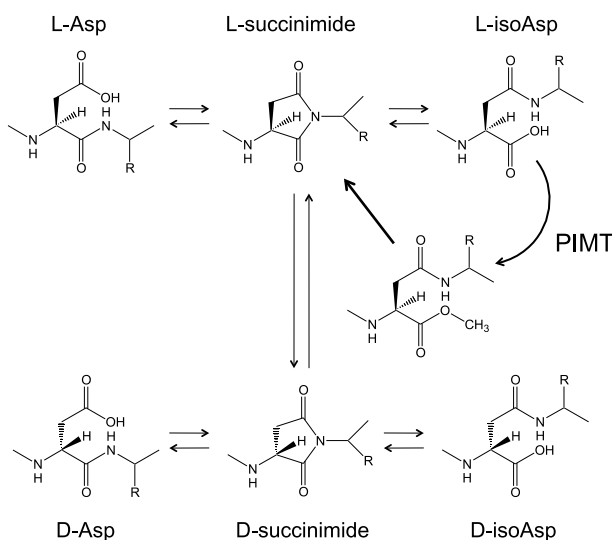


Fig. 1 The β -linkage isomerization and stereoinversion to D-form of aspartyl residue and PIMT repair system.

EXPERIMENTS: The recombinant PIMT protein was prepared by His-tag conjugated *E. coli* expression system, and the four isomerized peptides (HFSPEDLTVK) of crystalline (named as T10) were synthesized by using Fmoc amino acids. To be the substrate for PIMT, L-isoAsp was substituted for the aspartate residues. The T10 peptide bearing L-isoAsp residue was treated by PIMT for 60 min at 37 °C, and the reaction was stopped by addition of HCl. The reacted sample was analyzed by reversed-phase HPLC with acetonitrile/H₂O containing 15 mM phosphate buffer, detected by absorption at 215

nm. The peptides bearing methyl ester and succinimide were isolated and determined their molecular mass to identify the retention time of HPLC.

RESULTS: PIMT transfers the methyl group of S-adenosylmethionine onto a α -carboxyl group of L-isoAsp residue of T10 peptide, then resulted in generating the peptide bearing methyl ester. The methyl ester undergoes spontaneously and rapidly demethylation (30-60 min, half-time) to generate succinimide intermediate. The intermediate was hydrolyzed (3-20 hr, half-time) to produce the T10 peptide bearing L-Asp or L-isoAsp residue. To determine the amount of isomerized peptides above, we prepared the mixture of the T10 peptides bearing L-Asp, L-isoAsp, D-Asp and D-isoAsp, and PIMT-reacted solution, which included the peptide bearing methyl ester and succinimide. The mixture consisted of six peptides was separated by reversed-phase HPLC with 15 - 16 % acetonitrile with various pH of 15 mM phosphate buffer, and we found that the separation was accomplished in the condition using the eluent 15 % acetonitrile with pH 3.0 of 15 mM phosphate buffer (Fig. 2). We became able to determine easily the kinetics of cyclization and de-cyclization of succinimide intermediate.

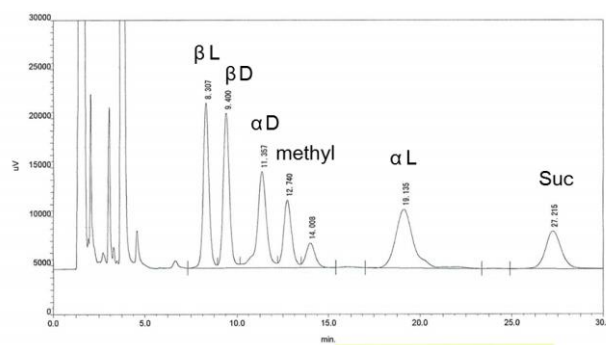


Fig 2 Separation of T10 peptides containing various isomerized Asp residue by standard HPLC. The mixture of six peptides were separated by reversed-phase HPLC with ODS column using the eluent 15 % acetonitrile with pH 3.0 of 15 mM phosphate buffer. β L: the peptide containing L-isoAsp residue, β D: D-isoAsp residue, α D: D-Asp residue, methyl: methyl ester, α L: L-Asp residue, Suc: succinimide.

PR4-4 Analysis of Environmental Stress-related Hearing Loss in Mice and Humans

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INTRODUCTION: Barium can be detected in tube well drinking water and foods including seaweeds or nuts. Thus, we eat or drink barium contained in drinking water and foods in our daily life [1]. However, it has not been recognized that the ingestion of barium contained in food and water can be a potential risk to our health. In a previous study, exposure to barium has been shown to impair physiological functions including blood pressure [2]. Our previous study further showed that exposure of mice to barium caused increased level of barium in inner ears, leading to hearing loss [3]. At present, however, it is not cleared about an association between barium and hearing loss in humans. Therefore, this study performed an epidemiological study in order to investigate an association of barium levels in biological samples and hearing levels in humans.

EXPERIMENTS: The study was performed for 145 subjects aged from 12 to 55 years who agreed in written form to participate in hearing examinations as previously described [4]. In short, this investigation was performed using a self-reporting questionnaire on smoking, age, clinical history, weight and height of subjects. The procedures were explained and informed consent was obtained from all of the subjects. This study was ethically approved by Nagoya University International Bioethics Committee following the regulations of the Japanese government (approval number 2013-0070). Auditory thresholds at frequencies of 1, 4, 8 and 12 kHz were measured by pure tone audiometry (PTA). We measured barium levels in biological samples by the method previously described [3]. In short, biological samples were put into a 15 ml polypropylene tube with 3 ml of HNO₃ (61%). The sample tubes were incubated at 80°C for 48 hours and then allowed

cool to room temperature for 1 hour. Then, 3 ml of H₂O₂ (30%) was added to each tube and the tubes were incubated at 80°C for 3 hours. After the samples had been diluted with ultrapure water, the Ba level in each sample was measured by using an inductively coupled plasma mass spectrometer (ICP-MS; 7500cx, Agilent Technologies, Inc.) with a reaction cell for absence of ArCl ion interference.

RESULTS: Our results showed hearing impairments in subjects with high barium levels in toenails. Our univariate analysis also suggested associations of barium levels in hair with hearing levels (1, 4, 8 and 12 kHz) in subjects. Multivariate analysis with adjustment for age, sex, BMI and smoking revealed that barium levels in hair had significant associations with hearing loss at 8 kHz (OR = 4.8; 95% CI: 1.4, 17.7) and 12 kHz (OR = 15.5; 95% CI: 4.0, 79.5).

Conclusions: Our study suggests that barium levels could be a risk factor for hearing loss in humans as well as in mice [4]. Results obtained in our previous study suggested that barium administered by drinking water specifically accumulates in inner ears resulting in severe impairments of hearing with degeneration of inner ears in mice [3]. A previous study has shown that exposure to a toxic element can cause aggregation of proteins resulting in neurodegeneration [5]. Therefore, we will further investigate whether exposure to barium causes aggregation of a specific protein in inner ears to clarify a mechanism of degeneration of auditory neurons.

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INTRODUCTION: We recently developed a method for four Asp isomers ($L\alpha$ -, $L\beta$ -, $D\alpha$ -, $D\beta$ -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 four 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. In this study, we developed an advanced method for identifying the β -Asp containing peptide using a LC-MS/MS.

EXPERIMENTS: The peptides containing four different Asp isomers were made using a Fmoc solid-phase chemistry. The synthetic peptides were human αA -crystallin sequence from residues 55 to 65 peptides ($\alpha AT6$ peptide) containing four different Asp isomers; TVL ($L\alpha$ -D)SGISEVR ($\alpha AT6$ - $L\alpha$), TVL($L\beta$ -D)SGISEVR ($\alpha AT6$ - $L\beta$), TVL($D\alpha$ -D)SGISEVR ($\alpha AT6$ - $D\alpha$), TVL($D\beta$ -D)SGISEVR ($\alpha AT6$ - $D\beta$).

Human lens was homogenized in buffer by ultrasonication and fractionated into water insoluble (WI) and water soluble fractions by centrifugation at 16,000 g for 20 min at 4 °C. The WI proteins were dissolved in 8 M urea, 50 mM Tris/HCl, pH 7.8, 1 mM $CaCl_2$, and then the urea concentration was diluted to less than 1 M. The denatured WI proteins were digested with trypsin for 17 h at 37 °C.

LC used a nano-flow HPLC system (Paradigm MS4, Michrom Bioresources). MS was performed on an ion trap system (LCQ Fleet, Thermo). The 1st and 2nd generation precursor ion of $\alpha AT6$ peptides were selected the $[M+2H]^{2+}$ ion at 588.5 m/z and the y_8 (DSGISEVR) fragment ion at 862.4 m/z.

RESULTS: Fig. 1 shows the 2nd generation product ion spectrum of $\alpha AT6$ peptide containing four different Asp isomers. The specific fragment ions of 789.6 m/z (y_8 -73) and 771.6 m/z (y_8 -91) were observed in the spectrum of $\alpha AT6$ - $D\beta$ and $\alpha AT6$ - $L\beta$. The y_8 -91 and y_8 -73 were observed in the 2nd generation product ion spectrum of β containing $\alpha AT6$ peptide (Fig 1b, d), while these product ions were not observed for α -Asp containing $\alpha AT6$ peptide (Fig 1a, c). These results indicated that the 2nd generation product ion of the peptides distinguish the β -Asp containing peptide from the α -Asp containing peptide.

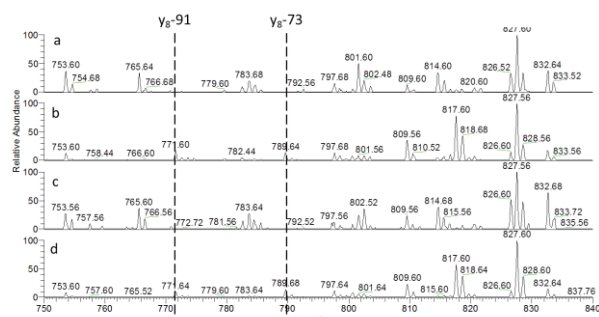


Fig. 1 Second generation product ion spectra of four isomeric Asp residues in $\alpha AT6$ synthetic peptides; a) $\alpha AT6$ - $L\alpha$, b) $\alpha AT6$ - $L\beta$, c) $\alpha AT6$ - $D\alpha$ and d) $\alpha AT6$ - $D\beta$.

Fig. 2 shows the mass chromatogram using a LC-MS/MS. Fig. 2a shows the set of mass chromatogram (588.0-589.5 m/z) of four Asp isomers $\alpha AT6$ synthetic peptides. Fig. 2b shows the set of mass chromatogram (789.0-790.0 m/z) of 2nd generation product ion spectrum of $\alpha AT6$ synthetic peptide containing four Asp isomers. The peaks of α -Asp ($L\alpha$, $D\alpha$) containing $\alpha AT6$ synthetic peptides were not observed by the mass chromatogram (Fig. 2b). Fig. 2c shows the set of mass chromatogram (588.0-589.5 m/z) of the $\alpha AT6$ peptide obtained from trypsin digestion of human lens protein. Fig. 2d shows the set of mass chromatogram (789.0-790.0 m/z) of 2nd generation product ion spectrum of $\alpha AT6$ peptide obtained from trypsin digestion of human lens protein. The peaks of α -Asp ($L\alpha$, $D\alpha$) containing $\alpha AT6$ peptide were not observed by the mass chromatogram (Fig. 2d).

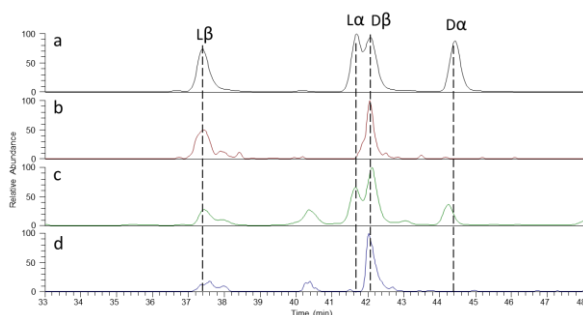


Fig. 2 Mass chromatograms (588.0-589.5 m/z) of LC-MS (a) $\alpha AT6$ synthetic peptides and (c) $\alpha AT6$ tryptic peptide of lens protein. Mass chromatograms (y_8 -73; 789.0-790.0 m/z) of second generation product ion of (b) $\alpha AT6$ synthetic peptide and (d) $\alpha AT6$ tryptic peptide of lens protein.

CONCLUSION: Biologically uncommon β -Asp containing peptides were identified by the analysis of the specific 2nd generation product ion of LC-MS/MS.

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PR4-6 D-β-Asp Containing Peptides are Resistant to Peptide Bond Cleavage Compared with L-α-Asp Containing Peptides

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INTRODUCTION: Natural L-α-Asp isomerize to the uncommon D-β-Asp form via a succinimide intermediate. D-β-Asp is responsible for the structural change of proteins or peptides, because D-isomers with different side-chain orientation and β-isomers which prolong main peptide bond can induce uncommon main chain structures, to trigger the abnormal unfolding or aggregation leading to a disease. D-β-Asp residues are accumulated in aged human-lens αA-crystallin [1] [2]. High resistance of D-β-Asp to biochemical reactions is predicted. However, no information has been provided about reactions such as bond cleavage next to D-β-Asp. In this study, spontaneous peptide bond cleavage next to Asp was compared between Asp isomers, by applying real-time solution-state NMR to eye lens αA-crystallin 51–60 fragment

(αA51–60), S⁵¹LFRTVLD⁵⁸SG⁶⁰ consisting of L-α- and αB-crystallin 61–67 analog (αB61–67), F⁶¹D⁶²TGLSG⁶⁷ consisting of L-α- and D-β-Asp 58 and 62, respectively.

EXPERIMENTS: Peptide synthesis Isomers of αA51–60 and αB61–67 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).

Real-time NMR measurement Real-time ¹H-NMR measurements were carried out on 400 MHz spectrometer (JEOL ECA400) equipped with a super-conducting magnet of 9.4 T. A high sensitivity probe (JEOL, NM40T10A/AT) for 10-mm diameter tube was used. About 3 mg of αA51–60 and αB61–67 consisting of L-α- or D-β-Asp residue was dissolved in 4 ml of 50 mM acetate buffer/D₂O (pD 4.0) and subject to NMR measurement at 70 °C. Free induction decays were accumulated at 512–1024 times, corresponding to 1–2 h intervals. Amounts of reactant and product peptides were quantified by using integral intensities of the respective NMR signals. For αA51–60, the signals of Asp58 H_α and Ser59 H_β, for αB61–67 Asp62 H_α and Thr63 H_γ were used [3].

side-chain conformers The population of staggered side-chain conformers (trans, gauche⁺ and gauche⁻) of αA51–60 and αB61–67 composed of L-α- and D-β-Asp residues was evaluated from vicinal spin-spin coupling constants H_α-H_{β1} (*J*_{αβ1}) and H_α-H_{β2} (*J*_{αβ2}) obtained by high-resolution ¹H NMR measurement at 10 °C [3] [4].

RESULTS: The reactivity of D-β-Asp residue to peptide bond cleavage was different from that of L-α-Asp in both peptides. When the cleavage reaction was treated as first-ordered, rate constants were estimated to be (6.9±0.5)×10⁻³ h⁻¹ for L-α-Asp58 and (2.2±0.4)×10⁻³ h⁻¹ for D-β-Asp58 in αA51–60. Similarly, the rate constants in αB61–67 were estimated (4.7±0.9)×10⁻³ h⁻¹ for L-α-Asp62 and (1.3±0.2)×10⁻³ h⁻¹ for D-β-Asp62 [3]. Uncommon D-β-Asp was less active to peptide bond cleavage than L-α-Asp residue. We consider why such difference in reactivity was found in L-α- and D-β-Asp residues. One is the difference in side chain conformers between L-α- and D-β-Asp isomers. The cleavage reaction proceeds as: (i) when the carbonyl carbon (C_{CO}) of Asp peptide bond is attacked by hydroxyl oxygen of carboxylic side chain (O_{COOH}), a cyclic anhydride intermediate is formed; (ii) another fragment is also cleaved as a result of intramolecular cyclization; and (iii) the fragment including C-terminal Asp is finally produced by hydrolyzing cyclic anhydride intermediate. D-β-Asp58 and D-β-Asp62 more than 50% of the Asp side-chain is trans. The trans conformer of D-β-Asp side chain is thought to be disadvantageous for such reaction pathway because the long distance between C_{CO} and O_{COOH} interferes with the attack of O_{COOH} on C_{CO} to initiate intramolecular cyclization. Second reason is that p*K*_a of α-Asp (p*K*_a 4.0) and β-Asp (p*K*_a 3.3) side chain carboxyl is different [5]. At pD 4 in this work, the carboxyl group of D-β-Asp side chain is more ionized than L-α-Asp. When the bond cleavage undergoes from unionized state of carboxyl, the ionized carboxylate in D-β-Asp side chain is disadvantageous for the cleavage reaction.

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PR4-7 One-shot LC-MS/MS Analysis of Deamidation of Rat Lens α - and β -crystallins Induced by γ -irradiation

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INTRODUCTION: The eye lens is a transparent organ that functions to focus light and images on the retina. The transparency and high refraction of the lens are maintained by the ordering of fiber cells and stable-long-lived proteins, comprising α -, β -, and γ -crystallins. Because the lens crystallins are long-lived proteins, they undergo various modifications including isomerization, inversion, deamidation, oxidation, glycation and truncation. These modifications are generated by ionizing irradiation, UV light and oxidative stress, and decrease lens transparency and ultimately lead to the development of age-related cataracts. Of the modifications, deamidation induces the negative charge into proteins and alter protein-protein interactions. There are many data on the deamidation sites of lens crystallins from age-related cataract, but there are few studies of the specific sites of deamidation in young rat lens crystallins that have been subjected to γ -irradiation. Thus we demonstrate the deamidation sites in rat crystallins after exposure to 5, 50, and 500 Gy of irradiation by one-shot LC-MS/MS.

EXPERIMENTS:

Application of γ -irradiation to rat lenses. Rat (Wistar rats, 4-weeks old, male) lenses in 50 mM sodium phosphate buffer (pH 7.4), 150 mM NaCl were irradiated with γ -rays at a dose of 5, 50 Gy or 500 Gy and a dose rate of 33.8 Gy/h. γ -Irradiation was carried out at the Co-60 γ -ray Irradiation Facility of the Kyoto University Research Reactor Institute.

Preparation of lens proteins. Rat lenses were homogenized and fractionated into water soluble (WS) and water insoluble (WI) fractions by centrifugation. The WI proteins were dissolved in 8 M urea for 1 h and then the final concentration of urea was diluted to less than 1 M in a buffer pH 7.8. The WS and WI proteins were digested with trypsin for 17 h at 37 °C at an enzyme-to-substrate ratio of 1:50 (w/w). The resulting peptides are applied to LC-MS/MS according to the previous method and analyzed by Proteome Discoverer 1.0 software. [1].

RESULTS:

Since deamidation of Asn or Gln residues generates Asp or Glu residues with an increase of 1 Da in the molecular mass of the tryptic peptide, LC-MS/MS analysis is very useful for the analysis of the deamidated sites in proteins. Figure 1 shows a typical example of the deamidation of

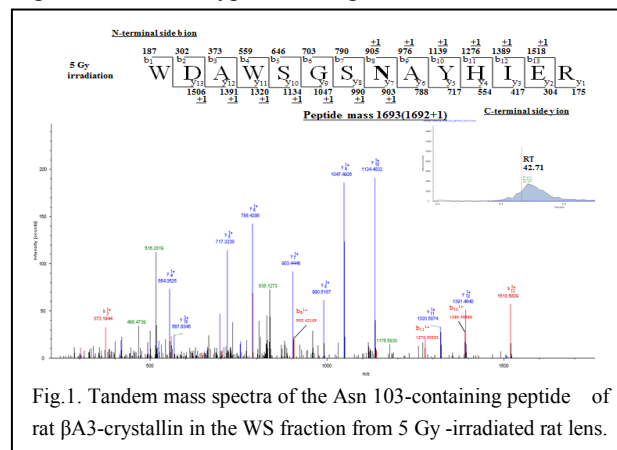


Fig.1. Tandem mass spectra of the Asn 103-containing peptide of rat β A3-crystallin in the WS fraction from 5 Gy-irradiated rat lens.

the Asn103 in β A3-crystallin 96-109 peptide (WDAWSGSAAYHIER, $[M+2H]^{2+} = 1692$) after 5Gy irradiated samples. The mass of the b1-b7 ions of the Asn 103-containing peptide shows the theoretical mass, but the mass of the b8-b13 ions was 1 Da greater in the 5 Gy-irradiated samples. Similarly, the mass of the y1-y6 ions shows the theoretical mass, whereas that of all y ions after y7 (i.e., y7-y13) was 1 Da greater for the irradiated peptides. The inset of Fig. 1 shows the elution time of deamidated peptides on LC. The elution time of the deamidated peptide (42.71 min) is different from the non-deamidated peptide (41.03 min). These results clearly indicate that the Asn 103 residue of β A3-crystallin was deamidated to an Asp residue by exposure to more than 5 Gy of γ -irradiation. Similarly, we found many deamidated sites in α -, β -crystallins from WI fractions.

CONCLUSION: The deamidation sites in rat irradiated crystallins resemble those reported in crystallins from human age-related cataracts. Thus, this study on deamidation of crystallins induced by ionizing irradiation may provide useful information relevant to the formation of human age-related cataracts.

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