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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 floral tissues of broccoli (*Brassica oleracea* var. *Italica*). (T. Kinouchi and N. Fujii)

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

ARS-3: Analysis of aspartate isomerization using protein L-isoaspartyl methyltransferase (PIMT) (Y. Sadakane and N. Fujii)

ARS-4: Analysis of imbalance in mice exposed to environmental stress. (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: Sidechain conformers of aspartyl isomers in crystallin mimic peptide (K. Aki, E. Okamura)

ARS-7: Rapid survey of Asp isomers in disease-related proteins by LC-MS/MS combined with Commercial Enzymes (H. Maeda, T. Takata, N. Fujii, H. Sakaue, H. Sasaki and N. Fujii)

Main Results and Contents of This Project

ARS-1: Kinouchi et al. detected high D-aspartyl endopeptidase (DAEP) activity in the floret of fresh broccoli (*Brassica oleracea* var. *Italica*). DAEP is commonly distributed in animals, and their testes and ovaries are especially shown to have the high DAEP activity. Broccoli floret is a cluster of numerous small flowers and also encloses the reproductive organs. Since DAEP activity in other parts of plants, such as stems, leaves and roots, was not detectable or quite low, it was suggested that the physiological function of DAEP would contribute to the early development.

ARS-2: Saito et al. revealed that carotenoids have no effect on the initial rapid peroxidation during the process of damage to lipid induced by gamma irradiation, but an optimum concentration of carotenoids inhibits the subsequent oxidative degradation involving radical reactions during this process.

ARS-3: Sadakane et al prepared a repair enzyme for aged proteins, protein L-isoaspartyl methyltransferase (PIMT) by E. coli expression system, and analyzed the effect of substrate size on the activity of PIMT. PIMT activity is affected by length of peptides and the activity is weaker in the shorter peptide. However, the peptides which consist of 5 to 9 amino acids are good substrates for PIMT regardless the position of L-isoAsp. The feature is useful to analyze various types of peptide because the shorter peptides are easily prepared.

ARS-4: Ohgami et al. showed that exposure of mice to low frequency noise (LFN) at moderate levels causes imbalance and tried to detect protein aggregation in vestibule of inner ears. Our results suggest that incidence of protein aggregation was undetectably low at least in the exposure condition to moderate LFN.

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. investigated populations of side chain conformers of Asp isomers (L- α -, D- α -, D- β -Asp) in α A-crystallin mimic peptides by using solution NMR. As a result, trans conformer was preferred in D- β -Asp, whereas gauche conformer was abundant in L- α - and D- α -Asp. Trans is thought to be disadvantageous to Asp isomerization. Therefore, the D- β -Asp is thought to be the most stable in the 3 isomers.

ARS-7: Fujii et al. demonstrated a new method for rap-

**PR10-1 Detection of D-Aspartyl Endopeptidases Activity in Floral Tissues of Broccoli
(*Brassica oleracea* var. *Italica*)**

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INTRODUCTION: D-isomer of aspartate (D-Asp) residue is detected in abnormally folded and aggregated proteins: i.e., crystalline, prion protein and β -amyloid protein. Accordingly, it is suggested that the formation of D-Asp in those proteins is responsible for the related diseases: cataract, prion disease and Alzheimer's disease, respectively. The D-aspartyl endopeptidase (DAEP), which we identified from mammalian liver, stereoselectively degrades its substrate at the internal D-Asp residue, and seems to physiologically serve as a scavenger against the noxious D-Asp containing-protein and to maintain the normal protein turnover [1]-[3]. However, the distribution of DAEP in other living-things was not as clear as in animals. As a result of searching the distribution of DAEP in various animals, reproductive organs were shown to have the high DAEP activity as a common feature. Especially this tendency was evident among aquatic animals, for example, in African clawed frogs (*Xenopus laevis*) and Japanese green sea urchins (*Hemiacentrotus pulcherrimus*). The high DAEP activity was practically detectable in their testes, ovaries and unfertilized eggs.

On the other hand, the existence of DAEP in plants is ambiguous. Considering the distribution of DAEP in animals, we searched the DAEP activity in the reproductive organ such as a floral tissue of plants.

EXPERIMENTS: Plant Material> Fresh broccoli (*Brassica oleracea* var. *Italica*) was purchased from a local supermarket and the floret was separated. After any damaged parts were removed from the floret, the remainder was cut into fine pieces and then homogenized by Polytron® PT2100 agitator in ice-cold sodium phosphate buffer (pH 7.0). 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 into it.

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 biological materials 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 $\lambda_{ex} = 380$ nm and $\lambda_{em} = 460$ nm.

RESULTS & DISCUSSION: As described in this last report, radish taproots were shown to have the DAEP activity but in quite low amounts [4]. And in tomato fruits, the DAEP activity was not detectable because endogenous pigments in the extract of tomato fruits would disturb the measurement. Although the supernatant extracted from broccoli floret also had a high amount of the green pigment, the DAEP activity showed 15 times higher than in radish taproots.

In this report, four broccoli florets were used for the DAEP assay, and each measured value of the specific activity was not stable ($\sim \pm 20\%$). Since each broccoli was purchased on different day, the different storage periods of those broccoli florets may have affected the postharvest senescence. Therefore we would like to make the next issue to observe chronological changes of the DAEP activity in plant embryo.

Table. Specific activities of DAEP in various samples.

Samples (tissue)	Specific activity (% of max in frog)
African clawed frog (Ovary)	100
Radish (taproot)	~ 1.5
Broccoli (floret)	~ 30

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INTRODUCTION: Some bacteria exhibit extreme resistance to ionizing radiation [1]. A common feature of these bacteria is that they contain red carotenoid pigments [1]. Colorless mutants of these radioresistant bacteria are more sensitive to gamma irradiation than wild types [1]. Therefore, carotenoids are thought to be involved in the bacterial defense mechanisms against ionizing radiation [1]. Biological effects induced by low-linear energy transfer ionizing radiation are mainly attributed to radicals generated by radiolysis. Carotenoids have high radical scavenging activity, and they are localized in cell surface lipids in prokaryotes. These facts indicate that carotenoids are likely to defend the cell surface lipids of radioresistant bacteria against ionizing radiation.

When considering the biological defense mechanism of these radioresistant bacteria against ionizing radiation, it is important to elucidate the effects of carotenoids on damage to biological molecules, especially biological lipids. In this study, we analyzed the effect of two typical carotenoids, β -carotene and astaxanthin, on the oxidative degradation as well as peroxidation of biological lipids, α -linolenic acid, induced by gamma irradiation.

EXPERIMENTS: Sample Preparation: α -Linolenic acid was dissolved in benzene at a final concentration of 5.0×10^{-1} M, and β -carotene and astaxanthin were added at a final concentration of 5.0×10^{-6} to 5.8×10^{-3} M and 5.0×10^{-8} to 5.0×10^{-4} M, respectively. Gamma Irradiation: The prepared solutions were irradiated with ^{60}Co gamma rays at a dose of 30 kGy and a dose rate of 400 Gy/min. Analysis of Oxidative Degradation of α -Linolenic Acid: The method described by Buege and Aust was used with some modifications [2]. TCA-TBA-BHT-HCl reagent (15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.04% butylated hydroxytoluene, and 0.25N hydrochloric acid) was prepared. The gamma irradiated sample was diluted 50-fold with benzene. The diluted solution (3.0 mL) was evaporated under reduced pressure. The resulting residue was dispersed in 9.0 mL of PBS(-) with a sonicator. The dispersed solution (1.0 mL) was combined with 2.0 mL of TCA-TBA-BHT-HCl reagent and thoroughly mixed. The mixed solution was heated in a boiling water bath for 15

min, and absorption at 535 nm was measured. The amount of malondialdehyde (MDA) formed was calculated using the molar absorption coefficient (ϵ) of the color substance formed by the reaction (i.e., 1.56×10^5 at 535 nm). Thus, the level of oxidative degradation of α -linolenic acid was evaluated by measuring the amount of MDA. Analysis of Peroxidation of α -Linolenic Acid: The method described by Kennedy and Liebler was used with some modifications [3]. The gamma-irradiated samples were diluted 600-fold with *n*-hexane, and then 5.0 mL of this solution was evaporated under reduced pressure. The resulting residue was dissolved in 5.0 mL of *n*-hexane, and the average absorbance of this solution in the 230–236 nm range, which was derived from the conjugated diene formed, was measured. In this study, the level of peroxidation of α -linolenic acid was evaluated by determining the relative amount of conjugated diene formed.

RESULTS: The analyses revealed that 8.5×10^{-3} M β -carotene and 5.0×10^{-4} M astaxanthin inhibited gamma radiation-induced oxidative degradation of α -linolenic acid; in contrast, 5.0×10^{-5} and 5.0×10^{-6} M β -carotene, and 5.0×10^{-7} and 5.0×10^{-8} M astaxanthin promoted its degradation. On the other hand, β -carotene and astaxanthin did not affect gamma radiation-induced peroxidation of α -linolenic acid. These facts indicated that carotenoids have no effect on the rapid initial peroxidation during the process of damage to lipid induced by gamma irradiation, but an optimum concentration of carotenoids inhibits the subsequent oxidative degradation during this process. The present study suggests that radioresistant bacteria possess the biological defense mechanism involving the scavenging of ionizing radiation-induced radicals in lipid regions, such as cell membranes, by red carotenoid pigments, thereby protecting biological lipids and other biomolecules in the vicinity of the carotenoid against damage induced by radical reactions.

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INTRODUCTION: 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).

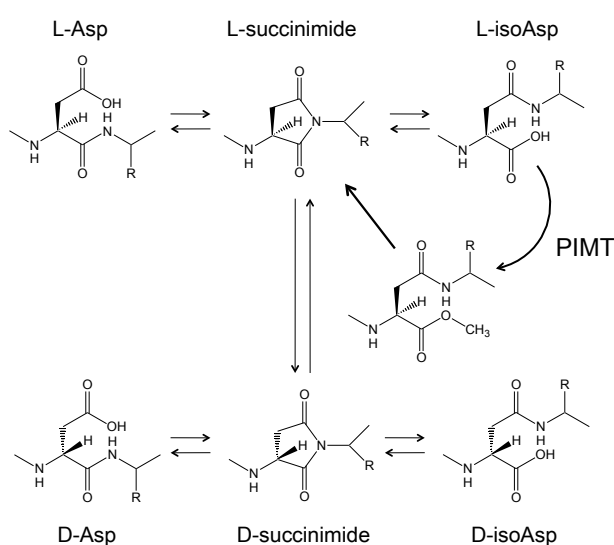


Fig. 1 The β -linkage isomerization and stereoinversion to D-form of aspartyl residue and PIMT repair system.

In this study, we prepared PIMT by *E. coli* expression system, and analyzed the effect of various length of substrate on the activity of PIMT.

EXPERIMENTS: The recombinant PIMT protein was prepared by His-tag conjugated *E. coli* expression system, and the various lengths of peptide fragments were synthesized by using Fmoc amino acids. To be the substrate for PIMT, L-isoAsp was substituted for the aspartate residues in these peptides. The PIMT activity was measured by HPLC analysis of S-adenosyl homocysteine (SAH), which was demethylated product of co-substrate S-adenosyl methionine for PIMT.

RESULTS: The various lengths of peptide bearing

L-isoAsp were incubated in recombinant PIMT with co-substrate S-adenosyl methionine and the production of SAH was analyzed by reversed-phased HPLC with 4 % acetonitrile solving in pH 4.6 acetate buffer (Fig. 2).

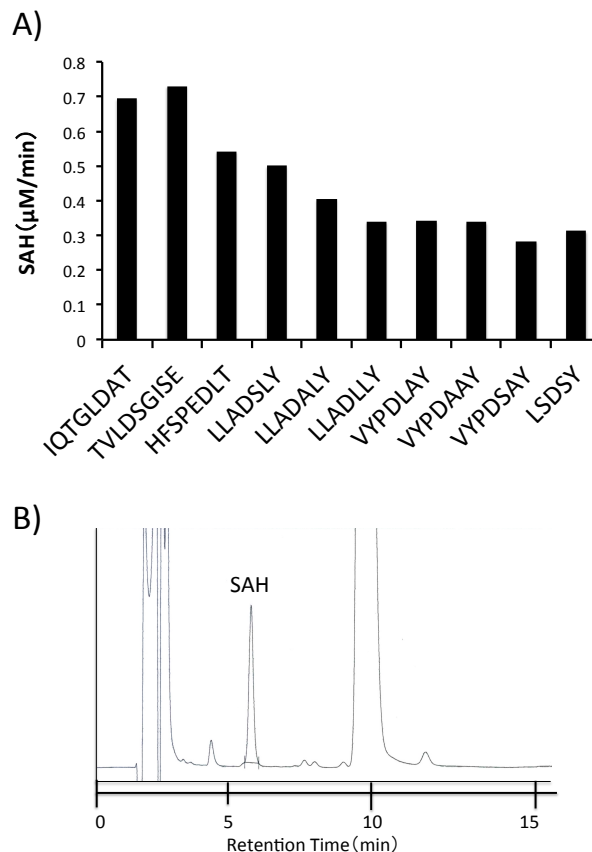


Fig 2 Effect of substrate length on the PIMT activity (A) and the determination of PIMT activity by HPLC (B). Various lengths of peptide were incubated with PIMT and the generation rates of S-adenosyl homocysteine (SAH) were determined by HPLC analysis.

The HPLC profile (Fig 2B) ensures quantitative analysis of SAH. Fig 2A shows that PIMT activity is affected by length of peptides and the activity is weaker in the shorter substrate. However, the peptides which consist of 9 to 5 amino acids are good substrates for PIMT regardless the position of L-isoAsp. Our recombinant PIMT is able to repair the L-isoAsp residue in the short peptide that consists of five amino acids. The feature is useful to analyze various types of peptide because the shorter peptides are easily prepared.

PR10-4 Analysis of Imbalance in Mice Exposed to Environmental Stress

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INTRODUCTION: Exposure to noise generated in occupational and daily environments is one of the risk factors threatening our health [1,2]. Although noise contains sound with broad frequencies, there is very limited information about the frequency-dependent influence of noise on health. Low frequency noise (LFN) is generated from industrial devices and home electrical appliances at all times. Thus, we are exposed to LFN generated from various devices on a daily basis. LFN is defined as noise having the frequency range below 100 Hz [3]. On the other hand, inner ears contain the vestibule as well as the organ of Corti. Vestibular hair cells covered with otoconia to sense gravity stimulus. Impairments of vestibular hair cells have been shown to cause imbalance [4]. Meanwhile, excessive exposure to noise at 1-20 kHz which is audible sound for mice and humans has been shown to induce damage of hair cells with enhanced oxidative stress in the organ of Corti in the inner ear in mice. Also, increased oxidative stress has been shown to cause aggregation of proteins in central nervous system. At present, however, most of the previous studies used broadband noise with no consideration of specific frequencies. Our previous study has shown that chronic exposure to LFN at moderate levels causes imbalance involving morphological impairments of the vestibule with enhanced levels of oxidative stress and positive signals stained by anti-D-beta-Asp antibody. However, there is limited information about whether exposure to LFN can cause protein aggregation in vestibular hair cells. In this study, therefore, we exposed mice to LFN (100 Hz) for exposure of mice to noise in order to detect the protein aggregation in vestibules caused by LFN stress.

EXPERIMENTS: Randomly bred wild-type mice

(ICR) at 6 weeks of age were used for exposure experiments. All experiments were authorized by the Institutional Animal Care and Use Committee in Nagoya University (approval number: 27241) and Chubu University (approval number: 2610016) and followed the Japanese Government Regulations for Animal Experiments. Mice were continuously exposed for 1 month to LFN as previously reported [5]. In order to morphologically detect protein aggregation, thioflavin-S staining was performed as previously reported [6].

RESULTS: After exposure for one month to LFN, behavior analyses including rotarod, beam-crossing and footprint analysis showed impaired balance in LFN-exposed mice but not in non-exposed mice. In contrast, thioflavin-S staining of vestibule in inner ears did not show positive signals in exposed mice at least in this exposure condition. Immunohistochemistry with anti-alpha-synuclein also did not show typical pattern of the aggregation. Our results suggest that incidence of protein aggregation was undetectably low at least in this exposure condition to moderate LFN. At present, output level of LFN is limited in our LFN generator system. Therefore, we will renew the generator system to output LFN at larger level. Also, we will set up an organ culture system of inner ears to directly expose to LFN. Sensitivity of thioflavin-S staining is affected by fixation conditions once in a while. Further study is needed to improve the detection system of protein aggregation in inner ears.

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PR10-5 Identification of Biologically Uncommon β -aspartyl Residues in Proteins Using MS

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INTRODUCTION: We recently developed a new method for determining peptides containing Asp isomers at individual sites and for detecting inverted Asp residues in any protein by using a liquid chromatography - tandem mass spectrometry system [1]. In order to determine which types of Asp isomer are present, however, standard peptides containing L α , L β , D α , D β isomers must be synthesized and their retention times on LC-MS must be compared with those of the sample peptides. The synthesis of the standard peptide contain 4 different Asp isomers per one peptide is time-consuming. Hence, we developed an advanced method for rapidly identifying the Asp isomers by detecting the specific 2nd generation product ions for the β -Asp containing peptides using an ion trap mass spectrometer (ion trap MS).

EXPERIMENTS: The peptides containing four different Asp isomers were made using a Fmoc solid-phase chemistry. The synthetic peptides were human α A-crystallin sequence from residues 146 to 157 peptides (α AT18 peptide) containing four different Asp isomers; IQTGLD(L α -Asp)ATHAER (α AT18 L α -Asp), IQTGLD(L β -Asp)ATHAER (α AT18 L β -Asp), IQTGLD(D α -Asp)ATHAER (α AT18 D α -Asp), IQTGLD(D β -Asp)ATHAER (α AT18 D β -Asp) and human α B-crystallin sequence from residues 57 to 69 peptides (α BT4 peptide) containing four different Asp isomers; APSWFD(L α -Asp)TGLSEMR (α BT4 L α -Asp), APSWFD(L β -Asp)TGLSEMR (α BT4 L β -Asp), APSWFD(D α -Asp)TGLSEMR (α BT4 D α -Asp), APSWFD(D β -Asp)TGLSEMR (α BT4 D β -Asp).

A mass spectrometry (MS) was performed on an ion trap system (LCQ Fleet, Thermo). The 1st and 2nd generation precursor ion of α AT18 peptides were selected the $[M+2H]^{2+}$ ion at 656.3 m/z and the y_7 fragment ion at 799.3 m/z. The 1st and 2nd generation precursor ion of α BT4 peptides was selected the $[M+2H]^{2+}$ ion at 748.8 m/z and the y_8 fragment ion at 908.4 m/z.

RESULTS: Fig. 1 shows the 2nd generation product ion spectrum of α AT18 peptide containing four different Asp isomers. The specific fragment ions of 726 m/z (y_7 -73) and 708 m/z (y_7 -91) were observed in the spectrum of α AT18 D β -Asp and α AT18 L β -Asp. Fig. 2 shows the 2nd generation product ion spectrum of α BT4 peptide containing four different Asp isomers. The specific fragment ions of 835 m/z (y_8 -73) and 817 m/z (y_8 -91) were observed in the spectrum of α BT4 D β -Asp and α BT4 L β -Asp. The y_7 -91 and y_7 -73 were observed in the 2nd generation product ion spectrum of β -Asp containing

α AT18 peptide (Fig 1a, c), while these product ions were not observed for α -Asp containing α AT18 peptide (Fig 1b, d). In addition, the y_8 -91 and y_8 -73 were also observed in the 2nd generation product ion spectrum of β -Asp containing α BT4 peptide (Fig 2a, c) but not for α -Asp containing α BT4 peptide (Fig 2b, d). These results clearly indicated that the $y_{(l-n+1)}-91$ and $y_{(l-n+1)}-73$ ions ("l" and "n" represent the length of the peptide and the position of the Asp residue) of the 2nd generation product ion of the peptides distinguish the β -Asp containing peptide from the α -Asp containing peptide.

CONCLUSION: Identification of biologically uncommon β -Asp containing peptide has been established by the analysis of the 2nd generation product ion spectrum of ion trap MS.

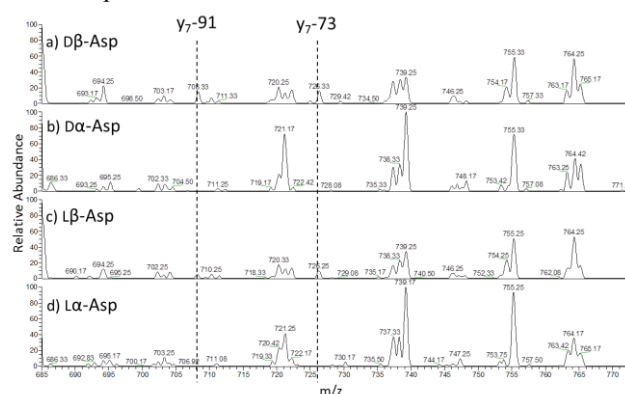


Fig. 1. Second generation product ion spectra of four isomeric Asp in α AT18 peptide. a) α AT18 D β -Asp, b) α AT18 D α -Asp, c) α AT18 L β -Asp, d) α AT18 L α -Asp

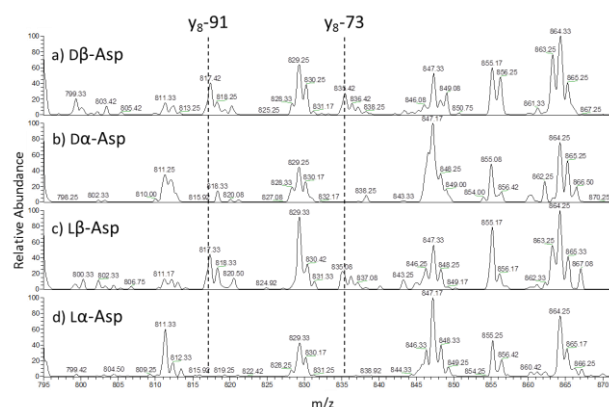


Fig. 2. Second generation product ion spectra of four isomeric Asp in α BT4 peptide. a) α BT4 D β -Asp, b) α BT4 D α -Asp, c) α BT4 L β -Asp, d) α BT4 L α -Asp

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INTRODUCTION: D-β-aspartyl (Asp) residues are accumulated in aged human-lens αA-crystallin [1] [2]. 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. Previous studies have suggested that D-β-Asp is more stable than L-α-Asp in human-lens αA-crystallin peptides [3]. However, it remains unsolved why the β-Asp is stable as compared to α-Asp in such peptides.

In this study, the stability between α- and β-Asp forms in the peptide is discussed in relation to the population of side-chain conformers (trans (T), gauche⁺ (G⁺) and gauche[−] (G[−])) of Asp isomers. By using human-lens αA-crystallin fragment, (T6f: T⁵⁵V CF₃-Phe D⁵⁸SGISEVR⁶⁵) composed of L-α-, D-α-, and D-β-Asp 58 residues, the vicinal spin-spin coupling constants (*J*) of Asp 58 H_α-H_{β1} (*J*_{αβ1}) and Asp 58 H_α-H_{β2} (*J*_{αβ2}) are quantified and compared by high-resolution solution NMR to calculate the population of side-chain conformers. Here, the difference in the population of side chain conformers between L-α-, D-α-, and D-β-Asp is interpreted by how easily the succinimide is formed.

EXPERIMENTS: The isomers of T6f in which L-α-Asp was replaced with D-α- and D-β-Asp at position 58 were synthesized by using an automated solid-phase peptide synthesizer (Shimadzu PSSM-8)[4]. Proton-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 o.d. sample tube was used. About 2 mg of T6f peptides was dissolved in 4 ml PBS/D₂O (pD 7.6) and subject to NMR measurement at 10-60 °C. Free-induction decays were accumulated 2048 times. The digital resolution was as high as 0.02 Hz to obtain the coupling constants with high accuracy[4]. The coupling constants of Asp H_α-H_{β1} (*J*_{αβ1}) and H_α-H_{β2} (*J*_{αβ2}) were evaluated by using Asp 58 H_β at 2.5–2.9 ppm. Using *J*_{αβ1} and *J*_{αβ2} obtained by the high-resolution 1H NMR measurement, the population of side-chain conformers of Asp in T6f isomers is calculated by the following equations [5].

$$J_{\alpha\beta1} = P(T)J_t + P(G^+)J_g + P(G^-)J_g$$

$$J_{\alpha\beta2} = P(T)J_g + P(G^+)J_t + P(G^-)J_g$$

$$P(T) + P(G^+) + P(G^-) = 1$$

Here *P*(*T*), *P*(*G*⁺), and *P*(*G*[−]) are the probabilities for conformers T, G⁺, and G[−], and *J*_{*t*} and *J*_{*g*} are the vicinal spin-spin coupling constants between the α- and β-protons in the trans and the gauche conformers, respectively[6].

RESULTS: In T6fLα at 37° C, the population of G⁺ is 39%, the highest in the side-chain conformers of Asp. In T6fDα at 37° C, the population of G[−] is the highest (45%), whereas the T conformer is the least (24%). In contrast, the population of T conformer is most preferable in T6fDβ at 37° C; more than 50% of the Asp side-chain is in the T state[4]. Such preferences are the case at all the temperatures examined. The result shows that the population of the gauche conformers, G⁺ and G[−] is relatively high in α-Asp containing T6fLα and T6fDα, as compared to the T conformer.

The racemization and isomerization of Asp proceed as: (i) when the carboxylate carbon (C_{COO}[−]) of the Asp 58 side chain is attacked by the nitrogen (N) of Ser 59, L(or D)-succinimide is formed by the intramolecular cyclization; (ii) L(or D)-succinimide is converted to D (or L)-succinimide through an intermediate that has the prochiral α-carbon in the plane of the ring; and (iii) the D- and L-succinimide are hydrolyzed at either side of their two carbonyl groups, yielding both α- and β-Asp residues, respectively [7]. The close distance between Asp 58 C_{COO}[−] and Ser 59 N should be advantageous to this cyclization. For G⁺ and G[−] conformers, the distance between Asp C_{COO}[−] and Ser N is, actually, 3.5 ± 0.7 Å, smaller than the distance 4.5 ± 0.5 Å for the T conformer. Therefore, Asp C_{COO}[−] in G⁺ and G[−] conformers are easily attacked by Ser N for the isomerization of Asp 58. In contrast, T6fDβ has the highest population of the T conformer of Asp 58 side-chain. In such case, Ser 59 N in T6fDβ is hard to attack Asp 58 C_{COO}[−] to form succinimide due to rather long distance between Asp C_{COO}[−] and Ser N. Thus, the Dβ form is thought to be the most stable in the 3 isomers of Asp 58.

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PR10-7 Rapid Survey of Asp Isomers in Disease-related Proteins by LC-MS/MS Combined with Commercial Enzymes

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INTRODUCTION: Until relatively recently, it was considered that D-amino acids were excluded from living systems except for the cell wall of microorganisms. However, D -aspartate residues have now been detected in long-lived proteins from various tissues of elderly humans. Formation of D -aspartate in proteins induces aggregation and loss of function, leading to age-related disorders such as cataracts and Alzheimer disease. A recent study used a liquid chromatography - tandem mass spectrometry (LC-MS/MS) to analyze isomers of Asp residues in proteins precisely without complex purification of the proteins. However, to identify the four Asp isomers (L α , L β , D β and D α) on the chromatogram, it was necessary to synthesize reference peptides containing the 4 different Asp isomers as standards. Here, we describe a method for rapidly and comprehensively identifying Asp isomers in proteins using a combination of LC-MS/MS and commercial enzymes without synthesizing reference peptides.

EXPERIMENTS:

Synthesis of peptides containing four different Asp isomers. The following peptides and their diastereoisomers corresponding to the human α A-crystallin sequence from residues 55 to 65 (α AT6 peptide) were synthesized by a Shimadzu PSSM-8 peptide synthesizer. The purity of each peptide was confirmed to be >95% by analytical RP-HPLC and mass spectrometry.

Preparation of lens proteins. Lens samples (one sample each) from elderly individuals (aged 80 years) were homogenized under physiological conditions and fractionated into water-soluble (WS) and water-insoluble (WI) fractions by centrifugation. The WS protein was dissolved in 50 mM Tris/HCl (pH 7.8), 1 mM CaCl₂ buffer before enzymatic digestion.

Identification of the tryptic peptides and the quantification of the Asp isomers. The protein sample is treated with trypsin, trypsin plus endoprotease Asp-N (Asp-N), trypsin plus L-isoaspartyl methyltransferase (PIMT) or trypsin plus paenidase, and the resulting peptides are applied to LC-MS/MS. Because Asp-N hydrolyzes peptide bonds on the N-terminus of only L α -Asp residues, it differentiates between peptides containing L α -Asp and those containing the other three iso-

mers. Similarly, PIMT recognizes only peptides containing L β -Asp residues, and paenidase internally cleaves the C-terminus of D α -Asp residues.

RESULTS: Figure 1 shows a typical example of the identification of the Asp 58 isomers in the α A 55-65 peptide (TVLD⁵⁸SGISEVR, [M+2H]²⁺ = 588.3) from α A-crystallin in lens from elderly people. This simplified approach has been successfully applied to the analysis of all tryptic peptides in aged lens.

CONCLUSION: This new method is able to search comprehensively for the Asp isomers in damaged or aged proteins from all living tissues and cells.

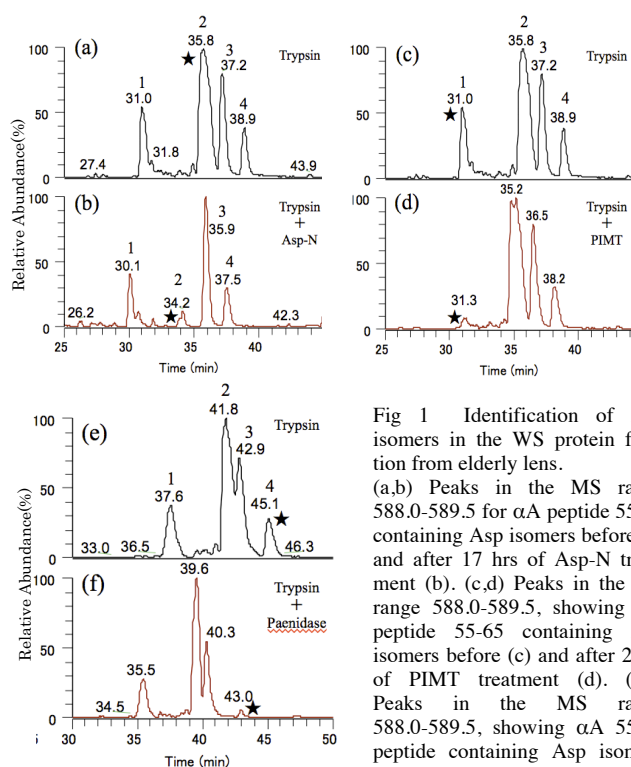


Fig 1 Identification of Asp isomers in the WS protein fraction from elderly lens. (a,b) Peaks in the MS range 588.0-589.5 for α A peptide 55-65 containing Asp isomers before (a) and after 17 hrs of Asp-N treatment (b). (c,d) Peaks in the MS range 588.0-589.5, showing α A peptide 55-65 containing Asp isomers before (c) and after 2 hrs of PIMT treatment (d). (e,d) Peaks in the MS range 588.0-589.5, showing α A 55-65 peptide containing Asp isomers before (e) and after 20 hrs of paenidase treatment (f). The asterisked peaks indicate peptide disappearance.

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