# VIII-II-1. Project Research

# Project 6

# PR6 Project Research on the Abnormal Aggregation of Proteins by UV and Gamma-Ray Irradiation and Study of Repair Mechanism

N. Fujii

*Research Reactor Institute, Kyoto University*

**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 irradiation such as gamma-ray and UV and protein function. We also investigate the repair mechanism for the damaged protein by irradiation. This research program has started in 2008. In this year, the 7 research subjects were carried out. The allotted research subjects (ARS) are as follows;

**ARS-1:** X-ray and neutron diffraction studies on multi-component enzyme.
(M. Sugiyama, N. Fujii and Y. Morimoto)

**ARS-2:** Denaturation of alpha-crystallin by replacement of amino-acid residue.
(M.Sugiyama, N. Fuji and N. Fuji)

**ARS-3** Screening of D-aspartate–containing proteins using the specific proteolytic enzyme, D-aspartyl endopeptidase. (T. Kinouchi and N. Fujii)

**ARS-4:** Damages of biological molecules induced by ionizing radiation and biological defense mechanisms.
(T. Saito and N. Fujii)

**ARS-5:** Preparation of recombinant Asp-isomerized crystallin and analysis of the effects of Asp alternation on protein function and structure using radiation
(Y. Sadakane and N. Fujii)

**ARS-6:** Improved Circular Dichroic Measurement of γ-Tryptophanase
**(**A. Shimada and N. Fujii)

**ARS-7:** Isomerization and racemization of aspartyl residues occur in proteins from elderly human eyes.
(N. Fujii and Y. Kaji)

**Main Results and Contents of This Project**

ARS-1: Y.Morimoto, et.al. has been studied a shape and an aggregate form of a proteasome activator (PA28) by the terms of X-ray and/or neutron scattering methods. The result shows that the PA28 forms a heptamer, and its oligomer makes itself as a dimer, so called dimer of heptamer form. It is very interesting form because of existing a self-assembly of thePA28 without proteasome, when at the presence of proteasome, such as inthe cell, how the PA28 binds or dissociate each other. Then we are nowin progress of a reaction scheme with proeasome and PA28 monomers.

ARS-2: Sugiyama et al. studied the mechanism of abnormal aggregation by observing the large scale structure of protein with Small-Angle Neutron Scattering (SANS). In this study, by replacing Asp with Asn in α
A crystallin and αB crystallin, the structural deformation of the proteins was observed with SANS. The SANS profiles clearly show that the mutants have the denatured structures and then the Asp could be one of key residues which maintain the normal structure.

ARS-3: Kinouchi *et al*. developed a screening system for D-Asp-containing proteins using D-aspartyl endopeptidase (DAEP), that specifically cleaves the proteins at the C-terminus, to clarify the role of these proteins in many aging-related disease. The digested proteins were detected by means of two-dimensional gel electrophoresis and identified using mass spectrometry. As the result, we detected tubulin beta-1 chain as D-Asp-containing protein in the brain tissues of mice. Therefore we expect that this method will be helpful for clarifying the mechanism of many of these diseases.

ARS-4: The lipid peroxidation of linolenic acid progressed with increasing dose of γ-irradiation in benzene. This dose-effect relationship was nonlinear. In this experimental condition, there was a inverse dose-rate effect. In linorenic acid micelle solution, a relative lipidperoxide amount increased linearly with increasing dose of γ -irradiation up to 0.5 kGy. In dose of γ -irradiation more than 0.5 kGy, a relative lipidperoxide amount decreased dose-dependently and gradually.

ARS-5: Sadakane *et al.* prepared the αA-crystallin in which specific Asp residue is altered by the method of intein-mediated protein ligation, which enables to the ligation of synthetic peptide to recombinant protein through a native peptide bond. The peptides have been prepared, and the condition of expression for the recombinant protein is almost determined.

ARS-6**:** Shimada *et al.* studied the secondary structure of tryptophanase by CD spectrum after γ-rays irradiation. The results showed that the secondary stereostructure of γ- tryptophanase changes more largely than tryptophanase in ammoniumphosphate solutions.

ARS-7: Fujii *et al*. found that biologically uncommon D-β-Asp-containing proteins appeared in various ocular tissues, such as the nuclei of the lens, non-pigmented ciliary epithelial cells, the drusens, and the sclera of elderly donors with age. This modification can be the result of the partial unfolding of protein leading to various age-related ocular diseases.

---

採択課題番号 20P6　放射線や紫外線照射によるタンパク質の異常凝集とその防御・修復機構に関する研究　プロジェクト

（京大・原子炉）藤井紀子

# PR6-1 X-ray and Neutron Diffraction Studies on Multi-Component Enzyme

M. Sugiyama, N. Fujii and Y. Morimoto

*Research Reactor Institute, Kyoto University*

**INTRODUCTION:** Mammals have defensive systems in their safety living. One of typical examples is a proteasome-ubiquitin system. In such a system, there are two main protein complexes: 20S proteasome, which is known as a degrading factory for intracellular proteins, and its activator protein. Invasion of foreign substances is a trigger to produce Proteasome Activator 28 (PA28) induced by an interferon $\gamma$, and then the 20S proteasome makes a complex with two PA28s. The target protein is specified by PA28 and degraded by the 20S proteasome. According to this, the 20S proteasome and PA28 complex regulates the processing of antigenic proteins for presentation by the MHC class I pathway. However, there is another activator protein, PA700 for the 20S proteasome. PA700 also connects to the 20S proteasome and then the complex has another function; the complex breaks a ubiquitinated protein up. Therefore, it is very important to elucidate the process of the connection to the 20S proteasome of PA28 in order to understand its regulation and/or connecting mechanism.

**EXPERIMENTS:** Sample preparation and various-sample condition (concentration, pH and so on) are carried out at the KUR D-shop Lab using a yeast expression system. SANS experiment was performed with SANS-U spectrometer of the Institute for Solid State Physics, University of Tokyo, installed at JRR-3M of Japan Atomic Energy Agency, Tokai, Japan (Okabe et al. 2005). SANS data were measured at the sample-detector positions of 8 m and 2 m with 6.7 ˚A neutrons: the total covered $q$ range was from 0.008 to 0.2 ˚A−1. The observed SANS data were corrected for background, cell, buffer scatterings and the transmissions.

**RESULTS:** In order to examine the experimental result, the SANS profile and the gyration radius of PA28 should be simulated according to its atomic coordinates. For this simulation, we should know the atomic coordinates of PA28 in a heavy water solution. At first, the atomic coordinates of PA28$\alpha$ which was solved by X-ray diffraction were obtained from Protein Data Bank (PDB): PDB ID is 1AVO (Knowlton et al. 1997). However, there is no atomic coordinate of hydrogen in this coordinates set even though neutron scattering cannot ignore hydrogen. Therefore, the hydrogen atoms were added at the most probable position to the data by using program CNS (Br¨unger 1998). In addition, the all replaceable hydrogen atoms were exchanged with deuterium atoms using CNS with a neutron-dedicated parameter file because the solvent of sample solution was heavy water.

We calculated the gyration radius of monomer PA28 (m-PA28), which is shown in Fig.1(a). The calculated $Rg$ of m-PA28 (34.7 ˚A) is relatively smaller than the experimental one (43.6 ˚A). It means that PA28 could make oligomers in an aqueous solution. Here, considering that PA28 connects to the 20S proteasome at the basal plane, we assumed that two PA28 make one dimer by connecting to each other at the basal plane: Figure 1 (b) shows this structural model (d1-PA28). The gyration radius of d1-PA28 is found to be 48.3 ˚A, which becomes closer to the experimental one (43.6 ˚A) than $Rg$ of m-PA28, but still 10% larger. Next, we assumed that each connecting PA28 could be embedded by the edge of columns of $\alpha$-helix each other because the out side chains are random coils and can move freely in aqueous solution. Figure 1 (c) shows this embedded dimer model (d2-PA28), in which, two PA28 get 2.75 ˚A closer to each other than those of d1-PA28. The gyration radius of d2-PA28 is found to be 44.6 ˚A, which is in good agreement with the experimental result.

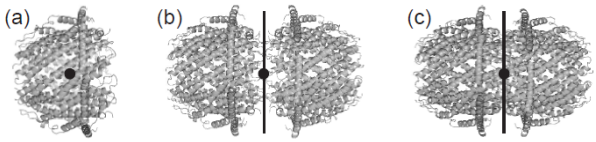


**Fig. 1.** Structural models of PA28 in aqueous solution. Closed circle and straight line show the center of distribution of the scattering contrast and a mirror plane, respectively. (a) m-PA28 (monomer). (b) d1-PA28: two PA28 connect to each other at the basal plane. (c) d2-PA28: PA28 is embedded by the edge of columns of $\alpha$-helix each other and then get 2.75 °A closer to each other than those of d1-PA28.

Recently, it was reported that free PA28 (un connecting to the 20S proteasome) and the complex coexist in a cell. It suggests that there a dissociation-binding mechanism exists between the 20S proteasome and PA28 in a cell. Our SANS result shows that free PA28 can exist by making a dimer in an aqueous solution. Therefore it could be considered that the dimer should be an intermediate state to store the free PA28 in a cell. The elucidation of the mechanism of dissociation and connection should be a next target of the investigation with existing the 20S proteasome.

採択課題番号 20P6-1　巨大で複雑なタンパク質複合体の動態と作動機構　　プロジェクト
（京大・原子炉）森本幸生、藤井紀子

## PR6-2 Denaturation of α-Crystallin by Replacement of Amino-acid Residue

M. Sugiyama, N. Fuji and N. Fuji

*Research Reactor Institute, Kyoto University*

**INTRODUCTION:** Protein aggregation diseases, for example variant Creutzfeldt-Jakob disease (caused by abnormal prion), Alzheimer's disease (caused by β-amyloid proteins) and so on, are becoming a serious problem. In order to investigate the mechanism of the abnormal aggregation of protein, we have focused on the eye lens protein, α-Crystallin. Because there is no metabolic system for proteins in eye lens, it is considered that the denatured protein without any repairing process is accumulated and then makes abnormal aggregation. Alpha-Crystallin is a major protein in eye lens. In a native state, α-Crystallin with its molecular weight of ca 800 kDa is a hetero-aggregate consisting of 20-30 subunits. There are two kinds of subunits, αA-Crystallin and αB-Crystallin. On the molecular level study, Fujii found that there exist racemized aspartyl residues (D-Asp) in the abnormal aggregates of α-Crystallin of Cataractous and elder eye lens. Therefore, we have supposed the racemization of aspartyl residue should be a trigger of abnormal aggregation. In addition, we have also proposed a pathway to Cataract as follows: Under external stresses such as UV irradiation, X-ray irradiation, low temperature and so on, aspartyl residues are racemized. The DAsp on the polypeptide chain induces the strain in the regular folding of the polypeptides and the strain makes the structural deformation of the subunits, αA-Crystallin and/or αB-Crystallin. The aggregates with these deformed subunits gather and make the abnormal aggregates.

**EXPERIMENTS:** As the first step to prove above the hypothesis, we prepared for two mutant samples of which an aspartyl residue was replaced with an asparagine residue. Mutant αA-crystallin (Asp → Asn@151) Mutant αB-crystallin (Asp → Asn@36) In addition, as a reference, we also prepared for normal αA-crystallin and αB-crystallin. It is espected that asparagine acid can make racemization more easily than aspartyl acid.

**RESULTS:** With these samples, SANS experiments were perfomed with SANS-U spectrometer (ISSP). Figure 1 shows the SANS profiles of normal and mutant αA-crystallins and those of normal and mutant αB-crystallins. The structual change of mutant αB-crystallin is larger than that of mutant αA-crystallin. It means that an aspartyl residue in αB-crystallin plays more important role than that in αA-crystallin.

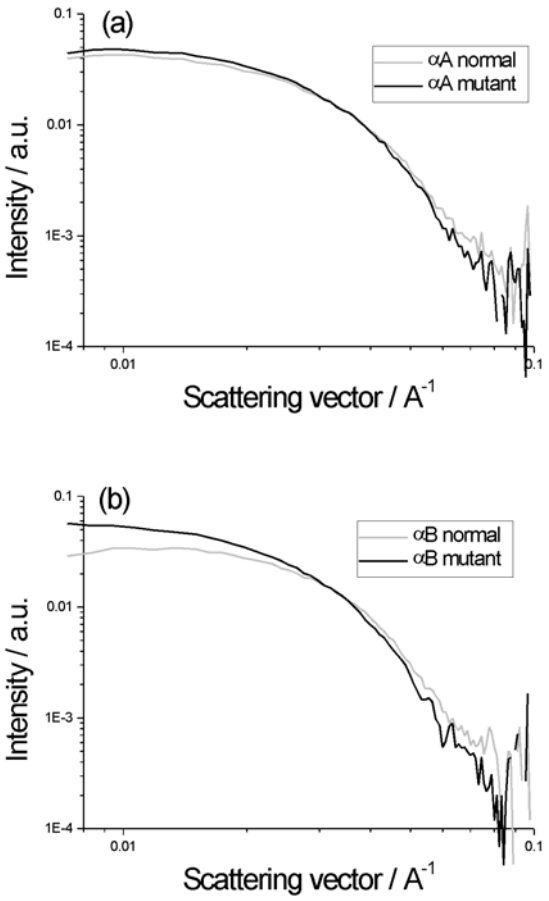


Fig. 1. SANS profiles of normal and mutant αA-crystallins (a) and those of normal and mutant αB-crystallins (b).

採択課題番号 20P6-2 蛋白質の凝集・会合過程の in situ 観測と動態解析 プロジェクト
（京大・原子炉）杉山正明、藤井紀子

# PR6-3 Screening of D-Aspartate–Containing Proteins Using the Specific Proteolytic Enzyme, D-Aspartyl Endopeptidase

T. Kinouchi and N. Fujii

*Research Reactor Institute, Kyoto University*

**INTRODUCTION:** The accumulation of D-isomers of aspartic acid (D-Asp) in proteins during aging has been implicated in the pathogenesis of Alzheimer's disease (AD), cataracts and arteriosclerosis. Our understanding of the relationship between racemized proteins and aging-related diseases would be increased if a screening system for novel D-Asp-containing proteins were developed. Until now, however, there has been no effective method for detecting D-Asp-containing proteins. Because there are thousands of proteins in the brain alone, any screening method would have to be highly selective. In this study, we developed a system for selective screening of D-Asp-containing proteins using D-aspartyl endopeptidase (DAEP) that specifically cleaves D-Asp-containing proteins at the C-terminus. We used a two-dimensional gel electrophoresis (2-DE) differential system, a high-resolution technique for protein separation and for visualizing differences in protein expression, to detect the products of protein cleaved by DAEP, and the protein was identified by means of mass spectrometry.

**EXPERIMENTS:** Mice brain samples were homogenized on ice in five volumes of lysis buffer (30 mM Tris/HCl (pH 8.5) containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% protease inhibitor cocktail, and 0.2 mM EDTA). The suspensions were sonicated and then centrifuged at 12,000g for 15 min at 4˚C. The supernatants were further centrifuged at 100,000g for 60 min at 4˚C. The resultant samples (1 mg total protein) were digested by 5 μL DAEP solution at 37˚C overnight. The control sample was treated in the same way with inactivated DAEP by heat treatment in boiling water for 10 min. Samples were isoelectrically focused on immobilized p*I* 3-7 strips and then performed on 12.5% polyacrylamide gels. Gels were then stained with coomassie brilliant blue (CBB) for visualization of the in-gel proteins.

**RESULTS & DISCUSSION:** Figures show the expression pattern in the cerebrum of a male mouse at 120 weeks old. Protein spot positions and intensities in the gel patterns of DAEP-treated and control samples were compared. By analyzing the protein spot expression on the gels, we identified one protein spot that was observed on the control gel. This spot was identified as tubulin beta-1 chain (TUB1) by means of mass spectrometry. Our results indicate that TUB1 containing D-Asp residues was digested by DAEP.

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

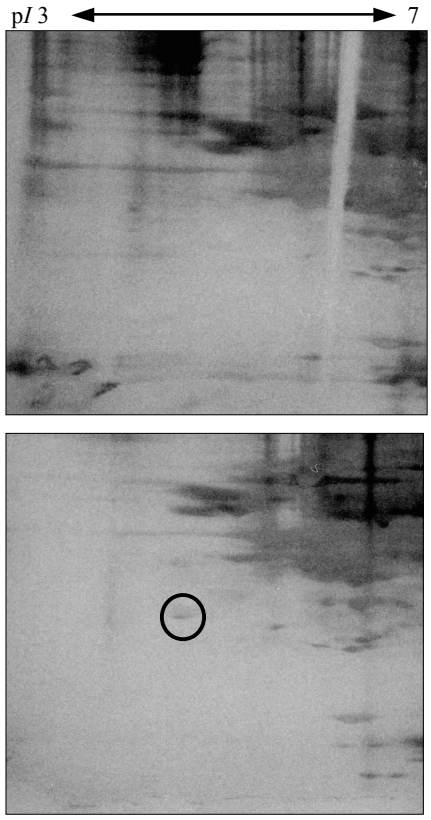


Fig.: The gel patterns for the sample treated with DAEP (**a**) and control sample (treated with inactivated DAEP) (**b**) are compared. The circle indicates the new spot not cleaved by DAEP.

---

採択課題番号 20P6-3　D-アスパラギン酸含有蛋白質の蓄積モデルの構築と　　プロジェクト
それに特異的な分解酵素による品質管理機構の研究

（京大・原子炉）木野内忠稔、藤井紀子

# PR6-4 Damages of Biological Molecules Induced by Ionizing Radiation and Biological Defense Mechanisms

T. Saito and N. Fujii

*Research Reactor Institute, Kyoto University*

**IINTRODUCTION:** Some bacteria show considerably high resistance to ionizing radiation [1]. A Common feature of these bacteria is that these bacteria contain red carotenoid pigments [1, 2, 3]. Colorless mutants of these radioresistant bacteria are more sensitive than wild types to gamma-irradiation [1]. Therefore, these red carotenoid pigments are thought to contribute to defense mechanisms of these radioresistant bacteria against ionizing radiation. It is well known that biological effects induced by low LET ionizing radiation are caused mainly by radicals that is generated by radiolysis. Carotenoid pigments possess high radical scavenging activity. Carotenoid pigments are localized within the lipids of the cell surfaces in procaryote. From these things, we consider that red carotenoid pigments in radioresistant bacteria must defense the lipids of the cell surfaces of these bacteria against ionizing radiation.

When this biological defense mechanism of these radioresistant bacteria against ionizing radiation is considered, elucidating damage process of lipids by ionizing radiation is important. In this study, a process of peroxidation of linolenic acid, which is one of fatty acids that is the simplest biological lipid, by gamma-irradiation was analyzed.

**EXPERIMENTS:** Sample Preparation: The linolenic acid was dissolved in benzene at a final concentration of 1 mM. The linolenic acid micelle was prepared as follows. The linolenic acid at a final concentration of 1 mM was added to PBS(-) with 0.8% nonaethylene glycol monododecyl ether. And, this solution was stirred vigorously. Gamma-Irradiation: The prepared solutions were irradiated with $^{60}$Co gamma-rays at a dose-rate 0.8 kGy/h or 30 kGy/h. Assay of Peroxidation: The modified method of Buege and Aust was used [4]. TCA-TBA-BHT-HCl regent which contained 15% trichloroacetic acid, 0.375% thiobarbituric acid, 0.04% butylated hydroxytoluene, and 0.25N hydrochloric acid was prepared. Three ml of gamma-irradiated benzene solution with linolenic acid was evaporated in vacuo. The residue was dispersed in 3 ml of PBS(-) by sonicator. One ml of the dispersed solution or gamma-irradiated micelle solution was combined with 2.0 ml of TCA-TBA-BHT-HCl regent and mixed thoroughly. The mixed solution was heated for 15 min in a boiling water bath. The malondialdehyde (MDA) amount was determined by measuring the absorbance of the reaction solution at 532 nm. In this study, the lipid peroxidation was evaluated by the MDA amount.

**RESULTS:** The lipid peroxidation of linolenic acid progressed with increasing dose of gamma-irradiation in benzene (Fig. 1). It was revealed that this dose-effect relationship was nonlinear. In this experimental condition, there was a inverse dose-rate effect. For example, a relative MDA amount value was 0.165 when a sample was irradiated with 5 kGy at 0.8 kGy/h. However, a relative MDA amount value was 0.079 with 5 kGy at 30 kGy/h.

In linorenic acid micelle solution, a relative MDA amount value increased linearly with increasing dose of gamma-irradiation up to 0.5 kGy. In dose of gamma-irradiation more than 0.5 kGy, a relative MDA amount value decreased dose-dependently and gradually. Difference between dose-effect relationship in a linolenic acid micelle solution and that in benzene shows that the molecular environment of linolenic acid affects the linolenic acid peroxidation by gamma-irradiation.

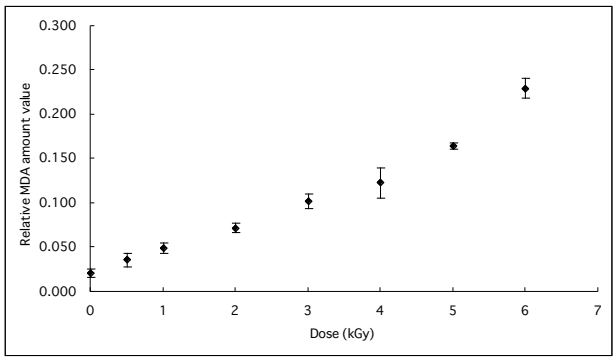


Fig. 1. The lipid peroxidation of linolenic acid in benzene by gamma-irradiation at a dose rate 0.8 kGy/h. Dose of gamma-irradiation is shown on the horizontal axis, and the relative MDA amount value is shown on the vertical axis. Each value is expressed as an average ± S.D. of three independent experiments.

**REFERENCES:**

[1] T. Saito, Viva Origino, **30** (2007) 85-92.
[2] T. Saito *et al*, Arch. Microbiol., **162** (1994) 414-421.
[3] T. Saito *et al*, Microbios, **95** (1998) 79-90.
[4] J. A. Buege and S. D. Aust, Meth. Enzymol., **52** (1978) 302-310.

採択課題番号 20P6-4　　放射線照射による生体分子の損傷と防護機構　　プロジェクト
（京大・原子炉）齊藤　毅、藤井紀子

# PR6-5 Preparation of Recombinant Asp-Isomerized Crystallin and Analysis of the Effects of Asp Alternation on Protein Function and Structure Using Radiation

Y. Sadakane and N. Fujii[1]

*School of Pharmaceutical Sciences, Kyushu Univ. Heal. Welf*
[1]*Research Reactor Institute, Kyoto University*

**INTRODUCTION:** The structural alternations of L-asparatic acid (Asp) and L-asparagine (Asn) residues are frequently found in the proteins or peptide, and believed to arise through intramolecular rearrangement, such as via a succinimide intermediate. Recently, isomerization of L-Asp and deamidation of L-Asn has been found in the key proteins related to neuronal disease, such as prion protein (PrP) and Alzheimer's β proteins (Aβ). Interestingly, the alternation of Asp or Asn in these protein was reported to cause these physiological activites, i.e. the aggregation properties *in vitro* of Aβ protein were changed by its isomerized form, and the channel activities in planar lipid bilayer were altered by Asn deamidation. These results show that the structural alternations of Asp and Asn residues are one of the key events to understand the physiological phenomena.

α-Crystallin, which is the major protein of the mammalian lens and is necessary for the focusing of the light on the retina, is one of the well-known proteins in which Asp residues are altered to D-form. Such modification of α-crystallin would affect the three-dimensional packing of the lens protein and may affect the transparency of the lens. Fujii *et al* (co-author of the article) have shown that a specific Asp residue located at 151 from N-terminus is highly changed to D-form in aged αA-crystallin protein, and more interestingly, D/L ratio of the Asp residues is more than 1.

To study the effect of such Asp alternation on the protein structure and functions, we need the artificial protein in which specific Asp have been altered. Since both β-linkaged Asp (isoAsp) and D-form Asp (D-Asp) are unnatual amino acids, these were not introduced by DNA point mutation strategy. Exquisite techniques of expanding genetic code have made possible the site-specific incorporation of unnatural amino acid into proteins. However, the method was very difficult and complicated. We planed in this study to prepare the αA-crystallin protein in which specific Asp residue is altered by intein-meditated protein recombination technique, and to analyze the effect of Asp alternation on protein functions and structure using radiation.

**EXPERIMENTS:** The Asp-isomerized αA-crystallin was prepared by intein-mediated protein ligation, which allows the ligation of synthetic peptide with an N-terminal cysteine residue to a protein with a C-terminal thioester through a native peptide bond. The experimental strategy was shown in Fig. 1. First, two types of the peptides of C-terminal region 142-173 of αA-crystallin ($^{142}$CGPKIQTGLD$^{151}$ATHAERAIPVSR EEKPTSAPSS$^{173}$) in which the Asp residue was L-Asp, L-isoAsp were synthesized by Fmoc chemistry using a Shimadzu PSSM-8 peptide synthesizer (Kyoto, Japan). Fmoc-L-Asp(OtBu)-OH and Fmoc-L-Asp-OtBu were used to introduce the L-Asp and L-isoAsp, respectively. The synthesized peptides were confirmed by mass spectrometry.

N-terminal region of αA-crystallin protein$^{1-141}$ was prepared as recombinant protein using bacterial expression system. Two primers, 5'-CATATGGACGT GACCATCCAGCAC-3' and 5'-GCTCTTCTGCAGAA GGTCAGCATGCCATCGG-3' were used for amplifying the related gene, and the PCR product were inserted into the pTWIN vector (NEB). The constructed vectors were transformed to *E. coli* strain BL21(DE3)pLysS, and cultured in Lenox broth containing 50 μg/mL ampicillin and 30 μg/mL chloramphenicol at 37 °C. Isopropyl thio-β-D-galactoside (IPTG) was added at a final concentration of 2 mM and the specimen was further cultured for 5h at 37 °C. The expressed proteins were separated by SDS-PAGE with 12.5% acrylamide gel, and stained with Coomassie Brilliant Blue.

**RESULTS and DISCUSSION:** Synthetic peptides of αA-crystallin$^{142-173}$ were prepared at ~10 mg order. The clone No. 5 and 20 *E. coli* expressed ~45 and 30 kDa proteins by IPTG induction (Fig. 2). The genetic and biochemical analyses revealed that these proteins were not fusion protein of αA-crystallin$^{1-141}$ and intein. We need further to isolate new *E. coli* clones that express the target fusion protein to prepare the αA-crystallin in which specific Asp residue is altered.

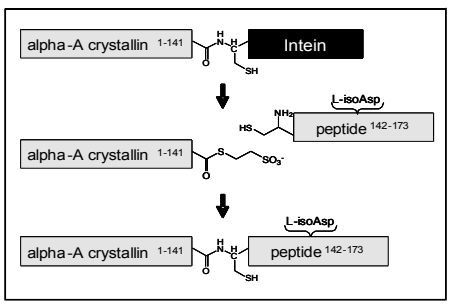


Fig. 1 Experimental Strategy

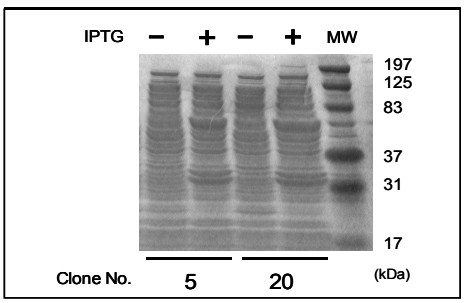


Fig. 2 SDS -PAGE analysis of expressed protein

採択課題番号 20P6-5　タンパク質中のアスパラギン酸残基付近の放射線による構造と機能変化の解析　　プロジェクト

（九州保福大・薬）定金　豊（京大・原子炉）藤井紀子

## PR6-6 Improved Circular Dichroic Measurement of γ-Tryptophanase

A. Shimada, N. Fujii[1] and T. Saito[1]

*Sustainable Environmental Studies, Graduate School of Life and Environment Sciences, University of Tsukuba,*
*[1]Research Reactor Institute, Kyoto University*

**INTRODUCTION:** Tryptophanase (TPase), whose stereospecificity is very exclusive to L-tryptophan, becomes active to D-tryptophan in highly concentrated ammonium phosphate solution. Kinetic data suggested this activity was induced by subtle and reversible conformational change. Ammonium phosphate, to which TPase activity on D-tryptophan was closely related, induced conformational changes of TPase. It is generally known that gamma-ray irradiation with protein can change its steric conformation. This will apply just as much to tryptophanase as to it, too. Tryptophanase might develop the D-tryptophan degradation activity without the help of ammonium phosphates when it receives an absorption dose of γ-ray that gives conformational change appropriate to produce the activity. However, there is few qualitative and quantitative data between γ-ray irradiation and their structural change. So, we aim to study in this short report to investigate how γ-ray irradiation influences on the stereostructure of tryptophanase. A level of usual dose such as several hundred kilograys leads TPase to irreversible denature. An optimal irradiation level of γ rays seems to be several hundred grays on the basis of preliminary experiments. In this study, the word γ-TPase means tryptophanase irradiated with different doses of γ ray irradiation. It is compared with TPase in ammonium monohydrogenphosphate (MAP), ammonium dihydrogenphosphate (DAP) and ammonium triphosphate (TAP). Previous studies used a 1 mm-path cuvette when using a circular dichroism (CD) spectrophotometer. As a result, unstable spectra with wobbly fluctuation were obtained to make reproducible measurement difficult. Thus present studies aim to obtain reproducible spectra by improving cuvettes for CD measurement of γ-TPase.

**EXPERIMENTS:** Source intensity and absorption dose of cobalt 60 was 151TBq and 11 kGy/h. Apotryptophanase purchased from Sigma Chem. Co. (St. Louis, USA) was prepared to a concentration of 200 μg/ml with 0.1 M potassium phosphate buffer solution of pH 8.3 including 1.1 mM pyridoxal 5′-phosphate. Tryptophanase was irradiated with dose of 0 – 11 kGy at room temperature. CD spectrum of γ-tryptophanase was measured in a 200 μℓ cell with 0.1 cm path length or a 3 mℓ call with 1 cm path length to analyze secondary structural change. Spectra were recorded at wavelengths from 200 to 400nm at room temperature. Scans were repeated five times per a spectrum, averaged, and expressed as molar ellipticity in degrees $cm^2\ dmol^{-1}$. The CD spectra were analyzed with a CDPro software package and calculated by subtracting a blank prepared identically but without TPase. They were compared with the CD spectra of TPase in three ammoniumphosphate solutions of 50 % saturation. Unless otherwise stated, reagents were obtained from Wako Pure Chem. Co. Ltd. (Osaka).

**RESULTS:** A 0.1 cm path cuvette was used in the previous researches. Here it was exchanged to a 1 cm path cuvette. Consequently, reproducible spectra were obtained when CD of γ-TPase was monitored. It is drawn as bold line in Figure 1. γ-TPase was compared with TPase in three ammoniumphosphate solutions. The stereostructure of γ-TPase changes more largely than TPase in ammoniumphosphate solutions. Future research should determine an optimal dose to provide a limited secondary structural change necessary for TPase activity on D-Trp.

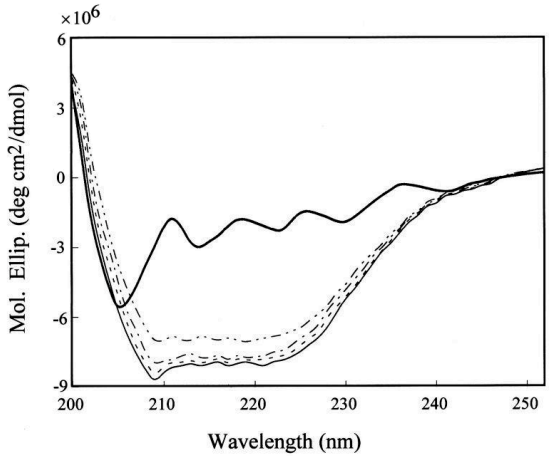


Figure 1 CD spectra of γ-TPase and TPase in ammoniumphosphate solutions.
γ-TPase: bold line, TPase in potassium phosphate buffer: fine line, dotted line: TPase in triammonium phosphate solution, single dash line: TPase in diammoniumhydrogen phosphate solution, double dash line: TPase in ammoniumdihydrogen phosphate solution.

採択課題番号 20P6-6　酵素の立体選択性に変化を及ぼす放射線照射の検討　　プロジェクト
（筑波大・生命環境科学）島田秋彦（京大・原子炉）藤井紀子、齋藤　毅

# PR6-7 Isomerization and Racemization of Aspartyl Residues Occur in Proteins from Elderly Human Eyes

N. Fujii and Y. Kaji[1]

*Research Reactor Institute, Kyoto University*
*[1]Department of Ophthalmology, University of Tsukuba Institute of Clinical Medicine*

**INTRODUCTION:** Protein composed of exclusively L-amino acids but biologically uncommon D-aspartic acids (D-Asp) have been detected in proteins of various human tissues from elderly donors. Our previous studies have identified D-β-Asp residues at four different specific sites in α-crystallin from aged human lenses and an increased amount of D-β-Asp residues with age. D-Asp formation was accompanied by isomerization from the natural α-Asp to the abnormal β-Asp via a succinimide. In the previous study, we prepared a polyclonal antibody against the peptide, (anti-peptide 3R antibody), which corresponds to three repeats of positions 149-153 of human αA-crystallin. This antibody can distinguish the configuration of the Asp-residue. As such, the antibody reacts very strongly against the D-β-Asp containing peptide but not with L-α-Asp, L-β-Asp or D-α-Asp contαining peptides. D-β-Asp is formed as a result of racemization during aging, therefore, it thought to be a potential marker of aging. Here, we report the detection of D-β-Asp containing protein in ocular samples such as those obtained from various ages of donors using the anti-peptide 3R antibody.

**EXPERIMENTS:**

***Eye Samples:*** Nine eyes of nine donors aged from 18 to 88 years old and two eyes from two fetuses were obtained at the time of necropsy from the division of pathology, University of Tokyo Hospital. None of the subjects had a history of any ocular diseases.

***Immunohistochemistry***

Immunohistochemical localization of D-β-aspartic acid containing peptides was investigated using the antibody mentioned earlier. After fixation with 10% formalin solution, 4 mm thick sections of paraffin-embedded eye samples were prepared. Following deparaffinization, the sections were treated with a polyclonal antibody to D-β-aspartic acid-containing peptides at a dilution of 1:500 dissolved with phosphate-buffered saline (PBS) containing 1% normal bovine serum albumin and kept at 4 °C overnight. After washing the section with the PBS, the were treated with the reaction solution containing a secondary antibody labelled with amino-acid polymer and horse radish peroxidase (Histofine Max-PO kit : NICHIREI Co Ltd., Tokyo, Japan) and were kept for 30 minutes at room temperature. The sections were then incubated with diaminobenzidine (DAB) in PBS. Finally, the sections were counterstained with hematoxylin. As a negative control, the primary antibody was replaced with normal rabbit serum IgG (1.0 mg/ml) diluted in PBS containing 1% bovine serum albumin.

To evaluate the specificity of the binding of the primary antibody to D-β-aspartic acid containing peptides, the sections were incubated with the mixture of the primary antibody and 10 mg/mL of peptide 3R instead of using the primary antibody alone. The following procedures were the same as the one described above.

The results of the immunohistochemistry was checked and graded in double-blinded manner.

**RESULTS:** The antibody showed negative reaction with any of the tissues in eye of human fetuses and under 18 years old donors. In contrast, strong immunoreactivity to D-β-Asp-containing peptides was seen in the nuclei of the lens, in non-pigmented ciliary epithelial cells, in the drusens, and in the sclera of elderly donors. In addition, moderate to weak immunoreactivity was seen in the cortex of the lens, in the blood vessels of the retina, in the optic nerve head, and in the lamina cribrosa of elderly donors.

**CONCLUSION:** D-β-Asp-containing proteins appeared in various ocular tissues with age. This study clearly demonstrated that D-β-Asp containing proteins are much more widespread in aged tissues than previously thought. The formation of D-Asp in protein can cause major changes in its structure since different side chain orientations can induce an abnormal peptide backbone and the main chain of peptide can become elongated by β linkage. Therefore, this modification can be the result of the partial unfolding of protein leading to various age-related ocular diseases.

---

採択課題番号 20P6-7　放射線や紫外線照射によるタンパク質の異常凝集と　　プロジェクト
アミノ酸のラセミ化
（京大・原子炉）藤井紀子（筑波大人間総合科学）加治優一