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Effect of Gamma Irradiation on the Structural and Physiological Properties of Silk Fibroin

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Abstract This study was conducted to examine the changes in the molecular structure and physiological activities of silk fibroin by gamma irradiation. The results of gel permeation chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that the molecular weight of fibroin was increased depending upon the irradiation dose. Secondary structure of fibroin determined by using circular dichroism revealed that the ratio of α-helix was increased up to 10 kGy and then decreased depending upon the irradiation dose. Whereas, the ratio of β-sheet, β-turn, and random coil were decreased and then increased with an alteration in the α-helix secondary conformation. The 2.2-diphenyl-1-picryl-hydrazil (DPPH) radical scavenging activity of fibroin was increased by gamma irradiation at 5 kGy, but was decreased above 10 kGy depending upon the irradiation dose. Also, the inhibition activities of tyrosinase and melanin synthesis of fibroin were increased by gamma irradiation. These results indicated that gamma irradiation could be used as an efficient method to make fibroin more suitable for the development of functional foods and cosmetics.

Keywords: fibroin, gamma irradiation, molecular weight, secondary structure, physiological property

Introduction

Silks are generally defined as protein polymers that are spun into fibers by certain *Lepidoptera larvae* such as silkworms, spiders, scorpions, mites, and flies (1). Silk derived from silkworm *Bombyx mori* is a natural protein that is mainly made of sericin and fibroin proteins (2).

In old days, fibroin was only used for clothing silk, but it has recently been suggested as a biomaterial and a matrix for biomedical applications. The unique physicochemical properties of fibroin (3) have enabled its various utilizations such as cell culture matrices (4), enzyme-immobilizing membranes (5), and an oral dosage gel form (6). Several biological activities of silk fibroin have also been reported. Lowered blood cholesterol, glucose levels, and alcohol absorption were observed in fibroin-fed rats (7), and sulphated fibroins showed an anti-HIV activity (8). In addition, the hydrolysates of silk fibroin have been considered for applications as a functional material for foods, cosmetics, and pharmaceutical preparations (9).

Gamma irradiation is a useful technology for an improvement of the storage and hygiene of food and also found to have importance in the medical and beauty care industry (10). Also, there have been several reports for a change of the structure and physiological properties of protein by an irradiation (11). Silk fibroin is a useful model to study for the changes of molecular structure and physiological activities by gamma irradiation. However, there are only a few reports on the effect of irradiation on the production of fine powder from silk (12,13).

Thus, this investigation was conducted to examine the changes in the molecular structure and physiological activities of fibroin by gamma irradiation and was undertaken to explore the possibilities of gamma-irradiated fibroin as a material for functional food stuff and cosmetics.

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Materials and Methods

Sample preparation Cocoons were supplied by Nuero Ltd. (Daegu, Korea). To remove sericin, the 50 g of cocoons were cut into small pieces and boiled in 2.5 L of 5%(w/v) Na_2CO_3 for 1 hr and filtered through a filter paper. The remaining sericin and Na_2CO_3 were removed by washing the residue with hot water 3 times. The fibroin was solubilized using a solution of $CaCl_2$-/H_2O-ethanol (14). A mixture containing 226.4 g $CaCl_2$, 346 g of distilled water, and 280 mL of ethanol was added to a 35 g fibroin residue and heated at 100°C for 2 hr. The dialysis (Mw cutoff of 1,000, Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) was carried out 5 times, and then the fibroin was dried in a vacuum-freeze dryer to obtain fibroin powder. The purified fibroin powder was stored in a refrigerator at 4°C for a further experiment.

Gamma irradiation Fibroin was dissolved into a concentration of 5 mg/mL (w/v) in deionized distilled water (DDW). Fibroin solution was irradiated at 0, 5, 10, 50, 100, 150, and 200 kGy in a cobalt-60 irradiator (IR-221; Nordion International Ltd., Kanata, ON, Canada) with an 11.1 *peta*-becquerel (PBq) source strength and operated at a dose rate of 10 kGy/hr. The gamma-irradiated fibroin solutions were stored in a refrigerator at 4°C for a subsequent experiment. The absorbed dose was measured using 5 mm

diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany). The dosimeters were calibrated as international standard set by International Atomic Energy Agency (Vienna, Austria).

Gel permeation chromatography The distribution of the molecular weight (Mw) of fibroin was determined by a gel permeation chromatography (GPC). GPC measurements were performed by a Waters GPC system equipped with a Waters 515 pump, 2×PLaqagel OH Mixed (7.8×300 mm) column and a Waters 2410 refractive index detector. The column was operated at 40°C and eluted with distilled water at a flow rate of 1 mL/min. The column was calibrated by a dextran standard at a concentration of 0.1%(w/w).

For the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 28.3 μg of the fibroin/lane, assayed by the BCA method (15) was loaded. Electrophoresis was carried out by using precasted 4-20% Nu-PAGE Bis-Tris gels (Invitrogen, San Diego, CA, USA) at 100 V in a Nu-Page MES SDS running buffer system (Invitrogen) according to the manufacture's instructions. A SeeBlue Plus 2 Pre-stained standard marker was purchased from Invitrogen and was used to determine the molecular masses of the protein bands. The gel was stained for visualization with Commassie brilliant blue R-250.

Circular dichroism spectroscopy Circular dichroism (CD) spectra were obtained using a Jasco J-715 spectropolarimeter (Jasco, Tokyo, Japan) fitted with a 150 W xenon lamp. Far-ultra violet (UV) spectra were registered in the range of 160-280 nm. Sample (0.2 mg/mL) was analyzed in DDW. A sample compartment was purged with nitrogen gas and a 1-mm cuvette was used. Triplicate scans were averaged, and the spectrum for the DDW background was subtracted. CD spectra were represented in terms of a mean residue ellipticity (degree cm^2/dmol).

UV spectroscopy Gamma-irradiated fibroin solutions were scanned using a spectrophotometer (UV-1601PC; Shimadzu, Tokyo, Japan). The UV-VIS spectra were recorded between 200 and 600 nm. A total of 3 mL of a sample (0.1 mg/mL) was assayed on a UV-VIS spectrophotometer with a quartz cell.

DPPH radical scavenging activity The free radical scavenging activity using the 2.2-diphenyl-1-picryl-hydrazil (DPPH) reagent was measured using the method described by Amarowicz *et al.* (16), with a slight modification. To 0.5 mL of fibroin dissolved in DDW at a concentration of 0.2 mg fibroin/mL, the same volume of freshly prepared DPPH radical in methanol (0.1 mmol) was added and vortexed. After a reaction for 25 min at room temperature (25°C) in the dark, the reaction mixtures were centrifuged at 4,000×g for 5 min. The decolorization of the supernatant was assayed at 517 nm ($A_{517\ nm}$) by a spectrophotometer (UV-1601PC, Shimadzu) and was compared with a blank control containing a fibroin solution and pure methanol instead of DPPH. A blind control containing DPPH and distilled water instead of a fibroin solution was also assayed. Equation was used for the calculation of the free radical scavenging activity;

$$\text{Scavenging activity } (\%)=(1-A^{sample}{}_{517}/A^{blind}{}_{517})\times 100$$

Where, the scavenging activity refers to the free radical scavenging percentages, A_{sample} refers to the absorbance of a sample, and A_{blind} refers to the absorbance of the blind control.

Anti-melanogenic activity To determine the tyrosinase inhibitory activity, a fibroin solution (0.2 mL) was added to a reaction mixture containing a 10 mM L-3,4-dihydroxy-phenylalanine (L-DOPA, Sigma-Aldrich, St. Louis, MO, USA) solution, a 67 mM sodium phosphate buffer (pH 6.8), and mushroom tyrosinase (final concentration of 100 unit/mL, Sigma-Aldrich). The reaction mixture was incubated at 25°C for 15 min. The amount of dopachrome produced in the reaction mixture was determined at 475 nm ($A_{475\ nm}$) by a spectrophotometer (UV-1601PC; Shimadzu). An equation was used for the calculation of the inhibitory effect on the tyrosinase (%).

$$\text{Inhibition } (\%)=(1-A^{sample}{}_{475}/A^{control}{}_{475})\times 100$$

Where, $A^{control}{}_{475}$ refers to the absorbance with DDW instead of the fibroin solution.

Determination of a melanin synthesis was performed by using a modified method of Bilodeau *et al.* (17). Briefly, B16BL6 cells (1×10^5) were plated on 24-well dishes and incubated in the presence of 100 nM α-melanocyte stimulating hormone (MSH, Sigma-Aldrich). Cells were then incubated for 72 hr with non-irradiated fibroin and gamma-irradiated fibroin at a concentration of 62.5 μg/mL. After twice washing with phosphate buffered saline (PBS), the samples were dissolved in 100 μL of 1 N NaOH and a water bath at 70°C. The absorbance at 405 nm was compared with a standard curve for synthetic melanin.

Statistical analysis The data were analyzed by the Statistical Package for the Social Science (SPSS Inc. Chicago, IL, USA) program. Differences among the mean values were obtained by Duncan's multiple-comparison tests at $p<0.05$.

Results and Discussion

Changes in the molecular weight distribution of fibroin by gamma irradiation The effect of gamma irradiation on the Mw distribution of fibroin was investigated by using a SDS-PAGE and a GPC. Electrophoretic pattern of fibroin irradiated at 5 to 200 kGy is shown in Fig. 1A. SDS-PAGE results suggested that the Mw distribution of fibroin was increased by gamma irradiation. Non-irradiated fibroin showed a broad smear at a position around 5 to 50 kDa. Gamma-irradiated silk fibroin at 5 kGy showed a higher Mw ranging from 38 to 188 kDa than the non-irradiated one, and gamma-irradiated fibroin at 10 kGy showed broad smear at a position around 90 to more than 188 kDa. But, fibroin irradiated at 50, 100, 150, and 200 kGy was not observed in any standard marker range except for the loading point (starting point).

Figure 1B represents the average Mw of fibroin as an expression of the radiation effect under different doses. The average Mw of fibroin irradiated at 0, 5, and 10 kGy was found to be 5, 320, and 576 kDa, respectively. The average

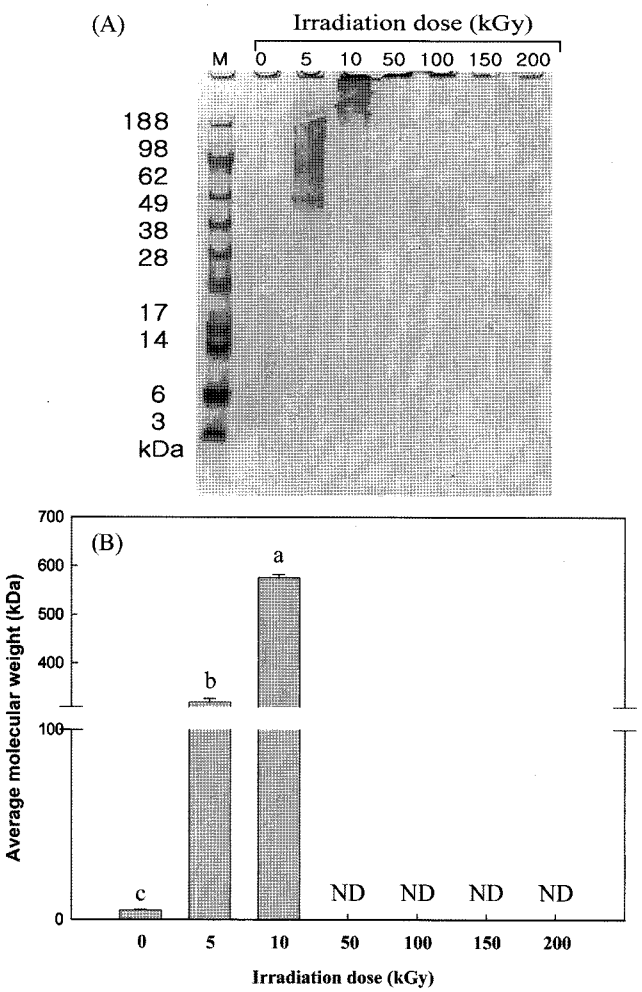

Fig. 1. (A) SDS-PAGE of fibroin irradiated at various doses. M indicates standard markers and the numbers on the left side indicate the Mw (kDa) of the standard marker. (B) GPC analysis for average Mw distribution of the gamma-irradiated fibroin. ND: can not determine due to out of determination range. Bars represent the mean±SD. The letters mean the statistically significant difference compared with the control ($p<0.05$).

Mw observed in fibroin irradiated at 50, 100, 150, and 200 kGy could not be calculated because of the upper limit of the GPC calibration range.

Irradiation exerts its effect through a radiolysis of water, which results in the production of radicals such as hydrated electrons, hydroxyl radicals, and hydrogen atoms (18). It appears that an irradiation could change the physicochemical and structural properties of various food components. Radiation-induced reactions in proteins are strongly influenced by their complex structure, that is, the folding of the peptide chains, disulfide bonds between the chains, secondary binding forces such as hydrogen bond, hydrophobic bond, or ionic bonds. The splitting reaction of disulfide bonds can cause degradation to smaller proteins and cross-linking among polypeptide chains via intermolecular bond can attribute to aggregation of protein (19-21). It has also been reported that the irradiation of proteins at a high dose induced a cross-linking and a hydrophobic interaction between proteins (22-25). Therefore, this data indicated

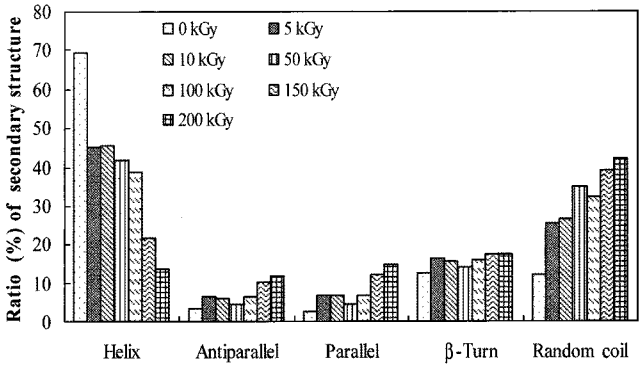

Fig. 2. Ratio (%) of secondary structural conformations from the far-UV CD spectra of fibroin irradiated at various doses.

that fibroin might be fairly aggregated into high Mw fragments by gamma irradiation.

Changes in the secondary structure of fibroin by gamma irradiation CD spectroscopy measures the differences in the absorption of a left-handed polarized light versus a right-handed polarized light and can be used to determine a protein's secondary structure in a far-UV spectral region (190-250 nm) (26,27). Figure 2 shows the change in the secondary structure of fibroin caused by gamma irradiation. When the radiation dose was increased, the ratio of α-helix was increased up to a dose of 10 kGy and then decreased. On the other hand, the ratio of β-sheet, β-turn, and random coil were decreased and then increased with an alteration in the α-helix secondary conformation.

Several researches reported that oxygen radicals, due to a radiolysis of water, subsequently destabilized the α-helical structure of proteins, and the changes in CD spectra by gamma irradiation were mainly due to a cleavage of the covalent bonds of proteins and the formation of aggregated products (28-30). In this study, CD results clearly supported that oxygen radicals, generated by gamma irradiation in solution, disrupted the ordered structure of fibroin, resulting in a change of the molecular properties of fibroin in solution. However, the reason for the increase of the α-helix content at a low dose, less than 10 kGy, and the decrease at a high dose should be elucidated with further experiments.

Changes in UV spectrum of fibroin by gamma irradiation UV absorption spectra show the conformational change in the secondary structure of proteins, particularly in the case of a change in the local environment of the ordered structure of a polypeptide chain (30). The UV spectra of fibroin solution irradiated at various irradiation doses are shown in Fig. 3. The data showed an increase in absorption intensity by gamma irradiation. There was a negligible shift in the absorption wavelength depending upon the irradiation dose. The UV results indicated that these changes were probably associated with a breakage or aggregation of the protein induced by the irradiation, suggesting that the internal aromatic amino acids of a protein were exposed due to a disruption in the conformation process and that the extent of the disruption depended on the irradiation dose (31).

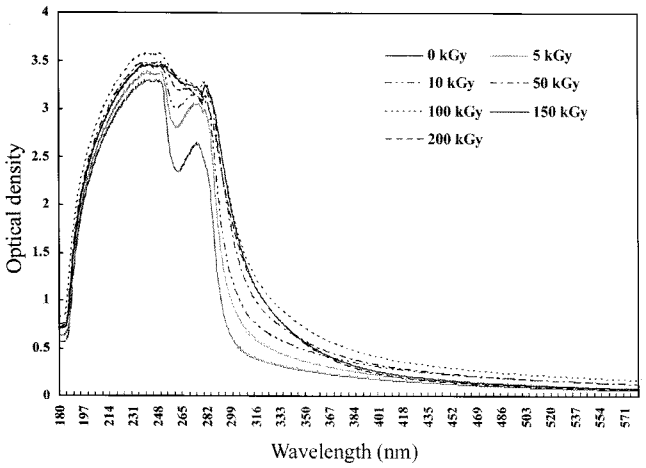

Fig. 3. UV absorption spectra of fibroin irradiated at various doses.

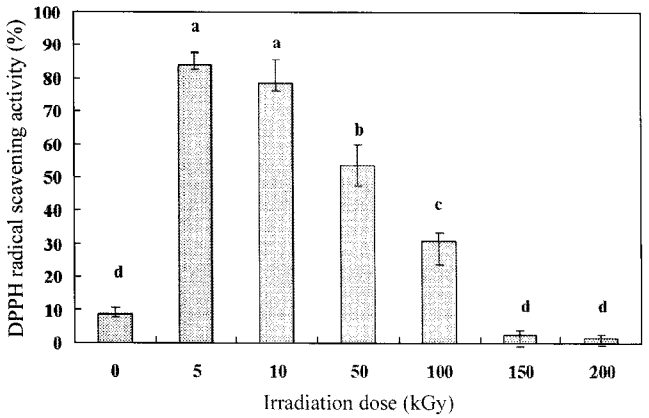

Fig. 4. Radical scavenging capacity of fibroin irradiated at various doses. Bars represent the mean±SD. The letters indicate the statistically significant difference compared with the control ($p<0.05$).

Change in DPPH radical scavenging capacity of fibroin by gamma irradiation The DPPH radical scavenging capacity of fibroin is shown in Fig. 4. The DPPH radical scavenging capacity of fibroin was increased by gamma irradiation at 5 kGy, but was decreased above 10 kGy depending upon the irradiation dose. The results indicated that the optimum dose for the highest antioxidant activity of fibroin is 5 kGy.

Some researches have reported that fibroin had an antioxidant activity by *in vitro* and *in vivo* experiments (32, 33). Although the effect of irradiation on the antioxidant activity of proteins has been not studied, irradiation could increase the antioxidant properties of various foods and natural extracts such as green tea leaf extract (34), phytic acid (35), and soybean (36). In the present study, gamma irradiation also increased the DPPH radical scavenging capacity of fibroin due to a structural change.

Change in anti-melanogenic effect of fibroin by gamma irradiation Tyrosinase is responsible for the biosynthesis of pigment melanin in human skin, and tyrosinase inhibitors have important roles in the cosmetics industry due to their skin-whitening effects (37,38). The inhibitory

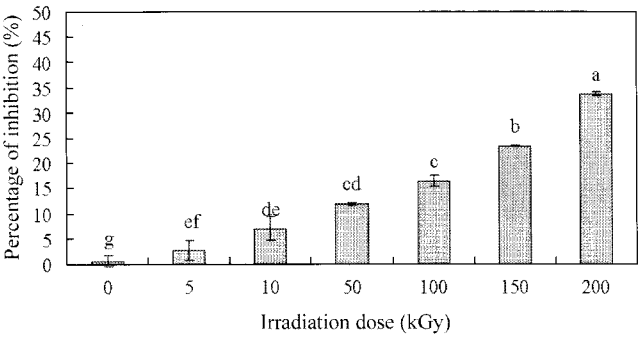

Fig. 5. Tyrosinase inhibition activity of fibroin irradiated at various doses. Bars represent the mean±SD. The letters mean the statistically significant difference compared with the control ($p<0.05$).

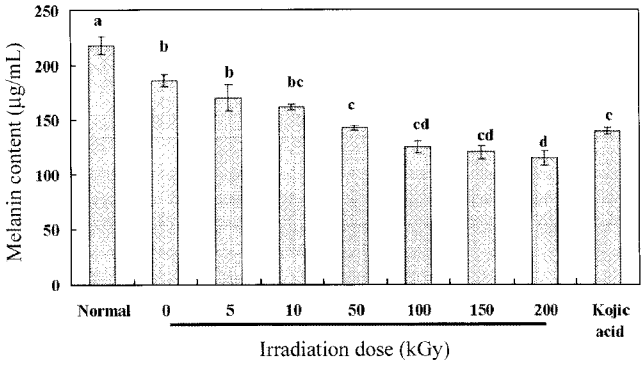

Fig. 6. Inhibition of melanin production of fibroin irradiated at various doses. Sample final concentration is 62.5 μg/mL. Normal include distilled water without fibroin solution. Bars represent the mean ±SD. The letters mean the statistically significant difference compared with the control ($p<0.05$).

effect of a gamma-irradiated fibroin on tyrosinase is shown in Fig. 5. Although the non-irradiated fibroin had a tyrosinase inhibition activity, the gamma-irradiated fibroin showed a stronger inhibitory effect on the activity of tyrosinase than the non-irradiated fibroin. The inhibitory effect of tyrosinase also increased depending on the irradiation dose.

The effect of fibroin on the inhibition of melanin production was conducted to support the result of tyrosinase inhibitory activity of irradiated fibroin (Fig. 6). The results showed that the inhibition effect of melanin synthesis was decreased as the irradiation dose increased and the fibroin irradiated at above 100 kGy had a stronger inhibitory effect than kojic acid, positive control.

Amino acids, peptides, and proteins can inhibit a tyrosinase activity by means of more complicated mechanisms including a reaction with intermediates, quinines (39), and a formation of stable Cu^{2+} chelates (40,41) and they have a different pathway due to their structure, composition, and Mw (42). It has also been reported that silk fibroin had a tyrosinase inhibition activity, because of its repetitive (-Gly-Ala-Gly-Ala-Gly-Ser-) structure (9,42,43). From these results, it could be concluded that the gamma irradiation change the conformational structure of fibroin, resulting in an increase of its physiological activities.

In conclusion, gamma irradiation could increase the physiological activities such as the antioxidant and anti-melanogenic effects of silk fibroin. This reason might due to the changes of conformational structure by gamma irradiation. Therefore, irradiation could be used as an effective method to produce fibroin, more suitable for the development of functional foods and cosmetics. Further studies should be carried out to elucidate the relationship between the molecular structure and physiological activities of fibroin.

Acknowledgments

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