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Effect of γ-Irradiation on the Molecular Properties of Bovine Serum Albumin and β-Lcatoglobulin

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To elucidate the effect of oxygen radicals on the molecular properties of proteins, the secondary and tertiary structure and molecular weight size of BSA and β-lactoglobulin were examined after irradiation of proteins at various doses. Gamma-irradiation of protein solutions caused the disruption of the ordered structure of protein molecules as well as degradation, cross-linking, and aggregation of the polypeptide chains. As a model system, BSA and β-lactoglobulin were used as a typical α-helical and a β-sheet structure protein, respectively. A circular dichroism study showed that the increase of radiation decreased the ordered structure of proteins with a concurrent increase of aperiodic structure content. Fluorescence spectroscopy indicated that irradiation quenched the emission intensity excited at 280 nm. SDS-PAGE and a gel permeation chromatography study indicated that radiation caused initial fragmentation of proteins resulting in a subsequent aggregation due to cross-linking of protein molecules.

Keywords: Irradiation, Molecular properties, Protein conformation.

Introduction

Radiation treatments of biological materials were applied in the fields of medical sterilization and food irradiation. The chemical changes that irradiation causes in biopolymers (such as proteins) are fragmentation, cross-linking, aggregation, and oxidation by oxygen radicals generated in the radiolysis of water (Schuessler and Schilling, 1984; Garrison, 1987; Davies and Delsignore, 1987c; Filali-Mouhim *et al.*, 1997). Assuming that these changes occur simultaneously, their rates depend on the chemical nature of the polymer, its physical state, and the irradiation condition (Woods and Pickaev, 1994). Especially, the effect of γ-irradiation on protein conformation appears to depend on several factors such as protein concentration, the presence of oxygen, and the quaternary structure of proteins. Davies (1987abcd) published a series of papers on protein damage and degradation by oxygen radicals. Hydroxy radical and superoxide anion radical generated by radiation could modify the primary structure of proteins, which would result in distortions of the secondary and tertiary structure (Davies and Delsignore, 1987c).

In general, radiation causes irreversible changes at the molecular level by the breakage of the covalent bonds of the polypeptide chains. Exposure of proteins to oxygen radicals results in both non-random and random fragmentation (Kempner, 1993). Fragmentation involves reaction of α-carbon radicals with oxygen to form peroxyl radicals, which decompose to fragment the polypeptide chain at the α-carbon. The protein fragmentation in aqueous solutions is affected by the local conformation of an amino acid in the protein, its accessibility to the water radiolysis products, and the primary amino acid sequence (Filali-Mouhim *et al.*, 1997). In Scuessler and Schilling's model (Schuessler and Schilling, 1984), bovine serum albumin (BSA) is cleaved by oxidative destruction of proline residues, thus yielding specific protein fragments. Also, there have been reports on the aggregation and cross-linking of proteins by irradiation (Garrison, 1987; Puchala and Schessler, 1993; Kume and Matsuda, 1995; Filali-Mouhim *et al.*, 1997). Covalent cross-linkages are formed between free amino acids and proteins and between peptides and proteins in solution after irradiation (Garrison, 1987).

The radiation-induced alteration of the protein structure is observed in a number of protein systems by measuring changes in the molecular properties of proteins. To further elucidate the effect of oxygen radicals on protein structure, this study describes the radiation effect on the secondary and tertiary structure, and molecular weight size of proteins using BSA and β-lactoglobulin as model proteins.

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Materials and Methods

Materials Bovine serum albumin and β-lactoglobulin were purchased from Sigma Chemical Co. (St Louis, USA) and used without further purification. Protein concentrations of BSA and β-lactoglobulin were determined by measuring the absorbance at 279 nm ($A^{1\%} = 6.67$) and 280 nm ($A^{1\%} = 9.5$), respectively. Standard marker proteins for SDS-PAGE were obtained from Bio-Rad, Inc. (Richmond, USA).

Sample Irradiation Ten ml solutions of BSA and β-lactoglobulin in 10 mM phosphate buffer (pH 7.0) were added in borosilicate glass vials (16×125 mm) and irradiated at room temperature using ^{60}Co gamma ray irradiation Type IR-79 (Nordion International Inc., Ontario, Canada) as source under air with 0, 0.5, 1, 5, and 10 kGy, respectively. ^{60}Co-exposure was varied from 6 to 189 cm in order to achieve total doses of 0.5-10 kGy and the dose rates were 0.5, 1, 5, and 10 kGy/h. The protein concentration was 65 μM and 190 μM for BSA and β-lactoglobulin, respectively.

Circular Dichroism (CD) Measurements CD spectroscopy was performed at 25°C with a JASCO J-720 spectropolarimeter according to the method reported previously (Lee and Song, 1997; Cho and Song; 1997). A 1-mm-pathlength cell was used. The protein solutions were diluted with a 10 mM phosphate buffer (pH 7.0) in order to achieve the desired protein concentration. The protein concentration for bovine serum albumin was 1.2 μM and for β-lactoglobulin was 7.6 μM. The reported CD spectra were the average of five scans, and these were smoothed by the polynomial curve fitting program and then analyzed by the method of Chang *et al.* (1978). CD data were expressed as molar ellipticity in deg cm^2 $dmol^{-1}$.

Fluorescence Spectroscopy Fluorescence emission intensity of BSA and β-lactoglobulin solution irradiated was measured using a spectrofluorometer (JASCO FP-750, Japan). The BSA and β-lactoglobulin irradiated solutions were excited at 280 nm and the emission spectra were recorded from 300 to 450 nm. The protein concentrations of BSA and β-lactoglobulin solutions were 2.4 μM and 7.6 μM, respectively.

SDS-PAGE SDS-PAGE was performed according to the method of Laemmli (1970).

Gel Permeation Chromatography The irradiated protein samples were loaded onto a Sephacryl S-200 HR column (1.5×170 cm) which had been equilibrated with a 10 mM phosphate buffer (pH 7.0). The protein solutions were eluted at 0.6 mL/min using a peristaltic pump. The absorbance of the fraction was measured at 280 nm.

Results and Discussion

Far-UV CD spectra show the conformational change on the secondary structure of proteins. In the case of change in the local environment of the ordered structure of a polypeptide chain, it is especially easily differentiated from the native one. Far-UV CD spectra of BSA solution, irradiated at various doses, were obtained (Fig. 1). The spectrum of native BSA had a typical α-helical structure, which has negative minimum ellipticity values at 207 and 221 nm and a positive maximum at 193 nm. However, γ-irradiation clearly affected the CD spectrum. Mostly, it decreased the ordered structure, resulting in a decrease of α-helical structure with a concomitant increase of aperiodic structure. With an increase in irradiation, ellipticity values at 207 and 221 nm were increased. Oxidative polypeptide chain fragmentation by oxygen radicals subsequently destabilized the α-helical structure of proteins. This was also confirmed by the estimation of a secondary structure content by Chang *et al.*'s method (1978). Table 1 clearly shows that the γ-irradiation decreased the α-helical structure content of BSA with a simultaneous increase of random coil structure. The same trend was observed for β-lactoglobulin, which is one of typical proteins having β-sheet structure. The CD spectrum of native β-lactoglobulin had a typical β-sheet structure, having a negative minimum around 216 nm (Fig. 2). The increase of the γ-irradiation shifted the negative minimum value of the spectrum to the lower wavelength. Also, the γ-irradiation disrupted the ordered structure of the protein, resulting in an increase of random coil structure (Table 2).

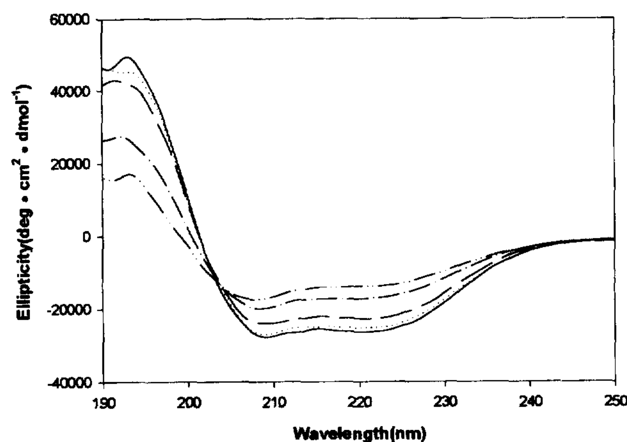

Fig. 1. CD Spectra of BSA irradiated. The protein concentration was 1.2 μM. —, 0 kGy; ------, 0.5 kGy; - - -, 1.0 kGy ; - · -, 5 kGy; - · · -, 10 kGy

Table 1. Estimation of secondary structure content of BSA irradiated.

Radiation Dose (kGy)	α-Helix	β-Sheet	β-Turn	Random coil
	(%)			
0	70	0	0	30
0.5	70	0	0	30
1	65	0	5	30
5	50	0	10	40
10	35	10	10	45

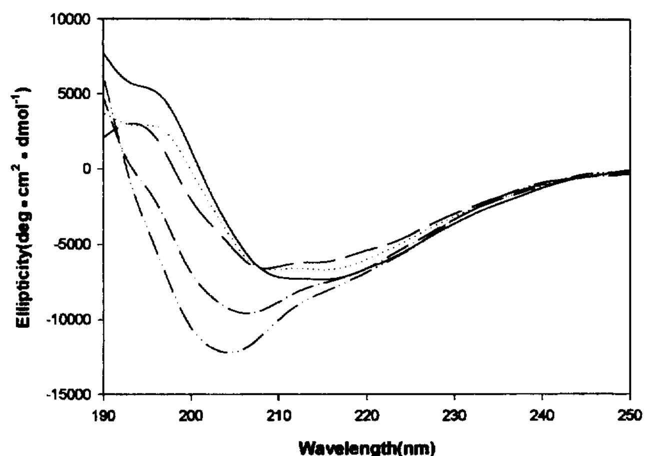

Fig. 2. CD Spectra of β-lactoglobulin irradiated. The protein concentration was 7.6 μM. —, 0 kGy; ------, 0.5 kGy; - - -, 1.0 kGy; - · · -, 5 kGy; - · · · -, 10 kGy

Table 2. Estimation of secondary structure content of β-lactoglobulin irradiated.

Radiation Dose (kGy)	α-Helix	β-Sheet	β-Turn	Random coil
	(%)			
0	15	55	5	25
0.5	15	45	10	30
1	10	55	5	30
5	5	55	0	40
10	10	30	10	50

The changes in CD spectra of these two proteins by radiation were mainly due to the cleavage of covalent bonds of proteins and the formation of aggregated products. The CD results clearly support the conclusion that γ-irradiation breaks covalent bonds easily and disrupts the ordered structure of proteins, resulting in unnatural products of proteins. This is also proved by the fact that some irradiated enzymes lose their activity as well as immunogenicity (Kume and Matsuda, 1995; Yang *et al.*, 1996). This indicates that even γ-irradiation of a very weak dose level alters the native function of a protein.

To examine further the change on the molecular properties of BSA and β-lactoglobulin solutions by irradiation, fluorescence emission intensity was measured. When excited at 280 nm, which excites both tryptophan and tyrosine residues of the protein, the tertiary structure of the protein is reflected. Figures 3 and 4 show that γ-irradiation caused the decrease of emission intensity of both BSA and β-lactoglobulin, due to the change of the local environment around tryptophan and tyrosine residues. An increase of radiation quenched the emission intensity of BSA and induced a blue shift (Fig. 3). In contrast, for β-lactoglobulin, the emission peak was changed from 342 to 338 nm, giving a slight red shift (Fig. 4). This discrepancy might be attributed to the difference in the tertiary structure of BSA and β-lactoglobulin.

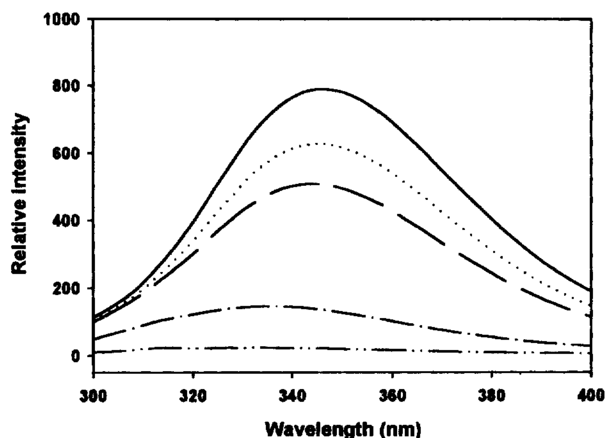

Fig. 3. Fluorescence emission spectra of BSA irradiated. The protein concentration was 2.4 μM. —, 0 kGy; ------, 0.5 kGy; - - -, 1.0 kGy ; - · · -, 5 kGy; - · · · -, 10 kGy

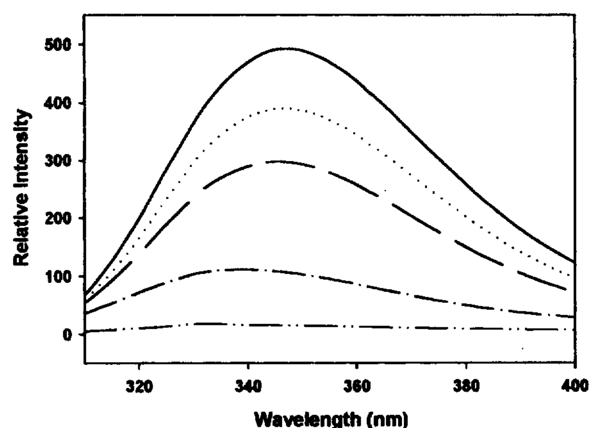

Fig. 4. Fluorescence emission spectra of β-lactoglobulin irradiated. The protein concentration was 7.6 μM. —, 0 kGy; ------, 0.5 kGy; - - -, 1.0 kGy ; - · · -, 5 kGy; - · · · -, 10 kGy

Regarding the radiation damage to proteins, two types of damages are observed: fragmentation and aggregation (Filali-Mouhim *et al.*, 1997). SDS-PAGE profiles of BSA and β-lactoglobulin showed that γ-irradiation at a low-dose range causes the breakdown of the polypeptide chain, resulting in the formation of small molecular weight molecules (Fig. 5 and 6). Similar results were observed in other studies (Schuessler and Schilling, 1984; Le Maire *et al.*, 1990). Le Maire *et al* (1990) suggesting that the locations of the break points, caused by radiation on the aspartate transcarbamylase, are fragile bonds in the polypeptide chain. Also, Schuessler and Schelling (1984) proposed that proline residues are the targets for chain scission caused by radiation. Wolff *et al.* (1986) reported that peptide bonds can be cleaved as a result of the direct oxidation of proline residues. Usually, breakage of covalent bonds in irradiated proteins is shown as new bands below the major band. At 10 kGy of dose on SDS-PAGE gel there was only a degraded pattern of protein molecules with

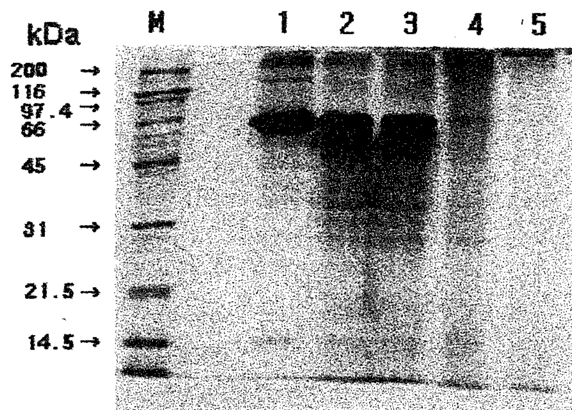

Fig. 5. SDS-PAGE Pattern of BSA irradiated. Molecular weight marker protein (M); O kGy (1); 0.5 kGy (2); 1 kGy (3); 5 kGy (4); 10 kGy (5)

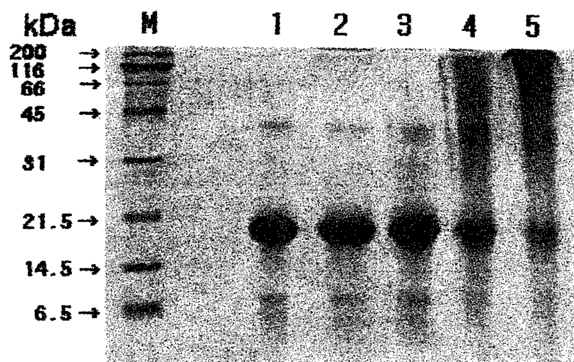

Fig. 6. SDS-PAGE Pattern of β-lactoglobulin irradiated. Molecular weight marker protein (M); O kGy (1); 0.5 kGy (2); 1 kGy (3); 5 kGy (4); 10 kGy (5)

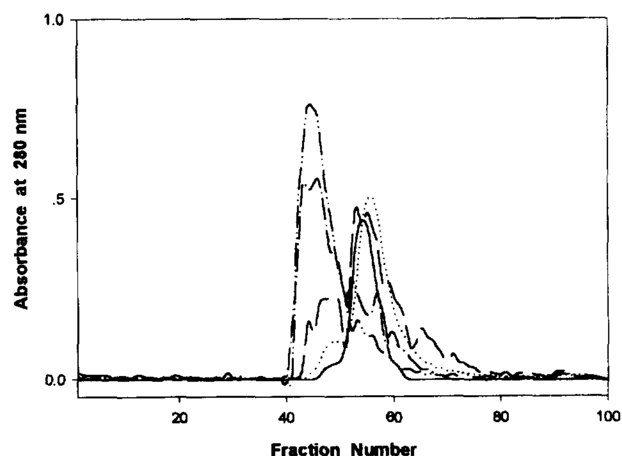

Fig. 7. Gel Permeation Chromatogram of BSA irradiated. —, 0 kGy; ------, 0.5 kGy; - - -, 1.0 kGy; - · -, 5 kGy; - · · -, 10 kGy

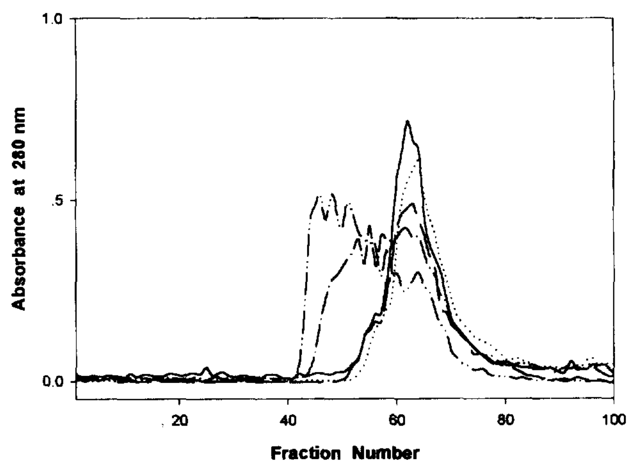

Fig. 8. Gel Permeation Chromatogram of β-lactoglobulin irradiated. —, 0 kGy; ------, 0.5 kGy; - - -, 1.0 kGy; - · -, 5 kGy; - · · -, 10 kGy

some of the aggregated molecules which could not penetrate the separating gel of PAGE (Fig. 5 and 6). This was also consistent with the GPC data of BSA and β-lactoglobulin (Fig. 7 and 8). The increase of γ-irradiation dose significantly and unambiguously showed the consistent pattern where initially γ-irradiation increased the number of small molecular weight molecules, indicating the degraded protein molecules. Eventually at a high dose range, such as 5 and 10 kGy, γ-irradiation significantly increased the size of protein molecules, which are cross-linked products of degraded protein molecules. A similar result was observed in the case of hemoglobin (Puchala and Schuessler, 1993). When hemoglobin was irradiated in a phosphate buffer at pH 7.0 under air, aggregation, as well as fragmentation, was observed.

Proteins may be converted to higher molecular weight aggregates due to the generation of inter-protein cross-linking reactions, hydrophobic and electrostatic interactions, and the formation of disulfide bonds (Davies and Delsignore, 1987c; Le Maire *et al.*, 1990). Any amino acid radical formed within a peptide chain could cross-link with an amino acid radical in another protein. At a high dose range, BSA and β-lactoglobulin exposed to radiation underwent covalent cross-linking. The formation of the high molecular weight aggregates was negligible at the low dose range, but increased significantly with higher doses (Fig. 7 and 8), partly caused by the intermolecular S-S bonds. A similar interpretation was reported previously for egg white lysozyme and BSA, where radiation damage was explained by the rupture of disulfide bonds and disulfide exchange (Schuessler and Schilling, 1984; Stevens *et al.*, 1967).

In conclusion, the γ-irradiation of protein solutions caused disruption of the ordered structure of protein molecules as well as degradation, cross-linking, and aggregation of the polypeptide chains. As a model system, BSA and β-lactoglobulin, as a typical α-helical and β-sheet structure protein, showed the conformational change and change of molecular weight size by γ-irradiation.

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