

Influence of Gamma Irradiation on Porcine Serum Albumin Structural Properties and Allergenicity

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Pork provides an ideal source of food energy; however, pork can elicit an allergic reaction, and porcine serum albumin (PSA) has been identified as a major allergen. This study examined the impact of gamma irradiation on the allergenicity and structural qualities of PSA; the PSA solution was gamma-irradiated at 1, 2, 4, 6, and 8 kGy. Allergenicity was investigated by immunoblotting and competitive indirect ELISA using serum from patients who are allergic to pork, and conformational changes in irradiated PSA were measured by circular dichroism, sulfhydryl group detection, and fluorescence emission spectra. The secondary and tertiary structures of gamma-irradiated PSA were altered, and the allergenicity of PSA was lowered by boosting the amount of irradiation. In addition, there is high correlation between depletion in the α -helix and immunoglobulin E-binding capability of PSA. The results show a new possibility in using gamma irradiation to reduce the allergenicity of pork products.

Food allergies are an emergent public health concern affecting approximately 5% of all adults and 8% of children (1). Clinical signs of food allergies differ, ranging from slight urticaria to life-threatening anaphylactic shock and asthma (2, 3). Most food allergies are mediated by antigen-specific immunoglobulin E (IgE); foods involved in IgE-mediated allergies commonly include milk (4), eggs (5), peanuts (6), soybeans (7), or wheat (8). According to national standards in China, GB/T 23779-2009 Allergenic Ingredients in Prepackaged Foods states that allergenic foods include wheat, crustaceans, fish, eggs, peanuts, soybeans, milk, and tree nuts. Patients who are allergic to pork are uncommon, but the

condition can manifest as atopic dermatitis and oral allergy syndrome (9). Porcine serum albumin (PSA) is a globular protein (67 kDa) that is identified as a major allergen in pork (10); however, the rationale for using food processing to reduce the allergenicity of pork has not been well demonstrated.

Nowadays, nonthermal techniques, including gamma irradiation, are regarded with growing interest by food industries. Gamma irradiation has been applied to enhance shelf-life and/or has been used to improve microbiological food safety without damaging nutritional quality (11). Recent studies have shown that gamma irradiation can influence the allergenicity of food allergens. Byun et al. (12) confirmed that gamma irradiation is regarded as an effective approach to reduce the allergenicity of shrimp, and similar findings have been reported for peanut, egg, and milk (13–15). At present, China remains the world's largest consumer and producer of pork, but few studies in China have been conducted on the influence of gamma irradiation on the allergenicity and structural properties of PSA, a major allergen.

Food-processing technologies can lower the chance of allergenicity by eliminating and/or altering allergens with a method through which allergenic epitopes are identified by the immune system. The goal of this study was to evaluate the impact of gamma irradiation on the allergenicity of PSA. Food allergenicity can be lowered or eradicated with processes that change allergen structures and cause them to be untouchable by antibody receptors. Thus, the effect of gamma irradiation on the structural properties of PSA [secondary structure, surface sulfhydryl (SH) content, and tertiary structure] will also be examined.

Materials and Methods

Allergen and Antibodies

PSA purified from porcine serum was obtained from Sigma Chemical Co. (St. Louis, MO), and goat antihuman IgE peroxidase conjugate was obtained from KPL (Gaithersburg, MD). Twenty patients (12 male, 8 female; age range: 18–45 years) were recruited at the People's Hospital of Jiangsu Province (Nanjing, China). Sera were obtained from all the patients diagnosed with IgE-mediated pork allergy through extensive history, physical

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examination, skin-prick test, and objective manifestations observations after pork ingestion.

Gamma Irradiation Treatment

PSA was disintegrated in 10 mM phosphate-buffered saline (PBS; pH 7.3) in borosilicate glass vials and transferred into a cobalt-60 gamma irradiator (Gaotong Isotope Co., Chengdu, Sichuan, China) with air. The dosages were 1, 2, 4, 6, and 8 kGy, and the temperature was set to $10 \pm 0.5^\circ\text{C}$ throughout the irradiation regimen.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was completed based on Laemmli (16) to establish the degree of PSA change by gamma irradiation. The concentration of the specimen was 4 mg/mL. The SDS-PAGE of the specimen was accomplished on 4.5% stacking gels and 12% separating gels. The gels were stained with Coomassie brilliant blue R250 (Sigma Chemical Co., St. Louis, MO) and destained in a destaining solution with 7% acetic acid and 5% methanol. Prestained protein MW markers were acquired from Fermentas Inc. (Glen Burnie, MD). Following destaining, the gels were scanned with a GeneGenius Bio Imaging System (Syngene, Frederick, MD).

Immunoblotting

Immunoblotting was completed based on Towbin et al. (17) with alteration. After SDS-PAGE analysis, the divided proteins were electrotransferred to methanol-activated PVDFs (Millipore, United States) at 250 mA for 1.5 h in a buffer of 25 mM Tris-base, 191 mM glycine, and 20% methanol. Protein transfer was verified by staining with 0.1% (w/v) Ponceau-S in 5% (v/v) acetic acid, and the background was destained with 7% (v/v) acetic acid. Each strip was obstructed with a 3% (w/v) gelatin in Tris-buffered saline (TBS) (20 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl), and incubated at 37°C for 3.5 h with pork-allergic patients' serum (1:10) diluted with 1% (w/v) gelatin in TBS. The strip was incubated at 37°C for 1.5 h with diluted goat antihuman IgE peroxidase conjugate (1:1000) in TBS of 0.1% (v/v) Tween-20 (TBST). The antigen-antibody complex was viewed using chromogen 3,3'-diaminobenzidine (Sigma Chemical Co.) and washed with deionized water. Washing for all steps was accomplished in TBST, except for the blocking step. The blots were visualized with the GeneGenius Bio Imaging System (Syngene, Frederick, MD).

Competitive-Inhibition (Ci) ELISA

The binding capabilities of the irradiated PSA to patients' serum were investigated by Ci-ELISA. A 96-well microtiter plate was covered with 100 μL of 10 $\mu\text{g}/\text{mL}$ PSA solution in a 0.2 M bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. The plates were obstructed with 1% gelatin in 10 mM PBS at 37°C for 2 h to lower nonspecific binding. We added 50 μL irradiated PSA solution (12 $\mu\text{g}/\text{mL}$) and 50 μL patients' serum (1:30) to each well, followed by incubation at 37°C for 2 h. We subsequently added 100 μL diluted goat antihuman IgE peroxidase conjugate

(1:250) to the wells, followed by incubation at 37°C for 2 h. We added 150 μL 3,3',5,5'-tetramethylbenzidine substrate solution (Promega Corp., Madison, WI) to the wells, followed by incubation at 37°C for 10 min. The reaction was halted with 50 μL of 2 M H_2SO_4 . The optical density (OD) at 450 nm was then read on an iMark microplate reader (Bio-Rad, Hercules, CA). The plates were washed three times with 10 mM PBS with 0.1% (v/v) Tween-20 following every step of the incubation process. We added 50 μL of each primary antibody and 10 mM PBS into each well for 100% binding (C_{max}) among the covered PSA solution and anti-PSA serum without competitive antigens. We used 100 μL of 10 mM PBS as the blank. Every sample was completed in triplicate. The IgE-binding capabilities were established with the following equation:

$$\text{IgE-binding capabilities (\%)} = \frac{\text{OD of } C_{\text{max}} - \text{OD of irradiated PSA}}{\text{OD of } C_{\text{max}} - \text{OD of untreated PSA}} \times 100\%$$

Circular Dichroism (CD) Analysis

CD spectra were recorded on a Chirascan spectrophotometer (Applied Photophysics Ltd, Surrey, United Kingdom) at room temperature under a constant flow of nitrogen gas. Subsequently, 0.2 mL irradiated PSA (0.05 mg/mL) or native PSA (0.05 mg/mL) in 10 mM PBS was heated to an ambient temperature ($25 \pm 1^\circ\text{C}$) prior to analysis. CD spectra were scanned in the range of 180–260 nm at a rate of 1 nm/s, with a bandwidth of 1 nm. Each spectrum was determined by taking an average of three scans and subtracting the 10 mM PBS background. The protein secondary structure of the CD spectra (190–260 nm) was acquired with CDNN CD Spectra Deconvolution Software (Version 2.1; Applied Photophysics Ltd, Leatherhead, United Kingdom). Each sample was established in triplicate.

Determination of Free SH Content

Alterations in the SH content in the protein were ascertained by the Ellman method (18). The samples were diluted with 10 mM PBS for a total volume of 2.5 mg/mL PSA. For the assay, 40 μL of 10 mM 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) solution (in 90 mM Gly, 4 mM EDTA, 86 mM Tris-HCl buffer, pH 8.0) were added to 4 mL sample, and the absorbance of the mixture was measured at 412 nm using a SpectraMax M2 microplate reader (Molecular Devices, Sunnyvale, CA) after incubating at an ambient temperature ($25 \pm 1^\circ\text{C}$) for 5 min. The values of SH content were acquired by dividing the value of the absorbance by a molar extinction coefficient of 13 600. The outcomes are in $\mu\text{mol SH/g PSA}$. Each sample was established in triplicate.

Fluorescence Spectroscopy Analysis

Fluorescence spectra were run on a Perkin Elmer Luminescence Spectrometer LS 50B (Norwalk, CT). Protein solutions (0.05 mg/mL) in 10 mM PBS were scanned at an ambient temperature ($25 \pm 1^\circ\text{C}$). Emission and excitation slits were both fixed at 5 nm. The emission spectra (λ_{em} : 300–550 nm) were acquired via scanning at the maximum excitation wavelength (295 nm). Each sample was determined in triplicate.

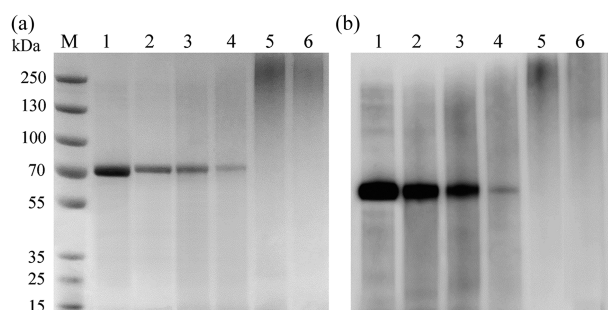


Figure 1. (A) SDS-PAGE and (B) immunoblotting of PSA irradiated at various doses. (A) Lanes: M, protein MW marker; 1, untreated PSA; 2, 1 kGy; 3, 2 kGy; 4, 4 kGy; 5, 6 kGy; 6, 8 kGy. (B) Lane 1–6: Immunoblotting of PSA with pork-allergic patients' serum.

Statistical Analysis

Data were statistically examined by one-way analysis of variance with Microcal Origin 8.0 (Microcal Software, Inc., Northampton, MA). Statistical differences with P -values of <0.05 were considered to be significant.

Results and Discussion

SDS-PAGE and Immunoblotting Profile of Gamma-Irradiated PSA

SDS-PAGE patterns of PSA at different irradiation doses are shown in Figure 1A. We noted two forms of irradiation damage to proteins: fragmentation and aggregation (19). The SDS-PAGE profiles of PSA suggested that gamma irradiation at dosages of 1, 2, and 4 kGy leads to the disintegration of the polypeptide

chain, which then causes a reduction in the intensity of the PSA band (67 kDa) with an identical quantity of the protein loaded (Figure 1A). Comparable findings were detailed in other evolutions (20, 21). Nevertheless, on the SDS-PAGE gel at dosages greater than 6 kGy, there were just smeared deteriorated patterns with aggregated protein molecules that could not infiltrate the separating gel when the identical quantity of the protein was loaded on each lane (Figure 1A). Irradiated proteins may be transformed into larger MW aggregates because of interprotein cross-linking reactions, hydrophobic and electrostatic interactions, and the creation of disulfide (SS) bonds (22, 23). Any amino acid radical created inside a peptide chain can cross-link with an amino acid radical in another protein. At large dosages, proteins exposed to radiation experienced covalent cross-linking. The formation of high-MW aggregates was inconsequential at minimal dosages, but grew significantly with larger doses (Figure 1A). Comparable findings were already noted for egg white lysozyme and bovine serum albumin (BSA; 24, 25). An immunoblotting method was implemented to detect reactivity against native PSA and gamma-irradiated PSA (Figure 1B). The results indicate that the density of immunoblotting gradually became weaker with the increase of the irradiation dose. As the irradiation dose increased up to 6 kGy, the density eventually disappeared. These results further suggested that gamma irradiation destroyed the protein's molecular structure, and polypeptide chains were broken or cross-linked, which could cause the epitopes to be masked or even destroyed.

Effect of Gamma Irradiation on Allergenicity

The allergenicity changes displayed by gamma irradiation were determined quantitatively. The IgE-binding capability of

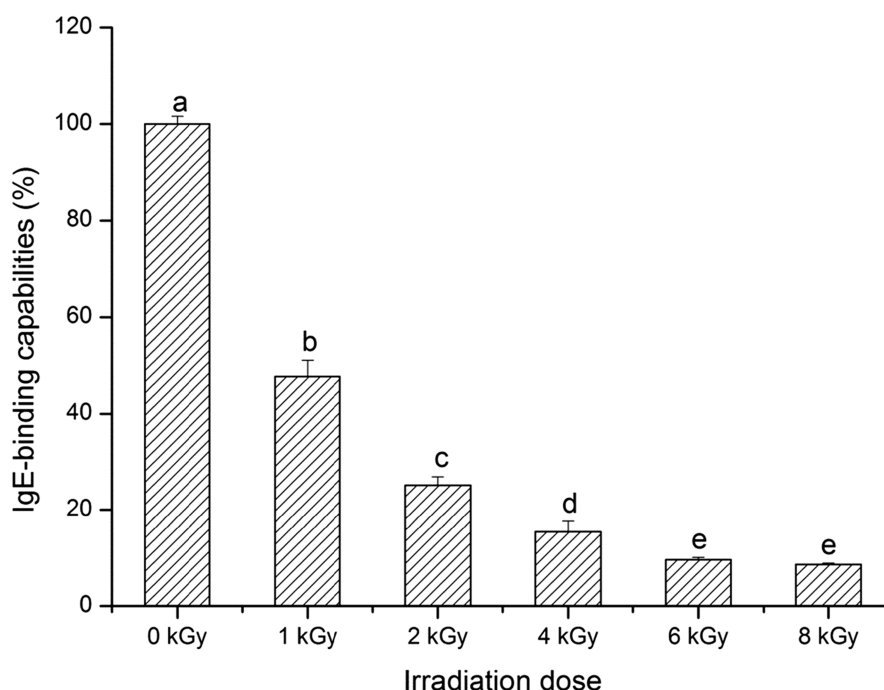


Figure 2. IgE-binding capabilities of PSA irradiated at various doses. (a–e) Means in the bars bearing different letters are significantly different at $P < 0.05$.

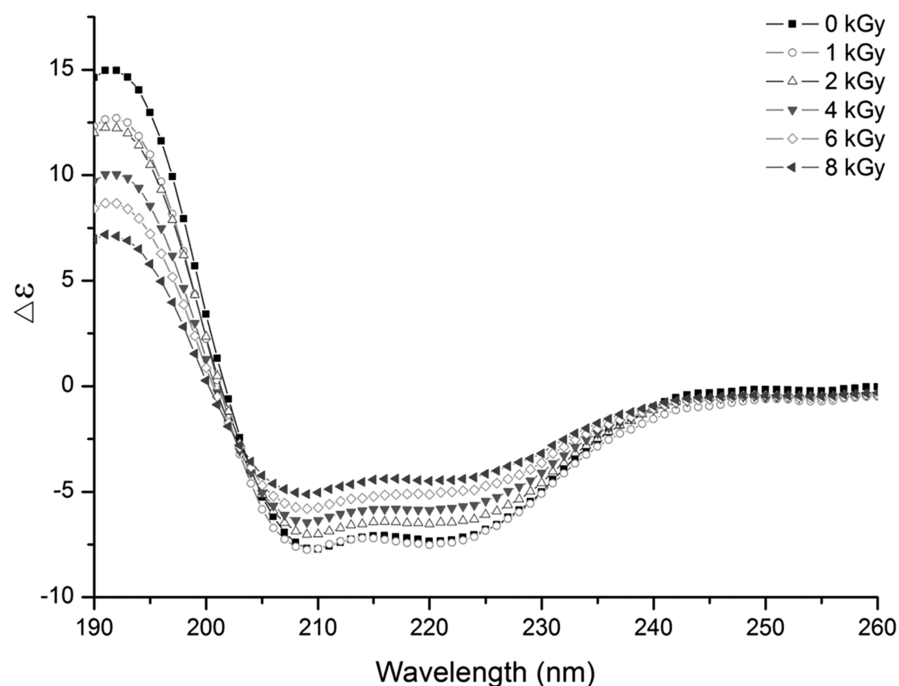


Figure 3. Far-UV CD spectra of irradiated PSA at various doses.

PSA samples treated with gamma irradiation was abruptly reduced to approximately 47.7% when irradiated at 1 kGy (Figure 2). The IgE-binding capabilities were reduced to about 25.2, 15.5, 9.7, and 8.6% in irradiated samples receiving doses of 2, 4, 6, and 8 kGy, respectively (Figure 2). Figure 2 reveals an abrupt reduction in allergenicity acquired from irradiated specimens at 0–6 kGy ($P < 0.05$); however, at 8 kGy, the allergenicity of PSA was not significantly different from 6 kGy. Additional food allergens have been also examined to lower their allergenicity by gamma irradiation, including shrimp tropomyosin (12, 26), peanut allergen Ara h 6 (15), BSA (27), and cow's milk allergen (28). The modification trends of the allergenicity of food allergens treated by gamma irradiation may occur from the protein structure and conformational alterations due to gamma irradiation. It is recognized that gamma irradiation prompts the decrease in the amount of epitopes by the denaturation of the protein, including fragmentation or conformational liberation of the molecule or accumulation of the molecules (27). Based on these notions, the impact of gamma irradiation on the conformational changes of PSA was completed in following experiments, which could additionally corroborate our findings.

Effects of Gamma Irradiation on the Secondary Structure

Usually secondary structures in proteins have α -helices, β -sheets, and β -turns with typical far-UV CD spectra that specifically typify the alteration of protein secondary conformation (29). Two negative peaks at 208 and 222 nm are typical of α -helix secondary conformation of proteins, and 214 nm is typical of β -sheet secondary conformation of proteins (29). Figure 3 shows the far-UV CD spectra of PSA irradiated at different dosages. The CD spectrum of 1 kGy-irradiated PSA revealed a minor change, whereas the spectra showed blatant alterations after irradiation dosages greater than 2 kGy. The negative ellipticity values in the span of 200 and 225 nm were mainly lowered following gamma irradiation, suggesting the reduction of the ordered structure. Assessment of the secondary structure revealed that gamma irradiation treatment led to a reduction in α -helices, a boost in the β -structure, and a variation in the unordered fraction of the PSA secondary structure (Table 1). The outcomes also suggested that the native PSA had 53.8% α -helices, 6.8% β -sheet, 13.6% β -turn, and 25.8% random coil. Following

Table 1. Secondary structure content of PSA irradiated at various doses^a

Treatment, kGy	α -Helices, %	β -Sheet, %	β -Turn, %	Random coil, %
0	53.8 $\pm$ 0.9	6.8 $\pm$ 0.2	13.6 $\pm$ 0.1	25.8 $\pm$ 0.6
1	51.0 $\pm$ 1.5	7.5 $\pm$ 0.4	14.1 $\pm$ 0.2	27.4 $\pm$ 1.0
2	46.9 $\pm$ 1.6	8.9 $\pm$ 0.6	14.8 $\pm$ 1.5	29.5 $\pm$ 0.8
4	41.3 $\pm$ 0.4	11.6 $\pm$ 0.2	15.8 $\pm$ 0.1	31.3 $\pm$ 0.3
6	37.0 $\pm$ 0.8	14.5 $\pm$ 0.6	16.5 $\pm$ 0.1	32.0 $\pm$ 0.1
8	32.7 $\pm$ 0.3	18.5 $\pm$ 0.2	17.3 $\pm$ 0.1	31.5 $\pm$ 0.1

^a All data are shown as means $\pm$ SD ($n = 3$).

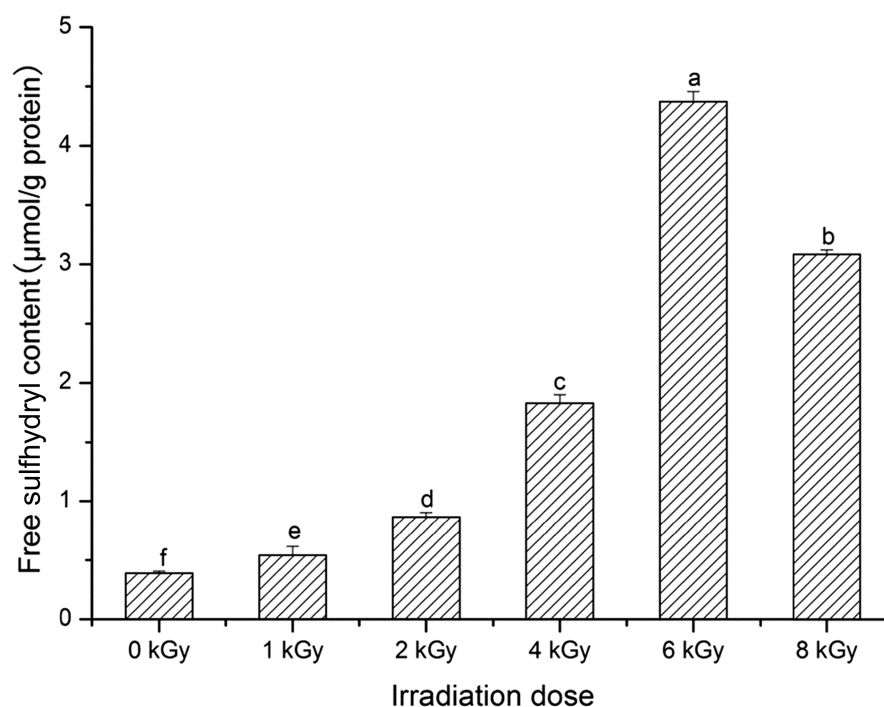


Figure 4. SH content on the surface of PSA irradiated at various doses. (a–f) Means in the bars bearing different letters are significantly different at $P < 0.05$.

gamma irradiation at 8 kGy, α -helix content of PSA was reduced to 32.7%, with the simultaneous growth of β -sheet content to 18.5%. Gamma irradiation caused the interruption in the ordered secondary structure of food allergens, as detailed in prior studies (15, 30, 31). Also shown in Figure 3, characteristic negative peaks at 208 and 222 nm in the native PSA are indicative of the predominant α -helix structures (32, 33). By linking the impact of gamma irradiation to the allergenicity of PSA with its secondary structures, these changes of secondary structures imply

that the allergenicity of PSA may be affiliated with the interruption of α -helix structures caused by gamma irradiation.

Effect of Gamma Irradiation on Surface SH Group Content

SH groups and SS bonds have a strong influence on the structure and allergenicity of food allergens (34). Gamma

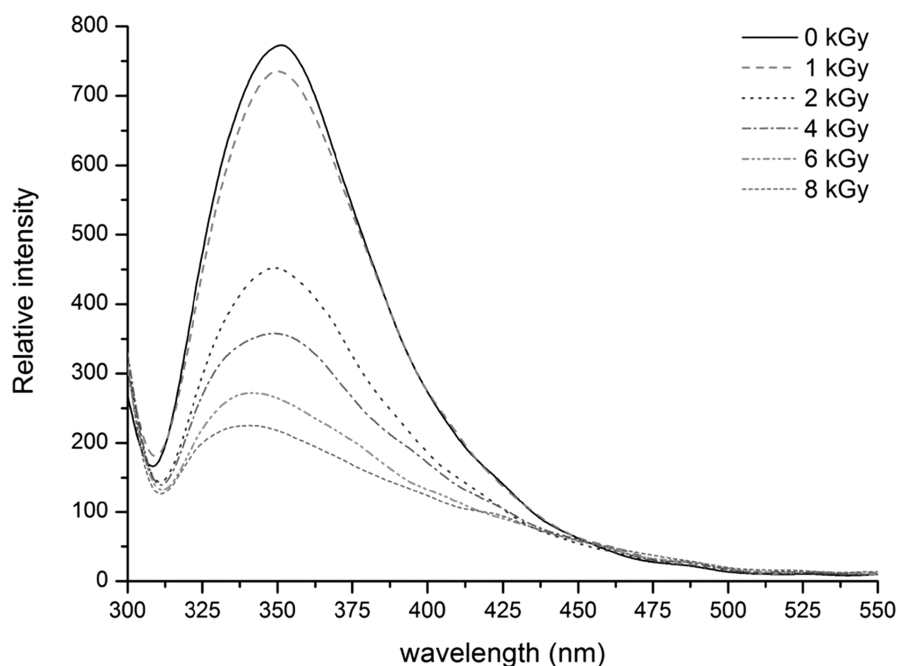


Figure 5. Fluorescence emission spectra of irradiated PSA at various doses.

Table 2. Effects of irradiation on the peak and emission intensity of PSA fluorescence spectra

Treatment, kGy	Peak/nm	Emission intensity
0	351	773.0
1	350	735.2
2	349	452.0
4	349	357.4
6	341	272.1
8	340	225.1

irradiation can interrupt noncovalent bonds, including hydrogen and SS bonds that anchor the protein structure by producing free radicals that assault protein molecules (35). The surface SH content of the PSA samples after gamma irradiation is shown in Figure 4. In the range of 1–6 kGy, the surface SH content of PSA increased significantly ($P < 0.05$). On the other hand, with irradiation at 8 kGy, the surface SH content decreased progressively and significantly ($P < 0.05$; Figure 4). The outcomes additionally verify the incidence of irradiation-induced protein unfolding and ensuing aggregation/reassociation of unfolded proteins. At larger dosages, the accumulation of unfolded proteins is distinguished because of hydrophobic interactions and/or SH–SS exchange reactions, thereby improving the reformation of SS bonds. The current examination suggests that PSA has a steady structure made of nine individual SS-bonded loops (36). Thus, it was hypothesized that these steady thioether linkages were paramount to keeping its indigenous conformation and allergenicity.

Effect of Gamma Irradiation on the Tertiary Structure

The fluorescence spectra were established primarily by the dichotomy of the surroundings of the tryptophan and tyrosine residues and by their particular interactions (37). The quantity of quantum yield of fluorescence is lowered when the chromophores interact with quenching mediums, with a solvent, or in the protein (38). The modifications in intrinsic tryptophan fluorescence emission correlate with alterations in the tertiary structure of the protein (39). The fluorescence emission spectra of gamma irradiation-treated PSA at $\lambda_{\text{ex}} = 295$ nm are revealed in Figure 5. Following irradiation processing at 1–6 kGy, the relative fluorescence intensity peak was lowered significantly when contrasted with the control. After the amount was strengthened to 8 kGy, the relative fluorescence intensity peak of PSA was solely lower. According to the change tendency of protein conformation as inferred from Figure 4, the decrease in the fluorescence intensity of PSA at irradiation doses of up to 6 kGy indicated that gamma irradiation induced the unfolding of PSA molecules and the exposure of amino acid residues such as tryptophan residues to the polar solvent environment; thus, the fluorescence was quenched by the environment. However, the decrease in fluorescence intensity of PSA at a higher dose of 8 kGy showed that the exposed hydrophobic groups may reassociate or aggregate to form a relatively stable structure. A blue shift of the maximum emission wavelength of PSA was also observed after gamma irradiation (Table 2), which indicates that the microenvironment of tryptophan residues was changed and the hydrophobicity

increased. These results show that gamma irradiation could induce changes in the tertiary structures of PSA, thus reducing the allergenicity of PSA.

Conclusions

The goal of this study was to evaluate whether gamma irradiation impacts the allergenicity and structural properties of PSA. The outcomes offer key insights into PSA's reaction to irradiation and the modification of allergenicity and the protein structure. As the allergenic decline and structure change coexisted in irradiated PSA, we could speculate that gamma irradiation treatment caused changes in the stereotactic conformation of the PSA protein molecule, masking or destroying the antigenic epitopes. These changes may be an important means of reducing the antigenicity of the allergen. Thus, PSA alteration by gamma irradiation may be appropriate not only for the generation of hypoallergenic pork products, but also to avert pork allergy.

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