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Influence of γ-Radiation on the Structure and Function of Soybean Trypsin Inhibitor

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ABSTRACT: Soybean trypsin inhibitor (STI) is a known antinutrient and food allergen present in soybean. γ-Radiation has the potential to inactivate the TI protein. However, a systematic study on the influence of different moisture levels during γ radiation on structure and function of the molecule has not been reported. Pure STI was irradiated up to 200 kGy, in dry state, with 50% moisture and in aqueous solution. The radiation damage in molecular structure was assessed using, SDS−PAGE, size exclusion chromatography, fluorescence measurement, and circular dichroism, while functional damage was assessed by the TI assay. In aqueous solution, both the structure and function of TI were almost destroyed at the 10 kGy dose. While with 50% moisture and in dry state, the loss in functional and structural attributes was discernible only at 30 and 100 kGy, respectively. The TI activity was found to be unaffected in dry and soaked seeds of soybean as well as other legumes up to irradiation doses of 100 and 50 kGy, respectively.

KEYWORDS: *soybean trypsin inhibitor (STI), γ-radiation, legume, fluorescence, circular dichroism*

INTRODUCTION

Soybean or soy proteins are widely used in human foods in a variety of forms including infant formulas, flours, protein isolates, and concentrates. Besides this, the consumption of soy foods, which includes, cheese, miso, tempeh, and tofu, is also increasing because of reported beneficial effects on nutrition and health.[1] However, antinutritional effects following the consumption of raw soybean meal have been reported and are attributed to the presence of the Kunitz trypsin inhibitor (KTI) and the Bowman−Birk inhibitor of chymotrypsin (BBI).[2] These inhibitors exert effects by inactivating gastric proteases, which in turn stimulate the pancreas for excess production of the gastric proteases, causing pancreas hypertrophy and hyperplasia. This effect alone could account for growth depression since the endogenous loss of nitrogen residing in the digestive enzymes would serve to drain the body tissue of proteins.[3] Stimulation of circulating cholecystokinin by trypsin inhibitor, which is responsible for pancreas hypertrophy and hyperplasia, is also reported.[3] Secondly, the possibility of inducing pancreatic cancer in rats due to the stress imposed on the pancreas by the long-term feeding of raw flour has been reported.[3] Moreover, KTI has been characterized as a food allergen causing type I allergy and is established as a minor component among the different soy allergenic proteins based on an IgE binding test. However, it is also reported to induce anaphylactic shocks.[4] The soybean trypsin inhibitor (STI) is also found as a contaminant in lecithin, which is widely used as an emulsifying agent in foods and pharmaceuticals. Thus, it presents a potential risk for allergic patients.

The overall structure of KTI is spherical with a diameter of 3−5 nm and it comprises 12 crisscrossing antiparallel β-strands, largely stabilized by hydrophobic side chains. The two disulfides bridges between Cys 39 and Cys 86 and between Cys 138 and Cys 145 are solvent-exposed and are essential for inhibitory function. The protein has been classified in β-II or the disordered class of all antiparallel β-sheet proteins on the basis of characteristic circular dichroism (CD) spectra.[5,6] The most interesting features of the molecule reported are its reversibility of thermal denaturation, resistance to chemical denaturants, and stability toward acidic conditions similar to that of stomach.[6−8]

γ-Radiation processing of cereals and legumes has been shown to inactivate antinutritional compounds, which also include trypsin inhibitors.[9] Literature on the effect of radiation processing on trypsin inhibitory activity is available for a few legume seeds, including soybean; however, a very wide variation in the reduction of protease inhibitor activity has been reported by different authors.[9−16] Moreover, there is no systematic study which relates the effect of γ-irradiation on the trypsin inhibitor in seeds to that observed with purified protein. There is a paucity of information regarding the change in functional aspects relating to structural changes of the trypsin inhibitor after irradiation. Present studies were thus undertaken to demonstrate the effect of γ-radiation on the trypsin inhibitor in seeds as well as in its pure form at different moisture levels.

MATERIALS AND METHODS

Soybean trypsin inhibitor, fast garnet GBC, and benzoyl-DL-arginine-β-naphthylamide (BANA) were purchased from Sigma Chemicals Co. (St. Louis, USA). All other chemicals were of analytical grade.

Gamma Irradiation Treatment. The dry and overnight soaked seeds of soybean, kidney bean, chickpea, and cowpea as well as pure trypsin inhibitor as an aqueous solution (10 mg/mL), in dry state, or with 50% (w/w) moisture were treated with γ-radiation at different doses (1, 10, 30, 50, 100, and 200 kGy) in a cobalt-60 gamma irradiator (Gamma Chamber 5000, Board of Radioisotope and Technology, Mumbai, India). Radiation processing of the pure trypsin

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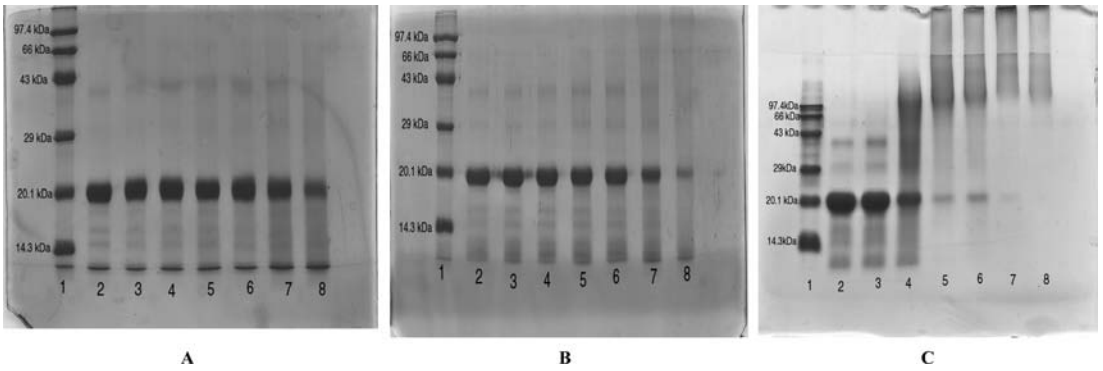

Figure 1. SDS−PAGE profile of pure STI irradiated at different doses in (A) dry state; (B) 50% moisture; and (C) aqueous solution. A protein sample of 8 μg was loaded in each well of a 15% polyacrylamide gel. Lane 1: molecular weight markers, rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine milk α-lactalbumin (14.3 kDa). Lane 2: native STI. Lane 3−8: STI irradiated at doses 1, 10, 30, 50, 100, and 200 kGy, respectively.

inhibitor was carried out in glass vials, while that of dry and soaked seeds in glass beakers under ambient conditions.

Sample Preparation. Pure soybean trypsin inhibitor (STI), in native form as well as in irradiated forms at different doses (1, 10, 30, 50, 100, and 200 kGy) in dry state or with 50% moisture level were reconstituted in phosphate buffered saline at a concentration of 10 mg/mL. For measuring the trypsin inhibitory activity in dry and soaked seeds, 8% (w/v) homogenate of the commodity was prepared in 10 mM NaOH, centrifuged at 10000*g* for 15 min, and the supernatant was used for determining trypsin inhibitory activity.

Determination of Trypsin Inhibitory Activity and Protein Estimation. Trypsin inhibitory activity assay was performed with the chromogenic substrate, benzoyl-DL-arginine-β-naphthylamide (BANA). An appropriate amount of trypsin inhibitor, either a solution of pure protein or a crude extract of legume seeds, was first incubated with 50 μL of trypsin (1 mg/mL) in 100 mM Tris buffer at pH 7.5 at ambient temperature (26 ± 2 °C) for 10 min. At the end of incubation, 1 mM substrate (BANA) was added to the reaction mixture, which was then incubated at 45 °C for 15 min. The reaction was stopped by the addition of 1 mM phenyl methyl sulfonyl fluoride (PMSF), and the color was developed using fast garnet GBC (0.1 mg/mL) in 4% Brij 35 solution. The absorption was measured at 520 nm. The amount of β-naphthylamine released was estimated from a standard curve. One unit of enzyme activity is defined as the amount of enzyme that liberates 1 nmol of β-naphthylamine per min under the assay condition. Trypsin inhibitory activity was expressed as the percent inhibition or number of trypsin units inhibited (TUI).

The protein content of pure STI samples was determined by measuring absorption at 280 nm, while that of the extracts was determined using the Bradford method.[17]

SDS−PAGE. SDS−PAGE was performed according to Laemmli.[18] Protein samples (8 μg) were mixed with loading buffer (50 mM Tris-Cl^-, pH 6.8, with 2% SDS, 10% glycerol and 0.1% bromophenol blue), resolved on 15% separating gel, and stained using a coommassie blue. The following standard molecular weight markers were used: rabbit muscle phosphorylase b (97.4 kDa), bovine serum albumin (66 kDa), egg albumin (43 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (20.1 kDa), and bovine milk α-lactalbumin (14.3 kDa).

Size Exclusion Chromatography. Pure trypsin inhibitor samples irradiated at doses of 1, 10, 30, 50, 100, and 200 kGy were reconstituted in buffer (10 mg/mL) and subsequently subjected to size exclusion chromatography. A 10 μL of the sample was loaded on a Superdex 200, 10/300 GL, (GE Healthcare, Hong Kong) column. The chromatography was carried out in 10 mM Tris buffer, pH 7.0, containing 0.1 M NaCl at a flow rate of 0.6 mL/min.

Measurement of Intrinsic Fluorescence of STI. The fluorescence emission spectrum of the irradiated samples of pure trypsin inhibitor was measured at a concentration of 1 mg/mL in phosphate buffer saline (0.1×) at 25 °C using a spectrofluorometer (JASCO, FP-6500, Tokyo, Japan). The measurement was carried out using a quartz cuvette with a path length of 10 mm. The excitation wavelength used was 295 nm, while the emission spectrum was recorded in the range of 305−405 nm with a slit width of 5 nm.

Measurement of Protein Surface Hydrophobicity (S_0). The relative surface hydrophobicity of the irradiated and unirradiated soybean trypsin inhibitor was determined by applying the optical method previously reported by Tubio.[19] The stock solution of 8 mM ANS was prepared in phosphate buffered saline (pH 7.4). Aliquots of the protein samples and ANS solution were mixed. The final concentration of ANS was 20 μM, while the protein concentration varied from 0 to 10 μM. The relative fluorescence intensity (RFI) of the solution was measured (360 nm, excitation, and emission, 470 nm) using a spectrofluorometer (JASCO, FP-6500, Tokyo, Japan). The initial slope (S_0) of the net RFI versus protein concentration was calculated by linear regression analysis and was used as an index of the protein surface hydrophobicity.

CD Spectroscopy. CD spectral measurements were performed on a JASCO 815 CD spectrometer equipped with a temperature jacketed cuvette holder. The entire instrument including the sample chamber was flushed with nitrogen gas during the operation. Far UV CD spectra (190−260 nm) were recorded at the protein concentration of 100−150 μg/mL at 25 °C. The solutions were centrifuged at 13000*g* for 20 min. The supernatants were used for recording the spectra as well as protein estimation ($A_{280\ nm}$). The optical path length was 0.1 cm. A total of 5 scans were taken for each measurement with a scan speed of 50 nm/min using a bandwidth of 1 nm. The results were expressed as the mean residue ellipticity $[\theta]$, defined as $[\theta] = \theta_{obs}/(10 \cdot C \cdot l \cdot n.)$, where θ_{obs} is the CD in millidegrees, C is the protein concentration (M), l is the path-length of the cuvette (cm), and n is the number of amino acid residues.

Statistical Analysis. Trypsin inhibitory activity assay and surface hydrophobicity determination were performed in duplicate. The values reported are the mean of the values of three independent experiments. The mean, standard deviation (SD), and standard error (SE) were computed using OriginPRO 7.5, Origin Lab Corporation, USA.

RESULTS

SDS−PAGE. Data presented in Figure 1A, B, and C shows the SDS−PAGE profile of irradiated samples. In dry state, a radiation dose up to 100 kGy exhibited no change in the intensity of protein band, while at 200 kGy, a decrease in the intensity of the protein band was observed (Figure 1A). However, the samples containing 50% moisture exhibited lower intensity of the band when irradiated at a dose of 100 kGy, and the band intensity was reduced drastically at 200 kGy (Figure 1B). Samples irradiated in an aqueous system, however, showed a strong decrease in the intensity of the band corresponding to native protein at a dose ≥10 kGy with concomitant increase in

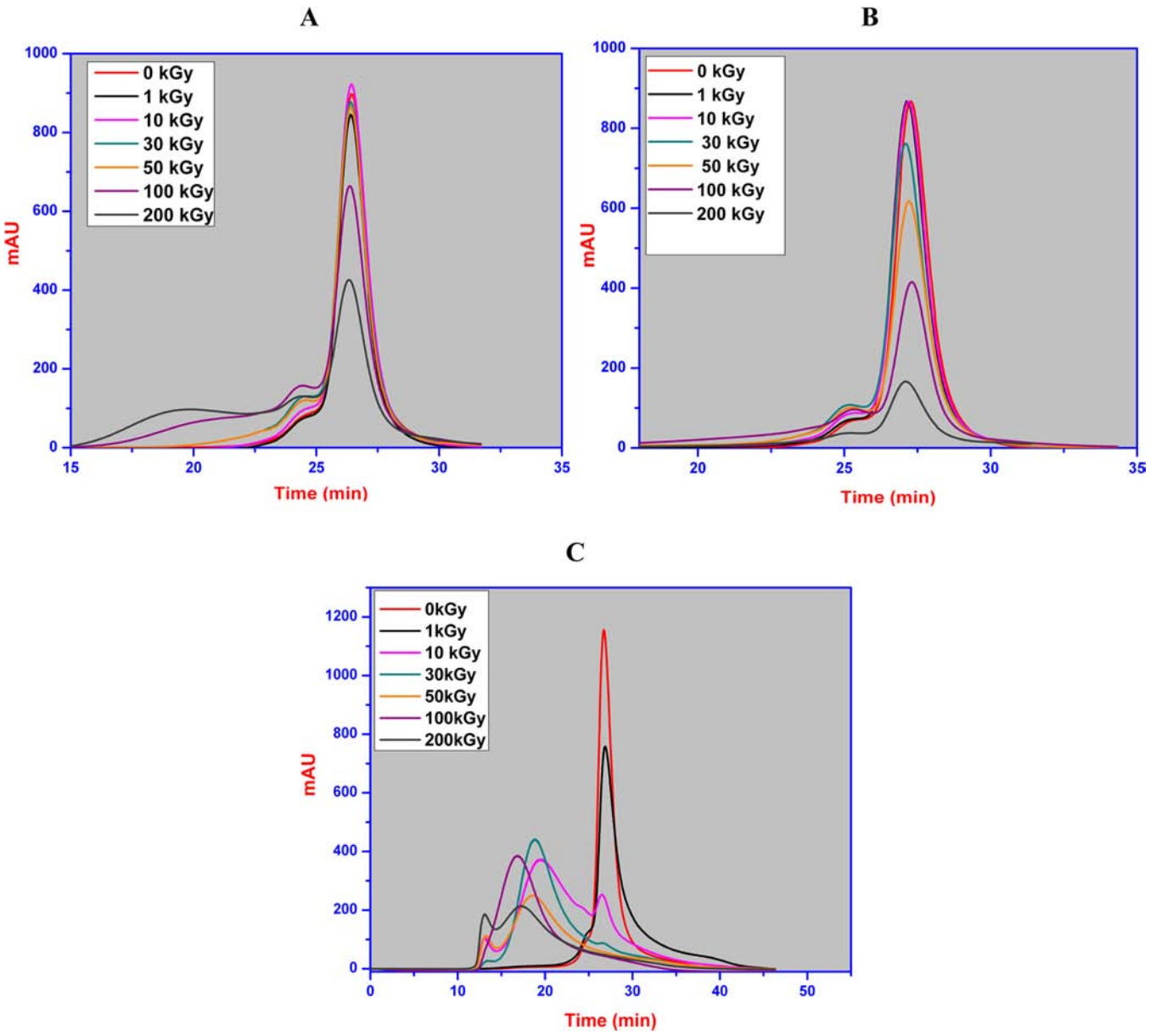

Figure 2. Size exclusion chromatography of pure STI irradiated at different doses in (A) dry state; (B) 50% moisture; and (C) aqueous solution. A 10 μL STI solution (10 mg/mL) was loaded on a Superdex 200, 10/300 GL, (GE Healthcare, Hong Kong) column already equilibrated with 10 mM Tris buffer at pH 7.0 containing 0.1 M NaCl and at a flow rate of 0.6 mL/min.

the intensity of the cross-linked products (bands corresponding to molecular weight >97.4 kDa) (Figure 1C). In addition to this, the stacking gel showed intense staining (samples 4 to 8, equivalent doses of 30 and 200 kGy, respectively) due to the nonpenetration of aggregates in the separating gel (Figure 1C).

Size Exclusion Chromatography. To study the aggregation or fragmentation products formed due to irradiation, size exclusion chromatography of the samples (pure STI) was performed. Figure 2A, B, and C show the gel filtration profile of pure STI, irradiated in dry state, with 50% moisture, and in an aqueous system, respectively. The results show that a radiation dose up to 50 kGy did not change the elution pattern of the STI molecule, when irradiated in a dry state (Figure 2A). However, at doses of 100 and 200 kGy, the peak height of the protein was reduced in a dose dependent manner with concomitant increase in the area under the curve in the region, where high molecular weight proteins (>600 kDa) get eluted. Irradiation in the moist condition, however, exhibited a dose dependent decrease in peak height at doses ≥30 kGy without appearance of any new peak. Since the samples separated by SEC are first dissolved in the elution buffer, centrifuged, filtered with 0.22 μm filter, and then injected into SEC system, it was not possible to analyze the aggregate by SEC. The decrease in peak height was also higher for the samples irradiated in moist conditions (Figure 2B). Figure 2C shows that irradiation of STI solution at a dose of 1 kGy decreased the peak height significantly without the appearance of any new peak. However, at doses ≥10 kGy the peak corresponding native STI disappeared with appearance of new peaks corresponding to high molecular weight proteins. Moreover, the position of the peak shifted toward void volume as the radiation dose increased from 10 kGy to 200 kGy.

Intrinsic Fluorescence. To examine the change in molecular properties of the STI irradiated at different doses

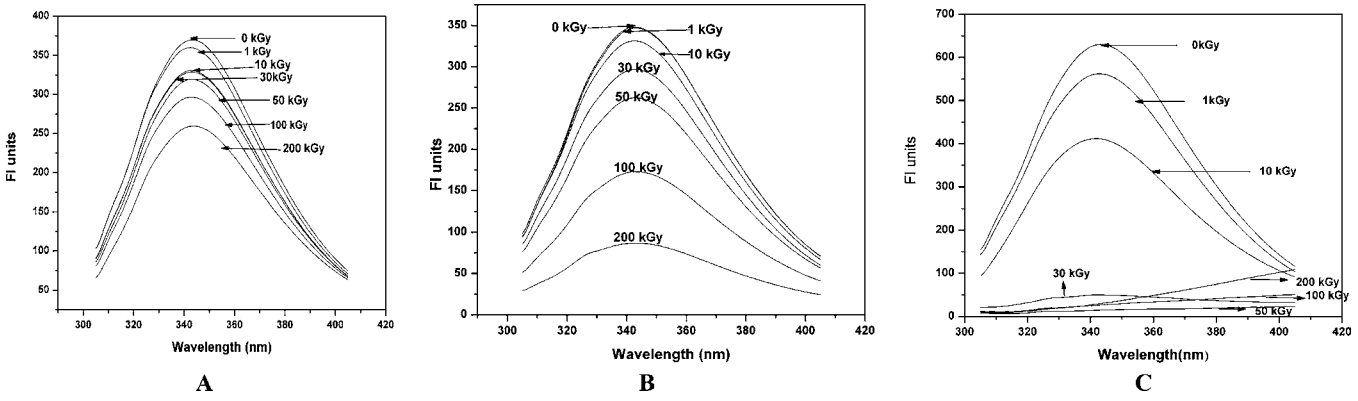

Figure 3. Measurement of intrinsic fluorescence of pure STI irradiated at different doses in (A) dry state; (B) 50% moisture; and (C) aqueous solution. The excitation wavelength used was 295 nm, while the emission spectrum was recorded in the range of 305−405 nm.

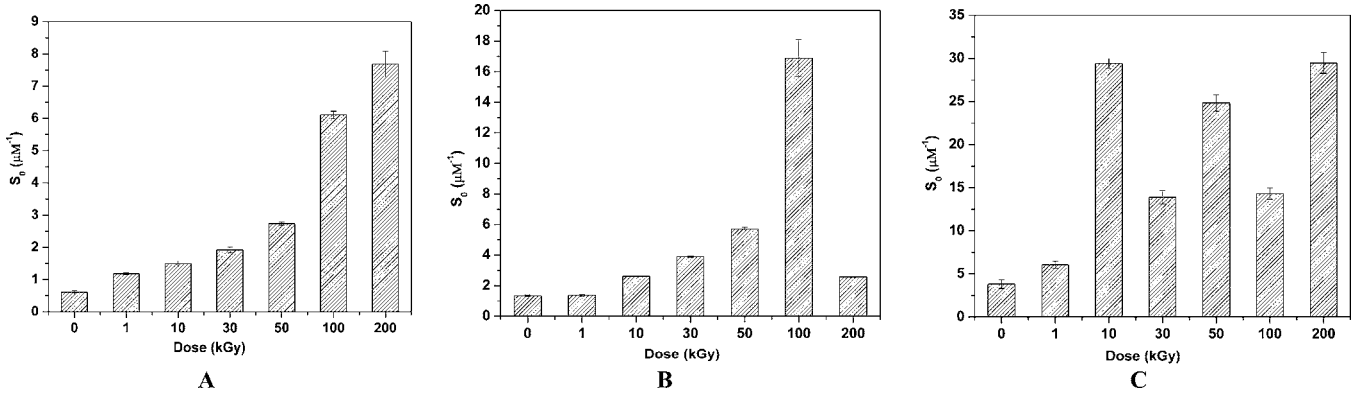

Figure 4. Measurement of surface hydrophibicity (S_0) of pure STI irradiated at different doses in (A) dry state; (B) 50% moisture; and (C) aqueous solution. Protein solution (0−10 μM) and ANS solution (20 μM) were mixed, and the relative fluorescence intensity (RFI) of the solution was measured (excitation, 360 nm, and emission, 470 nm). The initial slope (S_0) of the net RFI versus protein concentration was calculated by linear regression analysis and was used as an index of the protein surface hydrophobicity. The values are the mean ± SE of three experiments.

and different conditions, intrinsic fluorescence was measured. Figure 3A, B, and C show that γ-radiation causes a decrease in the emission intensity of STI due to the change in the local environment around tryptophan residues. Increase in the dose quenches the emission intensity of the proteins. The decrease in fluorescence intensity was observed in a dose dependent manner and was higher for the protein samples irradiated in moist conditions. Irradiation of STI solution at a dose of 1 and 10 kGy exhibited a decrease in fluorescence intensity in a dose dependent manner (Figure 3C), and for the samples irradiated at higher doses (≥30 kGy), the intensity of the fluorescence was almost nil.

Surface Hydrophobicity (S_0). The data presented in Figure 4A, B, and C show the fluorescence intensity of ANS bound to STI irradiated at different doses of γ-radiation in dry state, 50% moisture level, and in aqueous state, respectively. The unirradiated sample, i.e., the native protein, exhibited a negligible amount of dye binding, while irradiation of STI in dry state increased the S_0 values in a dose dependent manner (Figure 4A). At the dose of 50 kGy, there was nearly a 6-fold increase in surface hydrophobicity, while at 100 and 200 kGy the S_0 value was increased by nearly 12- and 16-fold, respectively. A similar pattern of increase in S_0 value was observed for the samples containing 50% moisture irradiated in the dose range of 1−100 kGy (Figure 4B). At a dose of 200 kGy, the S_0 value was found to be decreased. The STI solution exhibited increase in the S_0 value in the dose range of 1−10 kGy. At higher doses (≥30 kGy), the pattern was irregular suggesting a total loss of tertiary structure (Figure 4C).

CD Spectroscopy. Far UV CD spectra are indicative of the extent of secondary structural changes induced in proteins by environmental changes. Far UV CD spectra of STI solution irradiated at various doses are shown in Figure 5A, B, and C. Native STI has a CD spectrum (Figure 5A) characteristic of the class β-II proteins with a minimum ellipticity at about 200 nm and a slight positive shoulder at 225 nm.[6,7,20] γ-Irradiation of STI solution at a 1 kGy dose did not affect the CD spectrum of the molecule suggesting insignificant changes in the structure of STI. However, a dose of 10 kGy and higher indicated a progressive loss in the extent of coil structure as evidenced by the lower ellipticity at 200 nm. Major changes were also observed at 225 nm (Figure 5C). Figure 5A and B show CD spectra of STI irradiated in dry state and with 50% moisture level, respectively. The figure shows insignificant change in ellipticity in the regions 200 and 225 nm for both the samples irradiated at doses of 1, 10, 30, and 50 kGy with respect to the unirradiated sample. However, in the dry state, samples irradiated at doses of 100 and 200 kGy, exhibited a decrease in ellipticity at both the regions, presumably due to a loss of native conformation at and above a 100 kGy dose of γ-radiation. The changes were also prominent in the region of 220−225 nm. STI samples with 50% moisture also exhibited a similar pattern of CD spectra. However, an insoluble aggregate was formed in the samples irradiated at 100 kGy and 200 kGy,

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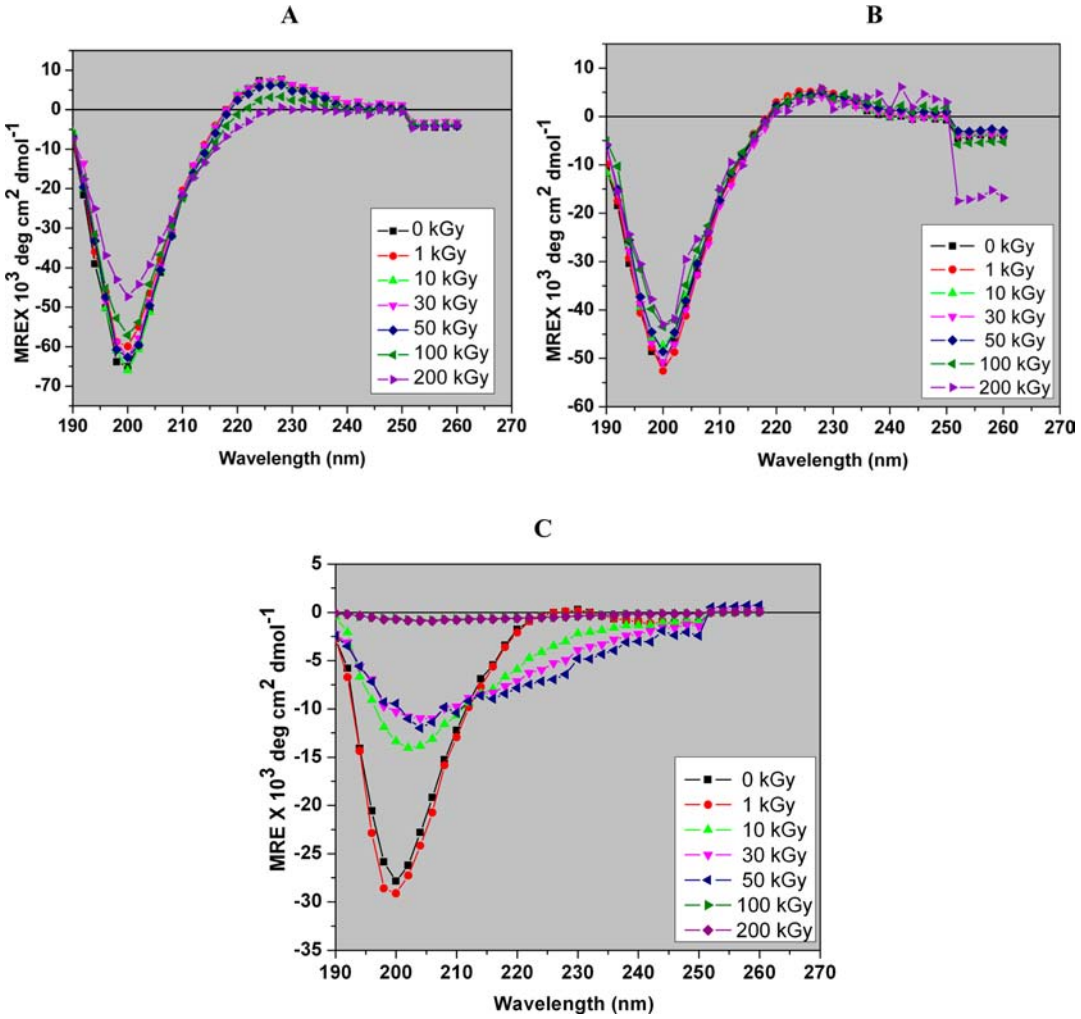

Figure 5. CD spectra of pure STI irradiated at different doses in (A) dry state; (B) 50% moisture; and (C) aqueous solution. All of the measurements were done at 25 °C. For far UV CD spectra (190−260 nm), the protein concentration was 100−150 μg/mL. The optical path length was 0.1 cm.

decreasing the concentration of soluble proteins. In these samples, a major part of the denatured protein was out of solution, and hence, even though the inactivation of protein was higher compared to the samples in the dry state, the CD spectra of these samples did not exhibit a drastic change in the overall pattern.

Trypsin Inhibitory Activity. The effect of γ-radiation on pure soybean trypsin inhibitor was also evaluated in terms of its potential to inhibit trypsin activity (Figure 6). A dose of 10 kGy could abolish nearly 80−85% of activity of the pure STI solution, while irradiation in dry state exhibited a significant loss in activity only at the doses ≥100 kGy. At 200 kGy, nearly 57% of initial activity was lost. The samples with 50% moisture showed higher inactivation as compared to that in dry state. A dose of 30 kGy could reduce the activity by 18%, which was further reduced by 38% and 59% at the dose of 50 and 100 kGy, respectively. At the dose of 200 kGy, total loss in activity was observed.

The trypsin inhibitory activity was also evaluated in dry as well as soaked legume seeds (soybean, kidney bean, cowpea, and chickpea) (Table 1). The highest activity was observed in soybean followed by kidney bean, cowpea, and chickpea. Soaking of the seeds per se reduced the activity by 50%. Irradiation of dry seeds up to 100 kGy did not affect trypsin inhibitory activity (Figure 7). At 200 kGy, the activity was reduced by 47% and 37% for soybean and kidney bean seeds, respectively. For cowpea and chickpea, no decrease in the activity was discernible. However, in soaked seeds, at 100 and 200 kGy the inactivation in soybean was found to be 22% and 89%, respectively, while in cowpea, the inactivation was 57 and 85%, respectively. There was no inactivation at 100 kGy in soaked kidney bean seeds, while at 200 kGy, the level of inactivation was 66%.

DISCUSSION

The radiation damaging effect on STI was monitored by SDS−PAGE and SEC. The SDS−PAGE profile demonstrated that in dry state, there was no decrease in the quantity of monomeric STI up to a radiation dose of 100 kGy. At a 200 kGy dose, there was a decrease in the band intensity of protein, however, without appearance of any fragmented or cross-linked products. The data corroborates the results obtained from size exclusion chromatography exhibiting a decrease in the height of the peak corresponding to the native STI molecule for the samples irradiated at 100 and 200 kGy doses. An additional small peak slightly earlier to the major peak of native protein was also observed at these doses. Samples irradiated in the presence of 50% moisture exhibited higher damage in comparison with

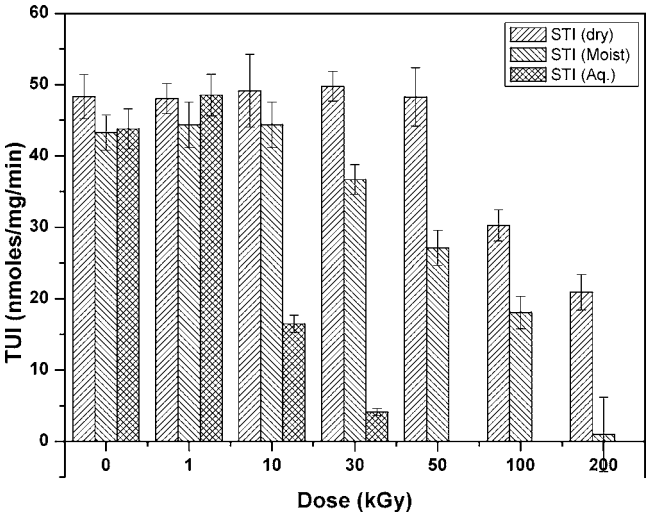

Figure 6. Trypsin inhibitory activity of pure STI irradiated at different doses in (A) dry state; (B) 50% moisture; and (C) aqueous solution. Trypsin inhibitory activity was carried out using the substrate benzoyl-DL-arginine-β-naphthylamide (BANA). One unit of enzyme activity is defined as the amount of enzyme that liberates 1 nmole of β-naphthylamine per minute under these assay conditions, while trypsin inhibitory activity was expressed as the number of trypsin units inhibited (TUI). The values represent the mean of three independent experiments ± SD.

Table 1. Trypsin Inhibitory Activity in Dry and Soaked Seeds of Commonly Consumed Legumes

	trypsin inhibitory activity (TIU/mg protein)[a,b]	
legume	dry	soaked
soybean	522.40 ± 20.3	272.82 ± 11.7
kidney bean	171.28 ± 13.2	96.53 ± 8.5
cowpea	166.18 ± 9.3	107.68 ± 9.3
chickpea	63.92 ± 7.81	35.71 ± 5.1

[a]One unit of trypsin activity was expressed as the amount of enzyme that liberates 1 nmole of β-naphthylamine per minute under the assay conditions, while trypsin inhibitory activity was expressed as the number of trypsin units inhibited (TUI). [b]Values are presented as the mean ± SD of three independent measurements.

their counterparts in dry state. However, in moist samples exposure to a high dose of γ-radiation (100−200 kGy) led to the formation of insoluble aggregates, and thus, a significant decrease in peak height was observed in SEC profile of these samples without the appearance of any additional peaks. The SDS−PAGE profile of these samples substantiates the results of SEC exhibiting reduced intensity of the band of the samples irradiated at 100 and 200 kGy. Radiation damage of STI in aqueous solution was observed as the formation of soluble and insoluble aggregates. The SDS−PAGE and SEC results suggested that irradiation of STI in an aqueous system abolished the native form of the molecule at dose of 10 kGy. Secondly, at doses above 10 kGy, the formation of high molecular weight soluble aggregates was observed. The radiation damage to the proteins is reflected as either fragmentation or aggregation of the molecules.[21] In the dry state, proteins are damaged directly by interacting with ionizing radiation, whereas that in the presence of moisture is mainly due to an indirect effect involving free radicals like $H^{\cdot}$, $^{\cdot}OH$, and e^{-}_{aq}, generated due to the radiolysis of water.[22] The direct effect causes protein fragmentation, while the latter causes cross-linking of the molecule leading to protein aggregation. In the SDS−PAGE profile, although we have seen reduction in the intensity of the protein band for the sample irradiated at 200 kGy in dry state, no low molecular weight products were observed, suggesting the requirement of a still higher dose to observe the said effect. The formation of insoluble aggregates due to cross-linking of protein molecules was observed only in those samples irradiated in the presence of moisture. Interestingly, irradiation of STI in low moisture conditions led to the formation of only insoluble aggregates, while in solution, the formation of soluble as well as insoluble aggregates could be seen. The individual molecules are well dispersed in the solvent, and hence, the probability of the formation of lower oligomeric cross-linked products (soluble aggregates) is higher. However, in low moisture conditions the protein molecules are so closely spaced that the possibility of the formation of insoluble aggregates due to extensive intermolecular cross-linking is higher.

The radiation induced structural changes were also monitored by taking the native fluorescence spectrum of the samples with excitation at 295 nm and emission in the range of 305−405 nm. Tryptophan residues that are exposed to water have maximal fluorescence at wavelengths around 340−350 nm, whereas completely buried residues fluoresce at about 330 nm.[23] The native molecule of STI contains two residues of Trp (Trp 93 and Trp 117). The crystal structure of the molecule shows that Trp 117 is present on the surface, while Trp 93 is buried inside.[24] The intrinsic fluorescence of irradiated as well as unirradiated samples of STI exhibited λ_{max} at 343 nm with a small shoulder at 330 nm. Irradiation of the samples in all the conditions exhibited a decrease in the intensity of the fluorescence without red or blue shift. Moreover, the quenching of the fluorescence was found to increase in a dose dependent manner, and the effect was enhanced with an increase in the moisture content of the sample. Further, at lower doses there was hardly any change in the trypsin inhibitory activity of STI, especially in dry samples. However, these samples depicted the quenching of Trp fluorescence. Thus, the results showed that at lower doses γ-radiation causes some changes in the structure of the molecule without affecting the function of STI. Irradiation of STI solution at a dose ≥30 kGy, however, caused a complete loss of fluorescence suggesting total destruction of the tertiary structure of the molecule and/or oxidative damage of the Trp residue. Similar results showing a decrease in the fluorescence of Trp residues of γ-irradiated proteins have been reported earlier.[25,26] Additional support for radiation induced structural changes was obtained from studies on ANS fluorescence in the presence of STI. The fluorescence yield of ANS, an extrinsic probe, increases upon its ability to partition into the hydrophobic core of the globular proteins.[27] Irradiation of the samples increased the fluorescence of ANS in a dose dependent manner. For the same dose of radiation, increase in fluorescence intensity was higher for the samples irradiated in moist conditions as compared to dry samples suggesting the higher susceptibility of the protein to radiation inactivation in moist conditions. A decrease in the fluorescence yield of ANS of the STI sample irradiated at a dose of 200 kGy in moist conditions was due to the formation of insoluble aggregates of the protein at this dose (Figure 4B). When irradiated in aqueous media, the highest value of S_0 was observed at 10 kGy, which was equivalent to the corresponding value at 200 kGy and 100 kGy of STI irradiated in dry conditions and with 50% moisture, respectively. Furthermore, increase of the dose did

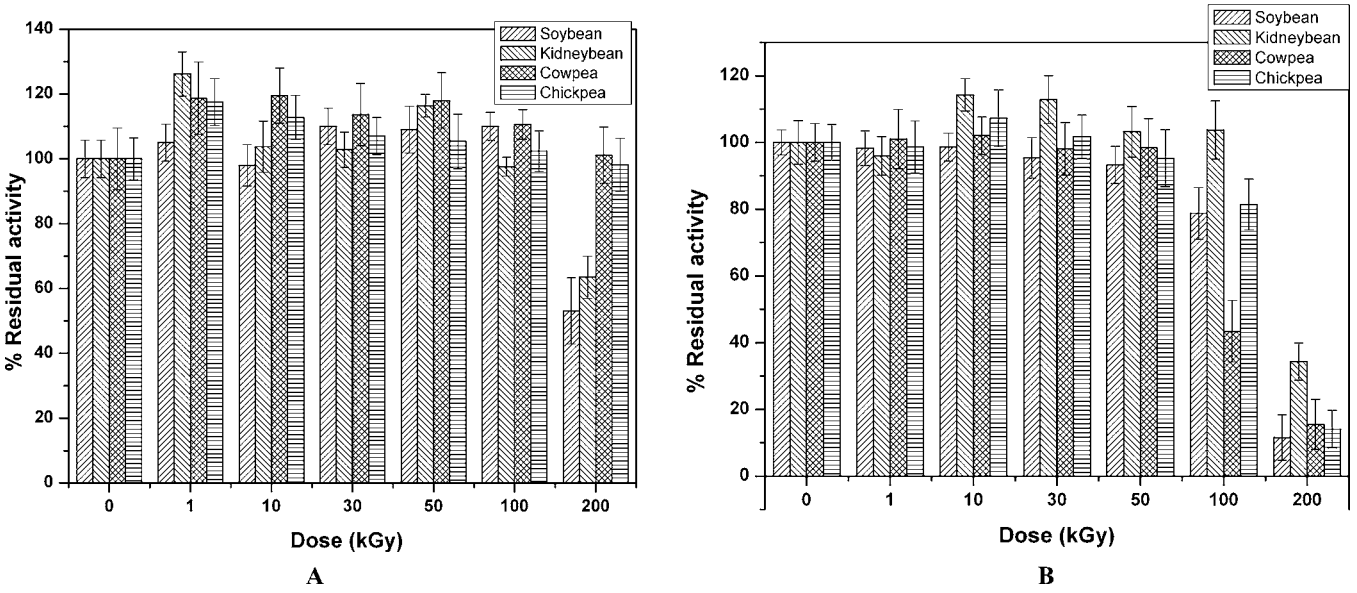

Figure 7. Trypsin inhibitory activity in the extract of dry (A) and soaked (B) seeds of soybean, kidney bean, chickpea, and cowpea irradiated at different doses. Trypsin inhibitory activity was carried out using the substrate benzoyl-DL-arginine-β-naphthylamide (BANA). One unit of enzyme activity is defined as the amount of enzyme that liberates 1 nmole of β-naphthylamine per minute under these assay conditions, while trypsin inhibitory activity was expressed as the number of trypsin units inhibited (TUI). The percent residual trypsin inhibitory activity of the seeds irradiated at different doses is plotted. The values represent the mean of three independent experiments ± SD.

not show a regular pattern of increase in the value of S_0 suggesting the total collapse of the structure. The reason for irregular pattern of yield of ANS fluorescence intensity at higher doses in aqueous solution could not be established. However, it might be due to the soluble aggregates formed at these doses.

The strong evidence that indicates global unfolding in STI was provided by CD spectra. Trp and Tyr are present in the interior core of the protein. Changes in the ellipticity of the samples in the region of 225−235 nm are attributed to aromatic and/or disulfide contribution,[6,7,20] thus suggesting the intactness of the secondary structure of the protein. Irradiation of STI solution at doses ≥10 kGy caused a decrease in ellipticity in both regions, i.e., 220−235 nm, as well as 195−200 nm, suggesting inactivation of the molecule. At doses of 100 and 200 kGy, the spectrum was almost flat, indicating total damage to the protein. In dry state, however, a slight change in ellipticity was observed in both regions at a dose ≥100 kGy, thus exhibiting resistance of the molecule toward a direct effect of γ-radiation. The samples irradiated in 50% moisture exhibited insoluble aggregates (>80% protein), and thus, the CD spectra of the residual (soluble) protein did not show major changes in the region of 225−235 nm. However, it showed lower ellipticity in the region of 195−200 nm with minimum red shift suggesting melting of the secondary structure.

We have irradiated STI at different doses in dry state or with 50% moisture level to simulate conditions of STI in dry and soaked seeds of soybean. Irradiation in aqueous solution was carried out to demonstrate the effect of excess moisture. The trypsin inhibitory activity of the protein was evaluated using the substrate Bz-DL-Arg-β-naphthylamide. The results of the study clearly showed that the radiation inactivation of STI increased with the increase in moisture level. In an aqueous system, a dose of 10 kGy was found to be sufficient to inactivate pure STI to a level >80%. However, this level could not be achieved in dry and soaked seeds, where the moisture level is much lower. The reduction in trypsin inhibitory activity due to radiation treatment at 100 kGy for pure dry STI, with 50% moisture, and dry and soaked (containing 50% moisture) soy seeds was 37, 59, 0, and 22%, respectively, while at 200 kGy the reduction was 57, 100, 47, and 89%, respectively. This clearly indicates the effect of moisture and food matrix on radiation inactivation of trypsin inhibitor. The difference in the degree of inactivation of TI in different legumes as a result of irradiation indicates variable sensitivity due to the structural differences of TI in different legumes and/or variation in the protective effect of the food matrix. In our studies, we have also demonstrated the protective effect of soy protein on STI. Soymilk (previously heat treated to abolish native STI activity) spiked with STI (10 mg/mL) and irradiated at different doses showed that complete inactivation of STI was possible in a radiation dose range of 30−50 kGy unlike that of pure STI solution at 10 kGy (data not shown).

The results of our studies showing the resistance of the trypsin inhibitor in seeds toward γ-radiation are in agreement with those of Hafez[12] and Nene.[16] However, a number of authors have shown that a radiation dose in the range of 0.25 kGy−5 kGy was sufficient to inactivate TI.[28−31] Farag[11] has shown in soybeans that at 10 kGy the loss of TI activity was nearly 54.5%. However, most of their studies are based only on trypsin inhibitory activity without any other supportive information. The present study gives insight in to the effect of γ-radiation on structure as well as on the function of STI. Moreover, radiation inactivation studies of a pure molecule carried out in an aqueous system overestimate the potential of the γ-radiation for abolishing these molecules in the food system.

In conclusion, STI was found to be stable toward the direct effect of γ-radiation. Inactivation of the molecule at lower doses was possible only in the presence of excess of moisture. Thus, the use of the γ-radiation processing to reduce or eliminate trypsin inhibitory activity in dry or soaked seeds would require

a sufficiently high dose of γ-radiation that may affect sensorial and other functional properties of the legumes.

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Notes

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