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Combinational Effect of Moist Heating and Gamma Irradiation on The Inactivation of Trypsin Inhibitory Activity in Soybean

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Abstract The combinational effect of gamma irradiation and moist heating on the trypsin inhibitor activity (TIA) in soaked and dried soybeans was evaluated by measuring the inhibition using N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) as substrate. Gamma irradiation significantly decreased the TIA level in soybean at doses above 5 kGy, and the ID_{50} (the gamma irradiation dose required to reach 50% inhibition) value for TIA was 13.53 kGy. Soaking prior to gamma irradiation significantly lowered the ID_{50} to 8.44 kGy, and the soaking process enhanced the efficiency to inactivate TIA by as much as 48%. When soaking prior to gamma irradiation was followed by subsequent mild heating (60°C) process, the IT_{50} (heating time required to reach the 50% inhibition of TIA) value at even 1 kGy (5.28 min) was greatly reduced by over 50% compared to the level for the no-soaking process. In addition, the activation energy of soaking prior to gamma irradiation at 1 kGy was 2.45 kcal/mole, which was also about 50% lower than the 5.10 kcal/mole of dried soybean gamma-irradiated. Based on these results, soaking prior to gamma irradiation is an effective method for TIA inhibition. Furthermore, a combination of two or more processing methods such as soaking, heating and gamma irradiation is much more effective than any single processing method.

Keywords: soybean, trypsin inhibitory activity (TIA), gamma irradiation, soaking

Introduction

Trypsin inhibitors are antinutrients commonly present in soybean that inhibit important pathways in the digestive metabolism and consequently reduce the maximum utilization of nutrients such as protein (1). Soybean (*Glycine* max.) contains 40% protein, 20% lipid and only a small amount of starch. Therefore, it can play a significant role in human nutrition, especially in developing countries where the average protein intake is less than recommended (2). Therefore, in order to inactivate or reduce the trypsin inhibitors, various conventional, simple processing methods have been used such as dry heating, roasting, boiling, soaking in water, alkali and acid, solvent extraction, germination and fermentation (3-7). However, none of these methods can completely remove all the antinutrients that are present in seeds, grains or feed materials. Another technique is the application of ionizing radiation, which has already been used for decontaminating food by killing bacteria, insects, other food born pathogens, and for increasing the shelf-life of fresh and dry food materials (8-10).

Recently, irradiated food is fast becoming popular due to its preservative function with minimal effect on the food components (11). Food irradiation is a physical process involving an energy-input that does not induce radioactivity in foods (12). There are some reports concerning the inactivation of trypsin inhibitor in soybean by gamma irradiation (13). Soybean is generally used after soaking and then processed into various types of foods. Soaking and then heating is the essential first step of processing for typical processed foods made from soybean such as soy milk, tofu or soybean paste. Moreover, a combination of processing methods is considered to be more effective than a single method. However, information on the effect of radiation processing combined with heating and soaking is still limited.

In present study, we evaluated the gamma irradiation effect of the soaking process prior to irradiation and the combinational effect of gamma irradiation and conventional moist heating on the trypsin inhibitor activity (TIA) in dried and soaked soybean.

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Materials and Methods

Materials Soybeans harvested in 2003 were purchased from a local store (Nonghyup, Daejeon, Korea). Soybeans of uniform size, absolutely sound and undamaged were used for analysis. Benzoyl-DL arginine p-nitroanilide (BAPNA) and purified trypsin were purchased from Sigma Chemical Co. (MO, USA), and Tris buffer from Research Organics, Inc. (Ohio, USA). All the chemicals used were of the highest quality.

Processing method Soybeans were freeze dried and then processed by two different methods as shown in Scheme 1. The dried soybean sample was prepared by gamma irradiation, followed by soaking in distilled water (1:2) for 6 hr at 10°C. The presoaked soybean sample was prepared by presoaking in distilled water (1:2) for 6 hr at 10°C, followed by gamma irradiation. Both samples were heated in distilled water (soybean:water, 1:5) at 60°C using a water bath (MCH-011D, Monotech Co., LTD., Korea) for different times (0, 5, 15, 30, 45, 60 or 120 min).

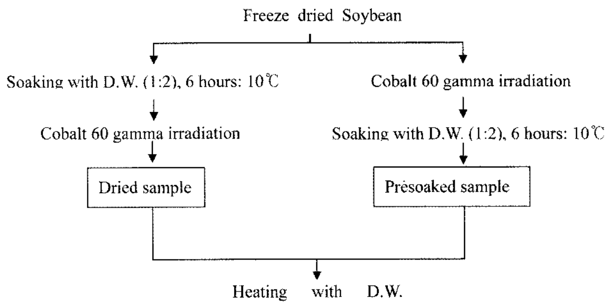
Gamma Irradiation One hundred grams of dried or soaked soybeans was packaged in oxygen-impermeable polyethylene/polypropylene bags (2 mL $O_2/m^2/24$ h at 0°C; 20 cm × 30 cm; Sunkyung Co. Ltd., Seoul, Korea) with a vacuum-packaging machine (Leepack, Hanguk Electronic, Kyungi, Korea). Each bag of soybeans was irradiated in a cobalt-60 gamma irradiator (Point Source, AECL, IR-79, Nordion, Canada) at the Korean Atomic Energy Research Institute, Daejeon, Korea. The applied dosages were 1, 3, 5, 7, 15 and 30 kGy. The source strength was approximately 100 kCi with a dose rate of 70 Gy/min at 15 ± 0.5 and the actual doses were within 2% of the target dose. Non-irradiated, control samples were vacuum packaged in the same bag without gamma irradiation. The absorbed dose was measured with both free radical and ceric/cerous dosimeters (Holm and Berry 1970). All soybean samples were stored at -70°C until use.

Sample extraction Treated soybean was homogenized and then extracted with 50 mL of 0.01N NaOH (pH 8.9) for 3 hr. The suspension was centrifuged for 20 min at 3,000 rpm, 4°C, and for another 1 hr at 15,000 rpm, 4°C. Finally, the resulting supernatant was used for the assay.

Moisture content Treated soybean was dried to a constant weight in an oven at 105°C and the moisture loss was determined gravimetrically according to the AOAC method (14).

Trypsin inhibition assay Trypsin inhibition by soybean trypsin inhibitor was assayed by the method of Kakade et al. (15). An aliquot of the sample extract was suspended in 0.05 M Tris buffer (containing $CaCl_2$, pH 8.2) and then mixed with a known volume of trypsin solution, and the mixture was incubated for several minutes to allow the trypsin-inhibiting factors to react with trypsin. Then an aliquot of BAPNA was added to the suspension, so that the non-inhibited trypsin catalyzed the hydrolysis of BAPNA, forming p-nitroaniline. After 10-min reaction, the hydrolysis was stopped by lowering the mixture pH with acetic acid, thereby denaturing the enzyme. The increase of absorbance was monitored at 410 nm by a spectrophotometer (Ultrospec 4300 pro, Amersham Bioscience, Uppsala, Sweden), and trypsin inhibition was evaluated from the difference in the degree of BAPNA hydrolysis between the sample solution and the uninhibited trypsin solution. Total TIA was expressed as trypsin unit inhibited per gram sample on a dry weight basis (16). One trypsin unit is defined as the increase equal to 0.01 absorbance units at 410 nm after 10 min in 2 mL reaction volume, where values originally expressed as trypsin units inhibited were converted to trypsin units with the relationship that 1mg of pure trypsin has an activity of 1.9 trypsin units (17). All assays were carried out at room temperatures ranging from 23-25°C.

Statistical analysis TIA analyses were performed in triplicate and all data were presented as mean ± standard error (S.E.). All statistical analyses were performed using an SPSS program for Windows. Statistical assessments were performed using ANOVA for the initial demonstration of significance at $p<0.05$, followed by post-hoc



Scheme 1. Processing method for soybean samples.

Duncan's multiple-range test (18). Student's T-test was applied to measure differences of TIA between dried and soaked soybeans prior to irradiation. Two-way ANOVA was applied to measure the interaction effect of heating time and gamma irradiation on soybean TIA.

Results and Discussion

Moisture content of processed soybeans The moisture content of control (freeze-dried soybean) was 9.8% and that of gamma-irradiated dried soybean ranged from 10.1 % to 10.4% (data not shown). The processed soybean samples were dried soybean and presoaked soybean as shown in Scheme 1. The moisture content of dried soybean (dried soybean was gamma irradiated and then soaked) and presoaked soybean (dried soybean was presoaked and then gamma irradiated) is presented in Fig. 1. In the case of control (dried soybean without irradiation), the moisture content was increased from 9.82 to 39.4% after presoaking. In addition, the moisture content of control was increased during moist heating at 60°C according to heating time, by 57.6% at 120 min. For the gamma-irradiated soybean, the moisture content was increased according to both radiation dose and heating time. Moreover, the moisture content was greater for the

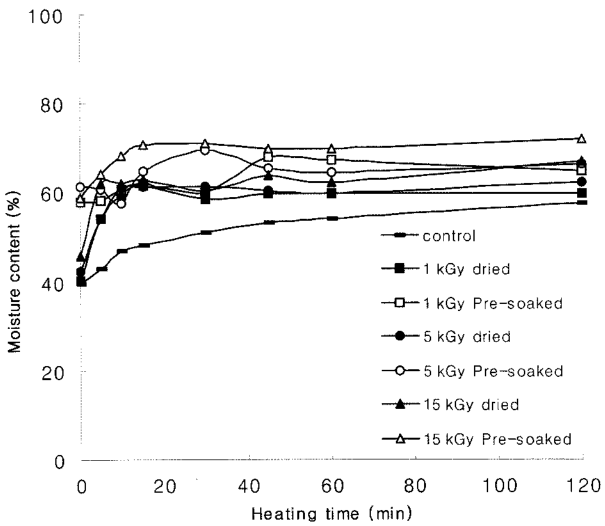


Fig. 1. Moisture content of dried soybean and presoaked soybean gamma-irradiated and then soaking for 6 hr at 10°C.

presoaked soybean than for the dried soybean. Especially the moisture content of presoaked soybean (ranged from 58.0 to 71.5%), gamma irradiated up to 15 kGy and not heated, was similar with that of control which was heated for 120 min.

Effect of gamma irradiation on TIA in dried soybean The effect of gamma irradiation on the TIA of soybeans is shown in Fig. 2. Gamma irradiation significantly decreased the soybean TIA at doses above 5 kGy, at which dose TIA decreased by about 10%. A remarkable decrease in TIA of 43% was observed at 7 kGy, compared to control. However, the TIA values from 15 kGy up to 30 kGy showed no significant difference. Based on our results, over 30 kGy was needed to destroy the TIA entirely. Farag (13) reported that the detoxification dose needed for complete inactivation of all the antinutritional factors naturally present in full-fat soybeans seemed to be higher than the maximal dose level of 10 kGy recommended by WHO in 1981 (19). This was attributed to the low water content (7.3%) of soybeans, which does not favor the production of enough radiolytic products for the denaturation of all antinutritional factors. The gamma irradiation dose required to reach 50% inhibition (ID_{50}) for TIA was calculated to be 13.53 kGy. These results were similar to those of reports that gamma irradiation dose at 10 kGy decreased the TIA by 54.5% in soybean (13) and by 35% in defatted soybean flour (20), while there was no change in TIA in dry kidney bean seeds gamma irradiated up to 5 kGy (21). However, elevation of gamma irradiation dose up to 30 kGy decreased the TIA to 10 mg/g (45%), at which high dose of gamma irradiation failed to entirely inactivate trypsin inhibitors in dry soybean. The differences in the reduction of protease inhibitor activity in soybean, when subjected to irradiation, maybe due to different moisture level or the different varieties of soybeans used. Significant changes were found by Haider *et al.* (22) in the TIA of green gram cultivars at a radiation dose of 4 kGy. Protease inhibitor inactivation may be attributed to the irradiation of high molecular weight carbohydrates present in soybeans in the solid state, as well as their aqueous solution that breaks the external ether bridges (12).

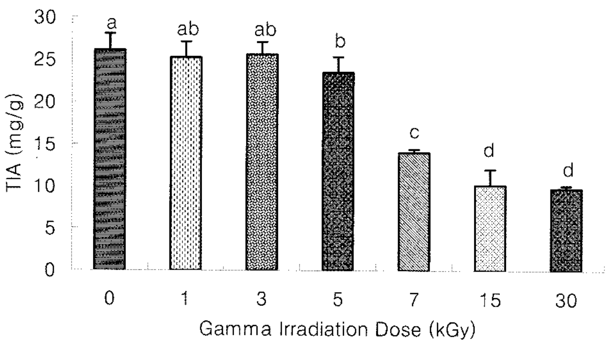


Fig. 2. Effect of gamma irradiation on the trypsin inhibitory activity (TIA) in dried soybean. [a]:Values having the same letters are not significantly different at *p<0.05* by Duncan's multiple range test.

Combinational effect of gamma irradiation and heating on the TIA in dried soybean Figure 3 shows the combinational effect of gamma irradiation and heating on dried soybean. Gamma irradiation and then mild moist heating (60°C) for 5 min significantly decreased the TIA to 79.6% even at the low dose of 1 kGy, while there was no significant decrease on the TIA of control after 15 min of heating at 60°C (Table 1). Even prolonged heating for 120 min, the control retained 31.7% of TIA, while gamma irradiation decreased the TIA to about 10% at the same heating condition as the control. The decreasing rate of TIA during the initial 15 min of moist heating, on exposure to gamma irradiation, was observed to be proportional to the radiation dose. Significant linear relationships between heating time and TIA (R^2 = 1.00, 0.99 and 0.98 at 1, 5 and 15 kGy, respectively) were observed in dried gamma-irradiated soybean as shown in Fig. 4. However, even after a prolonged heating up to 120 min, TIA was not completely destroyed but remained at 11.83, 6.58 and 5.05% for 1, 5 and 15 kGy, respectively, indicating that the remaining trypsin inhibitor was neither heat labile nor gamma irradiation susceptible. Our results concur with those of a previous report that gamma-irradiation (1-5 kGy) of dehydrated (10-12% final moisture level) samples followed by cooking was effective in reducing TIA in three bean varieties (23). Interaction for

Table 1. The trypsin inhibitory activity (TIA, %) in dried soybean or presoaked soybean treated with gamma irradiation during moist heating at 60°C.

Heating time (min)	Control	1 kGy		5 kGy		15 kGy	
		Dried	Presoaked	Dried	presoaked	Dried	presoaked
0	100.00^{a}	96.71^{a}	62.79^{a}*	88.32^{a}	51.38^{a}*	42.63^{a}	31.24^{a}*
5	95.72^{ab}	79.60^{b}	43.84^{b}*	65.16^{b}	43.84^{b}*	35.95^{b}	28.87^{a}*
10	95.71^{ab}	60.45^{c}	42.57^{b}*	51.38^{c}	35.26^{c}*	23.51^{c}	21.52^{b}*
15	95.20^{ab}	39.97^{d}	36.60^{c}*	32.89^{d}	29.71^{d}*	17.80^{c}	15.43^{c}*
30	86.14^{b}	29.52^{e}	21.82^{d}	26.95^{d}	17.80^{d}	14.62^{d}	10.53^{c}*
45	1.04^{c}7	24.20^{ef}	22.66^{d}	23.47^{d}	19.64^{e}	9.72^{d}	7.08^{d}*
60	60.95^{d}	15.43^{fg}	11.75^{e}	12.10^{e}	4.71^{f}	6.39^{c}	2.68^{e}
120	31.72^{e}	11.83^{g}	7.73^{f}	6.58^{f}	4.02^{f}	5.05^{d}	2.68^{e}

[a]Values in the same column with the same letters are not significantly different at $p<0.05$
*Values of TIA between dried soybean and presoaked soybean at the same heating time are significantly different at $p<0.05$ by 2-tailed student's t-test.

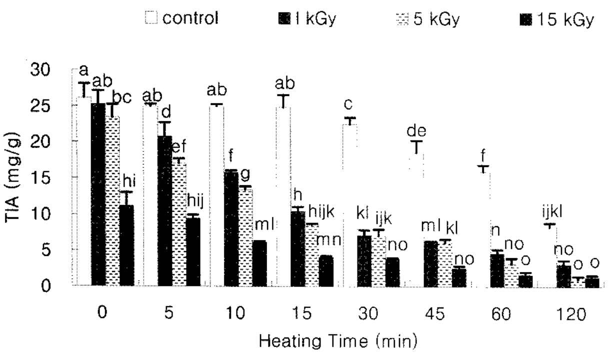


Fig. 3. TIA (mg/g) of dried soybean gamma-irradiated during moist heating at 60°C. [a]:Values having the same letters are not significantly different from each other at $p<0.05$ by Duncan's multiple range test.

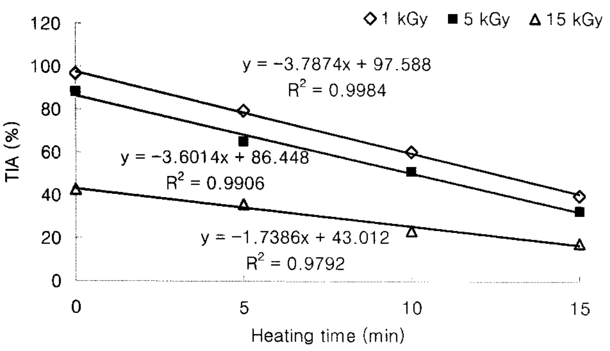


Fig. 4. Decreasing rate of TIA (%) of gamma-irradiated white soybeans (1, 5 and 15 kGy) during initial 15 min of moist heating.

gamma irradiation × heating time was observed at $p<0.05$ (Table 2).

Effect of gamma irradiation on the TIA in presoaked soybean Soybean is generally used after soaking and then processed into various types of foods. Soaking and then heating is the essential first step of processing for typical processed foods made from soybean such as soy milk, tofu or soybean paste. To evaluate the effect of gamma irradiation on the TIA in soaked soybean, we compared the TIA between dried soybean and presoaked soybean treated with gamma irradiation. The TIA in presoaked and then gamma-irradiated soybean without heating was greatly decreased compared to that in dried gamma-irradiated soybean. As shown in Table 1, at the low dose of 1 kGy, the TIA in presoaked soybean (62.8%) was much lower than that of dried soybean (96.7%). In addition, the inactivation of TIA, on exposure to gamma irradiation, was proportional to the radiation dose. Moreover, TIA value of presoaked soybean (presoaked gamma-irradiated soybean) without heating (1 or 5 kGy) was the similar to that of dried soybean that underwent 10-min heating (dried gamma-irradiated soybean). Based on these results, the soaking process prior to gamma irradiation has a sparing effect of heat energy on the reduction of TIA in soybean. ID_{50} values are represented in Fig. 5. Soaking prior to gamma irradiation significantly lowered the ID_{50} value to 8.44 kGy, from 13.53 kGy for drying and

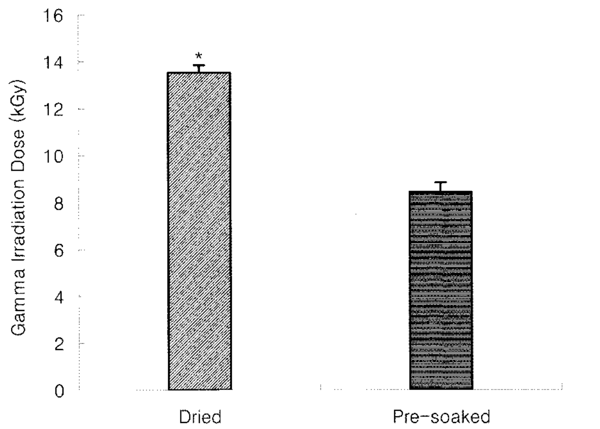


Fig. 5. ID_{50} (the gamma irradiation dose level required to inhibit 50% of TIA) values of dried soybean or presoaked soybean. *:Values between dried soybean and presoaked soybean are significantly different at $p<0.05$ by 2-tailed student's t-test.

gamma irradiation, and the soaking process enhanced the efficiency to inactivate TIA by as much as 48%. Our results were similar to those of a previous report that the moisture content was increased at low radiation dose (24).

Combinational effect of gamma irradiation and heating on TIA in presoaked soybean To maximize the efficiency for the inactivation of TIA by gamma irradiation, a moist heating process was combined with soaking. The effect of the soaking process prior to gamma irradiation on the TIA inhibition (%) in soybean at different conditions is shown in Fig. 6. The TIA inhibition (%) compared to control was greater in presoaked soybean (presoaked gamma-irradiated soybean) than in dried soybean (dried gamma-irradiated one) even at mild heating process at 60°C, as shown in Fig. 6. After initial 5-min heating at 60°C, TIA inhibition (%) in presoaked soybean was 56.1%, while that of dried soybean was 20.4%. Further prolonged heating of more than 15 min gave no significant difference in TIA (%) between dried soybean and presoaked soybean. In addition, there were significant interactions between gamma irradiation and heating time (gamma irradiation × heating time) for TIA(%) as reported in Table 2.

These results demonstrated that soaking prior to gamma irradiation was very effective for inactivating TIA in

Table 2. The effect of heating and gamma irradiation on TIA of dried soybean and presoaked soybean

Sources of Variation	Dependent Variable	D.F.	Mean Square	F value
Intercept	Dried	1	1459.5570	3585.155*
	Presoaked	1	2818.3975	2311.356*
Heating Time	Dried	5	153.9214	378.082*
	Presoaked	5	404.3476	331.604*
Dose	Dried	2	81.3340	199.783*
	Presoaked	2	135.4249	111.061*
Dose Heating time	Dried	10	6.9316	17.026*
	Presoaked	10	15.2717	12.524*

*Values are significantly different at $p<0.001$ by 2-way ANOVA

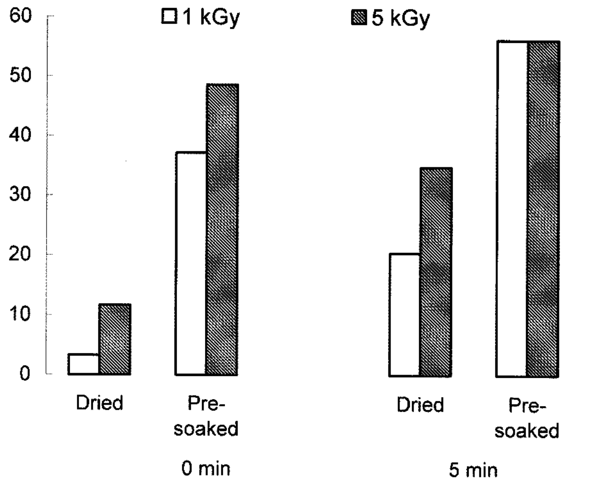


Fig. 6. Gamma irradiation effect of soaking process prior to irradiation on the TIA inhibition (%) in soybean at different conditions: Moist heating at 60°C for 0 and 5 min, and at the dose level of 1 and 5 kGy, respectively.

soybean. The heating time required to reach the 50% inhibition of TIA (IT_{50}) is represented in Table 3. The IT_{50} value was obtained from the linear plot based on the initial 15 min heating at 60°C. The IT_{50} value of dried soybean (dried gamma- soybean) at the dose level of 1 kGy was significantly decreased to 12.56 min, compared to the 85.69 min control. The IT_{50} value of dried soybean was decreased with increasing dose level of gamma irradiation. Furthermore, when soaking prior to gamma irradiation and subsequent heating process were combined, the IT_{50} value at even 1 kGy (5.28 min) was reduced by more than 50% compared to the presoaked level. The elevation of gamma irradiation in presoaked soybean was more effective in reducing the IT_{50} value than in the dried soybean. The effect of the combination of radiation plus moist heating on TIA was similar to a previous report that the combination of autoclaving for 10 min plus irradiation up to 20 kGy reduced the level of chlorogenic acid in sunflower meal more than other processing methods (25). A small decrease was observed by Hafez *et al.* (24) in soybean TIA (7.4% moisture) at low dose levels but an increased radiation dose of 100 kGy decreased the TIA by 25%. A similar reduction (25%) was observed by the same authors

Table 3. IT_{50} (the heating time required to reach the 50% inhibition of TIA) values of dried soybean or presoaked soybean at different doses of gamma irradiation. -: Starting values are below 50%

	Control	1 kGy	5 kGy	15 kGy
Dried	85.69	12.56	10.12	-
Presoaked		5.28	0.74	-

Table 4. Calculated activation energy (kcal/mole) for TIA decrease in dried soybean or presoaked soybean at different doses of gamma irradiation

	Control	1 kGy	5 kGy	15 kGy
Dried	12.13	5.10	3.18	1.02
Presoaked		2.45	1.92	0.69

for soybean when the moisture content was increased and the radiation dose was low. Sattar et al. (26) have reported a decrease in TIA during soaking of irradiated green grams. Significant linear relationships (*r*=-0.960 to -0.987) have been reported in chick pea between TIA loss and the soaking time of irradiated and unirradiated seeds and between TIA loss and increasing radiation dose (0.25-1.00 kGy) (27).

The activation energy (Ea) for the inactivation of the trypsin inhibitor was calculated according to the Arrhenius equation. The relation between the inactivation rate constant during the initial 5-min heating and the heating temperature was introduced to the activation energy calculation. As reported in Table 4, the activation energy was decreased according to the gamma irradiation dosage. The activation energy was 12.13 kcal/mole for the control, compared to 5.10 kcal/mole for gamma-irradiated soybean at a low dose level of 1 kGy. Interestingly, when soybean was soaked prior to gamma irradiation, the activation energy was decreased remarkably, compared to that of dried soybean gamma-irradiated. The activation energy of soaked soybean prior to gamma irradiation at 1 kGy was 2.45 kcal/mole, which was about 50% lower than that of dried soybean gamma-irradiated. Contrary to microwave cooking, a high moisture environment was needed to elevate the inactivation of TIA in soybean (28).

Based on the present results, soaking prior to gamma irradiation is an effective method for TIA inhibition. Furthermore, a combination of two or more processing methods such as soaking, heating and gamma irradiation is much more effective than any single processing method.

Acknowledgments

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