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Reduction of Egg White Allergenicity in Cake using Ionizing Radiation

Ju-Woon Lee, Jae-Hun Kim, Ji-Hyun Seo, Cheon-Jei Kim[1], Hong-Sun Yook[2], and Myung-Woo Byun*

Department of Radiation Food Science and Biotechnology, Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute

[1]Department of Animal Products Science, Konkuk University

[2]Department of Food and Nutrition, Chungnam National University

Abstract

This study was conducted to evaluate the effect of gamma irradiation for the reduction of an egg allergy in a cake with the irradiated egg white. A white layer cake was manufactured including 10- or 20-kGy-irradiated egg white. Enzyme-linked immunosorbent assays (ELISAs) with immunoglobulin (Ig) E from egg-allergic patients and with rabbit anti-ovalbumin (OVA) IgG were used to identify and quantify native OVA in the samples. Concentrations of native OVA detected by IgE and IgG in the control were 432.88 μg/g sample and 375.46 μg/g sample, respectively. However, native OVA in white layer cake prepared with 10- and 20-kGy-irradiated egg white were detected at low concentrations detected by IgE to 14.27 and 8.78 μg/g sample, respectively. Whereas, IgG recognized OVA more often in 10- and 20-kGy samples than in control. The results appear to suggest that irradiating egg white might reduce its allergenicity by the conformational change of OVA. Therefore, gamma irradiation could utilize reduce an egg allergy.

Key words : radiation technology, egg allergy, ovalbumin, cake, hypoallergenic food

INTRODUCTION

Egg allergy affects about 6% of infants less than 3 year of age (Sampson, 2004). Egg white, more so than egg yolk, is one of the most prevalent allergens in food hypersensitivity, particularly in atopic children (Taylor *et al.*, 2002). Egg white contains about 40 different proteins, some of which have been shown to be major allergens, including ovalbumin (OVA; 54 % of egg white proteins) and ovomucoid (11% of egg white proteins) (Holen and Elsayed, 1990; Yunginger, 1997).

Technologies for the reduction of a food allergy have been developed, which was reduced milk allergy by the proteolysis of milk allergen. Heat processing was used to reduce certain food allergy, but that did not decrease effectively. Meanwhile, the conformational changes of food allergens by gamma irradiation were recently observed in several studies. The change could reduce allergenicity by the modification or destruction of the binding epitopes of immunoglobulin E (IgE) to the food proteins (Byun *et al.*, 2000; Katial *et al.*, 2002; Kume and Matsuda, 1995; Lee *et al.*, 2001b). Previous studies reported that the abilities of egg-allergic patient IgE to bind OVA and ovomucoid treated with a combination of gamma irradiation with other processing treatments declined (Kim *et al.*, 2002; Lee *et al.*, 2002). Also, reduced allergenicity of irradiated OVA was also observed in *in vitro* skin prick tests (Jeon *et al.*, 2002).

Physicochemical and sensory qualities of cake with gamma-irradiated egg white in our previous work did not differ from that with non-irradiated egg white (Lee *et al.*, 2003). This work, therefore, was conducted to evaluate the effect of irradiation on the cake as practical hypoallergenic food to reduce an egg allergy.

* **Corresponding author** : Myung-Woo Byun, Department of Radiation Food Science and Biotechnology, Advanced Radiation Technology Institute, Korea Atomic Energy Research Institute, P.O. Box 1266, Jeongeup, Jeolabuk-do, 580-185, Korea. Tel: +82-63-570-3200, Fax: +82-63-570-3202, E-mail: mwbyun@kaeri.re.kr

MATERIALS AND METHODS

Protein and Antibodies

OVA was purchased from Sigma Chemical Co. (St. Louis, MO.). Human sera were obtained from 28 patients (< 3 years

of age; 18 boys and 10 girls) diagnosed with IgE-mediated egg allergy after open egg challenges, a convincing history of acute urticaria or diarrhea after egg ingestion, or both, and having not shown any diagnostic reactions to wheat flour or nonfat dried milk. Eighteen patients suffered from atopic dermatitis, acute urticaria, or both; six patients suffered from skin and gastrointestinal symptoms (diarrhea and vomiting); and four patients suffered from only chronic diarrhea. All the patients showed elevated egg-specific IgE (0.8 to 100 kU/L) measured by AlaSTAT RIA (DPC Co., Los Angeles, Calif.) or the Pharmacia CAP System FEIA (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). The sera were pooled to determine the ability of IgE to bind OVA in the samples. To measure the change in the ability of rabbit IgG to bind to OVA in cake sample solution, polyclonal rabbit IgG against OVA was produced from New Zealand white rabbit immunized to OVA. Biotinylated anti-human IgE developed in goat and horseradish peroxidase (HRP)-conjugated avidin D were purchased from Vector Laboratories Inc. (Burlingame, Calif.) to determine the binding reaction of IgE. Goat anti-rabbit IgG-HRP was purchased from Sigma Chemical Co. to trace the rabbit IgG.

Gamma Irradiation

Egg white separated from whole egg and OVA solution (2 mg/mL in 0.01 M PBS including 0.15 M NaCl, pH 7.4) were irradiated in a cobalt-60 irradiator (IR-79, Nordion International Ltd., Ontario, Canada) equipped with 100 KCi activity at 10 ± 0.5℃ and operated at a dose rate of 20 kGy/hr. The applied dose levels were 10 and 20 kGy. Dosimetry was performed with 5-mm-diameter alanine dosimeters (Bruker Instruments, Rheinstetten, Germany), and the free-radical signals were measured by a Bruker EMS 104 EPR analyzer (Bruker Instrumnets). After irradiation, the egg white and OVA solution were stored at 4℃.

Manufacturing the White Layer Cake and Preparation of Cake Sample Solution

White layer cake containing high content of egg white (19.6%) made in previous research (Lee *et al.*, 2003) was used in this study. The formula and preparation procedure applied was the commercial method used in the bakery industry and deli shops. Sample extracts were prepared by the method of Sajdok *et al.* (1990). Sample (20 g) was homogenized in 200 mL of an extraction buffer (0.1 M PBS, pH 7.4, containing 6 M urea, 0.01 M ethylenediaminetetra acetic acid disodium salt, and 0.01 M dithiothreitol) for 5 min. The homogenate was stirred at 4℃ for 4 hr and centrifuged at 9,000 × g for 30 min. Supernatant was filtered through a 0.45-μm filtration kit (MILLEX®-HV, Millipore, Molsheim, France) and dialyzed in a cellulose tube (pore size < 5,000, Spectrum Medical Industries Inc., Houston, Tex.) in 0.01 M PBS. After dialysis, the protein concentration of the extract solutions was determined with a bicicinchonic acid protein assay kit (Sigma Chemical Co.) and a bovine serum albumin (Sigma Chemical Co.) standard solution measured at 562 nm (UV-1600PC spectrophotometer, Shimadzu Corp., Kyoto, Japan) by a method described previously (Smith *et al.*, 1985). The protein concentrations of the extract solutions were measured and adjusted to 500.0 μg/mL by dilution with 0.01 M PBS for unifying the protein concentration. The diluted solutions were used in the electrophoretic analysis and ELISA as sample solutions.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; 5 to 15% gradient gel) for the sample solutions (500.0 μg/mL) was performed by the Laemmli's method (Laemmli, 1970) with the use of a Hoefer vertical electrophoresis apparatus (SE-600, Pharmacia Biotech, Uppsala, Sweden), and the gel was silver-stained as described previously (Heukeshoven, 1985), because of the low protein concentration of the sample solution. A prestained molecular weight marker (161-0372) was purchased from Bio-Rad Laboratories (Hercules, Calif.) and used to determine the molecular masses of the protein bands. The marker consisted of proteins with the following molecular masses: 250, 150, 100, 75, 50, 37, 25, 15 and 10 kDa. A molecular mass marker, two representative egg allergen solutions (1.0 mg/mL), OVA and ovomucoid, and the sample solutions were loaded into same gel.

Competitive Indirect ELISA

OVA was quantified by competitive indirect ELISA (Ci-ELISA), which was individually formatted with the patient IgE and polyclonal IgG to evaluate their ability to bind to the samples by the method reported previously (Kim *et al.*, 2002; Sajdok *et al.*, 1990). Briefly, polystyrene flat-bottom microtiter plates (Maxisorp, Nunc, Kamstrup, Denmark) were coated with 100 μL of OVA solution (10.0 μg/mL) in a 0.2 M bicarbonate buffer, pH 9.6, overnight at 4℃. The plates were washed three times with PBS containing 0.05% (v/v)

Tween 20 (PBST). To reduce nonspecific binding, the plates were blocked by incubation at 37℃ for 1.5 hr with 120 μL of PBS containing 1% (w/v) bovine serum albumin. After washing, 50 μL of the standard solution or sample solution was added to four coated and blocked wells, and 50 μL of the IgE solution diluted 25 times in PBS was added. The plates were incubated for 2 hr at 37℃ and then washed three times with PBST. For the detection of IgE bound to coated OVA, 100 μL of the biotinylated secondary Ab solution diluted 500 times in PBS was added to the wells and incubated for 1.5 hr at 37℃. Also, HRP-conjugated avidin D solution (100 μL of diluted 500 times in PBS) was added in the washed wells and incubated for 1.5 hr at 37℃. The plates were then washed, and 100 μL of the 0.04% *o*-phenylenediamine (Sigma Chemical Co.) in a 0.1 M phosphate-citrate buffer, pH 5.0, with 0.04% hydrogen peroxide (v/v, 35% H_2O_2) was added for the color reaction 20 min before the reaction was stopped with 2.0 M H_2SO_4 (50 μL per well). The absorbance was measured at 492 nm in an ELISA reader (CERES UV-900C, BIO-TEK instruments Inc., New York, NY.). For standards, a serially diluted allergen solution was prepared at concentration ranging from 0.12 to 1,000 μg protein per ml. Rabbit IgG solution and goat anti-rabbit IgG-HRP diluted 2,000 and 20,000 times in PBS, respectively, and used in ELISA formatted with rabbit IgG against OVA. The concentration of OVA in the sample solutions was calculated by an equation obtained from each standard curve.

Recovery tests to evaluate the precision of IgE- and IgG-ELISA were conducted by the method of Lee (1999). Native OVA solution was prepared at three different concentrations (5.0, 50.0 and 100.0 μg/mL) and added in the coated and blocked wells instead of standard solutions as standard condition. Sample solutions for the recovery test also were prepared by the procedure mentioned above, with extract buffer spiked with three different concentrations of native OVA (5.0, 50.0 and 100.0 μg/mL). When concentrations of OVA in nonspiked sample solution were individually determined by each ELISA, the concentrations were 43.2 (± 0.25) μg/mL in IgE-ELISA and 37.5 (± 0.47) μg/mL in IgG-ELISA. These values were used as references in both ELISAs.

Statistical Analysis

All samples were done in triplicate, and the experiments were repeated five times. Recovery tests were repeated five times. The means and standard errors were used to evaluate the differences in allergen concentrations obtained from the Ci-ELISA. The data were analyzed by general linear procedures, least square means, and Duncan's multiple range test (SAS software, 1988).

RESULTS AND DISCUSSION

SDS-PAGE Profile

The SDS-PAGE profile of the proteins in the sample solutions is shown in Fig. 1. Most bands in the samples have molecular masses of approximately 75 kDa or less. Many bands in all sample solutions appeared in the range of 12 to 70 kDa having similar separation patterns. Sample solution containing irradiated egg white showed smear appearance in high molecular weights. The result was demonstrated aggregation of protein molecules formed by covalent cross-linking with hydroxyl radical (HO□) induced from water radiolysis by irradiation (Davies, 1987; Tuce *et al.*, 2001). When compared with the standard OVA (43 kDa), the band with the same molecular mass as the OVA in all sample lanes was also observed. We could infer that OVA might be in the sample solutions. However, the existence of the allergens apparently

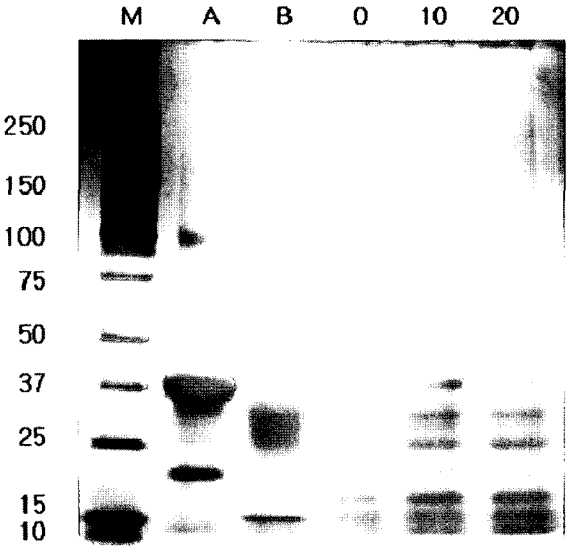

Fig. 1. SDS-PAGE profile of proteins in sample solutions prepared from white layer cakes with 0-, 10- and 20-kGy gamma-irradiated egg whites. M, molecular mass standard marker; A, ovalbumin; B, ovomucoid; 0, white layer cake with nonirradiated egg white; 10, white layer cake with 10-kGy- irradiated egg white; 20, white layer cake with 20-kGy-irradiated egg white. Numbers on the left side indicate the molecular mass (kDa) of the standard marker.

could not be. determined.

Standard Curves for the Quantification of OVA in Sample Solutions

Standard curves were used to quantify the OVA in the sample solutions (Fig. 2 and 3). OVA could be quantitatively determined at 3.9 to 125 μg/mL by patient IgE and at 0.05 to 250 μg/mL by rabbit IgG, and the concentration could be individually calculated by the equations ($x = e^{(0.3778-y)/0.1017}$ (IgE) and $x = e^{(0.4196-y)/0.0626}$ (IgG)) obtained from the curve. The x indicates the concentration of the allergen in the sample solutions and y represents the average optical density of a sample solution in the micro-wells at 492 nm. The limits of detection of OVA in the sample solutions were determined to be 0.2 μg/g in IgE-ELISA to 0.01 μg/g in IgG-ELISA. This result of the patient IgE-ELISA was similar to the previous reports (Kim *et al.*, 2002; Lee *et al.*, 2002), and the titration and affinity of IgE used in this study were not different from the studies.

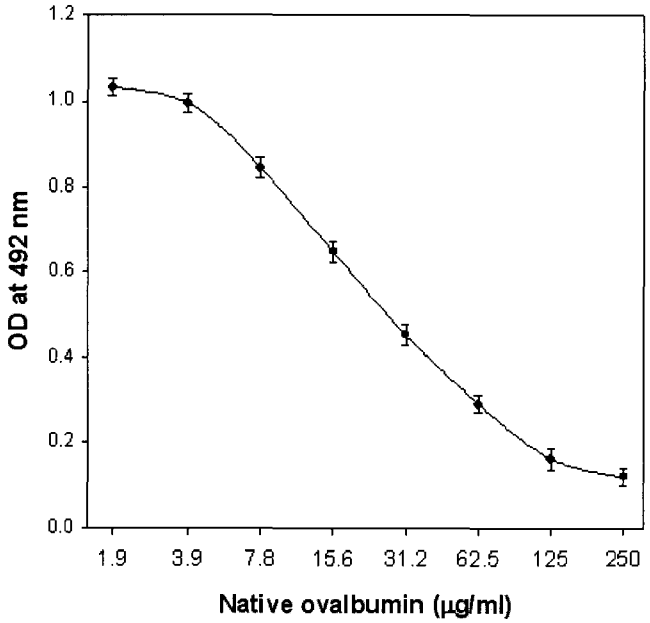

Fig. 2. Standard curves formatted with egg white-hypersensitive patient IgE for quantifying native ovalbumin. Standard solutions were prepared by dilution of native OVA (1.0 mg/mL in PBS buffer, pH 7.4).

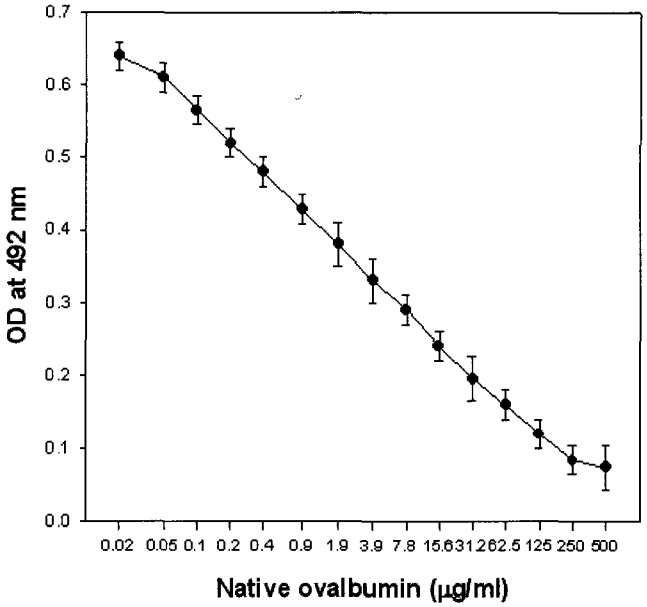

Fig. 3. Standard curves formatted with rabbit anti-OVA IgG (polyclonal) for quantifying native ovalbumin. Standard solutions were prepared by dilution of native OVA (1.0 mg/mL in PBS buffer, pH 7.4).

Binding Abilities of Patient IgE and Rabbit IgG to OVA Solution

Table 1 was showed the change of binding abilities to irradiated or irradiated/heat OVA solution. Egg allergic patient IgE detected the irradiated OVA solution to lower concentration than control. The concentration of OVA solution combined with irradiation and heat treatment recognized by patient IgE was reduced less and less. These results indicate that ionizing radiation could decrease egg allergy and is supported by other studies (Lee *et al.*, 2000; 2001a; 2001b,

Table 1. Binding abilities of egg-allergic patient IgE and rabbit anti-ovalbumin IgG to irradiated and irradiated/heat OVA solution[1)]

	Egg-allergic patient IgE	Rabbit anti-ovalbumin IgG
Control	9.7 ± 0.4	10.1 ± 0.6
Irradiation [2)]	1.5 ± 0.2	181.1 ± 7.3
Irradiation and heat treatment [3)]	0.5 ± 0.3	36.1 ± 2.5

[1)] The concentrations (μg/mL) were calculated by the equations of standard curves individually obtained from Ci-ELISA formatted with egg-allergic patient IgE and rabbit anti-ovalbumin IgG.

[2)] Irradiated dose was 10 kGy.

[3)] The sample treated at 100℃ for 15 min after irradiation.

Katial *et al.*, 2002).

OVA recognized by rabbit IgG in irradiated or irradiated/heat OVA solution increased more than control (Table 1). The result was supported by studies of Byun *et al.* (2002), Kim *et al.* (2002), and Lee *et al.* (2001a). They reported that antigenicity increased because irradiation might expose hidden epitopes by cleaving and unfolding the structure.

Binding Abilities of Patient IgE and Rabbit IgG to OVA in White Layer Cake

The concentrations of OVA in cake detected by egg-allergic patient IgE and rabbit IgG showed in Table 2. Egg-allergic patient IgE recognized less OVA in white layer cake containing irradiated egg white than control. The concentration of control, 10 kGy-sample, and 20 kGy-sample were 432.88, 14.27, and 8.78 μg/g, respectively. Gamma irradiation decreased significantly allergenicity to 96% in 10 kGy-sample and 98% in 20 kGy-sample against native OVA within control ($p<0.05$). This result showed that the binding ability of IgE to bind native OVA was reduced by irradiation, and is supported by the previous reports of Kim *et al.* (2002), Katial *et al.* (2002), and Lee *et al.* (2002).

The concentration of the OVA determined by rabbit IgG in the cake increased with the dependence on the irradiation dose. The determined concentrations indicated that the binding abilities of rabbit IgG increased to 124% in 10-kGy sample and to 231% in 20-kGy sample. This result caused by exposure of inner epitopes by irradiation as mentioned above.

Recovery Test of Patient IgE and Rabbit IgG-ELISA

Table 3 indicated the precision of the ELISAs by recovery tests with standard and sample solution. Recovery rates of

Table 2. Concentration (μg/g sample)[1] of ovalbumin detected by egg allergic patient IgE and rabbit anti-ovalbumin IgG in the sample solution prepared from white layer cake containing with 10 or 20 kGy-gamma-irradiated egg white

Type of antibody	Irradiation dose (kGy)		
	0	10	20
IgE	432.88±1.67[a2)]	14.27±1.16[b]	8.78±1.02[b]
IgG	375.46±2.68[b]	465.27±2.18[b]	867.32±3.07[a]

[1)] The concentrations were calculated by the equations of standard curves individually obtained from Ci-ELISA formatted with egg-allergic patient IgE and rabbit anti-ovalbumin IgG.

[2)] Different letter (a, b) within a same row differ significantly ($p<0.05$).

Table 3. Recovery by egg-allergic patient IgE-ELISA and rabbit anti-ovalbumin polyclonal IgG-ELISA

Detection method[1)]	Type of Ab	Concentration of added ovalbumin (μg/mL)	Recovery (μg/mL)[2)]		% recovered	Average % recovered[3)]	SD[4)]
			Expected	Detected			
Standard solution	IgE	5	5	4.6	92.0	97.6	0.01
		50	50	48.1	96.2		
		100	100	104.5	104.5		
	IgG	5	5	5.1	102.0	98.9	0.02
		50	50	48.6	97.2		
		100	100	97.5	97.5		
Sample solution	IgE	5	5	45.9	95.3	98.6	0.01
		50	50	90.8	97.4		
		100	100	147.8	103.2		
	IgG	5	5	43.9	103.3	101.2	0.01
		50	50	91.2	104.2		
		100	100	132.3	96.2		

[1)] Detection of recovery rates of IgE- and IgG-ELISA was performed with blank, instead of standard solution, and in sample solution (extracted cake) with the additions of three concentrations (5.0, 50.0 and 100.0 μg/mL) of native ovalbumin.

[2)] Recovery was calculated as percentage of the detected concentration to that expected by ELISA.

[3)] Average is the mean percent recovery rate obtained from three concentrations.

[4)] SD, Standard deviation ($p<0.05$).

IgE-ELISA and IgG-ELISA were 97.6% and 98.9%, respectively, in the intra- and interassays. When native OVA was spiked, the concentrations detected by both ELISAs differed by less than 5%. Kim (1983) and Lee (1999) reported a recovery rate of at least more than 90% should be needed when polyclonal Ab-based immunoassay is applied. These results showed that the IgE- and IgG-ELISAs used in this study were accurate and that the data obtained from the assays were considerably reliable.

In conclusion, the results obtained from this study present practical data for use in reducing allergenicity by modification of an egg white allergen with irradiation. Radiation technology might be used for inhibition of food hypersensitivity.

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