

Change of an Egg Allergen in a White Layer Cake Containing Gamma-Irradiated Egg White

JI-HYUN SEO,¹ JU-WOON LEE,¹ YOU-SEOK LEE,¹ SOO-YOUNG LEE,² MEE-REE KIM,³ HONG-SUN YOOK,³ AND MYUNG-WOO BYUN^{1*}

¹Department of Radiation Food Science and Biotechnology, Korea Atomic Energy Research Institute, P.O. Box 105, Yuseong, Daejeon, 305-600, Korea; ²Department of Pediatrics, School of Medicine, Ajou University, Suwon, Kyonggi-do, 442-749, Korea; and ³Department of Food and Nutrition, Chungnam National University, Yuseong, Daejeon, 305-764, Korea

MS 03-392: Received 10 September 2003/Accepted 29 February 2004

ABSTRACT

This study was conducted to evaluate the reduction of an egg allergen in a cake containing gamma-irradiated egg white. A white layer cake was manufactured by a commercial formula with 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 IgG were used to identify and quantify ovalbumin (OVA) in the samples. Concentrations of native OVA detected by IgE and IgG in the control were 432.88 and 375.46 µg/g sample, respectively. However, native OVA in samples with 10- and 20-kGy-irradiated egg white was detected at low concentrations (14.27 and 8.78 µg/g, respectively) by IgE ($P < 0.05$); IgG recognized OVA more often in 10- and 20-kGy samples than in controls. Conformational cleavage of OVA by irradiation could explain the IgG result. The results appear to suggest that irradiating egg white might reduce its allergenicity, which could be used in the production of baked goods of reduced allergenicity.

Egg is widely used as an ingredient in the production of bread, cookies and cakes, noodles and pasta, meat products, and so on, for specific applications such as flavor enhancing, gelation, foaming action, thickening, and emulsifying (19). However, egg white, more so than egg yolk, is one of the most prevalent allergens in food hypersensitivity, particularly in atopic children (12). 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) (4, 24).

In general, ionizing radiation used to free foods from pathogenic microorganisms is among the methods applicable in food preservation (11). Meanwhile, the conformational changes of food allergens by gamma irradiation were recently observed in several studies, and the change could reduce allergenicity or antigenicity by the modification or destruction of the binding epitopes of immunoglobulin E (IgE) to the food proteins (1, 7, 10, 14).

Previous studies reported that the ability of egg-allergic patient IgE to bind OVA and ovomucoid treated with a combination of gamma irradiation and other processing treatments declined (9, 15). Also, reduced allergenicity of irradiated OVA and ovomucoid was also observed in *in vitro* skin prick tests (5).

To evaluate the practical approach of using irradiation technology to eliminate or reduce egg allergens in processed foods, the allergenic and quality properties of cakes and cookies containing 10- and 20-kGy gamma-irradiated

egg white as an ingredient in a commercial formulation were determined (16). Physicochemical and sensory qualities of the products with irradiated egg white did not differ from those with nonirradiated egg white.

In this study, white layer cake was chosen because of its high egg white content (19.6%). Possible changes in the concentrations of native OVA, the targeted allergen, were determined by two enzyme-linked immunosorbent assays (ELISAs) formatted with patient IgE and rabbit anti-OVA immunoglobulin (polyclonal IgG), and the results were compared.

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/liter) 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. Ten age-matched subjects provided sera for the control. None had food allergy or any other atopic skin, respiratory, or gastrointestinal symptoms.

To measure the change in the ability of polyclonal IgG to bind to OVA in cake sample solution, two New Zealand white rabbits were immunized for production of polyclonal IgG against

* Author for correspondence. Tel: 82-42-868-8065; Fax: 82-42-868-8043; E-mail: mwbyun@kaeri.re.kr.

OVA. Before immunizing, 1 ml of blood was collected from the ear for preparation of a normal serum. The rabbits were immunized by intradermal injection (1 ml of 100 µg/ml) with Freund's complete adjuvant. The subsequent injection with Freund's incomplete adjuvant was performed three times every 2 weeks, and the antisera from each rabbit were checked for production of antibody (Ab) by indirect ELISA. The rabbits were finally boosted with 100 µg/ml of antigen mixed with 0.01 M phosphate-buffered saline (PBS) without adjuvant. After 1 week, the blood was collected by heart puncture and antiserum was separated by centrifuging at 3,000 rpm for 10 min. Rabbit anti-OVA polyclonal IgG was separated from the antiserum with the use of affinity chromatography with Protein A Hi-trap (5 ml; Sigma). The IgG solution was mixed with an equal volume of glycerol, the affinity and titration of IgG were checked by indirect ELISA, and the IgG solution was stored at -20°C .

Biotinylated anti-human IgE developed in goat (BA-3040) and horseradish peroxidase-conjugated avidin D (A-2004) were purchased from Vector Laboratories Inc. (Burlingame, Calif.) to determine the binding reaction of IgE. Goat anti-rabbit IgG-horseradish peroxidase was purchased from Sigma Chemical Co. to trace the polyclonal IgG.

Gamma irradiation. Egg white separated from whole egg was irradiated in a cobalt-60 irradiator (IR-79, Nordion International Ltd., Ontario, Canada) equipped with 100 kCi activity at $10 \pm 0.5^{\circ}\text{C}$ and operated at a dose rate of 20 kGy/h. 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 Instruments). After irradiation, the egg white was stored at 4°C .

Manufacturing the white layer cake. White layer cake made in previous research (16) was used in this study. The formula and preparation procedure applied was the commercial method used in the bakery industry and in deli shops. Briefly, shortening was softened by churning in a mixer (NVM2-14, Daeyung Bakery Machinery Ind. Co. Ltd., Seoul, Korea) and salt, sugar, and emulsifier were added. Soft cream was made by the gradual addition of egg white (irradiated or control) and cream of tartar to the mixture. About half of the water was gradually added to the creamed mixture at a low mixing speed, which was increased to around an average speed. Baking soda, wheat flour, and nonfat dried milk were added and mixed slightly, and the remaining water was added and mixed until a soft batter was made. The temperature of the batter was 23°C , and the specific gravity of the batter was adjusted to 0.8 ± 0.05 . Batter (550 g) was placed in a pan (24 by 4 cm) and cooked in a commercial oven (FDO-7104A, Daeyung Bakery Machinery) at 190°C for 25 min. After cooking, the cake was removed from the pan, cooled to an ambient temperature, and used for the subsequent experiments.

Preparation of cake sample solution. Sample extracts were prepared by the method previously described (20). 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°C for 4 h and centrifuged at $9,000 \times g$ for 30 min. Supernatant was filtered through a $0.45\text{-}\mu\text{m}$ 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 bicinchoninic acid protein assay kit (Sigma) and a bovine serum

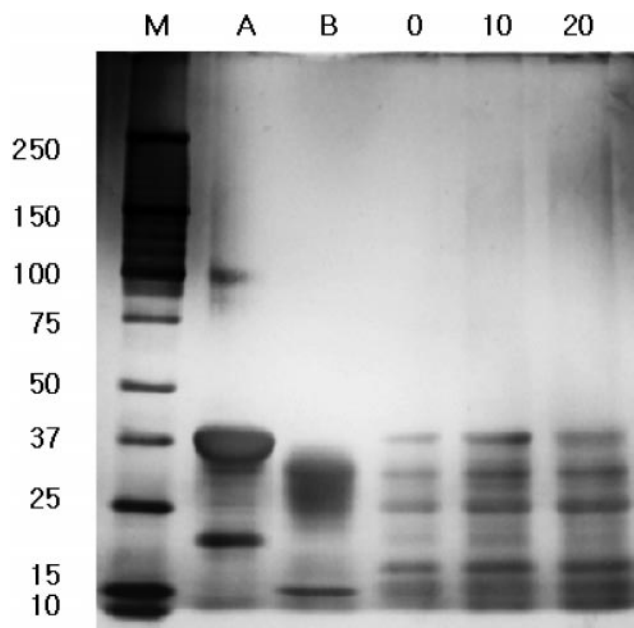


FIGURE 1. SDS-PAGE profile of proteins in sample solutions prepared from white layer cakes with 0-, 10-, and 20-kGy gamma-irradiated egg whites. M, molecular mass standard marker; A, ovalbumin; B, ovomucoid; 0, white layer cake with nonirradiated egg white; 10, white layer cake with 10-kGy-irradiated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

albumin (Sigma) standard solution measured at 562 nm (UV-1600PC spectrophotometer, Shimadzu Corp., Kyoto, Japan) by a method described previously (22). The protein concentrations of the extract solutions were measured and adjusted to $500.0 \mu\text{g/ml}$ 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 ($500.0 \mu\text{g/ml}$) was performed by Laemmli's method (12) with the use of a Hoefer vertical electrophoresis apparatus (SE-600, Pharmacia Biotech, Uppsala, Sweden), and the gel was silver-stained as described previously (3) because of the low protein concentration of the sample solution. A prestained molecular mass 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 the 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 (9, 15). Briefly, polystyrene flat-bottom microtiter plates (Maxisorp, Nunc, Kamstrup, Denmark) were coated with $100 \mu\text{l}$ of OVA solution ($10.0 \mu\text{g/ml}$) in a 0.2 M bicarbonate buffer, pH 9.6, overnight at 4°C . The plates were washed three times with PBS containing 0.05% (vol/vol) Tween 20. To reduce nonspecific binding, the plates were blocked by incubation at 37°C for 1.5 h with

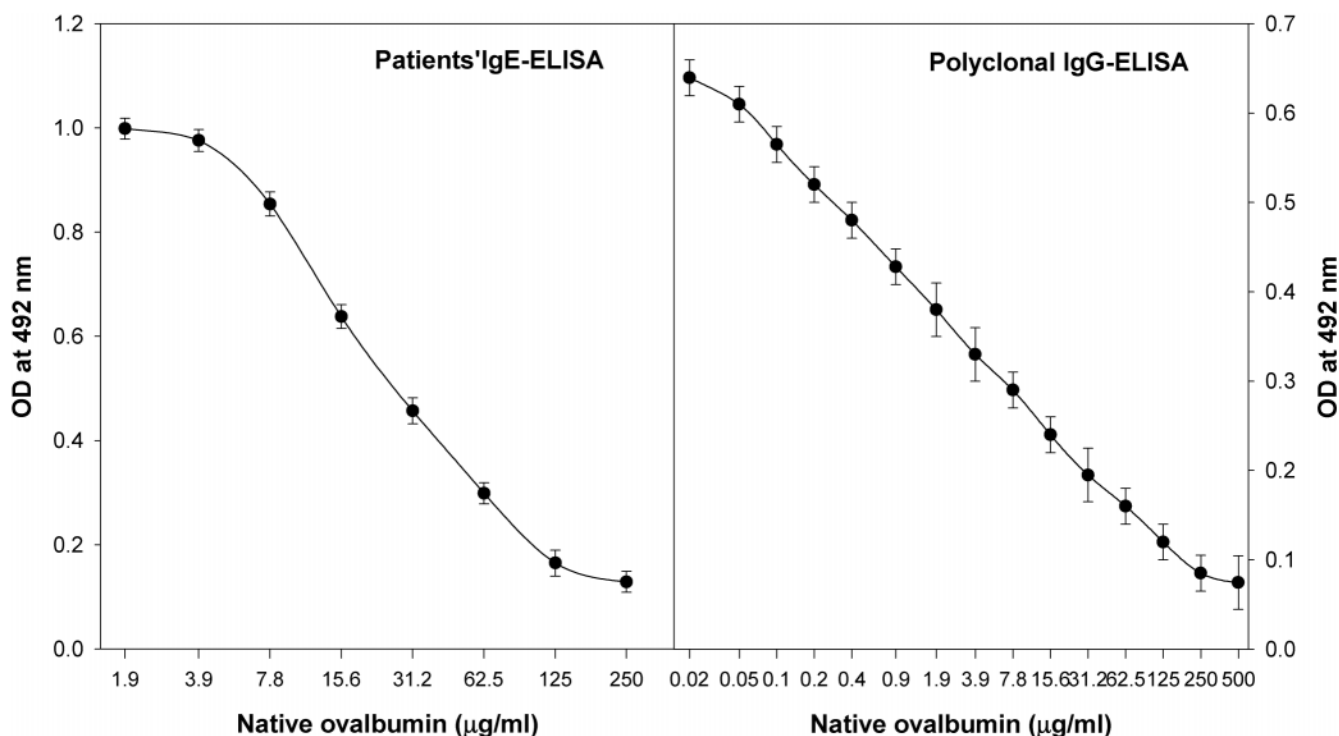


FIGURE 2. Standard curves for quantifying native ovalbumin (OVA). The curves were individually made by Ci-ELISA formatted with egg white–hypersensitive patient IgE and rabbit anti-OVA IgG (polyclonal) for OVA. Standard solutions were prepared by dilution of native OVA (1.0 mg/ml in PBS buffer, pH 7.4).

120 μ l of PBS containing 1% (wt/vol) bovine serum albumin (Sigma, lyophilized powder purified minimum 98%). After washing, 50 μ l of the standard solution or sample solution was added to four coated and blocked wells, and 50 μ l of the IgE solution diluted 25 times in PBS was added. The plates were incubated for 2 h at 37°C and then washed three times with PBS containing 0.05% Tween 20. For the detection of IgE bound to coated OVA, 100 μ l of the biotinylated secondary Ab solution diluted 500 times in PBS was added to the wells and incubated for 1.5 h at

37°C. Also, horseradish peroxidase–conjugated avidin D solution (100 μ l diluted 500 times in PBS) was added in the washed wells and incubated for 1.5 h at 37°C. The plates were then washed, and 100 μ l of the 0.04% *o*-phenylenediamine (Sigma) in a 0.1 M phosphate-citrate buffer, pH 5.0, with 0.04% hydrogen peroxide (vol/vol, 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 μ l per well). The absorbance was measured at 492 nm in an ELISA reader (CERES UV-900C, Bio-Tek Instruments Inc., New York,

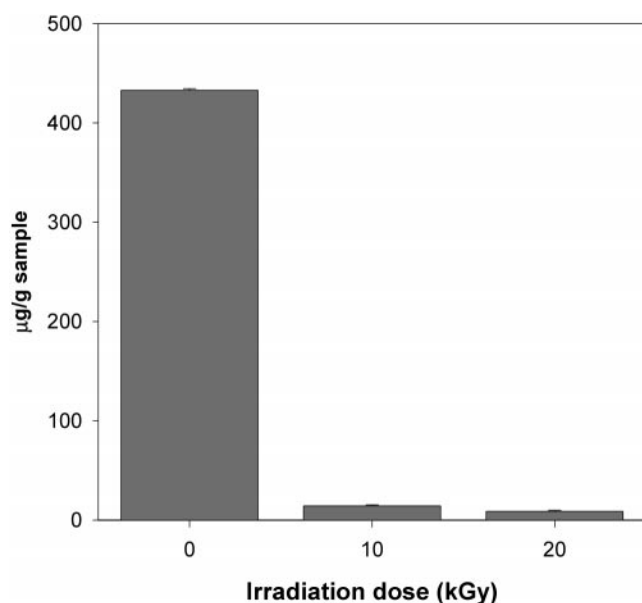


FIGURE 3. Detected concentrations of native ovalbumin in the cakes manufactured with 0-, 10-, and 20-kGy–irradiated egg white by patient IgE-ELISA.

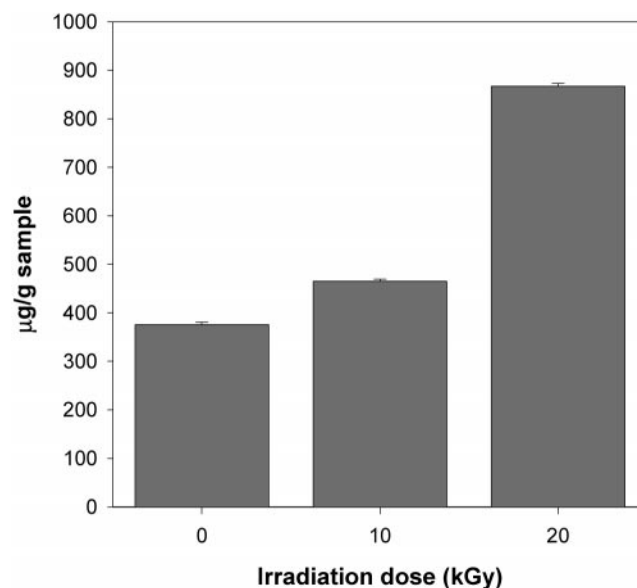


FIGURE 4. Detected concentrations of native ovalbumin in the cakes manufactured with 0-, 10-, or 20-kGy–irradiated egg white by rabbit anti-ovalbumin polyclonal IgG-ELISA.

TABLE 1. Recovery by egg-allergic patient IgE-ELISA and rabbit anti-ovalbumin polyclonal IgG-ELISA

Detection method ^a	Type of Ab	Concentration of added ovalbumin (μg/ml)	Recovery (μg/ml) ^b			Average % recovered ^c	SD ^d
			Expected	Detected	% recovered		
Standard solution	IgE	5	5.0	4.6	92.0	97.6	0.01
		50	50.0	48.1	96.2		
		100	100.0	104.5	104.5		
	IgG	5	5.0	5.1	102.0	98.9	0.02
		50	50.0	48.6	97.2		
		100	100.0	97.5	97.5		
Sample solution	IgE	5	48.2	45.9	95.3	98.6	0.01
		50	93.2	90.8	97.4		
		100	143.2	147.8	103.2		
	IgG	5	42.5	43.9	103.3	101.2	0.01
		50	87.5	91.2	104.2		
		100	137.5	132.3	96.2		

^a Detection of recovery rates for IgE- and IgG-ELISA was performed with blank, instead of standard solution, and in sample solution (extracted cake) with the addition of three concentrations (5.0, 50.0, and 100.0 μg/ml) of native ovalbumin.

^b Recovery was calculated as percentage of the detected concentration to that expected by ELISA.

^c Average is the mean percent recovery rate obtained from three concentrations.

^d Standard deviation ($P < 0.05$).

N.Y.). For standards, a serially diluted allergen solution was prepared at concentrations ranging from 0.12 to 1,000 μg protein per ml. Polyclonal IgG solution and goat anti-rabbit IgG horseradish peroxidase were diluted 2,000 and 20,000 times in PBS, respectively, and used in ELISA formatted with polyclonal IgG against OVA. The concentration of OVA in the sample solutions was calculated with 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 (13). 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 at standard conditions. 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 the 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-squares means, and Duncan's multiple range test (SAS software (21)).

RESULTS AND DISCUSSION

SDS-PAGE profile. The SDS-PAGE profile (5 to 15% gradient gel) of the proteins in the sample solutions is shown in Figure 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 12 to 70 kDa, and they had similar separation patterns. 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 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). OVA could be quantitatively determined at 3.9 to 125 μg/ml by patient IgE and at 0.05 to 250 μg/ml by polyclonal 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-based Ci-ELISA was similar to the previous reports (9, 15), and the titration and affinity of IgE used in this study were not different from those studies.

Changes in binding abilities of patient IgE and polyclonal IgG. Figure 3 shows the detected concentrations of OVA in the sample solutions by Ci-ELISA. In the control, the concentration of OVA was 432.88 μg/g. Native allergen considerably decreased in the cakes with irradiated egg white, and the concentration of OVA was 14.27 μg/g in the 10-kGy sample and 8.78 μg/g in the 20-kGy sample ($P < 0.05$). The content rate of the native OVA decreased to about 96% by 10 kGy of irradiation. Even though significant differences between the 10-kGy and the 20-kGy samples did appear ($P < 0.05$), the differences were not large. This result showed that the ability of IgE to bind native OVA was reduced by irradiation and is supported by the previous reports of Kim et al. (9) and Lee et al. (15). They reported that the ability of IgE to bind OVA that was

reduced by irradiation was not changed by heating. However, native OVA in the cake was not perfectly eliminated by the 20-kGy dose of irradiation. Meanwhile, Taylor et al. (23) reported that 0.13 mg of raw whole egg and 10 mg of cooked egg white for double-blind placebo-controlled food challenge with lowest provoking dose could induce an allergic response. Assuming that an average piece of cake is more than 100 g, the amount of irradiation-treated material in the allergic reduced cake is too close to the lowest provoking dose cited in Taylor et al. (23), and the allergic individual could respond to the material. Therefore, more research toward eliminating the ability of IgE to bind the allergens is needed.

The concentration of OVA in the sample solution was also determined by polyclonal IgG (Fig. 4). Binding reaction of polyclonal IgG to OVA increased with dependence on the irradiation dose. In controls, the concentration of OVA was 375.46 µg/g; however, 465.27 and 867.32 µg/g were determined in 10- and 20-kGy samples, respectively. These determined concentrations indicated that the binding abilities of polyclonal IgG increased 124% in the 10-kGy sample and to 231% in the 20-kGy sample. This result suggests that polyclonal IgG recognized epitopes in the inner part of native OVA that were exposed by irradiation and heating and is supported by previous studies (2, 9, 17) that reported that Ab immune reactions increased because irradiation exposed hidden epitopes by cleaving and unfolding the structure. The conformational change of physicochemically treated Ag (protein) with the use of Ab was also measured in several studies (6, 13, 18). Polyclonal IgG can be used effectively to monitor structural modifications of target allergens and antigens when studying how to reduce or eliminate allergenicity and antigenicity. However, The result obtained from polyclonal IgG should be compared with that of patient IgE because the changes in allergenicity (Fig. 3) and antigenicity (Fig. 4) of the target material are different, even when using the same treatments and processes.

Table 1 indicates the precision of the ELISAs by recovery tests with standard and sample solutions. Recovery rates of IgE-ELISA and IgG-ELISA were 97.6 and 98.9%, respectively, in intra- and interassays. When native OVA was spiked, the concentrations detected by both ELISAs differed by less than 5%. Kim (8) and Lee (13) 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 reliable.

The results obtained from this study present practical data for use in reducing allergenicity of an egg white allergen by irradiation.

ACKNOWLEDGMENT

This project has been carried out under the Nuclear R&D Program by the Korea Ministry of Science and Technology.

REFERENCES

- Byun, M. W., J. H. Kim, J. W. Lee, J. W. Park, C. S. Hong, and I. J. Kang. 2000. Effects of gamma irradiation on the conformational and antigenic properties of a heat-stable major allergen in brown shrimp. *J. Food Prot.* 63:940–944.
- Byun, M. W., J. W. Lee, H. S. Yook, C. Jo, and H. Y. Kim. 2002. Application of gamma irradiation for inhibition of food allergy. *Radiat. Phys. Chem.* 63:369–370.
- Heukeshoven, J., and R. Dernick. 1985. Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* 6:103–112.
- Holen, E., and S. Elsayed. 1990. Characterization of four major allergens of hen egg-white by IEF/SDS-PAGE combined electrophoretic transfer and IgE-immunoautoradiography. *Int. Arch. Allergy Appl. Immunol.* 91:136–141.
- Jeon, G. R., J. W. Lee, M. W. Byun, and S. Y. Lee. 2002. Reduced allergenicities of irradiated egg white ovalbumin determined by skin prick test and ELISA inhibition test. *J. Asthma Allergy Clin. Immunol.* 22:711–719.
- Kaminogawa, S., M. Shimizu, A. Ametani, M. Hattori, O. Ando, S. Hachimura, Y. Nakamura, M. Totsuka, and K. Yamauchi. 1989. Monoclonal antibodies as probes for monitoring the denaturation process of bovine β-lactoglobulin. *Biochim. Biophys. Acta* 998:50–56.
- Katial, R. K., T. J. Grier, D. M. Hazelhurst, J. Hershey, and R. J. M. Engler. 2002. Deleterious effects of electron beam radiation on allergen extracts. *J. Allergy Clin. Immunol.* 110:215–219.
- Kim, J. B. 1983. Development of chemiluminescence immunoassay for the measurement of plasma steroid and urinary steroid glucuronides. Ph.D. thesis. University of London, London, UK.
- Kim, M. J., J. W. Lee, H. S. Yook, S. Y. Lee, M. C. Kim, and M. W. Byun. 2002. Changes in the antigenic and immunoglobulin E-binding properties of hen's egg albumin with the combination of heat and gamma irradiation treatment. *J. Food Prot.* 65:1192–1195.
- Kume, T., and T. Matsuda. 1995. Changes in structural and antigenic properties of proteins by radiation. *Radiat. Phys. Chem.* 46:225–231.
- Kyzlink, V. 1990. Principles of food preservation. *Dev. Food Sci.* 22:337–355.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.
- Lee, J. W. 1999. Application of immunoassay for monitoring conformational changes of myosin under physicochemical treatment. Ph.D. thesis. Konkuk University, Seoul, Korea.
- Lee, J. W., J. H. Kim, H. S. Yook, K. O. Kang, S. Y. Lee, H. J. Hwang, and M. W. Byun. 2001. Effects of gamma radiation on the allergenic and antigenic properties of milk proteins. *J. Food Prot.* 64:272–276.
- Lee, J. W., K. Y. Lee, H. S. Yook, S. Y. Lee, H. Y. Kim, C. Jo, and M. W. Byun. 2002. Allergenicity of hen's egg ovomucoid gamma irradiated and heated under the different pH conditions. *J. Food Prot.* 65:1196–1199.
- Lee, J. W., J. H. Seo, Y. H. Kim, J. M. Choi, H. S. Yook, H. J. Ahn, and M. W. Byun. 2003. Quality properties of cakes containing gamma-irradiated egg white. *J. Korean Soc. Food Sci. Nutr.* 32:311–314.
- Lee, J. W., H. S. Yook, K. H. Cho, S. Y. Lee, and M. W. Byun. 2001. The changes of allergenic and antigenic properties of egg white albumin (Gal d I) by gamma irradiation. *J. Korean Soc. Food Sci. Nutr.* 30:500–504.
- Lee, J. W., H. S. Yook, K. H. Lee, J. H. Kim, W. J. Kim, and M. W. Byun. 2000. Conformational changes of myosin by gamma irradiation. *Radiat. Phys. Chem.* 58:271–277.
- Mine, Y. 1995. Recent advances in the understanding of egg white protein functionality. *Trends Food Sci. Technol.* 6:225–231.
- Sajdok, J., P. Rauch, E. Paluska, and J. Kas. 1990. Determination of egg and egg white content of food products by means of immunochemical assessment of ovalbumin. *J. Sci. Food Agric.* 53:253–259.
- SAS Institute. 1988. SAS/STAT[®] user's guide, release 6.03 edition. SAS Institute Inc., Cary, N.C.
- Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76–85.

23. Taylor, S. L., S. L. Hefle, C. Bindslev-Jensen, S. A. Bock, A. W. Burks, L. Christie, D. J. Hill, A. Host, J. O'B. Hourihane, G. Lack, D. D. Metcalfe, D. A. Moneret-Vautrin, P. A. Vadas, F. Rance, D. J. Skrypec, T. A. Trautman, I. M. Yman, and R. S. Zeiger. 2002. Factors affecting the determination of threshold doses for allergenic foods: how much is too much? *J. Allergy Clin. Immunol.* 109:24–30.
24. Yunginger, J. W. 1997. Food antigens, p. 50. *In* D. D. Metcalfe, H. A. Sampson, and R. A. Simon (ed.), *Food allergy: adverse reactions to food and food additives*. Blackwell Scientific Publications, Boston.