

## Research Note

# Allergenicity of Hen's Egg Ovomucoid Gamma Irradiated and Heated under Different pH Conditions

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## ABSTRACT

This study was conducted to evaluate the effect of a treatment combining gamma radiation and heating on the allergenic properties of hen's egg ovomucoid (OM) under basic pH conditions. OM solutions of 2.0 mg/ml with pHs of 7.0, 9.0, and 10.0 were gamma irradiated at 10 kGy, heated at 100°C for 15 min, or both. Half of the treated pH 10.0 sample solution was restored to pH 7.4 by dialysis. OM solutions were tested by a competitive direct enzyme-linked immunosorbent assay formatted with immunoglobulin E from egg-hypersensitive patients. An equation was obtained for quantifying intact OM from the standard curve, and the detected concentration of intact OM was calculated. The concentration of intact OM decreased with irradiation or heating, and the rate of the decrease was higher for a basic pH condition than for the physiological condition. The combination of irradiation and heating was very effective in reducing the amount of intact OM regardless of the pH condition. After treatment, the restoration of the pH to 7.4 did not affect the concentration of OM. The results of this study indicate that a combination of irradiation and heating might be an effective method for reducing egg hypersensitivity resulting from OM.

The egg is one of the most frequently encountered allergenic foods, and the consumption of minute amounts of egg can result in symptoms within minutes, including life-threatening anaphylaxis (12, 20). Egg white appears to be more allergenic than egg yolk. Among the egg white proteins, ovalbumin and ovomucoid (OM) have been recognized as major allergens (7). OM is a glycoprotein with a molecular weight of 28 kDa, consisting of three well-separated domains. OM is relatively unaffected by heating at 100°C under acidic and neutral pH conditions ( $\text{pH} \leq 7$ ); however, it is easily denatured by heating at  $<80^\circ\text{C}$  under basic conditions ( $\text{pH} \geq 9$ ) (1).

Meanwhile, conformational changes in food allergens brought about by gamma irradiation were recently observed in several studies (3, 5, 10, 13), and such changes could reduce allergenicity or antigenicity by the modification and/or destruction of immunoglobulin E (IgE) epitopes that bind to food proteins. Unfortunately, Byun et al. (4) observed that the antigenicity of OM was not easily changed by gamma irradiation at pH 7.4.

We hypothesized that gamma irradiation and heating under basic pH conditions ( $\text{pH} \geq 9$ ) would reduce the allergenicity of OM. This study was conducted to evaluate the effectiveness of gamma radiation in reducing egg aller-

gies by observing changes in the amounts of intact OM in samples treated with radiation, heating, or both under neutral and basic pH conditions.

## MATERIALS AND METHODS

**Proteins and antibodies.** Purified chicken egg white OM (type III-O, Sigma T2011) was purchased from Sigma Chemical Co. (St Louis, Mo.). Human sera used in previous studies (5, 14) were also used. Sera were obtained from 22 patients less than 3 years of age (15 boys and 7 girls) diagnosed with IgE-mediated egg allergies on the basis of open egg challenges and/or convincing histories of acute urticaria or diarrhea after egg ingestion. Fifteen patients had atopic dermatitis and/or acute urticaria, five patients had skin and gastrointestinal symptoms (diarrhea and vomiting), and two patients had only chronic diarrhea. All patients showed elevated egg-specific IgE (0.7 to 100 kU/liter), as measured by AlaSTAT RIA (DPC Co., Los Angeles, Calif.) or Pharmacia CAP System FEIA (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). The sera were pooled to determine the ability of IgE to bind to treated OM. Ten age-matched subjects provided control sera. None of the control patients had food allergies or any other atopy-related skin, respiratory, or gastrointestinal symptoms. Horseradish peroxidase conjugated rabbit anti-human IgE IgG was purchased from Sigma Chemical Co. for use as a secondary antibody with a chromogenic enzyme.

**Sample preparation and treatment.** OM was individually dissolved in 0.01 M sodium phosphate-buffered saline (PBS) with 0.15 M NaCl at pHs of 7.4, 9.0, and 10.0 and adjusted to a final

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concentration of 2.0 mg/ml with buffers of the same pHs. Each solution was divided into four portions: one portion underwent no treatment, one portion was subjected to irradiation, one portion was subjected to heating, and one portion was subjected to irradiation followed by heating. Each solution (10 ml) was put into a glass tube (diameter, 1.0 cm, glass thickness, 1 mm) with a cap and treated.

Gamma irradiation was carried out at an absorbed dose of 10 kGy at  $10 \pm 0.5^\circ\text{C}$  in a cobalt-60 irradiator (IR-79, Nordion International Ltd., Ontario, Canada) with an activity of 3.7 PBq (100 kCi). The dose rate, determined by Fricke dosimetry, was 10 kGy/h (8).

Heating was done in boiling water for 15 min. After heating, tubes were cooled in iced water for 15 min and then stored at  $4^\circ\text{C}$ .

For restoration of the pH of the basic (pH 10.0) solution to 7.4, half (5 ml) of the solutions treated as described above were put into a dialysis tube (pore size,  $<12,600$  Da; Spectrum Medical Industries, Inc., Houston, Tex.) and dialyzed in 2 liters of 0.01 M PBS (pH 7.4) overnight at  $4^\circ\text{C}$  (the buffer was changed twice). The protein concentration of the dialyzed solution was determined by an absorbance test at 280 nm and with a bicinchoninic acid protein assay kit (Sigma) with bovine serum albumin solution as a standard at 562 nm with a spectrophotometer (UV-1600PC, Shimadzu Corp., Kyoto, Japan) by the method described previously (19).

**Ci-ELISA.** A competitive indirect enzyme-linked immunosorbent assay (Ci-ELISA) was formatted with patients' IgE to evaluate the ability of IgE to bind to samples. For this evaluation, a slightly modified version of the method reported previously (14) was used. Briefly, polystyrene flat-bottom microtiter plates (Maxisorp, Nunc, Kamstrup, Denmark) were coated with 100  $\mu\text{l}$  of intact OM solution (10.0  $\mu\text{g}/\text{ml}$ ) in a 0.2 M bicarbonate buffer (pH 9.6) overnight at  $4^\circ\text{C}$ . All subsequent steps were carried out at  $37^\circ\text{C}$ . Plates were washed three times with PBS containing 0.05% (vol/vol) Tween 20 (PBST). To reduce nonspecific binding, the plates were blocked by incubation for 1.5 h with 120  $\mu\text{l}$  of PBS containing 1% (wt/vol) bovine serum albumin. After washing, 50  $\mu\text{l}$  of standard solution or sample solution was added to four coated and blocked wells, and then 50  $\mu\text{l}$  of IgE solution that had been diluted 25 times in PBS was added. The plates were incubated for 2 h and then washed three times with PBST. After the addition of 100  $\mu\text{l}$  of secondary antibody solution to the wells, the plates were incubated for 1.5 h. The plates were then washed, and 100  $\mu\text{l}$  of 0.04% *o*-phenylenediamine (Sigma) in 0.1 M phosphate-citrate buffer (pH 5.0) with 0.04% (vol/vol) hydrogen peroxide (35%  $\text{H}_2\text{O}_2$ ) was added for color reaction for 20 min before the reaction was stopped with 2.0 M  $\text{H}_2\text{SO}_4$  (50  $\mu\text{l}$  per well). The absorbance was measured at 492 nm by an ELISA reader (CERES UV-900C, BIO-TEK Instruments Inc., New York, N.Y.).

For a standard solution, serially diluted intact OM solution was prepared at concentrations of 1,000 to 0.12  $\mu\text{g}/\text{ml}$ , and sample solutions were individually diluted to 20.0  $\mu\text{g}/\text{ml}$  in PBS. The concentration of intact OM in a diluted sample solution was calculated by an equation obtained from the standard curve. The standard curve was prepared according to the reaction of IgE to intact OM for Ci-ELISA as discussed above.

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).** SDS-PAGE (5 to 15% gradient gel) for sample solutions (2.0 mg/ml) was performed by Laemmli's method (11) with a Hoefer SE-600 vertical electrophoresis apparatus (Pharmacia Biotech, Uppsala, Sweden), and a previously described staining and destaining method (6) was used. A molecular

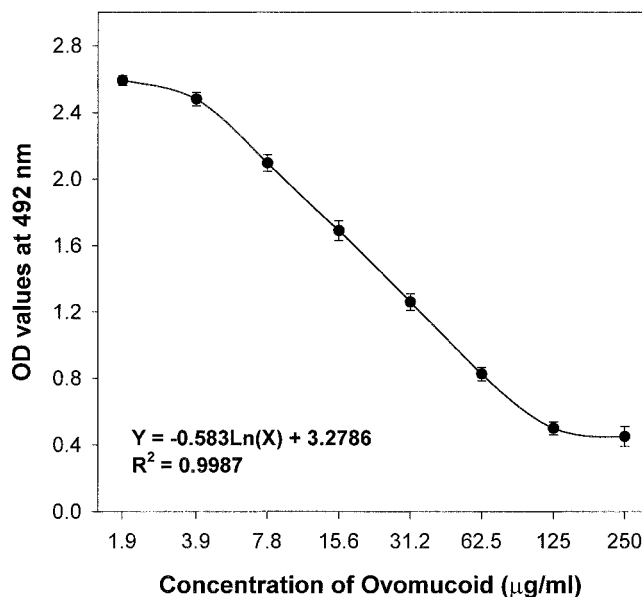


FIGURE 1. Standard curve for the quantification of intact OM. The curve was obtained by Ci-ELISA formatted with the IgE of egg white-hypersensitive patients. The standard solution was prepared by diluting intact OM (2.0 mg/ml in PBS buffer, pH 7.4). The equation shown was used to determine the amount of intact OM recognized by IgE in a sample solution. X and Y are the concentration of OM and the optical density value obtained from the immune reaction of IgE and OM coated onto microwells, respectively.

weight marker was purchased from Bio-Rad Laboratories (Hercules, Calif.) to determine molecular weights.

**Statistical analysis.** All experiments were done in triplicate and repeated five times. The means and standard errors were used to evaluate the differences in the concentrations of intact OM as detected by Ci-ELISA. The data were analyzed by general linear procedures, least-squares means, and Duncan's multiple-range test. SAS software (18) was used for the analysis.

## RESULTS AND DISCUSSION

**Standard curve for quantification of OM.** A standard curve was obtained for the quantification of the OM in a sample solution (Fig. 1). The curve, which took the form of a sigmoid line, could generally be obtained from a semi-logarithmic function, as discussed in several reports (3, 13, 14). Intact OM could be quantitatively determined in the range of 3.9 to 125  $\mu\text{g}/\text{ml}$ , and the concentration of OM could be calculated by the equation  $X = e^{((3.2786 - Y)/0.538)}$ , obtained from the curve, where X is the concentration of OM in a sample solution and Y is the average optical density value of the microwells of a sample solution. This equation was used to quantify intact OM in sample solutions treated by irradiation, solutions treated by heating, and solutions treated by irradiation followed by heating.

**Changes in detected concentrations.** Table 1 shows the concentrations of intact OM in sample solutions detected by Ci-ELISA. These concentrations were not changed by an increase in pH. The OM was denatured and the detected concentration was decreased by irradiation at 10 kGy or heating at  $100^\circ\text{C}$ . However, irradiation was more

TABLE 1. Detected concentrations of OM treated with radiation at 10 kGy, heating at 100°C for 15 min, or both under different pH conditions as detected by Ci-ELISA formatted with the IgE of egg white-hypersensitive patients

| Treatment <sup>a</sup>               | Concentration (μg/ml) for buffer pH |        |        |                             |
|--------------------------------------|-------------------------------------|--------|--------|-----------------------------|
|                                      | 7.4                                 | 9.0    | 10.0   | 7.4 (restored) <sup>b</sup> |
| No treatment                         | 10.36                               | 10.20  | 9.44   |                             |
| Irradiation                          | 3.56 A <sup>c</sup>                 | 2.76 B | 0.78 C | 0.81 C                      |
| Heating                              | 8.22 A                              | 4.37 B | 2.16 C | 2.13 C                      |
| Irradiation and heating <sup>d</sup> | 0.008                               | 0.010  | 0.009  | 0.008                       |

<sup>a</sup> Samples were prepared at a concentration of 2.0 mg/ml with different pHs (7.4, 9.0, and 10.0) and treated. The treated solutions were individually diluted to 20 μg/ml with buffers of the same pHs and tested.

<sup>b</sup> pH 10.0 samples were restored to pH 7.4 by dialysis after treatment, and the concentration of the solution was diluted to 20 μg/ml with a buffer of pH 7.4.

<sup>c</sup> Values with the same letter in the same row are not significantly different ( $P < 0.05$ ).

<sup>d</sup> After irradiation, samples were heated at 100°C for 15 min, cooled, and tested.

effective than heating in reducing intact OM ( $P < 0.05$ ). OM has been recognized as a heat-stable allergen (15–17), and our results indicate that OM can withstand heating at pH 7.4. The combination of irradiation and heating was a very efficient method for decreasing the amount of intact OM regardless of the pH condition. The detected allergen concentration decreased by 0.008 to 0.010 μg/ml with the combination treatment. This amount was about 0.001 times that for no treatment at pH 7.4 (10.36 μg/ml). Several clinical studies have shown that the reduction or inhibition of allergy expression can be expected when the amount of intact allergen might decrease 0.001- to 0.0001-fold (7, 22).

A pH increase in the OM solution could not change the reactivity of IgE to OM. Intact OM was more easily

denatured with irradiation or heat treatment under basic pH conditions than under physiological conditions. However, an obvious decrease in intact OM was not obvious for two individual treatments, even though the pH of the solution was 10.0. In a previous report, Byun et al. (4) reported that OM stood up well to gamma irradiation. Heating alone could not be expected to produce a clinically significant reduction in the allergenicity of OM under the basic pH conditions, even though the structural cleavage (conformational denaturation) of the OM was brought about (2, 16, 21).

When the pH of the basic solution (pH 10.0) was restored to the physiological condition, the allergenicity of OM did not revert to the original state; the decreased state was maintained. This result shows that conformational IgE epitopes that resulted from the pairing of structurally adjacent amino acids in domains I and II or in domains II and III in native OM were cleaved or destroyed in the basic condition and were not recovered; that is, the denaturation of OM in the basic condition was irreversible, and the adjustment of pH might be effectively used in the processing of foods, including egg whites, to reduce allergenicity. Besler et al. (2) and Mine and Zhang (15) reported that the binding activity of a patient's IgE for domain III was higher than its binding activity for other domains. These authors also reported that the amino acids within the binding IgE epitope of domain III were important in the allergenic reaction of OM. The combination treatment might mainly involve the destruction of the linear IgE epitope in domain III.

**SDS-PAGE.** An electrophoretogram for OM solution irradiated in the basic condition is shown in Figure 2. The band of about 28 kDa disappeared in irradiated samples. Traces spread from the starting line of the separating gel were observed in the sample irradiated at pH 7.4, and traces were below the starting line at <150 kDa for pHs of 9.0 and 10.0. By gamma irradiation, a protein molecule can be broken down into smaller molecules or coagulated into larger molecules by molecule interactions (3, 9, 13). OM molecules were coagulated into very large aggregates by gamma irradiation in the presence of oxygen. An increase in pH might induce the generation of smaller aggregates

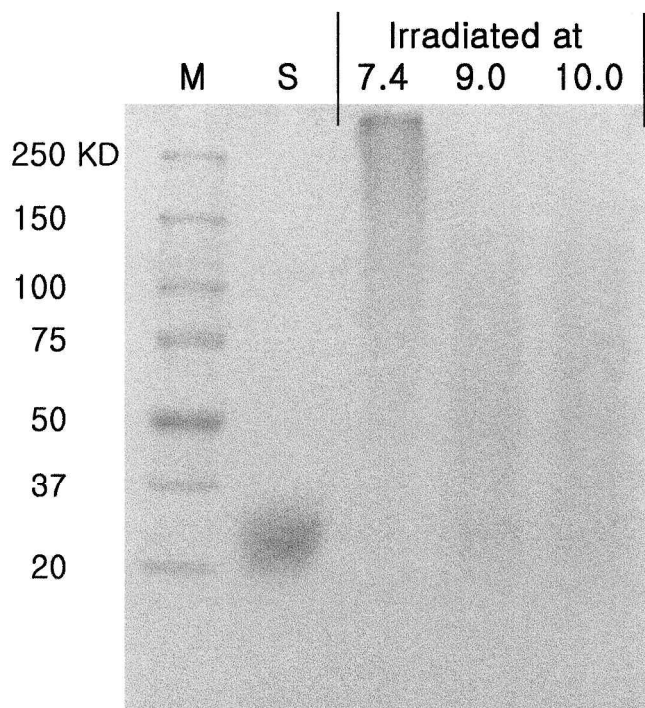


FIGURE 2. SDS-PAGE of OM in gamma-irradiated (10 kGy) solutions at different pHs. M, molecular weight standard marker; S, intact OM at pH 7.4; 7.4, OM irradiated at pH 7.4; 9.0, OM irradiated at pH 9.0; 10.0, OM irradiated at pH 10.0. Numbers on the left are the molecular weights (kDa) of the standard marker.

with wide molecular weight ranges by the hydroxyl group in the basic condition instead of the generation of a new heavy molecular weight in the neutral condition. Data obtained from this study suggest that the irradiation processing techniques available for the reduction of egg allergenicity can be used to treat raw materials in processed foods.

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