



## Short communication

Changes in the antigenicity and allergenicity of ovalbumin in chicken egg white by *N*-acetylglucosaminidaseHo-Young Park<sup>a</sup>, Taek Joon Yoon<sup>b</sup>, Ha Hyung Kim<sup>c</sup>, Young Shin Han<sup>d</sup>, Hee-Don Choi<sup>e,\*</sup><sup>a</sup> Division of Functional Food Research, Korea Food Research Institute, Gyeonggi 463-746, Republic of Korea<sup>b</sup> Department of Food & Nutrition, Yuhan College, Bucheon 422-749, Republic of Korea<sup>c</sup> College of Pharmacy, Chung-Ang University, Seoul 156-756, Republic of Korea<sup>d</sup> Environmental Health Center for Atopic Diseases, Samsung Medical Center, Seoul, Republic of Korea<sup>e</sup> Division of Strategic Food Research, Korea Food Research Institute, Gyeonggi 463-746, Republic of Korea

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## ABSTRACT

Ovalbumin (OVA), an (hen) egg allergen, is one of the most abundant glycoprotein allergens associated with IgE-mediated hypersensitivity through the T-helper type 2 immune response. The effect of deglycosylation of the *N*-terminal glycan in OVA on allergenicity and antigenicity after *N*-acetylglucosaminidase treatment was studied. *N*-acetylglucosaminidase-treated OVA (N-OVA) evaluated using an enzyme-linked immunosorbent assay, respectively. N-OVA significantly ( $p < 0.05$ ) OVA-specific IgE and histamine levels. In addition, N-OVA decreased the antigenicity of OVA 1000-fold. These results suggest that the degree of allergenicity and antigenicity reduced with deglycosylation of *N*-terminal glycan in OVA.

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## 1. Introduction

Egg whites isolated from hens' eggs are widely utilized in food processing because of their functional properties, specifically gel and foam formation. Furthermore, egg white proteins, such as ovalbumin (OVA; Gal d2, 54%), ovomucoid (Gal d1, 11%), ovotransferrin, conalbumin (Gal d3, 12%), and lysozyme (Gal d4, 3.4%), are a low-calorie source of complete high-quality protein (Lin, Wu, Huang, Cheng, & Yeh, 2016).

OVA, a 45-kDa glycoprotein with 385 amino acids and 3% carbohydrate content, (Nisbet, Saundry, Moir, Fothergill, & Fothergill, 1981), has been described as one of the major allergens in egg whites (Abeyrathne, Lee, & Ahn, 2013). Many immunoglobulin E (IgE) and immunoglobulin G (IgG) binding epitopes present on OVA have been identified in serum from patients with food allergies (Caubet et al., 2012).

As a result, egg white utilization is limited in infants and young children diets because of its high antigenicity (Eggesbo, Botten, Halvorsen, & Magnus, 2001; Sicherer & Sampson, 2006). It has been

reported that egg allergy affects 0.5–2.5% of young children (Rona et al., 2007) and is closely associated with atopic dermatitis (Niggemann, Sielaff, Beyer, Binder, & Wahn, 1999).

Most cases of OVA allergies are mediated by IgE antibodies (Caubet & Wang, 2011). Allergic responses are initiated by the binding of an antibody to an epitope. Several attempts have been made to remove or modify the antigenic determinant by heat treatment (Joo & Kato, 2006), enzymatic hydrolysis (Lopez-Exposito et al., 2008), and glycation (Zhang et al., 2014). Modification with terminal saccharides through deglycosylation and glycation seems to be a promising and safe method for attenuating glycoprotein-induced food allergenicity. Enzymatic deglycosylation is useful for cleaving the glycan epitope. Furthermore, many studies have reported beneficial effects associated with deglycosylation, including stability (Jafari-Aghdam, Khajeh, Ranjbar, & Nemat-Gorgani, 2005) and bioavailability (Park, Choi, Eom, & Choi, 2013).

This study compared the allergenicity of OVA and *N*-acetylglucosaminidase-treated OVA (N-OVA) by measuring histamine release and IgE levels using an antigen-sensitized mouse model. We also estimated antigenicity with a competitive indirect enzyme linked immunosorbent assay (ciELISA) using anti-OVA sera of rabbit and children.

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## 2. Materials and methods

### 2.1. Preparation of OVA and N-OVA

Hen egg white protein, OVA, was prepared as described by Roy, Rav Mv Fau, Gupta, and Gupta (2003) and further purified using cation-exchange chromatography after precipitating the unbound fraction with 5% trichloroacetic acid. The purity of OVA was 95%. The deglycosylated form of OVA was prepared as follows: OVA was incubated with 0.25 U/ml *N*-acetylglucosaminidase in 50 mM sodium phosphate buffer (pH 6.0) for 18 h at 37 °C. The reacted enzyme and released *N*-acetylglucosamines were separated from incubated buffer solution using an Amicon Ultra-4 unit with a 100-kDa cut off (Millipore, Billerica, USA), and then dialyzed against distilled water for 24 h to remove the buffer solution. The total protein concentration in each step was determined using a bicinchoninic acid assay kit (iNtRON Biotechnology, Seoul, Korea) with bovine serum albumin as a standard.

### 2.2. Animals and sensitization

Five-week-old female BALB/c mice weighing  $20 \pm 2$  g were purchased from SaeronBio Inc. (Gyeonggi, Korea), and grouped 12 mice per group. The animals were housed in an air-conditioned room ( $23 \pm 2$  °C, 12/12 h light/dark cycle) and allowed free access to food and tap water. All animal experiments were performed following established guidelines and the experimental protocol (KFRI-M-14013) in accordance with the Animal Care and Use Committee of Korea Food Research Institute.

As described in Fig 1A, mice were sensitized with 5 µg of OVA or N-OVA adsorbed in 2 mg/ml Imject Alum (Pierce, Rockford, USA) by intraperitoneal (i.p.) injection on days 7, 21, and 28. The normal control group received an i.p. injection of a 0.9% saline solution on days 7, 21, and 28.

### 2.3. Measurement of OVA-specific IgE and histamine levels

Seventy-two hours after the third sensitization, blood was sampled from the postcaval vein of each mouse to estimate the serum levels of IgE and histamine using a sandwich enzyme-linked immunosorbent (ELISA) assay kit (BD Biosciences, San Diego, USA) according to the manufacturer's protocols. Absorbance at 450 nm was measured in a microplate reader (EL 800, BioTek Instruments, Winooski, USA).

### 2.4. Serum samples of egg-allergic patients and OVA-sensitized rabbit

The pooled sera were made of equal parts of serum from four egg-allergic children aged 1–5 years whose levels of specific IgE to egg white were above 50 kUA/l (Table 1). The levels of OVA-specific IgE in patient sera were measured a fluorescent enzyme immunoassay (FEIA) method using UniCAP® (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). Egg allergies were diagnosed when the children had a history of aggravation of atopic dermatitis or anaphylaxis shortly after egg ingestion. After informed consent was received, blood samples were obtained and the sera were frozen at  $-80$  °C until use. This study was approved by the Samsung Medical Center Institutional Review Board (2008-11-049).

Sera of OVA-sensitized rabbits were kindly provided by Dr. Shon (Korea Food Research Institute) (Sung et al., 2014).

### 2.5. Competitive indirect ELISA

To evaluate the reduction of OVA allergenicity, a ciELISA was performed with both egg-allergic sera of children and rabbit antiserum to OVA. In brief, a microplate was coated with 100 µl of OVA (2 mg/ml) and incubated at 4 °C overnight. After discarded the coating buffer, different concentrations ( $10^{-5}$ – $10^{-4}$  µg/ml) of N-OVA were mixed with egg-allergic patient sera (diluted 1:100

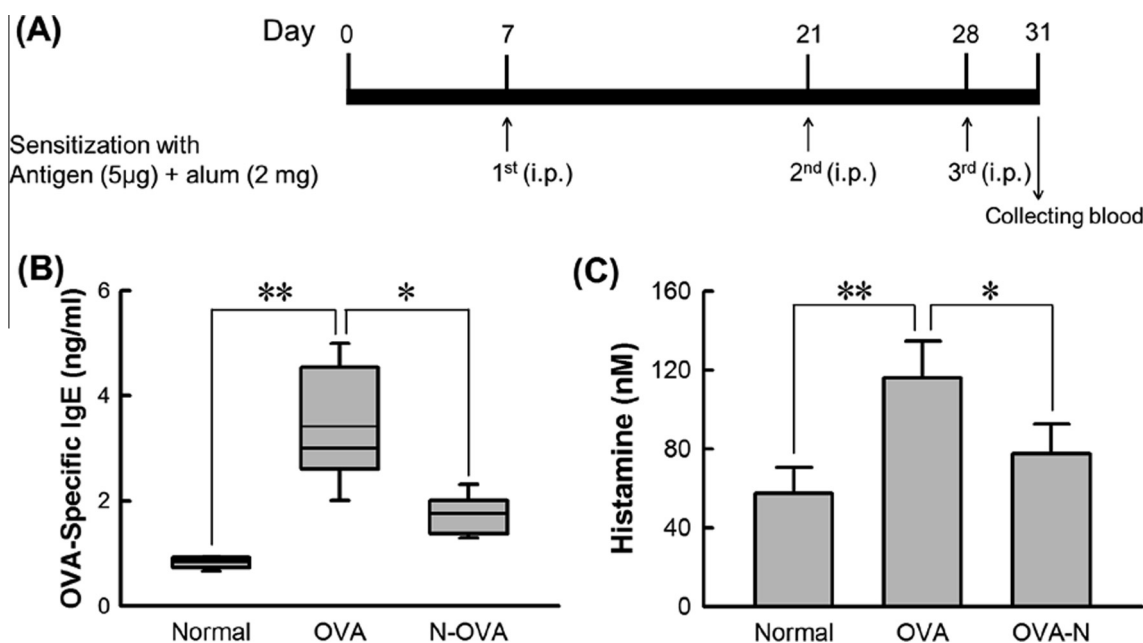


**Fig. 1.** Inhibitory effect of *N*-acetylglucosaminidase-treated ovalbumin (N-OVA) on specific IgE and histamine levels in serum of antigen-sensitized BALB/c mice. (A) Timeline of experimental procedures. (B) OVA-specific IgE levels and (C) histamine release were measured by ELISA. Collected sera were diluted 1:50 (v/v) for OVA-specific IgE detection. OVA-specific IgE and histamine levels are presented as mean  $\pm$  SD ( $n = 6$ , respectively). Data were assessed by ANOVA followed by Turkey's *post hoc* test. \* $p < 0.05$  and \*\* $p < 0.01$  vs. OVA-sensitized group.

**Table 1**

Clinical and immunologic findings of four patients with a convincing history and IgE binding to ovalbumin (OVA).

| Patient NO. | Age (year) | SEX | CAP IgE FEIA OVA (kUA/l) | Symptoms                          |
|-------------|------------|-----|--------------------------|-----------------------------------|
| 7645        | 5          | M   | 78.4                     | Other atopic dermatitis           |
| 2659        | 1          | M   | 95.1                     | Atopic dermatitis, unspecified    |
| 5486        | 3          | M   | 101.0                    | Other adverse food reactions, NEC |
| 2658        | 1          | M   | 101.0                    | Atopic dermatitis, unspecified    |

in PBST) or rabbit anti-OVA sera (diluted 1:50 in PBST) at a 1:1 ratio. The mixture (100  $\mu$ l) was added to each well and the plate was incubated for one hour for induction of competitive binding. After washing, 100  $\mu$ l of goat anti-human IgE-horseradish peroxidase (HRP) (diluted 1:10,000 in PBST) and goat anti-rabbit IgE-HRP diluted (1:3000 in PBST) as secondary antibody were added, followed by incubation for one hour.

The plate was further washed and developed with 100  $\mu$ l of 3, 3',5,5'-tetramethylbenzidine (TMB) substrate solution for 30 min,

and the reaction was stopped by addition of 50  $\mu$ l of 2 M  $H_2SO_4$ . Absorbance was measured at 450 nm in triplicate.

## 2.6. Statistical analysis

Data are expressed as mean  $\pm$  standard deviation (SD) of triplicate determinations, at least. Differences among experimental data were analyzed by an analysis of variance (ANOVA) followed by Tukey's *post hoc* test or Dunnett's multiple range tests using the

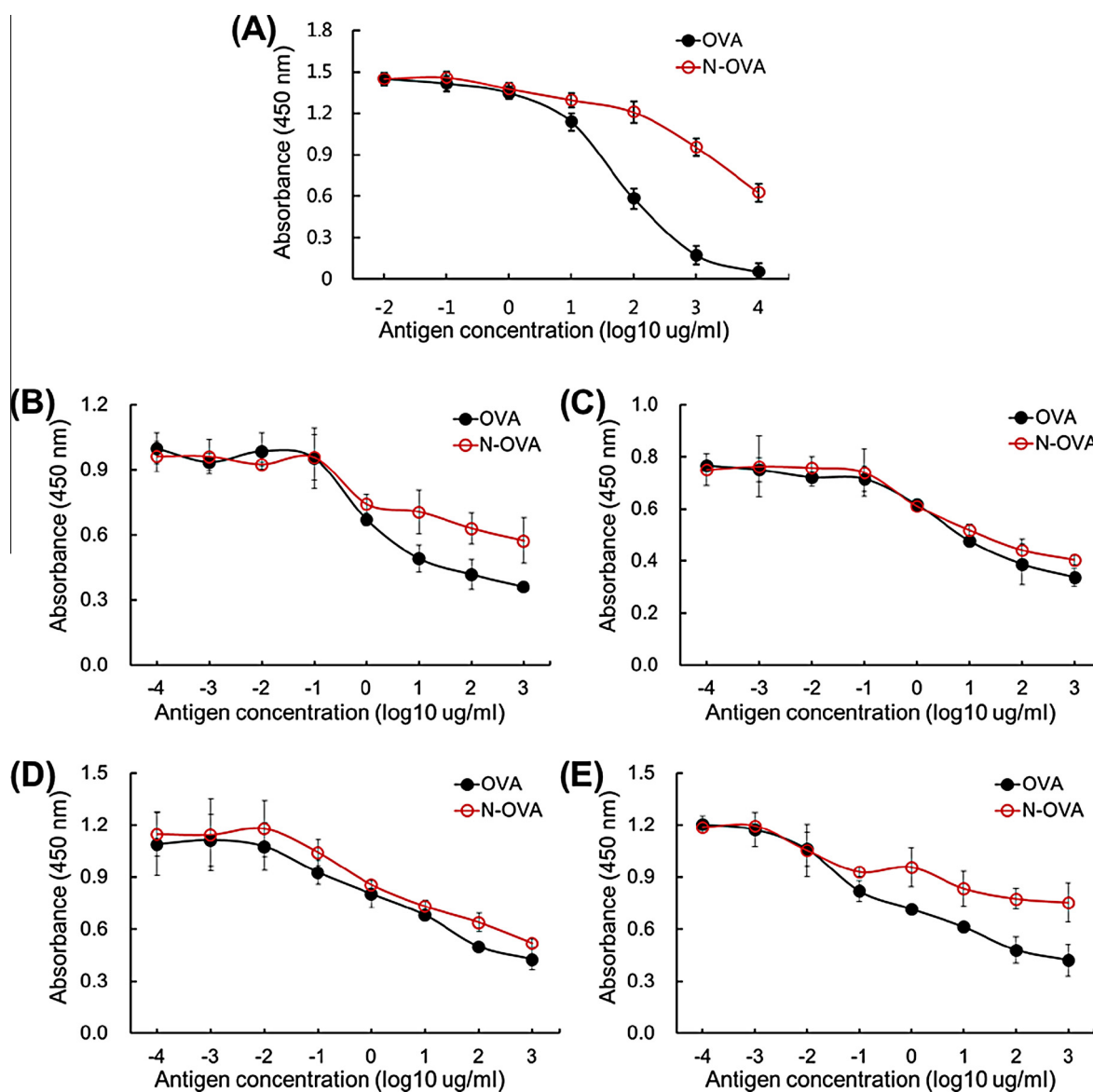


**Fig. 2.** Competitive indirect enzyme-linked immunosorbent assay inhibition analysis for detecting changes in the antigenic activity of ovalbumin (OVA) and N-acetylglucosaminidase-treated OVA (N-OVA) using (A) pooled sera of three sensitized-rabbits and (B–E) four egg-allergic children. Data shown are mean values  $\pm$  SD of three complete sets of experiments.

PASW Statistics 18 software (SPSS Inc., Chicago, USA). Significance was defined at  $p < 0.05$ .

### 3. Results and discussion

Glycan analysis and electrophoretical analysis of OVA and N-OVA confirmed that N-OVA was successfully cleaved at the terminal *N*-acetylglucosamins of OVA, with no accompanying degradation or contamination of the sample solution during the digestion (Hwang et al., 2014). To evaluate the allergenic immune response, we measured OVA-specific IgE levels in serum. As shown in Fig. 1B, OVA-sensitized mice had significantly higher OVA-specific IgE levels than the saline-treated normal control group (4.38 ng/ml vs. 0.86 ng/ml), while the N-OVA sensitized group had significantly lower OVA-specific IgE levels (1.75 ng/ml) than the OVA-sensitized groups. The immune system produces an allergic response upon exposure to an antigen. Thus, compared to the OVA-sensitized mice, those sensitized with N-OVA had significantly reduced OVA-specific IgE by (−60.1%). No significant increases in the serum levels of OVA-specific IgG1 and IgG2a were found when compared to the normal control group (data not shown).

Histamine release of mouse serum increased after sensitization with OVA compared to the normal control group. Histamine release decreased significantly after sensitization with N-OVA, almost to the level of the normal control group (Fig. 1C). N-OVA generally had much lower antigenicity values compared to the antigenicity values of the native OVA glycoprotein.

A ciELISA method was performed to evaluate the change in antigenicity after *N*-glycan hydrolysis with *N*-acetylglucosaminidase (Fig. 2). The antigenicity of N-OVA significantly decreased the antigenicity of OVA 124-fold when measured as  $IC_{50}$  using rabbit anti-OVA sera (Fig. 2A). In addition, we performed ciELISA using (allergic children) anti-serum with OVA to confirm the hypothesis (Fig. 2B–E). A decrease in the OVA antigenic activity was observed in half the OVA-allergenic sera samples from children. The antigenic activity of N-OVA was reduced compared to the antigenic activity of OVA in half the sera samples. This observation is in agreement with our previous study (Hwang et al., 2014) and is presumably caused by the cleavage of the terminal carbohydrate. The antigenicity of hydrolyzed samples significantly decreased, suggesting that the cleavage of *N*-acetylglucosamine might be an important factor to decrease OVA-induced antigenicity. In addition, glycosylation of OVA also decreased OVA antigenicity (Ma, Gao, & Chen, 2013). The reductions in antigenicity are thought to be due to the partial elimination of the exposed epitopes by hydrolysis and glycosylation.

Individuals have an increased risk of egg allergies when their serum concentrations of IgE antibodies specific to egg whites exceeded 7.38 kUA/L (Ando et al., 2008). In this study, allergenicity and antigenicity were reduced after *N*-acetylglucosaminidase treatment. Antigenicity declined by approximately 1000-fold in half of the sera samples from OVA-allergenic children. This may be related to differences in binding specificity of the saccharides to the allergenic sites of the protein. These results indicate that the reduction of OVA antigenicity might depend on a combination of individuality (genetics) and allergic symptom (severity).

In conclusion, the results from this study indicate that N-OVA decreased IgE levels and histamine release in OVA-sensitized BALB/c mice sera, and attenuated the antigenicity of rabbit anti-OVA sera. N-OVA treatment decreased the impact of OVA in egg-allergenic children. Thus, N-OVA might be used as an immunotherapy agent against the OVA-allergen. Moreover, it might also be used to produce non-allergic egg-products, if antigenicity could be eliminated. Further research should be conducted to elucidate what factors are modulated by N-OVA at cell membrane receptor level and control of gene expression of allergenic mediators.

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