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The Influence of the Presence of Wheat Flour on the Antigenic Activities of Egg White Proteins

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Purpose: It is known that ovomucoid, an egg allergen, is heat resistant and remains soluble after heating. However, a recent study showed that the antigenic activity of ovomucoid could be reduced by heating when egg white (EW) was mixed with wheat flour. This study was performed to determine the influence of wheat flour on the antigenic activities of EW proteins when EW is heated, and the influence of the duration of heat treatment. **Methods:** A mixture of EW and wheat flour was kneaded for 10 minutes and then baked at 180°C for 10 minutes and 30 minutes. The EW without wheat flour was also heated at 180°C for 10 minutes and 30 minutes. The proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and IgE immunoblotting was performed with the pooled sera of 5 egg-allergic patients. The antigenic activities of ovomucoid in different EW samples were measured by inhibition enzyme-linked immunosorbent assay (ELISA). **Results:** 1) SDS-PAGE: the intensity of the 37-50 kD bands (overlapped bands of ovomucoid and ovalbumin) decreased significantly in the mixture of EW and wheat flour baked for 30 minutes, compared with the mixture baked for 10 minutes, heated EW and raw EW. 2) IgE immunoblot: in the mixture of EW and wheat, a remarkable decrease of IgE reactivity to 37-50 kD was observed when baked for 30 minutes. 3) Inhibition ELISA: the antigenic activity of ovomucoid decreased significantly in the mixture of EW and wheat baked for 30 minutes, but not in the heated pure EW. **Conclusions:** This study showed that the antigenic activity of ovomucoid can be reduced by baking EW with wheat flour. The decrease in ovomucoid antigenicity in the baked mixture of EW and wheat flour was dependent on the time of heat treatment, indicating that heating should be prolonged to achieve a reduction in ovomucoid antigenic activity.

Key Words: Egg white; wheat flour; hot temperature; ovomucoid

INTRODUCTION

Hen egg is the most frequent cause of food allergy,[1,2] affecting approximately 1.6% of children.[3,4] Egg has been shown to be the most common food allergy in children with atopic dermatitis, and approximately two-thirds of children with atopic dermatitis are reactive to egg white (EW).[5-7] It has been reported that a majority of children develop tolerance within the first five years of life.[2,8] However, recent study has shown that tolerance to egg does not take place as early as was previously thought, and that the majority of children with egg allergy only developed egg tolerance by late childhood.[9]

Heat treatment has been recognized as a simple way of reducing allergenicity.[10] When a protein is denatured and undergoes random-coiled aggregation by heat, it becomes insoluble and most of the original tertiary structure is lost, causing a decrease in allergenicity.[11] Many studies have demonstrated that the allergenicity of EW proteins can be reduced by heating. Heating for 15 minutes at 95°C reduced IgE binding with ovalbumin[12] and ovomucoid in one study and in another EW proteins boiled for 5 or 60 minutes showed a much-decreased allergenicity, especially after prolonged boiling for 60 minutes.[13]

Ovomucoid is considered to be important clinically as a dominant allergen in hen EW. Although ovalbumin is the most abundant protein found in EW, it is sensitive to thermal denaturation, with a resultant decrease in allergenicity.[14,15] In contrast, ovomucoid is known to be heat resistant and is not coagu-

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lable, and therefore remains soluble after heating.[14-17] However, several recent studies have shown that the antigenic activity of ovomucoid can be reduced by heating when EW is mixed with wheat flour. Kato et al.[18] demonstrated a marked decrease in the solubility of ovomucoid when EW was mixed with wheat flour and then heated at 180°C for 10 minutes, mimicking the process of bread making. This decrease in the solubility of ovomucoid could explain why high levels of ovomucoid specific IgE are required to predict reactivity to heated egg in baked products.[19]

Wheat flour-based foods, including bread and cookies, are the most common form of egg ingestion worldwide. This study was therefore performed to determine the influence of the presence of wheat flour on the antigenic activities of EW proteins, especially ovomucoid, when EW is heated, and also to determine the influence of the duration of heat treatment.

MATERIALS AND METHODS

Serum samples of egg-allergic patients

A serum pool was made of equal parts of serum from five egg-allergic children aged 1 to 3 years, whose levels of serum specific IgE to EW were above 10 kU_A/L, with negative wheat-specific IgE and no clinical history of wheat allergy. The levels of serum specific IgE antibodies were measured with UniCAP® (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden). Egg allergy was diagnosed when the children have a history of aggravation of atopic dermatitis or urticaria/angioedma or anaphylaxis shortly after egg ingestion. Oral challenge tests were not performed. 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.

Preparation of samples and allergen extraction

We compared the samples baked for 10 and 30 minutes to ascertain whether or not prolonged heating could reduce the antigenic activity of both EW and EW mixed with wheat flour.

Hen eggs were purchased from a grocery store, stored at 4°C and used in this experiment within two days of purchase. After freeze drying, the preparations were milled into a powder by using a homogenizer. Raw EW was prepared by directly freeze-drying liquid EW before homogenization. Thirty-five gram of raw EW was mixed with 65 g of wheat flour and water. The mixtures of EW and wheat flour were kneaded for 10 minutes and then baked at 180°C for 10 minutes and 30 minutes. EW samples without wheat flour were also heated at 180°C for 10 minutes and 30 minutes. The proteins were extracted from raw EW, heated EW and the mixtures of EW and wheat flour.

EW protein extracts were prepared by adding 10 g of samples to 90 mL of phosphate-buffered saline (PBS) in sterile centrifuge tubes. The mixture was rotated for 90 minutes at 4°C, centrifuged at 9,000 rpm for 20 minutes, and then decanted and placed in sterile tubes. The supernatants were filter-sterilized through 0.45 μm filters (Carriglwohill, Co. Cork, Ireland) and lyophilized. Protein concentrations were determined by using microplate reader (VersaMax) with a Bradford protein assay (Bio-Rad cat #5000006). All extracts were stored at -80°C until use.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The extracts and purified proteins were separated by SDS-PAGE according to modified Laemmli methods. Extracts and proteins were reduced by heating with 4×SDS-PAGE gel loading buffer (LDS sample Buffer, Invitrogen cat. #NP0007; mixed 1:10 with reducing agent, Invitrogen cat #NP0004) and loaded (5 μg) into each well of a gel (4-12%Tris-Glycine gels, 1.0 mm× 10 wells, Novex pre-cast gel; Invitrogen cat #NC0321Box). The gel was electrophorized at 200 V for 35 minutes and strained with Coomassie blue. Precision plus protein standards (Bio-Rad, product #161-0374) were used as molecular weight markers to estimate protein size.

IgE immunoblot anlaysis

Immunoblotting was carried out with the samples subjected to SDS-PAGE followed by electrophoretic transfer to a supported PVDF membrane. IgE immunoblots were then performed with the individual serum of seven egg-allergic patients. Immunoblots were pre-incubated for 1 hour at room temperature with blocking reagent (PBST with 2% non-fat dry milk [NFDM]) and then incubated overnight with allergic and non-allergic sera diluted 1:10 v/v in blocking reagent. Unbound IgE was removed by repeated rinses with PBST. For detection of bound IgE the membranes were incubated with biotin-labeled goat anti-human IgE (KPL; Gaithersburg, MD, USA), followed by washing and then incubation in NeutrAvidin-horseradish peroxidase (HRP; Pierce Biotechnology, Rockford, IL, USA). Detection was achieved using enhanced chemiluminescence (ECL; Amersham, or Supersignal from Pierce), with multiple exposure times per membrane to provide optimal signal to noise ratio on the X-ray films. Blotted membrane was then exposed to High performance chemiluminescence film (GE Healthcare Limited, Buckinghamshire, UK) and the film was then developed. As a control, one membrane was incubated with the secondary antibody and ECL detection, but without human serum to evaluate the specificity of the detection system. Immunoblots were compared with a reflective densitometer (Chemi documentation UVP [DDS-8000]) and Quantity One software (BioRad).

Inhibition enzyme-linked immunosorbent assay (ELISA)

We performed inhibition ELISA with the pooled sera of the seven egg-allergic patients. The egg white proteins were dissolved in deionized water (1 mg/mL for final conc.), and then frozen until required. The OM (OM, Sigma T-2011) diluted (50

μg/mL) in coating buffer (0.05M carbonate-bicarbonate buffer, pH 9.6) was incubated in a 96-well microtiter plate overnight at 4°C to coat the wells. After washing the plate with 0.05% PBS-T (PBS containing 0.05% Tween-20), the plate was blocked with 2% BSA (bovine serum albumin, Carl Roth BmbH + Co.KG, Karlsruhe, Germany) in 0.05% PBS-T for 1 hour at room temperature to avoid nonspecific binding. Preincubation of the EW protein samples or OM with pooled sera was utilized to optimize the competition. The sera were diluted (1:20) with 2% BSA in 0.05% PBS-T. One volume (55 μL) of the pooled sera was added to one volume (55 μL) (vol/vol) of OM or sample extracts that had been previously diluted in assay diluents. The sera-protein mixtures were kept in tubes at 37°C for 1 hour. After washing the plate, the wells were incubated with 100 μL/well of preincubated sera-protein mixtures for 2 hours at 37°C and rinsed with 0.05% PBS-T. Next, the wells were incubated with a peroxidase labeled goat anti-human IgE (Sigma), which was diluted 1:2,500 v/v in blocking buffer, for 1 hour at room temperature. After washing with 0.05% PBS-T, 100 μL of tetramethylbenzidine (TMB) peroxidase substrate (KPL; Gaithersburg, MD, USA) was added. The optical density of each well was measured in a plate reader (VersaMax) at 595 nm.

RESULTS

SDS-PAGE

The intensity of protein fraction at 45.9 kD (ovalbumin) was decreased by heating EW even in the absence of wheat flour and regardless of the time of heat treatment. In SDS-PAGE, the protein fraction of ovomucoid overlaps that of ovalbumin in the range of 34-50 kD, but the strong broad band around 40 kD in the sample of raw EW corresponds mostly to ovomucoid.[15,20] The intensity of broad bands around 40 kD (ovomucoid) was not decreased by heating in the absence of wheat flour, even when EW was heated for 30 minutes (Fig. 1). However, in the presence of wheat flour the intensity of protein fractions around 40 kD was decreased by the heating of EW for 30 minutes. The lysozme fraction (14.4 kD) almost completely disappeared as a result of heating irrespective of both heating time and the absence or presence of wheat flour. The ovotransferrin fraction (76.6 kD) was significantly decreased in all heated EW samples. Multiple bands below 37 kD in the sample of EW mixed with wheat flour and baked for 10 minutes might be protein bands of wheat, which were diminished by heating for 30 minutes.

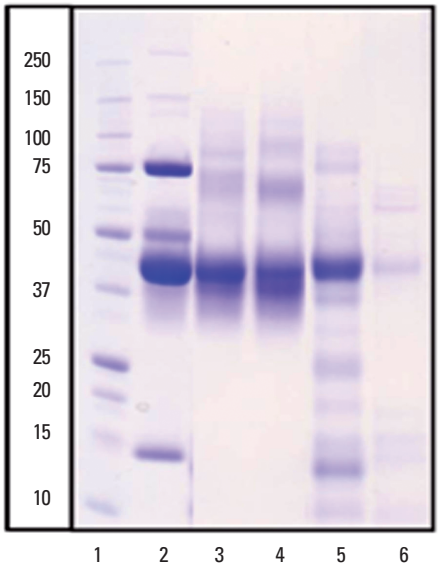

Fig. 1. SDS-PAGE analysis of heat treated egg white (EW) proteins and EW mixed with wheat flour proteins. Lane 1; Protein marker Lane 2; raw EW, Lane 3; baked EW for 10 min at 180°C, Lane 4; baked EW for 30 min at 180°C, Lane 5; baked EW mixed with wheat flour for 10 min at 180°C, Lane 6; baked EW mixed with wheat flour for 30 min at 180°C.

IgE immunoblot analysis

In the IgE immunoblots, heat treatment of only EW did not reduce IgE reactivities to ovomucoid and ovalbumin overlapping in the range of 37-50 kD. However, heating EW in the presence of wheat flour significantly decreased IgE reactivities to ovomucoid and ovalbumin at 37-50 kD, and this difference was greater when heat was applied for 30 minutes compared to 10 minutes (Fig. 2). These results imply that the heating of EW in the absence of wheat flour did not decrease the antigenicity of ovomucoid, while heating in the presence of wheat flour decreased the antigenicity of ovomucoid as well as of ovalbumin. When EW was heated in the absence of wheat flour, a wide band below 75 kD and a band at 100 kD were evident. These were thought to be new protein fractions produced by protein agglutination after heating.

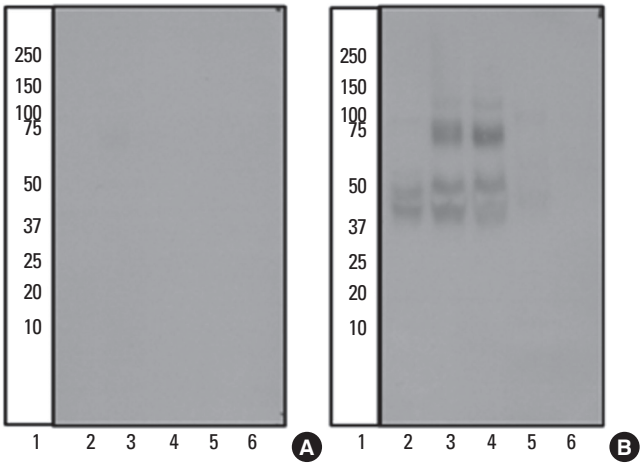

Fig. 2. IgE Immunoblotting analysis with pooled sera of egg-allergic patients. (A) shows an immunoblot result using native serum. (B) is a immunoblot result using the pooled sera of egg-allergic patients. Lane 1: Protein marker; Lane 2: raw egg white (EW); Lane 3: baked EW for 10 min at 180°C; Lane 4: baked EW for 30 min at 180°C; Lane 5: baked EW mixed with wheat flour for 10 min at 180°C and Lane 6: baked EW mixed with wheat flour for 30 min at 180°C.

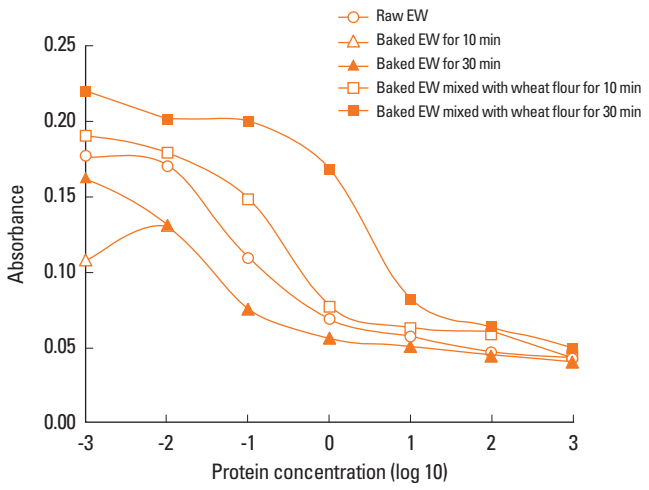

Fig. 3. Inhibition enzyme-linked immunosorbent assay analysis for heat treated egg white (EW) and EW mixed with wheat flour proteins for egg-allergic patients' sera.

Inhibition ELISA

The heating of EW with wheat flour induced a small decrease in IgE reactivity to ovomucoid even when heated for 10 minutes, and a significant decrease in the antigenicity of ovomucoid was observed when heated for 30 minutes. However, heating EW in the absence of wheat flour did not reduce IgE reactivity to ovomucoid even when heated for 30 minutes (Fig. 3).

DISCUSSION

Food processing is important for minimizing the allergenicity of food proteins. When food proteins are cooked or heated with other food proteins, antigenicity may be decreased through their interactions.[21,22] Egg proteins are usually subjected to heat treatment such as boiling or baking, either alone or mixed with wheat flour, and are likely to undergo significant structural changes during thermal treatment. Kato et al.[18] reported that when EW was heated in the presence of wheat flour, the solubility of ovomucoid was decreased and thus the antigenicity was markedly lowered. The current study was conducted to determine the effect of heating in the presence of wheat flour on the antigenicity of EW proteins, especially ovomucoid, which is known to be a heat resistant protein.

Our preliminary experiment indicated that antigenicity of EW proteins showed a greater decrease when heated at 170°C compared to 100°C, and was also more decreased when heated for 30 minutes rather than 10 minutes. The temperature of baking was determined considering the usual method of making cookies. Based on this, we conducted our current experiment using a 180°C temperature, and heated EW for 10 and 30 minutes to confirm the difference in the allergenicity of EW proteins according to time.

IgE immunoblots showed that the heating of EW alone did not decrease IgE reactivity to EW proteins at 37-50 kD regardless of heating time, suggesting that IgE reactivity to ovomucoid was not reduced by prolonged heating. However, when EW was baked in the presence of wheat flour, the IgE binding intensity to 37-50 kD (the overlapped band of ovomucoid and ovalbumin) significantly decreased compared with results for raw EW and EW heated alone. The decrease in antigenicity was greater when heat was applied for 30 minutes compared to 10 minutes. Reduced IgE reactivity to protein fractions at 37-50 kD in the mixture of EW and wheat flour may be due to a smaller amount of EW proteins than in the EW alone without wheat. However, because when the mixture of EW and wheat was heated for 30 minutes, IgE reactivity to the proteins at 37-50 kD reduced more than when heated for 10 minutes, it can be proposed that the presence of wheat flour facilitated a reduction in IgE reactivity induced by prolonged heating.

In inhibition ELISA, the antigenicity of ovomucoid was significantly reduced when heated for 30 minutes in the presence of wheat flour, not by heating of EW alone regardless of heating time. We found that the presence of wheat flour promoted a decrease in ovomucoid allergenicity induced by heating. In addition, the antigenicities of ovomucoid changed depending on the time of heat treatment and the presence of wheat flour. When heated for 30 minutes, ovomucoid antigenicity was reduced significantly more than when heated for 10 minutes, indicating that EW should be heated for a relatively longer time even in the presence of wheat flour in order to achieve a significant reduction in the antigenicity of ovomuoid.

The precise mechanisms by which the decrease in EW antigenicity by heating in the presence of wheat flour takes place have not yet been elucidated. Ovomucoid is a highly soluble glycoprotein, and is not rendered insoluble by heating. Kato et al.[18] suggested that the gluten in wheat flour agglutinates with EW proteins and increases the insolubility of EW proteins, thus decreasing EW antigenicity. Kato et al.[23] have also shown that the antigenicity of ovomucoid varies according to kneading time and wheat variety when heated. The interactions of proteins with other ingredients such as fat, sugar or other proteins, in processed foods are important, and in general result in a decreased availability of protein for interaction with the immune system.[22] For example, the heating of β-lactoglobulin results in the formation of intermolecular disulfide bonds, which cause β-lactoglobulin to bind to other food proteins. This change makes the β-lactoglobulin of cow's milk less allergic.[21] Further studies are needed to elucidate this mechanism and to explain why the effect of heating differs depending on heating time in the presence of wheat flour.

It has been reported that a considerable number of patients with egg allergy show allergic reactions to raw or lightly cooked eggs but not to eggs heated at a high temperature for a long time.[16,24] Lemon-Mulé et al.[24] recently reported that 64 of 117 subjects tolerated extensively heated egg in the form of muffins

(baked at 176°C for 30 minutes) and waffles (baked at 260°C for 30 minutes), but only 23 tolerated regular egg (in a form of scrambled egg or French toast). This suggests that the decrease in allergenicity of EW cannot be due to a reduction of antigenic activity only by prolonged extensive heating, but is also due to baking that involves mixing with wheat flour. Urisu et al.[16] reported that of 38 patients with egg allergy, 21 did not show allergic reactions to EW heated at 90°C for 60 minutes, and of remaining 17 patients who displayed allergy to heated EW, 16 did not show allergic reactions to heated and ovomucoid-depleted EW. Based on these results, a majority of allergic reactions to heated EW can be attributed to ovomucoid. To reduce allergic reactions to heated EW, it is thus necessary to reduce the allergenicity of ovomucoid. According to our study, prolonged extensive heating in the presence of wheat flour can be a good method for reducing ovomucoid allergenicity.

The standard treatment method for food allergy is currently elimination of the offending foods. Although oral immunotherapy has been performed on patients with peanut allergy as well as those with severe egg allergy, guidelines for this have not yet been established.

Lemon-Mulé et al.[24] demonstrated that the patients with egg allergy who did not display allergic reaction to muffins and waffles extensively heated at a high temperature also became tolerant to heated eggs after they ingested muffins and waffles regularly for more than three months. They also showed in another study that when patients with milk allergy continued to ingest extensively heated milk products, their skin test reactivity to cow's milk decreased. Taken together with the results of our study, which shows that extensive prolonged heating of EW in the presence of wheat flour can induce a decrease in the allegenicity of ovomucoid, which is known as a heat-resistant allergen, it is believed that continued ingestion of extensively heated EW products containing wheat flour, such as muffins and cookies, rather than strict avoidance of egg ingestion would be more helpful in treating patients with egg allergy. Thermal processing to decrease EW antigenicity has been used to treat patients with egg allergy. If heated EW products containing wheat flour keep the antigenicity level required for the induction of immunologic tolerance without inducing allergic reactions, they can be used for oral immunotherapy.

In conclusion, this study suggests that ovomucoid allergenicity could be reduced by prolonged heating in the presence of wheat flour. Instead of strict dietary avoidance, the introduction of extensively heated EW products containing wheat flour into the diet of egg-allergic children may be an alternative method for inducing oral tolerance.

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