

Effect of acid treatment on allergenicity of peanut and egg

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ABSTRACT

BACKGROUND: Many food experts have studied various treatments or processing techniques in order to develop hypoallergenic foods. In a previous study, acid treatment dramatically mitigated the allergenicity of peanut, especially Ara h 2.

RESULTS: Gel electrophoresis showed that most protein bands of acid treated peanut were not detected, but protein bands of egg white became weaker and broader by acid treatment. In immunolabeling using a rabbit antibody, the antigenicity against ovalbumin or ovomucoid in acid treated egg white was decreased but the antigenicity against Ara h 1 or Ara h 2 in peanut treated with pH 2 acetic acid was completely undetected. The allergenicity of ovalbumin and peanut fell significantly to $1/10^{22}$ and $1/5380$, respectively, when measured as IC_{50} in the sample treated with pH 2.0 acetic acid.

CONCLUSION: This study showed that acid treatment was more effective in peanut and barely effective in ovomucoid. This may contribute to the development of hypoallergenic food and clinical management of food allergy.

Keywords: Egg, Peanut, allergens, allergenicity, acetic acid, IgE

INTRODUCTION

Food allergy (FA) is a notable public health problem affecting both children and adults, and its prevalence has increased all over the world. In the 2013 report by CDC (Centers for Disease Control and Prevention), the prevalence of food allergy in children under 17 years old increased from 3.4% in 1997-1999 to 5.1% in 2009-2011¹. In Korea, the prevalence of FA is 5.3% in infants², and the mean lifetime prevalence of FA in 2000 was 4.7% for children aged 6 to 12 years and 5.1% for those aged 12 to 15 years which was higher than that in 1995 (4.2% among elementary school children, and 3.8% among middle school aged children)³. Besides, the survey in 2005 also indicates that the prevalence of diagnosed food allergy among the students in Seoul had increased to 6.2%⁴.

Major allergenic foods vary depending on countries, regions, and dietary cultures; however, more than 90% of all food allergies are caused by eight foods such as eggs, peanuts, soybean, milk, fish, crustacean, tree nuts, and wheat⁵. Among them, eggs and peanuts represent animal-origin food and plant-origin food, respectively. Eggs are the major offending food in young children⁶, and they have a strong correlation with atopic dermatitis. Peanuts are of a global concern due to the life-threatening anaphylactic reactions and a tendency to persist throughout adulthood⁷. The prevalence of egg allergy is similar all over the world, but peanut allergy is more common in the US than in Korea or other Asian countries. A discrepancy in the prevalence of peanut allergy between countries might stem from different processing methods. The antigenicity of peanut increases during roasting at high temperatures, whereas it decreases during acid treatment or frying⁸. Considering that the food is not eaten raw but it is consumed after

various processing techniques, the effect of processing on the food allergen should be studied in depth.

Many food experts have studied various treatments or processing techniques in order to develop hypoallergenic foods. Most of them focused on heating and enzymatic hydrolysis. Heating can destroy or generate new epitopes by conformational change in secondary or tertiary structure, rearrangement, cohesion or cross-linking of disulfide bonds, resulting in change in the allergenicity. A decline in allergenicity after heating has been reported in some foods such as milk, kiwi, and soybeans; on the other hand, increased allergenicity has also been reported⁹.

Enzymatic hydrolysis has also been used widely to decrease the allergenicity. Reduced allergenicity in fermented food is a good example of hypoallergenic food using enzymatic hydrolysis¹⁰. The allergenicity of soybean, buckwheat, peanuts, milk, rice, and eggs decreased significantly after enzymatic hydrolysis. Hydrolyzed cow's milk has already been used as a hypoallergenic food. Lately, nonthermal treatments such as high pressure processing, pulsed ultraviolet light, high voltage pulsed electric fields and gamma irradiation have been attempted. In comparison to thermal treatment, nonthermal treatment is better for preserving the natural properties of foods, organoleptic properties, and nutritional values, and it can be easily combined with other treatments, resulting in a synergistic effect of a decrease in allergenicity^{11, 12}. Cooking method using acid also produced an effective result in reducing the allergenicity. IgE binding to Ara h 2, the major allergen in peanuts, increased after roasting, but after acid treatment, IgE binding decreased significantly⁸.

The present study was conducted to determine whether the acid treatment produced the same results in all foods or not. The possibility of generalization for acid treatment

in reducing allergenicity has been investigated by comparing the results of acid treated eggs and peanuts, the animal originated foods and the plant originated foods, respectively.

MATERIALS AND METHODS

Patients' sera

Fifteen patients sensitized to peanut or egg and with a diagnosis of atopic dermatitis (AD) were included in this study (Table 1). Sera from patients sensitized to peanuts or egg were pooled. The patients had a peanut or egg white-specific IgE levels $>15 \text{ kU}_A \text{ L}^{-1}$, as measured using the Pharmacia CAP system (Uppsala, Sweden). This cut-off value was considered to be predictive of clinical reactivity. Normal human sera were used as negative controls. After receiving informed consent, 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 peanut or egg extracts

Raw in-shell peanuts grown in Korea were obtained from the Korean Rural Development Administration. Hen's eggs were purchased from a local grocery store, stored at 4°C , and used in this experiment within 2 days of purchase. Hen's eggs were soft boiled in boiling water for 10 min. Soft boiled hen's egg and peanut were submerged in acetic acid solutions with different pH values (pH 2, 3, or 4) for 24 hr. The peanut or egg protein extract of control and pickling samples was prepared by using the previously reported methods¹³. Protein concentrations were then determined using a BCA assay (Thermo scientific, Rockford, IL, USA) and a microplate reader (Bio-Rad,

Japan). All extracts were stored at -80°C .

SDS-PAGE and immunoblot analysis

Extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; Tricine System; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Extracts were then loaded into each well at $5\text{ }\mu\text{g well}^{-1}$. Precision Plus Protein standards (Bio-Rad) were used as molecular weight markers to estimate protein size. The separated proteins were transferred from the gel to a polyvinylidene difluoride membrane (PVDF) using the iBlot™ Dry Blotting System (Invitrogen). The PVDF membranes were then blocked with 20 mg mL^{-1} non-fat dried milk (NFDM) for 1 hour at room temperature, and then incubated overnight with the patients' pooled sera, which was diluted 1:10 in 20 mg mL^{-1} NFDM. After washing with $0.3\text{ }\mu\text{L mL}^{-1}$ PBST [Tween20 (Sigma) diluted in phosphate-buffered saline (PBS) (Amersco, Solon, OH, USA)], the membrane was incubated with biotin-labeled goat IgG-anti-human IgE (KPL, Gaithersburg, MD, USA), which was diluted 1:4,000 in 20 mg mL^{-1} NFDM, for 1 hour at room temperature. After rinsing with PBS-T, the membrane was incubated with NeutrAvidin-HRP (Pierce Chemical Co., Rockford, IL, USA) for 30 minutes at room temperature. After washing with PBS-T, the membrane was reacted with Amersham ECL reagent (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) for 2 minutes. The blotted membrane was then exposed to a high performance chemiluminescence film (GE Healthcare Limited, Buckinghamshire, UK) and the film was developed. The density of SDS bands and immunoblots were compared using a reflective densitometer (Bio-Rad) and Quantity One software (Bio-Rad).

Inhibition ELISA

Ninety-six-well ELISA plates (Nunc-Maxisorp NO FDF) were coated with 100 μL per well coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) containing 50 $\mu\text{g}/\text{mL}$ of crude peanut or egg white extract and incubated overnight at 4 ° C. Following incubation, the plates underwent 3 washes with the wash solution (0.5 $\mu\text{L mL}^{-1}$ PBST) using an automated plate washer. To avoid nonspecific binding, the plates were then blocked with 200 μL of 20 mg mL^{-1} bovine serum albumin (BSA) and incubated for 2 h at room temperature (RT). Preincubation of the raw and pickling samples in the presence of a commercial antibody (anti-OVA, anti-OM, anti-Ara h 1, and anti- Ara h 2) or pooled sera of egg or peanut sensitized patients was utilized to optimize the competition. The sera were diluted in PBS, pH 7.4, containing 0.5 $\mu\text{L mL}^{-1}$ Tween 20 and 20 mg mL^{-1} BSA (Sigma A-21536). One volume (55 μL) of antibody solution (anti-OVA, anti-OM, or anti-OT) was added to one volume (55 μL) (vol vol^{-1}) of raw or pickling extracts that had been previously diluted in assay diluents. The pooled sera were used at a final concentration of 1/40. The antibody-protein mixtures were kept in tubes at 37°C for 1 hour. After the blocking, plates were washed as described above. Then, 100 μL of inhibition samples was added to the plate in duplicate and incubated for 2 hr at RT. The plates were washed and 100 μL of a 1: 2,500 dilution of peroxidase labeled goat anti-human IgE (Sigma, Gaithersburg, MD, USA) was added to every well and incubated for 1 hr at RT. After washing with 0.5 $\mu\text{L mL}^{-1}$ PBS-T, 100 μL of tetramethylbenzidine (TMB) peroxidase substrate (KPL; Gaithersburg, MD, USA) was added. The optical density of each well was measured using a plate reader (Bio-Rad, Japan) at 595 nm. Optical density values were recorded and percent inhibition at each inhibitor concentration was calculated using the following equation: % inhibition = $[(A_0 - A_1)/A_0] * 100$, where A_0 is the absorbance without inhibitor (PBS only), while A_1 is the absorbance at each inhibitor concentration.

RESULTS

Changes in protein pattern of egg white (EW) and peanut by acetic acid treatment

The soft boiled EW and peanut were submerged in acetic acid solutions with different concentrations. The proteins were extracted from acid treated EW and peanut as described in the 'Materials and methods' section. Separation of these acid treated EW and peanut samples by SDS-PAGE was performed after protein concentration determination according to the Bradford assay (Fig. 1(a)). Several dominant protein bands were observed at 37-50 kD (overlapped bands of ovalbumin (OVA) and ovomucoid (OM)), 14 kD (lysozyme), and 76 kD (ovotransferrin (OT)) in raw EW. Overall, protein bands of EW became weaker and broader by acid treatment and the density of protein bands was strongly changed by increasing pH of treated solutions. The bands corresponding to lysozyme completely disappeared by acetic acid treatment, which signifies that lysozyme is very acid-labile and gets degraded by acetic acid treatment. Almost all of the peanut allergens were altered after incubation in the acidic solutions (Fig. 1(d)). The variably treated peanut samples in acetic acid solution with pH 4 showed no significant changes in the individual proteins as compared to the untreated sample. In samples treated with pH 2 or pH 3 acetic acid, 63kD (referenced to Ara h 1), 20kD (Ara h 2), and 15 kD (Ara h 6) appeared as a very thin, weak band than the bands of untreated peanut.

By western blotting, we identified that the reactivity between acid treated EW protein and rabbit OVA or OM specific antibody was decreased but it did not disappear (Fig. 1 (b), (c)). Otherwise, reactivity against OT specific antibody vanished completely by acid treatment.

Western blotting was repeated with acid treated peanut and the untreated peanut using a rabbit Ara h 1 or Ara h 2 specific antibody (Fig. 1 (e), (f)). Ara h 1 antigenicity in samples treated with the above pH 3 acetic acid was similar to that in untreated peanut. It is interesting to note that Ara h 1 antigenicity in samples treated with pH 2 acetic acid was completely undetected. Also, it was similar to the antigenicity change pattern of Ara h 1 and Ara h 2 in treated samples.

Change in antigenicity and allergenicity by acetic acid treatment

In competitive inhibition ELISA (ciELISA), we could confirm the individual antigenic activities of the 3 major EW allergens, especially differentiating the reactivity against OM from the reactivity against OVA, and the 2 major peanut allergens.

The results are depicted in Figure 2, and the corresponding IC₅₀ values are shown in Table 2. A decrease in the OVA antigenic activity was observed in the sample treated with pH 2.0 acetic acid. In EW treated using pH 3.0 acetic acid, OVA antigenic activity did not decrease when compared with that in raw EW. No reduction in the OM and OT antigenic activities in all kinds of pickled EW was observed (results of OT not shown). In peanut treated with pH 2.0 or pH 3.0 acetic acid, the antigenicity of peanut was decreased.

This result is underscored by the IC₅₀ values. The antigenicity of OVA in EW treated with pH 2.0 acetic acid was decreased to 1/10⁷ compared with that in raw EW. On the other contrary, the antigenicity of OM was not influenced in acid treated EW. Also, the pickling of peanut protein hardly changed the Ara h 1 and Ara h 2 antigenicity.

ciELISA was repeated with commercially available OVA, OM, and acid treated EW protein at pH 2.0 or 3.0 using pooled sera from egg white sensitized patients to

check allergenicity changes in 2 major EW allergens after acid treatment (Fig. 3).

Allergenicity of OM and OT was not changed by acetic acid treatment. But, the allergenicity of OVA was significantly reduced by treatment with pH 2.0 or pH 3.0 acetic acid, unlike the ciELISA results using a rabbit OVA specific antibody. The allergenicity of OVA and peanut fell significantly to $1/10^{22}$ and $1/5380$, respectively, when measured as IC_{50} in the sample treated with pH 2.0 acetic acid (Table 3).

DISCUSSION

Currently, the only way to manage FA is avoidance of the allergen. Strict avoidance of offending allergen-containing food products has been the standard of care for children with FA. However, Lemon-Mule et al.¹⁴ have demonstrated that ingestion of extensively heated egg products was well-tolerated and safe in some patients with egg allergy, which means that strict dietary avoidance of heated egg is not necessary in all patients with food allergy and partial dietary avoidance could be helpful in immune tolerance. Their results suggest that processed food is a useful way to treat and manage food allergy.

Our team has published several papers regarding the effect of food processing on allergenicity. One of the interesting findings was treatment with the acid. In a previous paper, acid treatment dramatically mitigated the allergenicity of peanut, especially Ara h 2⁸. Ara h 2 is known to be intrinsically more allergenic than other proteins due to structural features. Ara h 2 is composed of two different polypeptide chains which contain the conserved cysteine skeleton CXnCXn-CCXnCXnCXnCXnC. This skeleton contains two interchain and two intrachain disulfide bonds, which provide 2S albumins the characteristic of high stability against proteolytic digestion and heat treatment. The acid denaturation of proteins has been known to result in the less unfolded state due to

electrostatic repulsion¹⁷; hence, it has been considered to be a less useful method to reduce the allergenicity of food allergen. Treatment with acid seems to change the bioactivities of the protein surface of Ara h 2, resulting in reduction of allergenicity. We were curious if the result of treatment with acid is reproducible in other foods. We chose egg white because egg is a major casual food and a representative animal food and contains ovomucoid. Ovomucoid (Gal d 1) also has disulfide bonds allowing its tertiary structure to remain unaltered and is more allergenic than other proteins due to stability against proteolytic digestion and heat treatment¹⁸. In this study, IgE reactivity to proteins denatured by acid treatment appeared differently. IgE reactivity to Ara h 1, Ara h 2, and OVA decreased after treatment with acetic acid, whereas IgE reactivity to OM was barely changed, which means that treatment with acid could cause different consequences in each food antigen.

Acid treatment for mitigating allergenicity is a different approach compared with proteolytic digestion and heat treatment. Reduction of allergenicity by proteolytic digestion and heat treatment is mostly due to the cleavage in the epitope, whereas acidic treatment causes not only cleavage of the protein but also polarity change in the protein surface. Considering the degradation resistance of Ara h 2 to acidic treatment, reduction in allergenicity by acidic treatment could be due to the change in the bioactivity of the surface. Bogan and Thorn¹⁹ emphasize the role of several amino acids in the interaction of Ag-Ab, two hydrophobic aromatic residues Tryptophan and Tyrosine and one positively charged amino acid Arginine. Arginine is capable of multiple types of favorable interactions and has the ability to form a hydrogen bond network with up to five H-bonds and a salt-bridge with its positively charged guanidinium motif. Interestingly, Arginine appears frequently in the Ara h 2 epitope²⁰ but not in

ovomucoid²¹. Based on our results, we inferred that because each food has a different primary structure of the protein, acid treatment inevitably results in different levels of reduction in allergenicity of each food.

Currently, the standard treatment for food allergy is elimination of the offending foods until oral tolerance induction. Recently, there have been many trials for oral immunotherapy using a modified food allergen to induce tolerance. In traditional oral immunotherapy, peanut allergen can be delivered in various forms (crushed roasted peanuts, peanut flour, and whole peanut kernels). It has proven not feasible due to the high risk of severe systemic side effect but it is still a major concern. For this reason, new and innovative treatment options have been suggested for food-allergy immunotherapy including use of recombinant hypoallergenic peanut allergens, hypoallergenic protein with a bacterial adjuvant, combination with anti-IgE therapy²². Our results suggested another hypoallergenic peanut protein for food-allergy immunotherapy. Especially, reduction of Ara h 2, which is related to moderate to severe reactions and persistent peanut allergy, is promising. If a potentially safe peanut allergen could increase the tolerable concentration without life-threatening anaphylaxis, the results of this investigation provide considerable new insights into the allergenic potency of peanut allergens and will have an impact on the composition of allergen-specific immunotherapy. It indicates that reaction to IgE does not mean clinical allergy; hence, a careful clinical evaluation of each positive result is required with great focus on clinical tolerance status of food allergens. Future studies should focus on human cellular responses.

CONCLUSION

Acid treatment reduced the allergenicity of EW and peanut; however, the degree of reduction effect appeared different. Acid treatment was more effective in peanut and barely effective in OM. This may contribute to the development of hypoallergenic food and clinical management of food allergy.

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FIGURE LEGENDS

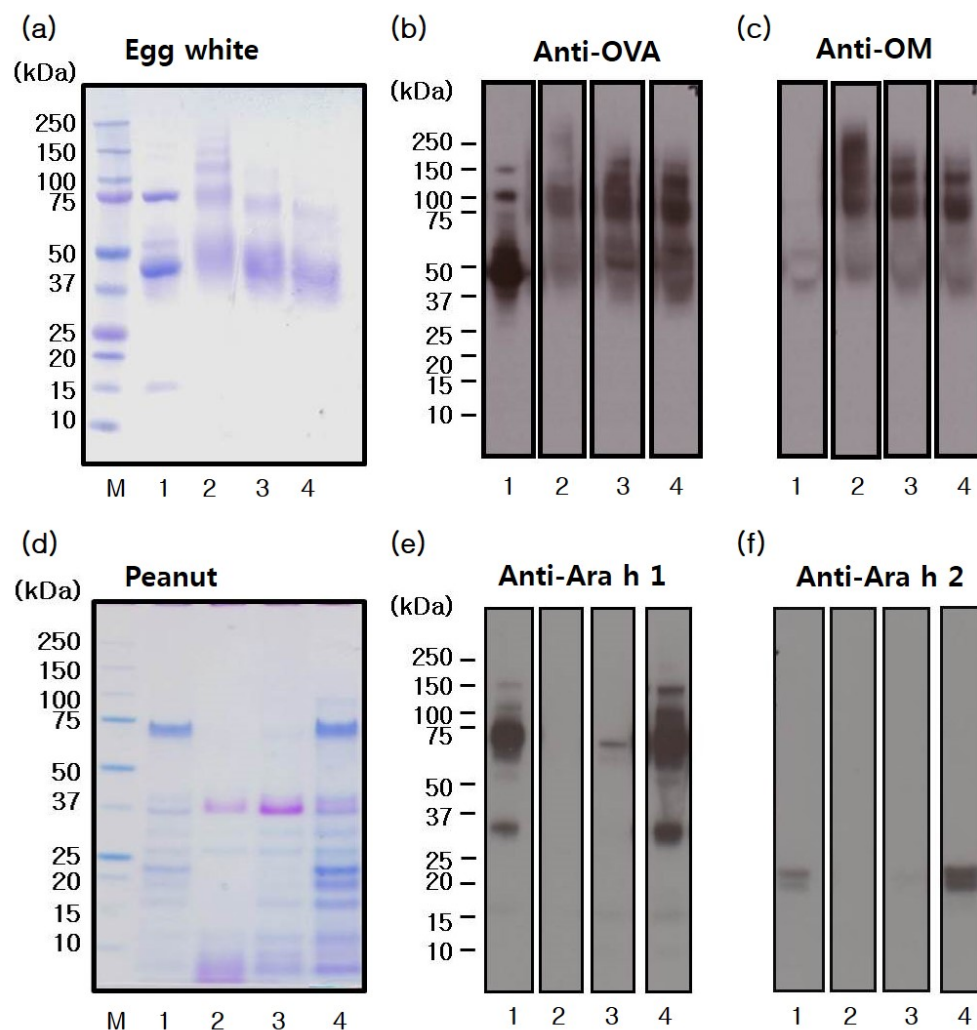


Figure 1. SDS-PAGE (a, d) and Western blots (b, c, e, f) of EW or peanut after various pH acetic acid treatments. The acid treatment of EW (a) and peanut (d) was performed at pH 2, 3, and 4 using acetic acid. Blots were reacted against rabbit anti-OVA sera (b), rabbit anti-OM sera (c), rabbit anti-Ara h 1 sera (e), and rabbit anti-Ara h 2 sera (f). M is MW markers. Lane 1; Raw EW or peanut, Lane 2; Pickled EW or peanut at pH 2, Lane 3; Pickled EW or peanut at pH 3, Lane 4; Pickled EW or peanut at pH 4.

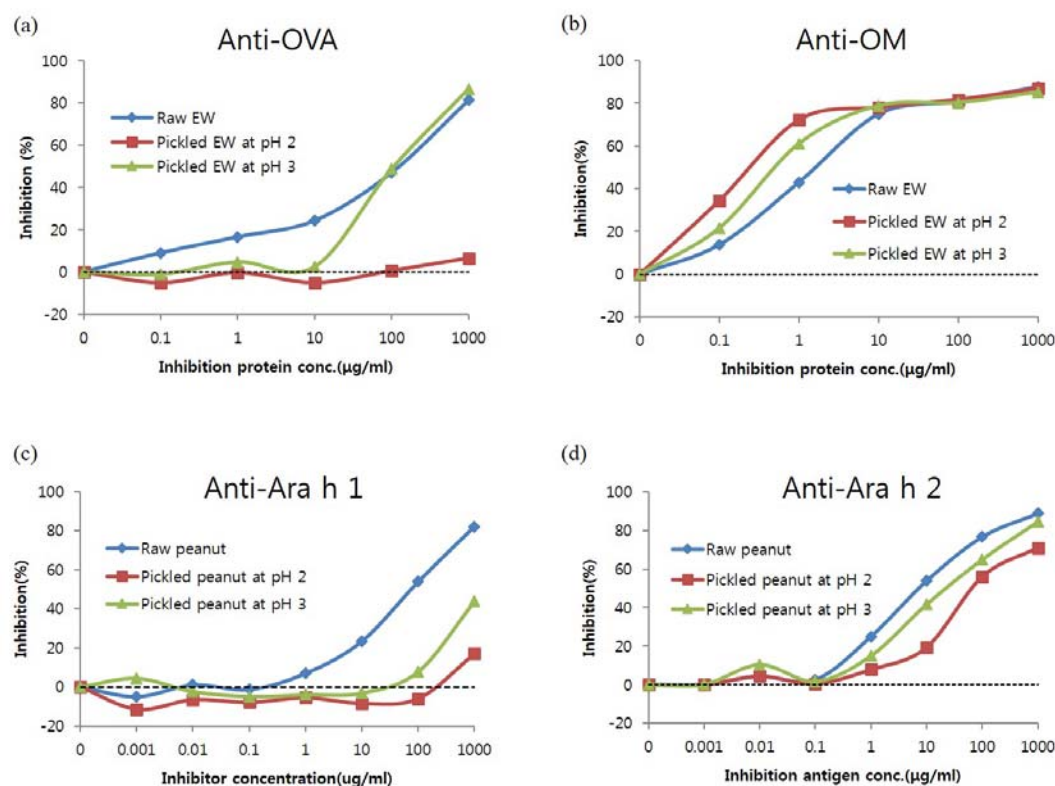


Figure 2. Inhibition ELISA results comparing pickled sample and controls. Inhibitions were conducted using rabbit anti-OVA (a), rabbit anti-OM (b), rabbit anti-Ara h 1 (c) and rabbit anti- Ara h 2 (d) with acid treated EW or peanut.

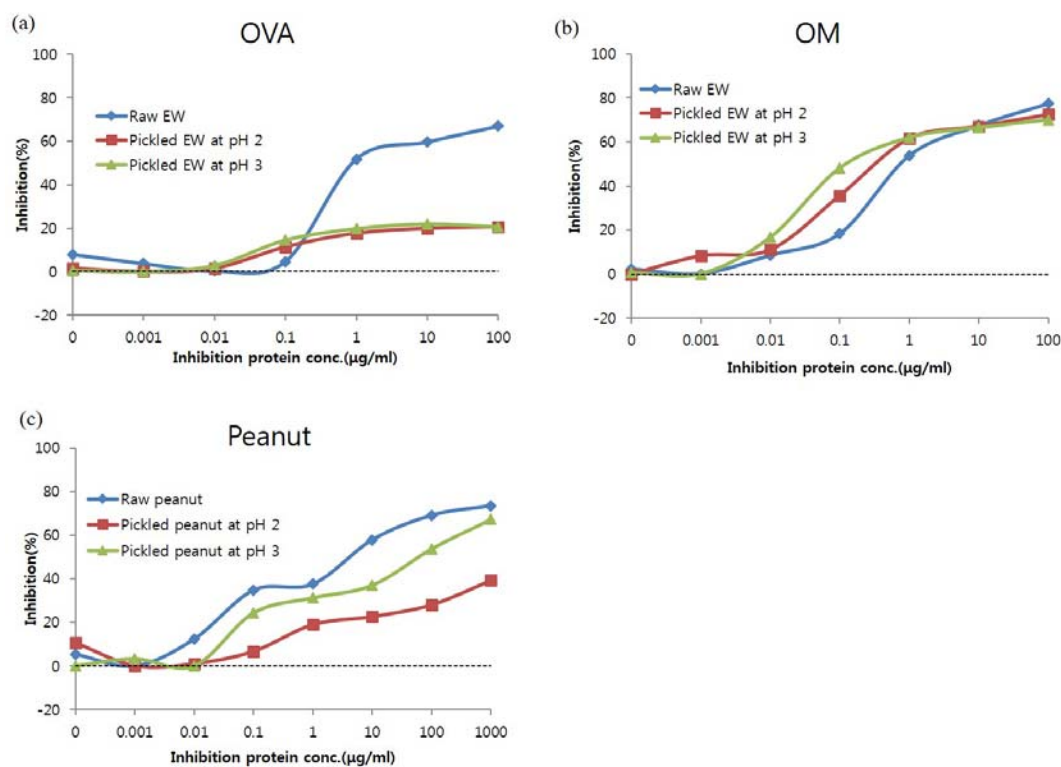


Figure 3. Inhibition ELISA using pooled sera of EW (n = 7) or peanut (n = 8) sensitized patients. Inhibitions were conducted using pooled sera with ovalbumin (a), ovomucoid (b), and peanut (c).

Table 1. Biological characteristics of sera from 15 patients sensitized to peanut or egg with a diagnosis of atopic dermatitis

	Patient number	Age (years)	Sex	Total IgE (kU_A L⁻¹)	sIgE (kU_A L⁻¹)
Patients with Peanut-IgE	1	5	M	755	21.9
	2	7	M	1529	21.5
	3	4	M	1520	19
	4	12	F	4808	62.8
	5	11	M	-	64.8
	6	7	M	2662	79.2
	7	7	M	-	18.7
	8	5	M	241	20.4
	9	3	F	307	14.3
	10	1	M	-	26.6
	11	1	M	98.5	13.3
Patients with Egg- IgE	12	7	M	120	31
	13	0	M	369	88.5
	14	3	F	356	38.8
	15	1	M	224	73.9

Table 2. IC₅₀ values of the ciELISA experiments using a commercial rabbit anti-specific antibody

Inhibition protein	Sample	IC₅₀	Reduction rate (%)
EW (OVA; ovalbumin)	Raw EW	0.20	1.00
	Pickled EW at pH 2	>10 ⁶	>10 ⁷
	Pickled EW at pH 3	0.16	0.82
EW (OM; ovomucoid)	Raw EW	1.68	1.00
	Pickled EW at pH 2	0.25	0.15
	Pickled EW at pH 3	0.5	0.30
Peanut (Ara h 1)	Raw peanut	74.3	1.00
	Pickled peanut at pH 2	25852.4	348.1
	Pickled peanut at pH 3	1496.2	20.1
Peanut (Ara h 2)	Raw peanut	20.9	1.00
	Pickled peanut at pH 2	90.1	4.31
	Pickled peanut at pH 3	52.9	2.53

Table 3. IC₅₀ values of the ciELISA experiments using pooled serum of patients

Inhibition			
protein	sample	IC₅₀	Reduction rate(%)
EW (OVA; ovalbumin)	Raw EW	0.001	1.00
	Pickled EW at pH 2	10 ¹⁹	10 ²²
	Pickled EW at pH 3	∞	∞
EW (OM; ovomucoid)	Raw EW	0.82	1.00
	Pickled EW at pH 2	0.36	0.44
	Pickled EW at pH 3	0.15	0.18
Peanut	Raw peanut	5.9	1.00
	Pickled peanut at pH 2	31622.8	5379.84
	Pickled peanut at pH 3	51.1	8.70