



Immunoglobulin E-binding Proteins of Cooked Walnuts in Korean Children

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Purpose: The immunological characteristics of young Korean children with walnut (WN) allergy and the influence of different cooking methods on WN proteins have not been evaluated to date. This study aimed to evaluate the major WN allergens identified among Korean children, together with changes in WN antigenicity caused by common cooking methods. **Methods:** We enrolled children under the age of 13 years with WN serum-specific immunoglobulin (Ig) E concentrations. The protein fractions of dry-fried and boiled WN extracts were compared with those of raw WNs using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), 2-dimensional gel electrophoresis (2DE) and a proteomic analysis using electrospray ionization (liquid chromatography-mass spectrometry [LC-MS]). An immunoblotting analysis was conducted to examine IgE reactivity toward raw WNs using serum samples from 6 children with a clinical WN allergy. To determine the processed WN proteins with IgE-binding capacity, a 2D-immunoblotting analysis was performed using the pooled sera of 20 WN-sensitized children. **Results:** Protein bands from raw WNs were identified at 9, 16, 28, 52, 58, and 64 kDa via SDS-PAGE. The 9- and 16-kDa protein bands were enhanced by boiling, whereas the 52- and 64-kDa bands were considerably diminished. On LC-MS analysis, of the 66 IgE-binding proteins present in raw WNs, 57 were found in dry-fried WNs, but only 4 in boiled WNs. The sera of 5 out of 6 participants reacted with the 52-kDa protein bands and those of 4 out of 6 participants reacted with the 16- and 28-kDa protein bands, respectively. Meanwhile, a 2D-immunoblotting result confirmed the presence of different binding patterns among children who consumed cooked WNs. **Conclusions:** The protein profile of boiled WNs is substantially different from that of raw WNs. However, 4 proteins including prolamins remained stable after dry-frying or boiling. Further studies are needed to evaluate the clinical relevance of these findings.

Key Words: Antigenicity; cooking; walnut

INTRODUCTION

Walnut (WN) allergies, which can be fatal or near-fatal, account for a large proportion of total cases of tree nut allergies that affect children.^{1,2} Approximately 1% of the general population³ is allergic to nuts including WNs, although a trend of increasing incidence has been observed. WN imports to Korea have tripled in the last 10 years,⁴ and the consumption of WNs has become more widespread. There has been an increase in WN allergies among young children,^{5,6} and WNs are currently the third most common food to induce anaphylaxis and the fourth most common food to induce food allergy among Korean children.^{2,7} Additionally, regional differences have been found among young children in WN antigenicity patterns and severe systemic reactions after WN consumption.^{7,8}

WNs, particularly English walnuts (*Juglans regia*), frequently undergo some form of thermal processing prior to consump-

tion. Food processing methods have the potential to modify food proteins, which can alter food antigenicity.⁹ Moreover, some treatments such as dry-roasting can reduce immunoglobulin (Ig) E-binding capacity *in vitro*, measured via immunoblotting analysis.^{9,10} Pressure¹¹ and thermal treatments^{9,10} have been used to diminish the cross-linking capacity of IgE to WN effector cells. However, previous studies which have focused on the decrease in allergenicity of WNs have employed

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thermal processing methods not used in domestic kitchens and have been performed using adult sera. The way Koreans cook is also different from Western countries. In Korea, WNs are mostly consumed raw, or eaten with rice. Often, WNs are boiled for 5 minutes in water and stir-fried with sugar, soy sauce and other seasoning for 10 minutes. Meanwhile, liquid chromatography tandem mass spectrometry (liquid chromatography-tandem mass spectrometry [LC-MS/MS])¹² can be used to assess changes in individual proteins or amino acid residues in a protein sequence, given the relative changes in key proteins as a result of thermal processing. Researchers have identified 5 WN allergens to date, namely, Jug r 1 (2S albumin), Jug r 2 (7S globulin), Jug r 3 (lipid transfer protein [LTP]), Jug r 4 (11S globulin), and Jug r 5 (profilin).

In the present study, we aimed to investigate the immunological characteristics of young Korean children with WN allergies. In addition, we identified the usual methods of cooking WNs in order to investigate changes in WN antigenicity caused by cooking. Furthermore, we used LC-MS/MS analysis to evaluate the antigenic changes in proteins from raw, dry-fried, and boiled WNs.

MATERIALS AND METHODS

Patients and sera

Between January 2011 and January 2016, we identified all children referred to Ajou University Hospital (Suwon, Korea) for the investigation of potential WN allergic reactions and enrolled patients under the age of 13 years who had WN serum-specific IgE (sIgE) concentrations, obtained via ImmunoCAP sIgE assays (Thermo Fisher Scientific, Waltham, MA, USA), of >0.35 kU/L (1.35-100.0 kU/L). The sera of the participants were stored at -20°C until further testing. The clinical characteristics of the participants were collected from medical records and via telephone interviews with parents. Children with a clinical WN allergy were defined as those participants with a history of anaphylaxis and repeated systemic adverse reactions within 2 hours of isolated WN exposure. Food challenges were not performed for the majority of participants as parents frequently refused to provide consent. In addition, sera from 2 atopic patients who were allergic to inhalant but not food allergens were used as a control for immunoblotting. The study protocol was approved by the Institutional Review Board of Ajou University Medical Center (MED-KSP-12-381), and informed consent was obtained from the parents of all participants.

WN preparation and processing

Whole natural English WNs (*Juglans regia*) (Atlas World Food & Agriculture, Inc., Visalia, CA, USA) purchased from a local grocery store were used to obtain the protein extracts. The WNs were either dry-fried at 110°C for 8 minutes in a frying pan, boiled in water at 100°C for 10 minutes or left uncooked. The

WNs were then ground in a blender until a smooth paste and defatted with cold (4°C) petroleum ether (1:1 w/v) stirred constantly for 1 hour, and then filtered through filter paper. The defatting procedure was repeated until the filtrate became clear. The defatted paste was air dried completely at ambient temperature, added to phosphate-buffered saline (PBS, pH 7.4; 1:10 w/v), and stirred for 48 hours at 4°C . The extracts were centrifuged twice at $14,000\times g$ for 30 minutes at 4°C . The supernatants were then filtered and dialyzed against several changes of distilled water for 48 hours at 4°C . The extracts were centrifuged once again, lyophilized, and stored at -20°C until use. Protein concentrations were then determined via a Bradford assay (Bio-Rad, Hercules, CA, USA) using a microplate reader (Bio-Rad).

One-dimensional polyacrylamide gel electrophoresis (PAGE) and immunoblotting analysis

The extracts were resuspended in PBS, and the protein concentration of each was measured using a Bradford assay. Equal concentrations of the samples were mixed with a loading buffer (60 mM Tris HCl, 25% glycerol, 2% sodium dodecyl sulfate, 14.4 mM 2-mercaptoethanol and 0.1% bromophenol blue) and heated for 10 minutes in a heating block at 100°C . The samples were then analyzed using sodium dodecyl sulfate (SDS)-PAGE, following which the gels were either stained with Coomassie brilliant blue or used for immunoblotting. After gel electrophoresis, the raw WN protein extract was transferred to a nitrocellulose membrane (Millipore Co., Bedford, MA, USA) and blocked in 3% bovine serum albumin (BSA) for 2 hours, washed 3 times in PBS-Tween, and placed in overnight incubation with clinically WN-allergic individual human and control serum (both 1:10 diluted with BSA) at 4°C . After washing, samples were conjugated with alkaline phosphatase-conjugated goat anti-human IgE (Sigma-Aldrich Co., LLC, St. Louis, MO, USA) diluted to 1:1,000. The membrane was then developed using SIGMA-FAST™ 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma-Aldrich Co., LLC) and washed, and the development was terminated using water. Immunoblotting images were then analyzed using ImageJ software (National Institutes of Health, Rockville, MD, USA).

Two-dimensional electrophoresis (2DE) and 2D-immunoblotting analysis

Protein extracts from raw, dry-fried and boiled WNs were diluted with 50 and 300 $\mu\text{g}/\text{mL}$ rehydration buffer (8 M urea, 2% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, 3-10 NL 0.5% immobilized pH gradient [IPG] buffer, 0.002% bromophenol blue, and 50 mM dithiothreitol). The first-dimension separation was carried out on an Ettan IPG-phor system (GE Healthcare, Chicago, IL, USA) in accordance with the manufacturer's instructions. The samples were loaded on 7- and 13- cm IPG strips, with pH 3-10 NL (GE Healthcare). Second-dimension separation was performed on 10% and 12%

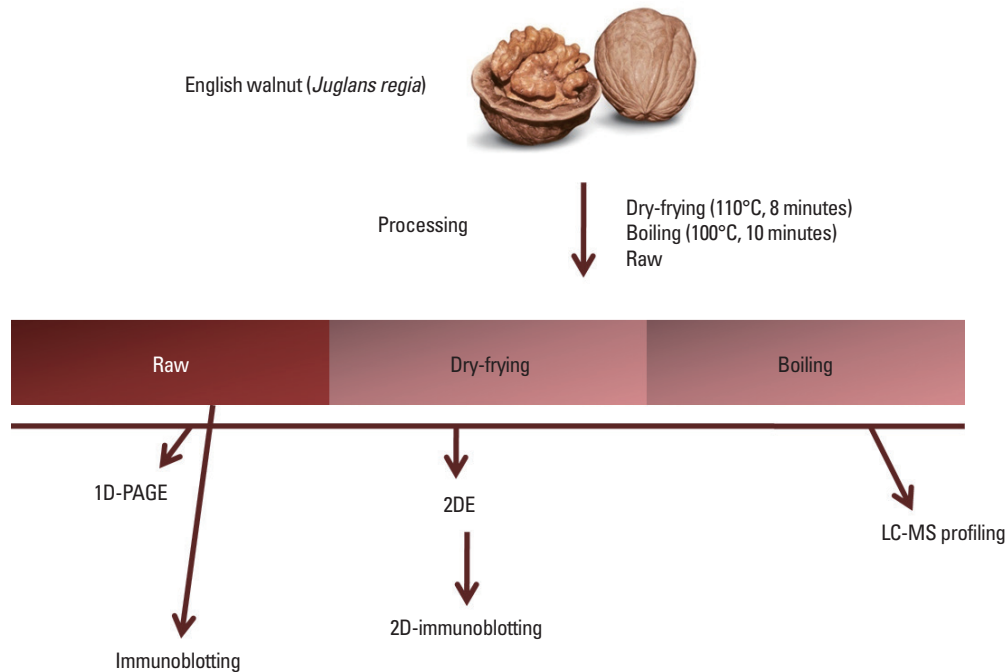


Fig. 1. Overview of the methodology used in this study to characterize the proteome of processed walnut.

acrylamide gels, and colloidal Coomassie brilliant blue staining or electroblotting used for visualization. After electroblotting, the membranes were blocked using a similar procedure to that described above and incubated with 25 mL of the serum pool from WN sensitized subjects at a dilution of 1:3. Protein spots were visualized and analyzed using Typhoon 7000 FLA series and ImageMaster 2D Platinum 7.0 software (GE Healthcare).

LC-MS/MS for peptide analysis

Nano-LC-MS/MS was performed using EASY n-LC™ and LTQ Orbitrap XL™ mass spectrometers (Thermo Fisher Scientific), which were equipped with a nanoelectrospray source. The raw, dry-fried, and boiled WN extracts were separated on a C18 nanobore column (150.0×0.1-mm, 3-μm pore size; Agilent, Santa Clara, CA, USA). Mobile phase A liquid chromatography separation consisted of 0.1% formic acid and 3% acetonitrile in deionized water, and mobile phase B separation of 0.1% formic acid in acetonitrile. The chromatography gradient was designed for a linear increase from 5% B to 30% B in 40 minutes, 30% B to 60% B in 4 minutes, 95% B in 4 minutes, and 3% B in 6 minutes. The flow rate was maintained at 1,500 nL/minutes.

The mass spectra were acquired using data-dependent acquisition with a full mass scan (350-1,200 *m/z*), followed by 10 tandem mass spectrometry (MS/MS) scans. For MS1 full scans, the orbitrap resolution and automatic gain control were 15,000 and 2×10^5 , respectively; for MS/MS the automatic gain control in the LTQ was 1×10^4 (Fig. 1).

Database search

The mascot algorithm (Matrix Science, Boston, MA, USA) was used to identify peptide sequences present in a protein sequence database. The database search criteria were: 1) taxonomy, fagales (National Center for Biotechnology Information non-redundant database, downloaded on Aug 4, 2016); 2) fixed modification, carbamidomethylation of cysteine residues, variable modification, oxidization of methionine residues; 3) maximum allowed missed cleavage of 2; 4) MS tolerance of 10 ppm; and 5) MS/MS tolerance of 0.8 Da. The peptides were filtered with a significance threshold of $P < 0.05$.

RESULTS

Participants and sera

A total of 20 participants who had sensitized to WNs (WN-sIgE > 0.35 kU/L) were enrolled in the study. The median age of the participants was 36 months, with a range of 8-132 months. The study group was composed mostly of boys (75.0%, 15/20). Of the participants, 12 (60%) who presented with immediate hypersensitivity on isolated exposure to WNs were allocated to the clinical WN-allergic group. Based on the diagnostic criteria of the World Allergy Organization,¹³ 3 participants had a history of anaphylaxis. Nine participants presented with repeated generalized skin rashes within 2 hours after WN exposure. In contrast, among all the sensitized participants, only 1 had no hypersensitivity reactions and 7 were too young to consume WNs due to the potential choking hazard (Table 1).

Comparison of the protein fractions of raw, dry-fried and boiled WNs via SDS-PAGE and 2DE

The protein fractions of dry-fried (3.4 mg/mL) and boiled (17.0 mg/mL) WN extracts were compared with those of raw (4.4 mg/mL) WNs using SDS-PAGE. The protein bands corre-

sponding to raw WNs were 9, 16, 28, 52, 58, and 64 kDa. The patterns of each WN protein differed depending on the cooking method. The intensity of the 9-kDa (likely Jug r 3) and 16-kDa (likely Jug r 1) protein bands were enhanced by boiling. In contrast, the intensity of the 52- and 64-kDa protein bands almost completely disappeared with boiling. In addition, the intensity of the 9-kDa protein band was enhanced, whereas that of the 16-kDa protein band was reduced, by dry-frying. However, the intensity of the 28-kDa protein band was unchanged and a 58-kDa protein band was newly presented with boiling. Notably, the intensity of the protein band corresponding to Jug r 3 was enhanced by all treatments (Fig. 2). When we compared the 2DE protein profiles of raw, dry-fried and boiled WNs, we found

Table 1. Characteristics of patients with a history of walnut ingestion

Patient No.	Sex	Age, mo	Total IgE, kU/L	WN-sIgE, kU/L	Symptoms	IgE immunoblot analysis of raw walnut (kDa)*
1	M	27	838	20.5	Anaphylaxis	16, 28, 52
2	M	24	267	8.36	Anaphylaxis	
3	M	15	N.D.	4.53	Anaphylaxis	
4	M	12	5,000	100	Urticaria	
5	F	58	301	91.3	Urticaria	16, 28, 52
6	F	84	494	73.8	Urticaria	
7	M	37	792	26.8	Urticaria	16, 28, 52
8	F	41	108	10.7	Urticaria	16, 52
9	M	132	448	8.98	Urticaria	
10	M	54	666	7.3	Urticaria	28
11	M	14	21	5.31	Urticaria	
12	F	36	157	1.52	Urticaria	52
13	M	63	540	5.64	None	
14	M	35	2,234	58.2	Unknown	
15	M	37	3,449	36.2	Unknown	
16	F	73	855	32.7	Unknown	
17	M	23	131	7.89	Unknown	
18	M	36	N.D.	4.41	Unknown	
19	M	8	2,157	2.88	Unknown	
20	M	65	91	1.38	Unknown	

N.D., not done.

*performed using serum samples from the 6 patients with a clinical walnut allergy (Patient No 1, 5, 7, 8, 10, 12).

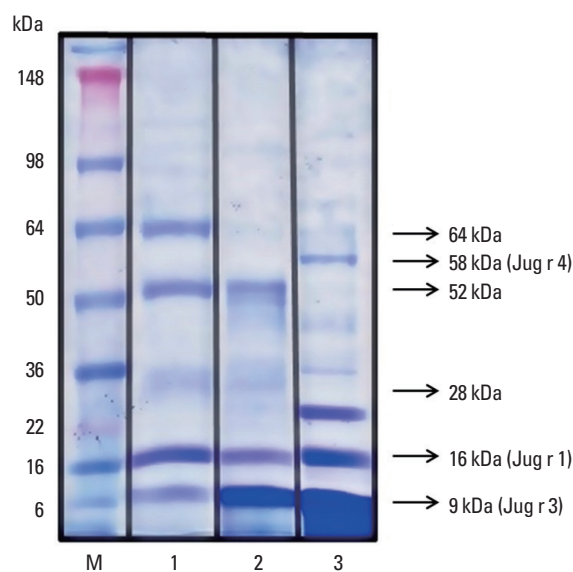


Fig. 2. SDS-PAGE analysis of processed walnut extract (M, marker; 1, raw; 2, dry-frying; 3, boiling). Concentration of extracts: raw 4.4 mg/mL; dry-frying 3.4 mg/mL; boiling 17.0 mg/mL. IgE bands identified: 9, 16, 28, 52, 58, 64 kDa.

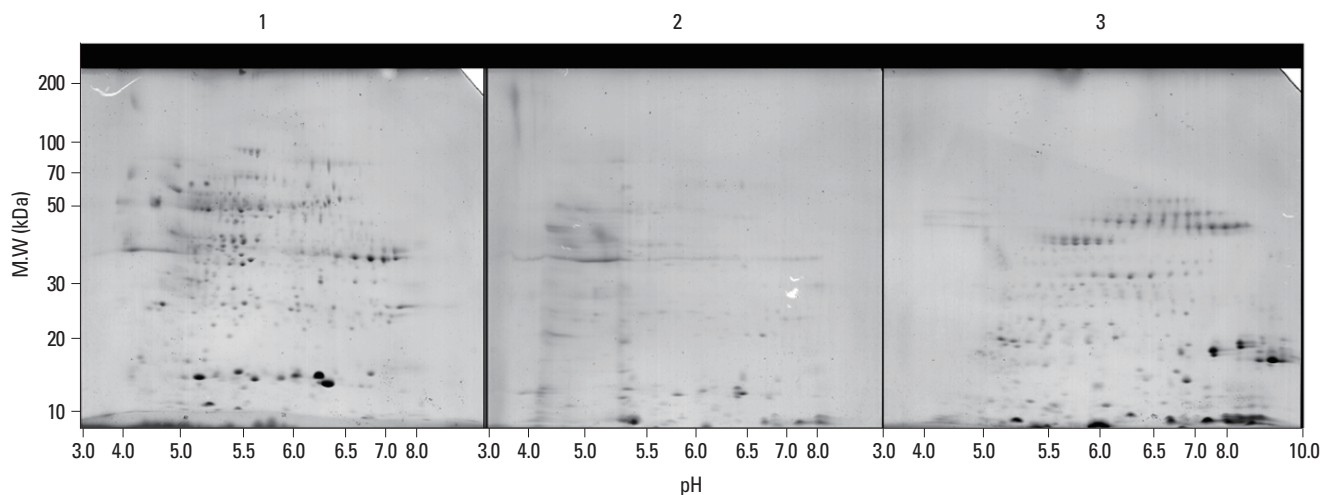


Fig. 3. 2DE analysis of processed walnut extract (1, raw; 2, dry-frying; 3, boiling; M.W, molecular weight).

that most protein spots dispersed after dry-frying. However, protein spots both dissolved and appeared spontaneously with boiling (Fig. 3).

IgE reactivity of each WN extract based on immunoblotting analysis

An immunoblotting analysis was conducted to examine IgE reactivity toward raw WNs using serum samples from the 6 patients with a clinical WN allergy (Patient Nos. 1, 5, 7, 8, 10, and 12; Table 1). The serum samples of 5 out of 6 participants (83.3%) reacted with the 52-kDa protein bands, and those of 4

out of 6 participants (66.7%) reacted with the 16- (likely Jug r 1) and 28-kDa protein bands, respectively, whereas none of the participants had reactions to the 9-kDa protein band, which we suggest was Jug r 3 (Table 1 and Fig. 4).

Meanwhile, to investigate the cooked WN extracts with IgE-binding capacity, a 2D immunoblotting analysis was performed using the pooled sera of the WN-sensitized patients (Patient Nos. 1-20; Table 1). The results confirmed the presence of different binding patterns among children who consumed cooked WNs (Fig. 5).

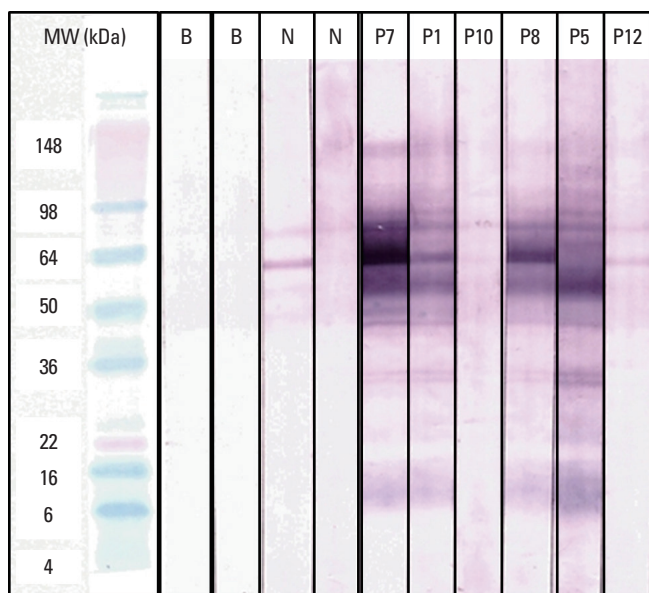


Fig. 4. Immunoblot analysis of raw walnut extract using the sera of patients with clinical walnut allergy (MW, molecular weight; B, BSA; N, negative control; P, patient).

Protein profiles of differently processed WN extracts

LC-MS/MS profiling of raw, dry-fried and boiled WN extracts revealed that 66 IgE-binding protein spots were observed in

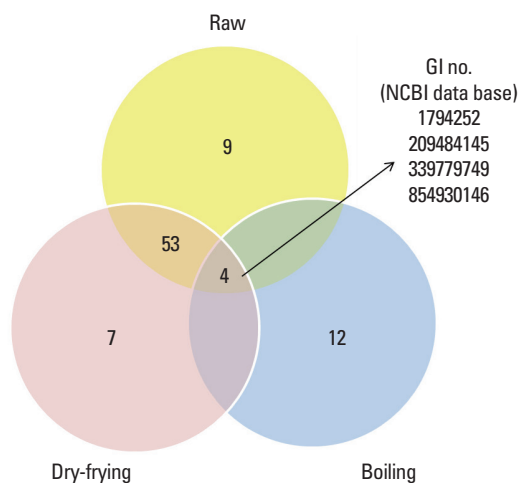


Fig. 6. Overview of the number of protein bands observed in processed walnut extracts via the proteomic approach. Out of the 66 proteins present in raw WNs, only 4 were observed in boiled WNs. GI, GenInfo; NCBI, National Center for Biotechnology Information non-redundant database.

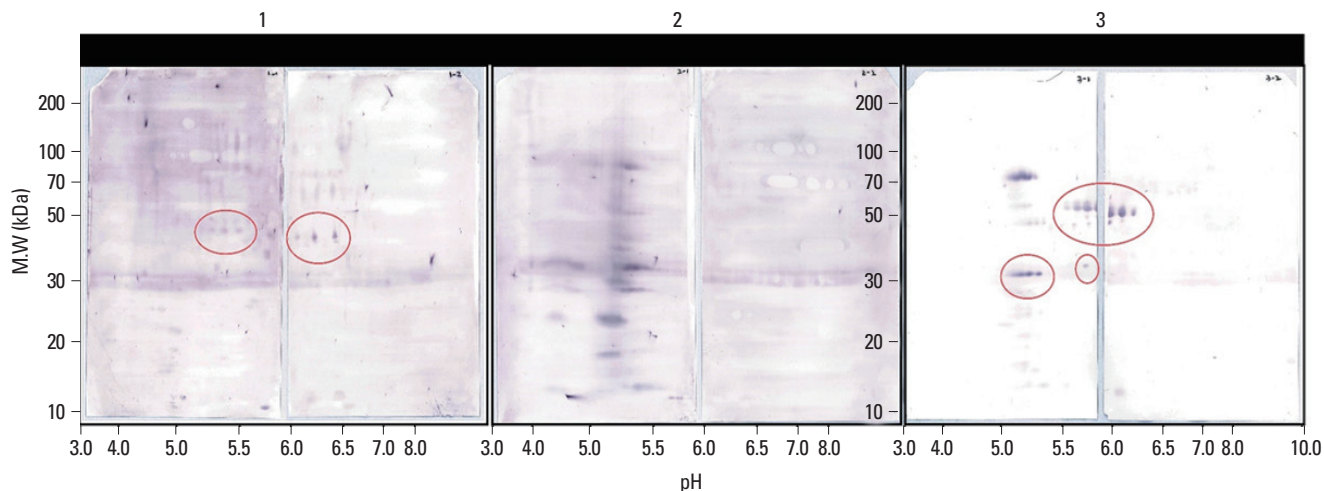


Fig. 5. 2D-Immunoblot analysis of processed walnut extract using walnut specific IgE positive pooled sera of patients (1, raw; 2, dry-frying; 3, boiling; M.W, molecular weight).

Table 2. Identification of differentially expressed proteins in processed walnut by LC-MS/MS

GI no.	1794252	209484145	339779749	854930146	56788031
Protein name	Albumin seed storage protein, Jug r 1	nsLTP, Jug r 3	Glyceraldehyde-3-phosphate dehydrogenase C subunit, partial	Superoxide dismutase 3 [Betula platyphylla]	Globulin seed storage protein, Jug r 4
Predicted molecular weight (kDa)	16.8	12.3	21.4	22.6	58.4
Number of peptides observed	4	5	3	3	58
% sequence coverage	88.9	60.0	66.7	100.0	84.4
Mascot score	68	76	74	116	1,731

LC-MS/MS, mass spectrometry, specifically liquid chromatography tandem mass spectrometry; GI, genInfo; LTP, lipid transfer protein; kDa, kilodalton.

raw WNs; of these, 57 were also observed in dry-fried WNs. In contrast, only 4 IgE-binding protein spots were found in boiled WNs (Fig. 6). The 4 proteins that remained stable after all forms of preparation had high identification scores based on the *J. regia* database and included the albumin seed storage protein (Jug r 1), nsLTP (Jug r 3), glyceraldehyde-3-phosphate dehydrogenase (G3PD) C subunit and superoxide dismutase (SOD). In addition, 7 proteins were newly observed in dry-fried WNs and 12 proteins that appeared spontaneously in boiled WNs included vicilin seed storage protein (Jug n 2) and globulin seed storage protein (Jug r 4) (Table 2).

DISCUSSION

Studies on antigenic changes in WNs due to heat treatments have been reported by a few institutions. The protein structure of Jug r 1 is known to be relatively stable at approximately 90°C, whereas Jug r 2 and Jug r 4 do not show antigenic changes when boiled for 5-10 minutes and are able to maintain their antigenicity when deep fried or microwaved at approximately 190°C.^{14,15} Nevertheless, another study reported that Jug r 2 and Jug r 4 lose their protein bands when roasted after boiling in water.¹¹ However, the aforementioned studies were not conducted on patients, and no studies have shown which allergenic components of cooked WNs cause serious reactions when consumed by children. In Korea, WNs are eaten with rice and usually boiled and stir-fried with seasoning. Accordingly, in this study, we investigated major WN allergens identified in Korean children and changes in the antigenicity of cooked WNs compared to raw WNs. Our aim was to identify the target component allergens in WNs in order to provide safer dietary advice to allergic children.

We analyzed antigenic changes in WNs cooked according to usual Korean methods (dry-frying and boiling) using SDS-PAGE, 2DE and LC-MS profiling. Our investigation of the effects of different cooking methods showed that 57 out of the 66 proteins found in untreated WNs were also identified in the dry-fried WNs, whereas only 4 out of the 66 proteins were identified in the boiled WNs. This shows that boiling resulted in lower antigenicity than dry-frying. Previous studies of component aller-

gens using recombinant allergen technology have found that storage proteins and LTP allergens in nuts are relatively stable to heat, which was consistent with the findings of the present study. After boiling, we were able to identify a 9-kDa protein (LTP), a 16-kDa protein (2S albumin), 21- and 23-kDa proteins (G3PD and SOD) remained unchanged. Also, Jug r 4, a member of the 11S globulin seed storage protein family, has been characterized by its distinct antigenicity, which newly appeared after boiling; this finding was consistent with our results from SDS-PAGE. The 2 allergens G3PD and SOD may have originated from birch or oak pollen, which acted as the primary sensitizer or may have cross-sensitized due to homology with WN allergens. Additional clinical studies on this topic are necessary.

When we analyzed individual immunoblots of IgE-binding proteins using blood serum from the 6 children with clinical WN allergies, our results indicated that the 16-, 28- and 52-kDa proteins, rather than the 9-kDa protein (LTP) of WN, are more likely to contribute to the adverse reactions among Korean children. In addition, in this study, the 52-kDa band almost completely disappeared with boiling. These findings suggest that when patients solely sensitized to 52-kDa protein, there is a low probability of consumption of cooked WNs prepared according to common Korean recipes causing serious allergic reactions and that the 16-kDa protein band (Jug r 1, storage protein) may be the most potent allergen among Korean children. Also, Jug r 1 was, as found in previous studies, generally stable under both cooking methods. Thus, clinicians should highlight this finding when educating their patients on the antigenicity of cooked WNs.

This study has several limitations. First, changes in antigenicity based on digestive enzymes were not considered. Thus, the study did not completely replicate in vivo conditions. Secondly and most importantly, although an *in vitro* study measuring IgE reactivity can give information on allergenicity, clinical studies using food challenges are considered to provide the highest level of evidence. Further research including prick-to-prick and food challenge tests, which can document changes in the eliciting cooked WN dose, are needed to determine the hypoallergenicity of processed WNs. Thirdly, the analysis had a limitation of including a small number of patients, thus being a

preliminary result.

Nevertheless, this study has several strengths. First, we have demonstrated that Korean children are highly likely to be sensitized to Jug r 1. We have also found that LTP is not a major allergen among this population. This result differs from those of previous studies mainly performed in Europe, and indicates which allergens need more focus in Korea. Secondly, when investigating antigenicity of the various WN proteins, we found that the storage protein Jug r 1 was stable under casual cooking methods. Previous studies that have focused on changes to the allergenicity of WNs have used cooking processes that do not replicate those used in everyday life. Although we found that the allergenicity of certain components of WN diminished, clearly cooked WNs must not be given to Korean children with WN allergies. Thirdly, the findings of this study support existing ones that the allergenic components of WNs have varying antigenicity depending on the cooking method. Therefore, the allergenic components of WNs identified via diagnostic tests of the allergic participants in this study could potentially be used to justify different dietary restrictions on WNs cooked in different ways.

In conclusion, we suggest that further studies are needed with a focus on the storage protein Jug r 1 in young Korean children for stable oral food challenges in high risk patients and the development of low-allergen WNs that can be cooked at home. In the future, these low-allergen WNs could be used for desensitization therapy of patients who are allergic to WNs. LTP allergen (Jug r 3) appears to be one of the candidate allergens causing severe reactions in patients with LTP syndrome,^{16,17} but not in this study. In order to draw more definite conclusions on LTP, further studies on older participants are needed. Additionally, since plant-derived panallergens, such as SOD and G3PD may be problematic for children allergic to nuts, future studies on these allergens, which have been neglected to date, are also necessary.

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REFERENCES

1. Jeong K, Lee SY, Ahn K, Kim J, Lee HR, Suh DI, et al. A multicenter study on anaphylaxis caused by peanut, tree nuts, and seeds in

- children and adolescents. *Allergy* 2017;72:507-10.
2. Lee SY, Ahn K, Kim J, Jang GC, Min TK, Yang HJ, et al. A multicenter retrospective case study of anaphylaxis triggers by age in Korean children. *Allergy Asthma Immunol Res* 2016;8:535-40.
3. Sicherer SH, Muñoz-Furlong A, Godbold JH, Sampson HA. US prevalence of self-reported peanut, tree nut, and sesame allergy: 11-year follow-up. *J Allergy Clin Immunol* 2010;125:1322-6.
4. Lee CH. Global tree nut statistical review. *World Agric* 2016;186:95-110.
5. Ahn K, Kim J, Hahm MI, Lee SY, Kim WK, Chae Y, et al. Prevalence of immediate-type food allergy in Korean schoolchildren: a population-based study. *Allergy Asthma Proc* 2012;33:481-7.
6. Kim M, Lee JY, Jeon HY, Yang HK, Lee KJ, Han Y, et al. Prevalence of immediate-type food allergy in Korean schoolchildren in 2015: a nationwide, population-based study. *Allergy Asthma Immunol Res* 2017;9:410-6.
7. Jeong K, Kim J, Ahn K, Lee SY, Min TK, Pyun BY, et al. Age-based causes and clinical characteristics of immediate-type food allergy in Korean children. *Allergy Asthma Immunol Res* 2017;9:423-30.
8. Pastorello EA, Farioli L, Pravettoni V, Robino AM, Scibilia J, Fortunato D, et al. Lipid transfer protein and vicilin are important walnut allergens in patients not allergic to pollen. *J Allergy Clin Immunol* 2004;114:908-14.
9. Downs ML, Simpson A, Custovic A, Semic-Jusufagic A, Bartra J, Fernandez-Rivas M, et al. Insoluble and soluble roasted walnut proteins retain antibody reactivity. *Food Chem* 2016;194:1013-21.
10. Cabanillas B, Maleki SJ, Rodríguez J, Cheng H, Teuber SS, Wallowitz ML, et al. Allergenic properties and differential response of walnut subjected to processing treatments. *Food Chem* 2014;157:141-7.
11. Su M, Venkatachalam M, Teuber SS, Roux KH, Sathe SK. Impact of γ -irradiation and thermal processing on the antigenicity of almond, cashew nut and walnut proteins. *J Sci Food Agric* 2004;84:1119-25.
12. Park KS, Kim H, Kim NG, Cho SY, Choi KH, Seong JK, et al. Proteomic analysis and molecular characterization of tissue ferritin light chain in hepatocellular carcinoma. *Hepatology* 2002;35:1459-66.
13. Simons FE, Ebisawa M, Sanchez-Borges M, Thong BY, Worm M, Tanno L, et al. 2015 update of the evidence base: World Allergy Organization anaphylaxis guidelines. *World Allergy Organ J* 2015;8:32.
14. Downs ML, Baumert JL, Taylor SL, Mills EN. Mass spectrometric analysis of allergens in roasted walnuts. *J Proteomics* 2016;142:62-9.
15. Sordet C, Culerrier R, Granier C, Rancé F, Didier A, Barre A, et al. Expression of Jug r 1, the 2S albumin allergen from walnut (*Juglans regia*), as a correctly folded and functional recombinant protein. *Peptides* 2009;30:1213-21.
16. Azofra J, Berroa F, Gastaminza G, Saiz N, Gamboa PM, Vela C, et al. Lipid Transfer Protein Syndrome in a non-mediterranean area. *Int Arch Allergy Immunol* 2016;169:181-8.
17. Pascal M, Muñoz-Cano R, Reina Z, Palacín A, Vilella R, Picado C, et al. Lipid transfer protein syndrome: clinical pattern, cofactor effect and profile of molecular sensitization to plant-foods and pollens. *Clin Exp Allergy* 2012;42:1529-39.