Clinical and immunological characterization of perilla seed allergy in children

Brief running title: Perilla seed allergy in children

Jeong K¹, Lee SY¹, Jeon SA¹, Gantulga P¹, Nam JY¹, Hong SJ², Lee SJ²
¹Department of Pediatrics, Ajou University School of Medicine, Suwon, Republic of Korea, ²Department of Pediatrics, Childhood Asthma Atopy Center, Humidifier Disinfectant Health Center, University of Ulsan College of Medicine, Seoul, Republic of Korea

Corresponding:

Sooyoung Lee
Department of Pediatrics, Ajou University School of Medicine
164 Worldcup-ro, Yeongtong-gu, Suwon, Gyeonggi-do, Republic of Korea 16499
E-mail: jsjs87@ajou.ac.kr

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.18176/jiaci.0756
ABSTRACT

Background: Perilla seeds are known to cause immediate allergic reactions. However, reports on perilla seed allergies are limited to a few case reports worldwide, and there is currently no diagnostic test for such allergies.

Objective: Our objective was to analyze the clinical and immunological characteristics of perilla seed allergy and to identify allergens for the development of diagnostic methods.

Methods: Twenty-one children with clinical perilla seed allergy were enrolled from two tertiary hospitals between September 2016 and June 2019. Using perilla seed extract, we developed a skin prick test (SPT) reagent and an IgE enzyme-linked immunosorbent assay (ELISA) for perilla seed allergy diagnosis. IgE immunoblotting was performed for identifying putative allergenic components, and amino acid composition analysis was performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Results: The median age of children with perilla seed allergy was 3 years; the proportion of children with anaphylaxis was 28.6%. Perilla seed SPT was performed in 15 of 21 children, all of whom tested positive. On ELISA, 85.7% of children tested positive for perilla seed-specific IgE. Proteins with molecular weights of 50, 31–35, and 14–16 kDa showed binding with the sera of >50% of children with perilla seed allergy. LC-MS/MS analysis of these three protein fractions indicated 8 putative proteins, including perilla oleosin (Accession No. 9963891), to be allergens.

Conclusion: This study documented the clinical characteristics and immunological profiles of 21 children with perilla seed allergy. Our results suggest that oleosin is one of the major allergens in perilla seeds.

Key words: allergen; seeds; perilla; anaphylaxis; amino acids; ELISA; Hypersensitivity; skin test
RESUMEN

Antecedentes: Las semillas de Perilla pueden causar reacciones alérgicas inmediatas. Sin embargo, existen escasos estudios, limitados a escasos casos clínicos, sin que existan pruebas diagnósticas para esta alergia alimentaria.

Objetivo: El objetivo de este trabajo es analizar las características clínicas e inmunológicas de los pacientes con alergia a semillas de Perilla e identificar los alérgenos responsables con el fin de desarrollar nuevos métodos diagnósticos.

Métodos: Se reclutaron 21 niños con alergia a semillas de perilla procedentes de dos hospitales entre septiembre de 2016 y 2019. Se realizaron prick test y determinación de IgE específica in vitro mediante Elisa utilizando un extracto de perilla. Igualmente se realizó immunoblotting para identificar los componentes alergénicos y determinar su composición mediante cromatografía líquida y espectrometría de masas (LC-MS/MS).

Resultados: Los niños con alergia a perilla tienen una mediana de edad de 3 años. El 28,6% de estos niños presentaron anafilaxia. Se realizó prick test con el extracto de perilla en 15/21 niños con resultado positivo en todos ellos. La IgE específica in vitro mediante Elisa fue positiva en el 85,7% de los casos. Más del 50% de los niños reconocían proteínas de 50, 31–35 y 14–16 kDa. El análisis mediante LC-MS/MS de estas tres fracciones identificó 8 proteínas diferentes, incluyendo una oleosina (Accesion No. 9963891), como posibles alérgenos.

Conclusiones: Este trabajo describe las características clínicas e inmunológicas de 21 niños con alergia a semillas de perilla. Nuestros resultados que una oleosina es uno de los alérgenos mayores en los pacientes con alergia a semillas de perilla.

Palabras clave: alérgeno; semillas; perilla; anafilaxia; aminoácidos; ELISA; Hipersensibilidad; pruebas cutáneas.
Background

Hypersensitivity reactions to seeds, including anaphylaxis, have been reported worldwide [1-3]. However, reports on seed allergy are limited and have mostly focused on sesame, sunflower, poppy, mustard, pumpkin, and flax seeds [4-8]. Clinical manifestations of seed allergy include immunoglobulin (Ig) E-mediated symptoms after ingestion, but various manifestations such as hypersensitivity reactions on inhalation, food-dependent exercise-induced hypersensitivity, and contact symptoms have been reported [9, 10].

Sesame and mustard seeds are widely consumed globally, and perilla seeds are commonly consumed in Asia including Korea. Perilla (Perilla frutescens) belongs to the Lamiaceae family (Supplementary Fig. 1), and perilla seeds are often added in homemade meals such as soups or porridges, and their continued intake gradually increases due to their nutritive health benefits. In a multicenter study of 126 cases of anaphylaxis due to peanuts, tree nuts, and seeds in Korean children, there were 7 cases of anaphylaxis triggered by seeds, with perilla seeds being the most common causative seed [11]. However, literature on perilla seed allergy is limited to a single case report of two anaphylaxis cases in English and a local case report in Korean [12, 13]. Other than reports on the anti-inflammatory or antioxidative effects of perilla seed oil, there are no reports on the immunologic properties of perilla seeds as allergenic sources [14, 15].

Skin prick test (SPT) and reasonably well-quantified serum specific IgE assays are mainly used for the diagnosis of food allergy, but there are no commercially available diagnostic kits for perilla seed allergies, unlike those for sesame, sunflower, and mustard other seeds. Detection of the major allergens in seeds is clinically important for the development of better diagnostic and therapeutic approaches. Most relevant studies have been conducted on sesame seeds, and
8 sesame seed allergens have been reported [16, 17]. The 11S globulin allergen is thought to be the potential major allergen in legumes, but reports suggest that oleosin may contribute to severe reactions to sesame seeds [17, 18]. To date, the major allergens in perilla seeds have not been registered by the World Health Organization and International Union of Immunological Societies Allergen Nomenclature Sub-committee.

Therefore, we aimed to analyze the clinical characteristics and immunological profiles of children with perilla seed allergy, develop appropriate diagnostic tests, and identify the major allergens in perilla seeds.

**Methods**

**Study subjects and sera**

Twenty-one children with clinical perilla seed allergy were included from the Department of Pediatrics in Ajou University Hospital and Asan Medical Center between September 2016 and June 2019. Diagnosis of perilla seed allergy based on a convincing history of immediate type allergic reactions, such as hives or anaphylaxis within 2 hours after perilla seed ingestion, was confirmed by experienced allergists after obtaining the patient’s detailed medical history. The diagnosis of anaphylaxis was based on the criteria published in 2006 by the National Institute of Allergy and Infectious Disease and the Food Allergy and Anaphylaxis Network. Patients were not included if perilla seeds were consumed with other foods for which previous tolerance was not clear or if only vague symptoms were observed. Six children who visited our institutions for atopic dermatitis or other food allergies but did not have any symptoms after
consumption of perilla seeds were also included as control subjects for SPT and immunologic studies. Blood samples were obtained from all participants, and the sera were frozen at -70 °C until use. The study was approved by the institutional review board (AJIUB-MED-KKKSP-19-545), and informed consent was received from the children’s parents.

**Preparation of perilla seed protein extract and development of a perilla seed-specific SPT**

Perilla (*Perilla frutescens*) seed powder was purchased from a local grocery store and was used for protein extraction. The powder was defatted using cold (4 °C) petroleum ether (1:1 w/v) by stirring constantly for 1 hour and was then filtered. The defatting procedure was repeated until the filtrate was clear. The defatted paste was air dried completely and added to phosphate-buffered saline (PBS) at 1:10 w/v and stirred for 4 days at 4 °C. The extracts were centrifuged at 19000 rpm for 1 hour, and the supernatants were dialyzed using 3.5-kDa pore size dialysis membranes for 56 hours. The obtained proteins were freeze-dried and stored at -70 °C until use. The protein concentrations were determined with the Bradford assay using a microplate reader (BioRad, Hercules, CA, USA). Participants underwent SPT using newly developed reagents, as perilla seed extracts for SPT are unavailable commercially. Pilot tests were performed using raw saline-squeezed perilla seed powder and perilla seed protein extracts at concentrations of 0.1, 0.5, and 1 mg/mL. Finally, perilla seed protein extract diluted with normal saline to 0.1 mg/mL was used for SPT in this study. SPT was performed according to the conventional method, and the elicited response was considered positive if the largest diameter was ≥3 mm larger than that of the negative control [19].
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting analysis, and perilla seed IgE-specific enzyme-linked immunosorbent assay (ELISA)

The perilla seed protein extract was analyzed using SDS-PAGE according to the protocol reported by Laemmli et al [20]. The procedures for SDS-PAGE and immunoblotting were performed in a similar manner to those in our study on walnuts, and detailed information on reagents and instruments is provided in Supplementary Table 1 [21]. Detection of allergen-specific IgE with ELISA was carried out using sera from 21 patients with perilla seed allergy and 6 control subjects in a similar manner to that in a previous study on chestnut allergy, and detailed information on reagents is provided in the Supplementary Table 1 [22].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) and quadrupole time-of-flight mass spectrometry (Q-TOF-MS)

Protein fractions with estimated molecular masses of 50, 31–35, and 14–16 kDa were excised from the SDS-PAGE gel, and their amino acid sequence was analyzed. Nano LC-MS/MS was performed using a nano high performance liquid chromatography system (Agilent, Wilmington, DE). A nano chip column (Agilent, Wilmington, DE, 150 mm × 0.075 mm) was used for peptide separation. Mobile phase A for LC separation was 0.1% formic acid in deionized water, and mobile phase B was 0.1% formic acid in acetonitrile. The chromatography gradient was designed for a linear increase from 3% to 45% of phase B in 30 minutes, 45% to 95% of B in 1 minute, at 95% of B in 4 minutes, and at 3% of B in 10 minutes. The flow rate was maintained
at 300 nL/min. Product ion spectra were collected in the information-dependent acquisition mode and were analyzed with Agilent 6530 Accurate-Mass Q-TOF using continuous cycles of one full scan TOF MS from 300 to 2000 m/z (1.0 s) plus three product ion scans from 150 to 2000 m/z (1.5 s each). Precursor m/z values were selected starting with the most intense ion, using a selection quadrupole resolution of 3 Da. The rolling collision energy feature was used, which determines collision energy based on the precursor value and charge state. The dynamic exclusion time for precursor ion m/z values was 60 seconds.

**Database search**

The Mascot algorithm (Matrixscience, USA) was used to identify peptide sequences present in a protein sequence database. Database search criteria included taxonomy (*Homo sapiens*), fixed modifications (carbamidomethylation at cysteine residues, variable modifications, oxidation at methionine residues), maximum allowed missed cleavage (2), MS tolerance (100 ppm), and MS/MS tolerance (0.1 Da). Only peptides resulting from trypsin digests were considered. The peptides were filtered with a significance threshold of p < 0.05, and those with Mascot scores > 54 were considered as promising hits.

**Results**

**Clinical characteristics of the patients**

A total of 21 children who had a convincing history of allergic reactions to a single ingestion episode of perilla seeds, without consuming other suspected foods at the same time, were
enrolled in this study. Age, sex, concomitant allergic history, and clinical manifestations are summarized in Table 1. The median age of the patients was 3 years (range, 14 months to 10 years). The proportion of boys was 66.7% (14/21). Six of 21 patients experienced anaphylaxis after exposure to perilla seeds. All 21 patients experienced immediate hypersensitivity reactions such as hives, angioedema, or anaphylaxis within 2 hours. Regarding medical history, atopic dermatitis was most the common condition in 71.4% of the children, followed by allergic rhinitis in 19.0% and asthma in 9.5%. Moreover, 18 of 21 children had food allergies other than perilla seed allergy, and 14 of them had a history of allergies to at least one plant food, including tree nuts, peanuts, fruits, and grains. The six control subjects were children (aged 1–5 years) who visited our Medical Center for atopic dermatitis or other food allergies, had no symptoms after ingestion of perilla seeds, and agreed to provide their sera.

Characterization of perilla seed extracts by SDS-PAGE

The electrophoretic separation of perilla seed protein extracts was determined by SDS-PAGE; analysis showed more than 10 protein bands (Fig. 1a), among which those with molecular weights of approximately 6, 16, and 26 kDa showed a relatively higher intensity.

SPT and detection of IgE by ELISA

The SPT was performed in 15 of 21 (71.4%) patients and in 1 of 6 (16.7%) control subjects. All 15 patients tested positive, whereas the control subject tested negative. The mean wheal
diameter induced by the perilla seed extract was 6.8 mm (range: 3.5 to 11.5 mm; Supplementary Table 2).

We found that sera from 18 of 21 (85.7%) were positive for perilla seed-specific IgE (median: 0.672, range: -0.015 to 2.530; Supplementary Table 2) using ELISA. The three patients who tested negative for perilla seed-specific IgE by ELISA all tested positive in the SPT and had wheal diameters greater than 3 mm. All six control subjects tested negative for the perilla seed-specific IgE by ELISA.

**IgE reactivity of perilla seed extract based on immunoblotting analysis**

Immunoblotting results using serum collected from each of the 21 patients with clinical perilla seed allergy showed that IgE-binding proteins had molecular weights ranging from 14 kDa to 55 kDa (Fig. 1b). The numbers of immunoreactive sera for each fraction are represented in Supplementary Table 3. The sera of all 21 patients had IgE-bound proteins of 31–35 kDa, and more than 50% of the patients had IgE-bound proteins of estimated molecular masses of 50 and 14–16 kDa. These fractions were considered potential major allergens, and therefore, were subsequently analyzed to determine their amino acid composition.

**Identification of immunoreactive peptides by LC-MS/MS**

LC-MS/MS analysis identified five putative proteins from the 50-kDa fraction (protein band No. 2 in Fig. 1b), three proteins from the 31–35-kDa fraction (protein band No. 3), and two proteins from the 14–16-kDa fraction (protein bands No. 7–8). The identified proteins are listed
in Supplementary Table 4, with the total scores and sequence coverage rates indicating the certainty of identification of each protein candidate. The protein with the highest Mascot score included spectrum matching peptides for dihydrolipoyl dehydrogenase 2 and chloroplastic isoform X1 of sesame (*Sesamum indicum*), based on the homology in amino acid composition. A comprehensive Mascot search against perilla proteins in the NCBI_nr database identified a peptide sequence matching oleosin (*Perilla frutescens*) (Accession No. 9963891), present in all three fractions.

**Discussion**

Our study describes the clinical and immunological aspects of perilla seed allergy. Considering that studies on perilla seed allergy are limited, our findings, which analyzed more than 20 patients with perilla seed allergy, substantially contribute to our understanding of the clinical and immunological aspects of this seed allergy.

Previously reported cases of perilla seed allergy mainly involved adults [12], whereas the median age of patients in this study was 3 years. Although the overall prevalence of food allergies is higher in children than in adults, that of sesame seed allergy was shown to be similar between adults and children [23]; thus, further observational studies are needed on the epidemiology of perilla seed allergies. The higher proportion of boys in this study is consistent with the previously reported higher prevalence of allergic diseases, including food allergy, in boys, although findings are often contradictory [24, 25]. Similar to that in reports on other plant food allergies in children, most children in our study had food allergies other than perilla seed allergy, and concomitant atopic dermatitis was more common than concomitant allergic rhinitis.
or asthma, which are common in young children [26-28].

The rate of anaphylaxis in this study was 28.6%, similar to the average rate of anaphylaxis in children with immediate type food allergies [29, 30]; however, the rate was slightly lower than that in patients with allergies to some plant foods, such as buckwheat and tree nuts, and higher than that in patients with common food allergies, such as allergies to cow’s milk, hen’s egg, and legumes [29]. The anaphylaxis rate among patients with other seed allergies has not been reported, but anaphylaxis cases have been described among those with allergies to sesame, poppy, and mustard seeds [2, 8, 31]. The severity of allergic symptoms is affected by various factors, including the patient’s age, the amount of intake, the degree of heating or processing of a specific food, cofactors such as exercise and acute viral illness, and the immunologic characteristics of the allergen itself. Among plant food allergens, storage proteins act as strong allergens and are highly likely to cause severe allergic reactions. Therefore, additional in-depth studies are required on patient symptoms and sensitization patterns in perilla seed allergy.

SDS-PAGE analysis of perilla seed extract followed by immunoblotting analysis revealed eight antigenic fractions with molecular weights ranging from 14 to 55 kDa, with the most common fractions (more than 50% of patients) having molecular weights of 14–16, 31–35, and 50 kDa. In contrast, a previous report of two cases of anaphylaxis caused by perilla seed reported one IgE-binding component with a molecular weight of 21 kDa [12]. This discrepancy might be attributed to differences in the age and number of study subjects between the studies as the two patients included in the previous report were both adults in their twenties while our study subjects were children with a median age of 3 years and the highest age of 10 years.

Allergenic proteins identified in seeds are restricted to Ses i 1-8 in sesame seeds, Sin a 1-4 in
yellow mustard seeds, and Hel a 3 in sunflower seeds [8, 17, 32]. Families of storage proteins, such as 2S albumin and 11S globulin, have been reported to be mainly involved in allergy as they are present in high amounts in seeds and are chemically stable. In this study, we did not identify proteins considered to belong to 2S albumin or 11S globulin families other than one protein with an estimated molecular weight of 56.7 kDa, which shared a peptide with the precursor of 11S globulin of pistachio (Pistacia vera).

Oleosins are alkaline-containing proteins with molecular weights of 15–30 kDa and constitute the most abundant proteins in oil bodies. Their functional role is to stabilize triacylglycerol-containing oil bodies in the aqueous cytoplasm, and they are known as one of the “lipophilic” allergens with hydrophobic binding sites for lipid ligands, inducing Th2 immunomodulation [33, 34]. Data regarding the allergenic potency and sensitization patterns of seed oleosins are generally rare. So far, only oleosins of sesame seeds, peanuts, and hazelnuts have been registered as allergens [18, 34, 35]. Our LC/MS-MS analysis confirmed considerable peptide sequence identity with the oleosin of perilla, suggesting that oleosin is the major candidate allergen in perilla seeds. Further research is needed to decipher the role of oleosins in allergy to perilla seeds and other plant foods and especially in inducing severe symptoms.

There are some limitations in our study. First, the selection of patients in this study was mainly based on the children’s clinical history rather than on DBPCFC test results. However, to minimize overdiagnosis or underdiagnosis, we selected patients through detailed history collection by experienced food allergists, and all 21 subjects tested positive for perilla seed-specific IgE either by ELISA and/or by SPT. Second, the molecular investigation of perilla seed allergen is yet at a preliminary stage and IgE reactivity of recombinant allergens was not

J Investig Allergol Clin Immunol 2023; Vol. 33(1) © 2021 Esmon Publicidad
doi: 10.18176/jiaci.0756
demonstrated; this should be addressed in future studies.

In summary, we reported the clinical and immunological profiles of children with perilla seed allergy in a systematic manner. The development of a perilla seed-specific SPT is also an important point of this study. Moreover, we suggested the novel perilla seed allergen candidate, which was presumed to be oleosin. Since the identification and characterization of new major allergens in food are the basis for improving diagnostic accuracy, major allergen quantification, and understanding of possible cross-reactivity at the molecular level, this study sets the basis for future studies in understanding and diagnosing perilla seed allergy.

**Funding source:** This study was supported by SAMA academic research grant from the Korean Academy of Pediatric Allergy and Respiratory Disease.

**Disclosure of potential conflict of interest:** All authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

**Presentation:** The abstract and results of this study were presented at the European Academy of Allergy and Clinical Immunology Annual Congress in 2020.
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Table 1. Clinical profiles of 21 patients with perilla seed allergy

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age at 1st Reaction (years)</th>
<th>Clinical phenotype</th>
<th>Food types ingested</th>
<th>Concomitant allergic history</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>M</td>
<td>3</td>
<td>Anaphylaxis (angioedema, cough, dyspnoea)</td>
<td>Seasoning of vegetables† (lightly roasted)</td>
<td>None</td>
</tr>
<tr>
<td>P2</td>
<td>M</td>
<td>4</td>
<td>Anaphylaxis (angioedema, dyspnoea)</td>
<td>Soup (boiled)</td>
<td>AD, FA (HE, peach, TNs)</td>
</tr>
<tr>
<td>P3</td>
<td>F</td>
<td>3</td>
<td>Urticaria</td>
<td>Oil</td>
<td>AD, FA (CM)</td>
</tr>
<tr>
<td>P4</td>
<td>M</td>
<td>5</td>
<td>Erythema, angioedema</td>
<td>Soup (boiled)</td>
<td>AD, AR, FA (HE)</td>
</tr>
<tr>
<td>P5</td>
<td>M</td>
<td>3</td>
<td>Erythema</td>
<td>Not available</td>
<td>AD, FA (HE, wheat)</td>
</tr>
<tr>
<td>P6</td>
<td>F</td>
<td>4</td>
<td>Erythema, urticaria</td>
<td>Seasoning of vegetables† (lightly roasted)</td>
<td>FA (HE, CM, barley, kiwi)</td>
</tr>
<tr>
<td>P7</td>
<td>F</td>
<td>3</td>
<td>Urticaria</td>
<td>Topping of noodles‡ (lightly roasted)</td>
<td>AD, AR, FA (HE, CM, PN, TNs, BW)</td>
</tr>
<tr>
<td>P8</td>
<td>M</td>
<td>4</td>
<td>Anaphylaxis (angioedema, vomiting)</td>
<td>Porridge (boiled)</td>
<td>FA (TNs, salmon)</td>
</tr>
<tr>
<td>P9</td>
<td>M</td>
<td>1</td>
<td>Urticaria</td>
<td>Soup (boiled)</td>
<td>AD, FA (HE, PN)</td>
</tr>
<tr>
<td>P10</td>
<td>F</td>
<td>1</td>
<td>Anaphylaxis (urticaria, dyspnoea)</td>
<td>Seasoning of vegetables† (lightly roasted)</td>
<td>AD</td>
</tr>
<tr>
<td>P11</td>
<td>F</td>
<td>2</td>
<td>Urticaria</td>
<td>Not available</td>
<td>AD, FA (HE, TNs, shrimp, fish)</td>
</tr>
<tr>
<td>P12</td>
<td>M</td>
<td>4</td>
<td>Urticaria</td>
<td>Not available</td>
<td>None</td>
</tr>
<tr>
<td>-----</td>
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<td>-----------</td>
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</tr>
<tr>
<td>P13</td>
<td>F</td>
<td>10</td>
<td>Anaphylaxis (urticaria, dyspnoea)</td>
<td>Soup (boiled)</td>
<td>FA (PN)</td>
</tr>
<tr>
<td>P14</td>
<td>M</td>
<td>4</td>
<td>Urticaria, angioedema</td>
<td>Seasoning of vegetables†</td>
<td>AD, AR, FA (HE, TNs)</td>
</tr>
<tr>
<td>P15</td>
<td>M</td>
<td>2</td>
<td>Erythema, urticaria</td>
<td>Soup (boiled)</td>
<td>AD, FA (Crustaceans)</td>
</tr>
<tr>
<td>P16</td>
<td>M</td>
<td>3</td>
<td>Anaphylaxis (urticaria, dyspnoea)</td>
<td>Seasoning of vegetables† (lightly roasted)</td>
<td>Asth, AR, FA (HE, CM, plum, watermelon)</td>
</tr>
<tr>
<td>P17</td>
<td>M</td>
<td>4</td>
<td>Urticaria</td>
<td>Soup (boiled)</td>
<td>AD, Asth, FA (HE)</td>
</tr>
<tr>
<td>P18</td>
<td>M</td>
<td>1</td>
<td>Urticaria</td>
<td>Topping of noodles‡ (lightly roasted)</td>
<td>AD, FA (HE, CM)</td>
</tr>
<tr>
<td>P19</td>
<td>F</td>
<td>2</td>
<td>Urticaria</td>
<td>Soup (boiled)</td>
<td>AD, FA (HE, BW, PN)</td>
</tr>
<tr>
<td>P20</td>
<td>M</td>
<td>2</td>
<td>Urticaria</td>
<td>Soup (boiled)</td>
<td>AD, FA (HE, CM)</td>
</tr>
<tr>
<td>P21</td>
<td>M</td>
<td>2</td>
<td>Urticaria</td>
<td>Not available</td>
<td>AD, FA (kiwi)</td>
</tr>
</tbody>
</table>

AD, atopic dermatitis; AR, allergic rhinitis; Asth, asthma; BW, buckwheat; CM, cow’s milk; F, female; FA, food allergy; HE, hen’s egg; M, male; PN: peanut; TN, tree nut

† “Seasoning of vegetables” means a recipe for adding lightly roasted perilla seed power to the seasoning of boiled vegetables

‡ “Topping of noodles” means a recipe for sprinkling lightly roasted perilla seed power on top of the noodles
Figure legends

Figure 1a. SDS-PAGE analysis of perilla seed protein extracts

Labeling with Coomassie Brilliant Blue R-250. M, standard molecular weight marker.
Figure 1b. Immunoblot membranes of 21 samples from patients with perilla seed allergy (P1-P21), 6 negative control samples (N1-N6), a negative pooled serum (NP) sample, and a positive pooled serum (PP) sample. The molecular weight corresponding to each protein band number is as follows; (1) 55 kDa, (2) 50 kDa, (3) 31-35 kDa, (4) 29 kDa, (5) 26 kDa, (6) 22 kDa, (7) 16 kDa, (6) 14 kDa. M, standard molecular weight marker; B, blank.