



Letter

Identification of peamaclein as a marker allergen related to systemic reactions in peach allergy

Peach is one of the most prevalent causes of plant-derived food allergies in Japan and Western countries. To date, most peach allergies have been observed in 1 of 3 distinct types of individuals¹: those sensitized primarily to birch pollen as the result of cross-reactivity between the major birch pollen allergen, Bet v 1, and a peach homologous protein, Pru p 1²; those characterized by IgE reactivity to multiple pollen extracts that corecognize the plant pan-allergen profilin, such as Pru p 4 in peaches³; and, particularly in Mediterranean countries, individuals who are allergic to the nonspecific lipid transfer protein (LTP) Pru p 3.⁴ Pru p 3 has been considered a severity marker for peach allergy in Europe. Peamaclein was recently reported as a new peach allergen, Pru p 7, in Italy.⁵ In the present study, we investigated the correlation between clinical severity and the sensitization to Peamaclein and LTP in Japanese patients with peach allergy.

Consecutive patients diagnosed as having peach allergy seen at the Dermatology Department of Yokohama City University Hospital in Yokohama during the years 2001 to 2010 were enrolled in the present study. When the patients with a convincing history of allergic reactions within 30 minutes after the ingestion of peach alone had positive results on skin prick tests (SPTs) with fresh peach, specific IgE measurements for peach using ImmunoCAP (Phadia, Uppsala, Sweden), and/or challenge tests with peaches, they were diagnosed as having peach allergy. This study was approved by our institutional review board, and written informed consent related to the study was given by all patients.

Depending on their symptoms after they ingested the peach sample, the patients were divided into 2 groups: the systemic reaction group, in which the patients developed symptoms in more than 2 organs (7 men and 7 women; age range, 18–60 years; mean age, 32.0 years; patients 1–14), and the oral group, in which oral symptoms alone were revealed after peach ingestion (4 men and 12 women; age range, 7–55 years; mean age, 32.3 years; patients 15–30). In 3 patients in the systemic reaction group and all 16 patients in the oral group, open challenge tests with fresh peaches were performed. Four systemic reaction patients (patients 1–4) experienced anaphylactic shock. Five of the 14 patients (35.7%) in the system reaction group and 15 of the 16 patients (93.8%) in the oral group were found to be sensitized to alder pollens. Allergy due to Rosaceae family fruits was accompanied by peach allergy in 12 of the 14 patients (85.7%) in the systemic reaction group and 14 of the 16 patients (87.5%) in the oral group. In the oral group, the reactions to fruits other than peaches

were similar to the reactions to peaches. In the systemic reaction group, Japanese apricots induced severe reactions, as did peaches, but other fruits induced oral symptoms or urticaria alone.

To prepare peach crude extract, whole peach fruits (*Prunus persica*, cultivar Asama-Hakutou strain) were homogenized with an extraction solution (see eMethods, eResults, and eFigs 1 and 2).⁶ Then Peamaclein and LTP were purified from the crude extract using monoclonal antibody technology.

In the IgE immunoblotting, the IgE-binding band to Peamaclein was more frequently detected in the systemic group (6 of 13 [46.2%]) than in the oral group (1 of 16 [6.3%]), but the IgE-binding band to LTP was not detected in either group (eMethods, eResults, and eFigs 1 and 2). The prevalence of systemic reactions showed a significant positive correlation with the positivity of Peamaclein ($P < .05$).

The prevalence of positive ImmunoCAP results for peach was lower in the systemic group (6 of 14 [42.8%]) than in the oral group (13 of 16 [81.3%]). As shown in Figure 1, the pattern for the positivity of IgE specific to the allergen components in the systemic group is in contrast to that in the oral group. The positivity shown by ImmunoCAP for rPru p 1 and rPru p 4 (68.7% and 56.2%, respectively) was higher in the oral group than in the systemic group (28.6% and 0%, respectively). The prevalence of oral symptoms alone was significantly positively correlated with sensitization to rPru p 4 ($P < .05$) but not to rPru p 1 ($P = .07$). A positive response for specific IgE against rPru p 3 was detected in only 1 patient (patient 8) in the systemic group. In contrast, in an enzyme-linked immunosorbent assay the positivity for IgE specific to Peamaclein was 64.3% (9 of 14 patients) in the systemic group and 12.6% (2 of 16 patients) in the oral group (E-material). The positivity for IgE specific to the purified LTP was 14.3% (2 of 14 patients) in the systemic group and 0% in the oral group. The prevalence of systemic reactions was significantly positively correlated with sensitization to Peamaclein ($P < .01$) but not to LTP ($P = .21$). In addition, 3 of the 4 patients who experienced anaphylactic shock had positive results for specific IgE for Peamaclein but not for LTP.

In the SPTs, positive responses for crude extract, the purified Peamaclein, and LTP were observed in 4 (80%), 4 (80%), and 2 patients (40%) of the 5 in the systemic group and in 2 (40%), 0, and 0 of the 5 in the oral group, respectively. The prevalence of systemic reactions in peach allergy indicated a significant positive correlation with positive results in SPTs with Peamaclein ($P < .05$) but not with LTP ($P = .44$).

We have identified Peamaclein as a marker associated with the systemic reactions of peach allergy rather than LTP in Japan because the prevalence of systemic reactions in peach allergy was significantly correlated with positive results for Peamaclein in IgE immunoblotting, enzyme-linked immunosorbent assay, and SPTs.

Peamaclein is a small basic protein (approximately 7 kDa) and the first allergen among antimicrobial peptides according to

Dr Inomata and Ms Okazaki contributed equally to this work.

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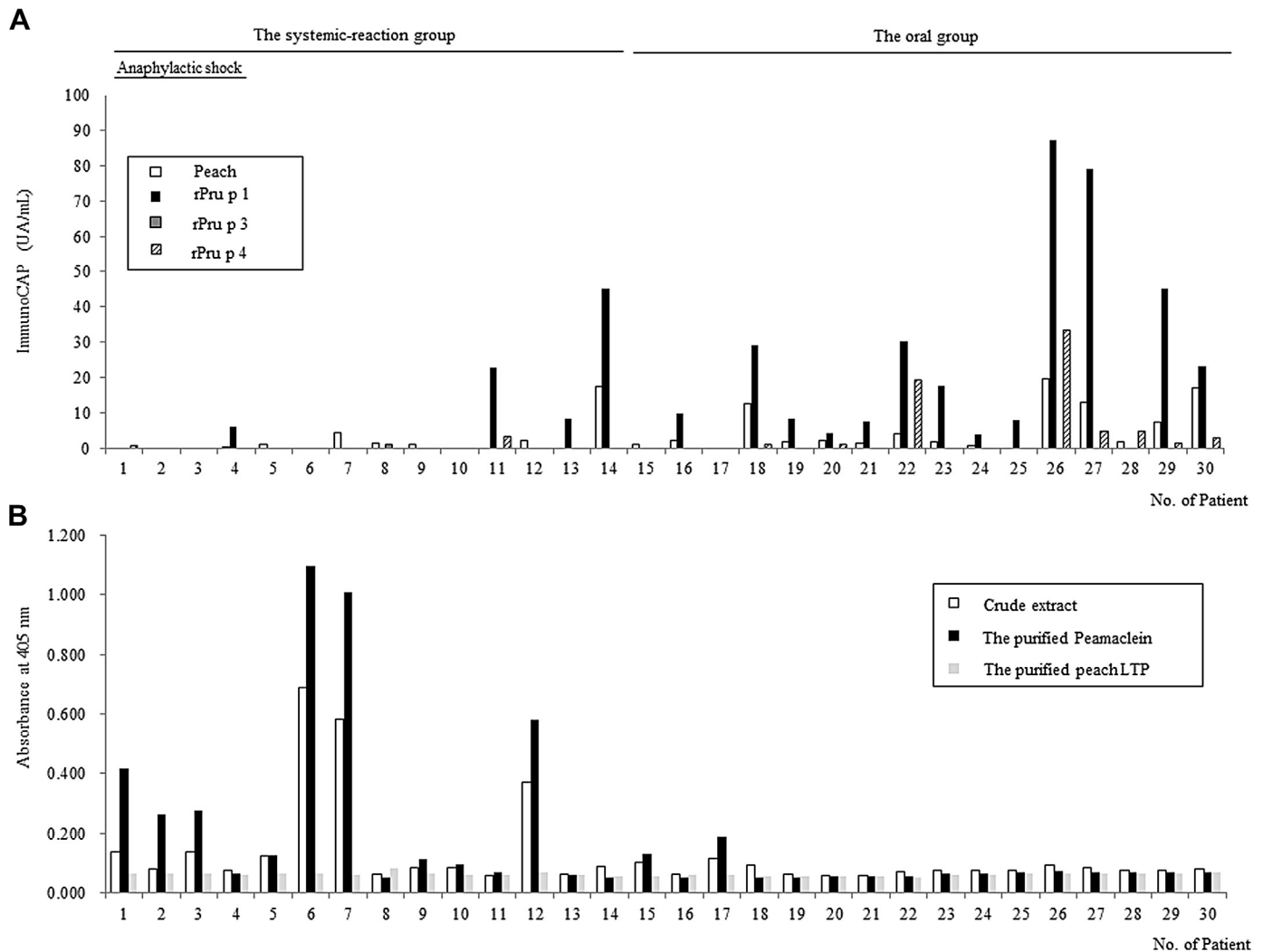


Figure 1. Measurements of IgE specific to peach, rPru p 1, rPru p 3, and rPru p 4 by ImmunoCAP (A) and allergic activity of the purified Peamaclein and lipid transfer protein (LTP) by enzyme-linked immunosorbent assay (B).

classifications of biological functions.^{5,7} Peamaclein has an unusually high content of cysteine (19% of total residues), resulting in heat stability and digestion resistance, as does LTP.^{8,9} Peamaclein, like LTP, is thus assumed to induce severe reactions after ingestion.

We speculate that the low prevalence of sensitization to LTP in Japan is due to differences in eating habits and the distribution of allergens in peaches rather than differences in peach cultivars. Interestingly, the distributions of Peamaclein and LTP in peaches were thought to differ. In an earlier study, Peamaclein was confirmed to be distributed in both the peach pulp and peel by both a homology search using the *N*-terminal sequence of Peamaclein to query the Expressed Sequence Tags database by the BLAST algorithm at NCBI (www.ncbi.nlm.nih.gov/Blast.cgi) and a microarray assay using purified Peamaclein.⁵ In contrast, LTP is located exclusively in the peel of peaches.¹⁰ The Japanese population generally eats the peach pulp alone after peeling the fruit and consequently may be rarely sensitized to LTP, contained exclusively in the peel. However, people who often eat the peach without peeling, as in Western countries, might be sensitized to LTP in addition to Peamaclein.

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Supplementary Data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.anai.2013.11.003>.

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e-Methods

Preparation of Peach Crude Extract

Peaches (*Prunus persica*, cultivar Asama-Hakutou strain) at the commercial ripening stage were obtained from a local store. Whole peach fruits (peel and pulp) were homogenized with an extraction solution (2-mmol/L disodium ethylenediamine tetracetate, 10-mmol/L sodium N, N-diethyldithiocarbamate, 3-mmol/L sodium azide, and 2% suspended solid polyvinyl polypyrrolidone at a 1:1 [wt:vol] ratio).¹ After filtration through gauze, the homogenate was centrifuged at 10,000g for 15 min at 4°C. To the supernatant, cation exchange resin (Toyopearl CM 650M; TOSO, Tokyo, Japan) was added and mixed overnight at 4°C. The resin was collected by centrifugation, packed, and washed with 20-mmol/L phosphate buffer at pH 5.0 in a column. The proteins absorbed to the resin were eluted with 0.5-mol/L sodium chloride in the same buffer as the crude extract. The protein concentration was quantified with a DC Protein Assay kit (BioRad, Bradford, United Kingdom). Crude extract was sterilized by filtration through a 0.22- μ m pore diameter membrane (Millipore, Bedford, Massachusetts) and frozen at –80°C.

SDS-PAGE and IgE Immunoblot Analysis

Extracted proteins from peach or purified peach proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (15% [wt:vol], 10 μ g per slot) under nonreducing or reducing conditions using the electrophoresis system MiniProtein-3 (BioRad, Hercules, California) according to the method of Laemmli.² For SDS-PAGE under reducing conditions, the samples were mixed with 0.1M Tris, pH 6.8, containing 4% (wt/vol) SDS, 20% (wt/vol) glycerol, 10% (wt/vol) 2-mercaptoethanol, and 0.02% (wt/vol) bromophenol blue. The samples were denatured by heating at 100°C for 5 minutes. To ensure proper protein separation and visualization, proteins on the gel were stained with Coomassie Brilliant Blue R-350 (GE Healthcare, Fairfield, Connecticut).

The proteins separated on the SDS-PAGE gel under nonreducing conditions were transferred onto an Immobilon-P polyvinylidene difluoride membrane (Millipore) by semidry blotting methods. The membrane was incubated in 10mM phosphate-buffered saline (PBS) (pH 7.5) that contained 0.1% Tween 20 and 5% skim milk for blocking. For the immunoblotting using patient sera, 1:20 diluted sera in the same blocking buffer was used as the primary antibody, and horseradish peroxidase–labeled goat anti-human IgE (KPL, Washington, DC) was used as a second antibody. Final signal detection was performed using the ECL Western blotting detection kit (GE Healthcare), following the manufacturer's instructions, and recorded onto X-ray film (Hyperfilm MP; GE Healthcare).

Purification of Peach Peamaclein and LTP

To obtain monoclonal antibodies (mAbs) specific to peach Peamaclein or LTP, we established hybridomas essentially as described previously.³ Eight-week-old, female, BALB/c mice (Nippon SLC Co, Shizuoka, Japan) were immunized by intraperitoneal injection of 50 μ g of proteins in crude extract emulsified in Freund complete adjuvant, 20 μ g in Freund incomplete adjuvant, and 10 μ g in PBS at 2-week intervals. Hybridomas that produced IgG reactive to crude extract were cloned twice with the limiting dilution method and inoculated into pristine-primed mice. Then mAbs were prepared from the ascitic fluids and purified on Protein G–Sepharose columns (GE Healthcare). The mAbs specific to peach Peamaclein and LTP were selected among them by an immunoblotting analysis with crude extract under nonreducing conditions. The animal experiments were performed under the guidelines of the Animal Experiment Committee of Kyoto Women's University following a bulletin (No. 71, 2006) from the Ministry of Education, Culture, Sports, Science, and Technology in Japan.

Peamaclein or LTP was purified from crude extract using a HiTrap NHS-activated HP Column coupled with each mAb, according to the manufacturer's instructions (GE Healthcare). The purified protein bands related to 9 kDa and 14 kDa on SDS-PAGE under nonreducing conditions were confirmed to be Peamaclein and LTP by N-terminal amino acid sequence analysis.

N-Terminal Amino Acid Sequence Analysis

Protein bands in SDS-PAGE under no-reducing conditions were electroblotted onto a polyvinylidene difluoride membrane as described above. After staining with Coomassie Brilliant Blue, the protein bands corresponding to 9 and 14 kDa were excised and analyzed on a 476A gas-phase protein sequencer (Applied Biosystems, Foster City, California), following the manufacturer's recommendations. The obtained sequences were analyzed by the Phytozome and BLAST programs to identify the proteins in the databases.

Determination of Specific IgE by Enzyme-Linked Immunosorbent Assay and ImmunoCAP

Specific IgE antibody to crude extract, purified Peamaclein, or LTP was detected using enzyme-linked immunosorbent assay as described with some modifications.³ The wells of a microtiter plate (Nunc A/S, Roskilde, Denmark) were coated with crude extract (10 μ g/mL), purified Peamaclein (2 μ g/mL in PBS), or LTP (2 μ g/mL in PBS) at 50 μ L per well.

After blocking, the individual sera (1:10 dilution with the solution containing 0.1% bovine serum albumin, 0.1% Tween 20, 0.15M sodium chloride, and 10mM Tris-HCl (pH 7.4) were added at 50 μ L per well and incubated for 1 hour at 37°C. Then IgE antibodies bound to the antigen were detected using alkaline phosphatase–labeled, anti-human IgE (American Qualex, San Clemente, California), and *p*-nitrophenylphosphate (1 mg/mL) as the substrate. Absorbance at 405 nm was read with a microplate reader (BioRad). Control sera from 3 nonatopic individuals without food allergies were used as negative controls. Specific IgE levels greater than 0.12, 0.08, and 0.07 absorbance units (a mean $\pm$ 10 SDs absorbance of the negative control value) were considered positive for crude extract, Peamaclein, and LTP, respectively. All tests were performed in triplicate.

Serum total IgE and specific IgE levels (ImmunoCAP; Phadia, Uppsala, Sweden) for foods, including peach, pollens of alder, and recombinant allergens, such as rPru p 1, rPru p 3, and rPru p 4, were measured. The cutoff value of the assay was 0.7 kUA/L as class 2.

Statistical Analysis

Correlations between the clinical characteristics of peach allergy and sensitization to the allergens were analyzed using the Fisher exact test. $P < .05$ was considered significant.

eResults

Identification of Peamaclein and LTP

Extracted proteins from peach were subjected to SDS-PAGE under reducing or nonreducing conditions. A single intense band at 9 kDa under reducing conditions, which was suspected to be LTP, was clearly divided into 2 bands at the molecular weights of 9 and 14 kDa under nonreducing conditions (eFig 1).

To identify these 2 protein bands they were subjected to N-terminal amino acid sequence analysis. The sequence of the 9-kDa protein band on the SDS-PAGE of crude extract under nonreducing conditions was GSXFXDSKXGVRHSKAGYTE, which corresponded to Peamaclein (accession No. P86888), registered to the World Health Organization and International Union of Immunological Societies Allergen Nomenclature Subcommittee in October 2012. The sequence of the 14-kDa protein band was ITXGQVSSXLAPPYVVPV, which was found to be clearly identical to peach LTP (accession No. P81402) by a homology search performed in UniprotKB. The same sequence was

obtained for Peamaclein or LTP purified on a respective immunoaffinity column (eFig. 1).

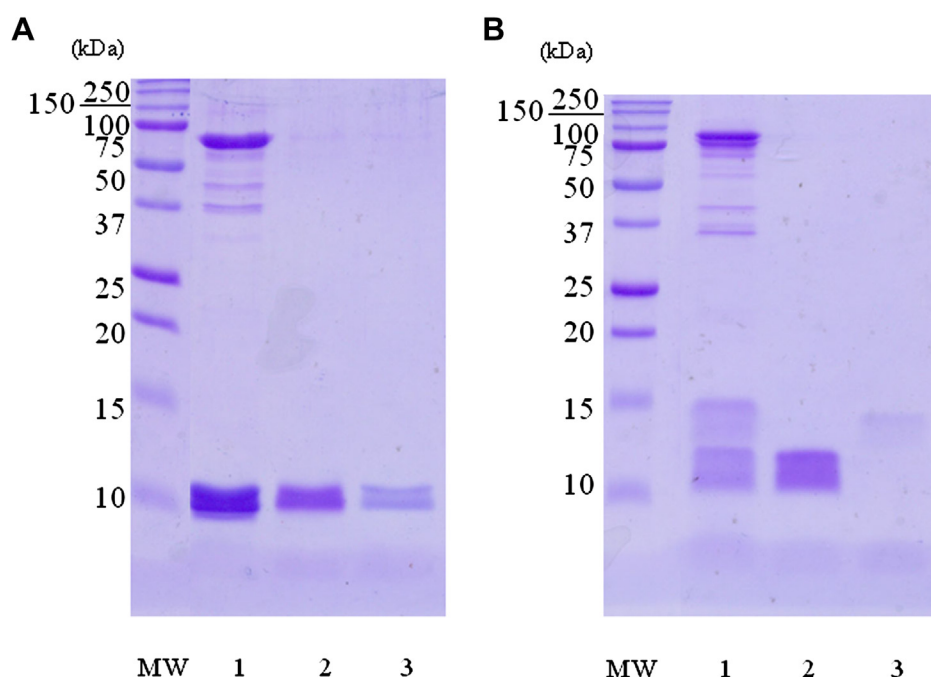
IgE Immunoblotting

As shown in eFigure 2, in the IgE immunoblotting, the serum IgE from 21 (72.4%), 5 (17.2%), and 0 (0%) of the 29 patients who provided sufficient serum samples for experiments (except patient 3) bound to proteins in crude extract, purified Peamaclein, and LTP, respectively, whereas that from the nonatopic controls did not. The IgE-binding band to Peamaclein was more frequently detected in the systemic group (6 of 13 patients [46.2%]; patients 1,

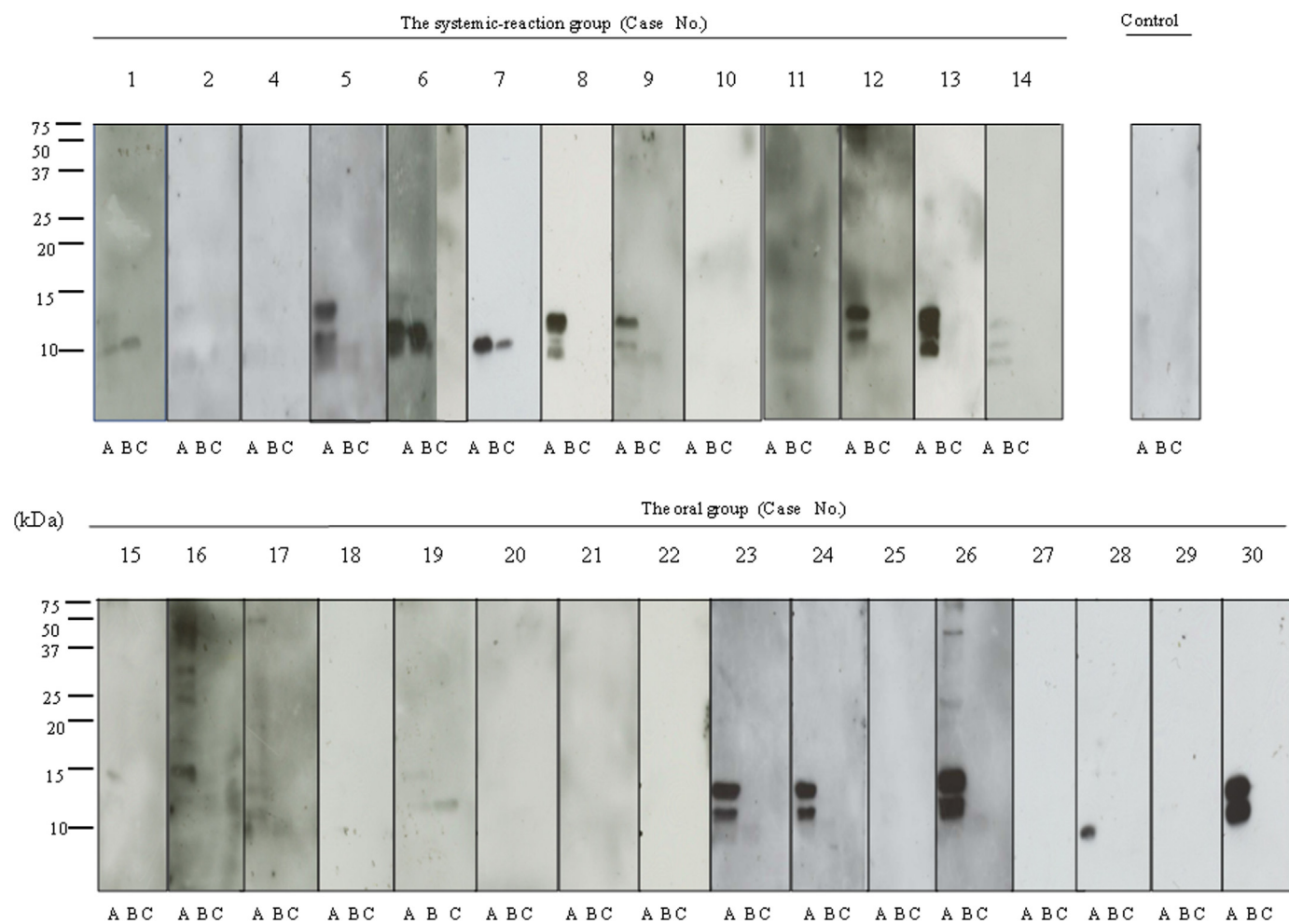
5, 6, 7, 9, and 12) than in the oral group (1 of 16 patients [6.3%]; patient 17).

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eFigure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis under reducing conditions (A) and nonreducing conditions (B). Separation of crude extract (lane 1), purified Peamaclein (lane 2), and lipid transfer protein (lane 3) from peaches. MW indicates molecular weight marker (in kilodaltons).



eFigure 2. eFigure 2 IgE immunoblotting with crude extract, purified Peamaclein, and lipid transfer protein. Top, Systemic group and control results. Bottom, Oral group results.