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Original Article

Protease-digested egg-white products induce oral tolerance in mice but elicit little IgE production upon epicutaneous exposure

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 EW, egg white; HPLC, high performance
 liquid chromatography; MLN, mesenteric
 lymph node; mMCP-1, mouse mast cell
 protease-1; OVA, ovalbumin;
 OVM, ovomucoid; PDEW, pepsin-digested
 egg-white product; qPCR, quantitative PCR;
 SDEW, Protin SD-AY10-digested egg-white
 product; TDEW, Thermoase PC10F-digested
 egg-white product; 3DEW, Protease P
 "Amano" 3SD-digested egg-white product

ABSTRACT

Background: Early food introduction induces tolerance, but epicutaneous exposure, especially via eczema lesions, promotes IgE sensitization. Aiming for safe and effective primary prevention of egg allergy, we examined several protease-digested egg-white (EW) products for three properties: 1) induction of oral tolerance that prevents IgE sensitization, 2) weak IgE binding that can prevent allergic reactions even in IgE-sensitized mice, and 3) minimal epicutaneous IgE sensitization even when in contact with inflamed skin.

Methods: Heated EW was digested with several proteases under optimal conditions. First, three-week-old BALB/c female mice were intragastrically administered EW or each protease-digested EW product, followed by intraperitoneal ovalbumin (OVA) or ovomucoid (OVM) injection with alum. Serum OVA- and OVM-specific IgE titers were measured. Second, six-week-old mice were sensitized with OVA/OVM, and the rectal temperature was measured after intraperitoneal administration of EW or each protease-digested EW. Third, EW or each protease-digested EW product was applied to the tape-stripped skin for 3 days/week for 3 weeks. Serum OVA- and OVM-specific IgE titers were measured.

Results: Orally administered pepsin-digested EW product (PDEW) and Thermoase PC10F-digested EW product (TDEW) significantly suppressed OVA-/OVM-specific IgE production. Neither product elicited a body temperature decline (anaphylaxis) in OVA-/OVM-sensitized mice. Serum OVA-/OVM-specific IgE levels were significantly lower in mice epicutaneously exposed to PDEW or TDEW than in EW-exposed mice.

Conclusions: Two protease-digested EWs showed potential as optimal EW products for early introduction for primary prevention of egg allergy.

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Introduction

The prevalence of food allergy has been increasing worldwide in recent decades.¹ Hen's egg is one of the most common food allergens in children.² Recent studies have elucidated the mechanisms behind the onset of food allergy: Early food introduction induces tolerance, whereas epicutaneous exposure, especially via eczema

lesions, promotes IgE-sensitization.³ Recent randomized controlled trials, i.e., the LEAP study⁴ and PETIT study,⁵ proved that early introduction of food proteins prevents the development of food allergies.

However, early introduction can encounter two problems. First, some babies—especially those with eczema—may already be sensitized and respond to foods even at the initial introduction.⁶ Second, exposure to food proteins—especially via eczema lesions—may cause new sensitization.⁶ The PETIT study succeeded in preventing egg allergy, at least in part, through successful treatment of eczema with a proactive regimen.⁵ However, complete eczema treatment is not always easy to accomplish in babies,⁷ because sensitization may occur due to exposure via

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erythematous lesions.⁸ To start early egg introduction safely and effectively, we need a new egg product having the following three properties. 1) Induction of oral tolerance that prevents IgE sensitization later in life. 2) Low IgE binding, which can prevent allergic reactions even in IgE-sensitized babies. 3) Low epicutaneous IgE sensitization even when in contact with inflamed skin.

Protease digestion is a potential approach: pepsin-digested ovalbumin (OVA; Gal d 2)⁹ and ovomucoid (OVM; Gal d 1)¹⁰ and endopeptidase-digested egg white (EW)¹¹ reportedly had lower IgE-binding activities than the intact proteins. In addition, oral administration of pepsin-digested OVA prevented cholera toxin-induced oral EW sensitization.¹² However, no protease-digested EW products have been tested for epicutaneous IgE sensitization. Moreover, several proteases other than pepsin have been approved for industrial use.

Here, we used mouse models to examine several protease-digested EW products for the aforementioned three properties.

Methods

Proteins and protease digestions

Porcine pepsin was purchased from Sigma–Aldrich; Merck (Kenilworth, NJ, USA). Food proteases (Thermoase PC10F, whose main component is thermolysin derived from *Bacillus stearothermophilus*; Protease P “Amano” 3SD derived from *Aspergillus melleus*; and Protin SD-AY10 derived from *Bacillus licheniformis*) were obtained from Amano Enzyme Inc. (Nagoya, Japan). EW powder (Kewpie Egg, Tokyo, Japan) was dissolved in sterile water (20 mg/mL) and heated at 100 °C for 10 min. Each digestive enzyme was used under its optimal conditions based on the recommendations of the manufacturers or our preliminary experiments. Pepsin-digested egg-white product (PDEW) was prepared by adding pepsin (2 wt%) to EW at pH 1.5 and 37 °C for 3 h. Thermoase PC10F-digested egg-white product (TDEW) was prepared by adding Thermoase PC10F (5 wt%) to EW at pH 8.0 and 65 °C for 3 h. Protease P “Amano” 3SD-digested egg-white product (3DEW) was prepared by adding Protease P “Amano” 3SD (6.7 wt%) to EW at pH 8.0 and 40 °C for 3 h. Protin SD-AY10-digested egg-white product (SDEW) was prepared by adding Protin SD-AY10 (8.3 wt%) to EW at pH 9.0 and 60 °C for 3 h. Each protease-digested EW product was adjusted to pH 7.0 after the enzymatic treatment and subsequently boiled for 15 min to inactivate the enzyme.

To evaluate the digestion, EW and the digested products were separated on a column (Superdex peptide 10/300 GL, GE Healthcare Life Science, UK) by high-performance liquid chromatography (HPLC, Shimadzu, Kyoto, Japan). The samples were eluted with 0.1% (vol/vol) trifluoroacetic acid and 30% (vol/vol) acetonitrile in distilled water and measured at a wavelength of 214 nm with LcSolution Software (Shimadzu).

Mice

Wild-type BALB/c female mice were purchased from SLC Inc. (Shizuoka, Japan) and housed under specific-pathogen-free conditions at the National Research Institute for Child Health and Development. All animal experiments were approved and performed in accordance with the animal care and use committees of the National Research Institute for Child Health and Development.

Oral tolerance induction and intraperitoneal egg sensitization

Figure 1A shows the experimental schedule for the oral tolerance tests. In brief, three-week-old mice were intragastrically

administered 2 mg/day of EW or each protease-digested EW product for 5 consecutive days. Two days later, the same product was intragastrically administered for 5 more days. The control group mice were administered sterile water. One week after the last administration, mice were injected intraperitoneally with OVA (grade V; Sigma–Aldrich; Merck) and OVM (Trypsin inhibitor from chicken Type II-O; Sigma–Aldrich; Merck) at 5 µg/g mouse body weight together with alum (Imject™ Alum Adjuvant; Thermo Fisher Scientific, Waltham, MA, USA), twice, 2 weeks apart. Sera were collected 13 days after the second injection. The mesenteric lymph nodes (MLN) were harvested from each mouse 7 days after blood collection.

Epicutaneous sensitization

Epicutaneous sensitization was performed as described elsewhere.¹³ In brief, the dorsal skin of six-week-old mice was shaved with electric clippers, depilated using a depilatory cream (Veet®; Reckitt Benckiser, Slough, Berkshire, UK), and then tape-stripped 3 times using a transparent film dressing (Cellotape™; Nichiban, Tokyo, Japan) on the same day. After that, a 1 cm × 2 cm lint fabric (Hakujiji, Tokyo, Japan) containing a 200-µL aliquot of 20 mg/mL EW, PDEW, TDEW or sterile water alone (control) was applied to the tape-stripped skin with a bandage (Multipore; 3M, St. Paul, MN, USA) for 3 days and then removed. One week later, a fresh lint fabric having the same content was applied to the same skin site. This treatment was repeated a third time. Sera were collected 8 days after the final treatment. Each mouse's spleen was harvested one day after blood collection.

Intraperitoneal sensitization and challenge for anaphylaxis (body temperature decline)

Six-week-old mice were intraperitoneally injected with OVA and OVM, both at 5 µg/g body weight together with alum, twice, two weeks apart. Pre-challenge sera were collected from the mice 12 days after the second injection. Only mice whose serum level of OVA-specific IgE was ≥50 ng/mL were used for the subsequent challenge (see below).

Two weeks after the last injection, mice were challenged intraperitoneally with EW, PDEW or TDEW at 200 µg/g body weight. The control group mice were challenged with sterile water (10 µL/g body weight). The rectal temperature of each mouse was measured using a digital thermometer (TD-300®; Shibaura, Saitama, Japan) at 0, 10, 20, 30, 40, 50 and 60 min after undigested- or digested-EW administration. Serum was collected from each mouse at 60 min post-challenge, and the concentration of mouse mast cell protease-1 (mMCP-1) was measured with an ELISA kit (Thermo Fisher Scientific).

Measurement of antigen-specific immunoglobulin levels in sera

The concentration of OVA-specific IgE in each serum was measured by ELISA, as described elsewhere.¹⁴ In brief, 96-well plates (Nunc-Immuno™ Plate; Thermo Fisher Scientific) were coated with 10 µg/mL OVA or OVM at 4 °C overnight. After washing with PBS containing 0.05% Tween 20 (Promega, Madison, WI, US), the wells were blocked with PBS containing 10% FCS. Optimally-diluted serum samples (IgE = 1:25; OVA-sIgG1 = 1:100,000; OVM-sIgG1 = 1:10,000; OVA-sIgG2a = 1:100 or 1:1000; OVM-sIgG2a = 1:100) were applied, and the plates were incubated at room temperature for 1 h. After washing, biotinylated anti-mouse IgE (clone R35-118; BD Biosciences, Franklin Lakes, NJ, USA), biotinylated anti-mouse IgG1 (clone A85-1; BD

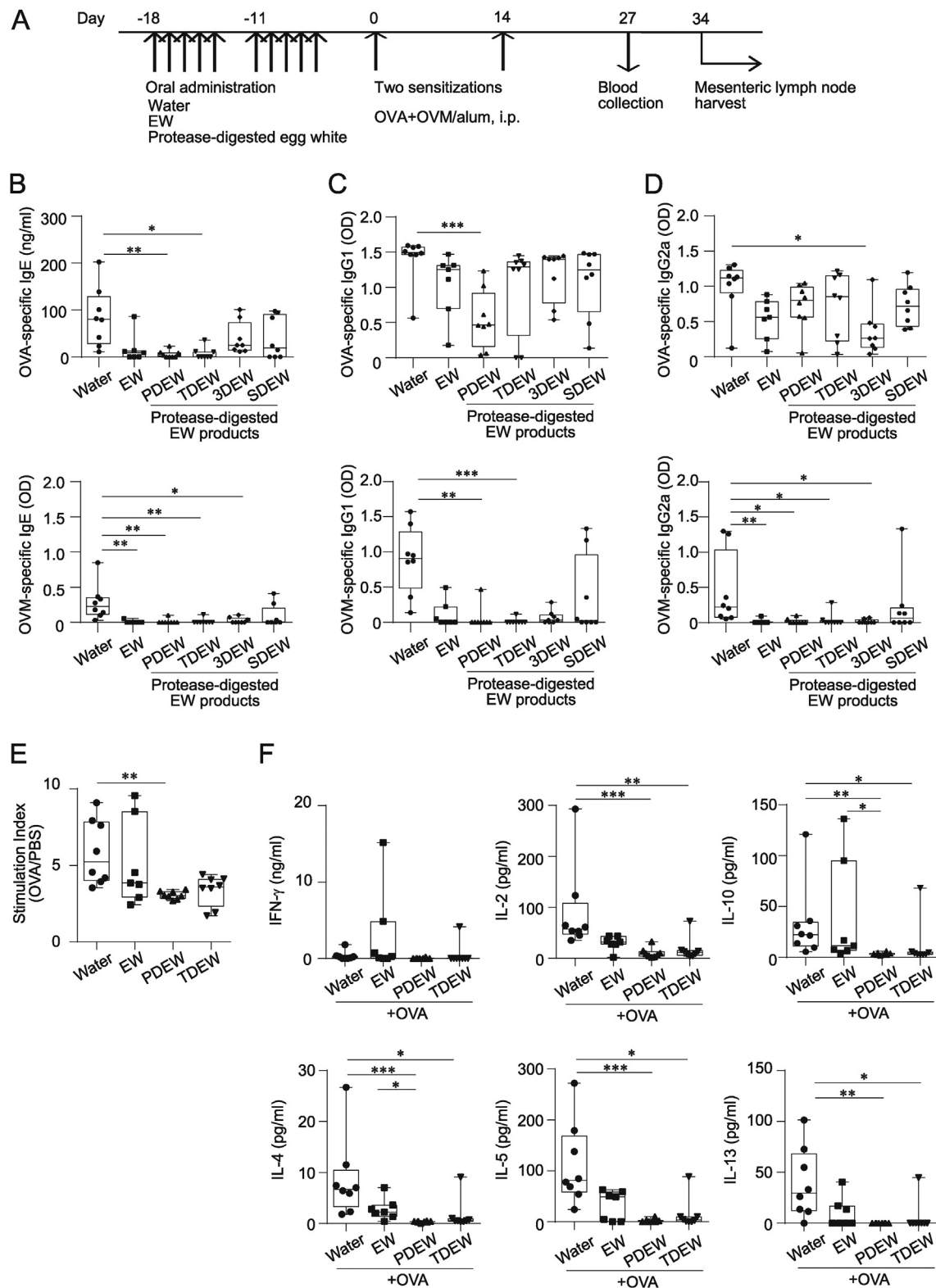


Fig. 1. Protease-digested egg-white products induced oral tolerance. (A) The protocol for oral tolerance induction and intraperitoneal sensitizations. Three-week-old female BALB/c mice were intragastrically administered heated egg white (EW), protease-digested EW products (pepsin-digested EW product: PDEW; Thermoase PC10F-digested EW product: TDEW; Protease P "Amano" 3SD-digested EW product: 3DEW; and Protin SD-AY10-digested EW product: SDEW) or water (control) 5 times/week for 2 weeks (day -18 to 0), followed by intraperitoneal injection of ovalbumin (OVA) or ovomucoid (OVM) together with alum twice, 2 weeks apart. Blood samples were collected from the animals' cheeks on day 27. All mice were sacrificed on day 34. Serum concentrations of OVA- or OVM-specific IgE (B), IgG1 (C) and IgG2a (D) antibodies. OVA-induced cell proliferation (E) and cytokine production (F) by mesenteric lymph node (MLN) cells from intragastrically-administered mice. The levels of cytokines in the culture supernatants were measured by ELISA or Multiplex. The data are shown as the median and the ranges; * $P < .05$, ** $P < .01$, *** $P < .001$ vs. indicated group ($n = 7-8$).

Biosciences) or biotinylated anti-mouse IgG2a (clone R19-15; BD Biosciences) was added, and the plates were incubated at room temperature for 1 h. Then, after washing, horse radish peroxidase-conjugated streptavidin (Merck) was added, followed by incubation at room temperature for 1 h. For enzymatic reaction, tetramethylbenzidine (TMB) substrate (SeraCare Life Sciences Inc., Milford, MA, USA) was used as a substrate, and 1 mol/L H_3PO_4 was used as a stop solution. The absorbance at 450 nm was measured using a plate reader (FlexStation 3; Molecular Devices, San Jose, CA, USA).

Antigen-induced cell proliferation and cytokine production of MLN cells and spleen cells

The MLN cells and spleen cells were filtered through a 70- μ m cell strainer (Falcon®; Corning, NY, USA). Only spleen cells underwent erythrocyte lysis with a lysing buffer (Red blood cell lysing buffer; Sigma–Aldrich; Merck) for 5 min at 4 °C. The MLN cells and spleen cells were suspended at 2×10^6 cells/mL in RPMI1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FCS (fetal calf serum; Biological Industries, Beit Haemek, Israel), 100 U/mL penicillin and 100 mg/mL streptomycin (Thermo Fisher Scientific) with/without OVA (200 μ g/mL) or with/without OVM (200 μ g/mL), and cultured for 72 h in 96-well plates. Half of each supernatant was collected, and the cytokine concentrations in each were measured by ELISA (Thermo Fisher Scientific) for IL-13 and IFN- γ , and with a multiplex bead array system (Milliplex®; Merck) for IL-2, IL-4, IL-5 and IL-10.

After the supernatant collection, the cells were cultured with 37 kBq [^3H] thymidine (Japan Radioisotope Association, Tokyo, Japan) for 16 h, and [^3H] thymidine incorporation was measured with a scintillation counter (PerkinElmer, MA, USA). The stimulation index was calculated as [^3H] thymidine incorporation in OVA- or OVM-stimulated cells/[^3H] thymidine incorporation in PBS-stimulated cells.

qPCR (TSLP, IL-25, IL-33)

Dorsal skin of mice was harvested 4 h after tape-stripping, exposed to samples and stored in RNeasy lysis buffer (Qiagen, Venlo, Netherlands). Total RNA was extracted from the dorsal skin samples using a miRNeasy mini kit (Qiagen). Quantitative PCR (qPCR) for cytokine mRNA expression was performed as described elsewhere¹³ using the following primer pairs: TSLP: Fwd 5'-CAATCC-TATCCCTGGCTGCC-3' and Rev 5'-TGTGCCATTCTCTGAGTACCGT-3'; IL-25: Fwd 5'-GGCATTCTACTCAGGAACGGA-3' and Rev 5'-GGTGGAGAAAGTGCTGTGC-3'; IL-33: Fwd 5'-CAGGCCTTCTTCGT CTTTAC-3' and Rev 5'-TCTCTCCACTAGAGCCAGCTG-3'; and GAPDH: Fwd 5'-CCACTCTTCCACTTCGATG-3' and Rev 5'-AGGTC-CACCACCTGTTGCT-3'. The mRNA expression levels were normalized to the GAPDH level in each sample. All primers were synthesized at FASMAC (Kanagawa, Japan).

Statistics

The data are shown as the median and the ranges. Differences between groups were examined for statistical significance by the Kruskal–Wallis test followed by Dunn's multiple comparisons test unless otherwise noted. Data on rectal temperature changes were analyzed by 2-way ANOVA with Tukey's post-test. Statistical evaluations were performed using GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA), and $P < .05$ was regarded as significant.

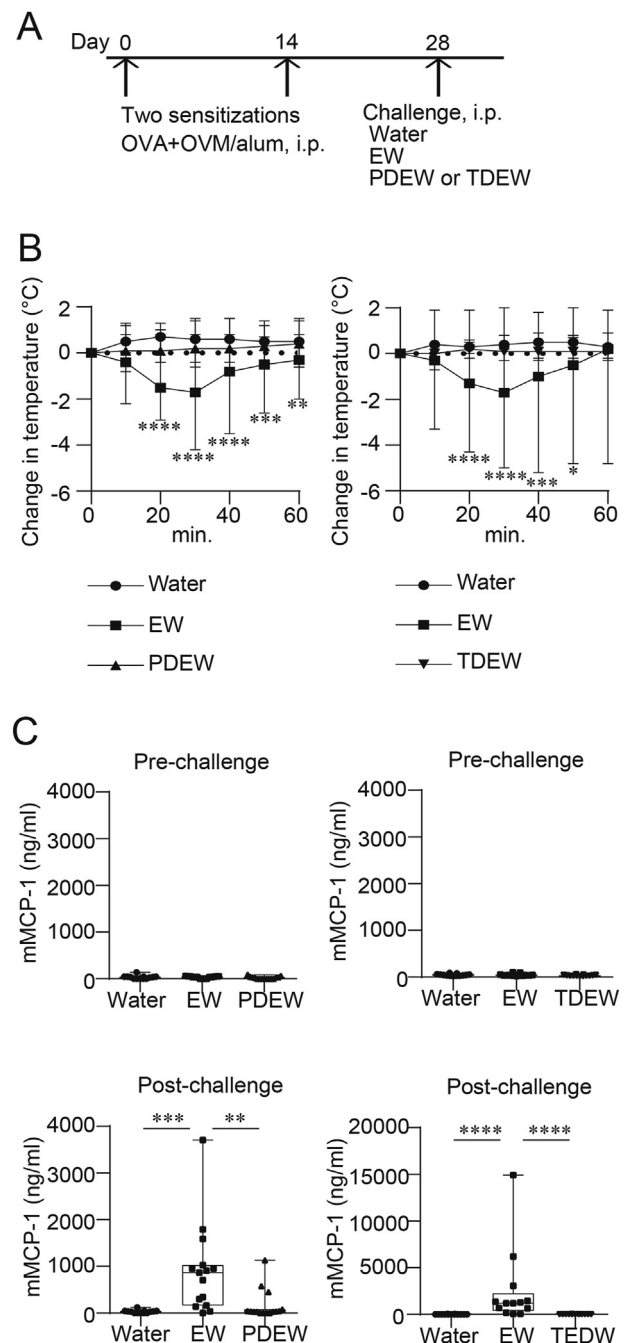


Fig. 2. Intraperitoneal challenge of IgE-sensitized mice with heated egg white (EW), pepsin-digested EW product (PDEW) or Thermoase PC10F-digested EW product (TDEW). (A) The protocol for intraperitoneal sensitizations and challenge. (B) The rectal temperature was measured after intraperitoneal challenge with EW and PDEW (left) or TDEW (right). (C) Serum mouse mast cell protease-1 (mMCP-1) concentrations of before (upper panels) and after (lower panels) challenge. The rectal temperature was measured after intraperitoneal challenge with EW and PDEW (left), or EW and TDEW (right). Blood was collected 2 days before (pre) and 60 min after (post) intraperitoneal challenge. The data are shown as the median and the ranges; * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$ vs. indicated group ($n = 13–15$).

Results

Digestion of EW protein by enzymes

EW, PDEW, TDEW, 3DEW and SDEW were analyzed by HPLC. A high-molecular-weight peak was seen in EW but not in any of the

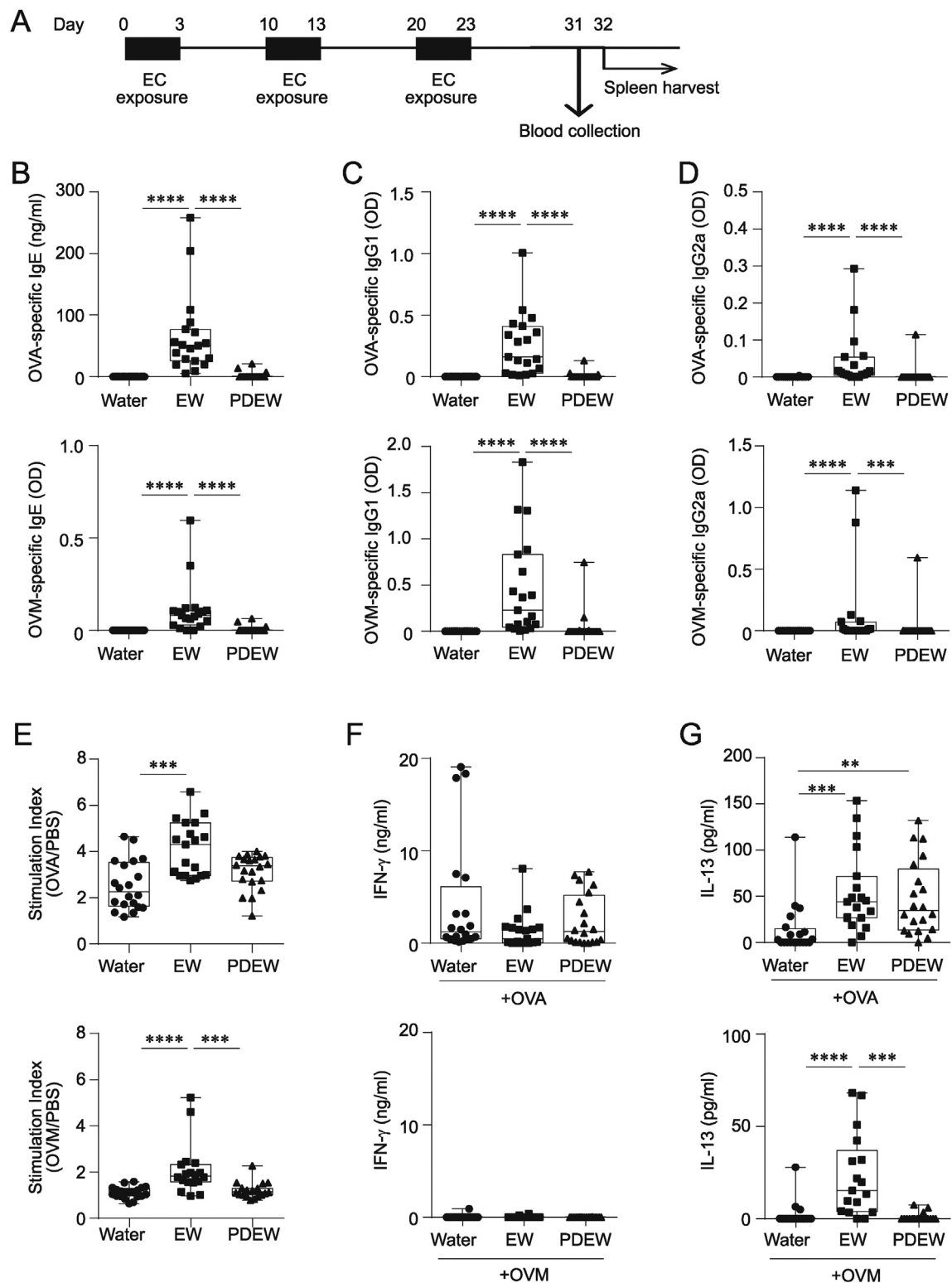


Fig. 3. The effects of pepsin digestion on epicutaneous sensitization with egg white (EW). (A) The protocol for epicutaneous sensitization. The dorsal skin of six-week-old female BALB/c mice was shaved and tape-stripped 3 times. Lint fabric containing heated EW or pepsin-digested EW product (PEDW) was applied to the skin site for 3 consecutive days repeatedly at 1-week intervals, for a total of 3 times. Blood samples were collected from the animals' cheeks on day 31. All mice were sacrificed on day 32. The serum concentrations of ovalbumin (OVA)- or ovomucoid (OVM)-specific IgE (B), IgG1 (C) and IgG2a (D) antibodies were measured by ELISA. OVA- or OVM-induced cell proliferation (E), IFN- γ production (F) and IL-13 production (G) by spleen cells from epicutaneously-sensitized mice are shown. The levels of cytokines in the culture supernatants were measured by ELISA. The data are shown as the median and the ranges; * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$ vs. indicated group ($n = 19-20$).

digested EW products, which showed various low-molecular-weight peaks (Supplementary Fig. 1).

Intragastric administration of protease-digested EW products prevented intraperitoneal OVA/OVM sensitization

Intragastric administration of PDEW or TDEW to mice prior to intraperitoneal OVA/OVM injection with alum significantly reduced their serum OVA- and OVM-specific IgE levels compared with the distilled-water-administered control mice (Fig. 1B). Intragastric administration of 3DEW also significantly reduced the OVM-specific IgE level, whereas the OVA-specific IgE level was unchanged. Intragastric administration of SDEW showed no such effects. These results suggest that PDEW and TDEW, but not 3DEW or SDEW, induce sufficient oral tolerance for achieving the objective of primary prevention. Therefore, in our subsequent experiments we investigated only PDEW and TDEW. Notably, PDEW and TDEW also significantly suppressed OVM-specific IgG1 and IgG2 (Fig. 1C,D), suggesting that this suppression is not IgE-specific.

MLN cells from intraperitoneal OVA-/OVM-administered mice showed significant proliferation and also significant IL-2, IL-4, IL-5 and IL-13 production when stimulated with OVA *in vitro* (Fig. 1E,F). Prior intragastric administration of PDEW showed significant suppression of MLN cell proliferation and also their IL-2, IL-4, IL-5 and IL-13 production upon OVA stimulation. Prior intragastric administration of TDEW also showed significant suppression of those cytokines, but MLN proliferation was not significantly suppressed. The groups showed no significant differences in IFN- γ production (Fig. 1F) or the proportion of Foxp3+ T cells in MLN (data not shown). Interestingly, IL-10 production was also suppressed in PDEW- and TDEW-administered mice, suggesting that this suppression is not due to induction of OVA-specific, IL-10-producing regulatory T cells.

Protease-digested EW products did not elicit a body temperature decline (anaphylaxis) in OVA-/OVM-sensitized mice

We next investigated whether there was a body temperature decline (anaphylaxis) when mice were intraperitoneally challenged 2 weeks after intraperitoneal OVA/OVM injection with alum (Fig. 2A). EW-challenged mice showed a significant body temperature decline, whereas PDEW- and TDEW-challenged mice did not (Fig. 2B). The serum mMCP-1 concentration increased after EW challenge but not after challenge with PDEW, TDEW or water (Fig. 2C).

Pepsin-digested EW product did not induce IgE production upon epicutaneous exposure

To investigate whether the protease-digested EW products induce epicutaneous sensitization, mice were exposed to EW, PDEW or TDEW via skin inflammatory lesions (Fig. 3A). The serum OVA- and OVM-specific IgE levels were significantly lower in the PDEW-exposed mice than in the EW-exposed mice (Fig. 3B). The serum OVA- and OVM-specific IgG1 and IgG2a levels were also significantly lower in the PDEW-exposed mice (Fig. 3C,D).

Upon stimulation with OVA or OVM *in vitro*, spleen cells from EW- but not PDEW-exposed mice showed significant proliferation (Fig. 3E). In addition, upon stimulation with OVA *in vitro*, spleen cells from epicutaneously EW- and PDEW-exposed mice produced significant IL-13 but not IFN- γ (Fig. 3F,G). The results for IL-4 and IL-5 were the same as for IL-13 (Supplementary Fig. 2). In contrast, upon stimulation with OVM *in vitro*, spleen cells from epicutaneously EW-exposed mice produced significant IL-13 but not IFN- γ ,

whereas spleen cells from PDEW-exposed mice did not produce either IL-13 or IFN- γ (Fig. 3F,G).

Thermoase PC10F-digested EW product did not induce IgE production upon epicutaneous exposure

Just like with PDEW, serum OVA- and OVM-specific IgE levels were significantly lower in mice epicutaneously exposed to TDEW than in EW-exposed mice (Fig. 4A). The serum OVA- and OVM-specific IgG1 and IgG2a levels were also significantly lower in the TDEW-exposed mice (Fig. 4B,C).

Upon *in vitro* stimulation with OVM, but not OVA, spleen cells from epicutaneously EW- and TDEW-exposed mice underwent significant proliferation (Fig. 4D). The OVA-stimulated cells from both mice produced significant IL-13 but not IFN- γ (Fig. 4E,F). On the other hand, the OVM-stimulated cells from epicutaneously EW-exposed mice produced significant IL-13 but not IFN- γ , while the cells from TDEW-exposed mice produced neither IL-13 nor IFN- γ (Fig. 4E,F). The results for IL-4 and IL-5 were the same as for IL-13 (Supplementary Fig. 3).

Discussion

To improve the safety features of early egg introduction aimed at primary prevention of egg allergy, we investigated protease-digested EW products for 1) induction of oral tolerance, 2) causation of a body temperature decline (anaphylaxis) and 3) causation of epicutaneous sensitization.

Immunoglobulins, including IgE, bind to a "B-cell epitope" that consists of the 3D structure of an antigen molecule, i.e., a so-called conformational epitope, or bind to a peptide sequence, i.e., a so-called linear epitope. In contrast, T-cell receptors specifically bind to a linear epitope (T-cell epitope) of the antigen molecule presented by an MHC class II molecule on antigen-presenting cells.

After digestion of EW with pepsin, Thermoase PC10F, Protease P "Amano" 3SD or Protin SD-AY10, no high-molecular-weight proteins were detected (Supplementary Fig. 1). Therefore, most conformational epitopes in the EW proteins were degraded in these optimally protease-digested EW products.

In our first series of experiments, intragastric administration of PDEW or TDEW significantly suppressed OVA- and OVM-specific IgE production (Fig. 1B,C), whereas both 3DEW and SDEW failed to prevent OVA-specific IgE-production. These results suggest that T-cell epitopes (linear epitopes) for oral tolerance exist in PDEW or TDEW but not in 3DEW or SDEW, presumably due to the amino-acid sequences created by each protease digestion.

Oral tolerance is reportedly mediated by two different mechanisms: anergy and active suppression. Lymphocyte anergy is induced by intake of a high dose of an antigen, whereas regulatory T cells actively suppress immune responses via IL-10 and TGF- β production.¹⁵ In this study, OVA-specific IL-10 production by MLN cells was not enhanced in PDEW or TDEW pre-administered mice, suggesting that oral tolerance induced by PDEW or TDEW may be mediated mainly by anergy. We then focused on PDEW and TDEW in our subsequent analyses.

Among previously performed early egg intervention studies aiming at primary prevention, 6–20% of the participants reacted to raw egg powder at initial introduction.⁶ Thus, new egg products need to induce weak IgE binding that can prevent allergic reactions even in IgE-sensitized babies. Whereas intraperitoneal heated-EW challenge elicited anaphylaxis (a body temperature decline) in sensitized mice in our present study, PDEW did not (Fig. 2B), which is consistent with a previous study.¹² In addition, we newly found that TDEW also did not elicit any body temperature decline (Fig. 2B). These results suggest that PDEW and TDEW contain no

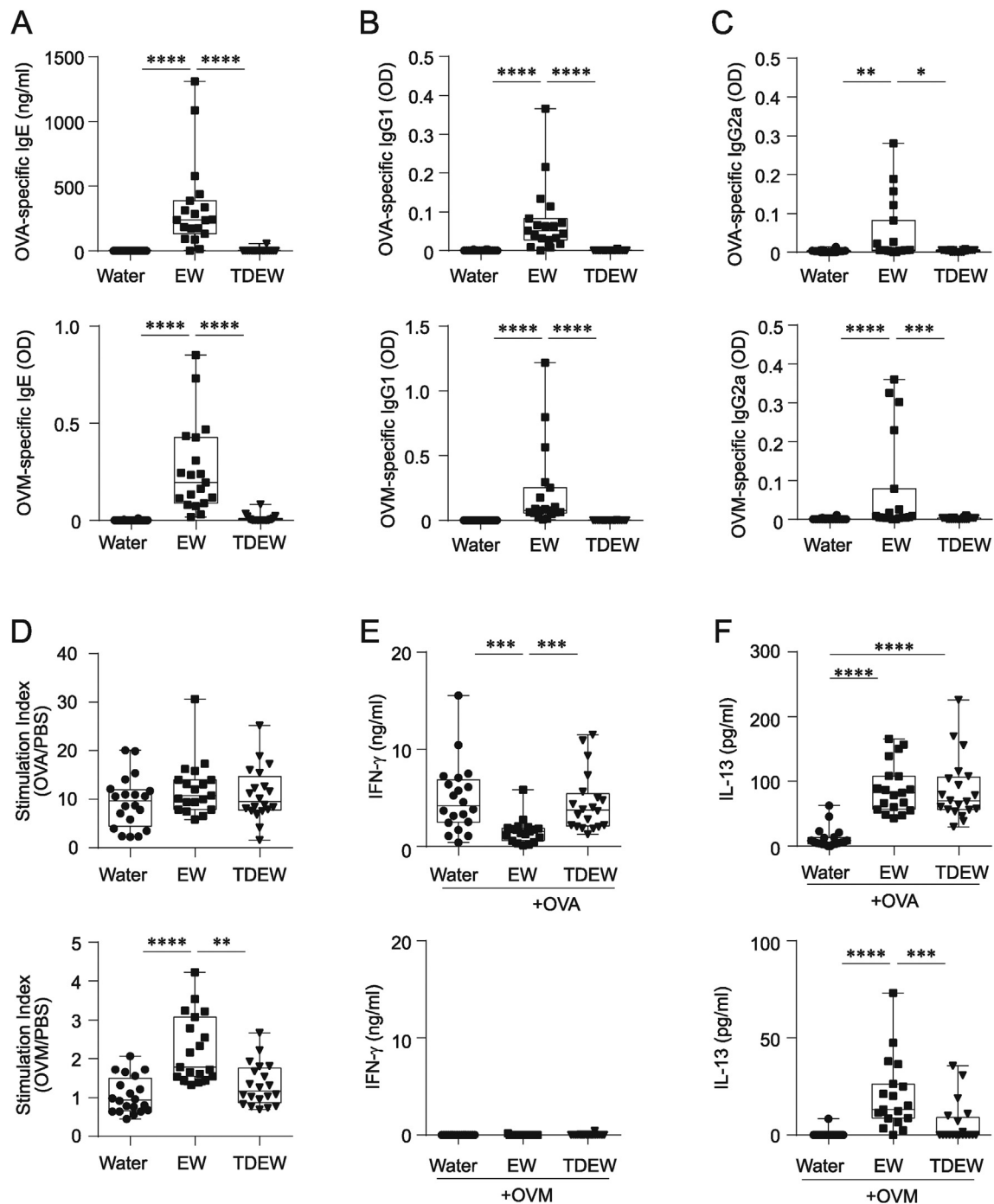


Fig. 4. The effects of heated egg white (EW) and Thermoase PC10F-digested EW product (TDEW) on epicutaneous sensitization. The serum concentrations of ovalbumin (OVA)- or ovomucoid (OVM)-specific IgE (A), IgG1 (B) and IgG2a (C) antibodies were measured by ELISA. OVA- or OVM-induced cell proliferation (D), IFN- γ production (E) and IL-13 production (F) by spleen cells from epicutaneously-sensitized mice are shown. The levels of cytokines in the culture supernatants were measured by ELISA. The data are shown as the median and the ranges; * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$ vs. indicated group ($n = 19-20$).

conformational or linear epitopes for IgE and are safer than heated EW, but they contain some T-cell epitopes for oral tolerance induction.

The effect of protease digestion of EW on epicutaneous sensitization has been totally unknown. TSLP, IL-25 and IL-33 reportedly play key roles in T2 responses and IgE sensitization of the skin.¹⁶⁻¹⁸ Therefore, we first examined mRNA expression for TSLP, IL-25 and IL-33 in tape-stripped skin that was exposed to EW or PDEW. We

found that the mRNA levels for TSLP, IL-25 and IL-33 were nearly equivalent among all the sample-exposed skins (Supplementary Fig. 4), which is consistent with our previous study.¹³

Repeated epicutaneous EW exposure caused T2-skewed sensitization with elevated serum OVA- and OVM-specific IgE, IgG1 and IgG2a levels and OVA-specific IL-13 production by spleen cells. In contrast, repeated epicutaneous PDEW and TDEW exposure did not lead to serum IgE elevation or OVA-specific spleen

cell proliferation. However, upon *in vitro* stimulation with OVA, spleen cells from PDEW- and TDEW-exposed mice showed significant IL-13 production, similar to in EW-exposed mice (Fig. 3G,4F). This result is not surprising since T-cell epitopes exist in PDEW and TDEW, as shown in the aforementioned oral tolerance-induction experiments (Fig. 1B). Thus, a longer period of repeated exposure to PDEW and TDEW might cause IgE production. Further experiments and careful observation will be necessary when such enzyme-digested EW products are tested in the clinical setting.

Although a well-known T-cell epitope in OVA 323–339 (ISQAVHAHAINEAGR)¹⁹ is reportedly cleaved by both pepsin⁹ and thermolysin,²⁰ PDEW and TDEW induced oral tolerance, suggesting that other T-cell epitopes remain intact in those products and are responsible for induction of oral tolerance and perhaps epicutaneous sensitization.

This study has several limitations to acknowledge. First, we used only one mouse strain, Balb/c, which is highly sensitive to IgE-mediated stimuli. Because MHC class II-restricted, T2-prone T-cell epitopes on antigen molecules may differ among mouse strains, other strains need to be investigated. Second, we investigated the digested EW products only in regard to prevention of intraperitoneal sensitization. Epicutaneous exposure reportedly elicits IgE production even after oral tolerance induction,²¹ so the products' ability to prevent epicutaneous sensitization also needs to be examined in the future. Finally, we did not investigate the appropriate dose of digested EW products for sufficient induction of oral tolerance.

In this study, we identified two protease (pepsin and thermolysin)-digested EW products that exhibited three properties required for an optimal food: sufficient induction of oral tolerance, weak IgE-binding that can prevent allergic reactions even in IgE-sensitized mice, and low epicutaneous IgE sensitization even when in contact with inflamed skin. Therefore, these protease-digested EW products seem to have potential as suitable foods for primary prevention of egg allergy in the future.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.alit.2022.03.006>.

Conflict of interest

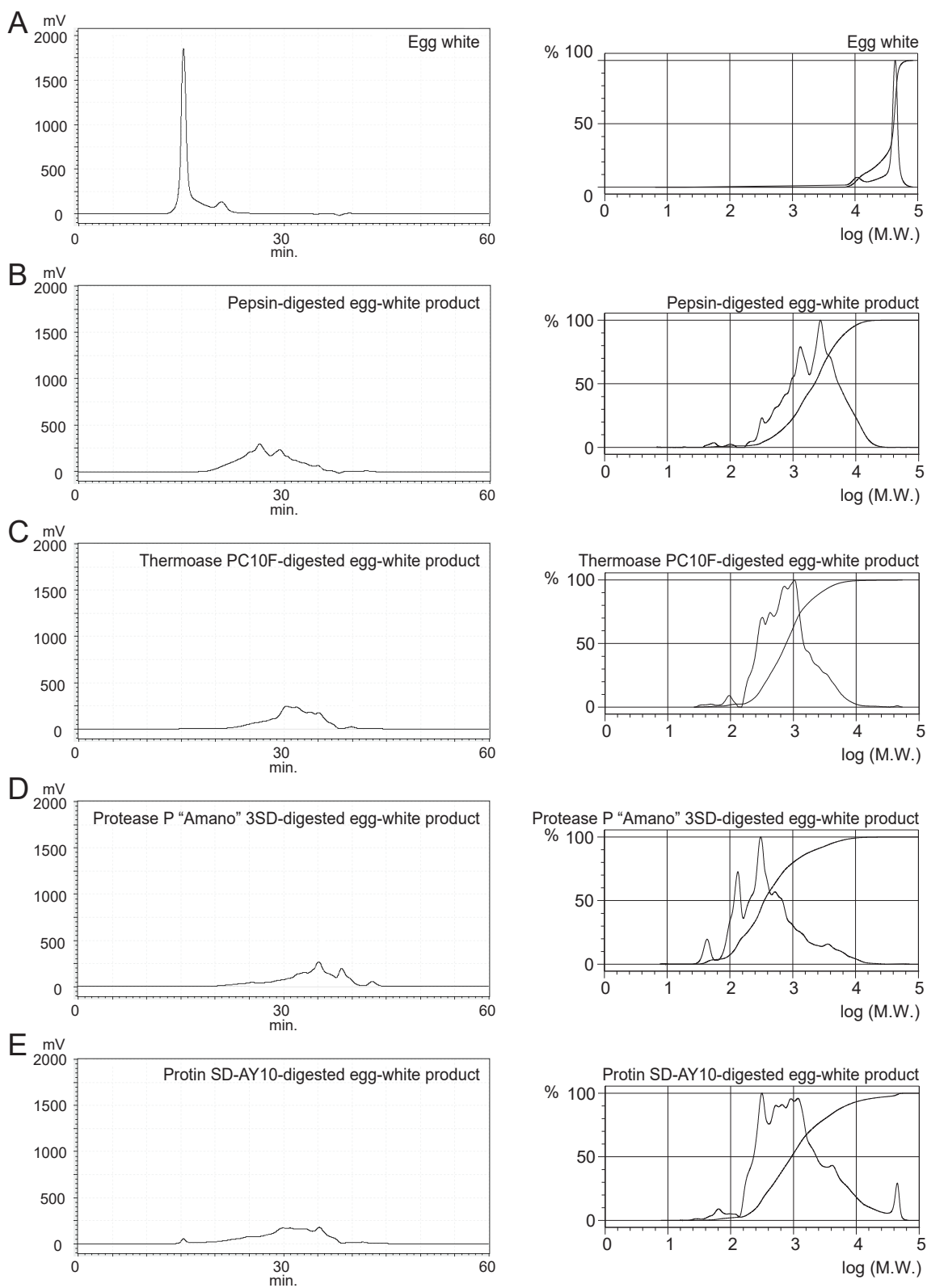
AY and TH are employees of NH Foods. The rest of the authors have no conflict of interest.

Authors' contributions

Conceived and designed the experiments: AY, TH, MF, HM, and KM. Performed the experiments: AY, TH, and HM. Analyzed the data: AY, TH, MF, HM, and KM. Wrote the paper: AY, TH, MF, HM, and KM. All authors read and approved the final manuscript.

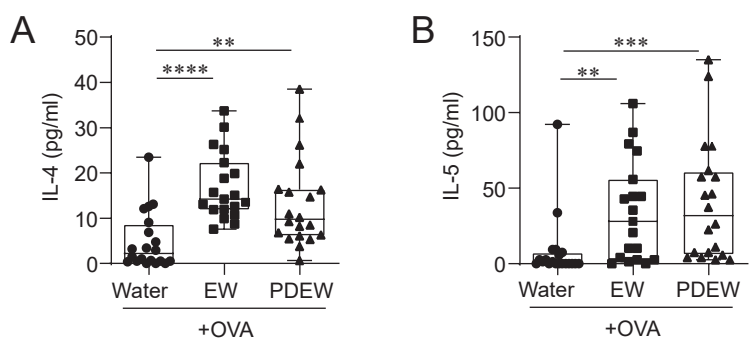
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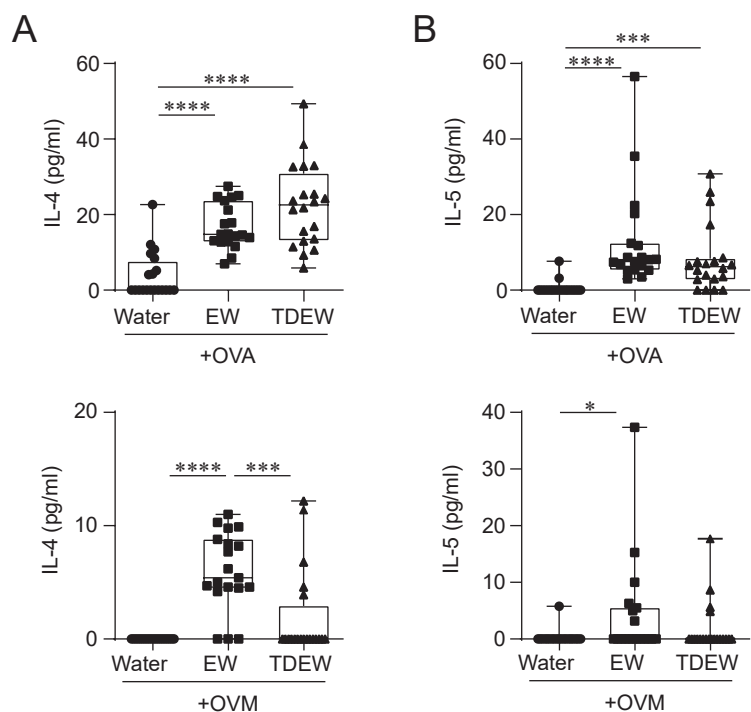
Supplementary Fig. 1. High-performance liquid chromatography (HPLC) chromatograms of heated egg white and protease-digested egg-white products.

The left panels show the raw data (retention times) obtained by HPLC. The right panels show the molecular weight (M.W.) distributions of the samples. HPLC chromatograms are shown for heated egg white (A), and the digests by pepsin (PDEW, B), Thermoase PC10F (TDEW, C), Protease P Amano 3SD (3DEW, D), and Protin SD-AY10 (SDEW, E).



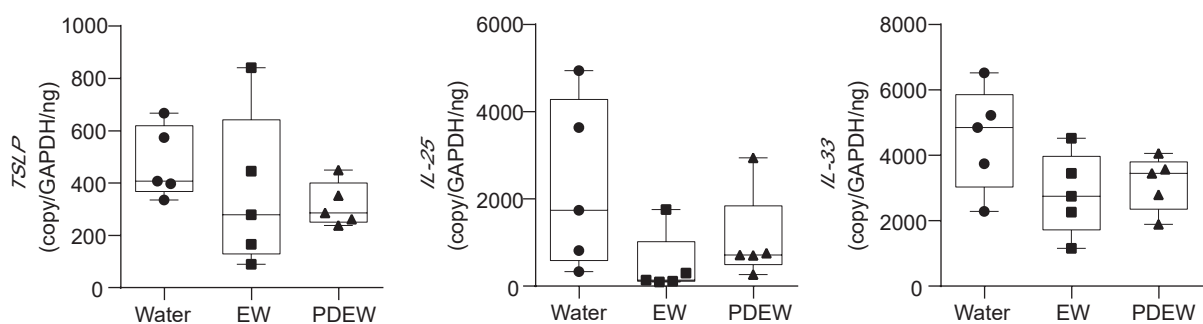
Supplementary Fig. 2. The effects of heated egg white (EW) and pepsin-digested EW product (PDEW) on epicutaneous sensitization.

The levels of OVA-induced IL-4 (A) and IL-5 (B) in the supernatants of cultured spleen cells from epicutaneously-sensitized mice were measured using a multiplex bead array system and are shown. The data are shown as the median and the ranges; ** $P < .01$, *** $P < .001$, **** $P < .0001$ vs. indicated group ($n = 19-20$).



Supplementary Fig. 3. The effects of heated egg white (EW) and Thermoase PC10F-digested EW product (TDEW) on epicutaneous sensitization.

The levels of OVA- or OVM-induced IL-4 (A) and IL-5 (B) in the supernatants of cultured spleen cells from epicutaneously-sensitized mice were measured by ELISA and are shown. The data are shown as the median and the ranges; * $P < .05$, *** $P < .001$, **** $P < .0001$ vs. indicated group ($n = 19-20$).



Supplementary Fig. 4. The mRNA expression levels for thymic stromal lymphopoietin (TSLP), IL-25 and IL-33 in the dorsal skin.

The dorsal skin of 6-week-old BALB/c mice was shaved, and 3-times tape-stripped, and then treated with heated egg white (EW), pepsin-digested EW (PDEW), or sterile water alone. Four hours later, the skin sites were harvested. The mRNA expression levels were measured by qPCR. The data are shown as the median and ranges (n = 5).