

RESEARCH ARTICLE

Heat treatment of egg white controls allergic symptoms and induces oral tolerance to ovalbumin in a murine model of food allergy

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Scope: Heated foods often present low allergenicity, and have recently been used in specific oral immunotherapy for food allergies. However, the influence of heating on tolerogenicity of food allergens is not well elucidated. Here, we investigated biochemical, allergenic, and tolerogenic properties of heated egg white (EW) using a murine model of food allergy.

Methods and results: Raw EWs were treated at 80°C for 15 min (80EW, mild heating condition), 100°C for 5 min (100EW, cooking condition), or 121°C for 40 min (121EW, retort pouch condition), and freeze-dried. A transgenic OVA23–3 mice model expressing T-cell receptor specific for ovalbumin (OVA, a major EW allergen) induced Th2 cells and IgE production, and presented intestinal inflammation when fed untreated EW diet. 80EW-fed mice presented only moderate inflammation but high Th2 responses. 100EW-fed mice did not present inflammation but induced tolerance as seen in reduced T-cell responses and IgE levels. 100EW demonstrated higher digestive stability and slower absorption in intestine, compared with untreated EW and 80EW. 121EW was strongly aggregated, was not absorbed well, and developed Th1 responses without tolerance induction.

Conclusion: OVA in EW treated only under a particular heat condition (e.g. 100°C for 5 min) lost allergenicity, but possessed tolerogenicity.

Keywords:

Egg white / Food allergy / Heat treatment / Mouse model / Oral tolerance



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Abbreviations: CN, casein; EW, egg white; LAL, lysinoalanine; OVA, ovalbumin; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SOIT, specific oral immunotherapy; TCR, T-cell receptor; 80EW, egg white heated at 80°C for 15 min; 100EW, egg white heated at 100°C for 5 min; 121EW, egg white heated at 121°C for 40 min

1 Introduction

The prevalence of food allergies has increased over the past decade [1–3]. So far, the only proven treatment for food allergies is elimination of the allergenic foods from the diet. To achieve induction of desensitization or clinical tolerance, specific oral immunotherapy (SOIT), involving oral administration of allergenic foods, has been assessed as an allergen-specific immunotherapy for food allergy patients [4–6]. However, occurrences of severe adverse reactions by allergenic

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foods have been observed in SOIT, indicating the need for improved treatment regimes.

Since allergens in their native conformation present IgE-reactive epitopes, they possess full allergenic potential and induce allergic symptoms through mediators released from mast cells and basophils. Strategies for disrupting IgE-reactive epitopes and preserving T-cell epitopes have been investigated to regulate T-cell responses without provoking IgE-mediated adverse reactions in allergen-specific immunotherapy. Notably, heat treatment reduces the allergenicity of some foods such as eggs by disruption of IgE-reactive epitopes due to protein denaturation and aggregation [7–11]. These observations facilitate establishing SOIT using heat-treated foods [12]. However, it is still not well elucidated whether and how heat treatment influences both allergenicity (an ability to cause allergic sensitization and allergic reactions in sensitized individuals) and tolerogenicity (an ability to induce tolerance) of food allergens.

In this study, we aimed at investigating the influence of heat treatment on biochemical, allergenic, and tolerogenic properties of ovalbumin (OVA, a major egg white (EW) allergen) comprehensively, using a murine model of food allergy. The experimental model is OVA23–3 mice expressing T-cell receptor (TCR) specific for amino acid residues 323–339 of OVA [13]. OVA_{323–339} acts as the dominant T-cell epitope not only in several mouse strains [14, 15] but also in food allergic patients reacting to the allergen [16, 17]. The advantage for the use of OVA23–3 mice is that feeding of a diet containing EW induces clinical symptoms (e.g. weight loss and diarrhea) and intestinal inflammation attributed to Th2-type immune responses, which mirrors pathological features of food-sensitive allergic enteropathy [18]. In addition, the mice tend to develop T-cell tolerance subsequently after an allergic phase when fed an EW diet for long-term continuously. Using this model, it is possible to assess both allergenicity and tolerogenicity of heat-treated EW.

According to commonly used food preparation methods [19] and based on conformational changes of heat-treated OVA [20], we heated EW under three conditions: at 80°C for 15 min (80EW, mild heating condition), 100°C for 5 min (100EW, cooking condition), and 121°C for 40 min (121EW, retort pouches condition). Here, we identified a heating condition that effectively induces tolerance without provoking allergic symptoms and inflammation.

2 Materials and methods

2.1 Animals

OVA23–3 transgenic mice expressing TCR $\alpha\beta$ (V α 3/V β 15) specific for OVA_{323–339} on a BALB/cA genetic background (provided by S. Habu, Tokai University School of Medicine) were maintained at Sankyo Labo Service Corporation (Tokyo, Japan). Heterozygous OVA23–3 mice were housed under specific pathogen-free conditions in the Kanagawa Prefec-

tural Institute of Public Health. For splenocyte proliferation and cytokine production assays, wild-type BALB/cA mice were purchased from Clea Japan (Tokyo, Japan) to prepare antigen-presenting cells as described in Section 2.10. Guidelines formulated by the Kanagawa Prefectural Institute of Public Health were followed for the care and use of animals.

2.2 Preparation of diet containing heated EW powder

Raw EWs were heated under three conditions: at 80°C for 15 min, 100°C for 5 min [20], or 121°C under 2.2 kgf/cm pressure for 40 min [19], and freeze-dried. Powder diets containing unheated, or heated EW, or unheated cow lactic acid casein (CN) as the source of 20% of the protein were prepared by Kewpie Corporation (Tokyo, Japan). CN was purchased from Fronterra Corporation (Auckland, New Zealand). The diets contained 20% protein, 57.47% starch, 5.0% sugar, 5.0% cellulose, 6.0% soy oil, 5.0% mineral mixture, 1.3% vitamin mixture (AIN-76 composition), and 0.23% chloride. Only CN diets contained 0.3% methionine to compensate the amino acid deficiency of CN. By feeding EW diet, it is calculated that a mouse consumes about 250 mg OVA per day.

2.3 Feeding of mice with heated EW diets

Heterozygous OVA23–3 mice (8–12 weeks) were fed a diet containing unheated, heated EW, or unheated CN as a powder for up to 28 days. During the feeding, mice were weighed and observed to check occurrence of diarrhea every 3 to 4 days.

2.4 Analysis of OVA aggregation in heated EW powder

Powder diets (1.0 g) containing unheated or heated EW were dissolved in a 19 mL Tris-HCl buffer containing 0.1% BSA and 0.05% polyoxyethylene-sorbitan monolaurate. The solutions were centrifuged at 3000 $\times$ g for 20 min. Concentrations of OVA in the supernatant were measured using a Morinaga High Sensitive Ovalbumin ELISA kit (Morinaga Institute of Biological Science, Yokohama, Japan) according to the manufacturer's instructions [21].

2.5 Analysis of lysinoalanine (LAL) and furosine concentration

The LAL content of unheated EW and heated EW powders was measured as a hallmark for the formation of cross-linked amino acids in heat-treated protein. Furosine, a reaction product formed from Amadori products during acid hydrolysis, served as a measure of protein glycation. LAL was

measured via cation-exchange chromatography after acid hydrolysis of EW samples with 6 N HCl at 110°C for 23 h. Aliquot samples were evaporated in vacuo, and suspended in 0.12 N lithium citrate buffer (pH 2.2). LAL content in the hydrolysates was determined with an amino acid analyzer S433 (Sykam, Fuerstenfeldbruck, Germany) with ninhydrin detection. Chromatographic conditions were as described in [22] with some modifications [23]. For analysis of furosine, the acid hydrolysates were subjected to SPE [24]. Extracted samples were then evaporated in vacuo and reconstituted in 0.4% acetic acid. Furosine content was measured by a HPLC on a RP-8 column and UV detection according to Resmini et al. [24].

2.6 OVA stability in EW powders heated in the presence of digestive proteases

The stability of OVA in heated EW powders in the presence of digestive proteases was assessed using simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) [25, 26]. For pepsin digestion, 80 μ L of OVA sample (1 mg/mL) was dissolved in 1520 μ L of prewarmed SGF (34 mM NaCl, pH 2.0) containing pepsin (3.8 mg/5 mL; Sigma-Aldrich, St. Louis, MO, USA). For pancreatin digestion, 90 μ L of OVA sample (2 mg/mL) was dissolved in 1710 μ L of prewarmed SIF (0.05 M KH_2PO_4 , pH 7.5) containing pancreatin (10 mg/mL, Sigma-Aldrich). The sample solutions were incubated at 37°C and an aliquot (100 μ L) was collected at 5, 10, 30, 60, 90, 120, and 180 min. Aliquots were quickly mixed with 35 μ L of Na_2CO_3 solution (400 mM) and 200 μ L of Laemmli buffer (Bio-Rad) containing 5% 2-mercaptoethanol. The mixture was boiled for 5 min and protein visualized by western blotting. Buffer without enzyme served as a control.

2.7 Western blotting

SDS-PAGE and western blotting were performed as previously described [19]. Digests of EW samples (15 μ L) were loaded onto slab gels (5–20% acrylamide gel, e-page, Atto, Tokyo, Japan). Following SDS-PAGE, proteins were transferred to polyvinylidene fluoride membranes, and OVA detected with the Morinaga Egg Protein Western Blot Kit (OVA; Morinaga Institute of Biological Science).

2.8 OVA levels in sera of mice administrated heated EW powders

Heterozygous OVA23–3 mice were administrated 50 mg of unheated or heated EW powders dissolved in 0.6 mL distilled water using intragastric feeding needles after 17-h depriving. After 10, 30, and 60 min, blood was collected for serum preparation. OVA concentrations in the sera were measured with the Morinaga FASPEK Egg Protein ELISA Kit (OVA,

Morinaga Institute of Biological Science), which detected both native and denatured forms of OVA in processed foods [21].

2.9 Antibodies levels in sera

Total IgE and OVA-specific IgE antibody levels in sera were measured by ELISA as described previously [18].

2.10 Splenocyte proliferation and cytokine production

Spleens were isolated from mice fed CN, unheated EW, or heated EW diets, and splenocytes were prepared. The cells (1×10^5 cells/well) were added to 96-well flat-bottom plates and cultured with mitomycin-treated syngenic splenocytes as antigen-presenting cells (4×10^5 cells/well) in the absence or presence of OVA (1 mg/mL; Grade III, Sigma-Aldrich). Cell proliferation was measured 48 h later using Amersham Cell Proliferation Biotrak ELISA System (GE Healthcare Japan, Tokyo, Japan). For cytokine assays, culture supernatants were collected at 48 h. Concentrations of IL-2, IL-4, and IFN- γ in the supernatant were measured by ELISA [18].

2.11 Histological analysis

Longitudinal sections of 10 cm from the upper small intestine were dissected. The tissues were opened longitudinally, fixed in 10% formalin, and embedded in paraffin. Three- or five-micrometer-thick sections were prepared and treated with hematoxylin and eosin for morphological analysis [18]. Micrographs of these specimens were observed by using an Axio Skop 2 Plus (Zeiss, Thornwood, NY, USA).

2.12 Statistical analysis

The Mann–Whitney *U* test was used for nonparametric analysis of weight change, serum antibody levels. OVA concentration and cytokine production were analyzed employing Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

3 Results

3.1 Aggregated OVA in heated EW powders

To assess the influence of heat treatment on potential allergenicity and tolerogenicity of OVA, EW was treated at 80°C for 15 min, 100°C for 5 min, or 121°C for 40 min, and freeze-dried. Heat treatment frequently induces protein aggregation and reduces its solubility. First, we wanted to examine the influence of heat treatment on aggregation of OVA, which is

Table 1. Solubility of OVA in heated EW diets

Diets	OVA contents (%)
EW	8.7 ± 0.4
80EW	0.294 ± 0.010*
100EW	0.163 ± 0.002*
121EW	0.0020 ± 0.0004*

OVA was extracted from diets containing powders of unheated EW (EW) or heated EW at 80°C for 15 min (80EW), 100°C for 5 min (100EW), or 121°C for 40 min (121EW) using ELISA extraction buffer with and without SDS. The concentrations of extracted OVA were determined by ELISA.

Data are mean ± SEM.

* $p < 0.01$ versus EW, ** $p < 0.05$.

contained in EW powder. The solubility of OVA was assessed using extract buffer. Quantitative ELISA to determine OVA contents in an extract buffer showed that contents of the allergen were $8.7 \pm 0.4\%$ from unheated EW diet (defined as EW diet), $0.294 \pm 0.010\%$ from 80EW diet, $0.163 \pm 0.002\%$ from 100EW diet, and $0.0020 \pm 0.0004\%$ from 121EW diet (Table 1). Moreover, total protein amounts in PBS extracts were 862 ± 16 mg/g from EW, 259 ± 41 mg/g from 80EW, 191 ± 35 mg/g from 100EW, and 83 ± 19 mg/g from 121EW powder (Supporting Information Table 1). Total protein amounts in PBS extract with SDS, a protein denaturant, was 858 ± 57 mg/g from EW, 410 ± 49 mg/g from 80EW, 367 ± 24 mg/g from 100EW, and 257 ± 10 mg/g from 121EW powder, respectively. The results suggest that (i) aggregation of OVA in 100EW powder was strongly induced relative to that of 80EW powder, and (ii) aggregation of the allergen in 121EW powder could be the highest among the EW samples.

We, then, examined the influence of heat treatment on modifying amino acids in EW powders. The levels of LAL (Fig. 1A), a product of cross-linked amino acids, and furosine (Fig. 1B), a product of the Maillard reaction [24], were higher in 80EW and 100EW powder, and significantly enhanced in 121EW powder, compared with unheated EW powder. These results indicate that heat treatment induces glycation and amino acid cross-linking, leading to protein aggregation [27, 28]. The modification was significant in 121EW powder, which presented a brown color, an

indication of the Maillard reaction [28] (Supporting Information Fig. 1).

3.2 The stability of OVA in heated EW powders in the presence of proteases

To examine the influence of heat treatment on the stability of OVA to digestive proteases, unheated or heated EW powders were incubated in SGF and SIF, models for gastrointestinal fluids [25, 26]. OVA proteolysis was then analyzed by western blotting. OVAs in unheated EW, 80EW, and 100EW powders were broken down into lower molecular mass molecules within 180 min in SGF treatment (Fig. 2A). SIF treatment rapidly degraded OVA in EW powder within 1 min, in 80EW powder within 5 min, and in 100EW powder within 10 min (Fig. 2B). 121EW powder did not dissolve in either SGF nor SIF (confirmed by SDS-PAGE, data not shown), and a weak OVA band disappeared within 30 min with SGF treatment but persisted 120 min with SIF treatment. These results suggest that (i) the stability of OVA in 100EW powder was slightly better than those in unheated EW and 80EW powders, and (ii) the allergen stability in 121EW powder may be the highest.

3.3 OVA absorption after ingestion of heated EW powders

To examine the influence of heat treatment in OVA absorption in vivo, we measured OVA concentrations in the sera of mice receiving oral administration of unheated or heated EW powders (Fig. 3). Approximately 100 ng/mL of OVA was detected in 10–60 min after feeding with unheated EW or 80EW powders. Following administration with 100EW powder, OVA was detected at approximately 100 ng/mL at 30 min, and increased approximately up to 230 ng/mL at 60 min, suggesting that slow absorption of 100EW powder induced high OVA serum levels. Administration with 121EW powder did not induce detectable levels of OVA absorption.

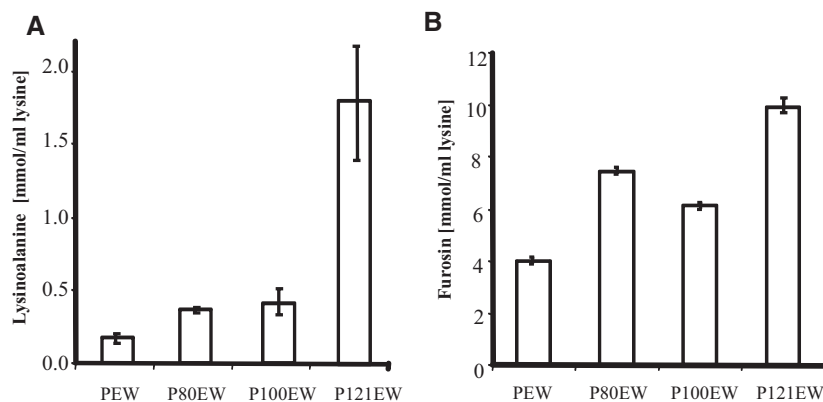


Figure 1. The contents of LAL and furosine in heated EW powders. (A) The contents of LAL and (B) furosine in powder of unheated (PEW) or heated EW at 80°C for 15 min (P80EW), 100°C for 5 min (P100EW), or 121°C for 40 min (P121EW) were measured. Data are mean ± SEM.

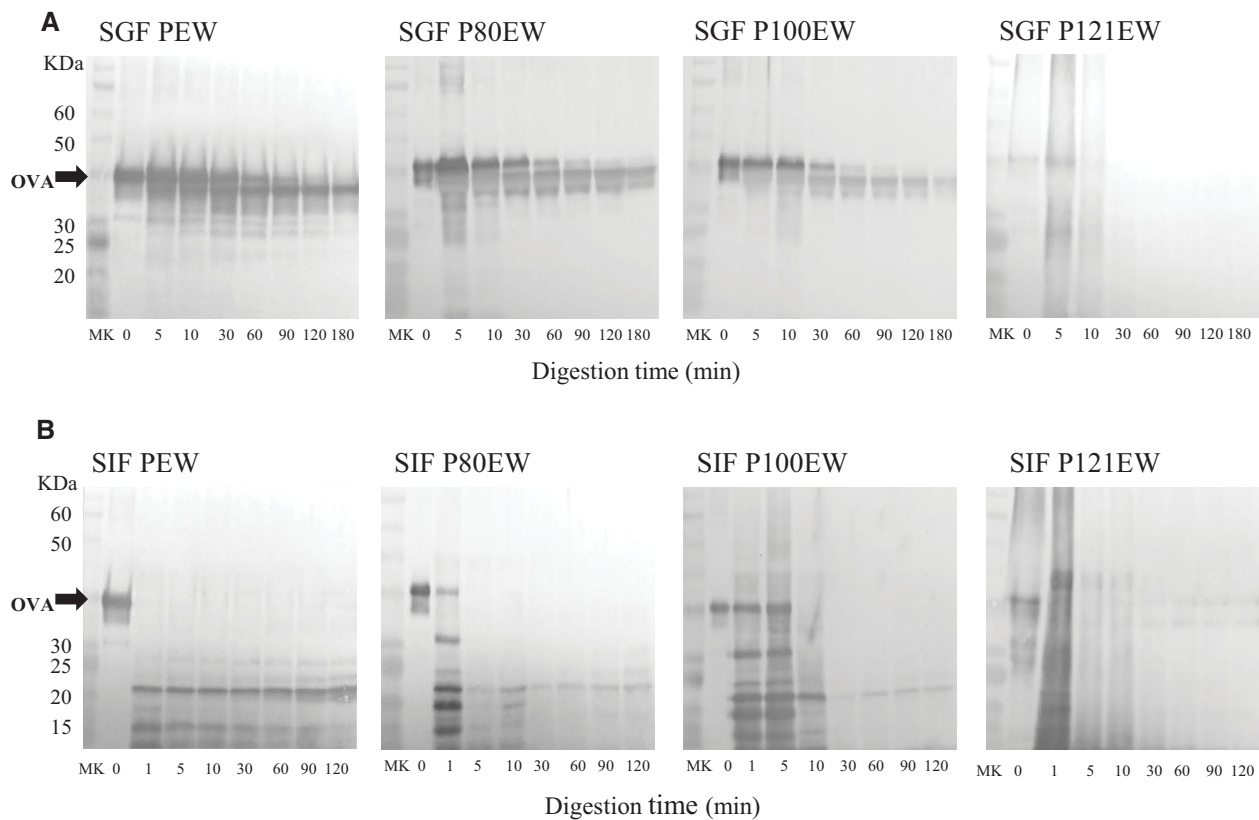


Figure 2. The influence of heat treatment on digestive stability of OVA in EW powders. Powders of EW unheated (PEW) or heated at 80°C for 15 min (P80EW), 100°C for 5 min (P100EW), or 121°C for 40 min (P121EW) were treated with (A) SGF for 180 min, and (B) SIF for 120 min. Samples were then subjected to western blotting and OVA was detected using polyclonal rabbit IgG. MK, molecular weight markers. Data are representative of two independent experiments.

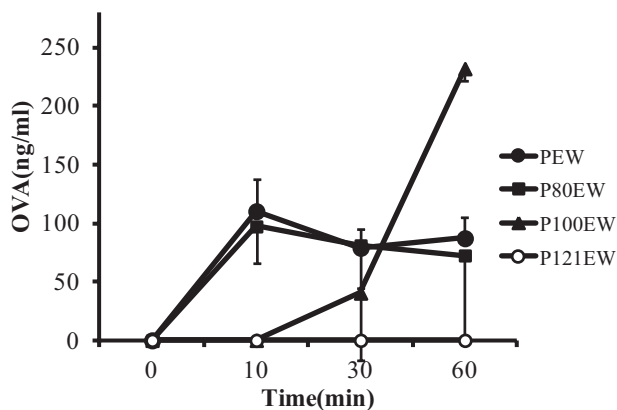


Figure 3. The influence of heat treatment on in vivo absorbance of OVA in EW powders. OVA23–3 mice were orally administered with diet containing powders of EW unheated (PEW) or heated at 80°C for 15 min (P80EW), 100°C for 5 min (P100EW), or 121°C for 40 min (P121EW). Blood was harvested at 10, 30, and 60 min after the administration for serum preparation. OVA concentrations in the sera were measured by ELISA. Data are mean $\pm$ SEM ($n = 4$).

3.4 Clinical symptoms and intestinal inflammation in OVA 23–3 mice fed with heated EW diets

We next examined the potential allergenicity and tolerogenicity of heated EWs by feeding OVA23–3 with a powder diet containing unheated or heated EW for up to 28 days. When mice were fed a diet containing unheated EW powder (EW diet), they started having diarrhea and losing weight on day 3 of the diet. Continuous feeding of mice with EW-diet for 28 days reduced their weight nearly 20% ($19.2 \pm 7.7\%$, Fig. 4). The concentration of histamine (Supporting Information Fig. 2A) and the number of mast cells (Supporting Information Fig. 2B) in small intestines significantly increased from day 10 of EW diet. Histological analysis of the small intestines revealed several signs of inflammation, including goblet cell hyperplasia, villous atrophy and branching, excessive cell infiltration into the lamina propria and a thickened muscular layer on day 10 (Fig. 5, EW 10 days), crypt elongation, and villous concentrations on day 28 (Fig. 5, EW 28 days).

80EW diet caused diarrhea and weight loss on days 7–14, which resolved transiently, but resumed to approximately 10% ($7.9 \pm 13.2\%$) of loss on day 28 (Fig. 4). Slightly increased histamine concentration, mast cell infiltration, and

Diarrhea symptom score

Feed	Days							
	3	7	10	14	18	21	25	28
CN	-	-	-	-	-	-	-	-
EW	+	++	++	++	+++	+++	++	++
80EW	-	+	++	++	+	+	+	++
100EW	-	±	-	-	-	-	-	-
121EW	-	-	-	-	-	-	-	-

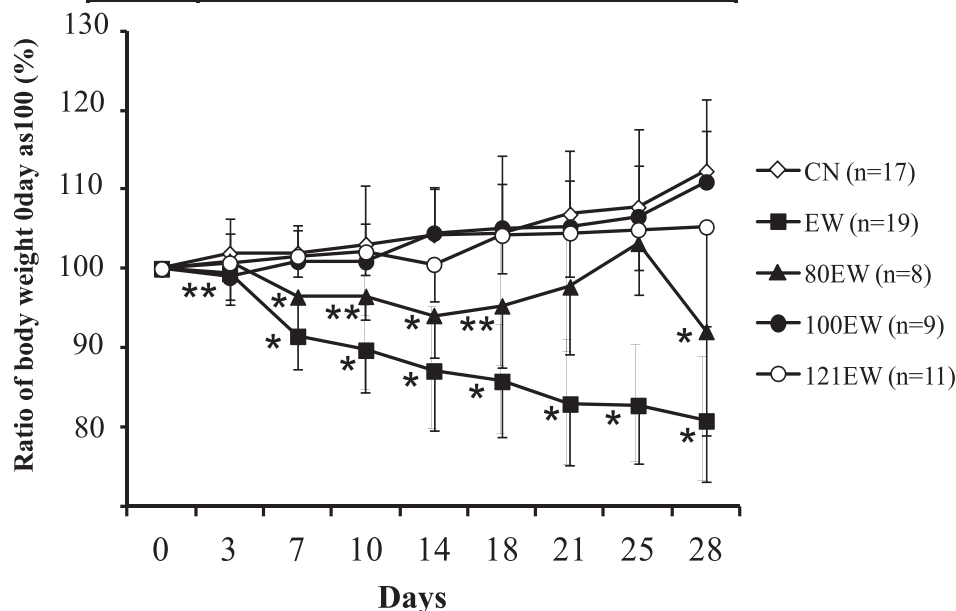


Figure 4. The influence of heated EW diets on body weight of OVA23–3 mice. OVA 23–3 mice were fed with diets containing powders of unheated EW (EW) or heated EW at 80°C for 15 min (80EW), 100°C for 5 min (100EW), or 121°C for 40 min (121EW), or unheated CN powder for 28 days. The weight of the mice was measured from the first day of the feeding. Occurrence of diarrhea was monitored and assessed: +++, severe liquid stool; ++, liquid stool; +, soft stool; ±, slightly soft stool. The number of mice used in each group is indicated in brackets. Data are mean ± SEM of three independent experiments. * $p < 0.01$, ** $p < 0.05$ versus mice fed with CN diet.

inflammatory features in the small intestines were observed on day 28 (Fig. 5 and Supporting Information Fig. 2). 100EW diet caused soft feces on day 7 without weight loss (Fig. 4). Histamine, mast cell infiltrations, and inflammatory features were not detected during feeding of 100EW diet for 28 days (Fig. 5 and Supporting Information Fig. 2). An increased number of goblet cells were detected on day 10 in the small intestines of mice fed with 100EW diet, whereas no inflammatory features were observed on day 28 (Fig. 5 and Supporting Information Fig. 2). The results suggested that 100EW diet induced an immune response but not allergic inflammation in the small intestines. 121EW diet did not cause inflammation, and slightly increased body weight on day 28 as seen in control mice fed with CN diet (Figs. 4 and 5, and Supporting Information Fig. 2). These results suggest that 80EW but not 100EW and 121EW powders retained potential allergenicity.

3.5 Serum antibody levels in OVA23–3 mice fed with heated EW diets

Next, we measured serum levels of total IgE, and OVA-specific IgG₁, IgG_{2a}, and IgE antibodies in mice fed with unheated or heated EW. Unheated EW diet significantly increased the levels of total IgE and OVA-specific IgE antibodies on day 10, and enhanced the levels on day 28 of feeding (Figs. 6A and B, EW1–3). 80EW and 100EW diets increased total IgE antibody levels on day 21, whereas only 100EW diet subsequently re-

duced the levels on day 28 (Fig. 6A, 80EW2 and 3, 100EW2 and 3). 121EW diet slightly increased total IgE levels on day 28 (Fig. 6A, 121EW3). 80EW, 100EW, and 121EW diets did not induce detectable levels of OVA-specific IgE antibodies (Fig. 6B).

Unheated EW diet also significantly increased the levels of OVA-specific IgG₁ and IgG_{2a} antibodies on day 21, and maintained it until day 28 (Supporting Information Fig. 3, EW2 and 3). 80EW and 100EW diets increased OVA-specific IgG₁ and IgG_{2a} antibody levels on day 21 but decreased it on day 28 (Supporting Information Fig. 3, 80EW2 and 3, 100EW2 and 3). These results suggest that long-term feeding of 80EW and 100EW diets induces systemic tolerance to reduce OVA-specific antibody production. 121EW diet did not induce detectable levels of OVA-specific IgG₁ and IgG_{2a} antibodies (Supporting Information Fig. 3).

3.6 Splenocyte responses of OVA 23–3 mice fed with heated EW diet

We also examined splenocyte responses in OVA23–3 mice with unheated or heated EW diets. In OVA-23–3 mice, naive T cells produce IL-2 and proliferate, whereas differentiated effector T cells produce IL-4 and IFN- γ upon OVA stimulation. Splenocytes from mice on 10 days of unheated EW or 80EW diets reduced IL-2 production, and increased IL-4 and IFN- γ production against OVA, compared to those from mice

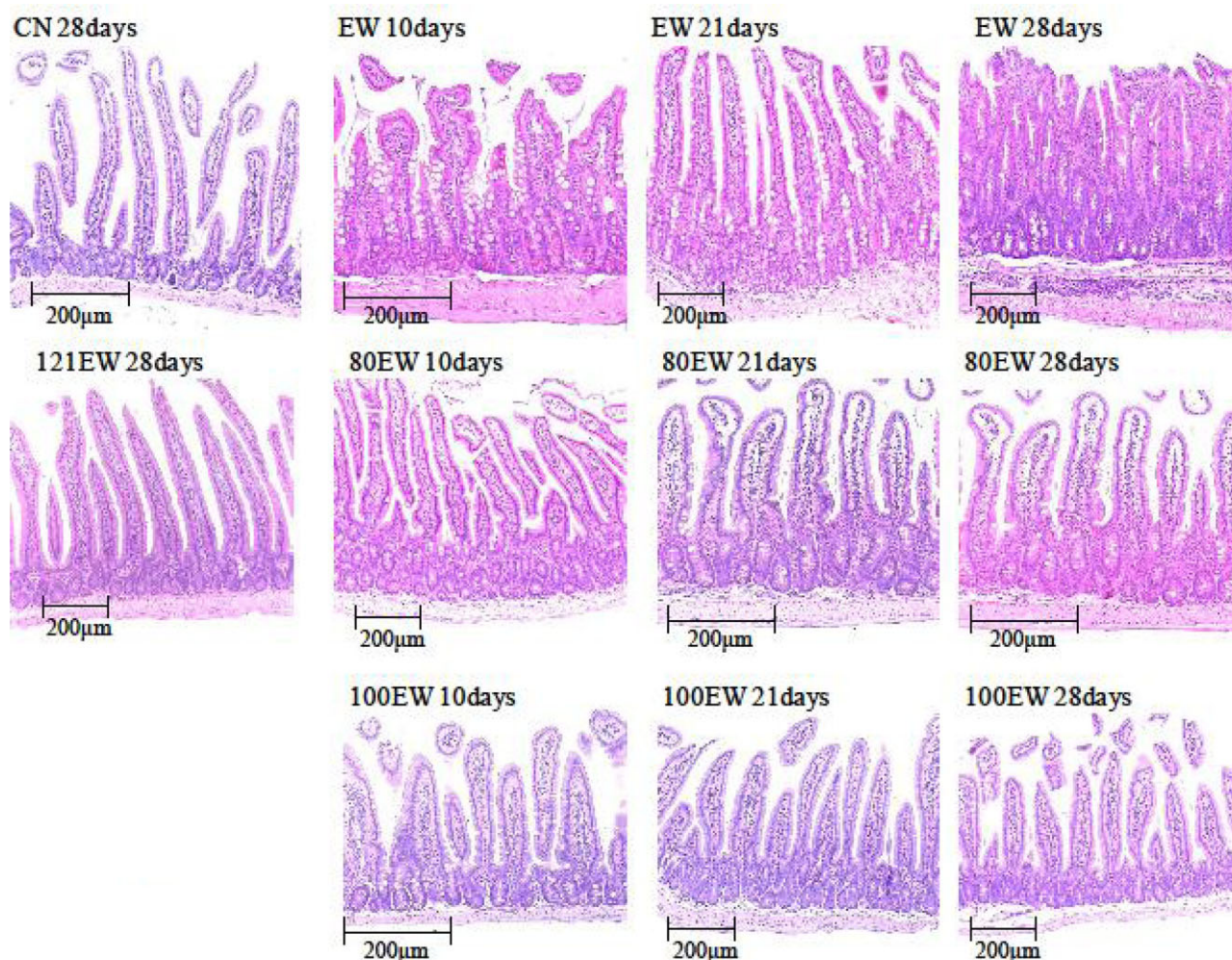


Figure 5. The influence of heated EW diets on intestinal inflammation in OVA 23–3 mice. OVA 23–3 mice were fed with diets containing powders of unheated EW (EW) or heated EW at 80°C for 15 min (80EW), 100°C for 5 min (100EW), or 121°C for 40 min (121EW), or unheated CN powder for 10 or 28 days. The small intestines were harvested for histological analysis and stained with hematoxylin and eosin. Data are representative of two independent experiments.

fed with CN diet (Fig. 7B, EW and 80EW). IL-4 production by splenocytes from mice fed with 80EW diet was significantly higher, compared with those from mice fed with unheated EW diet, suggesting that 80EW diet induced Th2 cells predominantly (Fig. 7B, 80EW). IL-4 and IFN- γ production and proliferation were significantly reduced in splenocytes from mice fed with unheated EW or 80EW diets on day 28, suggesting that long-term feeding with unheated EW or EW80 powder induced T-cell tolerance (Fig. 7, EW and 80EW).

Reduced IL-2 production and proliferation, and only marginal levels of IFN- γ and IL-4 production were observed in splenocytes from 100EW-fed mice on day 10 and 28 of feeding (Fig. 7, 100EW). These results suggest that 100EW diet induces T-cell tolerance by short-term feeding and maintains the tolerance by continuous feeding. Splenocytes from mice fed with 121EW diet showed slightly reduced IL-2 production and proliferation, and produced only IFN- γ but not

IL-4, indicating predominant induction of Th1 cells (Fig. 7, 121EW). Taken together, the results suggest that unheated EW, 80EW, and 100EW but not 121EW powder possess potential tolerogenicity.

4 Discussion

In this study, we investigated potential allergenicity and tolerogenicity of heat-treated EW powder using OVA23–3 mice as a model of food allergy. We demonstrated that EW treated under a particular heat condition (i.e. 100°C for 5 min) was capable of inducing oral tolerance against OVA without provoking intestinal inflammation in OVA23–3 mice. A mild heat treatment of EW at 80°C for 15 min retained the allergenicity of OVA, whereas a treatment at 121°C for 40 min under a pressure mirroring autoclave

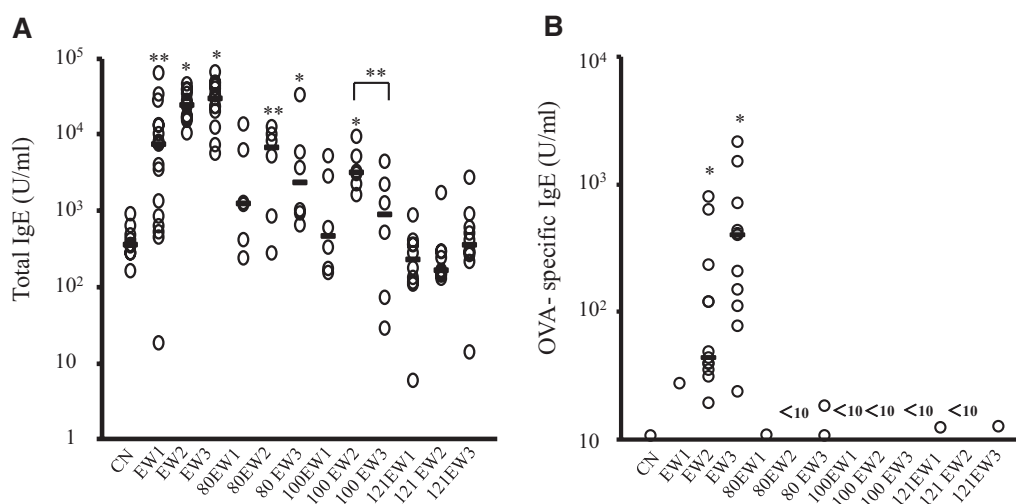


Figure 6. The influence of heated EW diets on serum levels of IgE antibodies in OVA23–3 mice. OVA 23–3 mice were fed with diets containing powders of unheated EW (EW) or heated EW at 80°C for 15 min (80EW), 100°C for 5 min (100EW), or 121°C for 40 min (121EW), or unheated CN powder for 10, 21, or 28 days. Serum was collected from mice fed with EW and heated EW diets. (A) Serum levels of total IgE and (B) OVA-specific IgE antibodies were measured by ELISA. The number of mice used in each group for total IgE (CN, $n = 10$; EW, $n = 14$; 80EW, $n = 6$; 100EW, $n = 6$; 121EW, $n = 10$) and OVA-specific IgE (CN, $n = 16$; EW, $n = 18$; 80EW, $n = 6$; 100EW, $n = 6$; 121EW, $n = 8$); 1, 10 days; 2, 21 days; 3, 28 days. * $p < 0.01$, ** $p < 0.05$ versus mice fed with CN diet.

condition almost completely reduced the allergenicity and tolerogenicity. Our findings recommend a careful heat setting to make low-allergenic OVA to induce oral tolerance against the allergen.

80EW, 100EW, and 121EW powders did not induce detectable levels of OVA-specific IgE antibody production in OVA23–3 mice. The results suggest that all heat conditions disrupted IgE-reactive epitopes in OVA. However, 80EW powder still presented allergenicity to induce clinical symptoms and intestinal inflammation. IgG antibodies would not be involved in the induction, since similar levels of IgG production were observed in OVA23–3 mice fed with 80EW and 100EW diet. In contrast, 80EW powder tended to elevate total IgE antibodies, compared with 100EW powder. Elevated total IgE antibodies might be induced by other heat stable EW allergens such as ovomucoid, and be linked to the allergenicity of 80EW powder. In this study, we used heterozygous OVA23–3 mice to assess the allergenicity and tolerogenicity of heated EW samples. Heterozygous mice express not only monoclonal TCR transgenic but also polyclonal and nontransgenic CD4⁺ T cells, which might be involved in induction of IgE antibodies against the other EW allergens than OVA. Notably, the levels of total IgE antibodies were significantly reduced in mice fed with 100EW diet for 28 days. This suggests that heating EW at 100°C for 5 min might be effective at suppressing IgE production not only against OVA but also against other EW allergens.

An alternative and more possible mechanism that explains for the allergenicity of 80EW powder would be T-cell-mediated inflammation. Short-term feeding of 80EW diet induced high levels of Th2 cells in the mice. Indeed, it is suggested that Th2 cells could provoke allergic inflamma-

tion even without involvement of IgE antibodies in some food allergies such as allergic enteropathy [2]. Moreover, consistent with our finding, clinical reports showed that heat-treated allergens triggered allergic symptoms without inducing allergen-specific IgE antibodies in some food allergic patients [29, 30]. These observations indicate that reduction in IgE reactivity of allergenic foods is not enough to create a safe material in SOIT, especially for non-IgE-mediated food allergies.

Why the 100EW but not the 80EW powder induced tolerance without triggering allergic symptoms and inflammation remains to be elucidated. Analysis of OVA solubility and digestive stability indicates that OVA in EW and 80EW powders might be less aggregated and rapidly absorbed in mice, compared with the allergen in 100EW powder. Several studies have shown that the amount of antigen and the duration of antigen stimulation for T cells influence the fates of immune responses [31–33]. 80EW diet feeding might induce rapid antigen presentation leading to Th2-cell activation, while 100 EW diet feeding induced slow OVA absorption resulting in T-cell tolerance. Further study is necessary to elucidate the detailed cellular and molecular mechanisms mediating the allergic symptoms and inflammation and developing oral tolerance by heat-treated EW diets.

OVA in 121EW powder was highly aggregated and did not induce allergen-specific IgE and IgG antibodies in OVA23–3 mice. It suggests that both IgE and IgG epitopes in OVA were almost completely disrupted in 121EW powder. Moreover, in vivo absorption of OVA in 121EW powder was low. The amount of OVA accessible to the immune system could be limited in mice fed with 121EW diet. Disruption of IgE- and IgG-reactive epitopes and low allergen absorption could

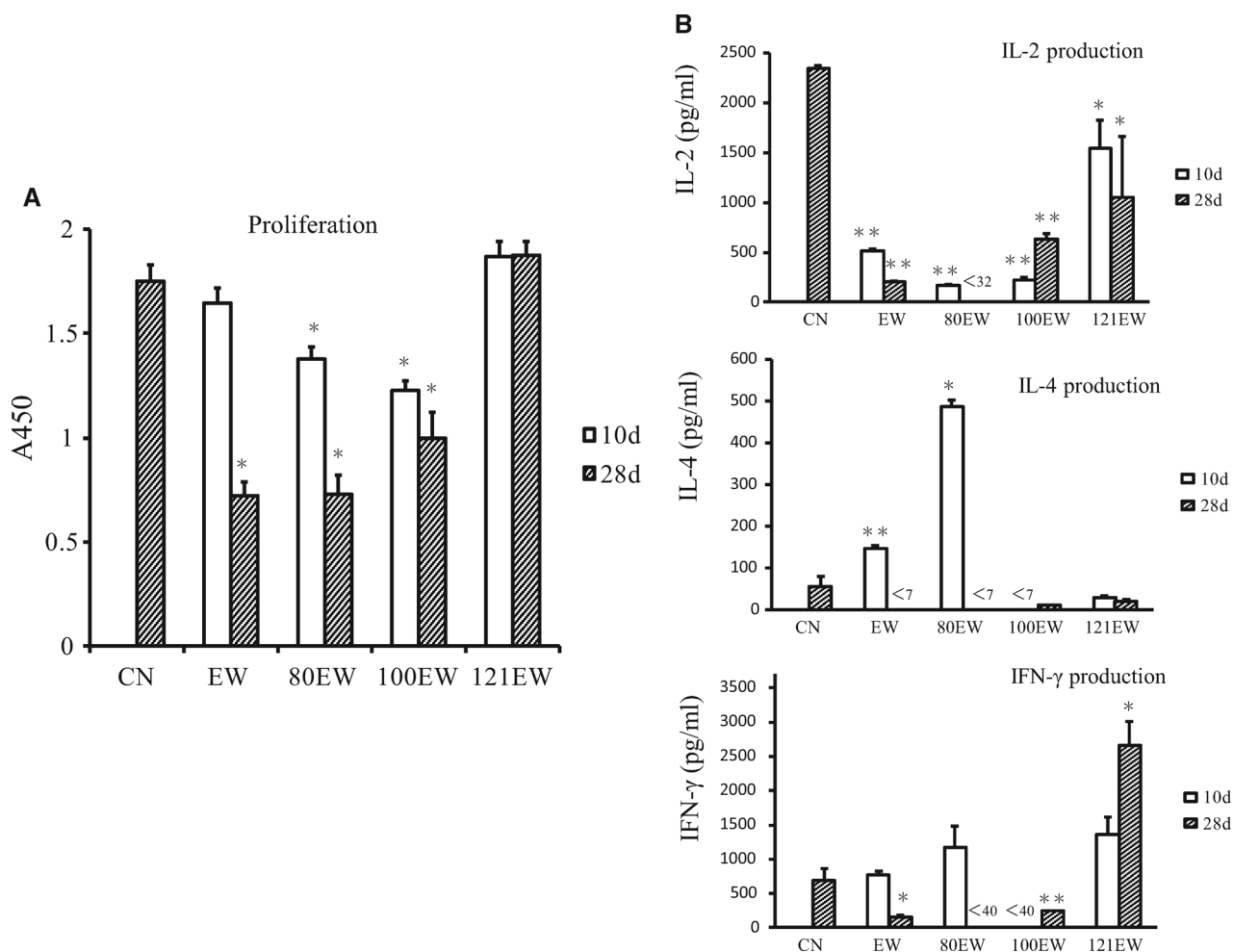


Figure 7. The influence of heated EW diets on splenic T-cell response in OVA 23–3 mice. OVA 23–3 mice were fed with diets containing powders of unheated EW (EW) or heated EW at 80°C for 15 min (80EW), 100°C for 5 min (100EW), or 121°C for 40 min (121EW), or unheated CN powder for 10 or 28 days. Splenocytes were isolated from mice and co-cultured with mitomycin-treated syngeneic splenocyte in the presence of OVA. (A) Proliferation was measured using BrdU. (B) Cytokine concentrations in cell culture supernatants were measured by ELISA. Data are representative of three independent experiments with two mice per group. White and gray bars represent data of mice fed the indicated diet for 10 and 28 days, respectively. * $p < 0.01$, ** $p < 0.05$ versus mice fed with CN diet.

contribute in the loss of allergenicity. However, although 121EW powder lost the allergenicity and tolerogenicity, it still possessed the immunogenicity to induce high levels of Th1 cells in OVA23–3 mice. The induction of Th1 cells by 121EW powder might be partially due to glycation of OVA by the Maillard reaction that occurred during the heat treatment. 121EW powder presented a brown color, a sign that the reaction had occurred. High levels of furosine, a product of the Maillard reaction [24], were also detected in the powder. Several studies indicate that some glycation structures may act as immune epitopes [34–37]. A previous study showed that glycated OVA was efficiently taken up by dendritic cells via scavenger receptor type A, which lead to enhanced T-cell immunogenicity [36]. Bhatia et al. reported that scavenger receptor type A engagement induced Th1-oriented immune responses [37]. Th1 cells are capable of inhibiting Th2 acti-

vation by IFN- γ production. The observations suggest that glycation by the Maillard reaction could be a factor to influence the allergenicity and tolerogenicity of food allergens.

Indeed, many factors influence the allergenicity and tolerogenicity of food allergens. One such factor is the form of allergenic foods. In this study, we showed that feeding of OVA23–3 mice with a powder form of EW diet for 28 days induced continuous weight loss and aggravated intestinal inflammation. When we fed OVA23–3 mice with a pellet form of EW diet in a previous study [18], the animals presented weight loss and inflammation during the first 3 to 10 days but subsequently resolved it by further feeding. The diet forms might influence on speed of eating and the digestibility and absorption of OVA in the gastrointestinal tissues, which could lead to the differential effects of powder and pellet forms of EW diet on induction of tolerance in OVA23–3 mice.

In summary, we show the importance of heat condition to reduce the allergenicity and gain tolerogenicity of OVA. We further indicate that disruption of IgE epitopes is not enough to reduce the allergenicity of OVA, since allergic inflammation in the intestinal tissues could be triggered by non-IgE-mediated mechanism. EW contains not only OVA but also other allergens such as ovomucoid. Future studies on the allergenicity and tolerogenicity of other heated EW allergens will elucidate whether a heated EW powder could be a safe therapeutic material in SOIT for egg-allergic patients.

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The authors have declared no conflict of interest.

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Supporting information

Materials and Methods

Analysis of total protein concentration in heated EW-powder

Powders of unheated or heated EW (2 mg) were dissolved 1 ml in PBS with or without 5% SDS. Protein concentrations in supernatants were measured using Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA, USA).

OVA-specific IgG Antibodies levels in sera

OVA-specific IgG₁, and IgG_{2a} antibody levels in sera were measured by ELISA as described previously [1].

Histamine levels in homogenates of small intestines

Ten centimeter sections of the upper small intestine were collected and homogenized according to the method of van Halteren *et al* [2]. Histamine levels in supernatants of homogenates were determined by high performance liquid chromatography (Post-Colum Reaction Systems, Waters Co., Milford, MA) [1]. Values were standardized to the weight of the jejunal tissue in each sample.

Histological analysis

Longitudinal sections of 10 cm from the upper small intestine were dissected. The tissues were opened longitudinally, fixed in 10% formalin and embedded in paraffin. Three- or 5-μm-thick sections were prepared and treated with Toluidine-blue to stain mast cells [1]. Micrographs of these specimens were observed by using an Axio skop 2 plus (Zeiss, Thornwood, NY, USA).

Statistical Analysis

The Mann-Whitney U test was used for nonparametric analysis of serum IgG antibody levels, histamine production and the number of intestinal inflammatory cells. Total protein was analyzed employing Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

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Supporting Information Table S1. Solubility of proteins in heated EW-powders.

Powders	Protein concentration (mg/g)	
	Extracts without SDS	Extracts with SDS
PEW	862 $\pm$ 16	858 $\pm$ 57
P80EW	259 $\pm$ 41*	410 $\pm$ 49*
P100EW	191 $\pm$ 35*	367 $\pm$ 24*
P121EW	83 $\pm$ 19*	257 $\pm$ 10*

Proteins were extracted from powders of unheated EW (PEW), or heated OVA at 80°C for 15 min (P80EW), 100°C for 5 min (P100EW), or 121°C for 40 min (P121EW) using PBS with and without SDS. The concentrations of extracted proteins were determined by BCA assay. The data are mean $\pm$ SEM. * $p < 0.01$ vs EW, ** $p < 0.05$

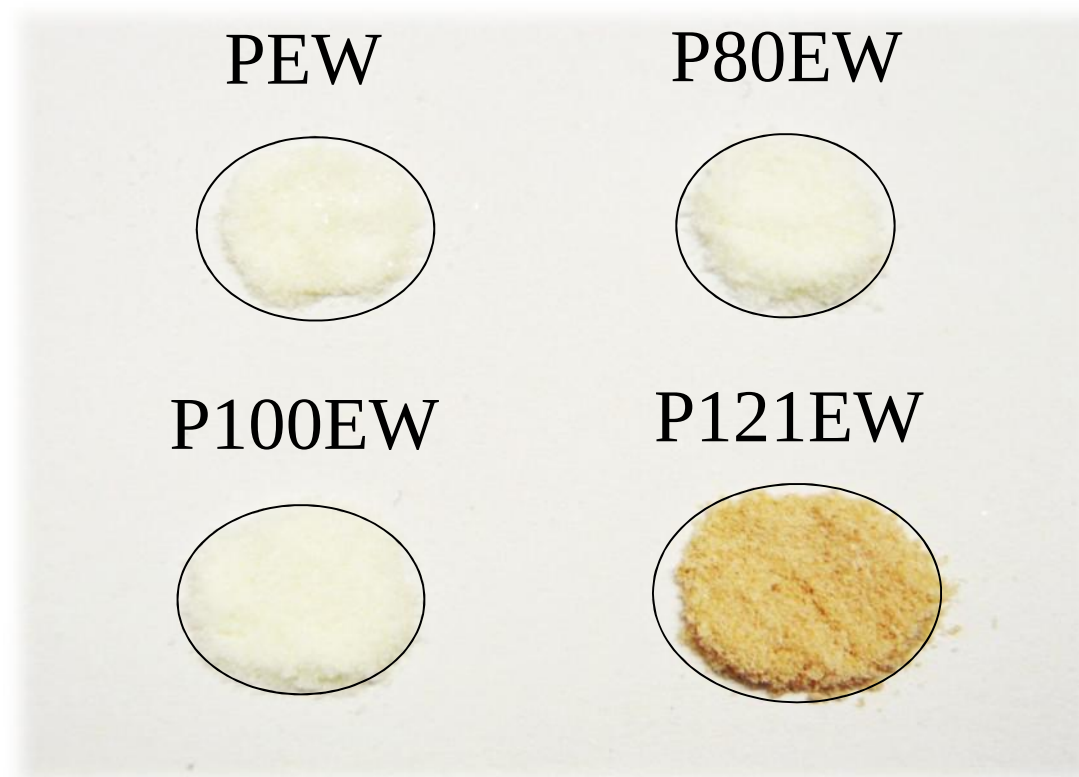


Fig. S1: Comparison of the colour of EW and heated EW powders.
EW was unheated (PEW), or heated at 80°C for 15 min (P80EW), 100°C for 5 min (P100EW), or 121°C for 40 min (P121EW), and freeze-dried.

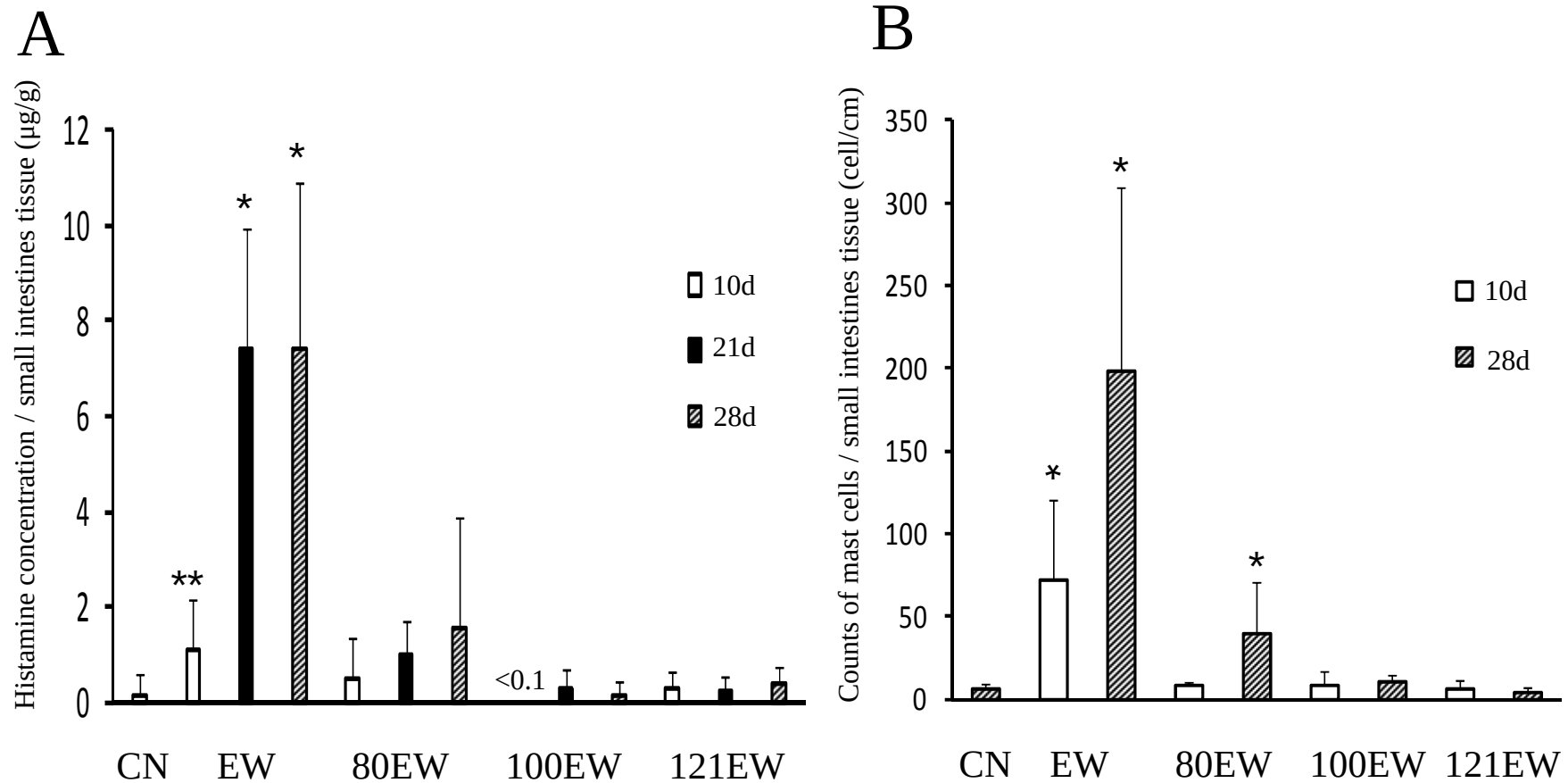


Fig. S2: The influence of heated EW-diets on intestinal mast cells in OVA23-3 mice. OVA 23-3 mice were fed with diets containing powder of unheated EW (EW), or heated EW at 80°C for 15 min (80EW), 100°C for 5 min (100EW), or 121°C for 40 min (121EW), or unheated casein (CN) for 10, 21, or 28 days. Small intestines were harvested from the mice. (A) Histamine levels in the intestinal homogenates were measured by HPLC. The number of mice used in each group (CN: n=17, EW: n=17, 80EW: n=6, 100EW: n=6, 121EW: n=6). (B) The number of mast cells in the small intestines was quantified. The number of mice used in each group (CN, n=8; EW1, n=6; EW3, n=5; 80EW1, n=4; 80EW3, n=4; 100EW1, n=4; 100EW3, n=4; 121EW1, n=6; 121EW3, n=6). White, black, and grey bars represent data of mice fed the indicated diet for 10, 21, and 28 days, respectively. * $p < 0.01$, ** $p < 0.05$ vs CN-diet fed mice

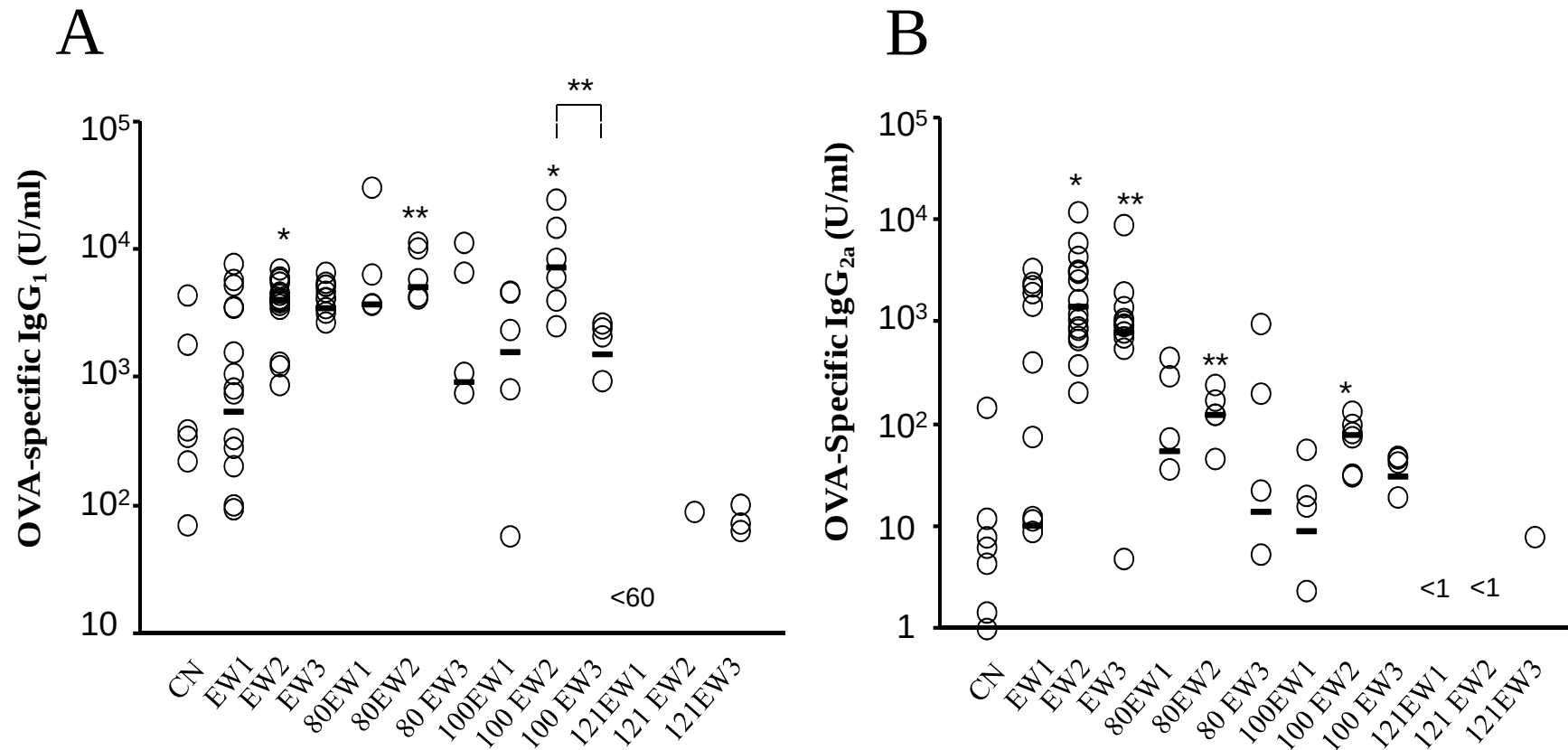


Fig. S3: The influence of heated EW-diets on serum levels of IgG antibodies in OVA 23-3 mice.

OVA 23-3 mice were fed with diets containing powder of unheated EW (EW), or heated EW at 80°C for 15 min (80EW), 100°C for 5 min (100EW), or 121°C for 40 min (121EW), or unheated casein (CN) for 10, 21, or 28 days. (A) Serum levels of OVA-specific IgG1 and (B) OVA-specific IgG2a antibodies were measured by ELISA.

The number of mice used in each group (CN, n=17; EW, n=16; 80EW, n=6; 100EW, n=6; 121EW, n=8). * $p < 0.01$, ** $p < 0.05$ vs CN-diet fed mice