

Low-Allergenic Hydrolyzed Egg Induces Oral Tolerance in Mice

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Key Words

Egg · Food allergy · Hydrolyzed proteins · Oral tolerance

Abstract

Background: Egg allergy is one of the most common food allergies in children. The standard therapy for egg allergy is strict avoidance. Yet, there is considerable clinical and scientific interest in primary or secondary prevention. A major drawback of oral tolerance (OT) induction protocols, however, is the possibility of severe side effects; thus, we have formulated a hypoallergenic egg product and demonstrate its in vivo capacity to modulate the immune system in the current study. **Methods:** Hydrolyzed egg (HE) was produced using a combination of moderate heat treatment and enzymatic hydrolysis. The capacity of HE to induce OT was tested in experimental models and compared to whole egg (WE). Delayed-type hypersensitivity (DTH) responses, immune markers and potential early markers of OT were analyzed. **Results:** Allergic responses, assessed by both DTH responses upon OVA challenge and serum OVA-specific IgE and IgG1, were decreased after treatment with HE and WE compared to the control group. Additionally, feeding WE and HE significantly decreased Th2 cytokine induction and cell proliferation, induced the activation of effector CD4⁺ T cells and increased numbers and percentages of ICOS⁺CD4⁺CD25⁺Foxp3⁺ cells. Furthermore, DO11.10

mouse experiments showed that HE contains other peptides than the OVA_{323–339} peptide that are able to induce tolerance to OVA. **Conclusions:** Altogether, results showed that HE induces OT in mice in a dose-dependent manner. Due to its low allergenicity compared to WE, it may represent a safer alternative for OT induction in at-risk subjects or oral immunotherapy in allergic patients.

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Introduction

Egg allergy is one of the most common food allergies in children, especially in those with atopic dermatitis [1]. It affects an estimated 1.8% of children younger than 5 years in the US [2], while a recent population-based study reported a prevalence of 8.9% in infants younger than 12 months in Australia [3]. Not all infants will spontaneously develop tolerance to egg later in life. A study including highly atopic children showed a resolution in 12 and 68% of patients by the age of 6 and 16 years, respectively [1], while a study including a milder atopic population showed a resolution of 66% of the patients by the age of 6 [4].

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There are several reports supporting that food hypersensitivity likely results from a failure in establishing oral tolerance (OT) or a breakdown of OT. OT refers to an immunological state of local and systemic nonresponsiveness to ingested protein antigens following prior administration of the antigens through the oral route [5, 6]. Today, the standard therapy for egg allergy is strict avoidance, which can be very complicated since egg is used as an ingredient in many food products. Thus, strategies that aim to prevent food allergy or induce desensitization in patients with food hypersensitivity could be attractive [5]. Indeed, in recent years, several pilot studies and randomized clinical trials have been performed on oral immunotherapy (OIT) for egg allergy. A review summarizing the recent advances made in OIT for egg allergy suggested the efficacy of this approach [7], which was substantiated by a recent multicenter, randomized, clinical trial from the US [8].

The cellular mechanisms behind OT induction have still not been fully elucidated. Only few data are available from human studies on egg OIT, but they imply an expansion of allergen-induced CD4⁺Foxp3⁺ regulatory T-cell (T_{reg}) levels [9, 10] and increased movement of these cells from the periphery to the lymph nodes, where dietary antigens are presented to immune cells [9]. Studies in mice showed that antigen is taken up rapidly after feeding [6, 11] and that T cells can be activated in peripheral and local lymphoid tissues within 6 h. T-cell accumulation upon antigen feeding can be detected simultaneously in peripheral lymph nodes (PLN), mesenteric lymph nodes (MLN) and Peyer's patches within 24 h. Furthermore, conversion of naïve CD4⁺ T cells into induced T_{reg} may be required to induce tolerance to ovalbumin (OVA) in mice [12].

There is still debate on where OT can be induced [reviewed in ref. 6]. There are indications that OT is induced locally in the intestinal immune system by dendritic cells migrating from the intestinal lamina propria to MLN [13]. Alternatively, antigens may escape capture by lamina propria phagocytes and reach the liver via the portal vein. The liver contains several subsets of antigen-presenting cells that may contribute to tolerance induction [11]. Beyond the liver, antigen reaching PLN and spleen might also induce tolerance through antigen presentation by resident dendritic cells, which, in the absence of co-stimulation, results in anergy of naïve T cells or induction of Foxp3⁺ T_{reg}. The fact that adoptive transfer of CD4⁺CD25⁺ T cells can induce OT in naïve animals and that depletion of CD25⁺ cells in vivo abrogates OT un-

derlines the importance of T_{reg} in the induction and/or maintenance of OT [14].

A major drawback of OIT, however, is that side effects are common, which prevents 10–20% of patients from continuing with therapy [7]. Hydrolysis of food-derived proteins has been successfully performed leading to less allergenic products that still induce OT in vivo [15, 16]. Hydrolyzed egg (HE) might be a promising candidate for OIT because a high reduction in the allergenic potential has been established in vitro [17] and in humans [unpubl. data]. However, it remained to be proven that HE can still induce OT. Recent studies already showed effective desensitization capacity of HE white [18] and heated ovomucoid-depleted egg white [19] in OVA-sensitized mice. However, it has not been studied yet whether prior oral administration of HE could also prevent egg allergy.

Clinical studies in humans have documented that OT to a dietary protein antigen manifests itself as suppression of T-cell-mediated delayed-type hypersensitivity (DTH), rather than a reduction in an antigen-antibody response [20, 21]. Also, several studies used animal models to study the induction of OT with intact proteins [22, 23], protein fragments [15, 24] or haptens [14]. A murine model of DTH mediated by antigen-specific T cells was adapted from Arnaboldi et al. [22] and used in our study to investigate the potential of HE and whole egg (WE) to induce tolerance by the oral route (i.e. by feeding). Systemic establishment of OT was measured by DTH response upon subcutaneous OVA challenge and by serum OVA-specific IgE and IgG1 levels.

In addition, early cellular mechanisms of OT induction were assessed following the oral administration of WE or HE. Our studies revealed that HE can induce OT in a dose-dependent manner and that OT might be associated with the induction of ICOS⁺CD4⁺CD25⁺Foxp3⁺ cells, which are thought to have a stronger suppressive activity than ICOS[−]CD4⁺CD25⁺Foxp3⁺ cells [25].

Methods

HE and WE Preparation

The process of HE preparation has been described in detail before [17] and was adopted with minor adjustments. Briefly, pasteurized liquid WE (FT/OVO/0105 R, ABCD S.A.; Avicole Bretonne Cecab Distribution, Plœrmel, France) was heated at 65°C for 10 min and after cooling to 55°C, 5% (E/S) Protamex enzymes (Novozymes, Bagsvaerd, Denmark) were added. The mixture was maintained at 55°C for 2 h and afterwards 1% (E/S) of Flavourzyme 1000L (Novozymes) was added and the mixture was heated at

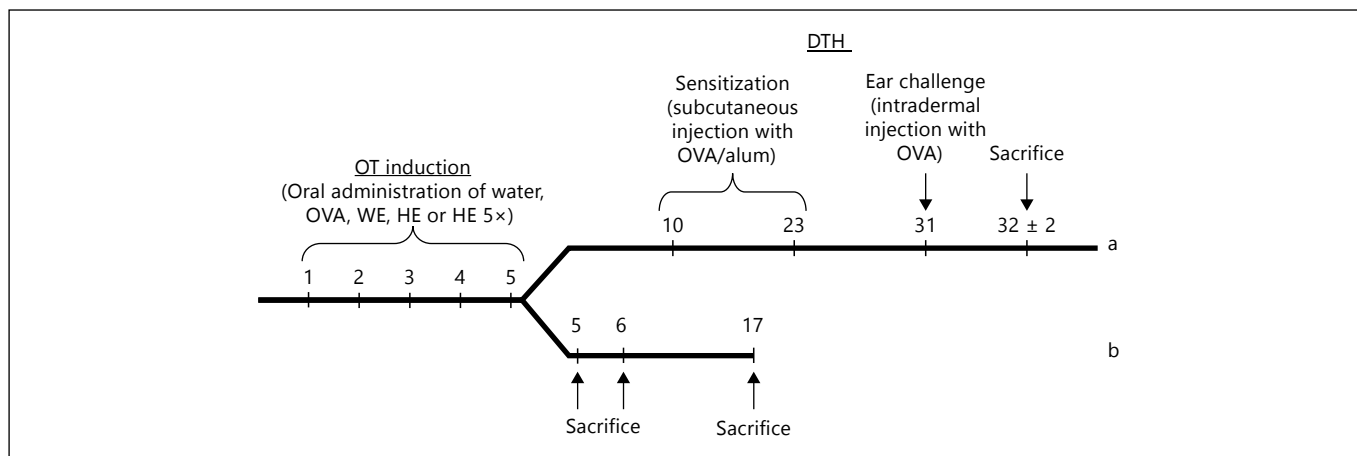


Fig. 1. Schematic representation of the in vivo models used in this study. In both models, 4- to 5-week-old C57BL/6J mice (models a and b) or BALB/c C.Cg-Tg(DO11.10)10Dlo/J mice (model b) were given water, OVA, WE, HE or HE 5× in the drinking water for 5 consecutive days in order to induce OT. In model a, OT induction was followed by a DTH model. Mice were immunized twice on days 10 and 23 subcutaneously with OVA in alum. On day 31, the DTH response was elicited by 20 µg OVA injected intrader-

mally in both ears, and ear thickness was measured before the challenge and each day during the 4 days after the challenge. Mice were sacrificed on day 32 ± 2, and blood, brachial, inguinal and axillary lymph nodes and spleen were collected from each mouse. In model b, mice were sacrificed after OT induction on day 5 or 6 (0 or 1 day after oral feeding) or on day 17 (12 days after oral feeding), and MLN were collected from each mouse.

75°C for 10 min. The mixture was then cooled to 55°C, 4% (E/S) of Flavourzyme 1000L were added again and the mixture was maintained at 55°C for 2 h. The mixture was heated at 90°C for 30 min to inactivate the enzymes and to pasteurize the product, and then spray dried to obtain a HE powder.

WE was obtained by spray drying the pasteurized liquid WE.

In vivo Assessment of OT Induction using a DTH Model

OT Induction. Four- to 5-week-old female C57BL/6J mice (Charles River, L'Arbresle, France) were given OVA (grade II; Sigma-Aldrich, Buchs, Switzerland), WE (NRC, Lausanne, Switzerland) or HE (NRC) by ad libitum feeding in the drinking water for 5 consecutive days. Solutions were freshly prepared every day and the amount of OVA in the drinking water was standardized in all groups to 10 g OVA (equivalent OVA in the case of HE) per liter. An extra group of mice receiving a five times higher concentration of HE (referred to as HE 5×) was included.

DTH. Mice (7–12/group) were immunized on days 10 and 23 by subcutaneous injection of 100 µg OVA (grade V; Sigma-Aldrich) with 1 mg aluminum potassium sulfate dodecahydrate (alum; Sigma-Aldrich) in 100 µl 0.9% NaCl (Sigma-Aldrich) in the neck region (injection of 50 µl/site at two different sites). On day 31, DTH response was elicited by ear challenge by intradermal injection of 20 µg OVA in 20 µl of PBS under isoflurane anesthesia. Ear thickness was measured under isoflurane anesthesia using a caliper (Interapid, Geneva, Switzerland) before the challenge and each day during 4 days after the challenge. Ear swelling (µm) was calculated by subtracting ear thickness measured before the challenge from that obtained after the challenge, and the results represent means of right and left ear swelling. The protocol was adapted from the DTH model described by Arnaboldi et al. [22]. Mice were sacrificed under isoflurane anesthesia on day 31 ± 2 and blood,

brachial, inguinal and axillary lymph nodes, and spleen were collected from each mouse (fig. 1a). All animal studies were approved by a Nestec internal ethics committee and the Service vétérinaire du canton de Vaud, Switzerland.

Assessment of Potential Early Markers of OT Induction to OVA

Four- to 5-week-old female C57BL/6J mice (Charles River; 6–10 mice/group) or BALB/c C.Cg-Tg(DO11.10)10Dlo/J mice [transgenic mice which express a T-cell receptor (TCR) specific to the OVA_{323–339} peptide; Jackson Laboratories; 6 mice/group] were given OVA, WE, HE, HE 5× or nothing in their drinking water for 5 days (fig. 1; OT induction). Mice were sacrificed under isoflurane anesthesia on day 5 or 6 (0 or 1 day after oral feeding) or on day 17 (12 days after oral feeding) and MLN were collected from each mouse for FACS staining (fig. 1b).

A single-cell suspension was prepared by a cell strainer and immunological phenotyping was performed on a FACS BD LSRFortessa (BD Biosciences, San Jose, Calif., USA); data were analyzed using FlowJo software (Tree Star Inc., Ashland, Oreg., USA). The following monoclonal antibody combination was used to assess T-cell activation and T_{reg} according to the manufacturer's instructions: α-CD4 (PE-Cy7; BD), α-CD62L (L-selectin, FITC; BD), α-CTLA-4 (PE; BD), α-Foxp3 (eFluor 450; eBioscience, San Diego, Calif., USA), α-CD278 (ICOS)-biotin followed by Strepta APC (APC; BD) and α-CD25 (APC-Cy7; BD).

Quantification of Serum Levels of OVA-Specific IgE and IgG1

OVA-specific IgE and IgG1 concentrations were measured in mouse serum by ELISA as described previously [26]. For OVA-specific IgE measurement, 96-well plates (NUNC Maxisorp; VWR, Dietikon, Switzerland) were coated overnight at 4°C with rat anti-

mouse IgE (2 µg/ml; BD Pharmingen, Allschwil, Switzerland) and afterwards blocked with PBS-1% BSA for 1 h at room temperature. Serially diluted sera and standard (monoclonal mouse anti-OVA; Serotec, Puchheim, Germany) were incubated for 2 h at 37°C. Then, biotinylated OVA (4 µg/ml; USBiological, Salem, Mass., USA) was added to the plate for 2 h at 37°C, followed by incubation with HRP-labeled streptavidin (1:200; R&D, Switzerland) for 30 min at 37°C. Plates were developed with tetramethylbenzidine substrate (KPL, Gaithersburg, Md., USA). The reaction was stopped with 1 M HCl (Merck, Darmstadt, Germany). Optical densities were measured at 450 nm. Concentrations were calculated by converting optical density values in picograms per milliliter using a polynomial standard curve.

For OVA-specific IgG1, microtiter plates were coated with 100 µg/ml OVA (Sigma-Aldrich) overnight at 4°C. Wells were blocked with PBS-1% BSA for 1 h at room temperature and, after washing, serially diluted sera and standard (monoclonal mouse anti-OVA IgG1; Antibody Shop, Switzerland) were added for 2 h at 37°C. After washing, wells were incubated for 2 h with an HRP-labeled goat anti-mouse IgG1 (1:5,000; Southern Biotech, BioConcept, Allschwil, Switzerland) and plates were developed with tetramethylbenzidine.

Isolation and Stimulation of Cells from Spleen and PLN

Spleens and PLN (a pool of inguinal, brachial and axillary lymph nodes) were collected at sacrifice and single-cell suspensions were prepared using a syringe plunger in a cell strainer (BD). Red blood cells were removed from the spleen cell suspensions by incubation with red blood cell lysing buffer (Sigma-Aldrich). Cells were cultured in flat-bottom 96-well plates (Corning, Switzerland) at 0.5×10^6 cells/well for splenocytes and 0.3×10^6 cells/well for PLN in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (BioConcept), 50 µM β-mercaptoethanol, 1% L-glutamine and 1% penicillin-streptomycin (all from Sigma-Aldrich). Cells from all mice were cultured individually in triplicate in the absence or presence of 100 µg/ml OVA.

Cytokine Quantification

After 70 h of culture, supernatants from triplicate wells were collected, pooled and frozen until further analysis. Innate and adaptive cytokines [IFN-γ, IL-1β, IL-2, IL-4, IL-5, KC/GRO, IL-10, IL-12 (total) and TNF-α] were measured by the mouse Th1/Th2 9-plex multiplex kit (Meso Scale Discovery, Gaithersburg, Md., USA) according to the manufacturer's protocol.

Proliferation Measurement

³[H]-thymidine (0.5 µCi/well, Amersham, Braunschweig, Germany) was added to the cells after collection of the supernatants at 70 h and plates were frozen after an extra 18 h of incubation. Proliferative responses of spleen and PLN cell cultures were determined in triplicate wells by scintillation counting.

Peptide Mapping by Liquid Chromatography Tandem Mass Spectrometry

WE or HE was resuspended in 2% (v/v) acetonitrile/0.1% (v/v) formic acid at a concentration of 5 mg/ml protein. The soluble fraction was then diluted to reach a final 1:1,000 dilution of the sample. This final solution was further filtered using 0.25-µm filter units. Samples were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). Briefly, samples (10 µl) were in-

jected into a CTC PAL injection system. Peptides were separated on a nano C18 column. Data were then searched with Sequest (Thermo) using a chicken (*Gallus gallus*) protein database with the following parameters: (i) no enzyme specificity, (ii) 5 ppm mass accuracy for the precursor, (iii) 0.8 Da for fragments and (iv) oxidation (M)/deamidation (NQ) set as variable modifications. Peptide hits were further statistically validated at an estimated false discovery rate of <1% (PeptideProphet). Finally, identified peptides in the HE hydrolysate and the OVA_{323–339} peptide were mapped together using an in-house tool programmed in Perl that allows displaying peptide sequences within any protein sequence [27].

Statistics

The software R 2.14.1 [28] was used for the analysis. Exact Wilcoxon tests have been performed for all the comparisons of interest. Two-sided tests have been performed without correction for test multiplicity. Results were considered significant if $p \leq 0.05$ and significant p values are shown comparing the water group with all treatment groups and comparing the WE, HE and HE 5× groups. Data are presented from 1 of 1–3 independent experiments.

Results

HE Induces OT in a Dose-Dependent Manner

As expected, oral administration of OVA induced OT to this antigen, as evidenced by reduced DTH responses to OVA (fig. 2a, b), and OVA-specific IgE (fig. 2c) and IgG1 (data not shown). Similar results were obtained with WE, while feeding HE was less efficient and resulted in a nonsignificant decrease in DTH ($p = 0.079$; fig. 2b) but a significant decrease in OVA-specific IgE ($p = 0.014$; fig. 2c). However, feeding a 5× higher dose of HE resulted in a significant decrease in the DTH response ($p < 0.001$; fig. 2b), and anti-OVA specific IgE ($p = 0.002$; fig. 2c) and IgG1 levels ($p = 0.006$; data not shown) compared with the water group. These data suggest a dose-dependent induction of OT by HE.

HE Decreases Th2 Cytokine Induction and Proliferation Capacity after Restimulation of Cells with OVA

As shown in figure 3a, feeding with OVA prior to sensitization with this antigen significantly blunted proliferation of spleen cells stimulated ex vivo with OVA, and similar results were obtained after feeding WE, HE and HE 5× (all $p < 0.001$). For OVA-restimulated PLN cells, significance was only reached in the group fed with WE ($p = 0.002$; fig. 3a).

Feeding with OVA as well as with WE, HE and HE 5× prior to OVA sensitization also significantly blunted IL-5 release by spleen and PLN cells after ex vivo restimulation

(fig. 3b). The amount of IL-5 in the supernatant of restimulated spleen cells was significantly higher in the HE group compared to the WE and HE 5× groups (fig. 3b). As observed for IL-5, IL-4 levels were significantly higher in the water group compared to all treatment groups: water (16.1 ± 1.7 pg/ml), OVA (11.2 ± 1.1), WE (12.4 ± 1.6), HE (13.6 ± 0.7) and HE 5× (10.6 ± 1.0). IL-4 levels in the supernatants of restimulated PLN cells were all <10 pg/ml, and most of them were below the detection limit. Feeding with OVA and HE 5× significantly decreased IFN- γ in the supernatant of restimulated spleen cells, while feeding with HE and HE 5× slightly increased the low levels of IFN- γ secreted by PLN cells (fig. 3c).

Overall, both a small decrease in IL-10 (fig. 3d) and an increase in IL-12 were observed for the treated groups, with strikingly higher levels of IL-12 in the WE group compared to the water group (data not shown). Altogether these results suggest that HE, like full-length egg antigens present in WE, significantly decreased both PLN and spleen antigen-specific restimulation responses.

HE Feeding Induces an Increase in ICOS+CD4+CD25+Foxp3+ Cells in MLN of Wild-Type Mice

Oral administration of WE, HE and HE 5× increased the total number of cells ($9.5 \pm 0.7 \times 10^6$, $8.5 \pm 1.2 \times 10^6$ and $7.5 \pm 1.0 \times 10^6$, respectively, data not shown) in the MLN (a major site of OT induction) compared to the water-fed group ($2.4 \pm 0.5 \times 10^6$). An increase in the absolute CD4+ cell number was observed ($3.9 \pm 0.2 \times 10^6$, $3.3 \pm 0.5 \times 10^6$, $2.9 \pm 0.4 \times 10^6$ and $1.1 \pm 0.2 \times 10^6$ for WE, HE, HE 5× and water, respectively; fig. 4a), while the percentage of CD4+ cells was significantly lower in the WE (41.1 ± 0.4), HE (38.5 ± 0.8) and HE 5× (39.5 ± 1.0) groups compared to the water group (44.1 ± 2.0 ; data not shown). The total number of CD4+CD25+Foxp3+ cells, as well as the number and the percentage of ICOS+ cells among CD4+CD25+Foxp3+ cells, was higher in the HE, HE 5× and WE groups compared to the water group (fig. 4b–d). No difference was observed between the different groups regarding the percentages of CD4+CD25+Foxp3+ cells or the percentages of CTLA4+ (CD152+) cells among CD4+CD25+Foxp3+ cells (data not shown).

OVA and WE but Not HE Induce a Transient Increase in ICOS+CD4+CD25+Foxp3+ Cells in MLN of DO11.10 Mice

To further support the increased number of ICOS+CD4+CD25+Foxp3+ cells observed in wild-type mice, we used DO11.10 TCR transgenic mice that express

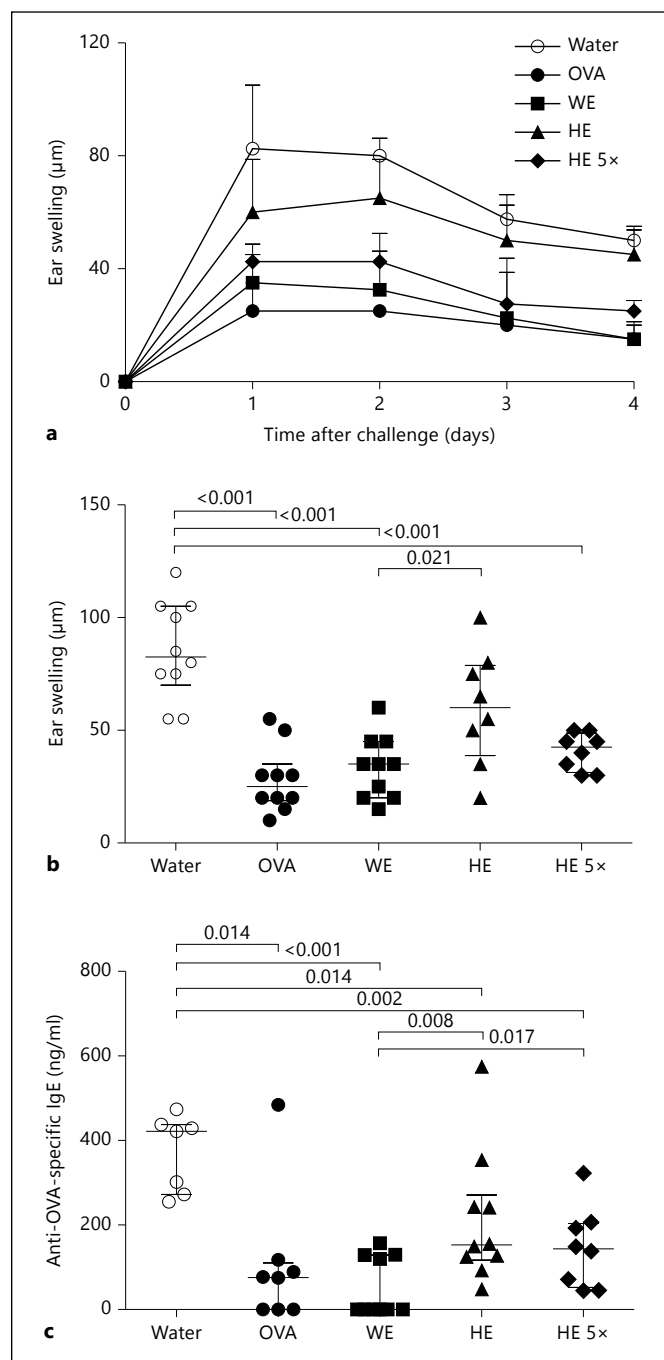


Fig. 2. Effect of WE and HE feeding on OT induction to OVA. C57BL/6J mice were fed with OVA, WE, HE, HE 5× or water for 5 consecutive days and subcutaneously immunized twice with OVA in alum. Intradermal ear challenges with OVA were performed on day 31. Ear swelling at various time points after challenge (a) and 24 h after challenge (b) is shown. Blood was taken on day 30 and levels of serum OVA-specific IgE were quantified by ELISA (c). Each dot represents an individual mouse. Bars represent medians and interquartile ranges; n = 7–10 mice/group. p values indicate significant differences between groups using an exact Wilcoxon test.

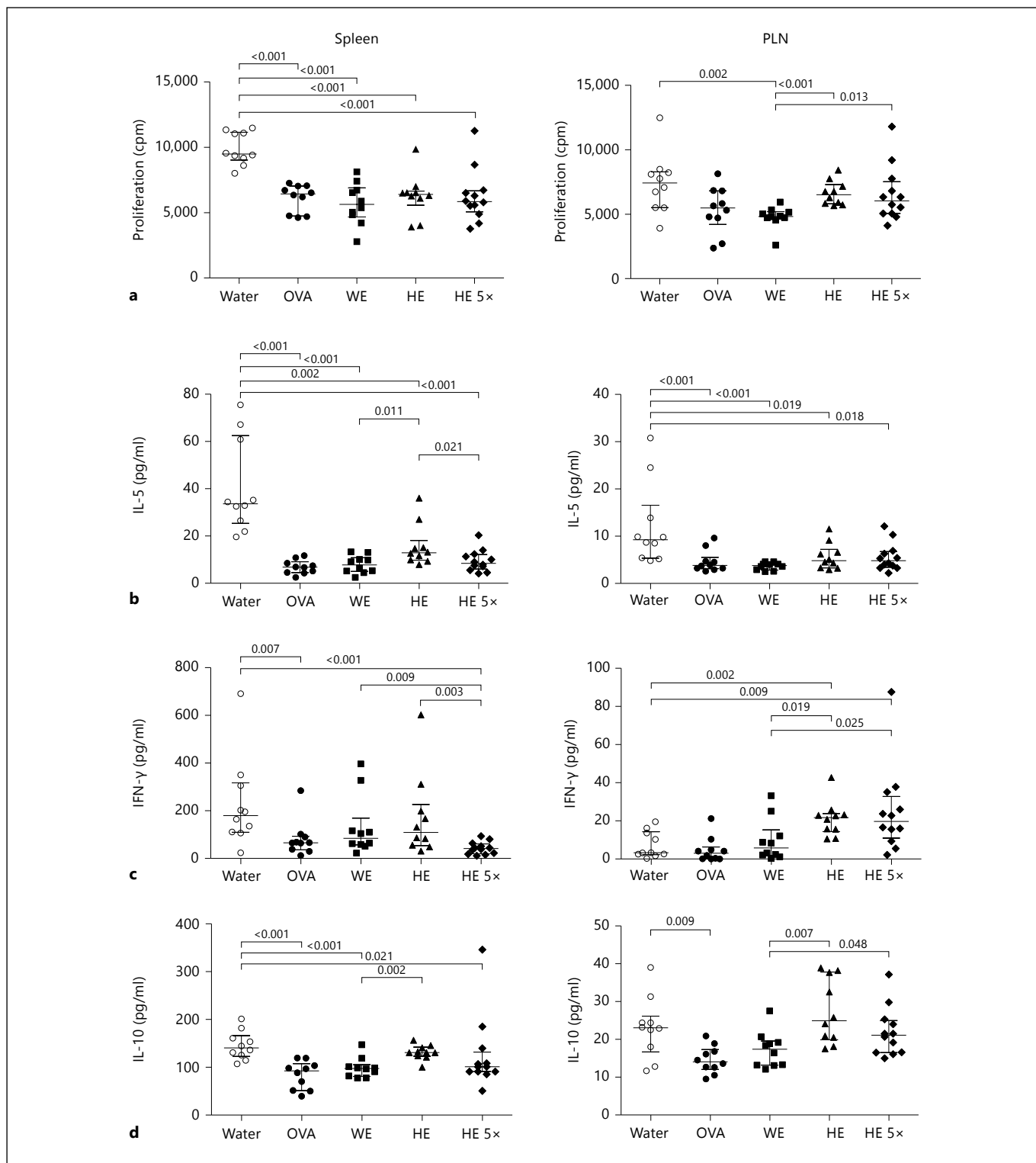
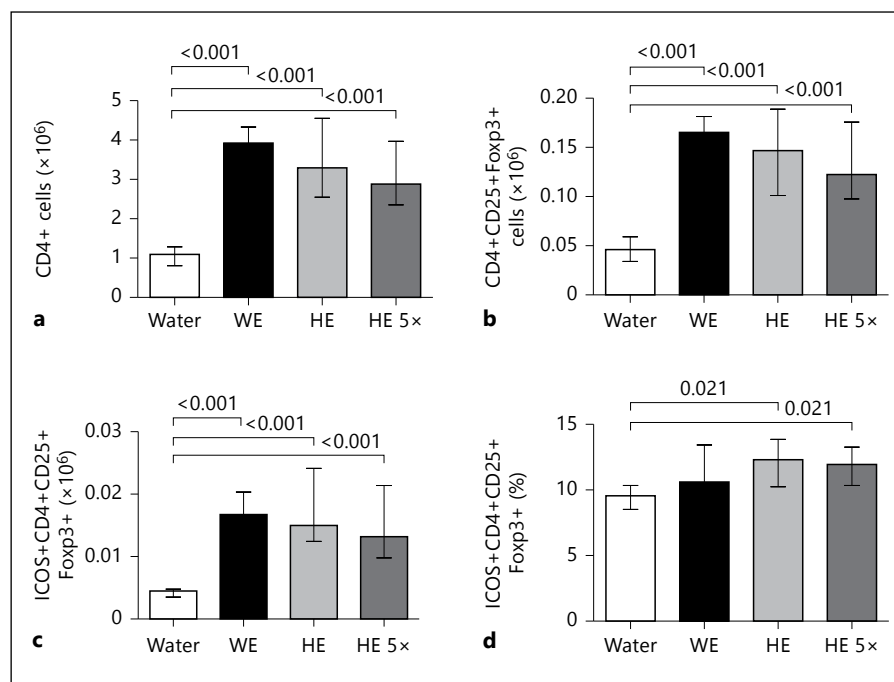


Fig. 3. Impact of prior WE and HE feeding on cell proliferation and ex vivo cytokine production in subsequently sensitized mice. C57BL/6J mice were fed with OVA (●), WE (■), HE (▲), HE 5× (◆) or water (○) for 5 days and subcutaneously immunized with OVA. Spleen (left panels) and PLN (right panels) cells were iso-

lated and restimulated with OVA. Proliferation (a) and cytokine release (b–d) were assessed. Each dot represents an individual mouse; bars represent medians and interquartile ranges; n = 10–12 mice/group. p values indicate significant differences between groups using an exact Wilcoxon test.

Fig. 4. CD4+, CD4+CD25+Foxp3+ and ICOS+CD4+CD25+Foxp3+ cells in MLN of wild-type mice fed with WE (black bars), HE (light grey bars), HE 5× (dark grey bars) or water (white bars) for 5 consecutive days and sacrificed on day 6 (i.e. 1 day after feeding). Results are expressed as medians and interquartile ranges of the absolute numbers of CD4+ cells (a), CD4+CD25+Foxp3+ cells (b) and ICOS+CD4+CD25+Foxp3+ cells (c), and percentage of ICOS+CD4+CD25+Foxp3+ cells (d) in MLN; n = 8–10 mice/group. p values indicate significant differences between groups using an exact Wilcoxon test.



a TCR recognizing specifically the OVA_{323–339} peptide. MLN cells from DO11.10 mice were obtained directly after (on day 5) or 12 days after (on day 17) the 5-day consecutive feeding with water, OVA, WE or HE to assess early mechanisms of OT induction (fig. 1b).

An increase in the absolute CD4+ cell number was observed ($15.6 \pm 1.3 \times 10^6$, $10.4 \pm 1.5 \times 10^6$, $7.3 \pm 1.4 \times 10^6$ and $3.6 \pm 2.4 \times 10^6$ for OVA, WE, HE and water, respectively; fig. 5a). The total number of CD4+CD25+Foxp3+ cells was increased after OVA and WE feeding, while this increase was significantly lower for HE (fig. 5b). A transient increase in the absolute number as well as the percentage of ICOS+ cells within CD4+CD25+Foxp3+ cells (fig. 5c, d) was observed on day 5 after both OVA and WE feeding; this increase was only minor for HE feeding compared to water. No difference in the absolute number or the percentage of ICOS+CD4+CD25+Foxp3+ cells was observed on day 17 (data not shown).

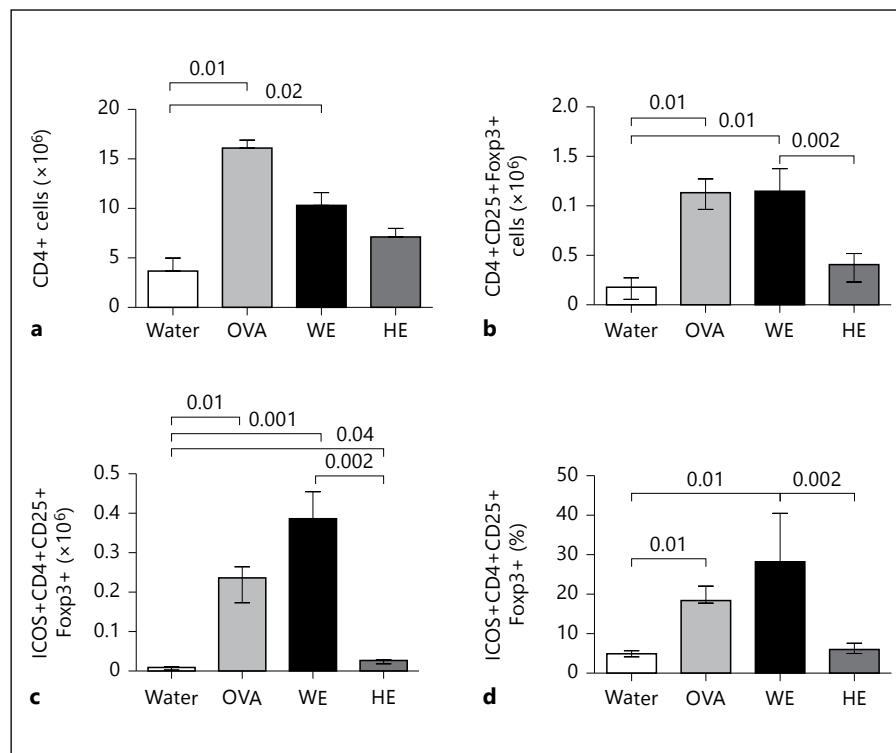
LC-MS/MS revealed that the OVA_{323–339} peptide, which is recognized by the TCR of DO11.10 mice, was not identified in the HE hydrolysate in its full length (fig. 6; online suppl. fig. 1; see www.karger.com/doi/10.1159/000363110). Only peptides sharing a maximum of 50% of the TCR OVA_{323–339} peptide sequence are present and this may explain why feeding DO11.10 mice with HE did not result in clear cellular immune events.

Discussion

This study showed that oral administration of both WE and HE induced OT to OVA. The allergic response, assessed by the DTH response upon OVA challenge and serum OVA-specific IgE and IgG1, was significantly decreased after treatment with WE and HE. In addition, a decreased antigen-specific ex vivo response was observed in mice fed with WE and HE compared to the water group, which consisted of a significant decrease in Th2 cytokine induction and cell proliferation capacity. Taken together, these data show OT induction to OVA after 5 days of OVA, WE and HE feeding. However, HE was less effective than WE, and a higher concentration of HE in the drinking water was needed to obtain a similar level of OT. While daily weighing of the water bottles demonstrated a lower preference for the OVA drinking water, no striking difference was observed between HE and WE. This lower efficacy of HE to induce OT might be caused by a reduced number of relevant epitopes recognized by the immune system after enzymatic and heat treatment of WE. Additionally, these processing conditions can influence absorption and transport of the allergens through the epithelial layer [29] as well as digestibility of the allergens [19], and thus impact on their presentation to the immune system.

Orally fed antigens can cross the intestinal epithelial-cell barrier, be presented to the immune system and in-

Fig. 5. CD4+, CD4+CD25+Foxp3+ and ICOS+CD4+CD25+Foxp3 cells in MLN of DO11.10 mice fed with OVA (light grey bars), WE (black bars), HE (dark grey bars) or water (white bars) for 5 consecutive days and sacrificed on day 5. Results are expressed as medians and interquartile ranges of the absolute numbers of CD4+ cells (a), CD4+CD25+Foxp3+ cells (b) and ICOS+CD4+CD25+Foxp3+ cells (c), and percentage of ICOS+CD4+CD25+Foxp3+ cells (d) in MLN; n = 6 mice/group. p values indicate significant differences between groups using an exact Wilcoxon test.



duce OT via different pathways [reviewed in ref. 6]. Induction of T_{reg} and subsequent migration of these T_{reg} back to the lamina propria, where they proliferate and may enter the circulation, is thought to be an important route to induce systemic OT [14]. We thus studied the effect of HE-induced OT on the regulatory phenotype of T cells from MLN at different time points after oral administration of the antigen, as the MLN represent one of the major sites of OT induction [6, 30]. It has been proposed that T_{reg} contain a predominant ICOS⁻ death-prone subset and a minor living ICOS⁺ hyperproliferative subset and that ICOS⁺ T_{reg} have much stronger suppressive activity than ICOS⁻ T_{reg} [25]. Moreover, it has been documented that ICOS-deficient mice are resistant to the induction of OT [31] and that ICOS+CD25+CD4+Foxp3+ T_{reg} represent a subset of activated T_{reg} that proliferate in vivo when in contact with their cognate antigen [32]. Interestingly, in our mouse model, a transient increase in ICOS+CD4+CD25+Foxp3+ cells was observed in both DO11.10 mice fed with OVA or WE, and in normal mice fed with WE, HE or HE 5 $\times$, suggesting that this T_{reg} subpopulation might be involved in OT induction by OVA, WE and HE. Studies using ICOS-deficient mice would give more information. DO11.10 cells are specific for a C-terminal epitope whose core encompasses amino acids

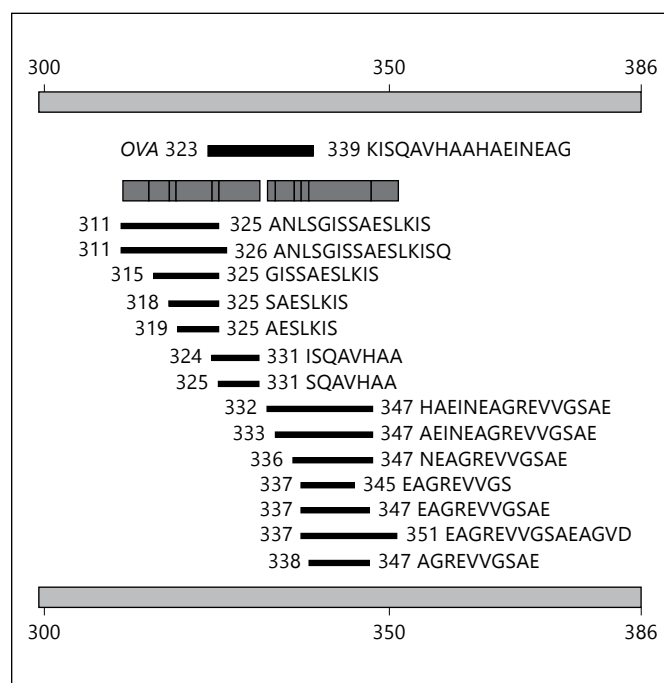


Fig. 6. Overlapping of the HE peptides with the OVA₃₂₃₋₃₃₉ peptide. Peptide OVA₃₂₃₋₃₃₉ was compared to HE peptides identified by LC-MS/MS using an in-house mapping tool.

329–337 [33]. Peptide identification showed that this specific peptide was not conserved in HE, which could explain why feeding DO11.10 mice with HE did not result in clear early cellular events. Interestingly, even though the full-length main OVA epitope was not present in HE, our studies showed that OT can be induced by feeding with HE. Therefore, other peptides than the OVA_{323–339} peptide seem to be able to induce tolerance to OVA.

As HE is able to modulate the immune system and induce OT to OVA, it could be of use in immunotherapy. Currently, OIT and sublingual immunotherapy seem to be the most promising approaches for food allergy; however, the challenge of immunotherapy is to achieve good safety and high efficacy. The safety of OIT might be improved using hypoallergenic processed materials such as HE [34]. Still, optimal administration schemes (e.g. dose, frequency and duration of treatment), identification of the most responsive target group (in terms of age and disease status) and long-term efficacy of OIT remain to be investigated [35].

In a recent paper, Watanabe et al. [36] showed the importance of specific hydrolysis conditions to generate HE exhibiting a reduced allergenicity but retaining tolerogenicity. Using a transgenic OVA23-2 mouse model expressing the OVA_{323–339}-specific TCR, they showed that long-term feeding of unheated egg white as well as egg white treated at 80°C for 15 min induced OT to OVA. However, the heat-treated preparation retained allergenicity.

In contrast, treatment at 121°C for 40 min almost completely reduced allergenicity but also tolerogenicity [36]. In our experiments, we used a combination of processing conditions leading to a hypoallergenic HE preparation [17], while preserving at least part of the tolerogenic properties of WE.

Taken together, our data demonstrated that HE is able to induce OT to OVA in mice in a dose-dependent manner. If proven also in humans, OT induction using HE could be a safer way to manage egg allergy and thereby broaden the diet of allergic or at-risk children while reducing the risk of adverse reactions.

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Disclosure Statement

All authors are or were employees of Nestec Ltd.

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Supplementary Material

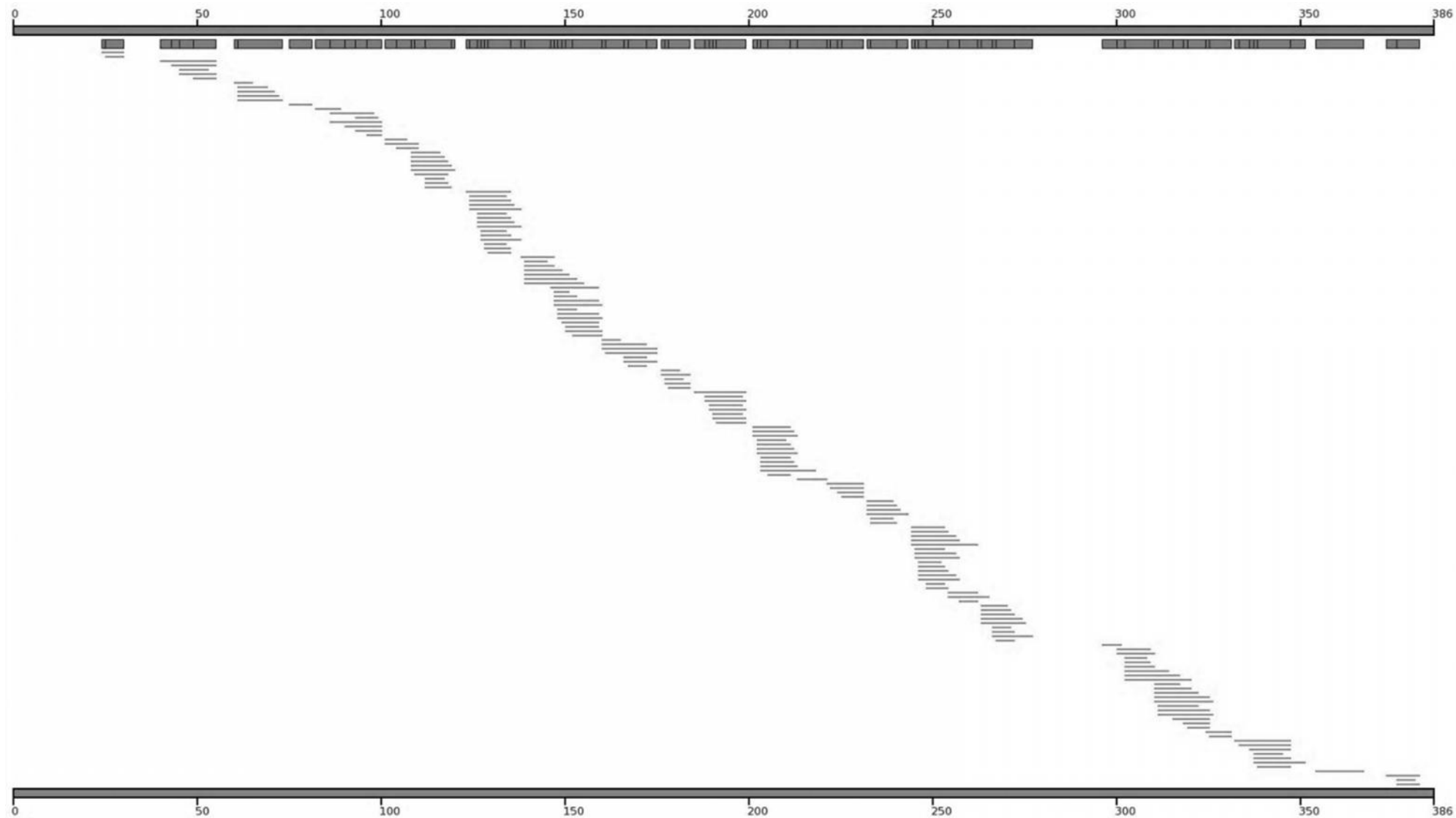


Fig. S1. Distribution of the hydrolyzed egg (HE) peptides in the native OVA sequence. Oligopeptide fragments of HE were identified by LC-MS/MS. Position of the peptides is shown in the native sequence of ovalbumin.