

Allergenicity of pasteurized whole raw Hen's egg compared with fresh whole raw Hen's egg

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Keywords

egg allergy; dehydrated whole egg; dehydrated egg white; desensitization; diagnosis of egg allergy; dried egg; egg oral immunotherapy; oral food challenge; oral tolerance induction

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Abstract

Background: Oral food challenges for diagnosis and management of egg allergy using fresh egg are common; however, to limit the risk of foodborne infection, many allergy units use pasteurized raw egg. Pasteurization and drying processes have the potential to affect the structure of egg proteins in egg powder and thus the allergenicity when compared to fresh egg. Our aim was to compare the binding of serum IgE from egg-allergic children to *in vitro* digested and undigested pasteurized whole raw egg powder with unpasteurized fresh whole raw egg.

Methods: Egg proteins from *in vitro* digested or undigested pasteurized whole raw egg powder, fresh whole egg, egg white and egg yolk were separated by SDS-PAGE, transferred onto nitrocellulose membrane and incubated overnight with pooled sera from egg-allergic children.

Results: In both the raw egg samples and the pasteurized whole egg powder, protein bands corresponding to known molecular weights of the major egg allergens were present. Pasteurized whole raw egg powder was bound by serum IgE in a similar manner to unpasteurized whole raw egg and was unaffected by *in vitro* digestion. Serum IgE also bound egg yolk, indicating sensitization to both egg yolk and egg white proteins.

Conclusions: The main egg allergens are present in pasteurized whole raw egg powder, and serum IgE of egg-allergic children binds to them in a similar pattern to those in fresh whole raw egg. Pasteurized whole raw egg powder is a suitable substitute for raw egg in clinical practice for oral food challenges.

The 'gold standard' for the diagnosis of a food allergy is a double-blind, placebo-controlled food challenge. The form of egg given for an oral food challenge for the diagnosis of egg allergy is important and will have an effect on the foods that the individual with the egg allergy is advised to consume. Many children who are unable to tolerate whole cooked egg may tolerate egg in well-baked goods (1). A cooked egg challenge reflects the way that egg is usually consumed in the diet. However, children who tolerate a cooked egg challenge may not tolerate raw egg and may react to common foods that contain raw egg such as gelato, mayonnaise and confectionary. As such, oral food challenges using fresh raw egg are often used, and many institutions challenge with pasteurized whole raw egg liquid or powder due to concerns regarding potential risk of infection with *Salmonella* or avian influenza (2, 3).

All egg proteins have the potential to induce an allergic reaction (4); however, six major egg protein allergens have been identified: ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), lysozyme (Gal d 4), as well as the egg yolk proteins α -livetins (Gal d 5) and glycoprotein 42 (Gal d 6) (4, 5). Food processing, in particular heating, disrupts the tertiary structure of egg proteins, affecting their allergenicity. It is assumed that pasteurized raw whole egg powder has equivalent allergenicity to fresh raw whole egg, but the pasteurization and drying processes employed in the manufacture of pasteurized raw egg powder have the potential to affect the structure of the egg proteins and their allergenicity when compared to fresh raw whole egg. Pasteurized egg white liquid (3) and powder (6) maintain their allergenicity compared to fresh unpasteurized egg white, but to date, there are no reports

comparing the allergenicity of pasteurized whole raw egg powder (which also contains the egg yolk allergens) with fresh, unpasteurized raw egg.

The aim of this study was to assess the allergenicity of pasteurized raw whole egg powder by comparing the IgE binding of pooled sera from egg-allergic individuals to *in vitro* digested and undigested pasteurized raw whole egg powder with that of fresh unpasteurized whole raw egg. The findings will validate current protocols for oral food challenges using pasteurized whole egg powder in the diagnosis of egg allergy.

Methods

Preparation of egg samples

Farm Pride whole pasteurized raw egg powder (pasteurized at 65°C for 4 min) was assessed (Farm Pride, Keysborough, Vic., Australia). Two grams of the egg powder contains 0.9 g protein, equivalent to one-sixth of an egg. For analysis, 0.5 g pasteurized egg powder was diluted in 5 ml of phosphate-buffered saline (PBS, Sigma, Sydney, NSW, Australia) and centrifuged for 5 min at 5000 *g*, and supernatants were collected. The protein concentration of the supernatants was determined using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc, Scoresby, Victoria, Australia) as per the manufacturer's instructions.

60 g free range fresh eggs (Coles Pty Ltd, Glen Iris, Victoria, Australia) were used for the fresh egg samples. It was estimated from the known protein content of fresh egg that 1.2 g of fresh egg would provide the equivalent amount of protein as 0.5 g pasteurized whole egg powder (7). 1.2 g samples of whole egg, egg white and egg yolk were diluted in 2.5 ml PBS and homogenized. The samples were centrifuged at 5000 *g*, the supernatants were collected, and the protein content of the supernatants was determined.

In vitro digestion of egg protein

The method used for the *in vitro* digestion was adapted from Martos et al. (8). To simulate gastric digestion samples of pasteurized egg powder, whole egg, egg white and egg yolk were diluted in 1% PBS adjusted to pH2 with 39% HCl and then incubated for 15 min at 37°C. Pepsin from porcine gastric mucosa (Sigma-Aldrich) was added at an enzyme to substrate ratio of 1:20 (wt:wt). The samples were then incubated for 60 min at 37°C. The reaction was stopped with 1 mol/l NaHCO₃ to give a final protein concentration of 5 mg/ml and pH7. To simulate duodenal digestion, the gastric digests were adjusted to pH7 with 1 mol/l CaCl₂ and 0.25 mol/l Bis-Tris pH6.5. 6.15 mmol/l of taurocholic acid sodium salt hydrate (Sigma-Aldrich) was then added, and the samples were incubated at 37°C for 15 min. Colipase from porcine pancreas (Sigma-Aldrich), prepared in 35 mmol NaCl adjusted to pH7, was then added at an enzyme: substrate ratio of 1:895. The protein content of the *in vitro* digested supernatants was then determined.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) of egg proteins

Egg proteins (whole egg, egg white, egg yolk, pasteurized egg, ovalbumin and ovomucoid) were electrophoresed on a 4–15% SDS–polyacrylamide gel (Mini-Protean TGX Precast gel, Bio-Rad Laboratories, Gladesville, NSW, Australia) as per the manufacturer's instructions. 60 µg of each egg sample was loaded into each lane. Gels were then fixed with isopropanol solution (25% isopropanol, 10% acetic acid) and stained with Coomassie Brilliant Blue stain (Sigma-Aldrich) for 90 min. The gel was destained overnight with 10% acetic acid solution and then photographed using GENESNAP analysis software program (Syngene, Frederick, MD, USA).

Pooled sera for use in immunoblotting

For the immunoassay, blood was collected from five children (aged 2.6–3.9 yr) with known egg allergy who failed an oral food challenge for egg in baked goods. Written informed consent was obtained before trial participation. Approval was granted by the local institutional review board (Human Research Ethics Committee) of the Women's and Children's Health Network, Adelaide. The average skin prick test results for this group of children were whole egg, wheal 8 mm; egg white, 12.3 mm; egg yolk, 11.2 mm; ovalbumin, 8.8 mm; and ovomucoid, 10.7 mm. Blood was centrifuged at 850 *g* for 10 min. Serum was collected and aliquots were stored at –80°C.

Western blotting of egg proteins using pooled sera from egg-allergic children

The egg proteins on the SDS-PAGE gels were transferred onto 0.2-µm-pore nitrocellulose membranes (Advantec MFS Inc, Dublin, CA, USA) by the process of wet transfer using the Bio-Rad Mini-Protean II Transfer Apparatus (Bio-Rad Laboratories). The membrane was then stained in Ponceau red stain (Sigma-Aldrich) to check for transfer efficiency.

Membranes were incubated overnight with the pooled sera followed by the secondary antibody goat anti-human IgE HRP (Invitrogen, Thermo Fisher Scientific Inc, Scoresby, Victoria, Australia). Membranes were developed using Super Signal West Femto Maximum Sensitivity Substrate Kit (Life Technologies, Thermo Fisher Scientific Inc, Scoresby, Victoria, Australia) according to the manufacturer's instructions, and signal capture was performed using GeneSnap analysis software program (Syngene).

Results

SDS-PAGE and immunoblotting: comparisons of fresh whole raw egg and pasteurized whole raw egg samples

Fig. 1a shows the comparisons of undigested fresh raw egg white (lane 1), raw egg yolk (lane 2), whole raw egg (lane 3) and pasteurized whole raw egg (lane 4) after SDS-PAGE and staining with Coomassie Blue. Fig. 1b shows the egg extracts

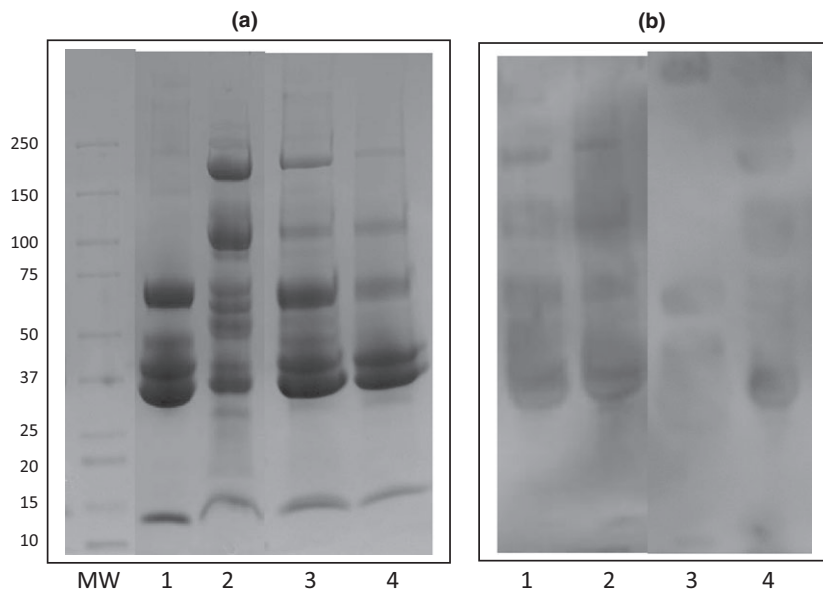


Figure 1 Analysis of fresh unpasteurized and pasteurized whole raw egg. (a) Coomassie Brilliant Blue stained SDS-PAGE gel. (b) IgE immunoblotting with a serum pool from egg-allergic children. Lane 1 contains raw egg white, lane 2 contains raw egg yolk, lane 3 contains raw whole egg, and lane 4 contains pasteurized raw whole egg. Molecular weight (MW) is indicated in kDa.

after electrophoresis and immunoblotting with pooled serum from egg-allergic children. Protein bands were apparent in both the whole egg and pasteurized egg samples around the 200 kD, 120 kD, 70 kD, 30–45 kD and 15 kD positions when compared with the protein standard (Fig. 1). These correspond with the known molecular weight of the major egg allergens ovotransferrin, (Gal d 3, 76.6 kD), α -livetin (Gal d 5, 65–70 kD), ovalbumin (Gal d 2, 45 kD), glycoprotein 42 (Gal d 6, 31 kD), ovomucoid (Gal d 1, 28 kD) and lysozyme (14.3 kD). After transfer and immunoblotting onto nitrocellulose, IgE from the pooled sera bound to the major protein bands (Fig. 1b, lanes 3 and 4). When the whole egg and pasteurized egg samples were compared, the binding patterns of the pooled serum IgE were similar.

The sera IgE also bound to the egg white and egg yolk fraction (lanes 2 and 3), indicating that children were sensitized to both egg white and egg yolk.

In vitro digested egg samples

Fig. 2a shows the comparisons of the *in vitro* digested egg samples after SDS-PAGE and staining with Coomassie Blue. Fig. 2b shows the egg extracts after electrophoresis and immunoblotting with pooled serum from egg-allergic children. For the *in vitro* digested whole egg and pasteurized egg samples, the major protein bands apparent after SDS-PAGE separation were between 60 and 75 kD, with 2–3 bands around the 37 and 50 kD positions, as well as another close

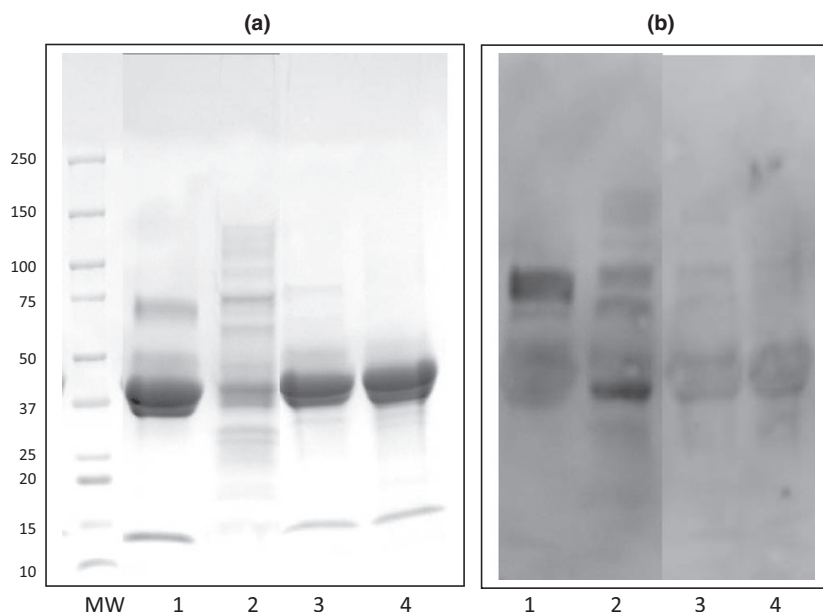


Figure 2 Analysis of *in vitro* digested samples of unpasteurized and pasteurized whole raw egg. (a) Coomassie Brilliant Blue-stained SDS-PAGE gel. (b) IgE immunoblotting with a serum pool from egg-allergic children. Lane 1 contains *in vitro* digested raw egg white, lane 2 contains *in vitro* digested raw egg yolk, lane 3 contains *in vitro* digested raw whole egg, and lane 4 contains *in vitro* digested pasteurized raw whole egg. Molecular weight (MW) is indicated in kDa.

to 15 kD (Fig. 2a). The bands apparent in the undigested egg samples at 250 and 120 kD were not present in the *in vitro* digested samples. After immunoblotting, each of the major protein bands from egg white and egg yolk bound IgE (Fig. 2b). When the *in vitro* digested whole egg and pasteurized egg samples were compared, the binding patterns were similar.

Discussion

Results from our study indicate that IgE of egg-allergic children binds to allergens present in pasteurized whole raw egg powder in a similar pattern to those in unpasteurized whole raw egg. *In vitro* digestion did not affect the IgE binding. This is consistent with results from other studies investigating pasteurized egg white liquid (3), pasteurized, dehydrated egg white (6) and *in vitro* digested ovalbumin and ovomucoid (8) and indicates that the major egg allergens in pasteurized and dried egg products remain intact during the manufacturing processes.

Jurado-Palomo et al. (3) compared pasteurized raw egg white liquid with fresh unpasteurized egg white and found equivalent allergenicity. The protein profile and ability to bind serum IgE from pooled sera of 11 egg-allergic children who failed an oral challenge were compared using SDS-PAGE and immunoblotting with serum IgE and shown to be almost identical. Open food challenges with both forms of raw egg white were also performed in 32 egg-allergic children (who were already tolerating cooked egg) and no significant differences in the results of challenges were noted. Similarly, Escudero et al. (6) reported no significant differences in reactivity to pasteurized and dehydrated egg white. The results from these two studies suggest that even if structural changes to egg white epitopes occur due to the pasteurization and drying processes, these changes do not affect the clinical allergenicity of the egg white.

Unlike previous studies, we investigated whole egg as this reflects the form of egg commonly present in the diet and used pasteurized whole raw egg powder for oral food challenges to raw egg. We identified 13 studies reporting raw egg food challenges (white, whole, pasteurized liquid or powder) (3, 6, 9–19). Six used whole egg (9–12, 16–18), and two used pasteurized whole egg (17, 18). Egg yolk contains the allergens Gal d 5 (α -livetin) and glycoprotein 42 (Gal d 6), and many children are sensitized to both egg white protein and egg yolk (20). Gal d 5 (but not Gal d 6) is relevant for bird-egg syndrome (5) and as yet the clinical relevance of Gal d 6 is unknown. As demonstrated by immunoblotting, allergic serum

IgE bound to allergens present in undigested and *in vitro* digested fresh egg yolk, indicating sensitization to egg yolk as well as egg white proteins.

A strength of this study is the well-defined cohort of 1- to 5-yr-old egg-allergic children from whom the serum pool used for the immunoblotting was obtained. These children have similar rates of tolerance to baked egg as described in cohorts of similar age (18, 21). A limitation is the lack of oral food challenges to both fresh raw egg and pasteurized raw egg, which were not performed for safety and ethical reasons in this group of egg-allergic children with egg white skin prick test results highly predictive of a reaction to raw egg (22).

In Australia, the risk of contracting Salmonella from contaminated raw egg is very low, provided that commercially produced and graded eggs are used (2). No foodborne infections from ingestion of raw egg during an oral food challenge have been reported. However, Salmonella is common in many other countries and there is also a slight risk of infection with bird flu from the consumption of raw egg (3). For these reasons, pasteurized raw egg products should be used for oral food challenges to raw egg.

Egg-allergic children may be sensitized to both egg white and egg yolk allergens, and thus, it is logical to use a product containing whole egg rather than just egg white alone for oral food challenges to raw egg. Pasteurized whole egg powder is palatable, and it is easy to deliver a standardized volume in a challenge making it a convenient and safe product to use for oral food challenges in egg-allergic children.

Conclusion

Pasteurized whole raw egg powder is readily available and is a safe, palatable and convenient challenge food for use in oral food challenges for children with egg allergy. The results of this study through *in vitro* digestions and IgE immunoblotting indicate that the main egg allergens are present in pasteurized whole raw egg powder and bind serum IgE from egg-allergic children in a similar pattern to those in unpasteurized whole raw egg, making it a suitable substitute for raw egg in clinical practice for oral food challenges.

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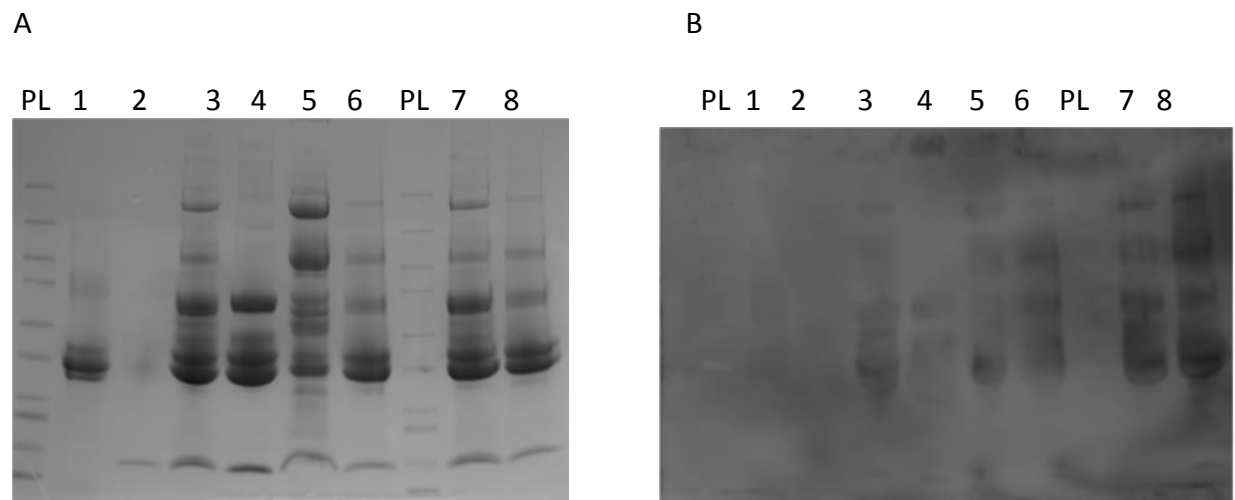
Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Undigested egg samples.

Figure S2. *In vitro* digested egg samples.

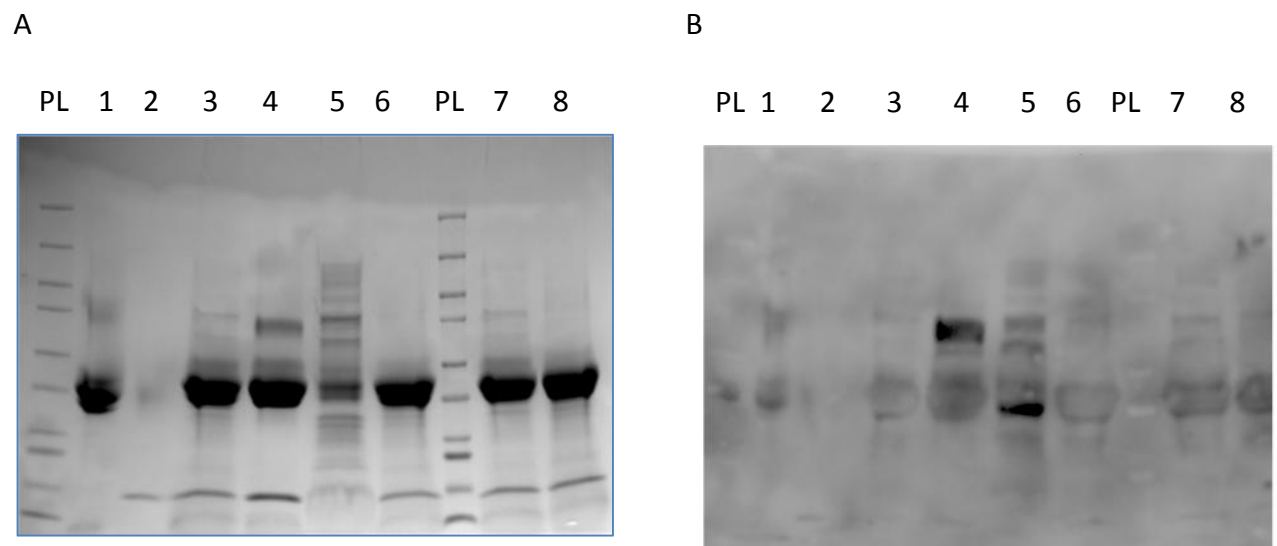
Figure S1. Undigested Egg Samples.



Undigested Egg Samples:

A: Coomassie Stained SDS-Page Gel of Whole egg extract and Pasteurised Whole egg. B: Immunoblot of A. Lanes PL) Precision Plus Protein Standard Kaleidoscope (BioRad #161-0375). 1) Ovalbumin 2) Ovomuroid 3) Whole Raw Egg 4) Egg White 5) Egg Yolk 6) Pasteurised Whole Raw Egg 7) Whole Raw Egg, 8) Pasteurised Whole Raw Egg. For immunoblot, nitrocellulose membrane was incubated overnight with a serum pool of allergic patients sera. Bound IgE antibodies were detected using Goat Anti-Human IgE HRP O (Invitrogen H15707), developed using Super Signal West Femto Maximum Sensitivity Substrate Kit (Pearce Biotechnology, Rockford, USA) and signal capture using GeneSnap analysis software program (Syngene, Maryland, USA).

Figure S2. *Invitro* Digested Egg Samples.



***Invitro* Digested Egg Samples:**

A: Coomassie Stained SDS-Page Gel of Whole egg extract and Pasteurised Whole egg. B: Immunoblot of A. Lanes PL) Precision Plus Protein Standard Kaleidoscope (BioRad #161-0375). 1) Ovalbumin 2) Ovomuroid 3) Whole Raw Egg 4) Egg White 5) Egg Yolk 6) Pasteurised Whole Raw Egg 7) Whole Raw Egg 8) Pasteurised Whole Raw Egg. For immunoblot, nitrocellulose membrane was incubated overnight with a serum pool of allergic patients sera. Bound IgE antibodies were detected using Goat Anti-Human IgE HRP O (Invitrogen H15707), developed using Super Signal West Femto Maximum Sensitivity Substrate Kit (Pearce Biotechnology, Rockford, USA) and signal capture using GeneSnap analysis software program (Syngene, Maryland, USA).