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Immunochemical detection of egg-white antigens and allergens in meat products

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Background: The purpose of this study was to detect antigens and allergens in egg-white byproduct ingredients and after their incorporation in processed pork meat pastes. Commercially prepared foods may have potentially allergenic ingredients (egg, milk, soybean, wheat, and peanut) added in processing. Since allergic patients may react to unidentified ingredients, it is important to assess the allergenic potency of these food proteins added during processing. Egg white was chosen as an experimental model, since egg is one of the most prevalent allergens in food hypersensitivity.

Methods: Experimental pork meat pastes containing egg white underwent pasteurization and sterilization. Ingredients derived from egg-white or paste extracts were isoelectrofocussed and then blotted onto cyanogen bromide-activated nitrocellulose membranes. Egg-white antigens were identified in ingredients and in meat products with rabbit anti-egg-white antiserum by isoelectric focusing immunoblotting. Allergens were identified with sera from sensitized patients. A sensitive ELISA test was developed to detect egg-white proteins in raw, pasteurized, and sterilized meat products.

Results: Antigens and allergens in four egg-white byproducts were detected. Egg-white antigens were detectable in all ingredients and meat pastes by ELISA. Allergens were detected in ingredients and in raw and pasteurized products by immunoprint techniques and ELISA.

Conclusions: Masked egg-white allergens are recognized by human serum IgE after pasteurization. Egg-white antigens are detectable in sterilized meat by ELISA techniques. Ingestion of processed foods could entail a risk of allergic reactions for sensitized consumers.

Abbreviations. ELISA: enzyme-linked immunosorbent assay;

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MaRG-AP: mouse anti-rabbit IgG alkaline phosphatase conjugate; RaEggW: anti-hen's egg-white antiserum; GaHE-AP: goat anti-human IgE alkaline phosphatase conjugate; RAST: radioallergosorbent test; IEF: isoelectric focusing; PBS: phosphate-buffered saline; SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NBT/BCIP: nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salts.

Modern food contains an increasing number of additives or ingredients. These products are generally used for specific applications, such as jellification (meat products), foaming action (biscuit), thickening (sauces), and emulsifying. They are derived from vegetable or animal sources: milk (whey or caseinate), soybean, egg (white and yolk), wheat, and peanut (1). Hidden allergens can be present in processed food when such an ingredient is added and can be a potential risk for atopic subjects (2–5). In 13 fatal or near fatal cases of anaphylaxis, six were due to food allergens not identified by the patients (peanut, nut, and egg) (6). Detection of the individual constituents of modern food products can be difficult due to the low concentrations or treatments involved in their production, which can modify allergen potency. It is important to assess the allergenicity of masked allergens for food-allergic consumers.

Egg white is one of the most prevalent allergens in food hypersensitivity, particularly in atopic children. Egg-white constituents appeared to be an excellent experimental model to assess the problem of masked allergens. Egg white contains about 40 different proteins, some of which have been shown to be major allergens: ovalbumin (Gal d 1), ovotransferrin (Gal d 2), ovomucoid (Gal d 3), and lysozyme (Gal d 4). Ovalbumin forms 54% of whole egg white and is the most extensively studied egg white allergen. It is very widely used in basic research as a model allergen (7–11). Ovomucoid has been described as noncoagulable by heat and is not denatured by 8 M urea (12, 13), whereas ovalbumin is thermolabile. In contrast, the heat stability of the other allergens has not been studied in detail.

This study investigated the ability of IgE from allergic patients to detect hidden egg white antigens and allergens in processed food, particularly in meat products. Four commercial ingredients were studied: new-laid egg white, and pasteurized powder, frozen pasteurized, and liquid pasteurized egg white. One of these ingredients was introduced into experimental meat products either raw, pasteurized, or sterilized. The products were then analyzed to detect egg-white antigens and allergens.

Material and methods

Antibodies and reagents

Anti-hen's-egg-white antiserum (RaEggW) was raised in New Zealand rabbits (14). The care and use of animals followed the guidelines of the École Vétérinaire d'Alfort (France). Secondary antibodies, mouse anti-rabbit IgG alkaline phosphatase conjugate (MaRG-AP) or goat antihuman IgE alkaline phosphatase conjugate (GaHE-AP), were purchased from Sigma Chemical Co. (St Louis, MO, USA). Biotinylated antihuman IgE was purchased from Genetic Systems Corp. (Redmond, WA, USA), and alkaline phosphatase streptavidin conjugate was purchased from Biosys (Compiègne, France).

Serum sources

Human sera were collected from 17 subjects with egg allergy and a positive RAST (radioallergosorbent test, CAP System, Pharmacia and Upjohn, Uppsala, Sweden), class 4, 5, or 6 for egg white (F1) or egg yolk (F75).

Egg-white byproducts

Purified egg-white proteins were a gift from Dr K. Ebbehøj (15). Four ingredients derived from egg white were studied: new-laid egg white (E1), and pasteurized powder (E2), frozen pasteurized (E3), and liquid pasteurized egg-white (E4). These ingredients were chosen because of the diversity of the production processes. Moreover, all of these ingredients are commercially available and commonly used in the food industry. Protein content was measured as described by Lowry et al. (16).

Experimental meat products

Experimental pork meat products (pastes) were prepared in the CTSCCV experimental processing laboratory. Their formulations are reported in Table 1. The control meat paste (C) was prepared without addition of egg-white ingredients. Paste E contained 12% of frozen pasteurized egg white (E3), corresponding to 2% dry powder, amounts often used in meat products. Frozen pasteurized and dry powder egg white are similarly used as food ingredients. The two meat products were distributed in 120-g cans. A first group (R) was kept raw, and the second group (P) was pasteurized at 70°C for 2 h. The third group was sterilized at 115°C for

Table 1. Composition of meat pastes. E3: frozen-pasteurized egg-white

	Control (C)		Egg (E)	
	(g)	(%)	(g)	(%)
Lean pork	1200	40	1200	40
Fatback	930	31	930	31
Ice	810	27	450	15
E3	–	–	360	12
Nitrite salt	45	1.5	45	1.5
Polyphosphates	10	0.33	10	0.33
Reddening salt	5	0.16	5	0.16
Total	3000	100	3000	100

90 min. After processing, the cans were kept at -20°C until protein extraction.

Sample extraction

Egg byproducts (0.6 g) or pork pastes (20 g) were homogenized in 20 ml of PBS (150 mM NaCl, 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 7.2) and incubated overnight at 4°C . Crude extracts were then centrifuged at 10000 g for 10 min and filtered through 0.2- μm filters. The resulting supernatants were stored at -20°C .

Isoelectric focusing (IEF) in acrylamide minigel (IEF/Phast)

IEF was performed on 4% acrylamide gel poured onto a Gelbond ($4.3 \times 5 \times 0.05$ cm) reswollen for 1 h in 10% glycerol, 2% (w/v) Pharmalytes (Pharmacia). Samples were applied on the anode side of the gel (1 μl , 1–10 mg/ml). The gel was focused with the Phast System apparatus (Pharmacia) according to the manufacturer's instructions until 300 V $\times$ h was reached. After blotting, the gel was stained with silver nitrate according to Rabilloud et al. (17).

IEF in agarose gel

IEF was performed according to Peltre et al. (18) on 0.8% agarose gel (Isogel, FMC, Rockland, ME, USA), 12% sorbitol mixed with 2% (w/v) carrier ampholytes (Pharmalytes, Pharmacia; Servalyt, Serva, Heidelberg, Germany). For separation of ingredients, 10 μl samples was applied to the acidic part of the gel. For screening of patient sera, 100 μl of pasteurized frozen egg white (E3), diluted 1/5 in water, was applied to the acidic part of the gel. For separation of meat samples, 10 μl was applied to the basic part of the gel. Runs

were performed with successive settings: 15 min at 300 V, 15 min at 2 W and 4 W until 1200 V $\times$ h was reached.

Transfers onto nitrocellulose membranes

After acrylamide IEF, the gel was submitted to a contact blot. The gel face was quickly washed with distilled water, and a wet CNBr-activated nitrocellulose membrane (19) was applied for 5 min. A glass plate was placed on the membrane to ensure good contact between the membrane and the gel. After agarose IEF, multiple successive immunoprints were performed according to Desvaux et al. (20) with slight modifications. Three pressure blots onto CNBr-activated membranes (10 s, 30 s, and 1 min) with a 1-kg weight were performed.

Antigen and allergen detection by immunoblotting

After transfers, the membranes were dehydrated for 20 min with hot air under a fan in order to improve the binding of components not covalently linked onto the membranes and to inactivate the endogenous alkaline phosphatases of ingredients or pork meat. Membranes were saturated for 1 h in PBS, 1% fish gelatin, and 0.1% Tween 20. They were then incubated with 500-fold diluted RaEggW. They were rinsed three times for 5 min in membrane washing buffer (PBS, 0.1% Tween 20) and incubated with 5000-fold diluted MaRG-AP. Primary and secondary antibodies were diluted in PBS, 1% fish gelatin, and 0.1% Tween 20. After three washings (3 $\times$ 5 min), antigens were revealed by addition of NBT/BCIP substrate, according to Leary et al. (21). The enzymatic reaction was stopped by dilution in distilled water. Patient sera (1/10) and GaHE-AP (1/1000) were used, as primary and secondary antibodies, respectively, to detect allergens. In the screening study, the specificity of IgE of patient sera was determined as described by Desvaux et al. (22).

Antigen and allergen detection by ELISA

Meat extracts (CR, CP, CS and ER, EP, and ES) and ingredients were diluted to 1/100 and 1/1000, respectively, in PBS. Volumes of 100 μl of these dilutions were coated onto microtitration plate wells (Maxisorp, Nunc, Roskilde, Denmark) overnight at 4°C . The remaining sites were blocked with 200 μl PBS, 1% gelatin, for 1 h at room temperature. Egg-white antigens were detected with RaEggW antiserum diluted to 1/1000 in PBS, 0.5% Tween 20, and 1% gelatin for 1 h. After three washes, MaRG-PA

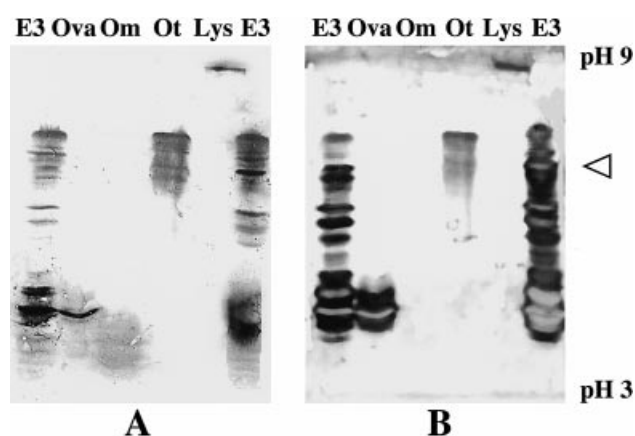


Figure 1. IEF patterns of purified egg-white proteins and antigens in Phast gel (pH 3–9 gradient). A) antigens revealed by rabbit anti-egg-white (RaEggW) antiserum; B) silver staining. E3: frozen pasteurized egg white; Lys: lysozyme; Ot: ovotransferrin; Ova: ovalbumin; Om: ovomucoid; $\triangle$: sample application.

diluted to 1/5000 was incubated for 1 h at room temperature. For allergen detection, sera (100 μ l, diluted to 1/10) from sensitized patients were incubated for 90 min after the blocking step. After three washes, biotinylated antihuman IgE diluted to 1/2000 was added and incubated for 1 h. Three washes were performed, and alkaline phosphatase streptavidin conjugate (1/5000) was incubated for 1 h. After washings, the enzymatic activity was revealed with 1 mg/ml of *p*-nitrophenyl phosphate in 50 mM $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$, pH 9.8, for 15 min for rabbit IgG detection and for 3 h for IgE detection. Absorbance was read at 405 nm with a reference at 630 nm.

Results

Egg-white proteins were first identified by IEF followed by immunoblotting (Fig. 1) with purified proteins prepared according to Ebbehøj et al. (15). Ovalbumin and ovotransferrin are composed of isoforms. Ovalbumin is localized around pI 4.5, and ovomucoid around pI 4. Lysozyme is a very basic protein with a pI greater than 9. Ovomucoid, a very acid protein, was not stained with silver salts, but was detectable with RaEggW antibodies.

The relative importance of the four major egg-white proteins was studied by IEF followed by immunoblotting. IgE specificity of 17 sensitized patient sera (H[1]xE to H[17]xE) were revealed (Fig. 2). The results showed that lysozyme is a minor allergen, as it was weakly detected by five sera (H[3, 4, 7, 8, and 10]xE). Ovotransferrin was very strongly recognized as allergen by five human sera (H[2, 3, 4,

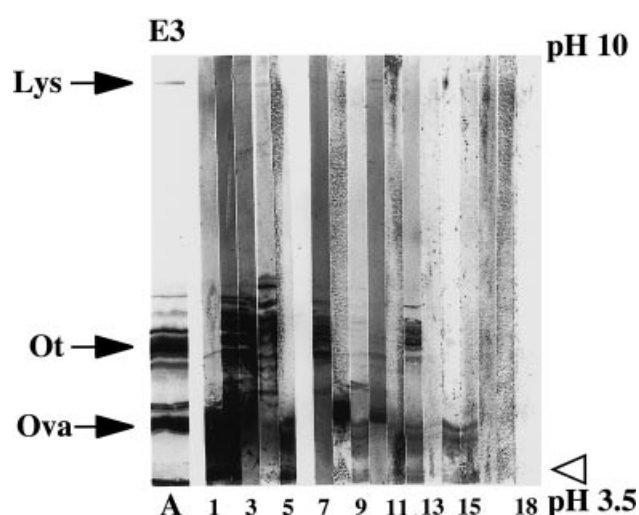


Figure 2. IgE specificity of 17 sensitized human sera H[1]xE to H[17]xE. Amount of 900 μ g of E3 was separated by IEF in agarose (pH gradient 3.5–10, Pharmalytes, Pharmacia). Immunoblot was cut into 3-mm-wide strips, each incubated with different serum diluted to 1/10. Strip 18 was incubated with PBS instead of human serum. A) Coomassie brilliant blue; $\triangle$: sample application.

7, and 12]xE) and weakly recognized by two sera (H[9, 10]xE). The pH range 4–5 contained proteins strongly recognized by the IgE of patient sera. In this pH range, two zones could be defined. The first zone was stained by Coomassie brilliant blue; allergens were detected in this zone by IgE of five sera (H[1, 2, 3, 8, and 10]xE). The other zone was slightly more acidic and not stained by Coomassie brilliant blue; this zone was revealed by IgE of 11 (H[1, 2, 3, 4, 5, 9, 11, 12, 13, 14, and 15]xE) of the 17 sera.

Four different ingredients (E1, E2, E3, and E4) were separated by IEF on agarose gel followed by immunoblots, on which allergens were detected by two different sensitized patient sera (Fig. 3). Coomassie brilliant blue staining revealed the protein diversity of these four ingredients. Most proteins were localized at pH 3–6. Lysozyme was detected at the extreme basic pH. Electrophoretic patterns of E1 and E3, and E2 and E4 appeared to be very similar. IgE from both human sera (H[3]xE and H[4]xE) also revealed this similarity, indicating that E3 is similar to native egg white (E1). An anodic shift of ovotransferrin was observed for E2 and E4. The analogy between E2 (dry powder) and E4 (liquid-pasteurized) could be due to thermal denaturation during the production process.

The frozen pasteurized egg-white ingredient (E3) was introduced into experimental meat pastes. Proteins were extracted with PBS from the six types of meat: raw (CR), pasteurized (CP) and sterilized (CS) control meat pastes, and raw (ER), pasteurized (EP), and sterilized (ES) meat pastes

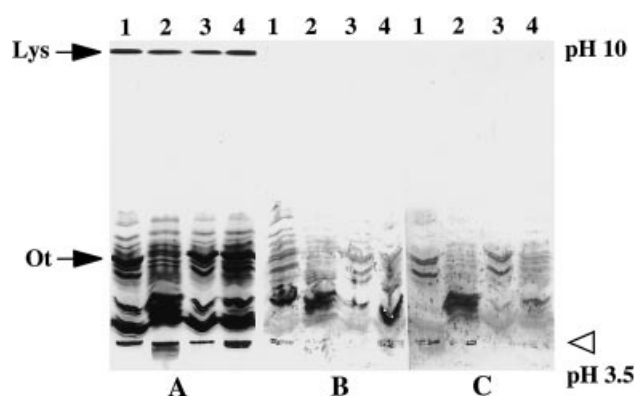


Figure 3. IEF patterns of egg-white byproduct components in agarose gel (pH 3.5–10 gradient). 1) new-laid egg white; 2) pasteurized powder egg white; 3) frozen pasteurized egg white; 4) liquid pasteurized egg white. A) Coomassie brilliant blue staining; B) allergens revealed by H[4]xE human serum; C) allergens revealed by H[3]xE human serum. Lys: lysozyme; Ot: ovotransferrin; Ova: ovalbumin; Om: ovomucoid; $\triangle$: sample application.

containing egg-white ingredient (E₃). Direct ELISA experiments were performed to detect egg-white antigens and allergens (Fig. 4). Rabbit RaEggW antiserum detected antigens in raw, pasteurized, and sterilized meat, indicating that some or all egg-white antigens are still recognized after thermal treatment. This also indicates that a satisfactory proportion of egg-white proteins is extracted from raw and cooked meat with PBS buffer, as the three absorbance values

(ER, EP, and ES) were comparable. The detection limit of egg-white antigens by this ELISA method was 0.03% of dry powder in raw and pasteurized products and 0.125% in sterilized product (data not shown). Furthermore, the RaEggW antiserum was specific for egg-white proteins, as no reaction was observed in control meats. Three sensitized human sera were tested by ELISA for egg-white allergen detection in meat extracts. All sera were able to detect allergens in raw and pasteurized, but not in sterilized products. These sera gave negative results on pork meat not containing egg-white ingredients.

IEF separation of meat extracts was performed, followed by immunoblots for identification of egg-white antigens and allergens after meat processing. The first immunoblot was revealed by RaEggW antibodies, which detected most egg-white proteins in raw and pasteurized meat (Fig. 5). No protein was detected in control meats, and a residual endogenous alkaline phosphatase activity was observed in raw-meat extracts. Lysozyme was no longer detectable in pasteurized products, and no egg-white antigens were detected in sterilized products with this technique. Two blots were revealed with patient sera chosen for their high level of egg-white recognition allergens and with different allergen recognition specificities. Human serum H[1]xE was able to detect egg-white allergens in raw and pasteurized pastes (Fig. 6) in the pH range 3.5–5, corresponding to the

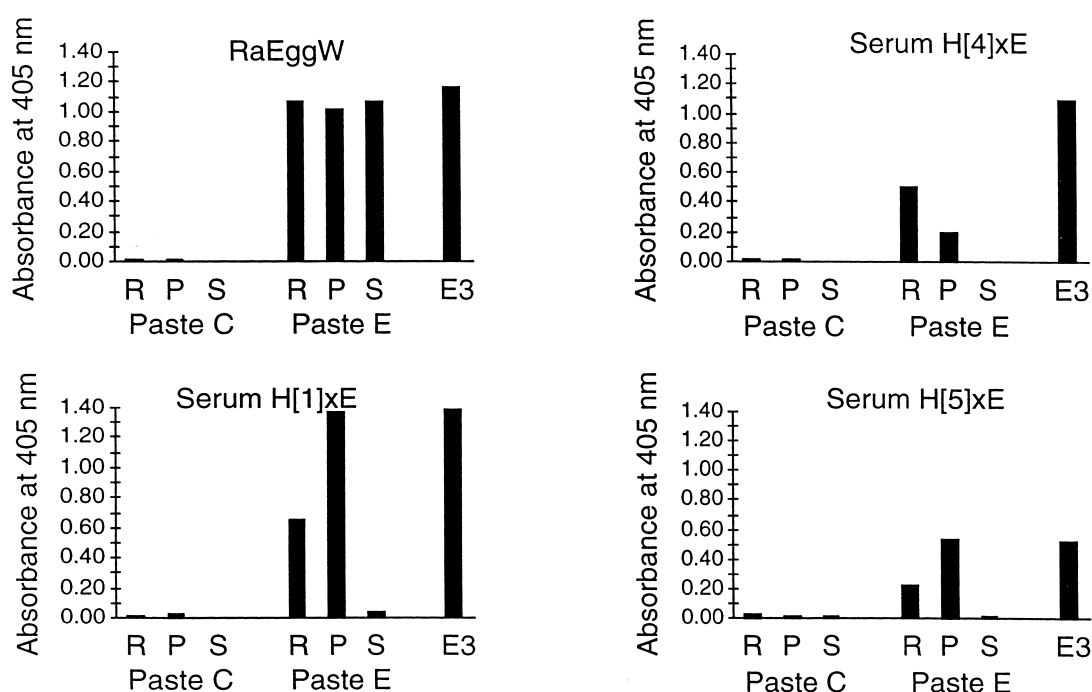


Figure 4. ELISA assays for egg-white antigens and allergen detection in soluble extracts. Paste C) negative control meat product; paste E) meat product containing egg white. R: raw; P: pasteurized; S: sterilized; E₃: frozen pasteurized egg white. Human sera H[1, 4, or 5]xE were diluted 1/10 and RaEggW 1/1000.

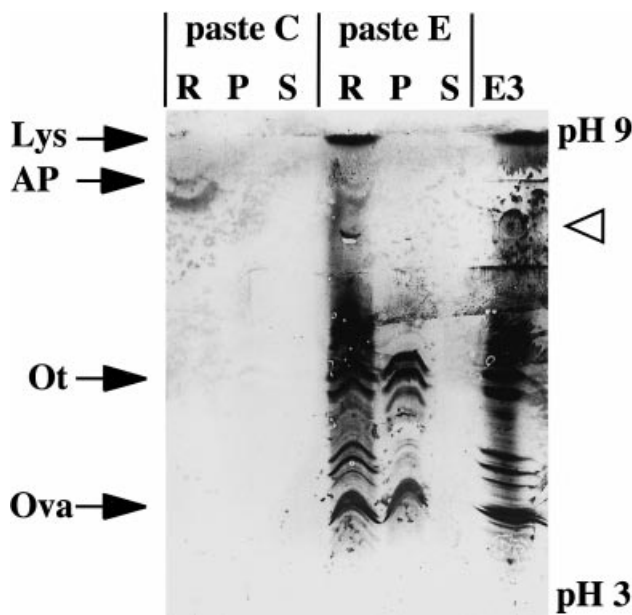


Figure 5. Immunoblot of IEF separation of meat extracts (pH 3–9, Servalyt, Serva). Egg-white antigens immobilized onto 10-s blot were revealed with RaEggW and MaRG-AP. Paste C) negative control meat product; paste E) meat product containing egg white. R: raw; P: pasteurized; S: sterilized; E3: frozen pasteurized egg white; Lys: lysozyme; AP: alkaline phosphatase; Ot: ovotransferrin; Ova: ovalbumin; Om: ovomucoid; <: sample application.

ovalbumin/ovomucoid pI zone. Human serum H[4]xE was able to detect ovotransferrin in raw and pasteurized pastes

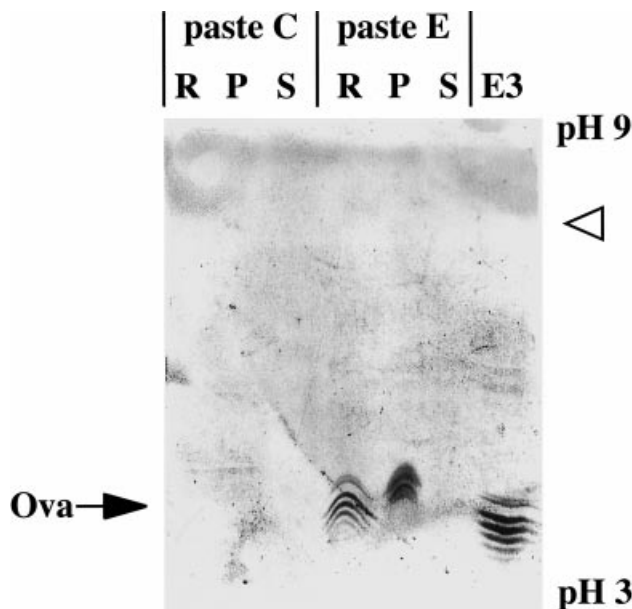


Figure 6. Immunoblot of IEF separation of meat extracts (pH 3–9, Servalyt, Serva). Egg-white allergens immobilized onto 30-s blot were revealed with serum from allergic patients H[1]xE and GaHE-AP. Paste C) negative control meat product; paste E) meat product containing egg white. R: raw; P: pasteurized; S: sterilized; E3: frozen pasteurized egg white; Ova: ovalbumin; Om: ovomucoid; <: sample application.

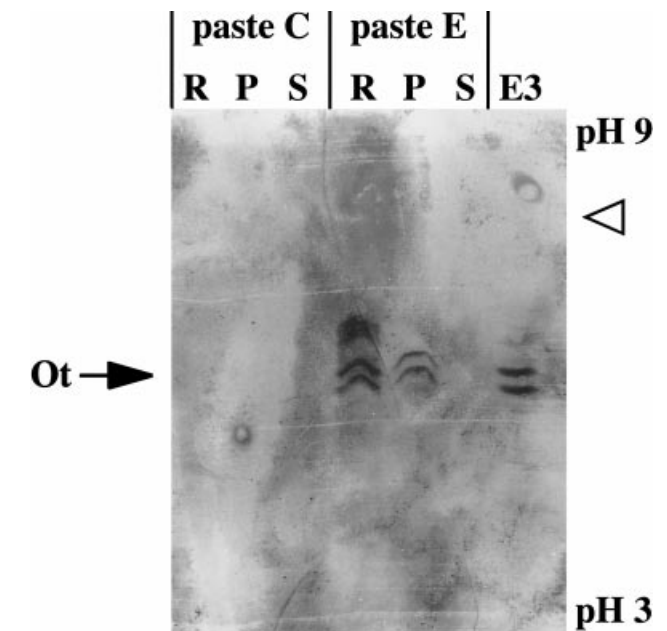


Figure 7. Immunoblot of IEF separation of meat extracts (pH 3–9, Servalyt, Serva). Egg-white allergens immobilized onto 1-min blot were revealed with sensitized human serum H[4]xE and GaHE-AP. Paste C) negative control meat product; paste E) meat product containing egg white. R: raw; P: pasteurized; S: sterilized; E3: frozen pasteurized egg-white; Ot: ovotransferrin; <: sample application.

(Fig. 7). No egg-white allergens were detectable in sterilized products with these two patient sera. Control products were still negative.

Discussion

The first objective of this study was to detect egg-white antigens and allergens in food ingredients, because they are common allergens and common components. Four egg-white-derived ingredients were chosen because of the different methods used during their production. Ingredient antigens were analyzed by IEF followed by immunoblotting revealed with rabbit antiserum raised against egg white. Allergens were also investigated with sera from egg-white-sensitized patients containing high levels of anti-egg-white-specific IgE. The second objective was to reveal the presence of egg-white ingredients incorporated in experimental pork meat products submitted to different cooking temperatures by detecting egg-white antigens and allergens.

The IEF separation technique was used for a full allergen detection, as it is a nondenaturing technique which keeps the native protein conformation intact. During SDS–PAGE, SDS denaturation could induce loss of conformational epitopes often involved in allergenic reactions (23). How-

ever, protein identification by IEF may reveal isoforms. Despite IEF separation of purified egg-white allergens, ovalbumin and ovomucoid could not be clearly distinguished. Immunoblotting was performed on CNBr-activated nitrocellulose membranes, which have been shown to increase protein binding (19, 24), and which allow multiple successive immunoprints (20) for better antigen description.

The study of ingredient allergens showed that, regardless of the nature of egg-white byproducts (new-laid, pasteurized powder, frozen pasteurized, or liquid pasteurized), their antigenicity and allergenicity were not diminished. Only the protein structure was modified by pI shift of ovotransferrin, which has been shown to be more prone to thermal denaturation than ovalbumin (25).

IEF/immunoblotting showed that egg-white ingredients mixed in pork meat in a 2% (w/v) ratio are detectable with rabbit immunoserum when the products are raw and pasteurized. Due to the high sensitivity of the technique, ELISA experiments were able to detect egg-white antigens under the three temperature conditions, indicating that egg-white proteins are thoroughly extracted by PBS and are coated onto ELISA plates. Sensitivity tests showed that egg-white proteins were detected at low levels in raw and pasteurized products (0.03% w/v) and at 0.125% in sterilized product (data not shown). Therefore, the rabbit anti-egg-white antiserum used in the present study is appropriate for assays performed by health boards concerned with food control.

Ovalbumin (42.7 kDa, pI 4.5; 54% of total protein in egg white) has long been known as the major allergen in egg white (8, 10). As shown in Fig. 2, IEF/immunoblotting showed that only five sera of the 17 studied could detect allergens in the intensely Coomassie brilliant blue-stained zone in the pH range 4–5 where ovalbumin is located, whereas slightly more acidic allergens were recognized by 11 sera in the Coomassie brilliant blue-unstained zone (Fig. 2) where ovomucoid (28 kDa, pI 4.1; 11% of total protein) focuses, too (Fig. 1). These results agree with other recent studies showing that ovomucoid is the major allergen of egg white, because most of the apparent IgE reactivity to ovalbumin is due to its minimal (less than 1%) contamination by ovomucoid (26). Urisu et al. (27) have also reported that ovomucoid has a more important role in egg hypersensitivity than other egg-white proteins. In the present report, antigens and allergens were detected on immunoblots after pasteurization, but not in sterilized meats (Figs. 5 and 6); they were labile at high temperature. Previous studies have shown that the native conformation of ovomucoid is resistant to extreme pH and temperature, its stability being

largely attributable to the presence of nine disulfide bonds in this glycoprotein (28). Therefore, the antigens and allergens revealed in the pH range 4–5 by rabbit IgG and IgE of the patient H[1]xE could be ovomucoid and/or could be other acidic molecules (Figs. 5 and 6). The weakness of ovomucoid detection by rabbit IgG, as shown in Fig. 1A, reflects the poor immunogenicity of this molecule in rabbit, as also previously noticed (29).

Ovotransferrin (77.7 kDa, pI 6.1; 12% of total protein), or conalbumin, has been shown by IEF to be composed of a large number of isoforms. These isoforms could be due to different levels of Fe saturation, as Wenn & Williams (30) showed that Fe depletion increased the pI of the protein from 5.78 to 6.73. This allergen was still recognized after meat pasteurization, but the IgE reactivity was decreased by sterilization. In the present study, ovotransferrin was a more important allergen than ovalbumin.

Lysozyme (14.3 kDa, pI 10.7; 3.4% of total protein) was no longer detected by rabbit antiserum after pasteurization and sterilization, possibly due to protein denaturation or to lack of extraction by PBS. The allergenicity of lysozyme has long been debated. Some studies reported that it was an important allergen for egg-allergic patients (31, 32). Holen & Elsayed (11) also showed that lysozyme was strongly recognized by IgE from all the individual or pooled egg-allergic patient sera. In contrast, recent studies showed that lysozyme is a weak allergen, suggesting that IgE reactivity to lysozyme could be due to nonspecific binding (26). In the present study, lysozyme was weakly recognized by five of the 17 patient sera chosen for their higher than class 4 RAST. Although lysozyme was a minor allergen in our study, caution is advised since egg lysozyme is used as a preservative in food and may be present in some cosmetics, shampoos, and drugs.

In conclusion, ELISA and IEF/immunoblotting demonstrated that pasteurization does not affect antigenic and allergenic properties of egg-white proteins. In contrast, sterilization seems to destroy egg-white allergens, even ovomucoid, which has been reported to be heat-resistant. A very sensitive ELISA was developed to detect egg-white proteins in meat products. With PBS extraction, egg-white antigens were detected in raw, pasteurized, and sterilized meats products by ELISA. This demonstrated good protein extraction and preservation of certain antigenic epitopes which could be a potential risk for allergic patients with different IgE reactivities. However, oral food challenge would need to be done in sensitive patients to assess the risk of allergic reactions to hidden egg white.

This study highlights the fact that food proteins added in processing may remain allergenic. The prevention of food disorders requires complete consumer information on product labels. For example, when egg white is present in prepacked foods, even as a “binder”, “emulsifier”, or “coagulant”, it should be declared in the list of ingredients (33). This principle could be applied to all other ingredients and to the potentially allergenic molecules used in specific

technical processes such as proteolytic or bacterial enzymes and genetically modified organisms (34–38).

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